# MERKEL CELL POLYOMAVIRUS POSITIVE MERKEL CELL CARCINOMA – FROM DIAGNOSIS TO THERAPY

by

**Reety Arora**

Bachelors in Engineering, Panjab University, 2007

Submitted to the Graduate Faculty of

University of Pittsburgh School of Medicine,

Program in Integrative Molecular Biology,

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Reety Arora

It was defended on

May 4, 2012

and approved by

Jeffrey L.Brodsky, Ph.D., Professor, Department of Biological Sciences

James M. Pipas, Ph.D., Professor, Department of Biological Sciences

Saleem A. Khan, Ph. D., Professor, Department of Microbiology and Molecular Genetics

Thomas E. Smithgall, Ph. D., Professor, Department of Microbiology and Molecular Genetics

Dissertation Advisor: Yuan Chang, MD, Professor, Department of Pathology

**MERKEL CELL POLYOMAVIRUS POSITIVE MERKEL CELL CARCINOMA**

**- FROM DIAGNOSIS TO THERAPY**

Reety Arora

University of Pittsburgh, 2012

Merkel cell polyomavirus (MCV) is one of the newer members of the polyomavirus family, recently discovered as clonally integrated into the genomes of a subset of Merkel cell carcinoma (MCC). MCV is the first polyomavirus that is widely accepted to cause a human cancer and its identification has resulted in a paradigm shift in the understanding of MCC biology. In the vast majority of the population, MCV is a harmless member of the normal human microbial flora, but can initiate an aggressive cancer if it integrates into the host genome and acquires a precise set of viral mutations that result in replication incompetence (in a susceptible host).

This dissertation describes how the identification of a new viral cause of MCC was harnessed to develop new diagnostic assays and therapeutic options for MCC. To assess MCV infection and its association with MCC as well as other human diseases, a monoclonal antibody that specifically recognizes both endogenous and transfected MCV large T antigen was developed. Using this antibody, specific nuclear localization of MCV T antigen in MCC tumor cells was demonstrated. A quantitative PCR assay revealed that MCV is present in MCC at > 1 copy per tumor cell. These assays were used to survey non-MCC tissues including hematolymphoid malignancies, neuroendocrine tumors, and various dermatologic cancers. None showed association with MCV infection. Restricted expression of the MCV LT oncoprotein to MCC tumor cells provides the mechanistic underpinning supporting the notion that MCV causes a subset of MCC.

To investigate treatment options for MCC two methods were used. An *in vitro* drug screen of 1360 chemotherapeutic and pharmacologically active compounds resulted in the

identification of a proteasome inhibitor, bortezomib, as a potent but nonselective candidate. To rationally and specifically target MCV positive MCC, deep-sequence profiles of MCV positive MCC tumors were compared to MCV negative MCC tumors. Among 64 cell death related genes, a seven fold differential expression of survivin was observed in MCV positive MCC. MCV T antigen knock down in MCV positive cell lines decreased survivin mRNA and protein expression. Also, exogenously expressed MCV T antigen increased survivin protein in non-MCC primary cells in an RB protein binding dependent manner. A survivin inhibitor, YM155 initiated selective non-apoptotic MCV positive MCC cell death. YM155 was nontoxic and halted MCV positive MCC xenograft tumor growth in mice while bortezomib was inactive and caused significant toxicity *in vivo.*

**TABLE OF CONTENTS**

## LIST OF TABLES

## LIST OF FIGURES

## ACKNOWLEDGEMENTS

The last five years of my life have been an incredible journey. I have faced many tough challenges, learned numerous skills, made lifelong friends, accomplished many goals, adapted and grown. Various fine individuals have helped me along graduate school to where I am today. I have been extremely fortunate to encounter such nice people and am grateful to all of them. I will not be able to list each of you by name but please accept my gratitude.

First and foremost I would like to thank my mentors Dr. Yuan Chang and Dr. Patrick S. Moore. I don't know where to begin to express my heartfelt gratitude for your time, guidance, patience and encouragement. Your insightful thoughts, constructive criticism and invaluable advice have helped me grow both professionally and personally. I will always be grateful for the opportunities and projects that you gave me, for all your support and the freedom to try some of my crazy ideas. I will always remember the “data walks” around the laboratory and the annual chinese new year parties. Thank you for being such wonderful people and such inspirational scientists. It has truly been an honor working with you.

I would also like to acknowledge my dissertation committee members: Dr. Jeffrey Brodsky, Dr. James Pipas, Dr. Saleem Khan and Dr. Thomas Smithgall for overseeing my doctoral work. I have enjoyed our scientific discussions and thank you all for your time, helpful criticisms and great ideas.

I owe my doctoral work to the amazing Chang-Moore laboratory members. With a lot of respect, I express my thanks to Dr. Masahiro Shuda and Dr. Huichen Feng. They are both brilliant humble scientists who have both been integral parts of my professional development. Masahiro Shuda has been almost like a third mentor to me and I express my deepest gratitude for all his help and guidance. I am also thankful to all the other post-doctorate members of the

laboratory: Dr. Yanis Tolstov, Dr Hyunjin Kwun, Dr. Zhihua Li, Dr. Tuna Toptan and Dr. Lindsay Dresang. Thank you all for answering all my silly questions and for all the support and encouragement. I learned a lot from all of you. My fellow graduate students and the medical students who rotated in the laboratory, Dr. Xi Liu, Ezra Mirvish, Mamie Thant and John Hughes made learning together a lot of fun. I would especially like to thank my partner in crime and *wunderbare Freundin* (German for wonderful friend) Anna Guastafierro. None of the mouse experiments would have been possible without her. Also, I will always remember and cherish the enjoyable brainstorming sessions over delicious homecooked dinners. *Danke!* Chrissie Usher and Susan Scudiere, the two administrators and managers of the laboratory, have been blessings in disguise and I am grateful for all the help with the technicalities and paperwork. The Chang-Moore laboratory has become like an extended family to me, a home away from home and I will cherish and miss all the great moments spent here.

I am very thankful to the PIMB program. All its affiliated members have created a warm and welcoming atmosphere for me over the years. This program is very special due to the few, well connected students and the close collaborations that form within. I would like to extend a special thanks to Jennifer Walker, her easy solutions and bright personality always fixed every hurdle. Thanks Jen!

I would also like to mention and extend my gratitude to our collaborators Dr. Andreas Vogt and Laura Vollmer for help with the high-throughput drug screen project, Dr. Daniel Normolle for the biostatistical analysis of the xenograft data, and Dr. Ole Gjoerup, Dr. Stergios Moschos and Dr. John Kirkwood for the detailed, helpful discussions.

The support of the Cancer Virology Program (CVP) and the ambience of the University of Pittsburgh Cancer Institute (UPCI) and all the core facilities available have been great. I also enjoyed participating in the UPCI summer academy and thank Phillip, William and Dr. Michael Lotze for the great program. I would also like to thank Dr Mary Ann Accavitti, Ms. Marie Acquafondata, Dr. Yuan-yuan Chen, Dr. Linda Gooding, Dr. Clayton Wiley, Dr. James Pipas, Dr. Jeffrey Brodsky, Dr. James Decaprio, Dr. Ole Gjoerup, Dr. Saumen Sarkar, Dr. Christopher Bakkenist, Dr. Dario Altieri, Dr. Jurgen Becker and NCI/DTP Open Chemical Repository for

reagents, antibodies, constructs, tissues, cell lines and compounds. A special thanks to all the members of the DLAR mouse facility at University of Pittsburgh, especially to Dr. Joseph T. Newsome, Dr. Frank Jenkins, Katie L. Leschak and Megan L. Lambert for all their help and guidance.

I have made some very valuable friendships in Pittsburgh and am thankful to all my friends in keeping me sane throughout the last five years: Janice, Jelena and Milos, Sandeep, Nisha and Balaji, John, Melanie, Supriya, Shruthi, Amrita, Eric, Madhav and K'Ann, Adriana, Kathleen, Amitabh, Alex, Aarti, Sergei, Gene, Prerna, Abhishek, Neha, Avantika, Sonam, Niti didi and all my AID (Association for India's Development) buddies. Thank you all for all the happy times and the great memories.

Finally and most importantly, I am forever grateful for my wonderful family. My father, whom jealous fates took away too early, and my mother taught me the importance of hard work and duty and to dream with no restrictions. They always did everything they could to guide me along the right path for me. I am very fortunate to have such supportive parents and am indebted to them for their truly unconditional love. I also want to extend my thanks to my brother Rahul, my sister Rupaly *didi*, my brother in law Bhupesh *jiju* and my nephew Rudra (our new source of joy). Lastly I would like to thank Karandeep S. Kapany, who has been so fantastically supportive and patient, and has encouraged me every step of the way.

This dissertation is dedicated to all those who are suffering from or have died of Merkel cell carcinoma. I hope to continue to work hard towards a better disease free tomorrow.

Thank you!

Sincerely,

Reety Arora

# 1.0 INTRODUCTION

The work described in this dissertation was initiated in 2008, when Merkel cell polyomavirus (MCV) had just been discovered as a new human polyomavirus clonally integrated into the Merkel cell carcinoma (MCC) genome. Studies over the past four years, including the ones described here, reveal various aspects of virus-mediated tumorigenesis of MCC. More than half a century of work studying simian virus 40 (SV40), a model polyomavirus, has produced important molecular and cellular tools to investigate MCV biology. Although polyomaviruses have been linked to human disease, BK virus to nephropathy and JC virus to progressive multifocal leukoencephalopathy as examples, MCV is the first polyomavirus convincingly linked to human cancer. MCV discovery has changed the perception of an intractable cancer MCC, leading to new opportunities for its early diagnosis and targeted treatment. MCV has become a new model for human cancer, in which a common, harmless member of the human viral flora can initiate cancer if it acquires a set of mutations in a susceptible host (immunosuppressed or elderly). Also, with the discovery of four additional human polyomaviruses since 2008, studies of polyomavirus basic science have become increasingly relevant, and future investigations of these viruses may give new insights into their connection with human disease.

## 1.1 VIRUSES AND CANCER

### 1.1.1 Discovery of link between viruses and cancer

Infections, mostly viruses, cause approximately 20% of cancer cases worldwide [1, 2]. It has been over 100 years since Francis Peyton Rous discovered the first tumor virus, and considerable research has been devoted to the understanding of this group of viruses [3]. Virus-associated cancers have identifiable viral targets amenable for diagnosis and therapy. Vaccination programs for the prevention of two common tumor virus infections, hepatitis B virus (HBV) and human papillomavirus (HPV) have begun to make a difference in tumor burden and the general health of the global population [4, 5].

In a groundbreaking discovery in 1911, Francis Peyton Rous showed that viruses could induce cancer. He successfully transplanted sarcoma from a 15-month old hen into other chickens through cell-free tumor extracts [3, 6]. The isolated etiologic agent, Rous sarcoma virus (RSV), was the first tumor virus identified. After an initial period of inattention, interest in the field re-emerged following Ludwik Gross's discovery of an acute transforming murine retrovirus and a polyomavirus that caused murine tumors [7]. The impact of these achievements was enormous. Soon after Rous received the Nobel Prize for his discovery, study of the RNA tumor virus-RSV lead to the discoveries of reverse transcription and the cellular origin of viral oncogenes (beginning with v-src of RSV) [8-11]. Subsequently, the study of tumor viruses has accelerated.

### 1.1.2 Human tumor viruses

Anthony Epstein, Bert Achong and Yvonne Barr discovered the first human tumor virus in 1964. They identified herpesvirus-like particles in cell lines from equatorial African patients with an unusual, geographically confined childhood cancer called Burkitt's lymphoma [12]. The virus was named Epstein-Barr virus [13]. Despite studies that demonstrated EBV infection as

persistent, asymptomatic and ubiquitous in adults worldwide, EBV-associated cancers were uncommon. This dissociation between prevalence of infection and occurence of cancer initially confused scientists' understanding of how viruses cause tumors [14, 15]. Additionally, sporadic Burkitt's lymphomas were observed in developed countries that frequently lacked EBV but retained a signature mutation of Burkitt's lymphoma c-myc IgG gene chromosomal translocations [16]. Although the proposed role of EBV in Burkitt's lymphoma was viewed with skepticism at first, its strong association with lymphomas and nasopharyngeal carcinomas, and follow-up studies of the tumorigenic potential of its viral proteins added more credence to the idea. The understanding of the "multistep" process of cancer development and the recognition that chronic viral infection functions together with various other non-viral host factors contribute to cancer (such as insect-borne parasitic infections like malaria, young age, status of immune system, nutrition, etc), finally led to the acceptance of EBV as a human tumor virus. The International Agency for Research on Cancer (IARC) declared EBV as a group 1 (*carcinogenic to humans*) human carcinogen in 1997 [14, 17].

Scientists have made numerous attempts to search for human tumor viruses using a similar electron microscopy approach. These efforts however, were disappointingly negative. It was later understood that cancer viruses generally do not replicate as virions in tumors, but exist as latent episomes or integrate into the host genome [18, 19]. Six other human cancer viruses have been identified since EBV and surprisingly all of them come from different classes of viruses (Table 1). Interestingly, almost all of these human tumor viruses have close relatives that do not cause human cancer.

The identification of the Australia (Au) antigen (antigen from the blood of an Australian aborigine found to cross react to an American hemophilia patient serum) by Baruch Blumberg in 1965 eventually led to the identification of the Hepatitis B virus. Persistent infection and asymptomatic carriage of the virus in healthy populations [20] obscured the association between HBV and disease, and several years elapsed before a link between the virus and hepatocellular carcinoma (HCC) was made in 1975 [21]. HCC is among the most

**Table 1 Human tumor viruses (Modified from Moore and Chang, Nat Rev Cancer 2010)**

| Virus | Year identified | Method of identification | Genome | Cancer associations | Reference |
|---|---|---|---|---|---|
| Epstein–Barr virus (EBV) | 1964 | Electron microscopy | Double-stranded DNA herpesvirus | Most Burkitt's lymphoma and nasopharyngeal carcinoma, most lymphoproliferative disorders, some Hodgkin's disease, some non-Hodgkin's lymphoma and some gastrointestinal lymphoma | Epstein *et al*., Lancet, 1964 |
| Hepatitis B virus (HBV) | 1965 | Serologic screening | Single-stranded and double-stranded DNA hepadenovirus | Some hepatocellular carcinoma | Blumberg *et al*., JAMA, 1965 |
| Human T-lymphotropic virus-I (HTLV-I) | 1980 | Tissue culture | Positive-strand, single-stranded RNA retrovirus | Adult T cell leukaemia | Poiesz *et al*., PNAS, 1980 |
| High-risk human papillomaviruses (HPV) 16 and HPV 18 | 1983–1984 | DNA cloning | Double-stranded DNA papillomavirus | Most cervical cancer and penile cancers and some other anogenital and head and neck cancers | Durst *et al*., PNAS, 1983 Boshart *et al*., EMBO J, 1983 |
| Hepatitis C virus (HCV) | 1989 | cDNA cloning | Positive-strand, single-stranded RNA flavivirus | Some hepatocellular carcinoma and some lymphomas | Choo *et al*., Science, 1989 |
| Kaposi's sarcoma herpesvirus (KSHV) | 1994 | Representational difference analysis | Double-stranded DNA herpesvirus | Kaposi's sarcoma, primary effusion lymphoma and some multicentric Castleman's disease | Chang *et al*., Science, 1994 |
| Merkel cell polyomavirus (MCV) | 2008 | Digital transcriptome subtraction | Double-stranded DNA polyomavirus | Most Merkel cell carcinoma | Feng *et al*., Science, 2008 |

EBV is also known as Human Herpesvirus 4 (HHV4) and KSHV is also known as Human Herpesvirus 8 (HHV8)

Besides HPV 16 and HPV 18, some other α-HPV types are also carcinogens

common malignancies in the world and is fatal. In adults, primary HBV infection either results in acute hepatitis or can be asymptomatic, if the immune system clears the virus. However in about 5% of infected neonates and adults where primary infection fails to resolve, long-term chronic infection with active viral replication in liver cells and viremia is seen. Persistent chronic infection as well as risk factors such as aflatoxin and hepatitis C virus or parasitic infections, increase the risk of HCC development [22-24].

HBV-induced liver cancer is responsible for >300,000 deaths per year worldwide and is a major public health concern. An important milestone in prevention of these cancers came with Merck's HBV vaccine in the late 1900s. The vaccine, still in use today, is a recombinant HBV surface antigen subunit vaccine produced in yeast cells, and protects against not only acute and chronic hepatitis but also the development of HCC [25, 26].

With the discovery of HBV and advancements in molecular biology, it became possible to screen for and identify virtually every patient infected with HBV. These assays unexpectedly distinguished a transfusion-associated serum hepatitis, not infected with HBV or hepatitis A virus (another close relative causing hepatitis in people). Non-A, non-B hepatitis is an acute disease that is relatively mild but ultimately results in chronic infections that lead to serious chronic liver disease and cirrhosis. Exploiting the observation that the disease could be transmitted to chimpanzees, Choo *et al*. generated a lambda phage cDNA expression library from nucleic acid extracted from the plasma of a chronically infected chimpanzee [27]. Using serum from a chronic non-A, non-B hepatitis patient to screen this library in 1989, Houghton and colleagues found an antigen encoded by an unknown RNA flavivirus. This virus was named Hepatitis C virus or HCV and is now recognized as another etiologic agent of chronic hepatitis, liver cirrhosis and HCC worldwide. Chronic HCV infection affects more than 170 million individuals, 20% of whom have or later develop cirrhosis and an increased risk of developing HCC (highest risk in Japanese individuals) [28-31].

In 1980, Bernard Poiesz, Robert Gallo and colleagues discovered the first and only known human retrovirus directly linked to a specific human cancer. HTLV-1 or Human T-lymphotropic virus-1 was discovered by searching for reverse transcriptase activity in a survey of adult T cell

leukemia/lymphoma (ATL) cell lines [32, 33]. Yorio Hinuma, Isao Miyoshi and colleagues visualized retroviral particles in ATL derived leukemia cell line and confirmed these findings the next year [34]. Over the following few years, the causal role of HTLV-1 in ATL was established. HTLV-1 infections correlated geographically with ATL cases distributed in Japan, central African coastal regions, Taiwan, the Caribbean, and Papua New Guinea. Cancer cells cultured from patients with ATL had proviral DNA and infection of normal, healthy human T cells with HTLV-1 induced cellular immortalization. Virtually every ATL patient demonstrated HTLV-1 infection and carried antibodies specifically recognizing HTLV-1 antigens. Other retroviruses closely related to HTLV-1, such as bovine leukemia virus and simian T-cell leukemia virus 1, caused leukemia in their respective animal hosts. In total, these epidemiologic and molecular studies implicated HTLV-1 as the etiologic agent of ATL [33, 35-37]. Intriguingly, by contrast to mechanisms typical of animal retroviruses, HTLV-1 does not cause tumors by insertional mutagenesis or by capturing and activating cellular proto-oncogenes. Instead, reminiscent of DNA tumor viruses, HTLV-1 produces an oncogenic protein called Tax that is essential for viral replication and perturbs various host cell growth regulatory pathways. Also, similar to other known human tumor viruses, HTLV-1 infects healthy individuals, but is kept in check by the immune system and remains latent/dormant for long periods. It is detected early in life and less than 5% of infected individuals develop ATL after 20-30 years.

Sexual activity was a known risk factor for the development of cervical cancer, the third most common cancer in women worldwide [38]. Based on the emerging evidence at the time, linking human papillomaviruses (HPV) to gential warts, Harald zur Hausen reasoned that HPV was the etiologic agent for cervical cancer, despite the general belief that herpes simplex virus 2 (a sexually transmitted virus) was the likely cause. In his landmark studies in 1983 and 1984, his group cross-hybridized known papillomavirus DNA to cervical cancer DNA and discovered two novel HPV genotypes (HPV16 and HPV18) [39, 40]. These genotypes, classified as high-risk HPVs, and are now confirmed as etiologic agents for 70% of cervical cancers worldwide. Subsequently, various other high-risk HPV genotypes were found to be associated with anogential cancers as well as head and neck cancers. HPVs are associated with more human

cancers than any other virus [41-44].

The success of the HBV vaccine in decreasing liver cancer prompted HPV biologists to develop a safe preventative HPV vaccine. Such an endeavor was successful when a quadrivalent virus like particle (VLP)-based vaccine (using the recombinant capsid protein L1 from HPV types 6, 11, 16 and 18) cleared clinical trials in 2006 conferring immunized women with type-specific protection against HPV infection, as well as associated cervical, vulvar and vaginal disease [45-50]. VLPs are non-infectious virions resulting from the self-assembly of viral capsid proteins, and are used in making conformational viral epitope-based vaccines [51]. The vaccines, marketed as Gardasil by Merck and Cervarix by GlaxsoSmithKline, are widely used and recommended for young individuals (both girls and boys, ages 9 through 26). Based on current estimates, these vaccines can lead to the prevention of greater than 300,000 cervical cancer cases per year globally.

Targetted searches for viruses as etiologic agents of human malignancies led to the discoveries of the two newest human tumor viruses, KSHV and MCV. Kaposi sarcoma herpes virus (KSHV) or Human Herpes virus 8(HHV-8) was discovered by Yuan Chang and Patrick Moore in 1994 using a molecular technique called representational difference analysis (RDA) [52]. Briefly, DNA sequences present in diseased tissue (Kaposi's sarcoma tumor tissue in this case), but absent in healthy tissue of the same patient, were specifically enriched and isolated following rounds of PCR amplification and subtractive hybridization. DNA fragments isolated by RDA were found to have homology to herpesvirus saimiri (HVS) and led to the identification and sequencing of KSHV. Although initially described in 1872 as an endemic tumor in the Mediterranean basin and Africa by Moritz Kaposi [53], Kaposi's sarcoma (KS) is now also recognized as an AIDS defining disease. A viral etiology of KS explains the increased prevalence of KS seen among men who have sex with men (MSM) with AIDS-associated immunosuppression following the HIV pandemic [54, 55]. Both AIDS related and unrelated forms of Kaposi's sarcoma invariably contain KSHV DNA and KSHV infection, measured by serology assays. Accumulated epidemiologic evidence demonstrated that KSHV is also sexually transmitted among homosexual men in the United States. Collective data from numerous

studies indicate a causal role of KSHV in conjunction with host factors (such as immunosuppression), in the development of Kaposi's sarcoma [56, 57].

Merkel cell carcinoma is an uncommon but aggressive primary cutaneous neoplasm frequently having a poor prognosis [58, 59]. The risk of developing MCC is 13-fold higher in AIDS patients and 10-fold higher after solid organ transplantation [60]. MCC is also more common in patients receiving immunosuppressive therapy, elderly individuals and patients with other cancers such as chronic lymphocytic leukemia, basal cell carcinoma and squamous cell carcinoma [61-67] (none of these cancers have been linked to MCV infection [68-73]). Based on a strong association with immunosuppression and its epidemiology, Merkel cell carcinoma was selected and subjected to a direct sequencing-based method called Digital Transcriptome Subtraction (DTS) [74, 75]. Complementary DNA (cDNA) libraries were created from mRNA of four MCC tumor tissues and exhaustively sequenced to generate a stringent high fidelity transcriptome database of MCC tumors. An *in silico* subtraction of known human sequences against curated and highly verified human genomic sequence databases (made available through the Human Genome Project) identified candidate nonhuman sequences for further examination. A transcript with homology to other polyomaviruses was identified and led the way to the discovery of a novel virus associated with MCC (GenBank accession numbers EU375803 and EU375804) [74]. For detailed description please see section 1.5.1 (Discovery of Merkel cell polyomavirs).

### 1.1.3 Human immunodeficiency virus: an indirect carcinogen

Discovery of human immunodeficiency virus (HIV), another human retrovirus discovered in the late 1900s [76-78], substantially increased our understanding of these viruses and their link to immunosuppression, specifically Acquired Immune Deficiency Syndrome (AIDS). The discovery resulted in a Nobel Prize award to Françoise Barré-Sinoussi and Luc Montagnier, in 2008 [79]. HIV is not a direct agent of carcinogenesis, but is responsible for a number of cancers by indirectly "setting the stage" through immunosuppression. AIDS patients usually have an

elevated risk for developing cancer, specifically virus-induced cancers, as compared to the HIV uninfected human population [80-82]. Due to these reasons, IARC designated HIV-1 as a group 1 carcinogen in 1996 [79].

## 1.2 POLYOMAVIRUSES

### 1.2.1 Introduction and phylogeny

Polyomaviruses are small, non-enveloped viruses which have icosahedral capsids and carry a double stranded, covalently closed, superhelical genome approximately 5200 bp in length. Distantly related to papillomaviruses, they were previously (now obsolete) taxonomically grouped together as papovaviruses. Polyomaviruses usually have restricted host ranges and have been identified in various species including humans, monkeys, rodents, rabbits, and birds. Recently the International Committee on the Taxonomy of Viruses (ICTV) has divided polyomaviruses into three groups: *Orthopolyomavirus* group, *Wukipolyomavirus* group (both of which contain mammalian species) and the *Avipolyomavirus* group (contains avian species) (Figure 1) [83]. They all encode a variably spliced hallmark oncoprotein called the tumor (T) antigen. These T antigens usually retain the 5'exon at the amino terminal region, but differ in the carboxy terminal region [84, 85]. The expression of these T antigens not only causes tumors in animal models, but often leads to malignant transformation of primary cells in culture [86].

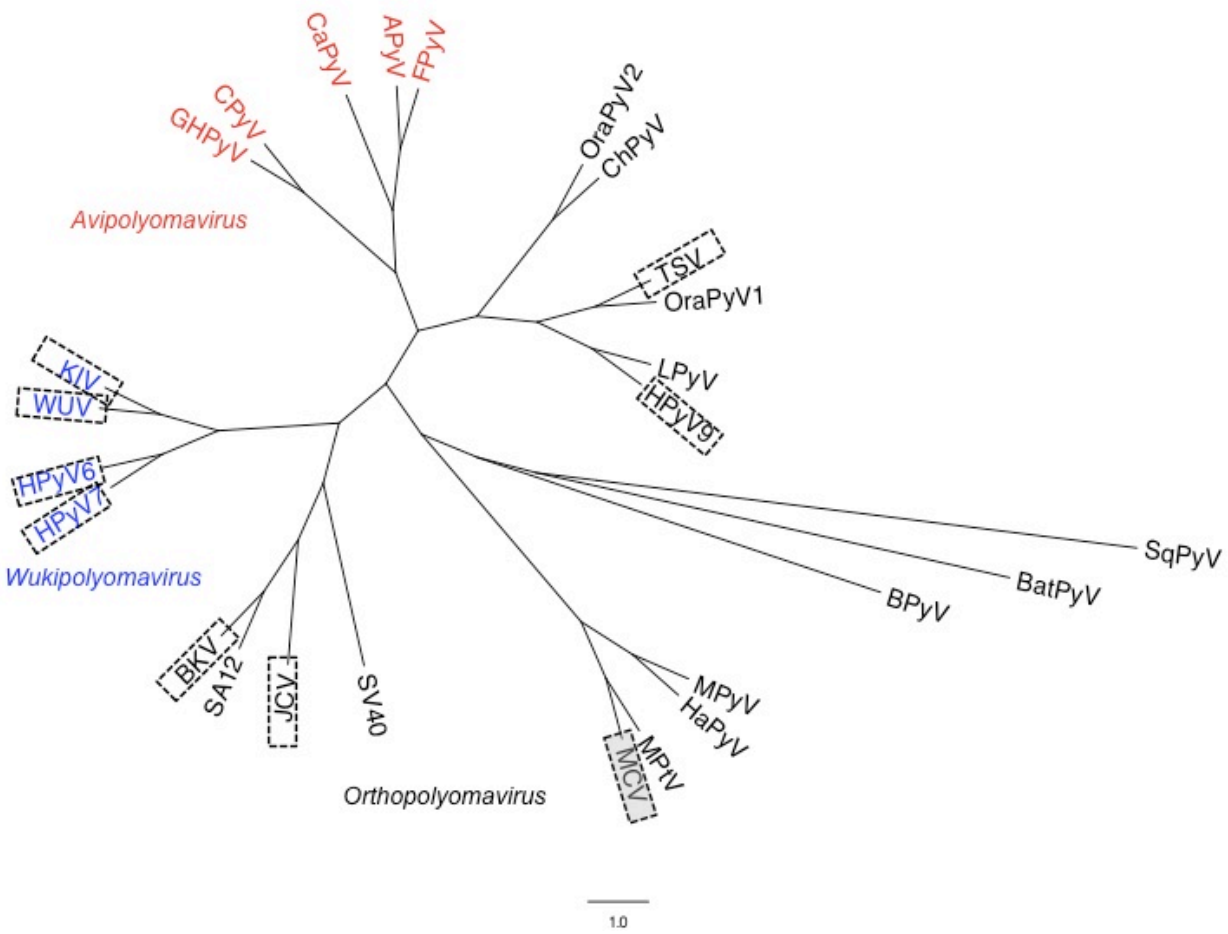



**Figure 1 Phylogenetic relationships and taxonomical classification of polyomaviruses based on whole genomic sequences**.

ClustalW alignment of the 26 known polyomavirus species was performed using Mac Vector software. The tree was created using neighbor joining method and FigTree software. Assignment of the viruses to the genera is indicated by the different colors used (red for *Avipolyomavirus* (Avian), blue for *Wukipolyomaviurs* and black for *Orthopolyomavirus* (Mammalian)). Boxes highlight human polyomaviruses. MCV is highlighted in a grey box. Information on the polyomaviruses, including their full names, abbreviations and Genbank accession numbers of the genome sequences is presented in Table 21 in Appendix A.

### 1.2.2 History and association with human cancer

The first known polyomavirus, called murine polyomavirus (MPyV), was discovered by Gross in 1953 as a filterable agent recovered from mouse leukemic extracts, that caused salivary gland carcinomas in inbred C3H mice [7]. The name “polyoma” virus (Greek terms: poly, meaning many; and oma, meaning tumors) was subsequently proposed due to its ability to cause multiple tumors in newborn mice, hamsters, and rats [87]. Since then the cell transformation and tumor formation abilities of polyomaviruses have been extensively studied and they have been proposed to play a causal role in human cancers.

Between 1959 and 1960, a scientist as the National Institute of Health (NIH), Bernice Eddy examined rhesus monkey kidney cells under the microscope and found that they would die without any obvious cause. She injected suspensions of these cells into hamsters, and observed that these hamsters developed tumors [88]. Shortly thereafter, in 1960 B.H. Sweet and M.R. Hilleman, scientists at the pharmaceutical company Merck & Co., discovered simian vaculoating virus (SV40), which was later determined to be the same virus identified by Eddy (*89)*. SV40 was soon found as a major contaminant of lots of poliovirus vaccine [88-90]. It is estimated that 10-30 million of the 98 million individuals who received injected polio vaccine, during the period (1955-1963), actually received a vaccine that contained SV40. Approximately 10,000 volunteers, who received an experimental oral polio vaccine between 1959-1961, may also have been exposed to SV40 [90]. In addition, SV40 was found in adenovirus vaccines given to more than 100,000 young men in army camps and as a contaminant of respiratory syncytial virus vaccine given to a few volunteers in a live virus infection experimental study during that period [86, 90, 91].

Although, it does not cause tumors in its natural Rhesus macaque host, SV40 causes tumors when injected into mice. Concerns regarding exposure of the human population to SV40 led to an extensive search for SV40 in brain cancers, osteosarcomas, non-Hodgkin lymphomas and mesotheliomas. Using PCR based analysis; SV40 T antigen sequences, but not viral genomes, were reported in these cancers. However, PCR contamination with laboratory

plasmids and cell lines (293 T cells) resulted in a lot of false positives in many tests, which have neither been reproducible nor consistent [86, 90, 92]. Antibodies to SV40 were detected in cancer patients, however subsequent case-control studies found equal prevalence in control cases. These antibodies were later found to cross-react to BKV and JCV viruses (infections are asymptomatic and common in the human population) [93, 94]. In 1998, the National Cancer Institute reported findings from an extensive study spanning decades of cancer incidence and mortality data (using SEER database and Connecticut Tumor Registry) and showed that, after 30 years, there was no increased incidence of cancer in persons who may have received SV40-containing vaccines [95]. Carbone *et al*. also specifically compared incidence of rare cancers in persons exposed as infants with non-exposed control populations and found no significant increase [96]. Various groups in United States and Europe have looked for, but have not found an elevated risk for cancer related to SV40 exposure [97, 98]. Although, there is strong corroboration from cell-culture studies and animal cancer bioassays that SV40 is oncogenic, numerous well-conducted studies show no convincing evidence that human cancers are caused by SV40 [86, 98]. Nonetheless, SV40 has become the prototype species and model virus for the Polyomavirus family. It is considered a workhorse for molecular biology and is extensively used for the study of DNA replication, transcription, oncogenic transformation and signal transduction pathways active in cancer [99-103].

Primate lymphotropic polyomavirus (LPyV) or African green monkey polyomavirus, known to infect only B-lymphoblast cells of African green monkeys, is another member of the mammalian polyomavirus family. Interestingly, zur Hausen *et al.* reported that 15-30 % of humans harbor specific antibodies to LPyV. This suggested the presence of a still unidentified polyomavirus in humans [104, 105]. A recently discovered polyomavirus (in 2011), Human polyomavirus 9 (discussed belowin section 1.2.5), shows a high level of sequence similarity to LPyV and may explain seropositivity against LPyV detected by zur Hausen *et al*.

### 1.2.3 Genome organization

Polyomavirus genomes (range = 4.6 kb - 5.4kb) consist of covalently closed, circular, double stranded DNA. Their genomes are often referred to as minichromosomes, since their DNA is closely associated with histones and resembles cellular chromatin. They are enclosed within nonenveloped, icosahedron capsids ~40-45 nm in size. Viral genomes are usually divided into three parts: the early region, the late region and the noncoding regulatory region (NCRR). The early and late nomenclature is based on the expression of transcripts before and after the onset of viral replication. The NCRR divides the early and late region almost equally and contains the origin of replication as well as the transcriptional control elements/ early and late promoters [106]. Representative genomes of SV40 and MPyV are shown in Figure 2.

The early message is differentially spliced to encode 2 to 5 proteins depending on the particular polyomavirus. These proteins are known as tumor (T) antigens, originally detected using antibodies from tumor bearing animals. Large T and small T are expressed in all known

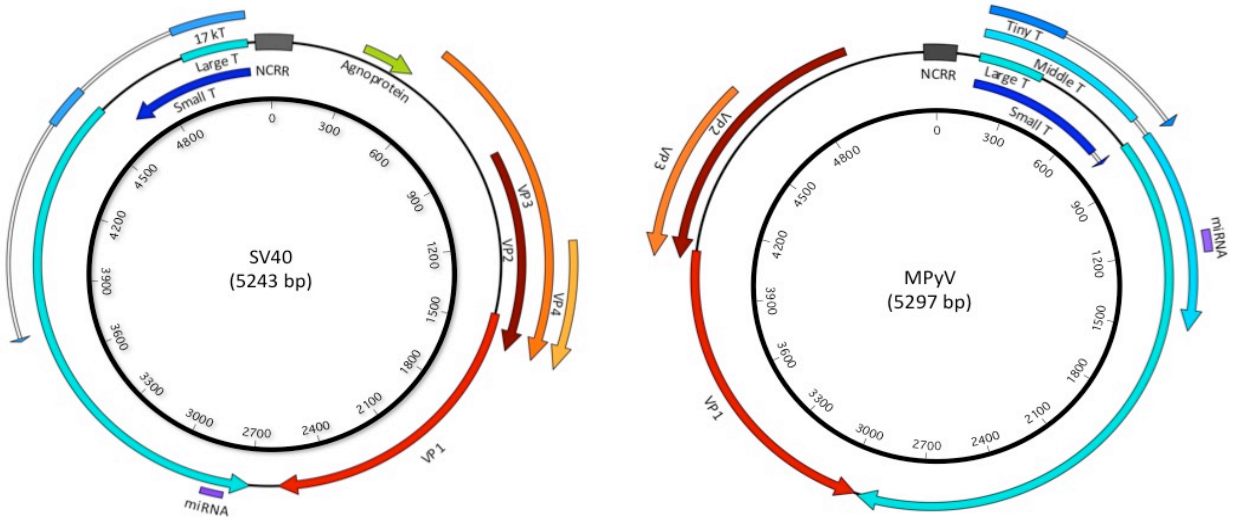



**Figure 2 Overview of SV40 and MPyV genome organization.**
The genomes of SV40 (5,243 bp)- NC_001669 and MPyV (5,297 bp)- NC_001515 are outlined. The early region encodes the T antigens and the late region encodes the capsid proteins VP1-3 (VP4 is unique to SV40). The noncoding regulatory region (NCRR) separates the early and late regions. Different open reading frames are generated through alternate splicing. A miRNA known to target the early message is also included. The numbering direction of nucleotide nomenclature is opposite for the polyomaviruses, and is studied as shown. Some key differences to note are the agnoprotein and VP4 in SV40 late region, and the middle T antigen and the tiny T antigen unique to MPyV. The complete genome sequences were obtained from NCBI database (NC_001669—SV40 and NC_001515– MPyV) and analyzed using Macvector and XPlasMap softwares

polyomaviruses, including all the nine human polyomaviruses (confirmed by protein expression or predicted via open reading frame analysis). Splicing results in additional T antigen isoforms including 17kT from SV40 [107], middle T [108] and tiny T from MPyV [109], T'165, T'136 and T135 from JCV [110], truncT from BKV [111] and 57kT from MCV [112] (Figure 3). These T antigens usually share the N terminal exon 1 regions and diverge in their alternately spliced C terminus regions (Figure 3). Except for MPyV MT's role in transformation [108, 113, 114] and JCV T antigens' roles in promoting efficient viral replication [115], the functions of these axillary T antigens remain largely unknown.

Through differential splicing and internal translation, the late region produces distinct capsid proteins (VP1, VP2 and VP3). The capsid is comprised of 72 pentamers of VP1 that contact VP2/3 proteins. VP1 is the major capsid protein and can spontaneously assemble into virus-like particles (VLPs) when expressed alone in cell culture. These VLPs are useful tools for serologic assays, which detect antibodies to polyomaviruses allowing estimates on exposure prevalence in human populations [116]. The VP4 capsid protein has so far been detected only in SV40, where it promotes egress of the virus after cell lysis [117]. In the cases of BKV and JC viruses the leader part of the late region also produces another protein called the agnoprotein, implicated in virion maturation. Although VP1 has been clearly defined as only a capsid protein, some studies have shown interactions of VP1 to c-myc, c-fos, poly (ADP-Ribose) polymerase 1 (PARP-1), caspases and Akt-1 proteins, indicating unexplored alternate functions [118, 119].

In 2005, Sullivan *et al*. discovered SV40 miRNAs using *in silico* screening followed by northern blot analysis and ribonuclease protection assays (shown in Figure 2) [120]. These miRNAs are perfectly complementary to the early viral mRNAs, targeting them for RISC-mediated cleavage. SV40 miRNAs accumulate late during infection and reduce the expression of viral T antigens. SV40 mutants lacking miRNAs have higher levels of intact early mRNAs, but similar total infectious virus yields as compared to wild-type virus. The functional significance of these miRNAs came from the observation that SV40-miRNA-lacking mutant infected cells had a higher susceptibility to cytotoxic T cell (CTL) mediated cell lysis and triggered more interferon-γ production [120]. The existence of these autoregulatory miRNAs indicates that viruses have

evolved to use the host RNAi machinery, to generate small RNAs that help evade the host immune system and increase the probability of a successful infection. JCV and BKV viruses also produce microRNAs homologous to SV40 miRNAs with a few nucleotide differences in their seed regions. They also direct the cleavage of early viral transcripts and downregulate large T antigen expression. JCV miRNAs were detected in brain tissues of PML patients, suggesting a functional role *in vivo [120, 121].* MPyV also encodes a microRNA that similarly cleaves early viral mRNAs but it is not essential for infection of cultured cells or experimentally inoculated mice [122]. MCV encoded miRNA is discussed Section 1.5.2.

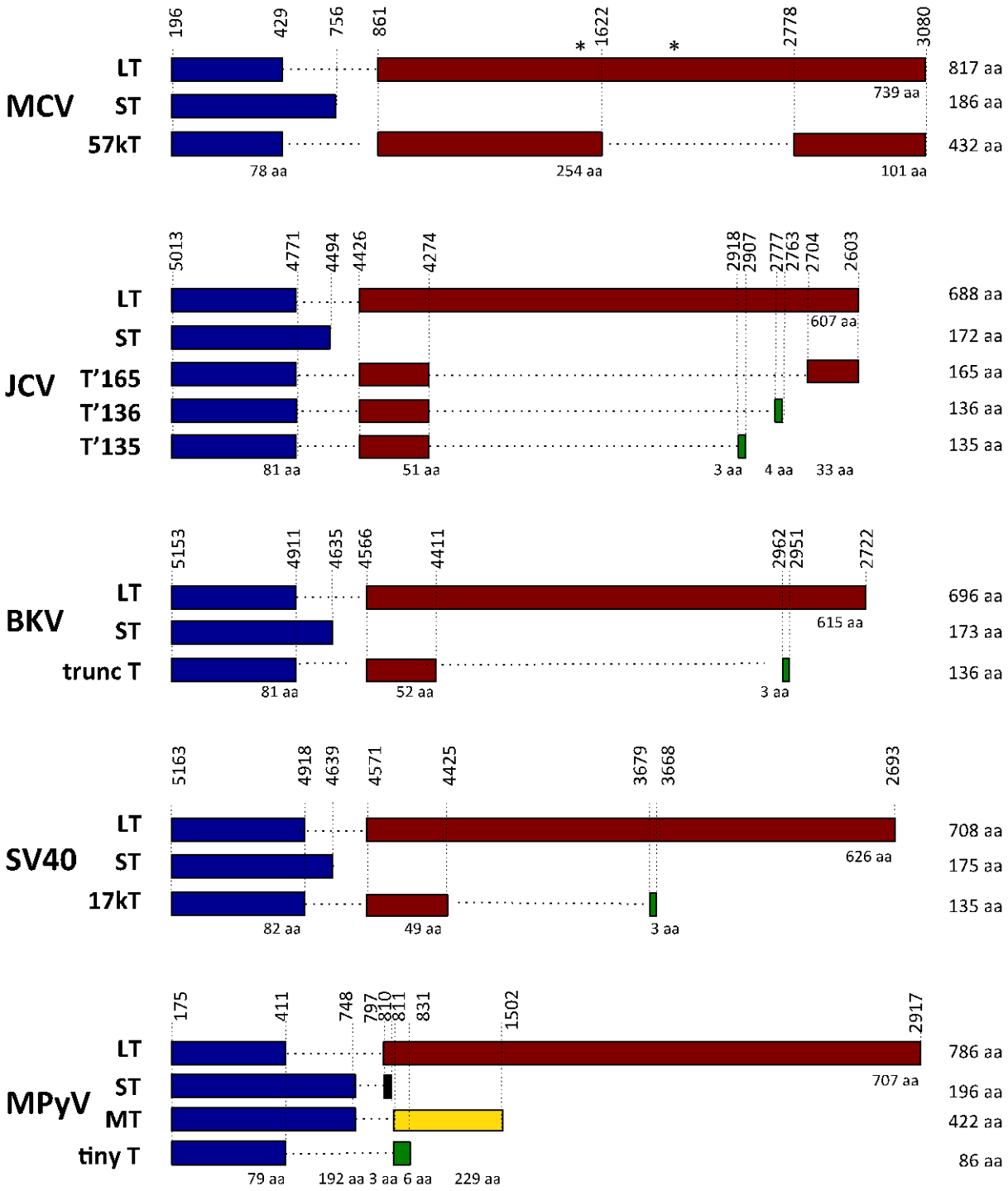



**Figure 3 Splicing patterns of various polyomavirus T antigens.**

The splicing patterns for JCV, BKV, SV40 and MPyV are compared to MCV T antigens. Different colors represent different reading frames and the dotted lines represent introns. The nucleotide positions and amino acid (aa) residue lengths of the different proteins are also shown. The N-terminus of each T antigen is shared (exon 1, range = 78-82 aa), but the C-terminus is most often unique (except 57kT and T'165). The asterisks on MCV LT reveal the premature stop codons found in truncated LTs (350 and 339 prototypes in that order respectively). (Modified from Gjoerup and Chang, 2010)

### 1.2.4 Viral life cycle and general properties

The polyomavirus life cycle begins with the interaction of the VP1 capsid protein with sialic acid or specific ganglioside receptors. Gangliosides GM1 are known receptors for SV40, GD1a and GT1b for MPyV, GD1b and GT1b are for BKV, GT1b and serotonin receptor 5HT2AR for JCV and co-receptors - GT1b and heparin sulfate (a glycosaminoglycan) - for MCV [123-126]. Gangliosides are components of the plasma membrane that comprise glycosphingolipids with one or more sialic acids. Once the virus binds to the receptor, the early stage of the life cycle proceeds via the virus being internalized through caveole for most polyomaviruses, or clathrin-dependent endocytosis pathways for JCV [127]. Cellular factors compromise the lipid bilayer and allow release of the viral genome into the cytoplasm. Viral DNA traffics to the nucleus where transcription factors are recruited to allow early gene expression. Translated Large T and small T antigens drive infected cells into S phase where LT promotes cellular expression of DNA replication factors and initiates DNA replication of the viral genome from the origin region in the NCRR [102]. Commencement of viral replication leads to transition into the late phase of the viral life cycle. Viral DNA replication proceeds bidirectionally on the circular DNA genome, involving leading and lagging strands. Replication terminates when the two-replication forks meet [128]. It is thought that LT transcriptionally activates the late gene promoter and represses the early promoter. This leads to the expression of the capsid proteins, which then assemble into capsids and encapsulate the viral genome. The virus then lyses cells and exits. An SV40 infection cycle takes 3-4 days in permissive cells (such as CV1 or BSC40 African green monkey cells) *in vitro*, eventually resulting in cell death and the production of 300 infectious progeny virions per infected cell [129] (Figure 4).

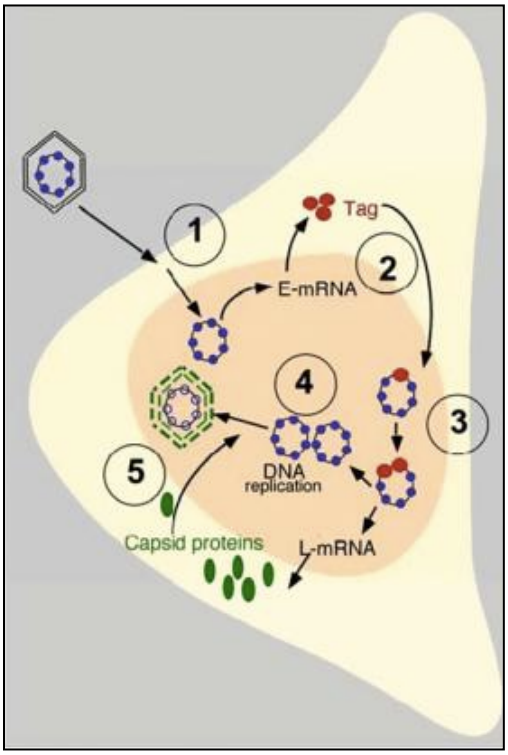



**Figure 4 Representation of the polyomavirus life cycle in a permissive host cell.**
1- Virus attachment, entry and intracellular trafficking to the nucleus. 2- T antigens expression from the early message (E-mRNA) and function, 3- T-antigens directed reprogramming of cells. 4- Viral DNA replication followed by capsid protein expression from the late message (L-mRNA). 5- Virion assembly and release. (Reproduced from Fanning *et al*., Chapter 1, DNA Tumor Viruses, 2009)

Both species and cell-type dictate the outcome of polyomavirus infection [130]. Polyomaviruses are able to productively infect their natural host, lyse cells and cause virion release. They usually establish a persistent infection that is lifelong and asymptomatic or subclinical. These viruses are usually kept in check by the immune system. Rarely are these infections associated with tumors or other diseases, except in immunocompromised or newborn animals. For example SV40 is found to cause polyomaviral disease in its natural host rhesus macaques only when they are immunosuppressed by simian immunodeficiency virus co-infection [131].

Three outcomes of SV40 infection have been observed (Figure 5).

1. SV40 infection of “permissive” cells, such as CV-1 or BSC40 lines of African green monkey kidney cells, results in cell death and production of infectious virions [132, 133].
2. SV40 infection of some rodent cells does not result in cell lysis and virion production. Although the early events of the virus infection occur normally and the cells are driven into the S phase, the virus fails to initiate DNA replication and activate transcription from the late promoter. For example, when mouse embryonic cells (MEFs) are infected with a high multiplicity of infection of SV40 all the cells become infected. However, due to lack of viral replication T antigen expression is often lost by cell division related dilution or degradation of viral DNA. Many cell types, in culture, support infectious entry of polyomaviruses but do not appear to support the late phase of the viral life cycle [99, 133].
3. An abortive infection, defined by normal viral entry and early gene expression but no viral replication and no late gene expression, is seen in some so-called “nonpermissive” cells. Initially, whole cell populations can become infected, but after subsequent cell divisions only rare transformants carrying an integrated viral genome grow out. Since this is a dead-end event that prevents the multiplication and spread of virions, it is often viewed as a “biological accident”. This accident is the mechanism believed to result in most tumors caused by polyomaviruses [19, 43, 99, 133]. The cells continue to express SV40 T antigens but are incapable of replicating the virus. A study by Gluzman and collaegues, in 1982, recognized this requirement for transformation of cells. They found that SV40 with mutations that disrupt viral replication and helicase activity show enhanced transforming activity [134].

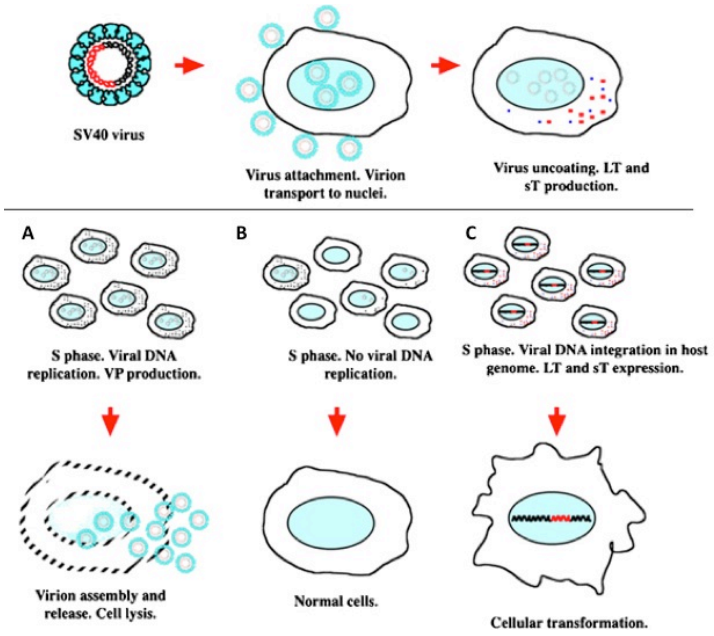



**Figure 5 Fate of SV40 in different cellular environments.**
(A) Infection of permissive cells results in cell lysis and virion production. (B) SV40 infection of some rodent cells induces S-phase, but no cell death or virus production is observed (C) Integration of viral DNA in very few nonpermissive cells and lack of replication lead to stably transformed cells (abortive infection, a biological accident) (Modified from Ahuja *et al*., Oncogene 2005)

### 1.2.5 Human polyomaviruses.

Nine human polyomaviruses have been identified to date (in 2012) (Table 2). JC virus (JCV) and BK virus (BKV), named after their source patient's initials, were the first of the human polyomaviruses to be discovered. They were culture isolated from diseased tissue of immunosuppressed individuals, JCV from brain tissue of a patient suffering progressive multifocal leukoencephalopathy and BKV from urine of a posttransplant nephropathy patient with poor renal function [135, 136]. JCV has a limited tissue tropism and infects only kidney, bone marrow, oligodendrocytes and astrocytes [137]. In animal models, JCV causes tumor formation in rodents as well as in non-human primates [138-143]. Similar to JCV, expression of BKV early region also transforms mouse and hamster cells and immortalizes human cells [144-

147]. Additionally, BKV inoculation of newborn mice, rats and hamsters causes various tumors including ependymoma, neuroblastoma, fibrosarcoma and osteosarcoma [148, 149]. Based on these results, although JCV and BKV are linked to and investigated in various brain cancers, colorectal cancers and gastric cancers, a consistent and robust association between the viruses and diseases has not been made [137, 148, 150-159]. Both the viruses however, are common in the general population and maintain lifelong disease-free infection in immunocompetent individuals [160]. Studies by Knowles *et al*., Egli *et al*. and Kean *et al*. show an overall seroprevalence rate of 81-82% for BKV and 35-58% among healthy blood donors from UK, Switzerland and the United States [160-162]. Seroconversion occurs early in childhood and dampens in elderly individuals for BKV but increases steadily upto ~50% in 60-69 aged individuals for JCV [161].

Two more closely related human polyomaviruses were found in 2007: Karolinska Institute (KIV) and Washington University (WIV) polyomavirus. Both were isolated from samples from children with acute respiratory tract infections by enriching for viral particles using DNase treatment, constructing deep sequenced cloned cDNA libraries and blasting against NCBI databases for non-human sequences [163, 164]. Current experimental evidence does not support causation of any respiratory illnesses and no other link to disease of symptomatic infection exists [165-168]. Both viruses show a wide geographic distribution and seropositivity estimates range widely from 1-65% among the general healthy population [160, 169-174].

Merkel cell polyomavirus was the fifth human polyomavirus identified. It is the underlying focus of this thesis and is discussed in detail in the subsequent sections (section 1.5).

Using a method called rolling circle amplification (RCA), three more polyomaviruses were discovered recently. RCA is a random primer extension technique that uses a DNA-dependent polymerase (such as phi29) that preferentially amplifies circular DNA and can displace an annealed DNA strand [175]. In 2010, during a search for MCV on skin surfaces using RCA, Schowalter *et al.* serendepitiously discovered Human polyomavirus 6 and 7 (HPyV6 and HPyV7) from skin swabs of healthy human foreheads. Follow up studies failed to reveal any

**Table 2 Human Polyomaviruses (Modified from Chang and Moore, Annual Reviews Pathology 2011)**

| Virus | Year identified | Method of identification | Prevalence in human population | Disease associations | Genome size (prototype) | GenBank | Reference |
|---|---|---|---|---|---|---|---|
| BK virus (BKV) | 1971 | Culture isolation from urine of renal transplant recipient | >90% of adults | Cystitis, polyomavirus-associated nephropathies, ureteral stenosis | 5153 bp | NC_001538 | Gardner *et al*., Lancet, 1971 |
| JC virus (JCV) | 1971 | Culture isolation from brain tissue with progressive multifocal leukoencephalopathy | >70% of adults | Progressive multifocal leukoencephalopathy | 5130 bp | NC_001699 | Padgett *et al*., Lancet, 1971 |
| Karolinska Institute polyomaviurs (KIV) | 2007 | Deep sequencing of DNAase-treated respiratory fluids | 55–70% of adults | Not defined | 5040 bp | NC_009238 | Allander *et al*., JVI, 2007 |
| Washington University polyomavirus (WUV) | 2007 | Deep sequencing of DNAase-treated respiratory fluids from children with upper respiratory infections | 69–80% of adults | Not defined | 5229 bp | NC_009539 | Gaynor *et al*., PLoS Pathogens, 2007 |
| Merkel cell polyomavirus (MCV) | 2008 | Digital transcriptome subtraction of Merkel cell carcinoma tissue | 42–70% of adults | Merkel cell carcinoma | 5387 bp | NC_010277 | Feng *et al*., Science, 2008 |
| Human Polyomavirus 6 (HPyV6) | 2010 | Rolling-circle amplification of skin and hair samples | Not defined | Not defined | 4926bp | NC_014406 | Schowalter *et al*., Cell host &microbe, 2010 |
| Human Polyomavirus 7 (HPyV7) | 2010 | Rolling-circle amplification of skin and hair samples | Not defined | Not defined | 4952 bp | NC_014407 | Schowalter *et al*., Cell host &microbe, 2010 |
| Trichodysplasia spinulosum polyomavirus (TSV) | 2010 | Rolling-circle amplification of trichodysplasia spinulosum lesion in transplant recipient | Not defined | Transplant-associated trichodysplasia spinulosum | 5232 bp | NC_014361 | Van der Meijden *et al*., PLoS Pathogens, 2010 |
| Human Polyomavirus 9 (HPyV9) | 2011 | Consensus PCR and deep sequencing | Not defined | Not defined | 5026 bp | NC_015150 | Scuda *et al*., JVI, 2011 |

link between HPyV6 and HPyV7 and basal cell carcinomas, MCV negative MCCs, melanomas, and cutaneous B and T cell lymphomas [176]. These two viruses are most similar to WUV and KIV and are classified into the *Wukipolyomaviruses* group in the new ICTV taxonomical classification [83].

In 2010, an eighth human polyomavirus was isolated from virus-associated trichodysplasia spinulosum (VATS), a rare type of dysplasia seen only in transplant and immunosuppressed individuals that manifests as hyperkeratotic spinous papules. Based on previous ultrastructural detection of polyomavirus like particles in VATS [177] and its close association with immunosuppression, van der Meijden *et al.* applied RCA to VATS tissue and found trichodysplasia spinulosum polyomavirus (TSaPyV) [178]. TSaPyV was detected at high viral DNA load showing abundant protein expression only in the affected hair follicles, suggesting a causative role in trichodysplasia spinulosa pathogenesis [179, 180].

Most recently (2011), Human Polyomavirus 9 (HPyV9) was identified from serum of an immunosuppressed kidney transplant patient by using consensus PCR [181]. The extraordinary homology between HPyV9 and LPV may explain the previously detected, but unexplained high human seropositivity to LPV [182, 183]

The majority of these newly discovered human polyomaviruses appear to be previously unrecognized, widespread infections. With improvement of detection techniques and assays the subfamily of human polyomaviruses is likely to expand revealing more viruses, either linked to disease or as a part of normal human microbial flora.

## 1.3 SV40 T ANTIGENS- CLASSIC MODELS FOR STUDYING CANCER

Polyomaviruses, specifically SV40, have been used as classic model systems for understanding basic cellular biology relating to cellular immortalization and oncogenic transformation. Over the last 50 years, since they were discovered, studies of polyomaviruses and their target pathways have lead to fundamental insights into key biological processes. Because of their

small genomes, these viruses rely heavily on their multifunctional T antigen proteins and the cellular machinery to perform various functions in their life cycle [85, 129]. They begin by reprogramming the host cell cycle to induce progression into S-phase, thereby creating an appropriate environment for viral replication. This is particularly important for SV40 infection of its natural host rhesus macaque, because SV40 is believed to infect non-cycling, often growth-arrested, epithelial cells of the kidney [99].

A tumor cell can be distinguished from its normal counterpart by a variety of indicators such as changes in cellular morphology, the ability to grow in the absence of anchorage, loss of density-dependent growth inhibition, decreased dependence on serum growth factors, and the capability to form tumors in animal hosts [184, 185]. Multiple assays have been established over the years to study transformation phenotype, *in vivo* and *in vitro,* elicited by SV40 and other polyomaviruses. In each of these assays, cells are placed under conditions of selective growth pressure so that only the transformed cells are able to survive and proliferate whereas the normal cells die or enter senescence. These form the basis by which SV40 and other polyomavirus' ability to transform cells have been assessed [184].

### 1.3.1 Large T antigen (LT)

The ability to transform cells is linked to SV40 T antigen's ability to target and alter different cellular pathways that are also important in preparing the cellular environment for replication.

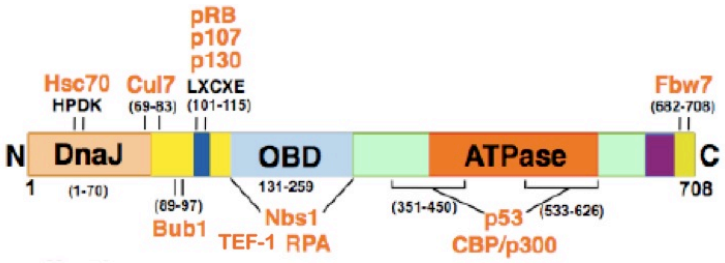



**Figure 6 Schematic of binding domains and interacting partners of SV40 Large T antigen.**
(Modified from Gjoerup and Chang, Adv Cancer Res 2010)

SV40 LT is multifunctional, nuclear phosphoprotein that is modular in structure. It has discrete regions corresponding to binding domains of its various interaction partners. Some of these interaction partners include pRB, p53, p107, p130 Hsc70, Cul7, Bub1, IRS1, p300/CBP, and FBW7 (Figure 6) [103, 186-197]. Two of the well-known tumor suppressor genes, p53 and the Retinoblastoma protein, were either discovered (p53) or functionally dissected (pRB) as a result of their interaction with SV40 [84, 85, 198]. The following sections will review and discuss the biochemical properties of SV40 LT and sT.

#### 1.3.1.1 Interaction with pRB family of proteins

We now know that a majority of tumor viruses target pRB or components of the pRB pathway, for inactivation. Polyomavirus T antigens share a highly conserved LXCXE motif, which binds to RB family proteins [195, 199-203]. Most cancers (viral and nonviral) contain aberrations in the regulation of the pRB pathway [204]. This reveals a common mechanism shared between viruses in targeting specific cellular proteins and demonstrates the importance of this protein family in tumorigenesis.

Knudson *et al.* first identified the retinoblastoma susceptibility gene in 1971. They statistically analyzed a genetic study, which suggested that childhood retinoblastoma had a hereditary component, and concluded that two independent mutation events were responsible for development of the disease. In hereditary retinoblastoma, the child inherited a mutated gene and acquired a second mutation in a somatic cell. In contrast, two independent mutations in the same somatic cell (later found to be two alleles of the same gene [205, 206] led to the development of sporadic retinoblastoma [17]. The gene was subsequently found and cloned from chromosome 13q14. The gene expresses a 110-kDa nuclear phosphoprotein [207].

A significant insight into pRb function was that it underwent phosphorylation in a cell cycle dependent manner. In the G0 state of quiescent or differentiated non-cycling cells and in the G1 phase of cycling cells, Rb protein is hypo- or under-phosphorylated. In this state pRb binds E2F transcription factors and represses transcriptional activity of genes having E2F-

dependent regulatory elements. As cells enter the S phase of the cell cycle, Rb is hyper-phosphorylated by sequential cyclin dependent kinases and dissociates from the E2Fs, permitting them to stimulate transcription of their target genes. The products of these genes then usher the cell from late G1 phase into S phase. The point of entry into early S phase of the cell cycle is often known as the “R point”/ “the restriction point” or the point of no return, as the hypophosphorylated form of pRb keeps the cells from entering into the cell cycle.

The Rb protein has been shown to bind over a hundred protein partners and mediates transcriptional regulation of numerous target genes [208]. These molecules mediate the various cellular functions of pRb, including temporary and permanent cell cycle arrest, differentiation, genomic stability and apoptosis. E2Fs are one of the most well studied and validated targets of pRb. E2F factors normally heterodimerize with DP family of proteins to facilitate DNA binding. The Rb protein binds this complex and effects its transcriptional repression by recruiting chromatin-remodeling elements, such as histone deacetylases [209].

The first demonstration of a viral oncogene targeting a known tumor suppressor came from the recognition of pRB as a critical transformation target of adenovirus E1A protein [203]. Following this, Decaprio *et al.* showed that SV40 T antigen binds pRB and more intriguingly T antigen LXCXE mutants, incapable of binding pRB, failed to transform cells (in a variety of cell types and assay systems) [195, 199]. The viral onocproteins bind and sequester hypophosphorylated form of pRb and free E2F transcription factors to activate cell cycle regulated genes, thereby steering the cell into the S phase and increasing cellular proliferation (see Figure 7 for a simplistic model) [195, 199].

The Retinoblastoma protein is structurally composed of two domains, A and B, forming a pocket conformation critical for tumor suppression and is thus often referred to as a pocket protein [204]. This region constitutes the binding site for the LXCXE motif and mutations in human malignancies usually map to this region. Although, the binding sites for both viral T antigens and E2F1 map to the pocket domain, they are distinct and pRB can bind both simultaneously.

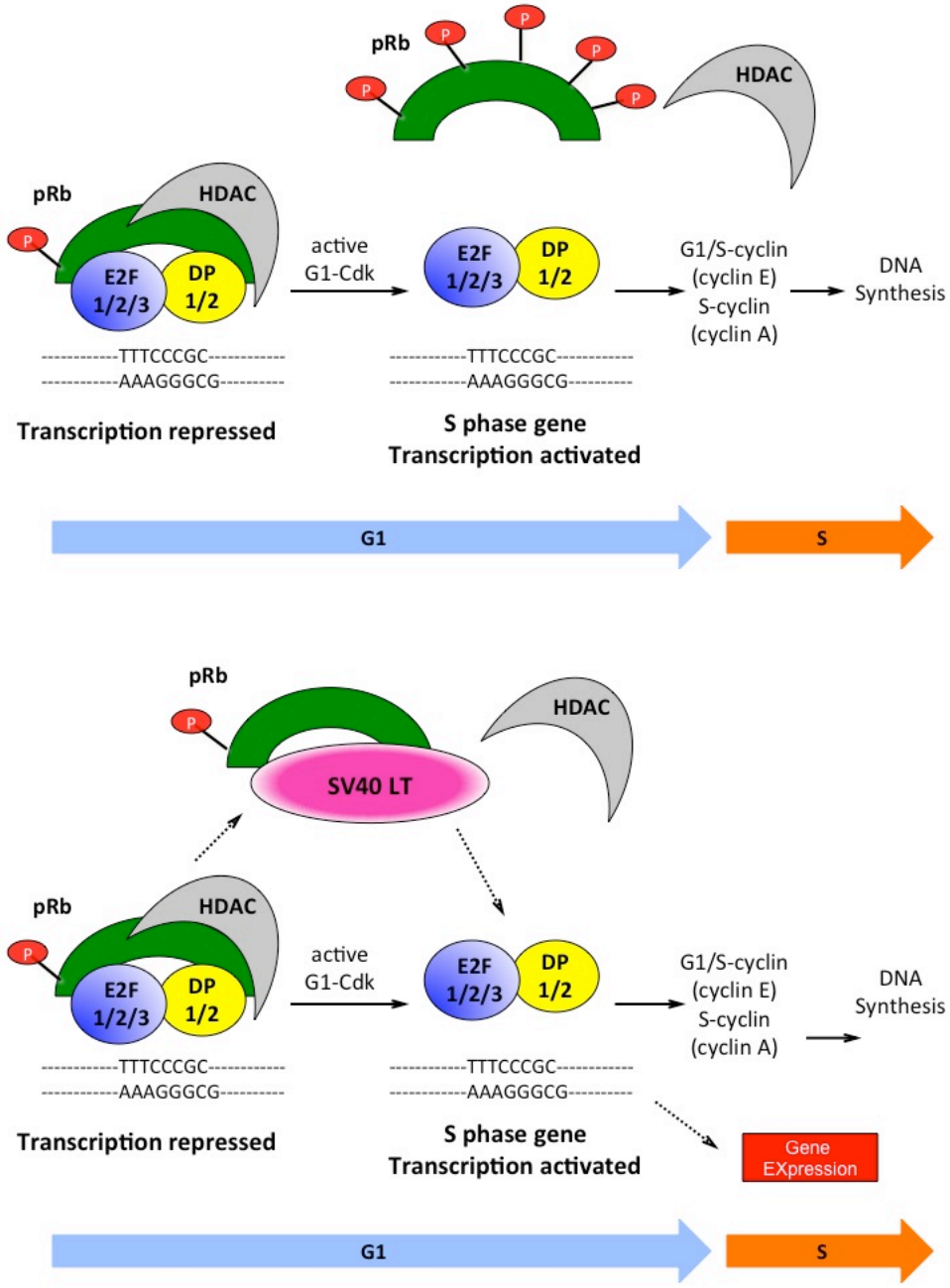



**Figure 7 Simplistic Model for SV40 LT mediated sequesteration of Rb and subsequent S phase related gene expression.**

Hypo-phosphorylated pRb (green) binds to the E2F transcription factor (which is usually bound to a DP1 protein (blue and yellow)) and discourages transcription of several S phase related genes by attracting histone deacetylase (HDAC, gray) enzymes to repress them. In normal cycling cells, when the cell enters the S phase, pRb becomes hyper-phosphorylated and is released from the complex, freeing E2F to transactivate and regulate gene expression (top). SV40 LT binds and sequesters pRb and is able to free E2F independent of the cell cycle phase. This helps activate cell cycle S phase related genes and progression (bottom).

Two other pocket proteins p107 and p130 were identified as additional cellular proteins in the RB family that bound to the transforming LXCXE domain of adenoviral E1A and SV40 LT [210-213]. Although partially redundant with pRB, a number of features distinguish the three proteins. A central spacer domain is present between the A and B pockets, in both p107 and p130. This region binds to cyclin A and E, when in complexes with CDK2 (cyclin dependent kinase 2). Also, different pRB family members bind different E2Fs. The pRB protein preferentially binds E2F1-3 (the "activating" E2Fs), in contrast the p107/p130 preferentially bind E2F4/5 (the "repressing" E2Fs) [199].

Studies of SV40 have revealed the importance of LT antigen HPDKGG/J domain in the inactivation of pRb. The model proposed is that large T antigen binds to the hypo-phosphorylated form of pRb and then recruits Hsc70 via its J domain. Once bound, the Rb-E2F complex first interacts with the substrate-binding domain of Hsc70 and then with its ATPase domain. Here, ATP hydrolysis and conformational changes lead to the dissociation of the complex [214, 215]. In addition to the LXCXE motif, the DnaJ domain is also essential for the inactivation of p107 and p130 [192, 216, 217].

Pocket proteins negatively regulate cell cycle and cell growth. With an enhanced understanding of RB and several of its cellular functions, the basic model proposed above seems an oversimplication. It is now known that different complexes of pRB and E2F form in different stages of the cell cycle and their absolute levels are also cell cycle dependent. Notably, p130-E2F4 complexes are found in G0 phase, E2F-pRB and E2F4-p107 complexes are found in G1 phase, whereas S phase has free E2F1, -2 and -3. [199]. Quiescence is maintained, in part, by the presence of a repressive complex called DREAM (DP1, p130 (p107 in p130's absence), E2F4 and MuvB like proteins: LIN9, LIN37, LIN52, LIN54, and RBBP4) [218], which is anchored on the promoters of E2F-responsive genes by E2F4. It is believed that SV40 LT binds to this complex and with the aid of Hsc70-mediated ATP hydrolysis, dislodges E2F from p130 and sends p130 to the proteasome for degradation [129, 191]. This eventually leads to de-repression of the target genes where activating E2Fs then bind and activate gene expression transitioning the cell into S phase.

#### 1.3.1.2 Interaction with p53

The p53 tumor suppressor protein was discovered (in 1979) as a cellular protein - binding partner of SV40 LT, both in SV40 transformed cells and infected cells [196, 197]. It is now recognized as a tumor suppressor found frequently mutated or deleted in approximately 50% of human cancers [219]. Also known as the "guardian of the genome", p53 is a DNA damage and cellular stress responsive transcription factor which affects DNA synthesis and repair, imposes cell cycle arrest or leads to apoptosis or senescence [220]. Most viruses inactivate p53. Two well-known examples are adenovirus E1B 55k which binds and transcriptionally represses p53; together with E4orf6 that targets p53 for degradation, and human papillomavirus E6 protein which targets p53 for proteasomal degradation [221, 222].

The p53 binding sites within SV40 LT is bipartite at residues 351-450 and 533-626 [223]. Reported in 2006, the co-crystal structure of LT ATPase domain bound to p53 revealed a region within the core DNA binding domain of p53 as the interaction site of LT [224]. By blocking p53 from binding to DNA promoter regions, the interaction between T antigen and p53 thus blocks p53- dependent gene expression [224, 225]. T antigen's ability to bind p53, though necessary is not sufficient in many cases for transformation [223, 226]. Interestingly, some studies found that the interaction of T antigen and p53 also blocked T antigen's replicative functions. Although one of the results of the interaction between LT and p53 is prevention of a DNA damage response, the consequences of this interaction are now known to be more complex. With the recent knowledge of the diverse downstream targets of p53, it is becoming clear that the effects of the LT and p53 interaction are not completely understood [227, 228]. Studies of LT transformation efficiencies and growth phenotypes in the presence or absence of p53 and p53 mutants led to the idea that LT stabilizes p53 and probably induces it to gain an unknown function [229-231]. The observation that presence of wild-type p53 enhances tumor formation in LT transgenic mice is consistent with this gain of function phenotype [232]. A popular hypothesis explaining this observation is that LT stabilizes p53 to gain access to p300/CBP, which then acts on promoters, or other LT bound proteins [233]. Several naturally occurring

p53 mutants seen in cancers exhibit a similar gain of function mutation [234, 235]. Hence, further studies of this effect are likely to reveal common mechanisms of tumorigenesis in nonviral malignancies as well.

#### 1.3.1.3 Other interactions

**DnaJ Domain**

The common region between LT and sT, implicated in transformation, includes a canonical HPDKGG motif [236]. This N terminus region of the two T antigens exhibits significant sequence homology and folds into a DnaJ domain like structure [236]. DnaJ proteins are co-chaperone proteins that recruit heat shock family proteins (DnaK family) for protein folding and protein transport. LT DnaJ domain binds to the constitutively expressed Hsc70 chaperone and stimulates its ATPase activity [103, 190, 236]. Classic D44N and H42Q mutants disrupt this binding and have been used to demonstrate the importance of the DnaJ domain in viral replication and in oncogenic transformation (via functional inactivation of pRB family members, as discussed in the previous section) [103, 190, 191, 217, 236, 237]. The DnaJ domain's contribution to LT mediated cellular transformation is limited to a subset of assays. The D44N mutant immortalizes mouse embryo fibroblasts normally and in anchorage- independent growth measuring assays it shows no defect [191]. However, LT requires the DnaJ domain in cis with the pRB family binding site to disrupt p107/p130 and E2F4 complexes and promote growth in low serum and growth to high saturation density [191, 216, 217].

Based on sequence homology, all polyomavirus T antigens appear to have a functional DnaJ domain. However, other viral oncoproteins such as human papillomavirus E6/E7 and adenovirus E1A/E1B do not have such a domain. Given the convergence of viral oncoproteins on other targets such as p53, pRB family and p300/CBP, this is surprising observation.

**CR1 region**

An adenovirus E1A conserved region 1 (CR1) like sequence is found in the N terminal region of SV40 LT. In E1A, this region is required for pRB and p300 binding, however there is no evidence that the CR1-like region in LT functions in a similar fashion [238]. In contrast, based on LT structural studies, it is likely that the CR1 domain is buried within a hydrophobic core. Further studies are required to determine the function of this region in SV40 and other polyomavirus T antigens [103].

**p300/CBP and p400**

Cyclic-AMP response element binding (CREB)- protein (CBP) and p300 are large scaffold proteins involved in transcriptional regulation. They act as co-activators, in part via their intrinsic histone acetylase (HAT) activity and mediate a number of biological processes, including cell growth and transformation and are thus considered tumor suppressors [239]. Adenovirus E1A was first shown to bind p300/CBP and this binding was linked to adenovirus transformation and cellular DNA synthesis [240, 241]. Subsequent studies by Yaciuk *et al.* showed that pRB binding-deficient SV40 LT, but not DnaJ domain mutants, complemented p300/CBP binding- defective mutants of E1A to restore transformation in primary baby rat kidney cells [242]. This suggests analogous targeting of p300/CBP by E1A and LT. Although initial studies suggested LT's CR1-like sequence as a binding site for p300/CBP, it was later demonstrated that LT C terminal residues 251- 708 are responsible for the interaction, although with decreased efficiency compared to wild-type LT [194, 243]. The binding is mainly indirect and is bridged by p53. LT-CBP interaction results in a K697 acetylation of LT in a p53-dependent manner [233, 244]. The significance of this remains unclear. Recent work by Ahuja *et al.*, based on structure-guided mutational analysis of LT, indicates a direct binding of LT and p300/CBP proteins that is essential for oncogenic transformation [186].

Like E1A, SV40 LT also binds to the p400 protein, through its C terminal 251-708 fragment, but the significance of this interaction in LT-mediated transformation has not been elucidated [194, 245]. The p400 protein is a SW12/SNF2 family of chromatin remodeling factor,

interacts with a c-myc binding protein called TRRAP and is likely to be involved in the p53-p21 cellular senescence pathway and p53-dependent apoptosis [246-248].

**Cul7**

Cul7 is a 185 kDa cellular protein that is a member of the SCF (skp1, cullin, F-box) type E3 ubiquitin ligase complex. It assembles into complexes with Skp1, Fbxw8 and Rbx1 ring finger proteins and targets cellular proteins for proteasomal degradation [249]. The Cul7 protein was identified from a large-scale immunoprecipitation experiments coupled with mass spectrometry of LT1-135 overexpressed in mouse NIH3T3 cells [187]. Subsequent genetic analysis identified the Cul7 binding site to residues 69-83 in LT [250]. Although a mutant defective in binding Cul7 exhibits normal binding to Bub1, pRB and p53, it is defective in soft agar and high-density growth assays [250]. LT transformation relevant substrates for Cul-7 mediated degradation have not been identified, but IRS1, involved in the insulin signaling pathway, and Mre11-Rad50-Nbs1 complex are reported candidates [251, 252].

**Bub 1**

Using LT1-135 as bait, a yeast two-hybrid screen revealed Bub 1, as an interactor for LT [188]. Bub1 is a mitotic checkpoint kinase that participates in the spindle checkpoint, at the metaphase to anaphase transition; ensuring correct attachment of kinetochores to spindle microtubules [253]. Coimmunoprecipitation experiments confirmed this interaction both *in vitro* (human cells) and *in vivo* (in mouse). Subsequent genetic mapping experiments identified residues 89-97 on LT as the Bub1 binding site. These sites contain a critical WEXWW motif, which is conserved in SV40, JCV, BKV and bovine polyomavirus T antigens. LT's interaction with Bub1 leads to a compromise of the spindle checkpoint and is also important for forming foci in Rat-1 cells (measure of independence from contact inhibition) [188]. Interestingly, reduced Bub1 expression in mice causes impaired chromosome segregation and aneuploidy that leads to tumorigenesis, and sporadic Bub1 mutations has been detected in human colorectal cancers [254, 255].

**IRS1**

Insulin receptor substrate 1 (IRS-1) is a downstream target of insulin growth factor I (IGF-I) and plays an essential role in the signal transduction pathway in transmitting signals from insulin and insulin-like growth factor I receptors (IGF-IR) [256]. LT binds IRS-1 and LT is unable to transform IGF-IR deficient cells in the absence of IRS-1 [189, 257]. The IRS-1 binding site is located in the first 250 amino acids of LT. This interaction implicates LT in IGF-I/insulin signaling and both SV40 and JCV LTs were shown to translocate IRS-1 from the cytoplasm to the nucleus [258, 259]. Although a specific IRS-1 binding mutant has not been identified, recently a pRB binding mutation was shown to disrupt LT interaction with IRS-1 and a consequent defect in PI3K/Akt activation [260].

**Nbs1**

Nbs1, the Nijmegen breakage syndrome protein is a component of the MRN (Mre11, Rad50, Nbs1) complex and functions in double strand break DNA repair, possibly as a sensor of the double strand breaks. Nbs1 is mutated in the Nijmegen breakage syndrome, which is associated with chromosomal instability and increased cancer susceptibility. SV40 LT binds to Nbs1 and allows unrestrained firing of the viral origin [261, 262]. This binding requires the LT origin-binding domain and a deletion mutant of residues 147-259 is defective in binding to Nbs1 [262]. Since the MRN complex is degraded by LT during infection, via its interaction with Cul7 [251], the significance of LT-Nbs1 interaction in genomic instability and oncogenic transformation is not completely understood.

**Fbw7**

The extreme C-terminus of SV40 LT binds to the F-box FBW7 protein via a phosphodegron, similar to the consensus found in FBW7 substrates. FBW7 is a component of the SCF complexes that recognizes the substrates and targets these cellular proteins for proteasomal degradation. LT was shown to mislocalize FBW7 from the nucleoli to the nucleoplasm, but this region is not

required for the full-length LT mediated transformation [263]. The extreme end of the C-terminus of SV40, BKV and JCV also has a host-range function, which allows SV40 to grow in cell lines such as CV-1, but is not required for growth in other cells such as BSC40 [264]. The host range function may be mediated by binding to an unknown cellular protein [265].

**TEF-1**

T antigen binds to the transcription factor TEF-1 through its origin-binding region [266]. This interaction plays a role in the early to late switch in viral transcription and appears to modulate transcription of cellular genes as well. Although, a TEF-1 binding deficient mutant (S189N) is less efficient than wild-type T antigen in focus formation assays, this has not been fully characterized. Further studies are required to assess the importance of T antigen TEF-1 interaction in the context of cellular transformation and viral replication [99, 267].

### 1.3.2 Small T antigen (ST)

SV40 viruses that fail to produce ST are viable, but grow slowly and produce fewer replicated virions [268, 269]. While LT expression is sufficient for focus formation in many rodent transformation assays, ST is required for anchorage-independent growth [269-271]. Hahn *et al.* first accomplished human cell transformation in 1999 [272]. Using a combination of LT, ST, oncogenic H-ras and hTERT (human telomerase reverse transcriptase) they transformed normal human embroyonic kidney cells (HEK) and primary foreskin fibroblast cells (BJ). Cells expressing all three genetic elements were anchorage independent and formed tumors in immunodeficient nude mice. This study demonstrated that both LT and ST are important for transformation but also established that hTERT is essential for this phenotype in human cells [272].

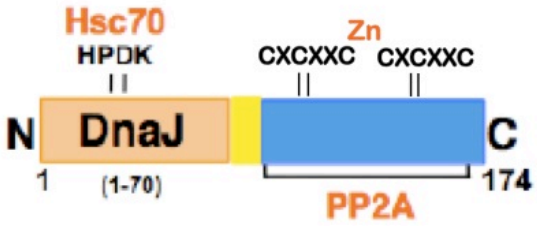



**Figure 8 Schematic of binding domains and interacting partners of SV40 Small T antigen**.
(Modified from Gjoerup and Chang, Adv Cancer Res 2010)

SV40 ST is a 174 amino acid long protein that shares its first 82 aa with LT, including a functional DnaJ domain. Unlike the LT DnaJ domain, the function or target of ST's DnaJ domain have not been elucidated nor implicated in ST-mediated transformation [190]. Although LT is a nuclear protein, ST is found both in the nucleus and cytoplasm. The C-terminus of ST has two CXCXXC clusters that bind Zinc and confer conformational stability (Figure 8) [273].

**PP2A**

Protein Phosphatase 2 A (PP2A) is a heterotrimeric serine-theronine phosphatase enzyme composed of an A scaffold subunit, a B regulatory subunit and a C catalytic subunit. There are two different A subunits, two different C subunits and 17 known B subunits that can assemble together, in various combinations, into more than 100 different holoenzyme complexes [274, 275]. The different isoforms of PP2A regulates a variety of proliferative and apoptotic pathways inside a cell.

SV40 ST C-terminal region binds to the PP2A family of proteins and a majority of its activities can be attributed to this association. ST residues 97-103 are crucial for this interaction with PP2A [276]. Human cell transformation requires ST and depends on its binding to PP2A. Mutants in ST that disrupt its PP2A interaction are defective in transformation. In addition, an ST truncation mutant containing only the PP2A binding domain (aa 88-174) retains the ability to promote transformation [270, 272, 277]. Effects of ST in anchorage-independent growth were mimicked by knockdown of the PP2A B56Y subunit in human embryonic kidney epithelial cells, indicating PP2A as a key target [278]. Mutations in PP2A subunits have been found in human cancers, suggesting a tumor suppressor function [274]. One of the many diverse targets of PP2A potentially involved in ST-involved tumorigenesis is Akt, a component of the

phosphatidylinositol 3-kinase (PI3K) pathway. ST inhibition of PP2A leads to the phosphorylation and activation of Akt, which further activates the mTOR complex resulting in increased cap-dependent translation. It was shown that in human mammary epithelial cell transformation assays constitutively active forms of PI3K or Akt and a small GTP binding protein named Rac could replace ST [279]. More than 30 kinases are regulated by PP2A [280]and the effect of ST-PP2A interaction on the PI3K pathway is likely to be a small part of its impact on ST-mediated transformation.

### 1.3.3 Insights into malignant progression from SV40 transgenic mouse models

T antigen has been expressed in a variety of different cell and /or tissue types including mammary glands, pancreas, liver, prostate, salivary glands, intestine, brain, lung, kidney, eye, smooth muscle, cartilage, and bone, both *in vitro* and *in vivo* [99, 281, 282]. The cell type plays an important role in the development of tumors. This is underscored by the observation that in different cell systems either LT alone or in conjunction with ST can induce hyperplasia, dysplasia, carcinoma, and metastasis or in contrast, no stimulation of proliferation at all. Targeted expression of only LT in the epithelium of choroid plexus, a structure in the brain that produces and filters the cerebrospinal fluid, is one of the most thoroughly studied SV40 mouse model systems [283-285]. The mice rapidly develop aggressive tumors and die within 1-2 months. Interestingly, use of a N-terminal fragment of the first 121 amino acids (T121) of LT, that lacks the p53 interacting function, produces identical, but slower growing tumors. However, in a p53 deficient background this T121 mutant causes rapid tumor growth with minimal apoptosis. A mutant T121 with a defective LXCXE pRB-binding motif is unable to induce tumors. Disruption of the Rb pathway causes abnormal entry of quiescent epithelial cells into the S-phase by upregulation of E2F1. The E2F1 transcription factor then triggers a p53-dependent checkpoint poised to eliminate tumor cells via apoptosis. However, T antigen prevents cell death by binding and inactivating p53 [284, 286, 287]. These experiments elegantly demonstrate the necessity of inactivating both the pRB and p53 pathways to induce

proliferation. Cell-type specific expression of T121 in the mammary gland also shows that p53-dependent apoptosis limits tumor development [288].

The requirement of p53 inactivation for tumor formation and development is cell context dependent. LT causes tumors in a variety of cell types and tissues, independently of its effect on p53. For example, targeted expression of T121 in astrocytes led to aberrant proliferation along with extensive apoptosis that was dependent on functional PTEN. A combination of pRB inactivation by T121 and PTEN loss accelerated astrocytomas in this model system [289]. In addition, expression of T121 in the liver causes hepatocellular carcinomas and requires an intact pRB and DnaJ domain [290].

Several studies indicate that p53 does not play a role in the tumorigenesis induced by LT in intestinal enterocytes. Expression of amino-terminus truncation mutants of LT in these cells leads to hyperplasia, that progresses to dysplasia with age [291-293]. LT fails to stabilize p53, which is undetectable in normal or T antigen expressing mice villi, and genetic removal of p53 also does not change or affect the frequency of this hyperplasia formation [228, 293, 294]. Microarray analysis by Rathi *et al.* indicate that LT and LT1-136 regulate a highly overlapping set of genes via their intact DnaJ and pRB binding motifs [228]. A majority of these are targets of the pRB/E2F pathway, underscoring its importance in hyperplasia formation in enterocytes.

Expression of both LT and ST has been successfully used in the prostate and in acinar cells of the pancreas to produce cancers. When expressed in the prostate epithelium under a probasin promoter, hyperplasia, adenocarcinoma and metastases are observed [295, 296]. PTEN, rather than p53, is the activator of apoptosis and inhibitor of tumor progression in this system. Truncated N terminal versions of T antigen are sufficient to induce pancreatic acinar carcinomas, again indicating that a disruption of p53 is not required to generate neoplasia [297].

Transgenic mouse models are valuable systems because they allow a direct assessment of the individual contribution of different genes to tumorigenesis in distinct cell and tissue types. In addition, they often provide a representation of human cancer useful for therapeutic compound testing. Although not entirely clear, the varied responses elicited by LT across

different systems may reflect the general heterogeneity of cancer. In their recent review, Robles and Pipas suggest two models to explain this observation. One model suggests that in addition to pRB and p53, LT could interact with other unique binding proteins within each cell type that contributes uniquely to specific aspects of tumorigenesis. A second model proposes that although LT interacts with the same set of proteins in each cell type, the responses differ because the cellular proteins and pathways are differentially regulated based on the cell system [281]. A combination of the two scenarios is also possibile. In mouse models of cancer, pRB inactivation appears to be more important than p53 inhibition, but the contribution of additional binding proteins (within the amino-terminus of LT, Bub1, Cul7, IRS1) remains unclear.

## 1.4 MERKEL CELL CARCINOMA

### 1.4.1 Origin and pathology

Merkel cell carcinoma (MCC) is an uncommon but aggressive, primary cutaneous neoplasm frequently having a poor prognosis [58, 59]. Initially named trabecular carcinoma of the skin, it was first described by Toker in 1972 [298]. MCC derives from mechanoreceptor Merkel cells (named after Friedrich Sigmund Merkel who first described them in 1875) located at the basal layer of the epidermis. These Merkel cells originate from epidermal progenitors during embryonic development and play a role in the sensory system of the skin [299-304]. Although MCC has variable manifestations, they most commonly appear as solitary purple to red nodules or plaques (in the early benign stages, Figure 9 A). MCC cells have round to oval nuclei with scant cytoplasm (Figure 9 B). Most common primary sites of MCC occurence are sun-exposed sites such as the head and neck, followed by the lower and upper extremities and the trunk region. Tumor cells are usually arranged in sheets, solid nests, or anastomosing trabeculae [62, 305]. Most tumor cells contain dense, membrane bound core structures in the cytoplasm similar to neurosecretory-type granules (80-120 nm). Sibley *et al*. and Tang *et al*. first identified

these through electron microscopy [305, 306]. Due to this expression of chromogranin, synaptophysin and other such neuropeptides in Merkel cell neoplasia, MCC is also referred to as a neuroendocrine carcinoma. Gene expression profiling and clinical analysis suggest that MCC comprises two clinically similar diseases with different causes [307].

Diagnosis of MCC has been challenging due to its similarity with other cancers, especially basal cell carcinoma [308, 309], metastatic small cell lung carcinoma [310, 311], and small cell sweat gland carcinoma [312]. MCC expresses a variety of neuroendocrine and epithelial markers. Positivity for Cytokeratin 20 (CK20) in a perinuclear dot-like pattern upon immunohistochemical staining (Figure 9 C), combined with negativity for thyroid transcription factor 1 (TTF-1), leukocyte common antigen (LCA) is the most distinguishing feature of MCC [313-315]. MCC shows variable positive staining with other epithelial markers - AE1/AE3, Cam5.2, neuroendocrine markers - neuron specific enolase and chromogranin, and neural marker CD56 and negative staining for synaptophysin and neurofilament. These markers are also clinically used to help confirm diagnosis [316-322].

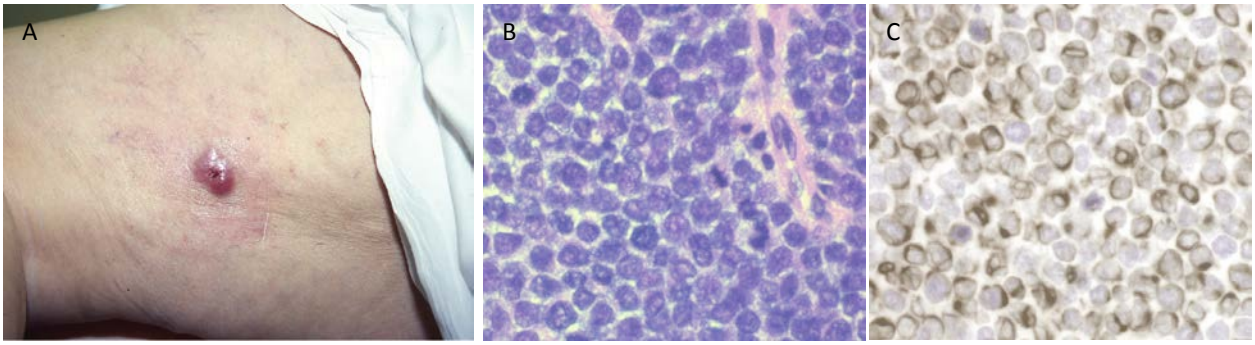



**Figure 9 Clinical appearance and pathology of Merkel cell carcinoma**
(A) Raised, red, nodular MCC lesion on arm of a patient. (Adapted from Chang and Moore, Annual. Rev. Pathol, 2012) Merkel cell carcinoma tissue staining with (B) Hematoxylin-eosin (H&E) and (C) Cytokeratin-20 (CK20). (Reproduced from Feng *et al*., Science, 2008)

### 1.4.2 Risk factors and clinical features

AIDS patients have a 13-fold higher age and sex-adjusted risk for developing MCC, compared with the general population [60]. MCC incidence rises sharply in elderly populations (median age =65 years) [62] and immunosuppression following transplantation and immune-related cancers is another recognized major risk factors for MCC [58]. Similar to melanoma, MCC also shows a strong correlation to UVB exposure [323]. The majority of the patients with MCC are fair skinned men [58, 59, 324]. Heath *et al*. have summarized the most significant features of MCC in an acronym: AEIOU (asymptomatic/lack of tenderness, expanding rapidly, immune suppression, older than 50 years, and ultraviolet-exposed site on a person with fair skin) [62].

### 1.4.3 Staging

MCC is divided into four clinical stages depending on the severity of disease. The stage determined at diagnosis informs prognostication, chance of spread (metastasis), and treatment options. According to American Joint Committee on Cancer's (AJCC) 2009 staging manual, MCC is defined as Stages I and II if the tumor is localized to the skin at the primary site. Stage I is for primary lesions less than or equal to 2 cm in diameter, whereas stage II is when primary lesions exceed 2 cm. Stage III is defined as disease that involves nearby/regional lymph nodes and stage IV, the most severe form, is found beyond the lymph nodes [325]. The 5-year disease specific survival rate for MCC ranges from 60% for stage I to only 18% for stage IV disease [325, 326].

### 1.4.4 Incidence

MCC age-adjusted incidence rates range between 0.18 to 0.4 per 100 000 persons [327]. Although rare, MCC's overall SEER (Surveillance, Epidemiology, and End Results Program of the National Cancer Institute (NCI), United States) study of 3870 cases collected from 1973-2006

[327], revealed tripling of incidence rates from 1986-2001. The incidence reached about 1,500 cases per year in the United States in 2006 [59]. A few other population-based studies in Scandinavian Europe [66, 328-330] also reveal similar patterns as in the USA.

### 1.4.5 Molecular studies

Although molecular studies of MCC have revealed amplification of L-Myc gene (~30% tumors) loss of pRB1 genetic region (~25%tumors), UV-B specific mutations in p53 and H-ras genes, activating c-kit mutations, VEGF alterations, and CD117 positivity, none of these distinct pathways have convincingly been linked to MCC tumorigenesis [331-336]. Cytogenetic deletions in chromosomes 3p, 4, 5q, 7, 10q, 13q and 17q and extra copies of regions on chromosomes 1q, 3q, 5p, 6 and 8q have also been observed [311, 332]. BRAF and MAPK signaling cascade alterations, although present, do not seem to play an important role in the biology of MCC [337-339].

### 1.4.6 Treatment

Treatment and optimal care for Merkel cell carcinoma patients is based on various issues that are highly variable: immune suppression, overall health of the patient, node status, tumor size and location, stage of the disease, age, lymphovascular invasion of primary lesion, etc. There are several treatment options for this disease. These include, surgical excision of the primary lesion, followed by (if needed) lymph node resection, radiation therapy and chemotherapy. Wide surgical excision (with greater than or equal to 2 cm margin) and sentinel node biopsy is usually the mainstay of treatment for patients with MCC. Due to the high radiosensitivity of MCC, radiation therapy is also used for unresectable or recurrent tumors. On the other hand, chemotherapy is uncommon and not usually effective. It is usually given for palliative therapy to patients with stage IV disease. Adjuvant chemotherapy is sometimes used to destroy any cancer cells that remain after surgery and/or radiation therapy. Although there is no well-

established combination chemotherapy for MCC, due to its similarities with other neuroendocrine carcinomas (such as small cell lung cancer), regimens that show good results in these diseases are used [340, 341].

A number of groups have tested and analyzed the chemosensitivity of commonly used chemotherapeutic cytotoxic drugs on MCC [342-344]. In 1999, Voog *et al.* [343] summarized 101 cases of locally advanced and metastatic MCC treated with a multiagent regimen. Cyclophosphamide, doxorubicin, and vincristine were the most frequently used regimen producing a response rate of 67%. The small-cell lung cancer regimen of cisplatin/etoposide produced a 47% response rate in MCC patients. The duration of these responses however was limited to 8 months overall and only 3 months for those with partial response. Full responders had a median survival of approximately 20 months and treatment toxicities were significant with a total of 9 treatment-related deaths (increasing age being a major risk factor).

The expression of the KIT receptor in MCC implicated the use of imatinib mesylate (Gleevec) in MCC [345, 346]. However, phase II trial of imatinib mesylate performed by Sondak *et al.* however did not show treatment effects in advanced MCC patients as anticipated [347].

Pascucci's group successfully showed an unusual compound selection to work. They treated 4 cases of stage-1 MCC with surgery followed by intralesional Bleomycin, and saw no evidence of recurrence or metastasis for 5 years [348]. This was a considerable improvement from a usual ~30% recurrence rate seen stage I-III MCC patients at a median follow up of 3.6 years [349]. Bleomycin is a glycopeptide antibiotic that induces DNA strand breaks and has antiviral properties against drug-resistant HIV strains [350]. Although the study has implications in viral associated diseases such as MCC, the sample size used by Pascucci *et al.* was limited and more data is needed to make any conclusion.

Due to the rarity of MCC cases clinical trials are difficult to perform. There are three recent ones that have generated interest among MCC clinicians and hold promise for future patients. One uses Oblimersen (also known as Augmerosen or Genasense), a bcl-2 antisense oligodeoxyribonucleotide developed by Genta Incorportaed [351] (Clinical Trail Identifier on clinicaltrials.gov: NCT00079131). In the second trial, patients are given interleukin-12 gene

therapy (NCT01440816) and the third clinical trial involves treating patients with the drug BB-10901/IMGN901 or Lorvotuzumab Mertansine (NCT00346385). IMGN901 is a CD56 antibody conjugated to a cytotoxic microtubule inhibitor (Mertansine of DM1). Since MCC is one of the few cancers that express CD56 on its surface, it was considered a good candidate for studying IMGN901 efficacy. This drug carries orphan drug status for MCC treatment and was developed by Immunogen Inc. While two out of the three trials are still recruiting patients, the Oblimersen trial was recently completed. In a study of 12 patients probable antitumor activity was documented in 1 patient, but no objective responses as per RECIST (Response Evaluation Criteria in Solid Tumors) criteria were observed [351]. A recent study by Willmes *et al*. shows inhibition of MCC cell line growth and regression of MCC mouse xenograft tumors by using interferon [352].

In Chapter 3 of this dissertation, the use of a recently developed survivin inhibitor YM155 in treating MCC cells both *in vitro* and in mouse xenograft models is described. The drug is in early stages of clinical development and a phase II clinical trial for MCC using YM155 is anticipated to begin patient recruitment in the fourth quarter of 2012 (ECOG # E1611).

## 1.5 MERKEL CELL POLYOMAVIRUS

Similar to other polyomaviruses (especially LPV [104] and SV40 [353]), MCV is also a non-enveloped, icosahedron shaped, small, double stranded DNA virus that is phylogenetically classified into the mammalian *Orthopolyomavirus* genera [83]. Polyomaviruses' ability to induce neoplastic transformation in cell culture and neoplasias *in vivo* have been well documented ([354]) however none have been consistently and convincingly linked with human cancer thus far. MCV discovery, as an integrated part of MCC represents the first widely accepted association of a specific human cancer with the consistent presence of a polyomavirus genome.

### 1.5.1 Discovery of MCV

MCV was discovered in 2008 by Feng *et al.* [74], using a technique called Digital Transcriptome Subtraction (DTS) [75]. As a result of the generation of curated and highly verified human genomic sequence databases (made available through the Human Genome Project) and the development of low-cost, high-throughput sequencing, DTS became a practical computational approach to detect new virus agents associated with human cancers. Complementary DNA (cDNA) was made from mRNA of four MCC tumor tissues through reverse transcription. These libraries were then exhaustively sequenced to generate a stringent high fidelity transcriptome database of MCC tumors. An *in silico* subtraction of known human sequences from human RefSeq RNA, mitochondrial, assembled chromosomes, and immunoglobulin sequences in NCBI databases identified candidate nonhuman (suspected pathogen) sequences for further examination. These candidates were then explored using lower-stringency alignment to virus databases. Of 395,734 MCC transcripts sequenced, 382,747 sequences formed the high-fidelity data set (HiFi) and after screening, 2395 were identified as non-human DTS candiadates. One transcript had 59% homology to the African green monkey lymphotropic polyomavirus (LPyV) tumor T antigen [104, 355] and human BK polyomavirus [135] T antigen sequences. Further experimental extension (RACE and genome walking) using this transcript completed the sequence of the entire genome of MCV (GenBank accession numbers EU375804) [74].

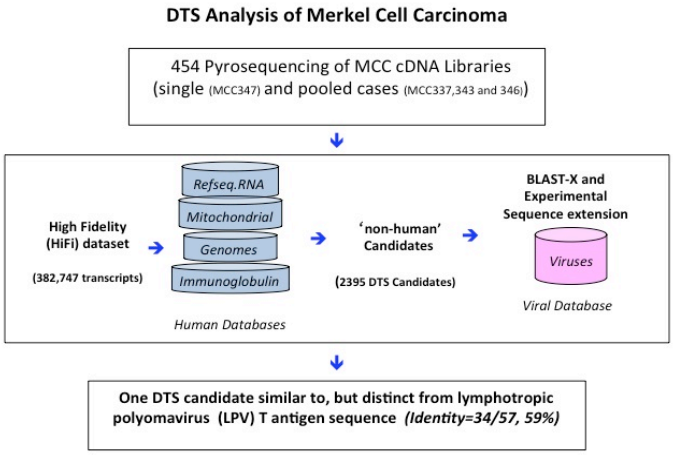



**Figure 10 Pictoral Representation of DTS Analysis of Merkel cell carcinoma**
(Modified from Feng *et al*, Science 2008, and Feng AACR Meeting presentation)

### 1.5.2 Genomic organization of MCV

MCV has a ~5.4 kb (range 5.2 – 5.4 kb) genome that is divided into early and late coding regions by a noncoding regulatory region (NCRR). The early region encodes a large T antigen (LT), a small T antigen (sT) and a 57kT antigen (analogous to the SV40 17-kT antigen [107]), all of which share a 78 amino acid stretch in the N terminus. These three proteins are expressed from a multiply spliced mRNA (with frame changes) as shown in Figure 11**.**

MCV late region encodes 3 capsid proteins (VP1, VP2 and VP3) that are expressed after the onset of viral DNA replication. These structural proteins self-assemble into a ~55-nm diameter icosahedron viral particles, that look similar to other polyomaviruses under electron microscopy [116, 356]. Unlike other polyomaviruses however, MCV does not encode an agnoprotein [357, 358] or VP4 [359]. Formally, little is known about the kinetics and regulation of MCV late gene expression because virus replication studies have been limited. Comparison of

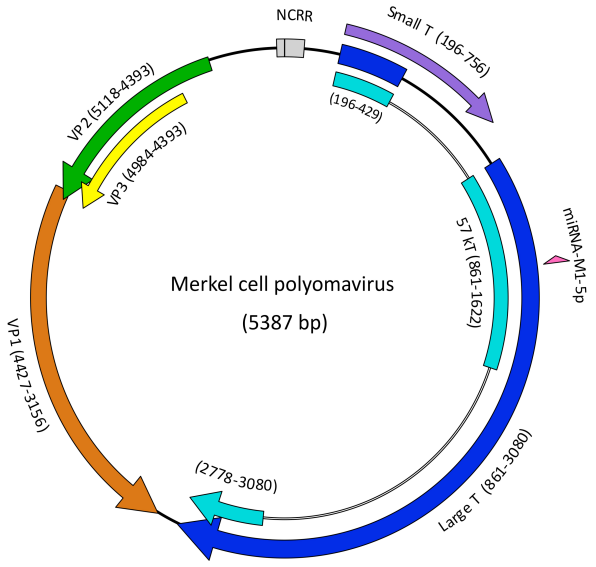



**Figure 11 Genome organization of Merkel cell polyomavirus.**
Merkel cell polyomavirus (wild-type) has a circular, 5387 bp genome divided into two halves by the noncoding regulatory region (NCCR). This NCCR contains the origin of replication of the virus as well as the promoters and regulatory elements to bidirectionally encode early and late viral proteins. The early region encoded proteins comprise the Large T, small T and the 57kT antigens. Viral protein 1, 2 and 3 (VP1, VP2 and VP3) constitute the gene products of the late region.

late gene expression for the MCV-HF molecular clone to a replication defective mutant clone having a point mutation in the viral origin suggests that MCV late gene expression depends on active DNA replication of the viral genome [360].

The NCRR region of MCV contains a 71-bp viral replication origin. This core sequence comprises of an AT-rich tract that helps in DNA melting and 8 GAGGC pentanucleotide sequences that bind MCV LT. Although the origin region maintains efficient full length (wild type) T antigen-directed DNA replication, tumor-derived LT is replication incompetent [361]. Interestingly, a naturally occurring point mutation in the core origin region that ablates replication was found in tumor derived viral genome (MCV350) [362]. The NCRR also contains bidirectional transcriptional promoters and regulatory elements for early and late viral gene expression.

Recent studies have identified an MCV encoded miRNA- MCV-mir-M1 [363]. It is encoded from the late strand (similar to MPyV) and is antisense to early transcripts (regions 1201-1245). Similar to the SV40 miRNA, it is predicted to cleave early viral RNAs during MCV infection and was shown to negatively regulate expression of chimeric luciferase reporters containing a portion of the early transcript *in vitro* [120]. Although MCV-mir-M1 was found using an *in silico* and *in vitro* approach, it was recently detected in 2/3 and 19/38 MCV positive MCC tumors by direct sequencing and real-time PCR methods respectively. A 2-nucleotide difference (shift) from the in silico predicted mature miRNA was found [364]. This microRNA shares no sequence identity with any other previously known miRNAs of viral and host origin. Further studies in MCV encoded miRNAs and their potential target viral and cellular genes in MCC tumors are needed to understand their function.

### 1.5.3 Tumor specific signature truncation mutations

MCV large T antigen retains conserved domains that are present across different virus families (including polyomaviruses), such as CR1, DnaJ and LXCXE Rb binding motifs [85, 99]. These are described in Figure 12. Besides tumor suppressor targeting domains, LT also encodes origin binding and helicase/ATPase regions needed for viral replication [112]. Recently Liu *et al.* found a novel interaction of MCV LT with human Vamp6 protein (hVam6p), a protein involved in lysosomal processing. The binding site was mapped to a unique region immediately adjacent to the Rb binding motif [365]. LT binds and sequesters the cytoplasmic Vam6p protein to the nucleus, causing a disruption in lysosomal clustering. Other consequences of this re-location are unknown, preliminary work indicates that Vam6p may regulate MCV replication [360].

MCC tumor-derived MCVs have premature stop codon mutations (substitutions, frameshift, missense, insertions and deletions) that truncate the LT protein, eliminating its C terminal domain [366, 367]. Although these truncation mutations ablate the replication functions of MCV T antigen in the tumors, they occur after the LXCXE retinoblastoma-binding (and other pocket protein binding) motif and do not interfere with other N-terminal LT domains [368]. However, a putative p53-binding domain is also disrupted by these mutations [223, 224]. These two independent mutation events – virus integration and T antigen truncation that leads to replication incompetence appear to play a mechanistic role in the development of MCC. Since both events are rare mutational events, but present in all MCV positive MCCs they may also explain MCC rarity.

MCV T antigen also has a nuclear localization signal mapping to aa 277-280 (Arg-Lys-Arg-Lys) [369]. This NLS is different from the KKKR (or K) KVEDP sequence that constitutes the SV40, JCV and BKV polyomavirus' NLS [370]. Although highly conserved and functional in MCC clinical samples there are exceptions where the NLS is lost in the truncated tumor Ts [74, 112, 369].

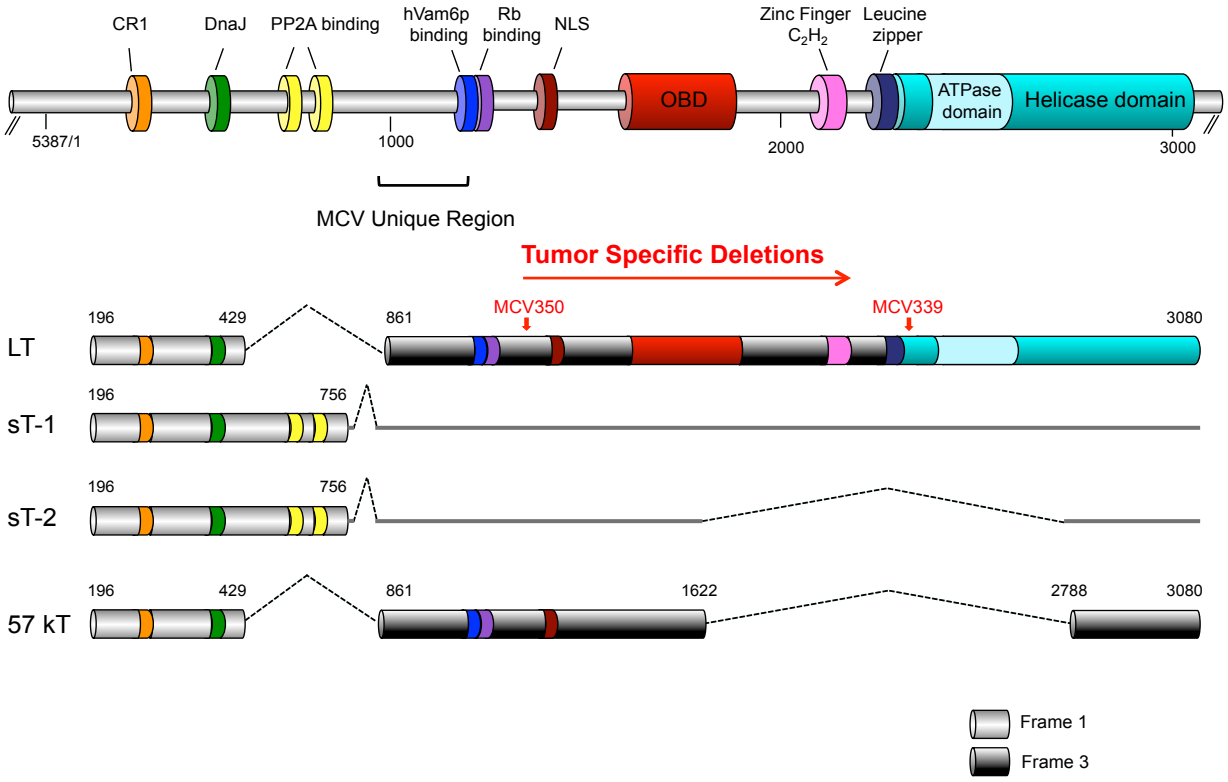



**Figure 12 T antigen locus genome, transcript and protein features.**
MCV T antigen contains conserved protein motifs, similar to other polyomaviruses. These include CR1 (conserved region 1), DnaJ, Rb (retinoblastoma) binding,hVam6p binding and a nuclear localization signal (NLS) in the N-terminal part of the proteins. The C terminal motifs (required for replication) :Origin Binding domain (OBD), Zinc finger, leucine zipper and helicase domain are lost in tumor Tags due to stop codon truncation mutations (red arrows mark the mutation sites for the two prototypes MCV350 and MCV339). The Rb binding site however is always retained. The small T antigen expresses a conserved PP2A (protein phosphatase) binding motif common with SV40 and MPyV small T antigens. The genomic T antigen locus encodes for 4 transcripts and three alternatively spliced proteins (LT, sT and 57kT). MCV expresses a unique set of amino acids in exon2 of its large T antigen that are distinct from other polyomaviruses.

### 1.5.4 T antigen dependency

An important affirmation of MCC tumorigenesis is the requirement of MCV protein expression to maintain the tumor phenotype. To assess the importance of MCV T antigens in MCC tumor cells both MCV LT and sT (panT) were knocked down in five different MCC cell lines. Three different exon 1 target sequences, and two independent selection methods were used. Knock down of both LT and sT caused cellular death, as measured by wst-1 cell proliferation and lactate dehydrogenase (LDH) release cell death assays. No late apoptotic markers, cleaved caspase3 or cleaved PARP expression, were observed and the mechanism of cell death is unknown [371]. Knockdown of sT alone, through the intron 1 sequence, does not fully recapitulate pan-T knockdown, but causes cell cycle arrest (not cell death) [372]. Figure 13 shows the effect of panT and sT knockdown on MKL-1, an MCV positive MCC cell line.

Due to the overlap of 3' coterminal transcripts and the sequence pattern of LT it is difficult to knockdown LT alone. However, by targeting the splice site between exon1 and exon2, Houben *et al.* succeeded in knocking down LT alone and observed a reduced growth phenotype. By expressing LT insensitive to the shRNAs used, they partially rescued the observed growth inhibition. This rescue was dependent on LT's ability to bind RB1, suggesting the importance of this interaction in MCC tumors [373].

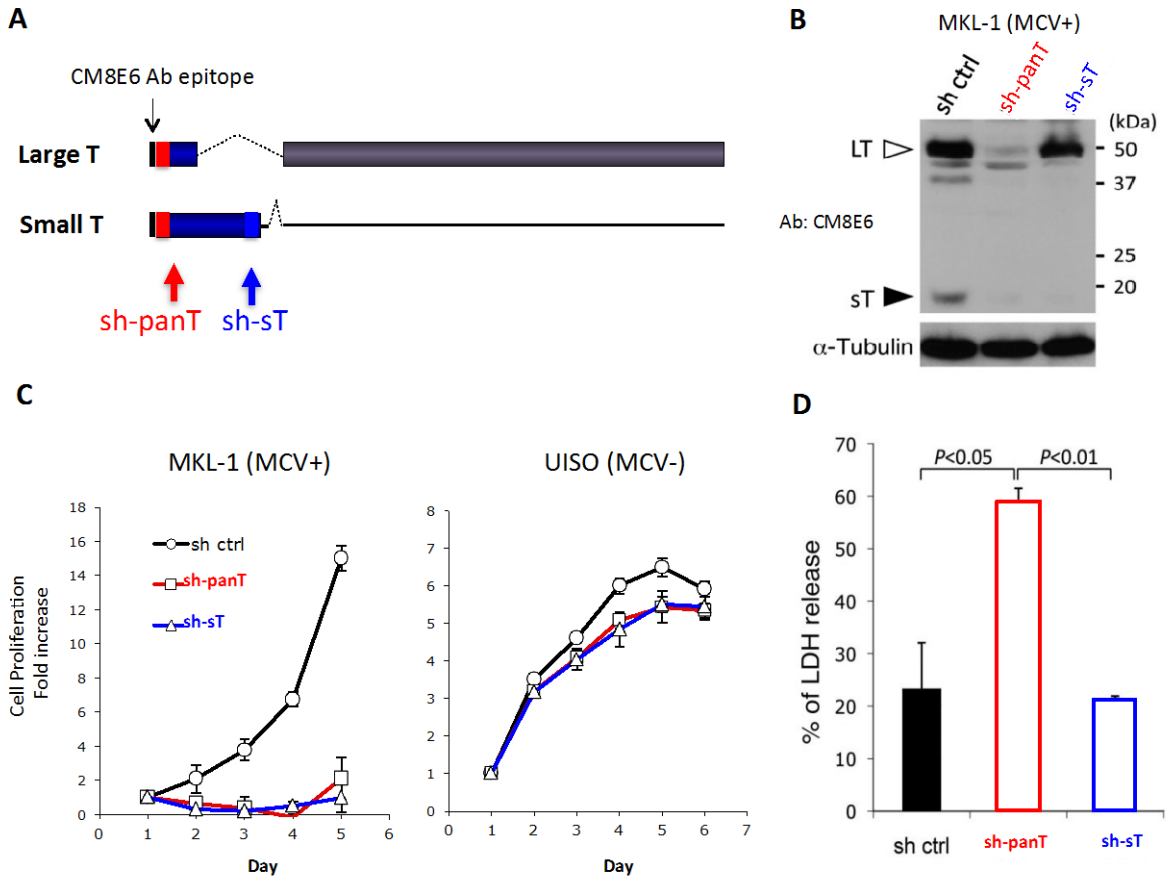



**Figure 13 T antigen is necessary for MCV positive MCC survival.**

**(**A) Location of two shRNAs targeting MCV T antigens for knockdown is shown. sh-panT targets the exon 1 region and thus both Large T and small T antigen. sh-sT targets the intron 1 region and thus only small T antigen. CM8E6 antibody epitope is part of exon 1 and is used to detect both sT and LT. (B) Infection with sh-panT lentivirus decreases both LT and sT expression in MKL-1 cells. However infection with sh-sT decreases only sT expression specifically. (C) Knockdown of T antigens cause a decrease in cell proliferation in MCV positive MCC cells (MKL-1), but not in MCV negative MCC cells (UISO) (D) Knockdown of both LT and sT causes cell death (measured via release of LDH), whereas knockdown of small T antigen alone does not kill cells but perhaps causes cell cycle arrest. (Modified from Shuda *et al*., JVI 2010, Shuda *et al*., JCI 2011 and Feng AACR Presentation 2011)

### 1.5.5 Transformation activity

Polyomavirus T antigens are extensively described in the literature as having the ability to overcome contact inhibition and anchorage dependence upon expression in rodent cell lines and display serum independence of human cell lines. These assays are well-established methods of measuring oncogenicity of candidate proteins [185]. Unlike SV40 and MPyV, MCV small T alone but not other T antigens, possesses this transforming activity [354, 374-376]. Recent studies by Shuda *et al*., describe both foci formation and soft agar growth caused by MCV sT expression in rodent fibroblast cells [372]. Although MCV sT transformation was independent of sT-PP2A (Protein Phosphatase 2A) interaction which is important in other polyomavirus mediated transformations, a constitutively active 4E-BP1 that could not be phosphorylated antagonized this phenotype. These results indicate the contribution of deregulation of cap-dependent translation through 4E-BP1 hyperphosphorylation to Merkel cell carcinogenesis [372].

### 1.5.6 MCV seroprevalence

MCV serology, largely based on antibodies reactive to the late structural capsid protein VP1, has revealed MCV to be a common human infection among both children and adults. Similar to other polyomaviruses primary infection may be asymptomatic. Initially Kean *et al.* [160] and Carter *et al.* [377] studied reactivity to GST-VP1 fusion proteins and found between 25-59% MCV prevalence in healthy donors. However, more sensitive and specific assays that use MCV VP1 conformational epitopes (present in assembled virus like particles (VLP)) as antigens found antibodies against MCV in 60-80% of healthy adults [116, 378, 379]. The rate of seroprevalence observed increased with age, from 9% in children younger than 4 years of age, 35% by 4-13 years of age, 49% in children and young adults between 15 to 26 years of age, to finally 80% in elderly individuals >50 years of age [378, 380, 381]. Unlike other polyomaviruses, antibody

responses to MCV VLPs are not diminished by cross-competition with other polyomaviurs antigens, indicating their specificity to MCV infection [182, 380, 381].

### 1.5.5 Role of MCV immune reactivity in MCC

Viral capsid protein 1 antibody titers, seen in healthy individuals, seem to correlate to the presence of MCV on the skin of healthy individuals [382, 383]. Despite the widespread VLP seropositivity seen among the healthy population, antibody titers are unusually high in MCV positive MCC patients than in non-MCC patients. However, in MCV positive MCC patients no correlation between tumor viral load and serum antibody titer has been observed [384]. Notably, Touze *et al*. did associate higher antibody titers with a better prognosis among MCC patients, implying a strong immune response to viral infection as a surrogate marker for prognosis [384]. However, a direct effect of the VP1 antibody response is unlikely because tumors do not express detectable amounts of MCV VP1 proteins [385]. In addition, in a recent longitudinal study of healthy adult gay and bisexual men, 18 years of age and older, 79% had antibodies to MCV at entry and subsequently 5.5% of the others seroconverted to MCV positivity. On analysis, no measurable signs or symptoms or common diagnostic test results were significantly associated with primary MCV infection (time of seroconversion) [386]. High serum IgG antibodies against MCV T antigen proteins (epiptopes match to the common/shared region between LT and sT) are also seen in MCV positive MCC patients, with a good correlation to viral load [116, 387]. These antibodies are rarely detectable in the general population and correspond to tumor burden and disease status. Although antibodies to the viral capsid indicate prior MCV infection and titers are higher in MCC patients, MCV T antigen antibody titers are likely to be more useful clinical indicators.

Additional indications of immune surveillance come from MCC gene expression profiling studies that show increased intratumoral CD8+ lymphocyte invasion in MCV positive MCC patients [388]. These studies also correlate signs of stronger immune function to better prognosis for MCC patients [388, 389]. Furthermore, researchers have also shown that MCV increases T-helper cell proliferation and related IFN-gamma responses in MCV seropositive

individuals [390].

Impaired immune function is an epidemiologically recognized risk factor for MCC. However, different degrees of immunologic impairment are observed among MCC patients. Among MCV positive MCC patients, it is likely that immunologic surveillance mechanisms actively inhibit MCV mediated carcinogenesis [19, 43]. Immune function may potentially be influencing both the induction of MCC by virus reactivation following immunosuppression and also MCC clinical outcome.

### 1.5.7 Transmission

Transmission routes have been proposed for different polyomaviruses, but none have been definitely established. BKV and JCV are likely to be transmitted by fecal-oral and oral routes, whereas even though KIV, WUV and MCV are found in urban sewage samples as well, they seem to have a respiratory route of transmission [167, 170, 391-395]. Interestingly, DNA from most human polyomaviruses can be detected in tonsillar tissue, a possible entry point. JCV is believed to begin its life cycle in humans by infecting tonisillar tissue, but it is probably disseminated by B-lymphocytes, which help it cross the blood-brain barrier into the central nervous system. BKV is found to persist in the kidneys and the urinary tract, and causes viruria in transplant patients [271, 396-399]. Transmission of MCV is likely through casual contact but the precise mode is not known.

MCV has been difficult to cultivate in the laboratory as a natural infection. Several independent attempts have been made to produce infectious MCV molecular clones [360, 400, 401]. In each case, primary low-level virion production can be achieved, however, secondary transmission to uninfected cells has not been successful. Early electron microscopy studies suggested that MCV virions might be seen in some MCC tumors [402] but the weight of evidence demonstrate that the structural proteins required for encapsidation are not expressed in MCC [385, 403].

## 1.6 MCC CELL LINES

Due to the uncommon occurrence of MCC, patient samples are limited. Hence, MCC cell lines are critical in studying MCV dependent and independent MCC tumorigenesis. Several Merkel cell carcinoma cell lines were established years ago and have recently been tested for MCV positivity, integration sites, viral sequence and truncation patterns [311, 404-406]. Recently, new MCC cell lines have also been created from Merkel tumor biopsies [371, 406]. These newer cell lines have the advantage of being in early passage, as compared to previously derived cell lines, which may have been adapted to changes after extended culturing. The following table summarizes the cell lines used in this study.

**Table 3 MCC Cell Lines used in this study (Modified from Houben *et al.*, JVI 2010)**

| Cell line | Source | | | Morphology | Doubling time (days) | Detection of MCV by - | | LT | | Expression of | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Age of patient | Sex of patient | Location | | | PCR | Southern blotting | Stop codon mutation | Predicted molecular mass (kDa) | CK-20 | pan-CK | NSE | Chromogranin A | Synaptophysi |
| **MKL-1** | 26 | M | Nodal metastasis | spheroidal | 3 | + | + | Deletion 1612-1657 | 36 kDa | + | + | ++ | + | + |
| **MKL-2** | 72 | M | ND | spheroidal | 5 | + | + | C1453A | 30 kDa | ND | ND | ND | + | + |
| **MS-1** | 59 | F | Adrenal metastasis | spheroidal | 4 | + | + | Deletion 1912-1951 | 47 kDa | + | + | + | + | + |
| **WaGa** | 67 | M | Ascites | single cell suspension | 4 | + | ND | C1461T | 30 kDa | + | + | + | + | + |
| **UISO** | 46 | F | Thigh | adherent | 1.5 | - | - | NA | NA | - | ND | ++ | - | - |
| **MCC13** | 80 | F | Nodal metastasis | adherent | 1 | - | - | NA | NA | - | - | + | + | - |

**Expression of cytokeratin 20 (CK-20), pan-cyto keratin (pan-CK), neuron-specific enolase (NSE), and Ki-67 was measured by immunohistochemistry. Synaptophysin chromogranin A expression was analyzed by quantitative real time PCR. ND- not determined or unknown, NA-not applicable. References : Ronan et al.,1993,Van Gele et al., 2 Rosen et al., 1987 and Shuda et al., 2009.**

## 2.0 MERKEL CELL POLYOMAVIRUS T ANTIGEN EXPRESSION IN MCC AND OTHER CANCERS

Work described in this section was published in the International Journal of Cancer, American Journal of Surgical Pathology and Blood

Int J Cancer. 2009; 125(6): 1243-9.

with authors Shuda M, Arora R, Kwun HJ, Feng H, Sarid R, Fernández-Figueras MT, Tolstov Y, Gjoerup O, Mansukhani MM, Swerdlow SH, Chaudhary PM, Kirkwood JM, Nalesnik MA, Kant JA, Weiss LM, Moore PS and Chang Y.

R. Arora, M. Shuda and H.J. Kwun contributed equally to this work.
R. Arora, M. Shuda and H.J. Kwun performed the qPCR studies and analysis. R Arora and M. Shuda performed the immunoblotting and immunofluorescence experiments. Dr Mary Ann Accavitti produced CM2B4 antibody. Ms. Mary Acquafondata and Ms. Yuan-Yuan Chen perfomed immunohistochemistry. R. Arora, M. Shuda, H.J. Kwun, Y. Chang and P. S.Moore conceived the project, analyzed the results and wrote the manuscript.

Am J Surg Pathol. 2009; 33(9): 1378-1385

with authors Busam KJ, Jungbluth AA, Rekthman N, Coit D, Pulitzer M, Bini J, Arora R, Hanson NC, Tassello JA, Frosina D, Moore PS and Chang Y.

R. Arora performed qPCR and data analysis for samples provided by Klaus Busam group.

and

Blood. 2010; 115(23): 4973-4

with authors Tolstov YL, Arora R, Busam KJ, Chaudhary PM, Chang Y and Moore PS

R. Arora, Y. L. Tolstov and S. Scudiere performed the PCR, ELISA and Immunohistochemistry respectively. R. Arora, Y.L. Tolstov, S. Scudiere, K.J. Busam, PM. Chaudhary, Y. Chang and PS. Moore analyzed and interpreted the data. Y.L. Tolstov, R. Arora, Y. Chang and P.S. Moore wrote the manuscript.

MCV was discovered as monoclonally integrated in the MCC tumor genome. To further assess T antigen expression in tumors and its association with human diseases, it became important to develop assays and tools of detection. To this effect we screened 33 antibodies to other polyomaviruses for cross reactivity to MCC. When none of them specifically detected MCV T antigen, we then developed a monoclonal antibody (CM2B4) using a peptide from a ~200 amino acid MCV unique region (absent in other viruses) in exon 2 of MCV large T antigen. We found that CM2B4 specifically recognizes both endogenous and transfected MCV large T (LT) antigens. We also found that MCV LT protein localizes to the nucleus of tumor cells from MCC. Of 62 CK20 positive MCC tissues tested for CM2B4 staining, 46 (74.2%) were positive.

We developed a qPCR assay for detection of MCV genome copy numbers in both MCC and other disease tissue. MCV genome is present at an average of 5.2 (range 0.8–14.3) T antigen DNA copies per cell, supporting a direct carcinogenic mechanism.

Since MCV is closely related to lymphotropic polyomavirus (LPV) and MCC occurs in association with many other malignancies, we used both qPCR based detection and CM2B4 staining to survey hematolymphoid malignancies (especially chronic lymphocytic leukemia), neuroendocrine tumors, and collision tumors tissues. Although 2.2% of 325 hematolymphoid malignancies surveyed showed evidence for MCV infection by DNA PCR, none were positive at high viral copy numbers. None of 173 lymphoid malignancies examined on tissue microarrays expressed MCV LT protein in tumor cells. None of the 26 pulmonary neuroendocrine tumors or 7 collision tumors showed signs of MCV T antigen protein expression either.

Expression of this viral oncoprotein in tumor cells indicates the mechanism supporting the notion that MCV causes a subset of MCC. As with some of the other human polyomaviruses, lymphocytes may serve as a tissue reservoir for MCV infection, but hematolymphoid malignancies associated with MCC are unlikely to be caused by MCV. MCV is thus specific to MCC.

## 2.1 MATERIALS AND METHODS

### 2.1.1 Human tissue samples

*For University of Pittsburgh study*

For Merkel cell carcinoma, fresh frozen tumor samples were obtained from the Cooperative Human Tissue Network [407]. An MCC tissue core microarray consisting of 36 MCC specimens was generated from archival paraffin-embedded tissues from the pathology departments at Hospital Universitari del Mar and the Hospital Universitari Germans Trias i Pujol, Barcelona, Spain as previously described [408]. Tissue microarrays for lymphoid malignancies and normal controls were purchased commercially (US Biomax, Inc.). Genomic DNA samples from consecutive hematolymphoid tumor tissues were collected and archived by the late Dr. Anne Matsushima, Columbia University, from excess tissue submitted for diagnostic pathology. This was supplemented with additional hematolymphoid tissues obtained from tissue banks at the Department of Pathology, University of Pittsburgh. For reasons of confidentiality, minimum patient identification and demographic data are available for most of these specimens. PBMC specimens were obtained from two sources: 1- excess samples submitted to the Division of Molecular Diagnostics, University of Pittsburgh Medical Center for genetic screening and 2- PBMC collected from HIV-positive persons participating in Kaposi's sarcoma epidemiologic studies [409]. None of these study subjects were diagnosed with Merkel cell carcinoma. All specimens were tested under University of Pittsburgh Institutional Review Board-approved guidelines.

*For Memorial Sloan- Kettering Study*

*Patients*

The study was approved by the Institutional Review Board. All patients were seen at or sought consultations from physicians at Memorial Sloan-Kettering Cancer Center (MSKCC). Consent

was obtained for use of tumor tissue for research. Clinical information and follow-up was abstracted from the medical records or by contacting the patient's clinician directly.

*Tumor Tissue*

Slides and tissues of primary and metastatic tumors were retrieved from the archives and the tissue bank of the institution's department of pathology. The material included 17 snap-frozen tumor samples of MCC, which had been stored at −70°C (MSKCC tumor bank) and formalin-fixed and paraffin-embedded (FFPE) archival tissue, which was used for immunohistochemical studies.

For a tumor to be accepted as MCC and selected for this study, the following criteria had to be present. The tumor had to be predominantly composed of nuclei with pale salt and pepper chromatin pattern, scant cytoplasm, and mitotic figures. Immunohistochemically, the tumor had to be positive for CK20, or, for the rare tumors, which lacked labeling for CK20, positive for the CAM 5.2 in a paranuclear dot-like pattern and/or positive for chromogranin and/or negative for thyroid transcription factor-1 (TTF-1). Furthermore, there had to be clinical evidence in support of a primary skin tumor or, for metastatic lesions, evidence in support of a derivation from a primary skin tumor or at least clear documentation that there was no extracutaneous primary in the rare cases of metastatic MCC with unknown primary. Cases of patients who carried a prior or concurrent diagnosis of an extracutaneous neuroendocrine carcinoma were excluded from this analysis.

In this study, immunohistochemical stains were performed on 3 sets of FFPE Merkel cell tumors:

1. Fifteen tumor samples corresponding to the same tumors, which were analyzed by quantitative PCR (qPCR; a total of 17 frozen tumor samples were tested by qPCR, but suitable archival material for a parallel analysis by IHC was available in 15 of 17 cases).
2. Thirty-six tumor samples spotted on a tissue microarray. Each case was represented in triplicate 1 mm punch biopsy samples from the donor tumor block.
3. A set of 7 combined primary skin tumors, which showed mixed features of squamous cell and CK-20-positive neuroendocrine carcinoma.

To test for the potential presence of MCV in neuroendocrine tumors noncutaneous origin, IHC with mAb CM2B4 was also performed on a series of 26 lung tumors, consisting of 16 primary small cell carcinomas and 10 large cell neuroendocrine carcinomas.

### 2.1.2 Real time quantitative PCR

For University of Pittsburgh study, quantitative PCR was performed using primers amplifying the MCV T antigen, TAg (1051–1131 nt; forward: 5′-cctctgggtatggg tccttctca-3′, reverse: 5′-atggtgttcgggaggtatatc-3′) and VP2 (4563–4472 nt, forward: 5′-agtaccagaggaagaagccaatc-3′, reverse: 5′-ggc cttttatcaggagaggctatattaatt-3′) loci with internal TaqMan probes (TAg: 5′-cccaggcttcagactc-3′, VP2: 5′-gcagagttcctc-3′) labeled with FAM and MGB quencher (Applied Biosystems). For the additional 10 peripheral blood samples with CLL, and Memorial sloan kettering MCC samples primers designed against MCV T antigen promoter region (98–184 nt forward: 5′-cccaagggcgggaaactg-3′, reverse: 5′-gcagaaggagtttgca gaaacag-3′) and internal probe (5′-ccactccttagtgaggtagctcatttgc-3′) labeled with FAM and BHQ quencher (Biosearch Technologies) was also used. Copy numbers were established from standard curves of Ct values from serial dilutions of known concentrations of MCV DNA originally amplified by PCR using contig 3 and contig 12 primer sets for TAg and VP2 detections, respectively [74]. Water was used as control to detect template contamination. No evidence of PCR template contamination was observed in the PCR reactions with water control. RNaseP (Applied Biosystems) or β-actin primer-probe mixtures (forward: 5′-cactggctcgtgtgacaagg-3′, reverse: 5′-cagacctactgtgcgcctacttaa-3′, probe: 5′-tggtgtaaagcggccttggagtgtgt-3′) (Biosearch Technologies) were used to determine cell genome copy number. qPCR reactions were performed using PRISM 7700 Detection System, PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems) and/or Smart Cycler 5RX4Z01 (Cepheid) with TaqMan reagents (UNG (+) TaqMan Universal PCR Master Mix). Amplification reactions of all target genes were performed with the following condition: 50°C for 2 min, denaturing at 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. Results were expressed as numbers of viral copies per cell calculated from Ct values

of viral and cellular gene standards. Cellular viral DNA copy number below $1.0 \times 10^{-3}$ per cell was considered negative.

### 2.1.3 Cell lines and transfection conditions

Human embryonic kidney 293 cells (ATCC) were grown in DMEM medium supplemented with 10% fetal calf serum. Cells were transfected with expression constructs using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions on 90% confluent cells. Cells were harvested 48 hr after transfection for analysis.

### 2.1.4 Plasmids

To clone 57 kT wt, the genomic T antigen expression plasmid constructed from appendix 206 [74] was transfected into 293 cells with Fugene 6 (Roche). After 48 hr, total RNA was extracted with Trizol (Invitrogen). First strand cDNA was synthesized with oligo-d(T) primer using Superscript III First-Strand Synthesis System (Invitrogen). Multiply spliced products were amplified by PCR with primers: MCV.*Eco*RV(S) (5'-ccgatatcatggatttagtcctaaaagg-3') and MCV.*Xho*I(AS) (5'-gggctcgagtattgagaaaaagtaccagaa tattggg-3'). A PCR fragment corresponding to 57 kT antigen was cloned into pcDNA6 expression vector with *Eco*RV and *Xho*I sites. To generate the pMCV TAg-EGFP expression constructs, pcDNA6 gLT206 encoding wild type full-length genomic T antigen7 was digested with *Nhe*I and *Sac*II and cloned into pEGFP-N1 (Clonetech) in-frame to C terminus GFP using same restriction sites. LT expression constructs for JCV and BKV were kindly provided by Dr. James DeCaprio[410]. SV40 T antigen cDNA cloned in pCMV vector is described elsewhere[411].

### 2.1.5 Generation of CM2B4 mAb

Monoclonal antibody CM2B4 (IgG2b isotype) was generated by standard methods of immunizing mice with KLH-derivatized SRSRKPSSNASRGA peptide from the MCV T antigen exon 2 with a C-terminal cysteine (Epitope Recognition Immunoreagent Core Facility, University of Alabama).

### 2.1.6 Immunofluorescence and immunohistochemistry

For immunofluorescence staining, cells were spotted on glass slides by Cytospin3 (Shandon), fixed with 10% buffered formalin for 20 min and permeabilized with phosphate-buffered saline (PBS) with 0.1% Triton X-100. After blocking, cells were reacted with CM2B4 (1:100 dilution) at 4°C overnight followed by secondary antibody (Alexa Fluor 488-conjugated anti-mouse, 1:1,000 Invitrogen) for one hour at room temperature. Stained cells were mounted in aqueous medium containing DAPI (Vector Laboratories, CA). For immunohistochemical staining of paraffin embedded tissues, epitope retrieval was performed using EDTA antigen retrieval buffer (Dako, Glostrup, Denmark) at 126°C for 3 min after deparaffinization and hydrogen peroxide treatment. After blocking with Protein Block (Dako), samples were reacted to primary antibody for 30 min at room temperature with dilutions described below. After washing, samples were incubated with Mouse Envision Polymer (Dako) for 30 min at room temperature for subsequent deaminobenzidine (DAB) reaction. mAbs used for immunohistochemistry were: CM2B4 (1:10–1:50 hybridoma supernatant), CK20 (Dako; 1:50), Chromogranin A (Dako, 1:600), Synaptophysin (Biogenex, San Ramón, CA; 1:100), and CD56 (Novocastra, Newcastle upon Tyne, United Kingdom; 1:50).

### 2.1.7 Immunoblotting

Transfected cells were lysed in buffer (10 mM Tris–HCl pH8.0, 0.6% SDS) containing proteinase inhibitor cocktail (Roche). Lysate was electrophoresed in 10% SDS-PAGE, transferred to nitrocellulose membrane (Amersham) and reacted with Pab416 (1:10) or CM2B4 mAb (1:10) overnight at 4°C, followed by anti-mouse IgG-HRP conjugates (Amersham, 1:5,000) for 1 hr at room temperature. Detection of peroxidase activity was performed by Western Lightning plus-ECL reagent (Perkin Elmer).

## 2.2 RESULTS

### 2.2.1 MCV T antigen expression in Merkel cell carcinoma tumors

To show that MCC tumor cells are infected with MCV and express MCV T antigen protein, we developed a monoclonal antibody (CM2B4) to a peptide epitope (SRSRKPSSNASRGA) in exon 2 of the MCV T antigen (Figure 14 A and Figure 37 in Appendix A). This epitope is located N-terminal to an LFCDE motif previously found to bind retinoblastoma protein and is likely to be conserved in viruses from both tumor and nontumor tissues[112]. CM2B4 is predicted to target the MCV LT protein and a T antigen isoform translated from a multiply spliced mRNA, but not the small T antigen (Figure 14 B). CM2B4 immunoblotting of lysates from 293 cells transfected with a wild-type genomic T antigen expression construct shows MCV LT protein migrating at ~120 kDa and an additional ~57kDa T antigen isoform translated from an alternatively spliced, SV40 17kT antigen-like mRNA (Figure 14 C). The identity of the 57 kDa protein was confirmed by transfection of a 57kT cDNA clone. T antigen loci cloned from tumor derived MCV sequences express faster migrating LT proteins with sizes corresponding to the presence of truncating mutations. The MCV infected MKL-1 cell line also expresses a truncated LT consistent with a

premature stop codon mutation, whereas MCV negative UISO, MCC13, and MCC26 cell line lysates showed no specific reactivity (Figure 14 C).

By immunofluoresence microscopy, precise nuclear colocalization of CM2B4 occurs with green fluorescence when an MCV LT-GFP fusion construct is expressed in 293 cells (Figure 15 A). CM2B4 is highly specific for MCV and does not react to T antigens from JCV or BKV by

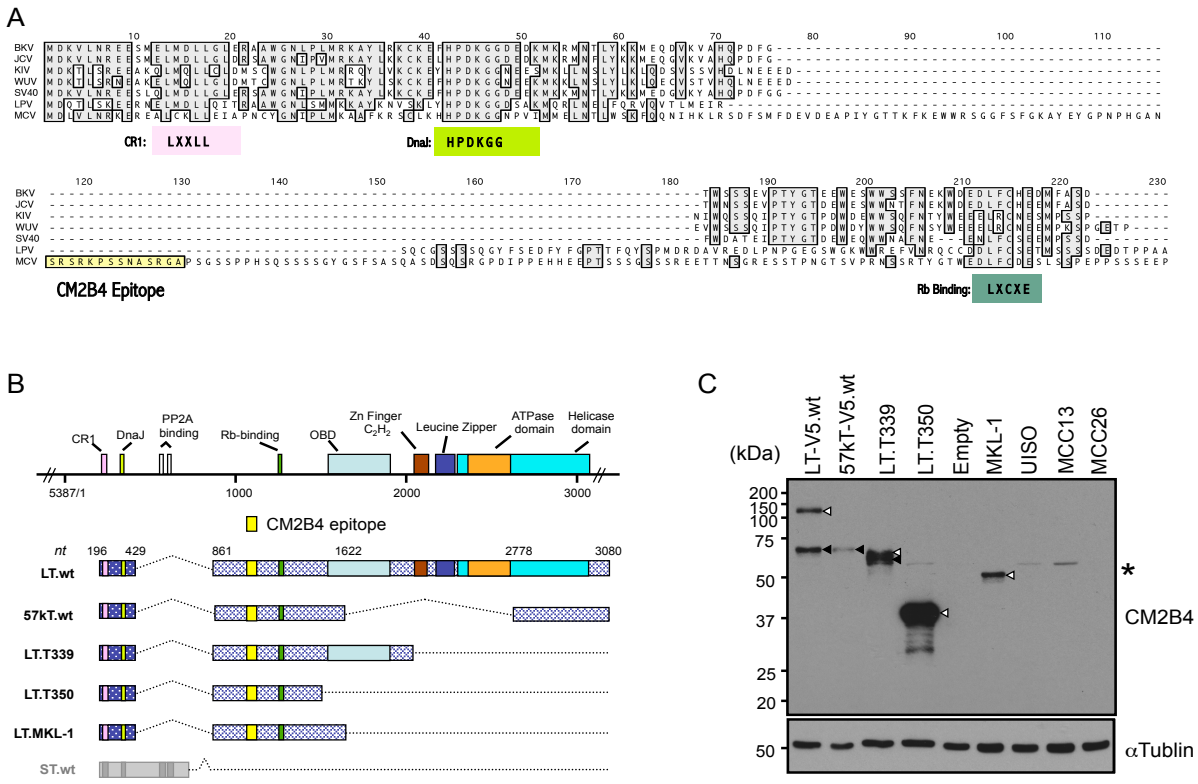



**Figure 14 CM2B4 – a monoclonal antibody detecting MCV T antigen**
(A) Amino acid alignment of MCV with known human polyomaviruses, SV40, and LPV. Region containing epitope site of CM2B4 is not conserved with these other polyomaviruses. (see Figure 37 in Appendix A for an alignment with all 9 human polyomaviruses) (B) Transcript diagram from various wild-type and tumor-derived MCV T antigen loci. Yellow box represents epitope site and does not overlap with putative small T antigen (gray). (C) CM2B4 western blotting of constructs diagrammed in (B) shows detection of full length or truncated tumor LT antigens (open arrowheads) and 57kT antigen (solid arrowhead). Only the truncated LT antigen is seen in the MCV infected MKL-1 cell line, whereas no specific protein is detected from lysates of MCV negative UISO, MCC13 and MCC26 cell lines. Asterisk (*) denotes non-specific reactivity.

immunofluorescence (Figure 15 B) or to T antigens from JCV, BKV or SV40 by immunoblotting (Figure 15 A). In contrast, an anti-SV40 T antigen mAb, PAb416, cross-reacts with T antigens from other SV40-group viruses including JCV and BKV, but not with MCV T antigen (Figure 15 B and 16 A). We examined 33 other anti-SV40 and anti-other polyomavirus T antigen mAbs [412], none show reactivity to MCV T antigen on immunoblotting (Table 4). Tissue immunohistochemistry with CM2B4 of an MCV DNA positive MCC biopsy also shows strong reactivity among tumor cells, but not surrounding tissues (Figure 15 C). CK20, a low molecular weight cytokeratin marker for MCC[316, 413-415] has a characteristic perinuclear dot-like pattern in CM2B4 positive cells (Figure 16 C). Similarly, examination of the MCV positive, MCC-derived MKL-1 cell line shows expression of LT protein in a diffuse nuclear distribution (Figure 16 B). CM2B4 does not react to JCV T antigen in a brain section with progressive multifocal leukoencephalopathy (Figure 16 C).

**Table 4 Polyomavirus antibodies tested against MCV T antigen**

| **Polyomavirus' T antigen Antibodies** | **References** |
|---|---|
| PAb101, 108 | Gurney, E.G. et al. , J Virol, 1986. |
| | Tack, L.C. et al., J Virol, 1989. |
| PAb 204,210, 211, 216 | Mole, S.E. et al. ,Philos Trans R Soc Lond B Biol Sci, 1987. |
| | Gannon, J.V. et al.,New Biol, 1990. |
| PAb 405,409,407,416,419,423,430, 431,433,441,442 | Harlow, E. et al., N.M. , J Virol, 1981. |
| PAb 602,603,605,606 | Mole, S.E.et al. , Philos Trans R Soc Lond B Biol Sci, 1987. |
| PAb 901,902 | Karjalainen, H.E. et al., J Virol ,1985. |
| | Thompson, D.L. et al.,Virology, 1990. |
| | Fu, T.M. et al., Virology, 1996. |
| Hamster Anti T antigen (HAT), Goat Anti T antigen (GAT) | Soule, H.R. and Butel, J.S., J. Virol, 1979 , Pipas, J. unpublished |
| 18-8-8 | Schaffhausen, B. et al., JBC, 1982 |
| F2A, F3 A/B, F4 A/B, F5 A/B and F8 A | Pallas, D.C. et.al., J. Virol, 1986. |
| Pab 762 | Dilworth, S.M. et al. , Nature 1994. |
| Anti LPV Hamster serum | Pawlita, M. et. al., Virol. 1985. |

Gifts of Dr. Gjoerup, Dr. Pipas, Dr. Pallas, Dr Pawlita and Dr Dilworth

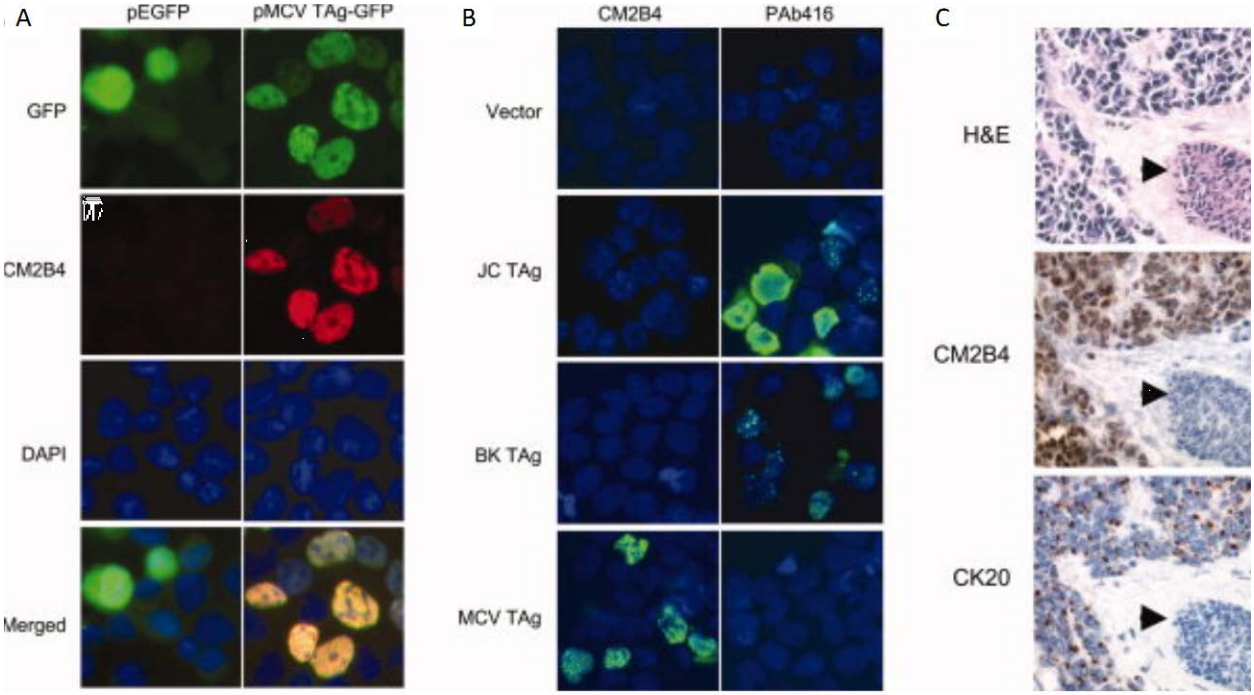



**Figure 15 CM2B4 immunofluorescence**
(A) MCV T antigen-EGFP fusion protein (green) colocalizes with CM2B4 staining (red) in 293 cells transfected with pMCV TAg-EGFP or pEGFP, an empty EGFP vector. (B) Immunofluorescence staining of 293 cells over-expressing T antigen constructs from JC, BK and MCV shows that CM2B4 specifically detects MCV T antigen. PAb416 shows reactivity with JC and BK T antigens, but not with MCV T antigen. (C) Sequential sections from an MCC skin excision shows small, round blue sheets of tumors cells (H&E) which express MCV LT (CM2B4) and CK20 proteins. Expression of MCV LT protein shows a diffuse nuclear pattern in MCC tumor cells but not in surrounding tissues including the epidermis, adnexal epithelium (arrow), endothelial cells or dermal fibroblasts.

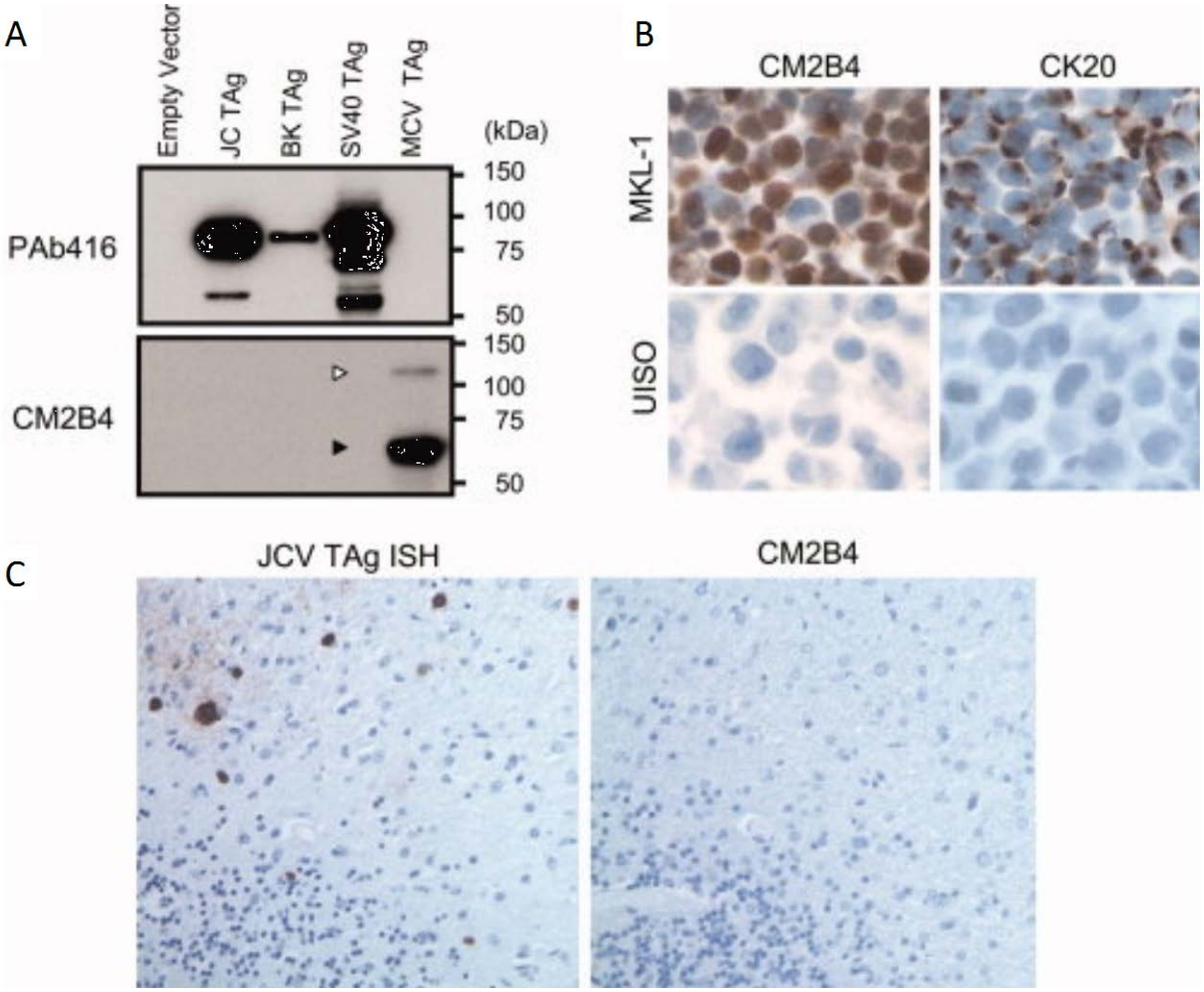



**Figure 16 CM2B4 does not crossreact with other polyomaviruses**

(A) Constructs encoding LT genes for JCV, BKV, SV40 and MCV were expressed in 293 cells and cell lysates were immunoblotted with CM2B4 or PAb416 antibodies. PAb416 cross-reacts with JCV and BKV LT proteins but not with MCV LT. (B) CM2B4 detects robust T antigen expression in a diffuse nuclear pattern in each cell from the MCV positive MKL-1 cell line. These cells retain perinuclear CK20 expression seen in Merkel cell carcinomas. (C) Brain tissue with progressive multifocal leukoenchephalopathy shows JCV infection of oligodendroglial cells by JCV specific *in situ* hybridization (left panel), and CM2B4 shows no reactivity to JCV antigens (right panel).

These results were extended by examining two tissue microarrays with CM2B4. Tissue microarray 1 (from Barcelona, Spain) contained 30 CK20-positive MCC, 6 CK20-negative but clinically suspect MCC, and 4 CK20-negative neuroendocrine control tumors. Of the 30 CK20 positive MCC, 21 (70%) were positive for LT protein expression. This suggests that the majority of MCC is caused by MCV infection whereas a subset of CK20+ MCC may have a different etiology. Of the 6 CK20-negative tumors diagnosed as MCC, none were positive for MCV LT protein expression but variably expressed neuroendocrine markers CD56, synaptophysin, or chromogranin (Table 5). A second tissue array (from Memorial Sloan-Kettering) with 36 cases of MCC was analyzed by IHC for CK20 and CM2B4. Thirty-two of the 36 tumors (89%) were positive for CK20 (Table 5). Positive staining was seen in 13 of 14 (93%) primary and 19 of 22 (86%) metastatic tumors. The staining was predominantly paranuclear dot-like, but membraneous staining was also focally seen.

Twenty-seven of the 36 tumors (75%) were immunoreactive for CM2B4. In the majority of tumors, the staining of tumor cell nuclei for CM2B4 was strong and homogeneous, labeling more than 75%, and up to 100% of the tumor cell population (Figure 15). Of the 14 primary tumors, 2 were immunonegative for CM2B4. One of them was a primary cutaneous neuroendocrine carcinoma, which had an associated in situ and invasive squamous cell carcinoma component (combined squamous cell and neuroendocrine carcinoma). Of the 22 metastatic tumors, 15 (68%) were immunopositive for CM2B4. One of the negative metastatic tumors was derived from a primary combined squamous cell and neuroendocrine carcinoma (the metastatic component had a pure neuroendocrine appearance).

Any combination of staining results for CK20 and CM2B4 was seen (Figure 17). Most (25 of 36) tumors expressed CK20 and stained with CM2B4. All but 2 tumors, which were positive for CM2B4, coexpressed CK20. Six CK20-positive tumors failed to react with CM2B4. Two tumors were negative for both CK20 and CM2B4 (Table 6).

The results of the immunohistochemical staining were compared with the patients' clinical information regarding potentially compromised immune status, and sun-exposure to the site of the primary tumor. Seven of the 12 CM2B4-immunopositive primary tumors were

from sites of chronic sun-damage (face and extremities with histologic evidence of marked solar elastosis in the adjacent non-neoplastic dermis). Five were from sun-protected sites (buttocks, with no significant solar elastosis). Of the group of patients with immunopositive tumors, one person was known to be clinically immunosuppressed (had chronic lymphocytic leukemia). There was also one patient with known immunosuppression (status post-organ transplant) in the group of patients with CM2B4-negative tumors (Table 6).

In combination, 46 out of 62 CK20 positive MCCs tested in the two tissue microarrays showed positive immunostaining for MCV LT by CM2B4. This 74.2% positivity is in sync with the initial finding that about 80% of MCC are positive for MCV (Table 7) [74].

**Table 5 Summary Of Merkel Cell Carcinoma Tissue Microarray I Staining**

| Merkel cell carcinoma | | | | | |
|---|---|---|---|---|---|
| **Case number** | **CM2B4** | **CK20** | **CD56** | **Chromogranin** | **Synaptophysin** |
| 1 | + | + | + | + | + |
| 2 | + | + | + | - | + |
| 3 | + | + | + | + | + |
| 4 | + | + | + | + | + |
| 5 | + | + | ND | ND | ND |
| 6 | + | + | + | - | ND |
| 7 | + | + | + | + | + |
| 8 | + | + | + | + | + |
| 9 | + | + | + | + | + |
| 10 | + | + | + | + | + |
| 11 | + | + | + | + | + |
| 12 | + | + | + | + | + |
| 13 | + | + | + | + | + |
| 14 | + | + | + | + | + |
| 15 | + | + | + | + | + |
| 16 | + | + | - | + | + |
| 17 | + | + | + | + | + |
| 18 | + | + | - | + | + |
| 19 | + | + | + | + | + |
| 20 | + | + | + | - | + |
| 21 | + | + | + | + | ND |
| 22 | - | + | + | + | + |
| 23 | - | + | + | + | ND |
| 24 | - | + | + | + | + |
| 25 | - | + | + | + | + |
| 26 | - | + | + | + | + |
| 27 | - | + | + | - | + |
| 28 | - | + | + | + | + |
| 29 | - | + | + | - | + |
| 30 | - | + | + | + | + |
| 31 | - | - | - | - | - |
| 32 | - | - | + | + | + |
| 33 | - | - | - | - | - |
| 34 | - | - | - | + | + |
| 35 | - | - | + | - | - |
| 36 | - | - | + | - | + |
| **Control: Neuroendocrine small cell carcinoma[†]** | | | | | |
| 37 | - | - | - | - | + |
| 38 | - | - | + | - | + |
| 39 | - | - | + | + | + |
| 40 | - | - | + | - | + |

ND, Not determined; CD56, chromogranin, synaptophysin staining performed on primary paraffi blocks prior to coring and microarray assembly.[†] 2 small bowel, one bladder, and one lung derived.

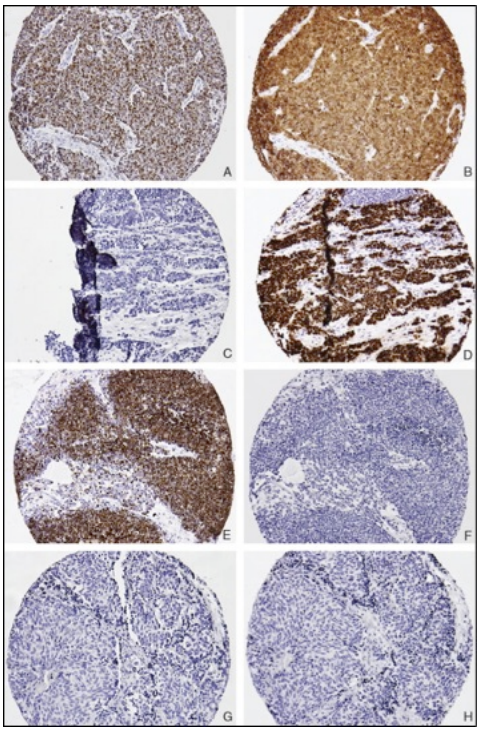


**Figure 17. Tissue microarray 2 staining (patterns of colabeling for CK20 and monoclonal antibody CM2B4).**
A and B, A tumor is positive for both CK20 (A) and CM2B4 (B)[most commonly seen]. C and D, A tumor negative for CK20(C), but immunoreactive for CM2B4 (D). E and F, A tumor expresses CK20 (G) and CM2B4(H). CK20 indicates cytokeratin 20.

**Table 6 Immunohistochemical detection of MCV with CM2B4 on tissue microarray-2 and comparison with CK20**

| | | |
|---|---|---|
| Primary MCC | CM2B4 (no. of pos cases/total no. of cases) 12/14 (86%) | CK20 (no. of pos cases/total no. of cases) 13/14 (93%) |
| Metastatic MCC | CM2B4 (no. of pos cases/total no.of cases) 15/22 (68%) | CK20 (no. of pos cases/total no. of cases) 19/22 (86%) |
| All MCCs | CM2B4 (no. of pos cases/total no. of cases) 27/36 (75%) | CK20 (no. of pos cases/total no. of cases) 32/36 (89%) |

CK indicates cytokeratin; FFPE, formalin-fixed and paraffin-embedded; MCC, Merkel cell carcinoma; pos, positive; no., number.

**Table 7 Summary of two tissue microarrays tested**

| Tissue Microarray | CK20+ | CM2B4+ | Both CK20+ and CM2B4+ |
|---|---|---|---|
| TMA 1 (from Hospital Universitari del Mar and Hospital Universitari Germans Trias I Pujol,Barcelona, Spain) | 30 | 21 | 21 (70%) |
| TMA2 (Memorial Sloan Kettering Hospital, New York, USA) | 32 | 27 | 25 (78.1%) |
| Total | **62** | 48 | **46 (74.2%)** |

*University of Pittsburgh study*

To correlate MCV LT protein expression with the level of MCV infection, we next developed a quantitative real-time PCR (qPCR) assay. DNA from ten tumors previously examined [74] were tested with primers designed to amplify regions of the T antigen and VP2. Seven of these Southern blot positive tumors had an average of 5.2 (range 0.8–14) T antigen DNA copies per cell consistent with clonal integration and viral genome concatenation (Table 8). Similar results were found using VP2 qPCR except for MCC345 and MCC347. These tumors had robust T antigen qPCR positivity (5 copies per cell) but minimal amplification of the VP2 gene, which may reflect integration and loss of this late viral capsid gene region as has been described by Kassem *et al.* [416] CM2B4 staining in CK20+ MCCs was concordant with qPCR results for all cases except MCC344, which showed abundant viral DNA but was negative with CM2B4 staining (Table 8).

**Table 8 qPCR Detection of MCV genome in MCC (University of Pittsburgh study)**

| MCC Tissue | MCV genome copies per cell[*] | | Genomic | Immunostaining | |
|---|---|---|---|---|---|
| | T Ag | VP2 | Southern[†] | CM2B4 | CK20[§] |
| MCC337 | $< 10^{-3}$ | 0 | - | - | + |
| MCC339 | 5.2 | 11.1 | + | + | + |
| MCC343 | 0 | 0 | - | - | + |
| MCC344 | 6.3 | 13.7 | + | - | + |
| MCC345 | 4.9 | $< 10^{-3}$ | + | + | + |
| MCC346 | $< 10^{-3}$ | 0 | - | - | + |
| MCC347 | 1.6 | 0 | + | + | + |
| MCC349 | 3.3 | 8.0 | + | + | + |
| MCC350 | 0.83 | 3.0 | + | NT[‡] | NT |
| MCC352 | 14.3 | 47.5 | + | + | + |

[*] RNaseP copy number was divided by two to determine cellular equivalent of DNA. [†] MCV positivity was previously examined by Southern blotting (Feng et al., 2008). [‡] NT, No paraffin embedded MCC tissues to evaluate. [§] CK20 expression was previously examined by immunostaining (Feng et al., 2008).

**Table 9 Comparison of MCV detection by PCR with CM2B4 staining (Memorial Sloan-Kettering Study)**

| Age | Sex | Site | Primary or Metastasis | PCR-MCV | IHC-MCV | IHC-CK20 |
|---|---|---|---|---|---|---|
| 80 | F | Skin | Primary | Pos | 4+ | 4+ |
| 56 | M | Skin | Primary | Pos | NA | NA |
| 87 | M | Skin | Primary | Pos | 4+ | 4+ |
| 58 | M | Skin | Primary | Pos | 3+ | 3+ |
| 86 | M | Skin | Primary | Pos | 4+ | 4+ |
| 88 | M | Skin | Primary | L Pos | Neg | 3+ |
| 62 | M | Skin | Primary | L Pos | Neg | 2+ |
| 68 | F | Skin | Primary | Pos | 3+ | 3+ |
| 85 | F | Skin | Primary | Neg | Neg | 3+ |
| 59 | M | Lymph node | Metastasis | Pos | 4+ | 3+ |
| 72 | F | Lymph node | Metastasis | Pos | 3+ | 3+ |
| 52 | M | Lymph node | Metastasis | L Pos | 4+ | 1+ |
| 60 | M | Lymph node | Metastasis | Pos | 4+ | 4+ |
| 77 | M | Lymph node | Metastasis | Pos | Neg | 4+ |
| 52 | F | Lymph node | Metastasis | Neg | Neg | Neg |
| 76 | M | Pancreas | Metastasis | Pos | 4+ | 3+ |
| 68 | M | Small Bowel | Metastasis | Pos | NA | NA |

CK20 indicates cytokeratin 20; F, female; FFPE, formalin-fixed paraffin-embedded; IHC, immuno-histochemistry; L Pos, Low positivity ( $< 10^{-2}$ copies/cell); M, male; MCV, Merkel cell virus; NA, not available for analysis (insufficient tissue); Neg, negative; PCR, polymerase chain reaction; Pos, positive.

*Memorial Sloan-Kettering study*

Frozen tumor tissue samples of MCC from 17 different patients were examined by qPCR for the presence of MCV DNA. The samples included 9 primary cutaneous tumors (5 from sun-exposed sites, 4 from sun-protected sites), 6 lymph node metastases, and 2 visceral metastases (Table 9). MCV was identified in 15 of 17 tumors (88%), with a positive PCR result seen in 8 of 9

primary and 7 of 8 metastatic tumors (Table 9).

The one MCV-negative primary tumor was from the right leg of an 85-year-old woman. Its histology was re-reviewed: the primary tumor was a combined superficial squamous cell and neuroendocrine carcinoma that had metastasized to the regional lymph node. The metastasis had a pure neuroendocrine phenotype.

The one MCV-negative metastatic tumor was from a neck lymph node of a 76-year-old man who presented with metastatic MCC in the absence of a known primary tumor. The lymph node metastasis was re-reviewed. The histologic and immunohistochemical features were typical of MCC with positive paranuclear dot-like staining for CK20.

Conventional tissue sections of FFPE portions from the same tumors that had been analyzed by PCR were examined by IHC, using mAb CMB24. An additional section from each tumor was also stained for CK20. Sufficient tissue for this analysis was available in 15 of the 17 cases. Thirteen of them were positive for MCV by PCR.

Ten tumors were immunoreactive for CM2B4 (67% of all tumors with tissue available for IHC, 77% of all PCR-positive tumors). All tumors, which were immunoreactive for CM2B4, were also positive for MCV by PCR. Seven of the CM2B4-positive tumors showed strong nuclear labeling in the vast majority (>75%) of the tumor cell population (Table 9). Three tumors showed only partial labeling for CM2B4 (3+ staining, that is, positive labeling of >=50% to 75% of tumor cells). All but one tumor was positive for CK20.

The one tumor, which was immunonegative for both CM2B4 and CK20, but contained MCV by PCR, was a lymph node metastasis. The clinical and histologic findings were re-reviewed. The tumor was judged to be metastatic MCC, because there was a known primary cutaneous tumor, which, like the metastasis, showed classic cytologic features of MCC. Furthermore, the tumor was immunohistochemically positive for CAM5.2 in a dot-like paranuclear staining pattern and also positive for chromogranin.

### 2.2.2 PBMC infection with MCV

To assess MCV lymphotropism as seen in other human polyomaviruses [417, 418], 83 whole PBMC DNA samples collected from persons undergoing genetic testing for Factor V Leiden deficiency were tested by qPCR. These samples were collected mainly from adults (average of 60 years, range 1–78 years) with 73 (88%) samples from persons over 18 years of age. None were positive for viral DNA. However, among 21 PBMC collected from adult HIV/AIDS patients without MCC, 2 (9.5%) were positive by either T antigen ($2.8 \times 10^{-3}$ copies per cell) or VP2 ($8.8 \times 10^{-3}$ copies per cell) primers and one (5%) was positive with both primers (T antigen, $7.9 \times 10^{-3}$ copies per cell; VP2, $6.0 \times 10^{-3}$ copies per cell). Although we are unable to verify MCV lymphotropism in this study since examining whole PBMC is unlikely to reliably detect MCV due to the dilution effect from nonpermissive PBMC, increased rates of detection in HIV positive individuals suggest that immunosuppression may lead to reactivation of virus.

### 2.2.3 Survey of hematolymphoid malignancies for MCV infection

qPCR was further performed on tissue DNA from 104 T cell-associated and 161 B cell-associated malignancies, 19 myeloid disorders and 41 other tumors including Hodgkin lymphoma and post-transplant lymphoproliferative disorders (Table 10). Of these 325 tumors, 7 (2.2%) were positive for either T antigen or VP2 DNA and two were positive for both. No consistent pattern of virus infection was found among these malignancies: 1 (3%) of 33 chronic lymphocytic leukemia, 1 (7.1%) of 14 non-Hodgkin lymphoma, not otherwise specified (NOS), 2 (3.1%) of 65 diffuse large B cell lymphoma, 1 (11%) of 9 marginal zone lymphoma and 1 (3.3%) of 30 Hodgkin lymphoma (Table 10). Copy numbers for these positive hematolymphoid malignancies however, were all 2–4 logs lower than MCV-positive MCC tumors (Table 11). These results were confirmed by CM2B4 staining of commercial tissue microarrays of hematolymphoid tumors. Of 122 B cell lymphomas, 17 T cell lymphomas, one myeloid disorder and 2 Hodgkin lymphomas

examined, none showed evidence for LT protein expression (Table 12). Thirty-one healthy lymphoid control tissues were also negative for MCV T antigen.

### 2.2.4 Immunohistochemial analysis of combined primary cutaneous squamous and neuroendocrine carcinomas

Seven combined primary skin tumors, which contained an intimately admixed component of both squamous cell and neuroendocrine carcinoma (Figure 18) were analyzed by IHC for CK20 and CM2B4. Although the neuroendocrine component of all tumors was positive for CK20 (Table 13), none of the cases was immunoreactive with CM2B4 in either the squamous or neuroendocrine component (Figure 18).

### 2.2.5 Immunohistochemical analysis of 26 primary pulmonary (small and large cell) neuroendocrine carcinomas

Twenty-six primary pulmonary tumors (16 small cell and 10 large cell neuroendorcrine carcinomas) were analyzed for immunoreactivity with CM2B4. All had been previously characterized by a panel of immunomarkers for clinical purposes and found to be positive for at least one neuroendocrine marker (chromogranin, synaptophysin, CD56). IHC for TTF-1 was performed on 21 of the 26 tumors. Sixteen of them (76%) were immunoreactive for TTF-1. All 26 pulmonary neuroendocrine carcinomas were retested for CK20 expression and confirmed to be negative. None of them showed any nuclear staining with CM2B4.

**Table 10 qPCR Detection of MCV genome in hematolymphoid malignancies.**

| Hematopathological samples studied | No. Tested | No. MCV positive (% MCV positivity) |
|---|---|---|
| **B cell-associated lymphomas** | | |
| Chronic lymphocytic leukemia | 33 | 1 (3.0) |
| Non-Hodgkin lymphoma, NOS | 14 | 1 (7.1) |
| Diffuse large B cell lymphoma | 65 | 2 (3.1) |
| Follicular lymphoma | 14 | 0 |
| Acute lymphoblastic leukemia | 11 | 0 |
| Primary effusion lymphoma | 2 | 0 |
| Mucosa-associated lymphoid tissue lymphoma | 5 | 0 |
| Mantle cell lymphoma | 8 | 0 |
| Marginal zone lymphoma | 9 | 1 (11) |
| **T cell-associated lymphomas** | | |
| Acute lymphoblastic leukemia | 10 | 0 |
| Large granular lymphocyte leukemia | 1 | 0 |
| Mycosis fungoides | 11 | 0 |
| T cell lymphoma, unspecified | 82 | 1 (1.2) |
| **Myeloid disorders** | | |
| Chronic myelogenous leukemia | 5 | 0 |
| Acute myeloid leukemia | 11 | 0 |
| Myelodysplastic syndrome | 3 | 0 |
| **Others** | | |
| Hodgkin lymphoma | 30 | 1 (3.3) |
| Post transplant lymphoproliferative disorder | 11 | 0 |
| Total | 325 | 7 (2.2%) |

**Table 11 MCV genome copies in MCV-positive hematolymhoid malignancies**

| Hematolymphoid malignancies positive for MCV | Copies per cell T Ag | VP2 |
|---|---|---|
| Chronic lymphocytic leukemia (#354*) | $1.2 \times 10^{-2}$ | $8.4 \times 10^{-3}$ |
| Non-Hodgkin lymphoma (#351) | $1.5 \times 10^{-3}$ | $< 10^{-3}$ |
| Diffuse large B cell lymphoma (#229) | $1.1 \times 10^{-3}$ | 0 |
| Diffuse large B cell lymphoma (#500) | $3.8 \times 10^{-3}$ | $1.1 \times 10^{-3}$ |
| Marginal zone lymphoma (#781) | $5.8 \times 10^{-3}$ | 0 |
| T cell lymphoma (#18) | $3.2 \times 10^{-3}$ | 0 |
| Hodgkin lymphoma (#86) | $1.8 \times 10^{-3}$ | $2.9 \times 10^{-3}$ |

* Blinded testing number.

**Table 12 MCV LT protein detection in hematolymphoid malignancies**

| | Result |
|---|---|
| **B cell malignancies** | 0/122 |
| **T cell malignancies** | 0/17 |
| **Myeloid disorders** | 0/1 |
| **Hodgkin lymphoma** | 0/2 |
| **Normal lymphoid tissues** | |
| Normal splenic tissue | 0/18 |
| Normal lymph node | 0/13 |

*These cases are derived from tissue microarray slides #SP482t, #LM801t, #NHL801t from BioMax. See http://www.biomax.us for subclassification and diagnosis.

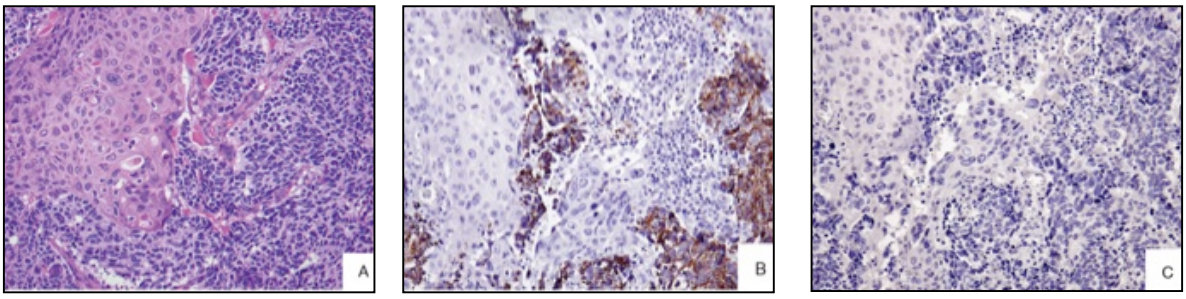



**Figure 18 Combined cutaneous squamous and neuroendocrine carcinoma.**
(A) The tumor shows a biphenotypic appearance (hematoxylin and eosin-stained section). It contains a pink squamous and a small cell neuroendocrine component. (B) The neuroendocrine component is immunoreactive for cytokeratin 20. (C) The neuroendocrine component fails to react with CM2B4.

**Table 13 Combined squamous and neuroendocrine carcinomas of the skin**

| Age (y) | Sex | Site | Associated Disease | IHC-CM2B4 | IHC-CK20 |
|---|---|---|---|---|---|
| 65 | M | Skin of scalp | None | Negative | Positive |
| 79 | M | Skin of face | None | Negative | Positive |
| 85 | F | Skin of face | None | Negative | Positive |
| 78 | M | Skin of calf | None | Negative | Positive |
| 74 | F | Skin of wrist | None | Negative | Positive |
| 67 | M | Skin of face | CLL | Negative | Positive |
| 75 | F | Skin of face | CLL | Negative | Positive |

CK indicates cytokeratin; CLL, chronic lymphocytic leukemia; F, Female; IHC, immunohistochemistry; M, Male.

### 2.2.6 MCV and chronic lymphocytic leukemia

Koljonen *et al* described an association between chronic lymphocytic leukemia (CLL) and MCV–positive Merkel cell carcinoma (MCC) in a recent large population-based survey from Finland [419]. CLL has been reported as a tumor significantly associated with MCC, validating the general findings of this study [66, 67]. This association suggests the possibility that MCV may play a role in CLL as well as in MCC.

As discussed above, we previously examined 33 CLL tissues by qPCR and 12 by MCV large-T antigen immunostaining. All specimens were negative for MCV genome or protein expression; however, none of these cases were associated with a history of MCC.

Given the epidemiological relationship between MCC and CLL, we examined additional CLL cases for evidence of MCV infection. Ten peripheral blood samples with CLL (WBC counts ranging from $13.2 \times 10^9 - 84.3 \times 10^9$ cells per L) were harvested and tested for the presence of MCV DNA. One displayed low MCV positivity VP2 ($2.0 \times 10^{-3}$ copies per cell). Twelve additional paraffin-embedded biopsies with CLL were examined by CM2B4 staining only. All CLL cases were uniformly negative for MCV T antigen protein expression.

Although the occurrences of these 2 neoplasms are significantly associated, the uncommon nature of MCC has resulted in only rare published cases [420, 421]. We obtained and tested 2 cases of concurrent CLL and MCV. We specifically sought evidence of MCV T antigen protein expression in CLL cells to circumvent the extremely high sensitivity of PCR, which can detect incidentally infected cells in tissues.

**Case 1.**

A 74-year-old female with CLL presented with a right thigh mass and enlarged right groin mass. Pathologic examination of thigh mass reveals immunohistochemically confirmed MCC. The groin resection contained metastatic MCC to lymph nodes with CLL. Examination of a lymph node by CM2B4 and CD20 immunohistochemical staining shows MCV T antigen protein expression only in the metastatic MCC (Figure 19 A, C) but not in CD20-positive CLL cells (Figure 19 B).

**Case 2.**

A 66-year-old male diagnosed with CLL 12 years earlier presented with a left forearm bump (diagnosed as MCC). Biopsy and left axillary dissection reveals cytokeratin-20–positive MCC metastatic to 9 of 80 lymph nodes, virtually all having effacement of normal architecture by CLL. A node containing metastatic MCC illustrates robust nuclear expression of MCV T antigen in tumor cells (Figure 19 D), whereas the CLL component is negative (Figure 19 E).

We also collected 18 CLL and 18 acute lymphoblastic leukemia (ALL) age-matched sera. Ten of

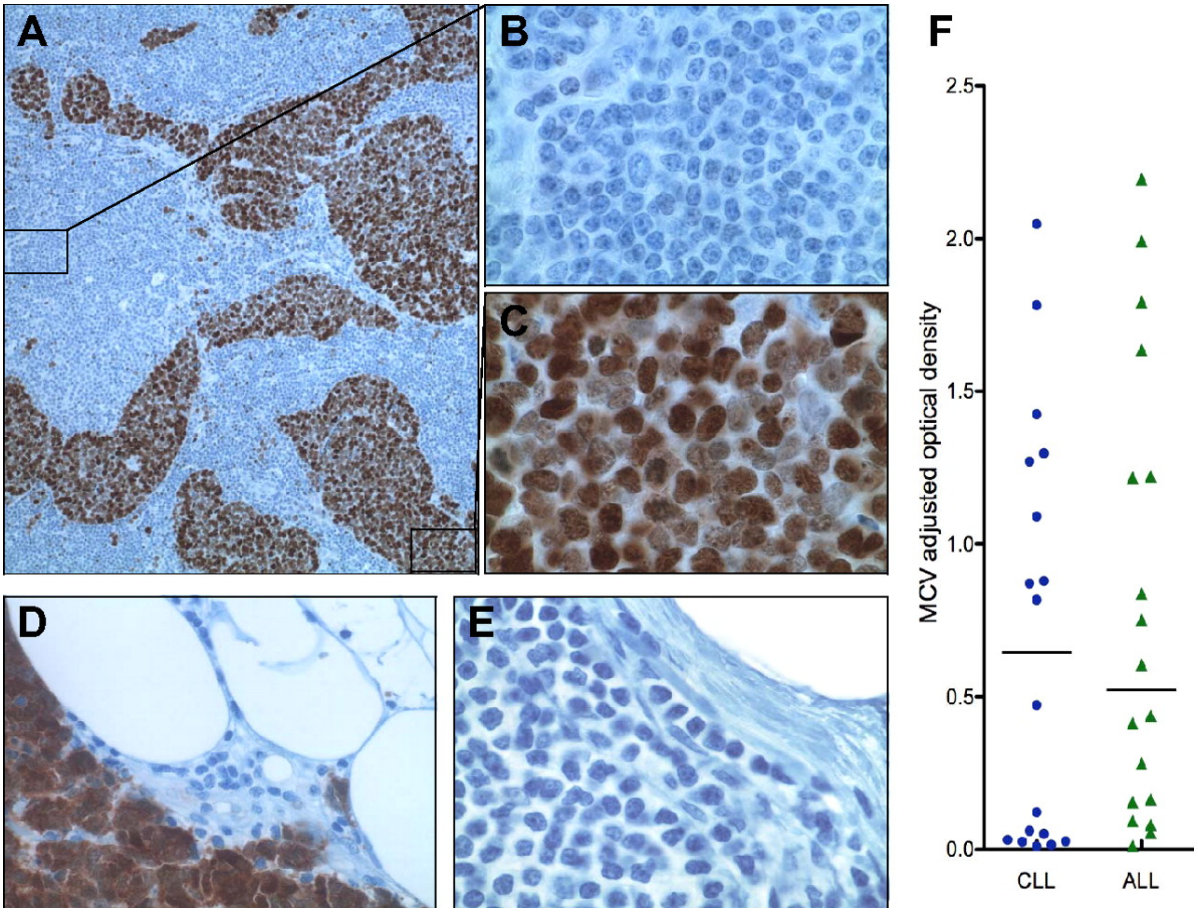



**Figure 19 Lack of evidence for direct involvement of MCV in CLL**

(A) Case 1: lymph node with both CLL (10x/0.30 NA; B) and metastatic MCC (100x/1.40 NA oil objective; C) immunohistochemically stained (Dako EnVision+ Kit) with CM2B4, and counterstained with hematoxylin. Case 2: MCC metastatic to lymph node showing strong nuclear expression of MCV T antigen expression (40x/0.75 NA; D) and CLL (40x/0.75 NA; E) demonstrating effacement of normal nodal architecture with a population of cells comprised predominantly of small lymphocytes with scant cytoplasm, round nuclei, and clumped chromatin. Images were acquired on an Olympus Provis AX70 using Spot 3.5.9 software for Mac OS (Diagnostic Instruments). All images were processed using Adobe Photoshop CS3. (F) Anti-MCV antibody levels (colored dots and triangles), as measured with MCV virus-like particle enzyme immunoassay (VLP ELA), are not significantly different in sera from CLL and ALL patients. Median MCV optical density values are indicated by horizontal lines, with bars representing interquartile values.

18 (55.6%) CLL and 12 of 18 (66.7%) ALL patients showed serologic evidence for MCV exposure (Figure 19 F). The median MCV IgG titer for the CLL sera (0.645 OD units) was similar to, and not statistically different from, ALL patients (0.521 OD units).

These findings together indicate that MCV is not likely to be directly involved in chronic lymphocytic leukemogenesis. We also do not find MCV infection more commonly among CLL patients than among a representative control group [116, 160]. Other possible explanations for the association between these 2 cancers include CLL-related immunosuppression predisposing to MCC or a pathologic immune response to MCV infection in persons predisposed to CLL.

## 2.3 DISCUSSION

Several independent groups have confirmed that MCV infection is present in the majority of MCC but not in most tissues from control patients [74, 416, 422, 423] (also see Chapter 3). The monoclonal pattern of MCV infection [74] and the pattern of tumor-virus derived mutations in T antigen genes [112] provide strong evidence that MCV causes a subset of MCC. We now show that MCC tumor cells, but not adjacent non-neoplastic cells, are infected with MCV and express MCV T antigen protein in most MCV-positive MCC tumors. This provides additional evidence that MCV is a human tumor virus.

Quantitative PCR shows that MCC biopsies are generally positive for the virus at >1 copy per tumor cell. This is consistent with clonal integration being common in MCV-positive MCCs and with antibody staining results that revealed nearly all MCV-positive MCC tumor cells harbor virus. Expression of the MCV T antigen in tumor cells may provide clues on the molecular origins of these tumors since T antigens from other polyomaviruses have been found to be oncoproteins. Animal models show that continued expression of polyomaviral T antigen, for example, is required to induce and maintain viral transformation [424, 425]. Additional studies are needed to determine whether MCV LT protein or other alternatively spliced T antigen

isoforms contribute to cell transformation [112]. Our study does not provide this formal proof but it does demonstrate that T antigen is a useful marker for MCC tumor cells.

Although a substantial fraction of adult humans may be infected with MCV [74], we only find evidence for high virus copy number infection in MCC. Approximately 74.2% CK20+ MCCs were positive for LT protein expression in our study. This suggests that the majority of MCC is caused by MCV infection whereas a subset of CK20+ MCC may have a different etiology. Other clinically suspect MCCs lacking CK20 expression, were all negative for LT protein expression. Additional clinical validation is needed for CM2B4 staining as a tissue marker for MCV, but this preliminary evidence suggests it is useful in identifying subsets of tumors associated with MCV infection.

There was no apparent association of MCV detection with anatomic site/mode of sun exposure and major clinical immune suppression of the patients. This is not a surprise. Less than 10% of MCC occur in patients with major immunosuppression, such as HIV, associated lymphoma or status postorgan transplantation [62]. As the majority of MCCs had previously been found to be positive for MCV [62, 68, 74, 416, 422] it was already evident that MCV-associated MCC could not be restricted to severely immunosuppressed patients. Similarly, given the high frequency of MCV in MCC and the known anatomic distribution of the tumor [426], it was also expected that MCV could be found in tumors of both sun-exposed and sun-protected sites.

With regard to its potential diagnostic use, our findings suggest that mAb CM2B4 will likely emerge as a valuable adjunct reagent for the differential diagnosis of MCC from histologic mimics, in particular for the distinction of MCC from a cutaneous metastasis of a pulmonary neuroendocrine carcinoma, when current panels of immunomarkers do not allow a definitive diagnosis [310, 313, 314, 318, 427-429]. None of the 26 neuroendocrine carcinomas of the lung, which we examined in this series, labeled with CM2B4. Further investigations, however, are needed to examine the sensitivity and specificity of CM2B4 on a larger set of tumors, especially neuroendocrine carcinomas from sites other than skin and lung.

Although our results support prior observations that the majority of MCCs are

associated with MCV, not all of them are. Lack of virus detection may in part be due to technical reasons, for example, tissue preservation of the specimen, or virus mutation that does not permit PCR-amplification with the primers used, and/or may also lead to lack of immunoreactivity for CM2B4. However, it is also possible that there is a subset of MCCs, which may not be associated with MCV. Consequently, MCC may be divided into 2 broad categories—MCV-positive type, representing the majority of case, and MCV-negative type.

It is of interest in this regard that none of the 7 combined cutaneous squamous and neuroendocrine carcinomas were immunoreactive with CM2B4. Furthermore, the one PCR-negative primary MCC tumor turned out to be a combined squamous and neuroendocrine carcinoma. Such combined tumors are rare and account for only a minor portion of MCCs. Population-based data are lacking. In the patient review of 29 cases of MCC, Walsh found 3 tumors, in which MCC was associated with a superficial squamous cell carcinoma [430]. In the institutional data set of Merkel cell tumors from MSKCC, approximately 5% of patients with a diagnosis of MCC have a neuroendocrine tumor that is associated with a superficial invasive squamous cell carcinoma (KJ Busam, unpublished observations). Although it is difficult to draw definitive conclusions from such as small sample size, it is tempting to speculate that neuroendocrine carcinomas arising in association with a squamous cell carcinoma may develop via a MCV-independent pathway and are different from “classic” MCCs. One may also question whether or not such combined MCV-negative tumors should be designated as “MCC.”

Historically, pathologists have classified the combined tumors as variants of MCC, because phenotypically, they are primary cutaneous carcinomas with a predominant neuroendocrine phenotype (ie, after the differentiation pathway of Merkel cells). Except for the associated presence of a (usually minor) superficial squamous cell carcinoma component, the histologic and immunohistochemical features of the dominant tumor component are indistinguishable from de novo “pure” MCC [430-433]. Furthermore, the combined tumors tend to behave clinically similar to pure MCCs [430], and their metastases tend to have a pure neuroendocrine appearance. It is of interest that among the patients with combined tumors, the proportion of those with chronic lymphocytic leukemia was higher than expected [62], but

this may be related to referral bias to a large cancer center.

We find little support for the possibility that MCV also contributes to CLL, a malignancy that can be associated with MCC, or other common hematolymphoid malignancies. Methods in sample collection did not allow us to screen specimens simultaneously for both viral DNA and T antigen expression, but results from both assays were consistent and no CLL cases were MCV positive at levels found among MCCs infected with the virus. In concurrent cases of CLL and MCC we showed that although the MCC part stains positive for MCV LT expression, the CLL part does not. MCC arising in the setting of CLL may result from CLL-induced immunosuppression [65]. These results do not exclude the possibility that less common hematolymphoid or nonlymphoid malignancies might be caused by this virus, and surveys of other tumors and diseased tissues are warranted.

Quantitative PCR detects MCV infection in peripheral blood cells from infected persons but at levels that approach the technical limit of our technique ($\sim 10^{-3}$ DNA copies per cell). This is similar to other lymphotropic viruses, such as Kaposi's sarcoma herpesvirus, in which only a fraction of PBMC are positive from persons known to be infected with the virus [409, 434]. Examining whole PBMC is unlikely to reliably detect MCV in most infected persons due to the dilution effect from nonpermissive peripheral blood cells. For this reason, it is not surprising that low rates of positivity are present for whole PBMC samples from persons screened for an unrelated genetic marker (Factor V Leiden) or HIV/AIDS patients. Determining true human prevalence for MCV infection requires the development of serologic tests that can accurately measure exposure to this virus [380]. Tolstov *et al*. and others achieved this and the prevalence of MCV in adults is determined to be between 42 to 70% [160, 380].

## 3.0 LITERATURE REVIEW OF MCV POSITIVITY IN MCC AND OTHER TISSUES

Various groups confirmed our study and found MCV in about 70-80 % of MCC tissues tested. Researchers from around the world also tested MCV status in MCC, other diseases and healthy human tissues by PCR, quantitative PCR, immunostaining for MCV T antigen (with CM2B4 and other antibodies), and Southern blotting techniques. The following tables summarize a literature review describing the results of the different groups.

### 3.1 MONOCLONAL INTEGRATION OF MCV IN MCC AT >1 COPY PER CELL

In the initial investigations Feng and colleagues found 7 out of 10 MCC tumors from different patients to be positive for MCV by Southern blotting. Subsequently, we showed these had high copy numbers in the range of 1.6 to 48 copies per tumor cell [68, 74]. This high rate of MCV positivity in MCC has been confirmed by many other laboratories in various countries (including USA, Germany, France, Italy, Finland, Switzerland, Spain, Canada, Australia, Japan and Korea) with 74.04% (1743/2354) of MCC cases being positive for MCV DNA (Table 14).

Clonal integration of MCV in MCC tumor genome was confirmed by Southern blotting and 3'-RACE in both primary tumors and their metastasis [74]. This suggests that MCV infection occurred prior to initial monoclonal expansion of cells and tumor development. This viral integration pattern is analogous to that seen in high-risk human papillomavirus integration in cervical cancer cells and supports the belief that virus–associated tumors are biological

accidents [19]. Although virus concatemers are found integrated at a single integration site in each tumor tested, there is no consistent or preferential site of integration in the cellular genome. In the viral genome however, the breakpoints occur mainly in the second exon of the LT, after the pRb-binding site [74, 367, 435, 436].

Monoclonal integration and replication incompetence refutes the possibility of MCV as a coincidental passenger virus in MCC. Although, one study did report MCV viral particles in 2 of 5 MCCs by electron microscopy [402], antibodies against the VP1 protein has not detected any expression in tumors so far [356, 437].

## 3.2 MCV IS SPECIFIC FOR MCC

Numerous surveys on other tumor types have been performed including various hematolymphoid malignancies, neuroendocrine carcinomas, various skin disorders, other cancers (including breast, prostate, lung, liver and colon), CNS (central nervous system) tumors, other virus-related disorders and many other diseases (Table 15). MCV was found in only 8.5% of 6470 non-MCC disease samples tested. In cases where MCV was found in non-MCC tumors it is generally at low abundance (2-3 logs lower in copy per cell than found in MCC) and the data do not point to any association between the two but may suggest incidental infection [68, 70-73, 382, 394, 435, 438-447].

Researchers also tested samples from HIV+ individuals and found that 25.5% cases harbored MCV at low copies (Table 16). This is not surprising, as immunosuppression is a major risk factor for MCC and AIDS patients have a 13 fold higher risk of developing MCC [60]. The increased detection of MCV in HIV positive individuals suggest that the immune system actively keeps MCV under check in healthy individuals and this policing is lost in the immunosuppressed.

Since 2008, various studies on MCV detection show MCV to be a widespread but previously unrecognized human infection. Other than MCC, MCV was detected in 9/84 non-

MCC tissues in the initial study [74] at low levels (at the limits of PCR sensitivity). Since then various studies have detected MCV in healthy skin flora [369, 394, 440, 448, 449], respiratory tract samples and nasopharyngeal aspirates [391, 450-452], saliva [394], gut [328, 453], lymphoid tissue [68, 328], urine [393, 444, 447, 454] and whole blood from healthy donors [444, 453, 455-457] (Table 17). Skin showed the highest level of MCV positivity (56.4%) as compared to the digestive system, respiratory system, lymphatic system, blood, urinary system and other tissues. These studies claim MCV as a part of the healthy skin flora, which otherwise harmless, can undergo certain mutational events and cause tumors in susceptible hosts (immunosuppressed, older in age).

## 3.3 PROGNOSTIC VALUE OF MCV DETECTION IN MCC

MCV status in MCC has become an important clinical determinant and may even have prognostic value. Sihto *et al*., found that MCV positive MCC patients from Finland have a significantly reduced disease-specific mortality, as compared to MCV negative MCC patients [328]. Laude *et al*. also analyzed associations between MCV viral load and survival [436]. They reported similar results, demonstrating that patients with more than 1 copy per cell spent longer periods in complete remission. However, upon analyzing viral presence in peripheral blood cells, they concluded that MCC patients with MCV in PBMC samples had shorter overall survival times and fared worse than MCC patients without virus shedding.

Using PCR based assays such as the ones described in this dissertation, researchers have detected MCV abundantly in MCC tumors. Various other cancers - including skin cancers, hematolymphoid tumors, neuroendocrine tumors, and other cancers - have been surveyed and found to be negative for MCV. However, MCV has been detected at various human body parts, making it more evident that it is a part of the normal human viral flora [68, 74, 394, 440, 448-450]. This complicates the association between viral DNA load and MCC prognosis.

The use of MCV immunohistochemistry with CM2B4 antibody is a less sensitive but more robust technique than PCR. It has the advantage of detecting a functional outcome of MCV presence, namely expression as well as nuclear localization of MCV LT oncoprotein. Bhatia *et al*. used CM2B4 based detection of MCV and reported that MCV positive tumors are associated with better prognoses [458]. However, Schrama *et al*. did not find any differences in clinical behaviors and prognoses between MCV positive and MCV negative MCCs [459]. Further studies with larger sample sizes are still needed to clearly determine any association between the two.

Recent studies have also found a link between increased serum antibodies to MCV T antigen and disease progression in MCV positive MCCs [384, 387]. Accordingly, disease progression may be analyzed and marked by a loss of immune surveillance control, leading to viremia and increased antibodies against viral T antigens.

Nonetheless, MCV status has become an important diagnostic tool in the field of MCC study. Most importantly, determining whether an MCC tumor is MCV positive or not, gives us a clue about its etiology. Although both virus positive and virus negative MCCs are clinically comparable, it is only a matter of time before more specific, MCV-positivity-based disease management regimes will become useful.

**Table 14 MCV Presence in MCC Tissues (Review of Literature)**

| Country | Total | MCV +ve | Method Used | Reference |
|---|---|---|---|---|
| USA | 10 | 8 (80%) | PCR (LT and VP1) sequence analysis, Southern hybridization,and CM2B4 Staining | Feng *et al*, Science 2008 and Shuda *et al*.,Int J Cancer 2009 |
| USA | 16 | 11 (68.7%) | qPCR (LT) | Garneski *et al*., J Invest Dermatology 2009 |
| USA | 13 | 7 (53.8%) | PCR (LT and VP1) | Ridd *et al*., J Invest Dermatology, 2009 |
| USA | 41 | 32 (78%) | PCR (LT and VP1) | Duncavage *et al*., Modern Pathology 2009 |
| USA | 53 | 42 (79.2%) | qPCR and CM2B4 staining | Busam *et al*., Am J Surg Pathol 2009 |
| USA | 31 | 24 (77.4%) | qPCR (VP1) | Carter *et al*., J Nat Cancer Institute 2009 |
| USA | 22 | 13 (59.1%) | qPCR (LT) | Paulson *et al*., J Invest Dermatol 2009 |
| USA | 23 | 17 (73.9%) | PCR (Tag) | Bhatia *et al*., Int J Cancer 2010 |
| USA | 7 | 6 (85.7%) | qPCR (LT and VP1) | Loyo *et al*., Int J Cancer 2010 |
| USA | 6 | 4 (66.7%) | PCR (LT) | Lewis *et al*., Oral Surg, Oral Medicine, Oral Pathol, Oral Radiolo and Endodon 2010 |
| USA | 20 | 15 (75%) | CM2B4 Staining | Reisinger *et al*., J Am Acad Dermatol 2010 |
| USA | 4 | 2 (50%) | qPCR (LT) | Toracchio *et al*., Emerg Infect Diseases 2010 |
| USA | 80 | 59 (73.7%) | qPCR (LT) | Paulson *et al*., J Clin Oncol 2011 |
| USA | 4 | 4 (100%) | Hybrid capture and next-gen sequencing | Duncavage *et al*., J Molecular Diagnostics 2011 |
| USA | 51 | 47 (92.2%) | CM5E1 and CM2B4 Staining | Shuda *et al*., JCI 2011 |
| USA | 52 | 38 (73.1%) | qPCR (LT) | Lee *et al*., J Clin Virology 2011 |
| USA | 58 | 38 (65.5%) | PCR (LT) | Nardi *et al*., Clin Cancer Research 2012 |
| Switzerland | 30 | 20 (66.6%) | PCR (LT and VP1) | Mangana *et al*., Dermatology 2010 |
| Spain | 36 | 21 (58.3%) | CM2B4 staining | Shuda *et al*., Int J Cancer 2009 |
| Netherlands | 7 | 3 (42.8%) | PCR and sequence analysis (LT) | Wetzels *et al*., PLoS ONE, 2009 |
| Korea | 7 | 7 (100%) | PCR (LT) | Woo *et al*., J Plastic Reconstructive and Aesthetic Surg 2010 |
| Korea | 14 | 12 (85.7%) | qPCR (LT and VP1) and CM2B4 Staining | Jung *et al*., Histol Histopathol 2011 |
| Japan | 11 | 6 (54.5%) | PCR and qPCR (Tag and VP1) | Katano *et al*., J Medical Virology 2009 |
| Japan | 14 | 11 (78.6%) | PCR (LT) | Nakajima *et al*., J Dermatol Sci 2009 |

| | | | | |
|---|---|---|---|---|
| Japan | 19 | 11(57.8%) | qPCR (LT) and LT Ab Staining | Nakamura *et al*., Virology 2010 |
| Japan | 26 | 20 (76.9%) | qPCR (LT) and CM2B4 Staining | Kuwamoto *et al*., Human Pathology 2011 |
| Italy | 9 | 8 (88.8%) | PCR (LT and VP1) | Paolini *et al*., Virology Journal 2011 |
| Italy | 70 | 60 (85.7%) | PCR (LT) | Asioli *et al*., Modern Pathology 2011 |
| Italy | 18 | 18 (100%) | PCR (LT) | Biase *et al*., Human Pathology 2011 |
| Hungary | 8 | 7 (87.5%) | PCR (LT) and Sequence analysis | Varga *et al*., Br J Dermatol 2009 |
| Austalia | 38 | 33 (86.8%) | qPCR (LT and VP1) and CM2B4 Staining | Schrama *et al*., J Invest Dermatol 2011 |
| Germany | 138 | 116 (84.1%) | qPCR (LT and VP1) and CM2B4 Staining | Schrama *et al*., J Invest Dermatol 2011 |
| Germany | 39 | 30 (76.9%) | PCR(LT and VP1) and sequence analysis | Kassem *et al*., Cancer Research 2008 |
| Germany | 53 | 45 (84.9%) | qPCR (LT) | Becker *et al*., J Invest Dermatology 2009 |
| Germany | 39 | 35(89.7%) | PCR (LT) | Wieland *et al*., Emerg Infect Dis 2009 |
| Germany | 98 | 90 (91.8%) | PCR (LT) | Helmbold *et al*., Mol Carcinogenesis 2009 |
| Germany | 33 | 21 (63.6%) | PCR (Tag) and Southern hybridization | Andres *et al*., J Cut Pathol 2010 |
| Germany | 50 | 43 (86%) | PCR (LT) and CM2B4 staining | Houben *et al*., Int J Cancer 2010 |
| Germany | 59 | 34 (57.6%) | PCR (LT and VP1) | Handschel *et al*., Int J Oral and Maxillofacial Surg 2010 |
| Germany | 52 | 43 (82.7%) | qPCR (LT and VP1) | Werling *et al*., Histopathology 2011 |
| Germany | 3 | 2 (66.7%) | Polyomavirus genotyping assay | Schmitt *et al*., Cancer Epid Biomarker and Prevention 2011 |
| Germany | 34 | 22 (64.7%) | PCR (Tag) | Andres *et al*., Acta Dermato-Venereologica 2011 |
| Germany | 43 | 37 (86%) | qPCR(LT) | Wieland *et al*., J Am Acad Dermatol 2011 |
| Germany | 142 | 111 (78.2%) | qPCR (LT and VP1) | Vlahova *et al*., Br J Dermatology 2012 |
| France | 9 | 8 (88.8%) | PCR (LT and VP1) | Foulogne *et al*., Emerg Infect Dis 2008 |
| France | 10 | 10 (100%) | PCR (Tag and VP1) | Sastre-Garau *et al*., J Pathology 2009 |
| France | 32 | 21 (65.6%) | PCR (LT and VP1) | Touze *et al*., Emerg Infec Diseases 2009 |
| France | 18 | 14 (77.8%) | qPCR (LT and VP1) and Sequence analysis | Foulogne *et al*., Br J Dermatol 2009 |
| France | 43 | 41 (95.3%) | qPCR (LT) | Laude *et al*., PLoS pathogens 2010 |
| France | 68 | 51 (75%) | qPCR (LT) | Touze *et al*., J Clin Oncol 2011 |
| France | 113 | 70 (61.9%) | PCR (LT) and qPCR (sT) | Martel-Jantin *et al*., Virology 2012 |

| | | | | |
|---|---|---|---|---|
| Finland | 114 | 91 (79.8%) | qPCR(LT)and DNA sequencing | Sihto *et al*., J Natl Cancer Inst 2009 |
| Finland | 3 | 1 (33.3%) | qPCR (LT) | Koljonen *et al*., Nephrology Dialysis Transplantation 2009 |
| Finland | 5 | 5 (100%) | qPCR (LT) | Koljonen *et al*., Br J Cancer 2009 |
| Finland | 91 | 61 (67%) | qPCR (LT) and CM2B4 Staining | Sihto *et al*., Clin Cancer Research 2011 |
| Finland | 87 | 67 (77%) | qPCR(LT) | Waltari *et al*., Int J Cancer 2011 |
| Canada | 27 | 17 (62.9%) | CM2B4 Staining | Ly *et al*., Human Pathol 2011 |
| Canada | 30 | 29 (96.7%) | CM2B4 Staining | Erovic *et al*., Head and Neck 2012 |
| Australia | 21 | 5 (23.8%) | qPCR (LT) | Garneski *et al*., J Invest Dermatology 2009 |
| Australia | 104 | 19 (18.3%) | CM2B4 Staining | Palik *et al*., Human Pathology 2011 |
| **Total** | **2354** | **=1743 (74.04%)** | | |

Methods used were - PCR, qPCR, CM2B4 Staining and Southern blotting

**Table 15 MCV Presence in non- MCC Disease Tissues (Review of Literature)**

| Disease | Total | MCV +ve | Reference (s) |
|---|---|---|---|
| Lymphomas | 1105 | 44 | Shuda *et al*., Int J Cancer 2009,Kantola *et al*., J Clinical Virology 2009, Toracchio *et al*., Emerg Infect Diseases 2010, Andres *et al*., Am J Dermatopathology 2010, Mirvish *et al*.,J Am Acad Dermatol 2011 |
| Myeloid disorders | 401 | 33 | Feng *et al*, Science 2008, Shuda *et al*., Int J Cancer 2009,Kantola *et al*., J Clinical Virology 2009,Pantulu *et al*., Blood 2010,Teman *et al*., Leukemia Research 2011,Toracchio *et al*., Emerg Infect Diseases 2010 |
| Neuroendocrine cancers | 196 | 1 | Shuda *et al*., Int J Cancer 2009,Touze *et al*., Emerg Infec Diseases 2009, Busam *et al*., Am J Surg Pathol 2009, Touze *et al*., Emerg Infec Diseases 2009, McCluggage *et al*., Am J Surg Path 2010,Duncavage *et al*., Am J of Surgical Pathology 2009, Lewis *et al*., Oral Surg, Oral Medicine, Oral Pathol, Oral Radiolo and Endodon 2010, , Ly *et al*., Human Pathol 2011, Schmitt *et al*., Cancer Epid Biomarker and Prevention 2011, Chernock *et al*., Am J Surg Pathol 2011 |
| Skin diseases (cancers and others) | 1925 | 329 | Feng *et al*, Science 2008,Katano *et al*., J Medical Virology 2009,Kassem *et al*., Int J Cancer 2009, Wieland *et al*., Emerg Infect Dis 2009,Becker *et al*., J Invest Dermatology 2009, Sastre-Garau *et al*., Journal of Pathology 2009, Andres *et al*., J Cut Pathol 2009, Dworkin *et al*., J Invest Dermatol 2009, Foulogne *et al*., Br J Dermatol 2009, Garneski *et al*., J Invest Dermatology 2009, Varga *et al*., Br J Dermatol 2009 , Ridd *et al*., J Invest Dermatology, 2009, Mangana *et al*., Dermatology 2010, Handschel *et al*., Int J Oral and Maxillofacial Surg 2010, Loyo *et al*., Int J Cancer 2010, Reisinger *et al*., J Am Acad Dermatol 2010, Andres *et al*., Am J Dermatopath 2010, Mertz *et al*., J Investigative Dermatol 2010, Mertz *et al*., Human Pathology 2010,Kassem *et al*., J Dermatolg Science 2010, Faust *et al*., J Infect Diseases 2011 ,Rollison *et al*., Cancer Epid Biomark and Prevention 2011, Wieland *et al*., J Am Acad Dermatol 2011, Ly *et al*., Human Pathol 2011, Murakami *et al*., J Clin Virol 2011, Mertz *et al*., Dermatology 2011, Jung *et al*., Histol Histopathol 2011, Ly *et al*., Human Pathol 2011,Koburger *et al*., Experimental Dermatology 2011 |
| Central Nervous System diseases | 291 | 1 | Feng *et al*, Science 2008, Giraud *et al*., PLoS ONE 2009,Sastre-Garau *et al*., Journal of Pathology 2009, Katano *et al*., J Medical Virology 2009, Rubin *et al*., Anticancer Research 2011, Jung *et al*., Histol Histopathol 2011, Dang *et al*., PLoS ONE 2011 |
| Other Virus related diseases | 146 | 9 | Shuda *et al*., Int J Cancer 2009, Wieland *et al*., Emerg Infect Dis 2009, Katano *et al*., J Medical Virology 2009, Sastre-Garau *et al*., Journal of Pathology 2009 Mertz *et al*., Human Pathology 2010, Mertz *et al*., Dermatology 2011, Dang *et al*., PLoS ONE 2011 |

| | | | |
|---|---|---|---|
| Other cancers | 1807 | 79 | Feng *et al*, Science 2008, Bhatia *et al*., Int J Cancer 2009,Giraud *et al*., PLoS ONE 2009, Sastre-Garau *et al*., Journal of Pathology 2009,Bluemn *et al*., J Clinical Virology 2009, Katano *et al*., J Medical Virology 2009,,Bhatia *et al*., Int J Cancer 2009,Kassem *et al*., Int J Cancer 2009, Joh *et al*., Exper and Mol Pathol 2010, Bhatia *et al*., J Clinical Virology 2010, Loyo *et al*., Int J Cancer 2010,Toracchio *et al*., Emerg Infect Diseases 2010, Mertz *et al*., Dermatology 2011, Ly *et al*., Human Pathol 2011, Jung *et al*., Histol Histopathol 2011, Jung *et al*., Histol Histopathol 2011, Campello *et al*., J Medical Virology 2011, Helmbold *et al*., Eur J Cancer 2009,Andreas *et al*., Thorax 2009,Wetzels *et al*., PLoS ONE, 2009,Ly *et al*., Human Pathol 2011, Biase *et al*., Human Pathology 2011, Palik *et al*., Human Pathology 2011 |
| Other diseases | 599 | 55 | Wieland *et al*., Emerg Infect Dis 2009, Ridd *et al*., J Invest Dermatology 2009,Kassem *et al*., Int J Cancer 2009, Katano *et al*., J Medical Virology 2009, Loyo *et al*., Int J Cancer 2010, Toracchio *et al*., Emerg Infect Diseases 2010, Mertz *et al*., Human Pathology 2010, Mertz *et al*., J Investigative Dermatol 2010, Babakir-Mina *et al*., J Medical Virology 2010, Dang *et al*., PLoS ONE 2011, Kiasari *et al*., J Medical Virology 2011 |
| **Total** | **6470** | **550 (8.5%)** | |

Methods used were - PCR, qPCR, CM2B4 Staining. The qPCR copy numbers in the positive cases were 2-4 logs lower than that seen in MCC.
For details of the disease categories see Appendix section A1.

**Table 16 MCV Presence in Samples from HIV + Individuals (Review of Literature)**

| Tissue | Total | MCV +ve | Method | Reference (s) |
|---|---|---|---|---|
| Normal skin from HIV Positive individual | 1 | 0 | PCR | Feng *et al*, Science 2008 |
| Kaposi Sarcoma from HIV + individuals | 4 | 0 | PCR | Feng *et al*, Science 2008 |
| Lymph node and spleen tissues from HIV +ve individuals with AIDS | 42 | 0 | PCR | Sharp *et al*., J of Infectious Diseases, 2009 |
| Mucosal samples from HIV +ve MSM | 120 | 37 | PCR | Wieland *et al*., Emerg Infect Dis 2009 |
| Cerebrospinal fluid from HIV+ve MSM with nervous problems | 7 | 0 | PCR | Wieland *et al*., Emerg Infect Dis 2009 |
| Eyebrow hair plucked from HIV + MSM | 14 | 7 | PCR | Wieland *et al*., Emerg Infect Dis 2009 |
| AIDS related Lymphoma | 11 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| HIV associated non small cell lung cancer | 8 | 0 | PCR | Stebbing *et al*., AIDS 2010 |
| Cerebrospinal fluid from HIV +ve Individuals | 36 | 0 | qPCR | Dang *et al*., PLoS ONE 2011 |
| HIV Positive PML tissue | 50 | 0 | qPCR | Dang *et al*., PLoS ONE 2011 |
| HIV+ large cell lymphoma | 1 | 0 | PCR | Feng *et al*, Science 2008 |
| Cutaneous Swabs from HIV Positive MSM | 210 | 124 | qPCR | Wieland *et al*., Archives of Dermatol 2011 |
| PBMC from HIV/AIDS patients | 21 | 2 | qPCR | Shuda *et al*., Int J Cancer 2009 |
| AIDS autopsies below : | | | | |
| Brain | 15 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Tongue | 5 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Submandibular gland | 5 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Lung | 15 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Lymph node | 12 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Heart | 9 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| GI Tract | 13 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Liver | 16 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Spleen | 19 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Pancreas | 12 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Kidney | 14 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| Adrenal gland | 7 | 0 | qPCR | Katano *et al*., J Medical Virology 2009 |
| **Total** | **667** | **170 (25.5%)** | | |

The qPCR copy numbers in the positive cases were 2-4 logs lower than that seen in MCC.

**Table 17 MCV Presence in Healthy Tissue (Review of Literature)**

| Tissue | Total | MCV + ve | Method used | Reference(s) |
|---|---|---|---|---|
| **Skin** | | | | |
| Skin (normal/healthy) | 618 | 291 | qPCR, PCR and LT Ab (rabbit polyclonal) staining, rolling circle amplification | Sastre-Garau *et al*., Journal of Pathology 2009,Sitho *et al*., J Natl Cancer Inst 2009,Helmbold *et al*., Mol Carcinogenesis 2009,Foulogne *et al*., Br J Dermatol 2009,Mangana *et al*., Dermatology 2010,Nakamura *et al*., Virology 2010,Mertz *et al*., J Investigative Dermatol 2010,Loyo *et al*., Int J Cancer 2010,Mogha *et al*.,PLoS ONE 2010,Dworkin *et al*., J Invest Dermatol 2009,Wieland *et al*., Emerg Infect Dis 2009,Feng *et al*, Science 2008,Bhatia *et al*., Int J Cancer 2009,Schowalter *et al*., Cell Host and Microbe 2010,Foulongne *et al*., Emerg Infect Diseases 2010, Wieland *et al*., Archives of Dermatol 2011,swin swabs from Healthy volunteers 47 44 qPCR Pastrana *et al*., Medical Microbio and Immunol 2011 |
| Cutaneous swabs from MCC patients | 27 | 29 | qPCR | Foulongne *et al*., Emerg Infect Diseases 2010 |
| Cutaneous swabs from patients with cutaneous diseases | 58 | 78 | qPCR | Foulongne *et al*., Emerg Infect Diseases 2010 |
| Infalmmatory skin | 3 | 0 | | Feng *et al*, Science 2008 |
| Total | 706 | 398 (56.4%) | | |
| **Digestive System** | | | | |
| Appendix | 9 | 2 | PCR, southern hybridization | Feng *et al*, Science 2008 |
| Buccal Mucosal swabs from patients with MCC and cutaneous disorders | 42 | 2 | qPCR | Foulongne *et al*., Emerg Infect Diseases 2010 |
| Colon | 111 | 12 | PCR and qPCR | Campello *et al*., J Medical Virology 2011, Sitho *et al*., J Natl Cancer Inst 2009, Feng *et al*, Science 2008,Loyo *et al*., Int J Cancer 2010 |
| Esophagus | 6 | 1 | qPCR | Loyo *et al*., Int J Cancer 2010 |
| Saliva from patients without cancer | 10 | 10 | qPCR | Loyo *et al*., Int J Cancer 2010 |
| Small bowel | 3 | 1 | PCR, southern hybridization | Feng *et al*, Science 2008 |
| Stomach | 1 | 0 | qPCR | Sitho *et al*., J Natl Cancer Inst 2009 |

| | | | | |
|---|---|---|---|---|
| Tonsillar tissues | 234 | 8 | PCR | Feng *et al*, Science 2008,Kantola *et al*., J Clinical Virology 2009 |
| Mouth, mouthwash DNA | 13 | 2 | PCR | Feng *et al*, Science 2008, Dworkin *et al*., J Invest Dermatol 2009 |
| Mucosa (normal/healthy) | 10 | 2 | qPCR | Loyo *et al*., Int J Cancer 2010 |
| Total | 439 | 40 (9.1%) | | |
| **Respiratory System** | | | | |
| Respiratory tract specimens | 612 | 8 | PCR and qPCR | Sharp *et al*., J Infectious Diseases, 2009,Bialasiewicz *et al*., Emerg Infect Diseases 2009 |
| Nasopharyngeal aspirate samples | 775 | 30 | PCR and qPCR | Kantola *et al*., J Clinical Virology 2009, Goh *et al*., Emerg Infect Diseases 2009 |
| Lung | 15 | 1 | qPCR | Loyo *et al*., Int J Cancer 2010 |
| Transbronchial biopsies from lung transplant patients | 65 | 22 | qPCR | Bergallo *et al*., J Clinical Path 2010 |
| Total | 1467 | 61 (4.2%) | | |
| **Blood** | | | | |
| Whole Blood | 317 | 12 | PCR and qPCR | Feng *et al*, Science 2008,Helmbold *et al*., Mol Carcinogenesis 2009,Campello *et al*., J Medical Virology 2011,Helmbold *et al*., Eur J Cancer 2009,Pantulu *et al*., Blood 2010,Mertz *et al*., J Investigative Dermatol 2010,Kassem *et al*., Cancer Research 2008,Dworkin *et al*., J Invest Dermatol 2009 |
| Buffy coats | 60 | 13 | qPCR | Pancaldi *et al*., Blood 2011 |
| Sera | 725 | 0 | PCR | Kantola *et al*., J Clinical Virology 2009 |
| PBL | 40 | 2 | PCR and qPCR | Bhatia *et al*., J Clinical Virology 2010,Bhatia *et al*., Int J Cancer 2009 |
| PBMC | 123 | 0 | PCR and qPCR | Shuda *et al*., Int J Cancer 2009, Duncavage *et al*., Modern Pathology 2009 |
| Inflammatory Monocytes of MCC Patients | 2 | 2 | PCR | Mertz *et al*., J Investigative Dermatol 2010 |
| Total | 1267 | 29 (2.9%) | | |
| **Lymphatic System** | | | | |
| Lymph node | 143 | 11 | qPCR, CM2B4 staining | Feng *et al*, Science 2008, Sitho *et al*., J Natl Cancer Inst 2009 ,Shuda *et al*., Int J Cancer 2009, Toracchio *et al*., Emerg Infect Diseases 2010 |
| Bone marrow | 2 | 0 | qPCR | Sitho *et al*., J Natl Cancer Inst 2009 |
| Total | 145 | 11 (7.6%) | | |
| **Urinary System** | | | | |
| Bladder | 2 | 0 | qPCR | Loyo *et al*., Int J Cancer 2010 |

| | | | | |
|---|---|---|---|---|
| Kidney | 1 | 0 | PCR | Feng *et al*, Science 2008 |
| Urine | 69 | 10 | PCR and qPCR | Wieland *et al*., Emerg Infect Dis 2009,Bofil-Mas *et al*., Virology Journal 2010,Husseiny *et al*., J Clin Virol 2010,Mertz *et al*., J Investigative Dermatol 2010 |
| Total | 72 | 10 (13.9%) | | |
| **Other Tissues** | | | | |
| Brain tissues | 300 | 0 | PCR | Lam *et al*., J Clinical Virology 2010 |
| Fetal autopsy | 535 | 1 | qPCR and Sequence analysis | Sadeghi *et al*., Virology Journal 2010 |
| Fibrous tissue | 2 | 0 | PCR | Feng *et al*, Science 2008 |
| Fistula track | 1 | 0 | PCR | Feng *et al*, Science 2008 |
| Gall Bladder | 7 | 1 | PCR, southern hybridization | Feng *et al*, Science 2008 |
| Heart | 1 | 0 | PCR | Feng *et al*, Science 2008 |
| Hemorrhoid | 1 | 1 | PCR, southern hybridization | Feng *et al*, Science 2008 |
| Hernia | 2 | 0 | PCR | Feng *et al*, Science 2008 |
| Liver | 15 | 5 | qPCR | Loyo *et al*., Int J Cancer 2010 |
| Prostate | 29 | 0 | PCR and qPCR | Feng *et al*, Science 2008, Bluemn *et al*., J Clinical Virology 2009 |
| Prostate tumor adjacent stroma | 6 | 0 | qPCR | Bluemn *et al*., J Clinical Virology 2009 |
| Skeletal muscle | 1 | 0 | qPCR | Sitho *et al*., J Natl Cancer Inst 2009 |
| Total | 900 | 8 (0.9%) | | |

The qPCR copy numbers in the positive cases were 2-4 logs lower than that seen in MCC.

# 4.0 SURVIVIN AND MERKEL CELL CARCINOMA

## 4.1 THE DUAL ROLE OF SURVIVIN

In response to a variety of different stimuli including UV radiation, toxins, cytokines, and DNA damage cells usually initiate an evolutionary conserved process of autodestruction called apoptosis. Both intracellular and extracellular factors exist that control apoptosis, triggering the intrinsic and/or the extrinsic pathways respectively. Extracellular activators of apoptosis include death receptors such as Fas and tumor necrosis factor receptors, whereas intracellular activators include stress-activated factors controlling mitochondrial activity. Proteolytic caspases, activated upon cleavage, are the main effectors of apoptosis. Both caspase-8 (extrinsic pathway) and caspase-9 (intrinsic pathway) stimulate the effector serine protease-caspase 3, which activates a DNAse that eventually degrades cellular DNA and kills the cells. Due to the importance of apoptosis in both normal and diseased tissues, the process is intricately regulated by a number of anti- and pro- apoptotic molecules. One such group is called the inhibitor of apoptosis (IAP) family; one of its most important members is survivin [460-462].

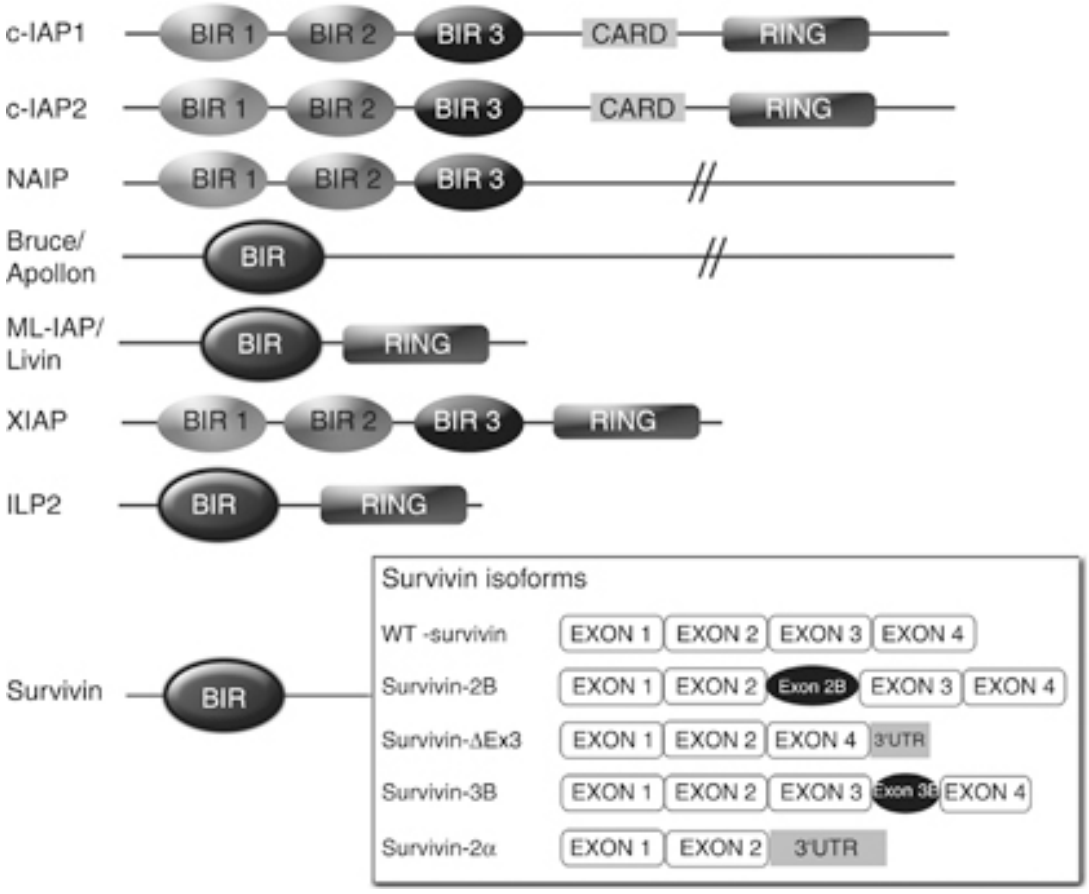



**Figure 20 The inhibitor of apoptosis (IAP) family members and survivin isoforms.**
The IAP family comprises of eight members: XIAP (X-linked IAP), c-IAP1, c-IAP2, ILP2 (IAP-like protein-2), ML-IAP (melanoma IAP)/Livin, NAIP (neuronal apoptosis-inhibitory protein), and survivin. All these proteins exert antiapoptotic functions. Survivin is an exception as it also regulates the cell cycle. Alternative splicing of survivin transcript generates five major isoforms characterized by different functions. BIR, baculovirus inhibitor of apoptosis repeat; CARD, caspase recruitment domain; RING, really interesting new gene; 3'UTR, 3' untranslated region; WT, wild type. (Reproduced from Dallaglio *et al*., JID 2012)

The IAP family is comprised of structurally different proteins, sharing one or more copies of a domain called the BIR (baculovirus IAP repeat), which binds and blocks caspase activity. The BIR domain containing proteins include Bruce, ILP2, Livin, survivin, NAIP, c-IAP1, c-IAP2, and XIAP (X-linked IAP) (Figure 20). Unlike other IAP members, the smallest member, survivin also plays an important role in the regulation of mitosis. This dual nature of survivin can be attributed to its unique distinguishing structure. Survivin has only one BIR domain, which it uses

to homodimerize, and interact with other chromosome passenger proteins (phosphorylation of threonine 48 in the BIR domain is essential for this)[460, 461, 463]. In addition, survivin also has a coiled-coil α-helix C-terminal domain. Unlike other IAPs, survivin lacks a caspase recruitment domain (CARD) [344] and does not directly bind caspases *in vivo*. It primarily forms a complex with XIAP, stabilizes it and thereby increases XIAP's ability to inhibit caspases and thus apoptosis[464, 465]. In addition, survivin also interacts with Smac/DIABLO (second mitochondria-derived activator of caspases expressed from the *DIABLO* gene) and thus prevents it from activating caspases at the postmitochondiral level[466]. Finally, survivin is dynamically regulated by multiple post-translational mechanisms, and its various functions are also spatially and temporally regulated[467]. There are several other molecules that have been identified in complex with survivin, but their significance and functions are still under evaluation. The gene encoding for the 16.5kD (142 amino acids) wild type survivin, called BIRC5, spans 14.7 kb at the telomeric end of chromosome 17. It generates five major transcripts by alternative splicing: wild-type [75]-survivin, survivin-2B, survivin-**Δ**Ex3, survivin-3B, and survivin-2**α** (Figure 20). Survivin-2**α** and survivin-2B favor induction of apoptosis, whereas WT-survivin, survivin-**Δ**Ex3, and survivin-3B appear to be antiapoptotic and cytoprotective[461, 467]. More studies are still needed to determine the expression, localization and functions of all these different isoforms. Analysis of human survivin protein on NCBI website shows that it shares 84% and 91.5% sequence identity with mouse and canine survivin respectively, suggestive of a conserved function in the mammalian species.

Survivin is unique in its ability to regulate mitosis. One of the ways in which it mediates this is by binding to microtubules through its C-terminal domain and ensuring correct cell division. Survivin also associates with the chromosome passenger proteins Aurora B, INCENP, and Borealin, and the complex is recruited to the mitotic centromeres to assist and ensure proper chromosomal segregation[468, 469]. Additionally, survivin localizes to the centrosomes of dividing cells. It is here that it binds to cyclin dependent kinase 1 (Cdk1), the activation of which allows the cells to enter mitosis (specifically S phase)[470].

Within a cell, survivin exists in different subcellular compartments where it interacts with various effector molecules and executes its functions. Nuclear, cytoplasmic, and mitochondrial pools of survivin protein have been reported. Scientists have linked nuclear localization with its mitotic regulation function and mitochondrial survivin with its inhibition of apoptosis function. Cytoplasmic survivin on the other hand, loses its cytoprotective ability after exiting from the mitochondria once apoptosis is initiated[471, 472]. Survivin induced by stress stimuli, is also found in the extracellular space. This pool seems to retain both antiapoptotic and proliferative activities[473]. A role in for survivin in the pathogenesis of rheumatoid arthritis is being explored [474].

Although regulatory mechanisms of survivin are not yet fully understood at the transcriptional level, survivin levels has been demonstrated to be cell cycle dependent. The BIRC5 promoter possesses a cell-cycle dependent element/ cell cycle gene homology region (CDE/CHR) G1 repressor element. Furthermore, Raj *et al.* reported that survivin expression in melanocytes is cell cycle dependent and regulated by both p53 and Rb. In particular, wild-type p53 is able to repress mitochondrial apoptosis-preventing survivin (both mRNA and protein) [475]. The stabilization of p53 following DNA damage leads to subsequent repression of survivin and the activation of cell cycle checkpoints. Conversely, overexpression of survivin is able to rescue cells from p53-induced apoptosis. The loss of p53 is often observed in cases of protracted UV irradiated skin (sun-exposed skin). This results in uncontrolled survivin expression, impaired checkpoint control and accumulation of genomic mutations that eventually lead to the development of a neoplasm [476, 477].

Among the interactor proteins of survivin, another worth mentioning is the heat shock protein 90 (Hsp90). The interaction and formation of the survivin-Hsp90 complex appears to stabilize survivin and prevent its proteasomal degradation [478].

Survivin tissue expression is ubiquitous and abundant in embryonic and fetal development; and in a variety of malignant tumors including melanomas, gastric, colon, uterine, pancreatic, ovarian and bladder carcinomas [476, 479-489]. However, it is almost undetectable in most adult, differentiated tissues. Overexpression in most tumor tissues and

cell lines, independent of mitotic index, is what has earned survivin the status of a putative " tumor-specific antigen". Although, survivin has been detected, and postulated to play a role, in some adult tissues (epidermal stem cells, keratinocyte stem cells, neural stem cells, mesenchymal stem cells, CD34+ haematopoietic progenitors, thymocytes, adult colonic epithelium, pancreatic beta cells, outer root sheeth and matrix cells of hair follicles, etc.) its level of expression is much lower compared to levels in cancer cells [460, 467].

Studies of tumor viruses have shown interesting links between important viral proteins and survivin. Hepatitis B X-interacting protein forms a complex with survivin, which in turn binds to pro-caspase-9, preventing the activation of apoptosis [490]. Human papillomavirus E6 oncoprotein transactivates the survivin promoter in HeLa and human embryonic fibroblast cells. This transactivation effect of E6 on survivin seems to be dependent on E6's ability to interact with p53 and causes its ubiquitination-dependent degradation. The downregulation of p53 directly derepresses survivin expression [491]. Consistent with this, high levels of survivin protein have also been found in HPV-induced warts [492]. Other than that, upregulated survivin was found in oligodendrial and astrocytic cultures infected with JCV and in JCV positive PML (progressive multifocal leukoencephalopathy) cases. Gualco *et al.* recently showed that this effect might be insulin-like growth factor-1 receptor (IGF-IR) dependent [493]. Induction of JCV T antigen expression caused an increase in survivin expression in wild type IGF-IR embryo derived neural progenitors, but not in IGF-IR knockout embryo derived cells. A single study by Raj *et al*. also explored the increase in survivin by SV40 large T antigen overexpression [475]. Chapter 5 describes the newfound link between MCV large T antigen and survivin.

## 4.2 SURVIVIN EXPRESSION IN MERKEL CELL CARCINOMA

To clinical investigators survivin has become a diagnostic cancer biomarker and also a molecular signature of poor disease outcome in a majority of human cancer cases. Two separate groups have validated survivin as a prognostic biomarker in Merkel cell carcinomas as well. Both nuclear and cytoplasmic survivin was detected in MCC, with higher expression correlating to tumor recurrence and metastasis. Kim *et al*. reported that in the nucleus, survivin colocalizes with the mitotic spindle markers. On the other hand, cytoplasmic survivin is also detected and found in large cell foci but predicts better outcome (Figure 21). We also analyzed 5 MCC cell lines and found nuclear localization of survivin expression (Figure 22). Small case numbers and short clinical follow up periods limited these studies. More evaluations are thus required to make any stronger correlation [494, 495].

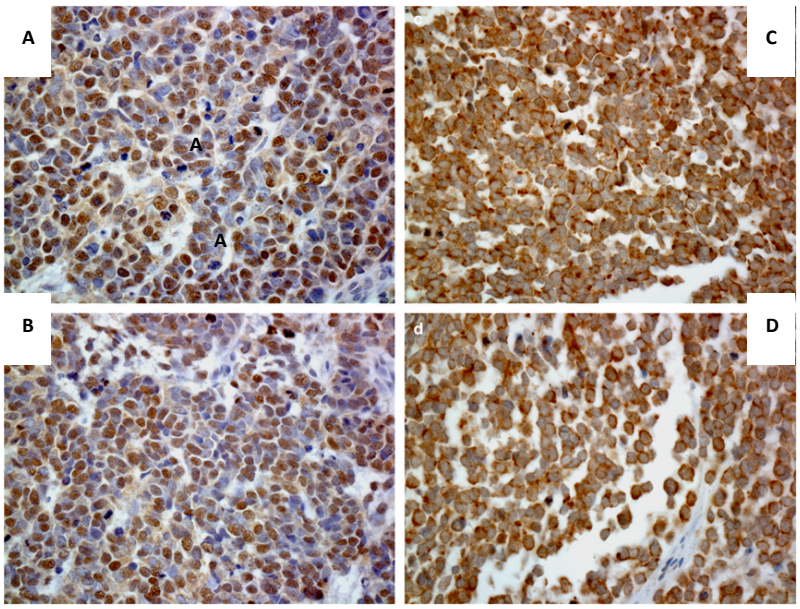



**Figure 21 Immunohistochemical staining for survivin expression in Merkel cell carcinoma.**
(A and B) Survivin localization in the nuclear compartment. (C and D) Survivin localization in the cytoplasmic compartment. Original magnification 200X. (Kim *et al*. Modern Pathology 2008)

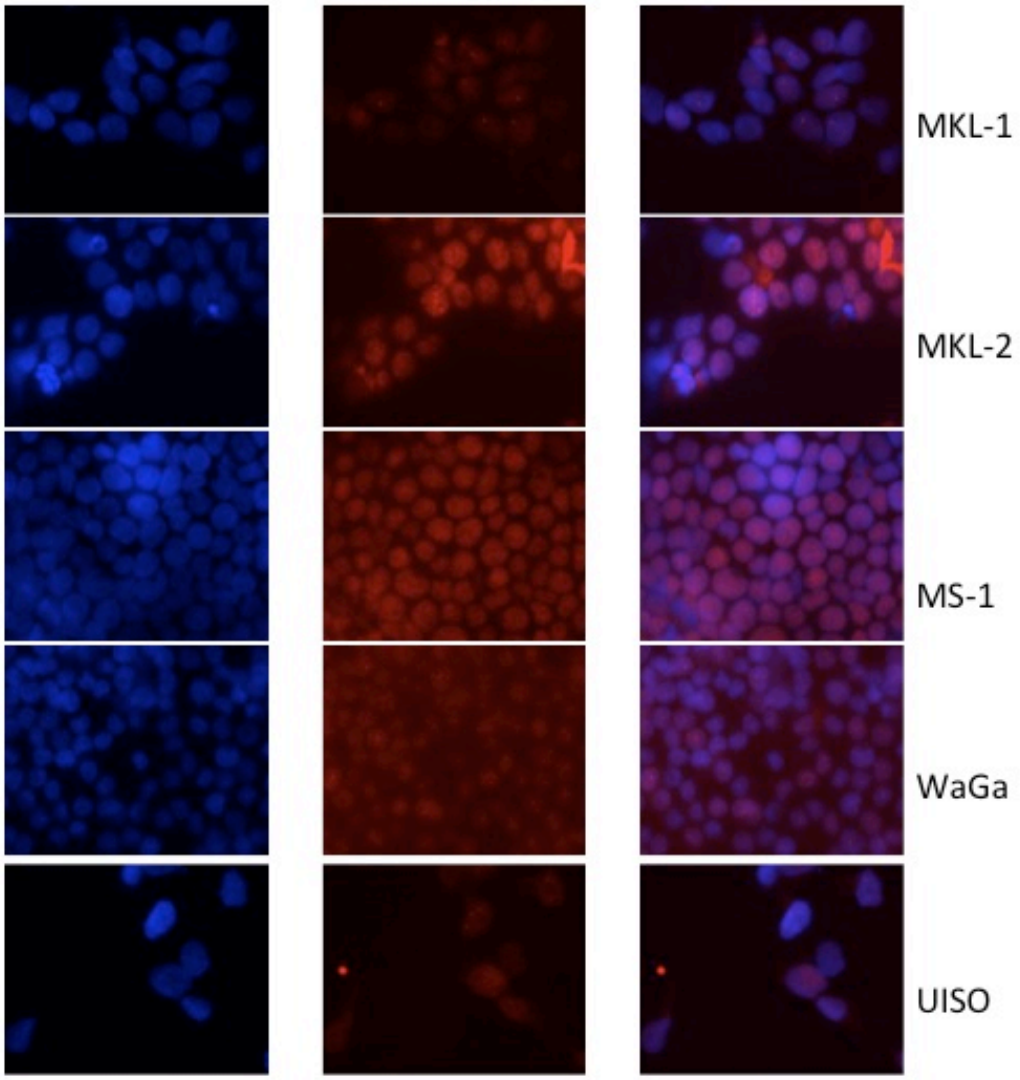



Figure 22 **Nuclear localization of survivin in MCC cell lines**.
Immunofluorescence analysis of MCC cell lines, both MCV positive (MKL-1, MKL-2, MS-1, WaGa) and MCV negative (UISO) reveals nuclear staining of survivin. Survivin antibody (Cell signaling) was used at a 1:250 dilution and anti-rabbit Alexa 568 (red) was used as secondary antibody. DAPI was used as a counter stain for the nucleus.

## 4.3 SURVIVIN AS A CHEMOTHERAPEUTIC TARGET

Survivin is a promising chemotherapeutic target in multiple cancers for several reasons. First, this small inhibitor of apoptosis protein is differentially over-expressed in cancer. Other than inhibiting apoptosis it is also actively involved in cell cycle progression and proliferation. Most importantly, it involved in multiple signaling mechanisms in tumor maintenance (considered a nodal protein), and most of the survivin-binding partners are oncoproteins themselves, as either they are mutated, overexpressed or functionally altered in cancer. Hence, targeting survivin for treatment confers the advantage of eliminating tumor cells while sparing normal tissues. Targeting this nodal protein also yields the benefit of avoiding resistance to single molecule/pathway directed therapy that often develops in heterogenous cancers. Further, survivin promotes tumor-associated angiogenesis, considered a hallmark of cancer[185], and acts as a resistance factor to various anticancer therapies. Lastly, retrospective analyses of patient cohorts have consistently found that an increased level of survivin expression predicts poor prognosis and recurrence[476, 480, 496, 497].

Survivin, has thus, emerged as an important target for cancer drug discovery. Unfortunately, despite the enormous amount of knowledge gained and the interest generated in survivin, the portfolio of survivin antagonists available for clinical testing is small. Currently used survivin antagonists and their respective clinical trial information are summarized in the following table.

**Table 18 Survivin antagonists and their clinical development**

| Compound Name | Therapeutic approach | Reference(s) | Clinical Development (Identifier# from clinicaltrials.gov) |
|---|---|---|---|
| LY2181308 | Antisense oligonucleotide | Tanioka, M., et al. Cancer chemotherapy and pharmacology, 2011<br>(Developed by Elli Lilly and Company) | 1. Phase II for relapsed or refractory acute myeloid leukemia (NCT00620321)<br>2. Phase II in combination with docetaxol for non-small cell lung cancer (NCT01107444)<br>3. Phase II in combination with docetaxol and prednisone for hormone refractory prostate cancer (NCT00642018) |
| SPC3042 / EZN-3042 | Antisense oligonucleotide | - Hansen, J. B., et al. Molecular cancer therapeutics, 2008<br>- Sapra, P., et al. Nucleosides, nucleotides & nucleic acids,2010<br>(Developed at Santaris and Enzon Pharmaceuticals Inc.) | At preclinical stage completed<br>Phase I for Leukemia |
| Shepherdin | Peptidomimetic (combined survivin and HSP90 antagonist) | Plescia, J., Cancer cell,2005 | At preclinical stage |
| Dominant interfering mutants (C84A, T34A and TC34,84AA) | Gene Therapy | - Mesri, M., The Journal of clinical investigation, 2001<br>- Tu, S. P., Cancer research 2003<br>- Zhang, R., Cancer biology & therapy 2008 | At preclinical stage |
| BIRC5 promoter for tumor-specific transcription of cytotoxic genes | Gene Therapy | Chen, J. S.,Cancer gene therapy, 2004 | At preclinical stage |
| Ribozyme RNA Interference (RZ-1, RZ-2) | Molecular antagonist | Choi, K. S., Cancer gene therapy, 2003 | At preclinical stage |
| YM155 | Small molecule transcriptional repressor | Nakahara, T., Cancer research, 2007<br>(Developed by Astellas Pharmaceuticals Inc) | 1. Phase II for Lung cancer (NCT00328588)<br>2. Phase II for prostrate cancer (NCT00257478)<br>3. Phase II for Melanoma (NCT00281541)<br>4. Phase II in combination with docetaxel and prednisone for prostrate cancer. (NCT00514267)<br>5. Phase II for Diffuse large B-cell lymphoma (NCT00498914) 6. Phase II in combination with paclitaxel and carboplatin for non-small cell lung carcinoma. (NCT01100931)<br>7. Phase II in combination with docetaxel for breast cancer. (NCT01038804)<br>8. Phase II in combination with rituximab for Non-hodgkin's lymphoma. (NCT01007292)<br>9. Phase II in combination with docetaxel for melanoma. (NCT01009775)<br>10. Phase II for advanced cancer. (NCT01023386 and NCT00818480) |
| EM-1421 (Terameprocol) | Small molecule transcriptional inhibitor | Heller, J. D.,Cancer research. 2001<br>(Developed by Erimos Pharmaceuticals) | 1. Phase I for refractory solid tumors, leukemias and lymphomas<br>2. Phase I and II for Cervical Intraepithelial Neoplasia (NCT00154089)<br>3. Phase I and II for Brain and central nervous system tumors (NCT00404248) |

| | | | |
|---|---|---|---|
| Autologous Cytotoxic T cells with survivin-primed dendritic cells | Immunotherapy | Svane, I. M., Cancer immunology, immunotherapy, 2004 | 1. Phase I and II in Melanoma (NCT00197912)<br>2. Phase I and II in renal cell carcinoma. (NCT00197860)<br>3. Phase II in combination with docetaxel for prostrate cancer. (NCT01446731)<br>4. Phase I and II in combination with hTERT tumor vaccine for multiple myeloma. (NCT00834665)<br>5. Phase I and II for Multiple myeloma and plasma cell neoplasm. (NCT00499577)<br>6. Phase I and II for prostrate cancer. ( NCT01197625)<br>7. Phase I and II for Melanoma. (NCT00074230)<br>8. Phase I and II for ovarian cancer. (NCT01334047) |
| DPX-Survivac | Immunotherapy | Karkada, M., Journal of immunotherapy 2010<br>(Developed by ImmunoVaccine Technologies Inc.) | Phase I and II in combination with cyclophosphamide for ovarian, fallopian tube and peritoneal cancer. (NCT01416038) |

Sequence data and chemical structures were not publically available for most of the drugs enlisted and hence are not included in this table.

The Clinical Development column is mainly focussed on Phase II trials and all the information is from www.clinicaltrials.gov and www.cancer.gov. The table includes a summary of trials only in the USA, and hence is not an exhaustive list.

# 5.0 VIRAL RNAI KNOCKDOWN AND RATIONAL DRUG SCREENING REVEALS SURVIVIN AS A THERAPEUTIC TARGET FOR MERKEL CELL CARCINOMA

Work described in this section is in press in Science Translational Medicine Journal

with authors Reety Arora, Masahiro Shuda, Anna Guastafierro, Huichen Feng, Tuna Toptan, Yanis Tolstov, Daniel Normolle, Laura L. Vollmer, Andreas Vogt, Alexander Dömling, Jeffrey L. Brodsky, Yuan Chang and Patrick S Moore

R.Arora and M.Shuda performed the T antigen and survivin knock down experiments and immunoblots as well as cell cycle analysis. H.Feng performed the DTS analysis. T.Toptan and R.Arora created BJ stable cell lines and tested for survivin, cyclin E, E2F1 and MCV T Ag expression. R.Arora, Y.Tolstov and L.Vollmer performed the drug screen. R.Arora tested individual drugs and performed cell titer glo studies. A.Guastafierro and R.Arora performed mouse xenograft experiments and qRT-PCR studies. D.Normolle performed xenograft tumor volume analysis. A.Vogt, J.Brodsky, A.Domling provided compounds and supervision on drug screen. R. Arora, Y.Chang , and P.S.Moore interpreted the data and wrote the manuscript.

Merkel cell carcinoma is an aggressive, often lethal cancer. There is no optimal therapy for MCC beyond surgery. Also, MCC are highly chemoresistant tumors and either respond poorly to standard chemotherpies or lose sensitivity within a few treatment rounds.

Before the discovery of MCV, the prospects of this cancer seemed dismal as MCC based mortality was increasing; however no pathway or important target had been identified. The discovery of MCV changed that perception and revealed the integrated virus as a target for therapy.

To seek for a treatment module for this cancer, post the discovery of MCV, we experimentally exploited the molecular biology of this virus. By comparing digital transcriptome subtraction (DTS) deep-sequencing profiles, we found cellular survivin oncoprotein (*BIRC5a*) transcripts upregulated seven-fold in virus-positive compared to virus-negative MCC tumors. We also knocked down MCV T antigen in MCV positive MCC cell lines and found an associated decrease in survivin mRNA and protein expression.

To follow this up we exogenously expressed MCV large T antigen (LT) in non-MCC primary cells. This caused an increase in survivin protein expression and required an intact retinoblastoma (RB) protein-targeting domain in MCV T antigen. This RB binding domain not only activated surivivin gene transcription but also increased expression of other G1/S-phase proteins including E2F1 and Cyclin E. Also shRNA based knockdown of survivin alone caused cell death in MCV positive MCC cells.

We next tested an experimental drug called YM155, known to target survivin, on MCV positive MCC cells. YM155 selectively initiated irreversible, non-apoptotic MCV-positive MCC cell death. A compound library screen of 1360 other chemotherapeutic and pharmacologically-active compounds *in vitro*, resulted in only one compound named bortezomib (Velcade) with similar potentcy. Bortezomib however was not selective for killing MCC cells.

We created an MCC xenograft model by injecting MCV positive MCC cell line MKL-1 subcutaneously in NSG mice, and used it for YM155 and bortezomib preclinical evaluation. YM155 was nontoxic and halted MCV-positive MCC xenograft tumor growth in mice. On the other hand, bortezomib was significantly toxic and was not active *in vivo*. Xenograft tumors

resumed growth once YM155 treatment ended suggesting that YM155 may be cytostatic rather than cytotoxic *in vivo*.

Collectively, this study identified an important pathway (survivin) targeted by MCV T antigen to initiate tumor formation. Using this protein we were able to rationally design a precisely targeted, less-toxic therapy for MCC in less than four years. Thus, identifying the cellular circuits, such as survivin, that are targeted by tumor viruses can lead to rapid and rational identification of drug candidates for viral cancers that are otherwise difficult to treat. Multicenter clinical trials for these drugs are scheduled to open soon in the USA.

## 5.1 MATERIALS AND METHODS

### 5.1.1 Cell culture

Seven Merkel cell carcinoma cell lines: MKL-1, MKL-2, MS-1, UISO, MCC13, MCC26 and WaGa (kind gift of Jurgen Becker [405, 498, 499]; NCI-H69 small cell lung cancer cell line (ATCC); 293 human embryonic kidney cells (ATCC); U2OS osteosarcoma cell line (kind gift of Ole Gjoerup); BJhTERT immortalized foreskin fibroblast cell line (kind gift of Ole Gjoerup); and BJ primary foreskin fibroblasts (ATCC) were used to screen and evaluate the small molecules examined in this study [405, 498, 499]. The Merkel cell lines and NCI-H69 were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin and streptomycin at 37 °C in humidified air containing 5% $CO_2$. The remaining cell lines were grown in DMEM supplemented with 10% fetal calf serum.

### 5.1.2 Compounds

A total of 1,360 compounds were used in the screening survey and are listed in Appendix A-

Table 22: 1,280 compounds from LOPAC1280 library (Sigma Aldrich, accessed through University of Pittsburgh Drug Discovery Institute), 89 compounds from NCI's Approved Oncology Drug Set II (from The NCI/DTP Open Chemical Repository, http://dtp.cancer.gov), 6 LT ATPase inhibitors (MAL2-11B, MAL3-101, MAL2-51, DMT3084, bithionol and hexachlorophene [500, 501]) and 4 compounds that target and inhibit p53 and MDM2 binding (Nutlin-3, YH264A, Y2H265A and KK_NW_16A [502, 503]). 19 compounds that were in common between the LOPAC 1280 and the NCI Approved Oncology Drug Set II library were screened twice with comparable results. NCI Approved Oncology Drug Set II, SV40 LT ATPase inhibitors and MDM2 inhibitors were reconstituted as recommended by supplier. YM155 (4,9-Dihydro-1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(2-pyrazinylmethyl)-1H-naphth[2,3-d]imidazolium bromide) was purchased from Active Biochemicals Co. Ltd. (Hong Kong, China). Reconstituted compounds were diluted in cell culture media to obtain a 100x stock concentration prior to addition to cells. Doxorubicin (positive control) was obtained from Sigma-Aldrich and DMSO (negative control) was obtained from Fisher Bioreagents.

For EC50 measurements, dose-response curves were established for 17 drugs obtained individually in bulk stocks. Iodoacetamide (I1149), sanguarine chloride (S5890), NSC95397 (N1786), chelerythrine chloride (C2932), calmidazolium chloride (C3930), tetraethylthiuram disulfide (T1132), bay 11-7085 (B5681), quinacrine dihydrochloride (Q3251), ellipticine (E3380), amsacrine hydrochloride (A9809) and nutlin-3 (N6287) were purchased from Sigma Aldrich, USA. Mitoxantrone (NSC279836), daunorubicin HCl (NSC82151), valrubicin (NSC246131), topotecan HCl (NSC609699), teniposide (NSC122819) and bortezomib (NSC681239) were kindly provided by the NCI/DTP Open Chemical Repository, http://dtp.cancer.gov.

### 5.1.3 MKL-1 cytotoxicity screen

MKL-1 cells were seeded at a density of 6000 cells in 50 ml of medium per well (120 cells/ml) in opaque polypropylene 384-well microplates (#781080, Greiner Bio-One, Germany). Cells were incubated at 37°C in humidified air containing 5% $CO_2$ for 24 hours. Thereafter, 25 ml of

medium containing 3X drug per well was added to the plates, which were incubated for an additional 48 hours. Cell viability was measured using Cell Titer Glo (Promega) following manufacturer's instructions. The validity of Cell Titer Glo results in measuring cell viability was confirmed by trypan blue exclusion staining and WST-1 assays (Roche) in pilot studies.

The LOPAC1280 library was screened at a final concentration of 10 mM for each compound and the NCI library was screened at a final concentration of 1 mM for each compound. MAP-C (Titertek Instruments Inc) and Janus MDT (PerkinElmer Inc) were used for automated resuspension and the addition of LOPAC library drugs to assay plates. NCI library compounds were added to wells by manual pipetting.

Cell Titer Glo assays were performed in duplicate using 384 well plates, each containing 24 wells with 1% DMSO (negative control) and 32 wells with 200 mM doxorubicin (positive control). Screening results were evaluated on the basis of percentage cell survival normalized to the DMSO control (100%). Positive candidates were identified using a cut-off value of <10% cell survival. The average Z factor was 0.61 (range 0.34-0.74) for the LOPAC library screen and 0.82 for the NCI library screen (range 0.75-0.91).

### 5.1.4 Dose-response studies

Compounds that met the selection criteria < 10% cell survival were purchased or obtained in bulk from NCI/NIH Developmental Therapeutics Program. Serial drug dilutions from $10^{-4}$ M to $10^{-9}$ M were used on MCC and non-MCC cell lines. Cells were seeded into 384-well plates at 6000 cells in 50 ml of medium per well. After 24 hours, 25 ml of 3X drugs were added at increasing concentration to each well. Cell viability was then measured using Cell Titer Glo (Promega) kit following manufacturer's instructions as described previously. Each drug concentration was tested in triplicate for each cell line and experiments were repeated twice. $EC_{50}$ doses for the drugs were calculated using a four parameter logistic equation (GraphPad Prism).

### 5.1.5 Trypan blue dye exclusion assay

Cells were equally seeded and treated with YM155 for 48 hours. To quantitate cell death, cells were treated with Accutase (Millipore), collected, resuspended in PBS, mixed with equal volume of Trypan blue (Lonza, 0.4%) and counted using a hemocytometer under the microscope. Counting was performed three times, in triplicate.

### 5.1.6 Expression and shRNA lentivirus construction

To express codon optimized full-length MCV large T antigen, the gene was synthesized (DNA2.0) from the MCV-HF strain large T antigen sequence template [360] (GenBank ID: JF813003) and cloned into the lentiviral pLVX EF puro vector [504]. Truncated tumor LT339 (representing the MCV339 strain, amino acid 1-455) and LT339.LFCDK were cloned by site-directed mutagenesis from the codon-optimized full-length LT into pSMPUW-hygro vector (Cell Biolabs Inc.) [504]. MCV small T codon optimized was also cloned into the lentivirus vector. Cells were infected with lentiviruses in the presence of 1-4 mg/ml polybrene for 24 hours, followed by media change. Stable selection with either puromycin (1 mg/ml) or hygromycin (200 mg/ml) was initiated 48 hours after infection.

shRNA for MCV T antigen knockdown was generated and used as previously described [498, 505], we renamed shT1 and shScr in Houben *et al*. to panT1 and shCntrl in this study. To knockdown survivin gene expression, shRNA sequence (shsur1-5' ccggCCGCATCTCTACATTCAAGAACTCGAGTTCTTGAATGTAGAGATGCGGtttttg-3' and shsur2- 5' ccggCCTTTCTGTCAAGAAGCAGTTCTCGAGAACTGCTTCTTGACAGAAAGGtttttg-3' (Lower cased nucleotides indicate linker sequences used for cloning)) was cloned into a pLKO.1puro lentiviral vector. shCntrl is a nontargeting shRNA negative control [498]. Cells were infected with lentiviruses, washed after 24 hours and then harvested for immunoblotting six days after infection.

### 5.1.7 Immunoblotting

Cells were lysed in buffer (RIPA or 10mM Tris-HCl pH 8.0, 0.6% SDS) containing protease inhibitor cocktail (Roche). Lysates were electrophoresed in 10% SDS-PAGE, transferred to nitrocellulose membrane (Amersham) and reacted with specific antibodies CM2B4 (1:5000 dilution) [506], CM8E6 (1:500 dilution), cleaved PARP, cleaved caspase3, survivin, XIAP, p53, Bcl-2, Bax (1:1000 dilution, Cell Signalling Technologies), E2F1, cyclin E (1:1000 dilution, Santa Cruz Biotechnology), LC3 (1:1000 dilution, Novus Biologicals) or $\alpha$-tubulin (1:5000 dilution, Sigma)) overnight at 4°C, followed by anti-mouse (1:5000 dilution, Amersham) or anti-rabbit IgG-HRP conjugates (1:3000 dilution, Cell signaling) for 1 hour at room temperature. Peroxidase activity was detected using Western Lightning plus-ECL reagent (Perkin Elmer). For quantitative immunoblotting, Odyssey Infrared Imaging system (LI-COR) was used with IRDye 800-conjugated secondary antibodies (1:5000 dilution, Rockland Immunochemicals).

### 5.1.8 qRT-PCR

RNA was extracted from cell lysates using Trizol reagent (Invitrogen) and cDNA was synthesized using SuperScript III First Strand Synthesis (Invitrogen). Quantitative real-time PCR for survivin was performed on cDNA using the SYBR green method (based on manufacturer's protocol, Applied Biosystems). Primers used were 5'-CTGCCTGGCAGCCCTTT-3' (Forward) and 5'-CCTCCAAGAAGGGCCAGTTC-3' (reverse) for surviving [507] and 5'-CACTGGCTCGTGTGACAAGG-3' and 5'-CAGACCTACTGTGCGCCTACTTAA-3' for β-actin. The relative change in expression was calculated using the Pffal method [508]. Experiments for MCC cell lines were repeated six times (2 biological repeats done in triplicate). Experiments for BJ cell lines were repeated six times (3 biological repeats done in duplicate). Mean and standard error of mean was calculated and plotted as column graphs for comparison.

### 5.1.9 Cell cycle analysis

MKL-1 cells were treated with Accutase (Millipore) to break clumps and then resuspended in fresh medium containing drug and treated for 12 hours. Bromodeoxyuridine (10 µM concentration) was added three hours before harvesting. Cells were then harvested and fixed in chilled 70% ethanol overnight. The cells were then washed, resuspended in 200 ml of 2M HCl/Triton X (1%) and incubated for 30 mins at room temperature. Cells were centrifuged at 2000 rpm for 10 mins and neutralized in 200ml of 0.1M sodium tetraborate (pH 8.5). Cells were then washed, suspended in 20ml of PBS containing 0.5% tween20, 1% donkey serum and 2ml of anti-BrdU antibody (1:10 diution, BD Biosciences) and incubated overnight at 4°C. Cells were washed and incubated with secondary anti-mouse IgG Alexa488 (1:1000) for 1 hour at room temperature. Cells were washed, suspended in PBS containing 100mg/ml of RNase A, 50mg/ml of PI (propidium iodide) and 0,05% Triton X and incubated for 30 mins at 37°C in the dark and then analyzed using an Accuri C6 flow cytometer.

### 5.1.10 Cell death evaluation by CFDA and PI staining

After harvesting, cells were resuspended in 2 ml of PBS containing 4mg/ml PI (propidium iodide, Sigma) and 10 mM CFDA (Carboxy fluorescin diacetate, Invitrogen) at room temperature for 10 mins. Cells were then rinsed in 1X PBS and examined under the microscope. Quantitation was performed using ImageJ software.

### 5.1.11 Mouse xenograft studies

**Compounds**

For *in vivo* experiments, clinical-grade bortezomib (Velcade) was purchased from the University of Pittsburgh Cancer Institute Pharmacy and YM155 was purchased from Active Biochemicals Ltd. (Hong Kong, China). Compounds were dissolved in sterile 0.9% saline

solution for administration to animals.

**Animals**

Six-week-old female triple immune-deficient NSG (NOD scid gamma) mice (Jackson Laboratory) were maintained in a specific pathogen-free environment at the Hillman Cancer Center Mouse Facility, University of Pittsburgh. All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the University of Pittsburgh (IACUC Protocol # 1102226).

**Xenograft Drug Treatments**

MCC cells were checked for viability >90% by trypan blue staining resuspended in PBS ($2 \times 10^7$ cells in 100 µl) and inoculated subcutaneously into the right flank of mice. Once tumors were palpable (2-4 weeks after injection), mice were assigned sequentially into receiving either bortezomib, YM-155 or saline treatment arms.

All treatments were delivered for three consecutive weeks. Bortezomib treatment was delivered subcutaneously twice weekly at 1 mg/kg per mouse. To avoid previously observed side effects, mice were given hydrogel (Clear$H_2O$) and kept at 30°C (using a heating blanket to heat up half of the cage) during bortezomib treatment. YM-155 (2 mg/kg) was given intraperitoneally on five consecutive days, followed by a two-day treatment-free interval. The control group received saline alone (21 mice on the same dosing schedule as bortezomib and 10 mice on the same dosing schedule as YM155). Day 19 was the last day of drug delivery for both schedules and hence the end of treatment. Caliper measurements of the longest perpendicular tumor diameters were performed every other day and tumor volumes were calculated using the formula: $(\text{width})^2 \times (\text{length}/2)$. Animals were sacrificed when tumors reached 2 cm in any dimension, >20% weight loss or when they became moribund. Survival was defined as time from the first day of treatment until death/sacrifice.

### 5.1.12 Statistical analysis

Two-tailed paired Student's *t* test was used to analyze statistical differences in qRT-PCR results. Mouse survival curves were estimated using the Kaplan–Meier product-limit method and were compared using the log-rank test (GraphPad Software, La Jolla, CA). A piecewise linear hierarchical Bayesian model [509] was used to characterize differences in tumor volumes and growth between treatments.

### 5.1.13 Immunohistochemistry

Immunohistochemical staining of mouse tumor tissues was performed as previously described in Shuda *et al*. 2009 [506].

## 5.2 RESULTS

### 5.2.1 Survivin expression in MCV-positive MCC

To identify pathways perturbed by MCV infection in MCC, we analyzed DTS datasets to identify cellular genes differentially regulated between MCV-positive and MCV-negative MCC [74]. We compared 400,000 cellular transcripts from MCV-positive and MCV-negative MCC tumors and found 1096 of 11,531 (9.5%) genes elevated >3 fold for the MCV-positive compared to the MCV-negative library.

We next identified sixty-four genes using Gene Ontology (GO) gene definitions [510]

directly involved in programmed cell death or cell cycle regulation (Table 19 and Figure 23A). *BIRC5a* (Baculoviral inhibitor of apoptosis repeat-containing 5) mRNA encoding the survivin oncoprotein were increased 7 fold ($p=2.90xe^{-10}$) for virus-positive compared to virus-negative MCC tumors (Figure 23A and Table 19). Other genes regulating programmed cell death, including TP53, cIAP2, XIAP, BAX, BCL2 and Caspase 3/6 transcripts, were not differentially expressed (Figure 23A and Table 19). Notably, some genes including TRAF2 and PI3K were significantly reduced in virus-positive compared to virus-negative MCC tumor libraries.

### 5.2.2 MCV large T induces survivin through retinoblastoma protein targeting

To determine if MCV T antigen increases survivin expression in MCC, we used a short-hairpin (shRNA) lentivirus (panT1) to perform RNAi targeting of the MCV T antigen exon1 sequence that selectively knocks down all MCV T antigen isoforms in MCC cells [498]. MCV-positive MCC cells infected with this lentivirus undergo non-apoptotic cell death (necroptosis) when MCV oncoprotein expression is inhibited [498]. MCV T antigen reduction correlated with survivin reduction in all MCV-positive MCC cell lines, but not in any of the MCV-negative cell lines (Figure 23B). The degree of survivin decrease with T antigen knockdown ranged from modest in MS-1 to near complete in WaGa cell lines (Figure 23B). This effect is at the level of

## Table 19 Gene expression patterns in MCC Libraries using DTS comparison

| Gene symbol | Gene name | NCBI Accession | MCC347 Library (MCV +ve)* | MCC337.343.346 Library (MCV -ve)* | Normalized ratio (MCV +ve / MCV -ve) | Log2 value # |
|---|---|---|---|---|---|---|
| Apotosis-associated genes | | | | | | |
| *BIRC5* | Baculoviral IAP repeat containing 5 | NM_001012271 | 17 | 2 | 6.79 | 2.76 |
| *DFFB* | DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase) | NM_004402 | 7 | 1 | 5.59 | 2.48 |
| *CHUK* | Conserved helix-loop-helix ubiquitous kinase | NM_001278 | 7 | 1 | 5.59 | 2.48 |
| *ENDOG* | Endonuclease G | NM_004435 | 6 | 1 | 4.79 | 2.26 |
| *AIFM1* | Apoptosis-inducing factor, mitochondrion-associated, 1 | NM_145813 | 9 | 2 | 3.59 | 1.85 |
| *PPP3CA* | Protein phosphatase 3, catalytic subunit, alpha isozyme | NM_000944 | 36 | 8 | 3.59 | 1.85 |
| *BIRC2* | Baculoviral IAP repeat containing 2 | NM_001166 | 8 | 2 | 3.19 | 1.68 |
| *CASP10* | Caspase 10, apoptosis-related cysteine peptidase | NM_032977 | 8 | 2 | 3.19 | 1.68 |
| *ATM* | Ataxia telangiectasia mutated | NM_138292 | 37 | 10 | 2.96 | 1.56 |
| *TNFRSF1A* | Tumor necrosis factor receptor superfamily, member 1A | NM_001065 | 19 | 6 | 2.53 | 1.34 |
| *IRAK2* | Interleukin-1 receptor-associated kinase 2 | NM_001570 | 3 | 1 | 2.40 | 1.26 |
| *MYD88* | Myeloid differentiation primary response gene (88) | NM_002468 | 6 | 2 | 2.40 | 1.26 |
| *PRKAR2B* | Protein kinase, cAMP-dependent, regulatory, type II, beta | NM_002736 | 3 | 1 | 2.40 | 1.26 |
| *PIK3R3* | Phosphoinositide-3-kinase, regulatory subunit 3 (gamma) | NM_003629 | 15 | 6 | 2.00 | 1.00 |
| *CHP* | Calcium binding protein P22 | NM_007236 | 12 | 5 | 1.92 | 0.94 |
| *BCL2* | B-cell CLL/lymphoma 2 | NM_000657 | 2 | 1 | 1.60 | 0.68 |
| *CASP3* | Caspase 3, apoptosis-related cysteine peptidase | NM_032991 | 4 | 2 | 1.60 | 0.68 |
| *TRADD* | TNFRSF1A-associated via death domain | NM_003789 | 2 | 1 | 1.60 | 0.68 |
| *ENDOD1* | Endonuclease domain containing 1 | NM_015036 | 11 | 6 | 1.46 | 0.55 |
| *MAP3K14* | Mitogen-activated protein kinase kinase kinase 14 | NM_003954 | 8 | 5 | 1.28 | 0.35 |
| *PRKAR1A* | Protein kinase, cAMP-dependent, regulatory, type I, alpha | NM_212472 | 48 | 30 | 1.28 | 0.35 |
| *PRKX* | Protein kinase, X-linked | NM_005044 | 8 | 5 | 1.28 | 0.35 |
| *CAPN1* | Calpain 1, (mu/I) large subunit | NM_005186 | 18 | 12 | 1.20 | 0.26 |
| *PIK3R2* | Phosphoinositide-3-kinase, regulatory subunit 2 (beta) | NM_005027 | 61 | 43 | 1.13 | 0.18 |
| *CYCS* | Cytochrome c, somatic | NM_018947 | 51 | 40 | 1.02 | 0.03 |
| *DFFA* | DNA fragmentation factor, 45kDa, alpha polypeptide | NM_213566 | 23 | 18 | 1.02 | 0.03 |
| *RIPK1* | Receptor (TNFRSF)-interacting serine-threonine kinase 1 | NM_003804 | 12 | 10 | 0.96 | -0.06 |
| *AKT2* | v-akt murine thymoma viral oncogene homolog 2 | NM_001626 | 23 | 20 | 0.92 | -0.12 |
| *APAF1* | Apoptotic peptidase activating factor 1 | NM_013229 | 8 | 7 | 0.91 | -0.13 |
| *PRKY* | Protein kinase, Y-linked, pseudogene | NM_002760 | 9 | 8 | 0.90 | -0.15 |
| *AKT1* | v-akt murine thymoma viral oncogene homolog 1 | NM_001014432 | 36 | 34 | 0.85 | -0.24 |
| *CASP7* | Caspase 7, apoptosis-related cysteine peptidase | NM_033338 | 2 | 2 | 0.80 | -0.32 |
| *EXOG* | Endo/exonuclease (5'-3'), endonuclease G-like | NM_005107 | 1 | 1 | 0.80 | -0.32 |
| *IRAK4* | Interleukin-1 receptor-associated kinase 4 | NM_016123 | 5 | 5 | 0.80 | -0.32 |
| *NTRK1* | Neurotrophic tyrosine kinase, receptor, type 1 | NM_001012331 | 1 | 1 | 0.80 | -0.32 |
| *PIK3CB* | Phosphoinositide-3-kinase, catalytic, beta polypeptide | NM_006219 | 4 | 4 | 0.80 | -0.32 |
| *PPP3CB* | Protein phosphatase 3, catalytic subunit, beta isozyme | NM_021132 | 15 | 15 | 0.80 | -0.32 |
| *TNFRSF10B* | Tumor necrosis factor receptor superfamily, member 10b | NM_147187 | 2 | 2 | 0.80 | -0.32 |
| *cIAP2* | Cellular inhibitor of apoptosis 2 | NM_001165 | 1 | 1 | 0.80 | -0.32 |
| *IKBKG* | Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma | NM_001099857 | 8 | 9 | 0.71 | -0.49 |
| *BCL2L1* | BCL2-like 1 | NM_138578 | 24 | 28 | 0.68 | -0.55 |
| *PRKACB* | Protein kinase, cAMP-dependent, catalytic, beta | NM_002731 | 11 | 13 | 0.68 | -0.57 |
| *PRKACA* | Protein kinase, cAMP-dependent, catalytic, alpha | NM_207518 | 25 | 31 | 0.64 | -0.63 |
| *CFLAR* | CASP8 and FADD-like apoptosis regulator | NM_003879 | 5 | 8 | 0.50 | -1.00 |
| *PIK3CA* | Phosphoinositide-3-kinase, catalytic, alpha polypeptide | NM_006218 | 3 | 4 | 0.60 | -0.74 |
| *PPP3R1* | Protein phosphatase 3, regulatory subunit B, alpha | NM_000945 | 6 | 8 | 0.60 | -0.74 |
| *TP53* | Tumor protein p53 | NM_000546 | 15 | 21 | 0.57 | -0.81 |
| *BAX* | BCL2-associated X protein | NM_138761 | 2 | 3 | 0.53 | -0.91 |
| *PIK3CD* | Phosphoinositide-3-kinase, catalytic, delta polypeptide | NM_005026 | 2 | 3 | 0.53 | -0.91 |
| *PRKAR1B* | Protein kinase, cAMP-dependent, regulatory, type I, beta | NM_002735 | 3 | 5 | 0.48 | -1.06 |
| *XIAP* | X-linked inhibitor of apoptosis protein | NM_001167 | 10 | 18 | 0.44 | -1.17 |
| *CASP9* | Caspase 9, apoptosis-related cysteine peptidase | NM_032996 | 1 | 2 | 0.40 | -1.32 |
| *BID* | BH3 interacting domain death agonist | NM_197967 | 1 | 2 | 0.40 | -1.32 |
| *CHP2* | Calcineurin B homologous protein 2 | NM_022097 | 1 | 2 | 0.40 | -1.32 |
| *NFKBIA* | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | NM_020529 | 10 | 22 | 0.36 | -1.46 |
| *IL1R1* | Interleukin 1 receptor, type I | NM_000877 | 3 | 7 | 0.34 | -1.55 |
| *IKBKB* | Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta | NM_001556 | 5 | 12 | 0.33 | -1.59 |
| *AKT3* | v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma) | NM_005465 | 2 | 5 | 0.32 | -1.65 |
| *CASP6* | Caspase 6, apoptosis-related cysteine peptidase | NM_001226 | 2 | 5 | 0.32 | -1.65 |
| *BAD* | BCL2-associated agonist of cell death | NM_032989 | 5 | 13 | 0.31 | -1.70 |
| *IRAK1* | Interleukin-1 receptor-associated kinase 1 | NM_001025243 | 9 | 24 | 0.30 | -1.74 |
| *NFKB1* | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 | NM_003998 | 1 | 3 | 0.27 | -1.91 |
| *PIK3R1* | Phosphoinositide-3-kinase, regulatory subunit 1 (alpha) | NM_181504 | 3 | 9 | 0.27 | -1.91 |
| *PPP3CC* | Protein phosphatase 3, catalytic subunit, gamma isozyme | NM_005605 | 2 | 9 | 0.18 | -2.49 |
| *CAPN2* | Calpain 2, (m/II) large subunit | NM_001748 | 3 | 21 | 0.11 | -3.13 |
| *TRAF2* | TNF receptor-associated factor 2 | NM_021138 | 1 | 11 | 0.07 | -3.78 |
| Control genes | | | | | | |
| *ACTB* | Actin, beta | NM_001101 | 544 | 668 | 0.65 | -0.62 |
| *B2M* | Beta-2-microglobulin | NM_004048 | 94 | 60 | 1.25 | 0.32 |
| *CK20* | Keratin 20 | NM_019010 | 15 | 13 | 0.92 | -0.12 |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | NM_002046 | 438 | 228 | 1.53 | 0.62 |
| *HPRT1* | Hypoxanthine phosphoribosyltransferase 1 | NM_000194 | 7 | 9 | 0.62 | -0.69 |
| *PPIA* | Peptidylprolyl isomerase A (cyclophilin A) | NM_021130 | 143 | 75 | 1.52 | 0.61 |

** "MCV +ve" stands for MCV positive in the MCC347 library with 143,373 gene reads; "MCV -ve" stands for MCV negative in the MCC337.343.346 library with 114,514 gene reads*

# "+" stands for gene upregulation in the "MCV +ve" library compared with the "MCV -ve" one; "-" stands for gene downregulation ratios.

(Feng et al. Science 319:1096-100, 2008).

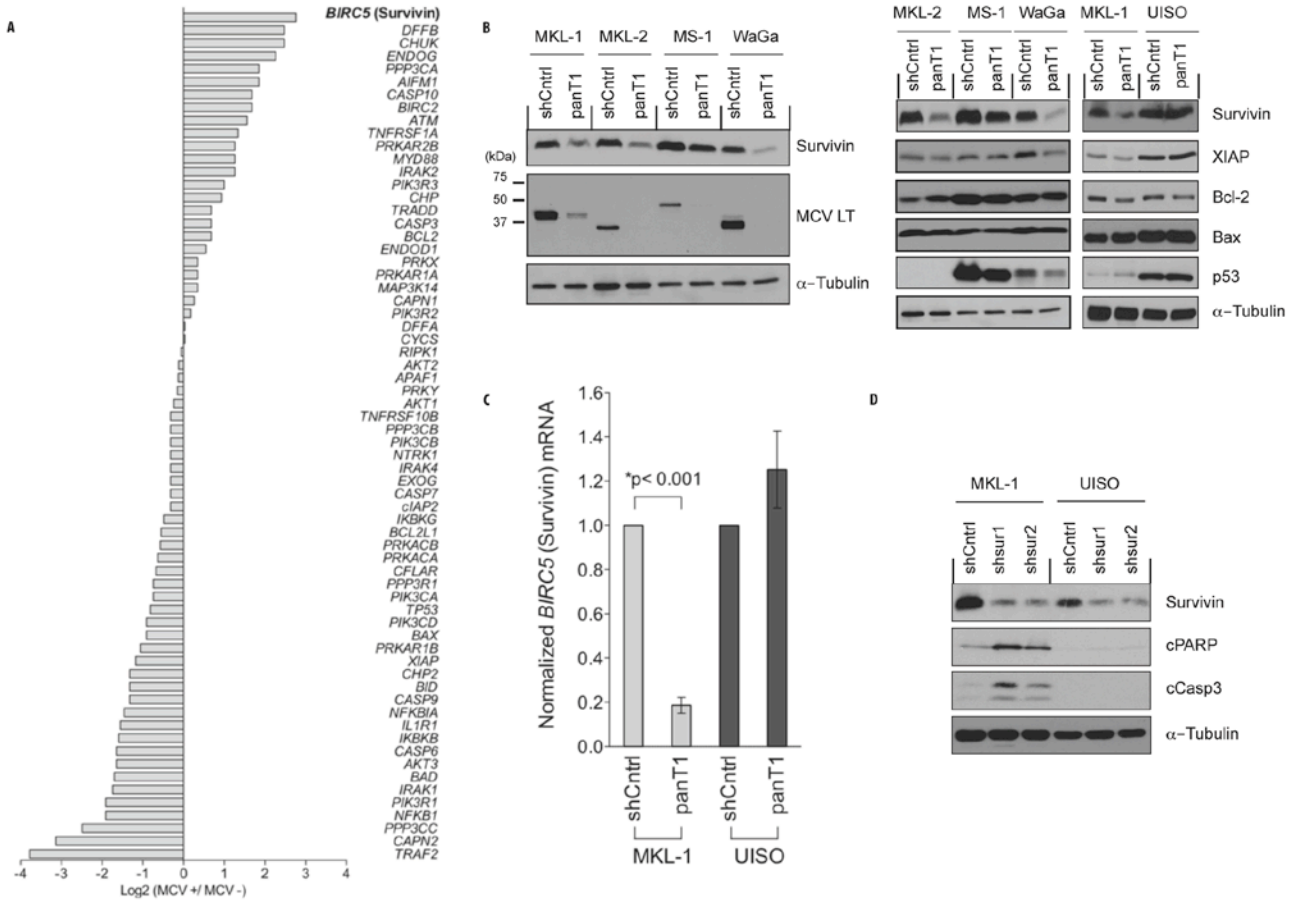



**Figure 23 Survivin oncoprotein mRNA expression is increased in MCV-positive MCC.**

(A) DTS comparison of 64 genes involved in programmed cell death and cell cycle regulation, showing that survivin (*BIRC5*) mRNA transcripts (highlighted in bold) were seven-fold higher in a MCV-positive than an MCV-negative MCC DTS cDNA library. The relative expression of genes was normalized to total sequence reads for each MCC library (also see Table 19 for more details). (B) MCV T antigen is required for survivin expression. Lentiviral MCV T antigen exon1 knockdown (panT1) decreased survivin protein expression among four MCV-positive MCC cell lines (left panel). shCntrl is a scrambled shRNA control lentivirus. No consistent changes in XIAP, BCL-2, Bax or p53 protein levels are seen after MCV T antigen knockdown among MCC cell lines (right panel). MKL-1, MKL-2, MS-1 and WaGa are MCV positive and UISO is MCV negative. (C) MCV T antigen is required for survivin transcription. Survivin mRNA levels were reduced in MKL-1 but not UISO cells after T antigen knockdown indicating that T antigen acts survivin transcription. Survivin mRNA was measured by qRT-PCR and normalized to β-actin mRNA. The experiments were performed in triplicate and repeated two times (mean±SEM). (D) Survivin expression is required for MCV-positive MCC cell survival. Survivin was targeted for knock down with two shRNA lentiviral vectors, shsur1 and shsur2 in MKL-1 cells and UISO cells. MKL-1 cells initiate apoptosis after survivin knockdown, with increased expression of cleaved polyADP ribose polymerase (cPARP) and caspase 3 (cCasp3), whereas UISO cells are resistant to survivin knock down-induced apoptosis. α-Tubulin is used as a loading control.

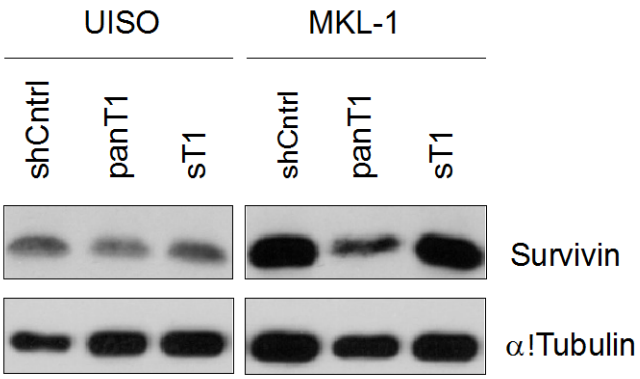



**Figure 24. MCV sT antigen knockdown does not affect survivin expression**
UISO and MKL-1 cells infected with sT1, which knockdown sT alone (Shuda *et al*., JCI 2011) as well as panT1 shRNA lentiviruses were tested for immunoblotting for survivin and α- tubulin. Lanes were run on the same gel, but were not contiguous.

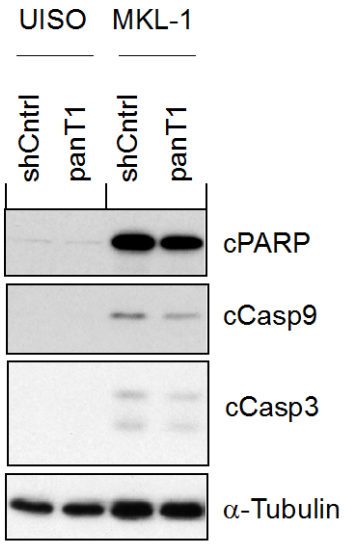



**Figure 25. T antigen knockdown does not affect caspase and PARP cleavage.**
UISO and MKL-1 cells with panT1 knockdown were tested for immunoblotting for cleaved caspase 3 and 9, cleaved PARP and α- tubulin. Also see Figure 20.

transcription rather than translation since *BIRC5α* mRNA is significantly reduced by T antigen knockdown (Figure 23C). Use of an shRNA lentivirus that selectively targets only the small T antigen isoform [504], however, did not affect survivin expression (Figure 24), indicating that increased survivin transcription was dependent on MCV LT but not small T antigen (knockdown of LT alone is difficult due to the overlapping structure of the T antigen cistron). In contrast to

survivin, no consistent changes in protein expression for p53, XIAP, Bcl-xL, Mcl-1, Bad, Bik, Bmf, Bim, Puma, Bcl-2 or Bax proteins (Figure 23B and [498]) or for cleaved polyADP ribose polyomerase (PARP), cleaved caspase 3 or cleaved caspase 9 proteins (Figure 25) were seen after panT1 knock down in MCV-positive cells. These results are consistent with our DTS findings suggesting that MCV T antigen selectively activates *BIRC5α* transcription. We next sought to determine the importance of survivin expression to survival of MCV-positive and MCV-negative MCC cells. Although only partial suppression of survivin expression was achieved by shRNA *BIRC5α* targeting, MCV-positive MKL-1 cells, but not MCV-negative UISO cells, underwent apoptosis (Figure 23D).

To confirm that LT is the MCV T antigen isoform responsible for survivin activation, we cloned and transduced a tumor-derived LT (LT339) cDNA into nontransformed, primary human BJ fibroblasts. Survivin protein levels increased three-fold when LT was expressed compared to the empty vector control (as measured by quantitative LICOR immunoblotting, Figure 26 A and 26 B). Also consistent with our knockdown experiments (Figure 23C), LT directly activates *BIRC5α* promoter transcription in BJ cells (Figure 26C). This is mediated by a specific domain (LXCXE) in LT responsible for sequestration of RB1. Both cyclin E and E2F1, required for cell cycle S phase entry [511], are repressed by active RB1 and were used as markers for RB-regulated gene expression. MCV LT339 activates expression of both cyclin E and E2F1 proteins in BJ cells whereas LT339, having a point mutation that prevents RB1 binding (changing LFCDE to LFCDK) [368], abolishes induction of cyclin E, E2F1 and survivin (Figure 26 A). MCV small T expressed alone in BJ cells did not increase survivin oncoprotein expression (Figure 27). In contrast to primary BJ fibroblasts, we did not find that MCV LT markedly increased survivin expression in cell lines that had been already transformed, including 293HEK and U2OS cells (Figure 28).

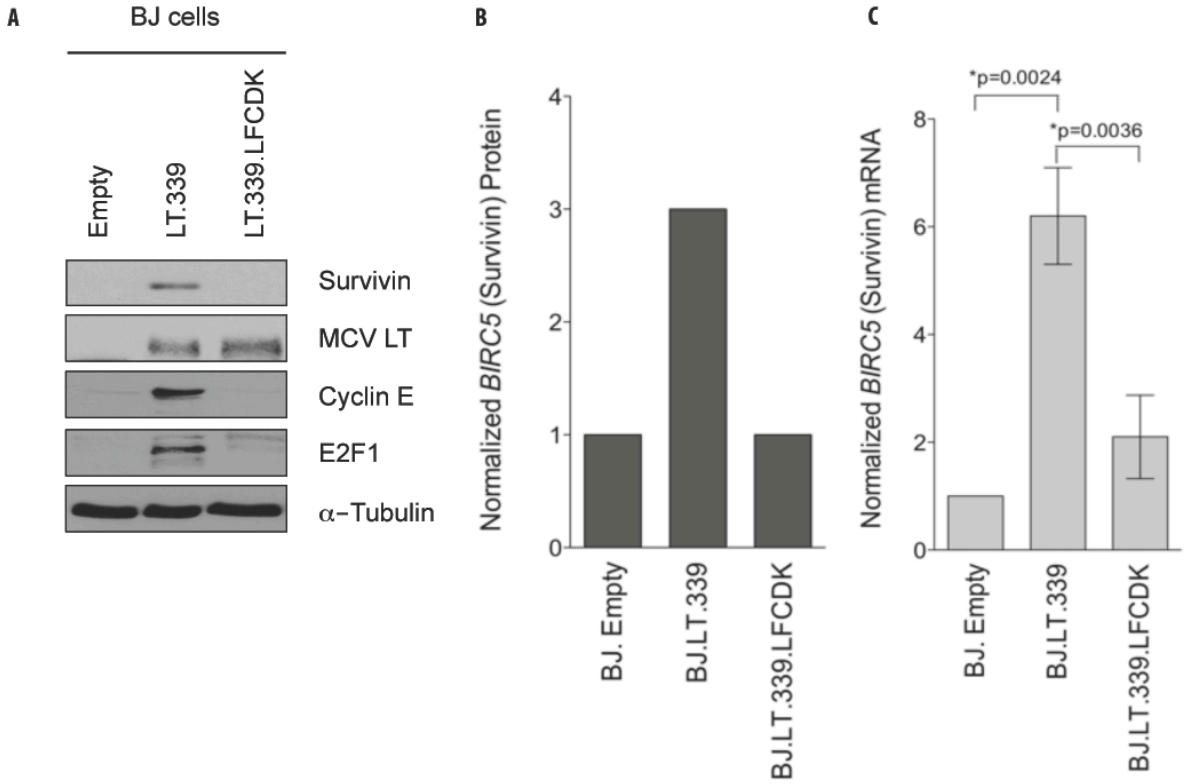



**Figure 26 MCV LT protein isoform induces survivin oncoprotein expression in human BJ cells by targeting retinoblastoma protein (RB)**

(A) BJ cells were transduced with either empty vector, a tumor-derived LT cDNA (LT.339) or an LT cDNA with an inactive RB binding domain (LT.339LFCDK). Immunoblotting reveals that MCV LT.339 induces survivin expression but LT.339LFCDK does not. A similar pattern is seen for other S phase cell cycle proteins such E2F1 and cyclin E that are also transcriptionally repressed by RB. (B) LICOR quantitative immunoblotting for survivin in (A), normalized to ±tubulin (arbitrary units). (C) Survivin mRNA levels increased in BJ cells expressing LT.339 protein but not in cells expressing the RB1 binding mutant LT.339LFCDK. BJ cells expressing either empty, LT.339 or LT.339LFCDK were serum starved for 48 hours and then harvested for RNA. Survivin mRNA was measured by qRT-PCR and normalized to β-actin mRNA. The experiments performed three times in duplicate (mean±SEM).

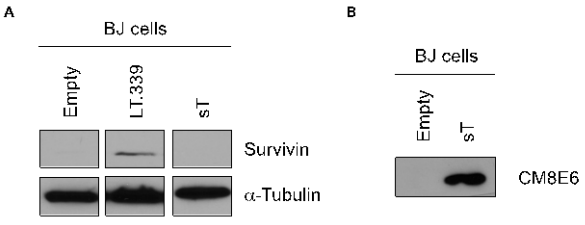



**Figure 27 MCV sT isoform doe not induce survivin expression in BJ cells.**

BJ cells were transduced with either empty vector, tumor derived LT cDNA (LT.339) or small T antigen cDNA and immunoblotted for (A) survivin and α-tubulin (B) CM8E6 (MCV T antigen exon 1). Lanes were run on the same gel, but were not contiguous.

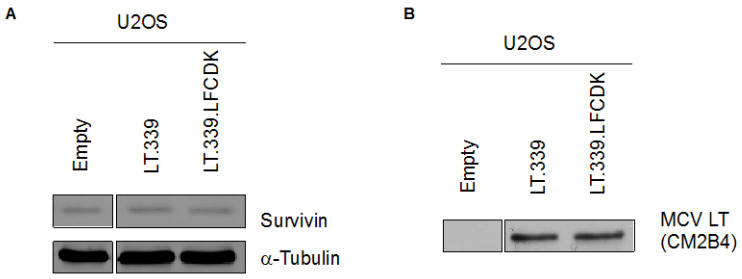



**Figure 28 MCV LT protein does not affect survivin oncoprotein expression in U2OS cells.**
U2OS cells were transduced and stably selected with empty vector, tumor derived LT cDNA (LT.339) or an LT cDNA with an inactive RB binding domain (LT.339.LFCDK) and immunoblotted for (A) survivin and α-tubulin (LICOR) (B) MCV LT (using CM2B4 antibody). Lanes were run on the same gel, but were not contiguous.

### 5.2.3 Survivin as a target for MCC chemotherapy

Given the apparent importance of MCV-induced survivin expression to MCC cell survival, we examined YM155, an imidazolium small molecule inhibitor (Figure 29) of the survivin promoter that is currently undergoing Phase II trials for prostate cancer [507, 512, 513]. YM155 is both highly active and selective for inhibiting MCV-positive MCC cell growth *in vitro* as measured by Cell-Titer Glo assays ($EC_{50}$ 1.34 nM to 12.2 nM) (Figure 30A). MCV-negative MCC cell is also inhibited by YM155 but this occurs at concentrations 1-2 orders of magnitude higher than for MCV-positive MCC cells. YM155 treatment for 48 hours at 10-100 nM preferentially killed MCV-positive MKL-1 compared to MCV-negative UISO cells as measured by trypan blue staining (Figure 30B). Survivin protein was reduced in MKL-1 cells after YM155 treatment, consistent with YM155's proposed mechanism of action in inhibiting the BIRC5 prmoter (Figure 30C).

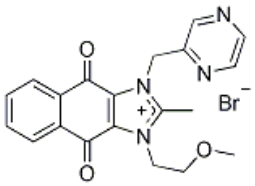



**Figure 29 YM155 chemical structure. Scanned from MSDS sheet of YM155**
(Activebiochemicals Inc.)

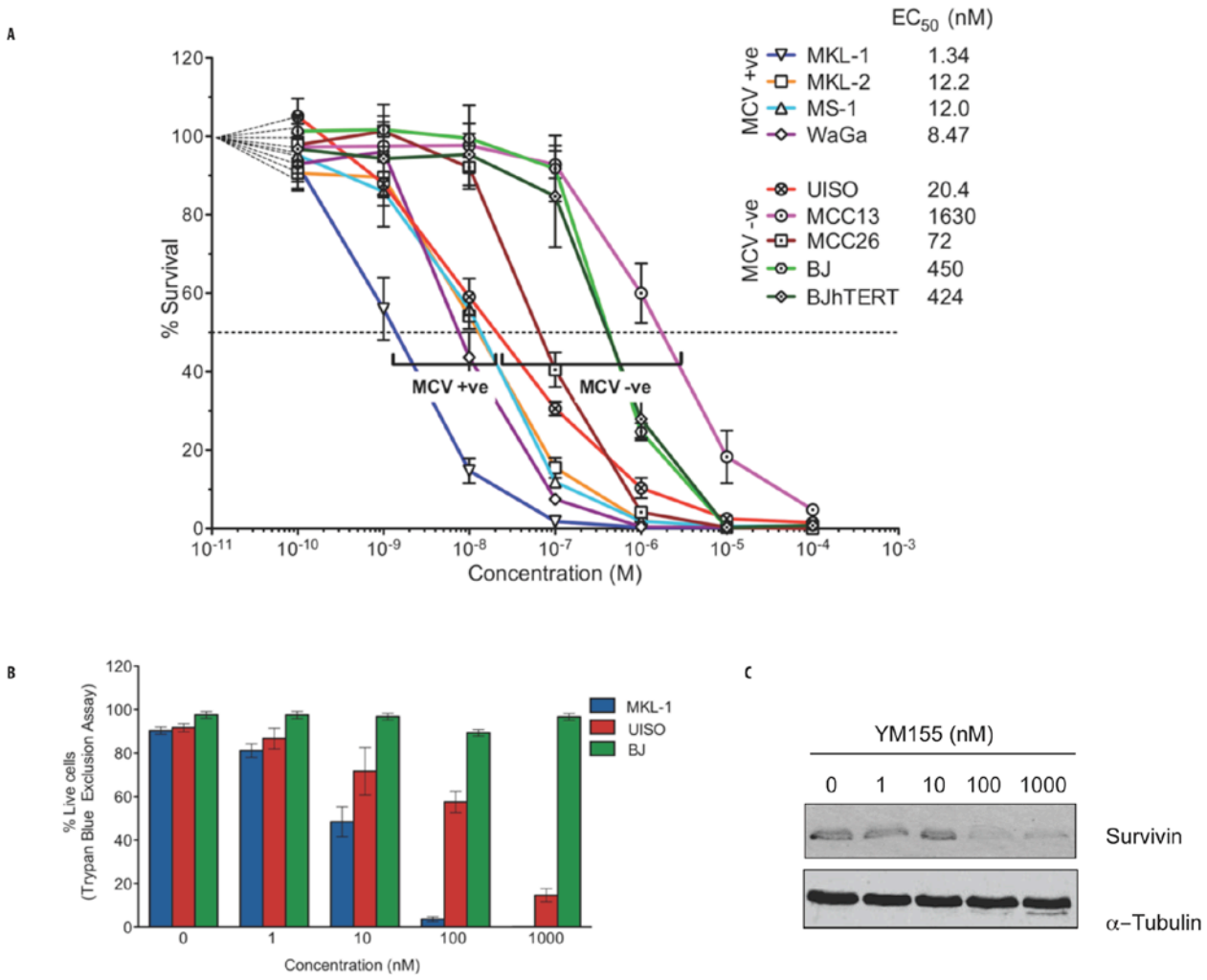



**Figure 30 The survivin promoter inhibitor YM155 inhibits MCV-positive MCC cell line growth.**
(A) Dose-dependent growth curves at 48 h for YM155-treated cell lines. MCV-negative MCC13, BJ and BJhTERT cells showed relative resistance to YM155 treatment, whereas all MCV-positive cell lines (MKL-1, MKL-2, MS-1 and WaGa) were sensitive to YM155. MCV-negative UISO and MCC26 had intermediate sensitivity to the YM155. (B) Trypan blue vital dye exclusion assay showed dose-dependent cell killing at 48 h for MKL-1 cells (blue bars) whereas UISO cells (red bars) are relatively less sensitive and BJ cells (green bars) are resistant to YM155. (C) Dose-dependent decrease in MKL-1 cell survivin protein expression after 12 h YM155 treatment.

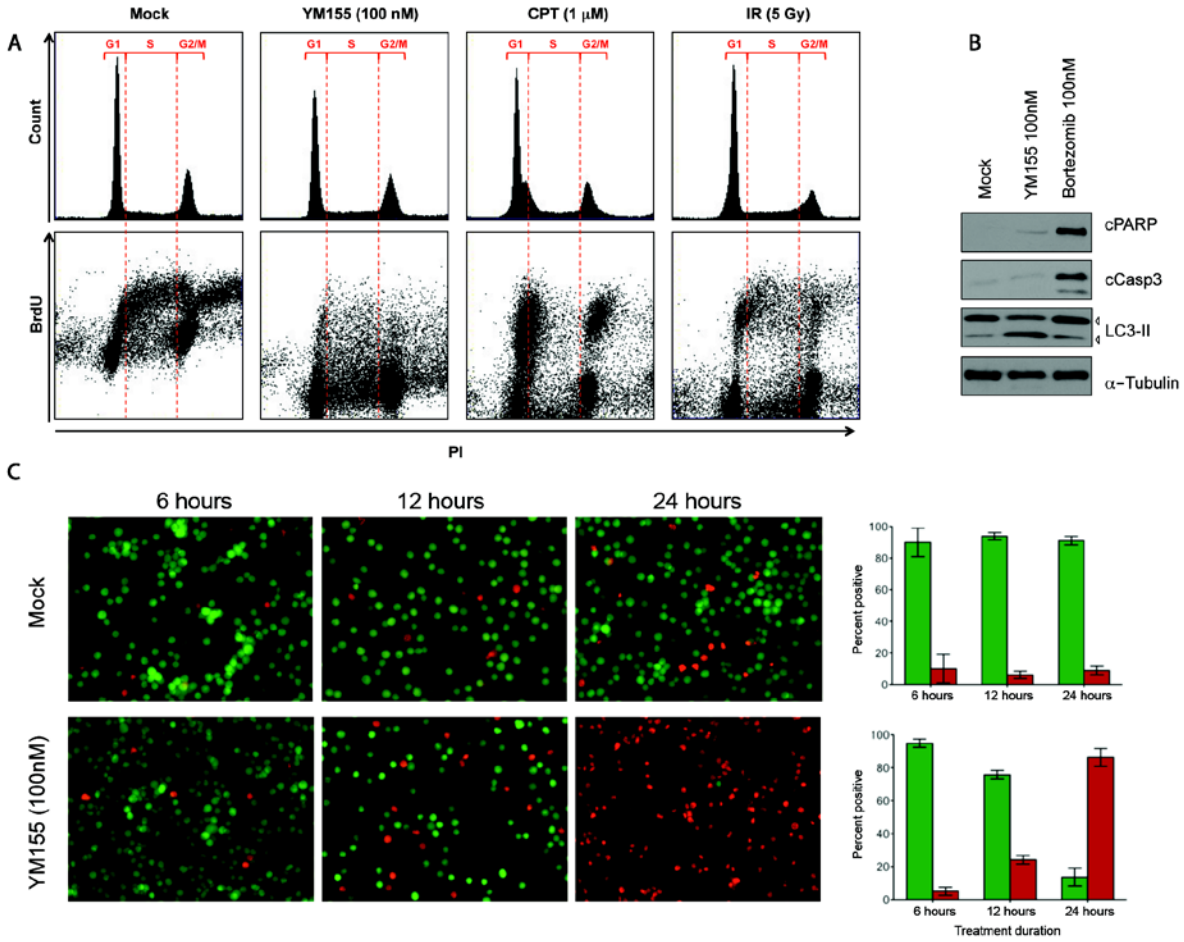



**Figure 31 Cell death phenotype of YM155-treated MCV-positive MCC.**

(A) Cell cycle analysis reveals YM155 treated cells do not undergo mitotic catastrophe. MKL-1 cells were treated with DMSO, YM155 (100nM) and camptothecin (CPT, 1 [ BrdU staining (lower panel). Boxes show S phase BrdU incorporation without treatment, accumulation at G1 and G2/M/G1 tetraploid during CPT treatment, and near-complete loss of BrdU incorporation during YM155 treatment. Box a represents G1 phase, box b represents S phase and box c represents G2/M phase and M1-M4 describe the corresponding percentages of cells in these phases (B) YM155 induces nonapoptotic cell death associated with autophagy in MKL-1 cells. MKL-1 cells were treated with DMSO, YM155 (100nM) or bortezomib (100nM) and immunoblotted for (c) cleaved PARP, cCaspase 3, LC3 and -tubulin. In contrast to YM155, the bortezomib proteasome inhibitor activates MKL-1 cell apoptosis (also see Fig S2). (C) YM155 treatment initiates programmed cell death within 12-24 hours after treatment. MKL-1 cells were co-stained with CFDA (green, live) and PI (red, dead). Column graphs (right panel) represent mean and range of % CFDA-positive (green) and % PI-positive cells (red).

We next examined mechanisms of cell killing by YM155 in MCV-positive MCC cells (Figure 31). Survivin plays a role in mitotic progression and loss of survivin can lead to cell death through mitotic catastrophe in some tumor cells [514, 515]. MCV-positive MCC, however, are slowly cycling cells (doubling time of 3 days, [498]) and do not undergo G1 arrest or G2/M pileup as would occur with mitotic checkpoint activation in rapidly cycling cells (Figure 31A, top panels). Instead, bromo-deoxyuridine (BrdU) incorporation into DNA reveals a profound inhibition of DNA synthesis by YM155. Figure 31 A (boxes, bottom left panel) shows the normal inverted-U pattern for BrdU incorporation during S phase DNA synthesis in MKL-1 cells. When the topoisomerase I inhibitor camptothecin is added (Figure 31A, bottom right panel), accumulation of BrdU-positive cells in G1 and G2/M/G1-tetraploid becomes evident, and S phase incorporation is lost, consistent with repair polymerase activation during DNA damage response signaling. YM155 treatment, however, ablates BrdU incorporation (Figure 31A, center panel), most consistent with disruption of DNA replication forks and inhibition of new S phase and repair DNA synthesis, but not mitotic catastrophe.

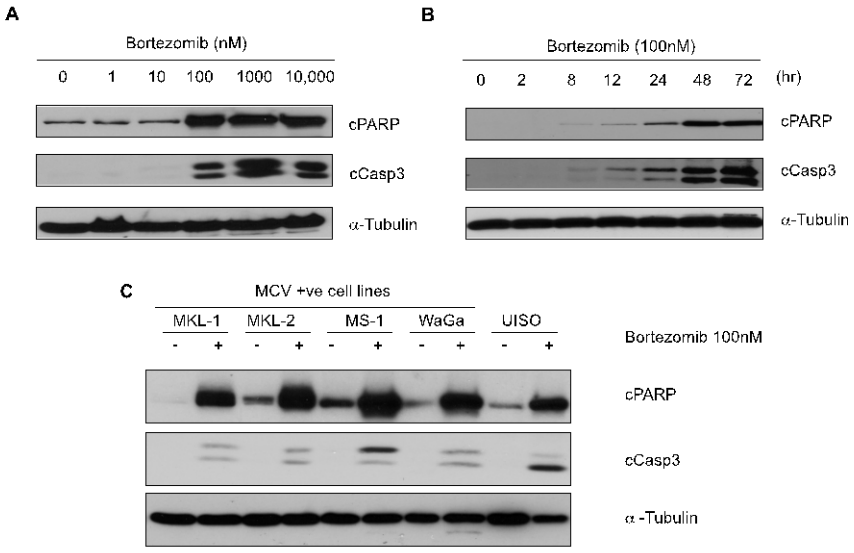



**Figure 32 Bortezomib induces apoptotic cell death in MCC cells.**
(A) MKL-1 cells were treated with Bortezomib (1,10,100,1000 and 10,000 nM) for 48 h or (B) MKL-1 cells treated with 100nM bortezomib for different time points, or (C) other MCC cell lines (MKL-1, MKL-2, MS-1, WaGa and UISO) treated with 100nM bortezomib for 48 h were treated for immunoblotting for cleaved caspase 3, cleaved PARP apoptotic markers and α-tubulin. 0.01% DMSO was used for mock-treated control.

YM155 treatment results in early commitment to nonapoptotic programmed cell death. No evidence of apoptosis was present, measured by caspase 3 and PARP cleavage (Figure 31B), when MKL-1 cells were treated with 100 nM YM155 for 48 hours, but YM155 did initiate LC3-II accumulation, a marker for cell autophagy [516]. This is not due to loss of apoptosis pathway signaling since treatment with the proteasome inhibitor bortezomib (Velcade) activated PARP and caspase cleavage (Figure 31B and Figure 32). Commitment to YM155-dependent cell death occurs relatively quickly and irreversibly: when MKL-1 cells were treated with 100 nM YM155 for 3, 6 or 12 hours, followed by wash out with complete cell culture medium, only 51%, 3% and 1.8% of cells respectively, remained viable at 48 hours, as measured by trypan blue dye exclusion. This was confirmed by a cell viability assay using propidium iodide (PI, dead) and carboxyfluorescein diacetate (CFDA, alive) co-staining (Figure 31C). Twelve to 24 hours of 100 nM YM155 treatment causes MKL-1 cells to lose membrane integrity, becoming positive for PI and negative for CFDA staining.

To search for other MCC chemotherapeutics, we performed a two-stage cytotoxicity library screen on 1,360 pharmacologically-active drug compounds (see Table 22 and Figures 33A and 33B) including 1,280 drugs from the Library of Pharmacologically Active Compounds (LOPAC) (Sigma Aldrich), 89 drugs from the National Cancer Institute's Approved oncology drug set II (19 compounds in common with LOPAC1280), 6 compounds targeting SV40 LT ATPase activity [500, 501] and 4 compounds targeting MDM2 to activate p53 [502, 503]. These compounds were screened at $10^{-5}$ M for >90% inhibition of MKL-1 cell growth in a Cell-Titer Glo assay (Promega). Notably, mTOR inhibitors (everolimus, rapamycin etc), antiviral compounds (ribavirin, acyclovir) and MDM-2 inhibitors active in other viral cancers (e.g., nutlin-3 [517, 518]) were not active in our screen. This is consistent with previous findings that MCV loses replication activity [368] and activates cap-dependent translation downstream of mTOR [504] in MCC tumor cells.

Eighteen (1.3%) of these 1360 drugs met our initial screening criterion for anti-MCC activity and were selected for secondary dose-dependent screening on MCV-positive and MCV-

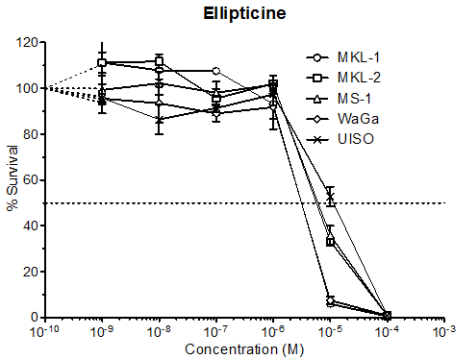
negative cell lines (Table 20). Only the proteasome-inhibitor bortezomib [519] was active *in vitro* at low doses ($EC_{50}$ 1.3-13.2 nM, Figure 33C and Figure 32). This activity, however, was not selective for MCV-positive cells (Figure 33). Other agents, particularly topoisomerase I and II inhibitors, also inhibited MCC cell growth but were generally far less potent or had variable activity among different MCC cell lines (Table 20 and Figures 33A and 33B).

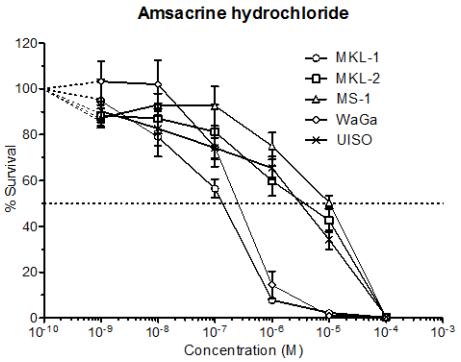
**Table 20 $EC_{50}$ (µM) concentrations for MCC cell lines**

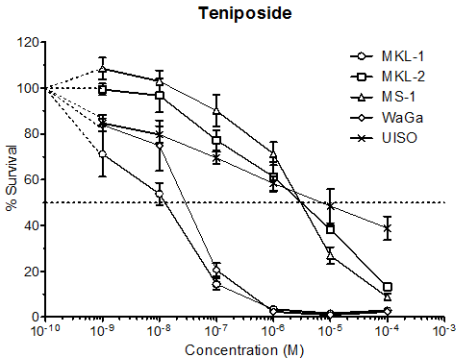
| Drugs | MKL-1 | MKL-2 | MS-1 | WaGa | UISO |
|---|---|---|---|---|---|
| **PROTEASOME INHIBITOR** | | | | | |
| Bortezomib | 0.013 | 0.005 | 0.002 | 0.001 | 0.003 |
| **TOPOISOMERASE INHIBITORS** | | | | | |
| Ellipticine | 3.2 | 6.5 | 6.9 | 3.0 | 1.1 |
| Amsacrine hydrochloride | 0.11 | 3.0 | 7.5 | 0.25 | 2.6 |
| Teniposide | 0.010 | 2.8 | 3.0 | 0.026 | 7.3 |
| Valrubicin | 0.23 | 2.8 | 9.9 | 0.32 | 4.0 |
| Mitoxantrone | 0.006 | 1.3 | 1.6 | 0.014 | 0.97 |
| Daunorubicin | 0.015 | 0.086 | 0.15 | 0.018 | 1.4 |
| Doxorubicin | 0.21 | 0.022 | 0.37 | 0.022 | 0.25 |
| Topotecan | 0.028 | 0.43 | 0.62 | 0.015 | 0.17 |
| **OTHERS** | | | | | |
| Iodoacetamide | 0.29 | 0.30 | 0.64 | 0.37 | 2.5 |
| Sanguinarine chloride | 5.3 | 8.4 | 4.9 | 2.5 | 6.5 |
| NSC 95397 | 1.2 | 1.5 | 1.8 | 0.73 | 2.8 |
| Chelerythrine chloride | 0.60 | 0.65 | 0.52 | 0.70 | 3.7 |
| Calmidazolium chloride | 2.2 | 1.7 | 2.1 | 2.1 | 2.0 |
| Tetraethylthiuram disulfide | 0.49 | 0.19 | 6.3 | 1.1 | 13 |
| Bay 11-7085 | 1.4 | 1.7 | 2.7 | 1.2 | 4.5 |
| Quinacrine dihydrochloride | 5.1 | 4.6 | 4.9 | 5.2 | 7.1 |

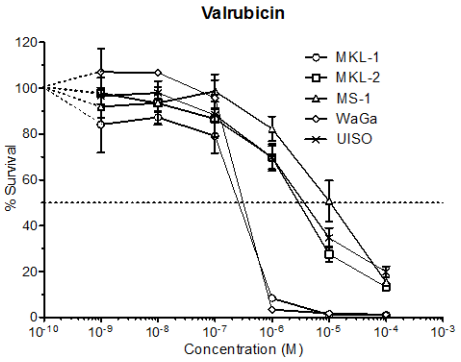
**A**

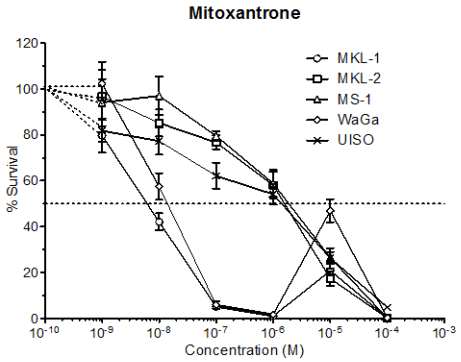
## Topoisomerase Inhibitors

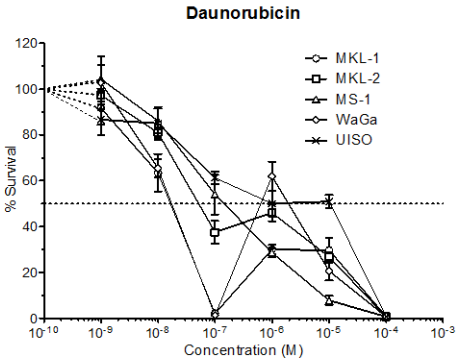


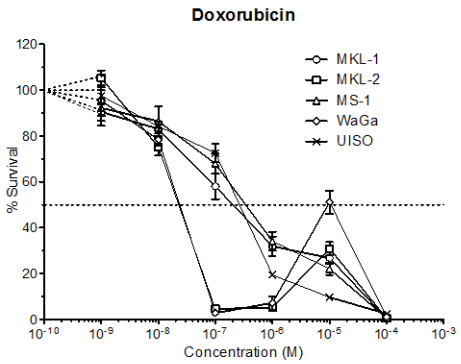



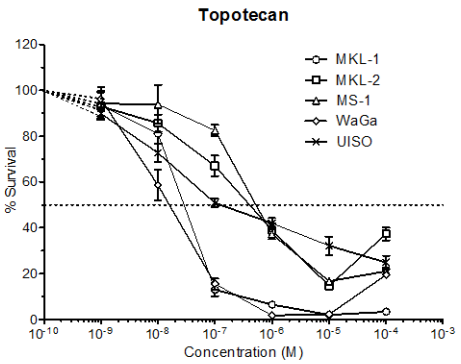



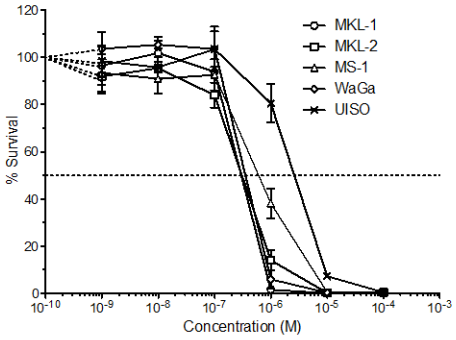



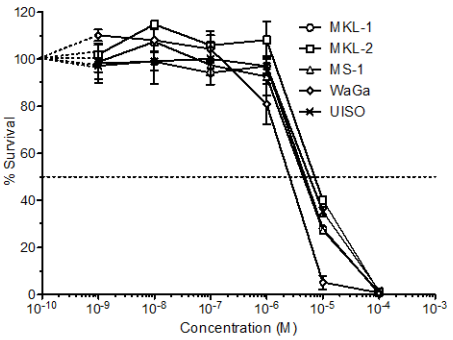
**B**

## Other Drugs

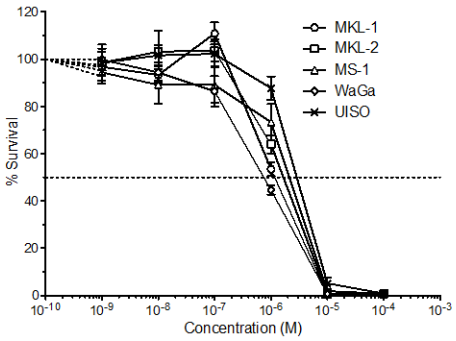
### Iodoacetamide



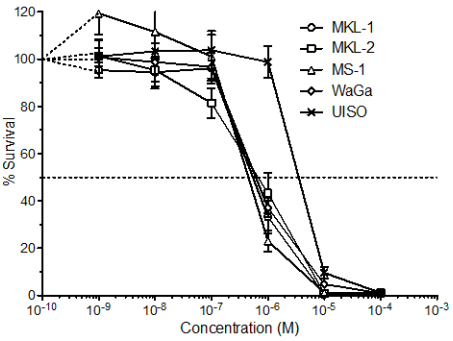

### Sanguinarine chloride



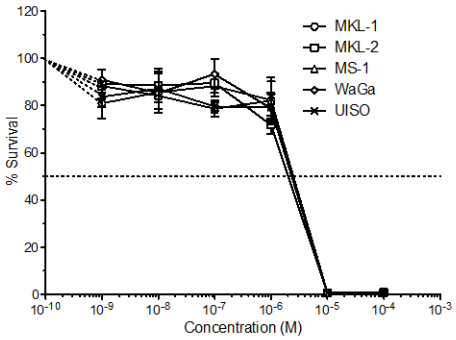

### NSC 95397



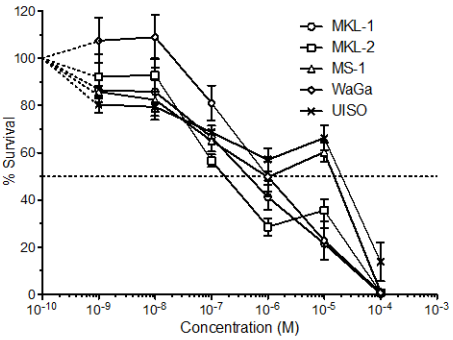

### Chelerythrine chloride



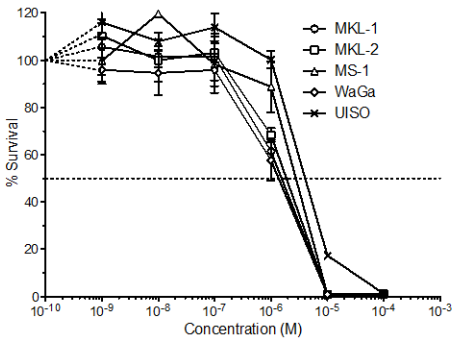

### Calmidazolium chloride



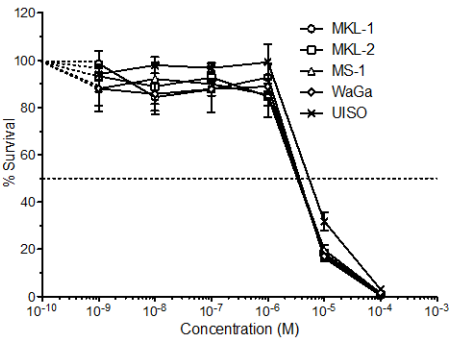

### Tetraethylthiuram disulfide




### Bay 11-7085




### Quinacrine dihydrochloride



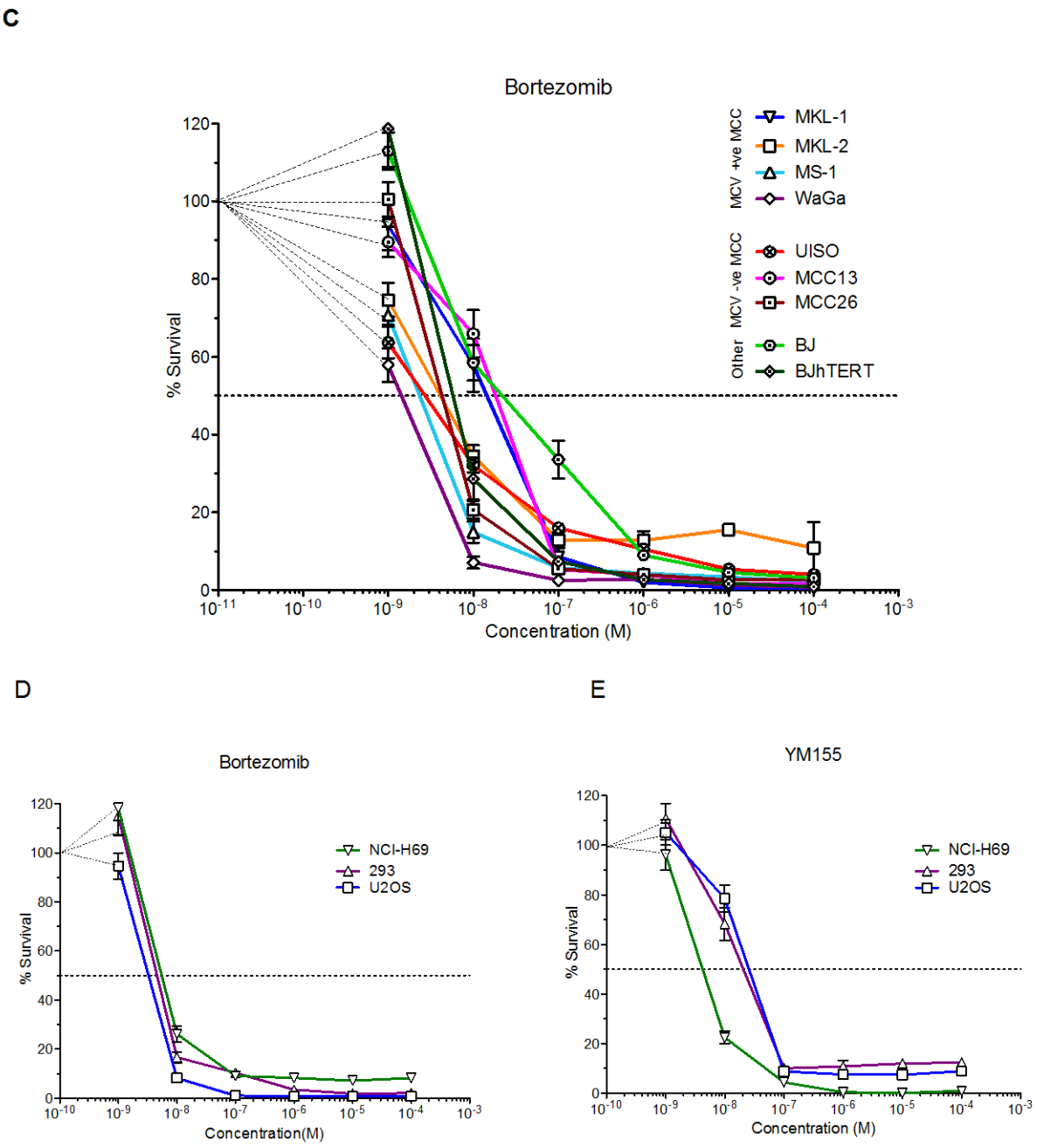


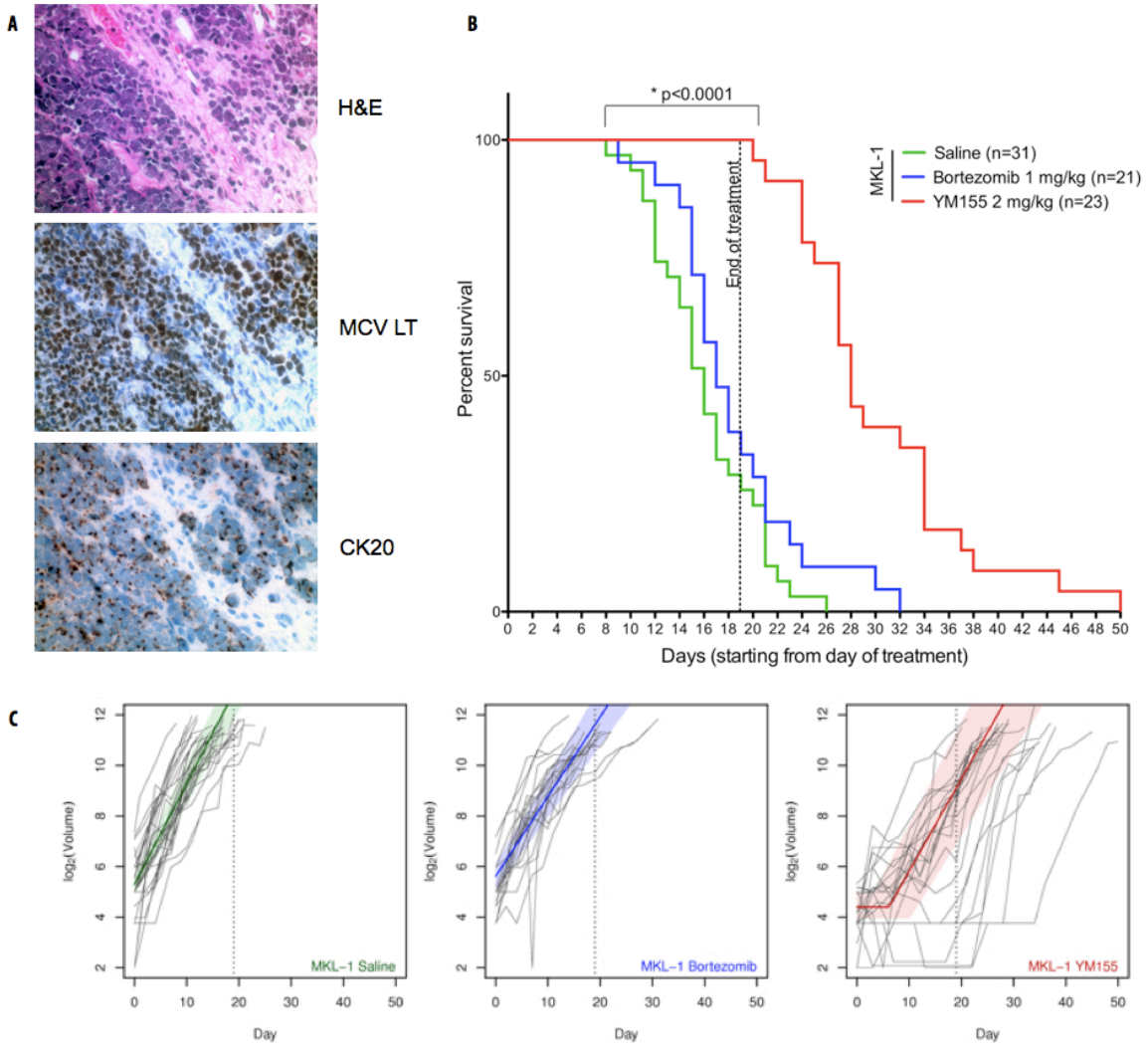

**Figure 33 Dose response curves for drug screen candidates.**

MCC cell lines and other human cancer cell lines were treated with compounds identified from the screen at increasing doses. Cell viability was normalized to no drug conditions (%survival=100). Shown is the average and standard deviation for 2 independent experiments tested in triplicate. (A) Topoisomerase inhibitors (B) other drugs (C) Bortezomib and (E) YM155 tested on NCI-H69 and U2OS cells.

### 5.2.4 Effect of YM155 on Human MCC Xenografts in Mice

We developed an MKL-1 xenograft model to test the *in vivo* efficacy of YM155 and bortezomib on human MCC in NOD scid gamma (NSG) mice. Subcutaneous injection of MKL-1 cells generates tumors that are positive for MCV LT and the MCC diagnostic marker CK20 (Figure 34A), and progress to endpoint (2 cm tumor diameter) within 2-4 weeks after tumors are first detected. We treated these mice with bortezomib, YM155 or saline for three weeks once tumors became palpable. Bortezomib was administered at levels effective on multiple myeloma xenografts [520] (1 mg/kg, twice-weekly subcutaneous injections). This did not significantly affect MCC tumor progression or volume compared to treatment with saline alone ($p=0.53$, log-rank test). Bortezomib administration at this level was associated with mouse lethargy and weight loss, requiring temporary use of heat blankets and Hydrogel (Clear$H_2O$) to prevent animal loss during initial treatment.

In contrast to bortezomib, YM155 (2 mg/kg, subcutaneous 5 times weekly [507, 521]) markedly delayed MKL-1 xenograft growth and significantly prolonged survival compared to either saline or bortezomib ($p<0.0001$, log-rank test, Figures 34B and 34C). This YM155 treatment regimen is the standard dosing for mouse xenograft experiments based on pharmacokinetic and treatment response studies of Nakahara *et al*. on six different human cancer xenografts (prostate, bladder, melanoma, breast and lung cancer) in mice and >100 human tumor cell lines [522, 523]. While 66.7-74.2% of bortezomib or saline-treated mice reached the euthanasia endpoint during the three-week treatment period, none of the YM155-treated mice reached this endpoint during treatment. Partial tumor regression occurred among some YM155-treated mice but all tumors resumed growth once YM155 was stopped, indicating that a single 3-week treatment course was insufficient for tumor eradication (Figure 34B). For the majority of the mice, MKL-1 tumor volumes were unchanged or showed delayed growth during YM155 treatment, suggesting this drug may be cytostatic rather than cytocidal for MKL-1 xenografts (Figure 34C and Appendix B). YM155 was well-tolerated and no adverse effects or acute toxicities were noted. In smaller cohorts of mice bearing MS-1 (MCV-positive) and UISO




D

| Cell line | Number of mice | Treatment | Day of termination | | | Tumor Volume day 19 (mm^3) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Average | Median | Range | Average | Median | Range |
| MS-1 | 5 | Saline | 34.6 | 34 | 26 -36 | 698.11 | 825 | 126-1080 |
| | 6 | YM155 | 37 | 37.5 | 24 - 44 | 380.02 | 352 | 171-600 |
| UISO | 5 | Saline | 52.4 | 47 | 37 - 81 | 122.5 | 24.5 | 13.5-486 |
| | 5 | YM155 | 53 | 54 | 41 -58 | 11.9 | 13.5 | 4-24.5 |

**Figure 34 YM155 inhibits growth of human MKL-1 MCC xenografts in NSG mice**.
(A) MKL-1 xenograft tumors stained with hematoxylin and eosin, MCV LT (CM2B4 antibody) and CK20 (magnification 40X). (B) MKL-1 xenograft survival curves after drug treatment. Mice were subcutaneously injected with 20 million MKL-1 cells and assigned to a three-week drug treatment after tumors became palpable (see text). No significant difference was found between saline and bortezomib treatment. Tumor progression was significantly delayed by YM155, with none of the YM155-treated mice dying during treatment (up to day 19) compared to 23 of 31 (74%) saline and 14 of 21 (67%) bortezomib-treated mice. Tumor progression recurred for all YM155-treated mice once treatment was stopped. (C) Piecewise linear hierarchical Bayesian model for tumor volumes in treated mice (see supplemental appendix for details). Colored lines show estimated central population tumor volumes with shaded regions representing 95% credible intervals. Actual tumor volumes (grey lines) for each mouse are shown for comparison. YM155 treatment retards tumor growth compared to saline or bortezomib treatment. (D) Table showing day of termination and tumor volumes for MS-1 and UISO xenograft mice treated with YM155 or saline.

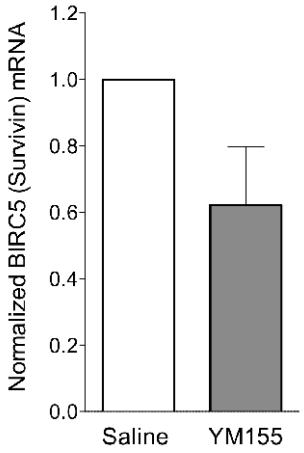



**Figure 35 Lower survivin levels in mice xenograft tumors treated with YM155.**
qRT-PCR for survivin from saline and YM155 (2 mg/kg) treated tumor tissues. Column represents mean and error bars represent standard error. Experiment done in triplicate (mean+ SEM)

(MCV-negative) cell xenografts, final tumor volumes for YM155-treated mice were 43-55% (median) of saline-treated control mice at the end of the three-week treatment period (Figure 34D).

### 5.2.5 DISCUSSION

We show here that discovery of a viral cause for most MCC in February 2008, followed by the description of the virus' oncogenes one year later, has now been used to rationally identify a survivin inhibitor that may have activity against this cancer. Our library screen of 1360 drugs, including the entire NCI Approved Oncology Drug Set II, confirmed that MCC is very chemoresistant and identified only one drug (bortezomib) that was highly active *in vitro*. Bortezomib however was not active *in vivo* against MCC xenografts. DTS, a quantitative cDNA deep sequencing method, not only identified MCV as a new human polyomavirus in MCC but also helped to uncover cell signaling pathways that are potential targets for MCV-positive MCC treatment.

Survivin, an inhibitor of apoptosis protein (IAP) family member[524], contributes to chemoresistance of melanoma[525] and is overexpressed in many cancers including MCC[494].

It increases during non-neoplastic JC polyomavirus infection[480, 524, 526], and by SV40 LT through an E2F-regulated mechanism [475, 527]. Our MCV T antigen knockdown and expression experiments confirm survivin to be activated by MCV LT's sequestration of RB-family transcription repressors. Survivin has pleiotropic activities in both preventing apoptosis and activating cell cycle entry[480]. Our findings are consistent with retinoblastoma protein or other pocket proteins repressing survivin expression in primary cells[527], which can be relieved when MCV LT is expressed. MCV LT did not further increase survivin levels for transformed cells having the retinoblastoma protein pathway signaling blocked by mutation or E1A. MCV activation of survivin through retinoblastoma protein targeting may typically serve to promote virus replication but is aberrantly activated during MCV-tumorigenesis (*10*).

YM155 is highly cytotoxic *in vitro* to MCV-positive MCC cells, causing these cells to initiate a necroptotic cell death routine. Loss of survivin is commonly linked to mitotic catastrophe in cancer cells [515]; however, we did not find this to significantly contribute to MCC cell death. There was no distinct G1 or G2-M phase accumulation of cells during YM155 treatment and irreversible commitment to cell death occurs too quickly for most MCC cells to have an opportunity to transit the cell cycle. YM155 does increase LC3-II, implicating autophagy in YM155-induced cell death, but may reflect a consequence rather than a cause for cell death [528]. Intriguingly, a very similar cell death phenotype occurs when T antigens are knocked down in MCV-positive MCC cells[498], whereas we found that knockdown of survivin alone causes apoptosis rather than necroptosis. As with YM155 treatment, this effect was predominant in an MCV-positive but not MCV-negative MCC cell line. Since knockdown of survivin causes apoptosis rather than necroptosis, we cannot exclude the possibility that YM155 targets other molecules in addition to survivin, or that different level of survivin inhibition by YM155 and survivin knockdown result in different programmed cell death responses.

There are several important caveats to our current study. While YM155 was highly active *in vitro*, it was only cytostatic—not cytotoxic—in most mouse xenografts. Once YM155 treatment was stopped, tumors re-emerged in all of the treated mice. Either prolonged YM155 treatment or combined use with other drugs (e.g., bortezomib, topoisomerase inhibitors) may

more effectively control MCC. YM155 was relatively nontoxic in our study, making it potentially more suitable for prolonged or combined therapy. Practical limitations prevented us from extensively measuring YM155 activity on a variety of MCC cell line xenografts and we cannot exclude the possibility of resistance among other MCC. But it is encouraging that YM155 reduced tumor masses in two additional MCC xenografts, including one from a MCV-negative tumor. Finally, MCV sT (also expressed in MCC cells) increases cap-dependent protein translation [504], and could contribute to YM155 resistance[529].

DTS [75] is a useful method to discover[74] or exclude [75] viruses being present in human cancers. In this study, we show that DTS is also useful in measuring cellular gene expression by demonstrating that *BIRC5* transcription is activated in MCV-positive MCC. If carefully performed and rigorously analyzed, DTS data yields useful information even when no cancer viruses are discovered [75]. Prior to discovery of MCV, few clues were available about the molecular causes for MCC[333]. Now that MCV has been shown to be central to most MCC, rational targeting of survivin and other cellular pathways perturbed by MCV may lead to discovery of additional treatments that may be more effective and less toxic than current therapies.

# 6.0 CONCLUSIONS AND PERSPECTIVES

## 6.1 MCV – A NEW HUMAN CARCINOGEN

There is substantial evidence that supports MCV's causal role in ~70-80% of MCC. In the 2012 IARC (International Agency for Research on Cancer) Monograph meeting MCV was classified as a group 2A carcinogen ('probably carcinogenic to humans')[530]. MCV's strong and specific association with MCC, consistently found by research groups around the world, fulfills a number of A. B. Hill's criteria for establishing causality between a factor and a human disease. Clonal integration of MCV within tumors and prior exposure to the virus both establish a significant temporal–relationship requirement. Although experimental evidence supporting MCV's contribution to MCC is critical, similar to other viral cancers additional factors and cellular changes are also needed for the outgrowth of the tumor. Following is a summary of the current experimental evidence supporting the notion that MCV is a direct carcinogen.

| **MCV and MCC ( Evidence for Causality in a subset of MCC)** |
|---|
| - Monoclonal Integration (Temporality) |
| - 74.2% (1743/2354) of MCC is positive for MCV (Association) |
| - MCV is not found in other cancers (Specificity) |
| - T antigen protein expression in all tumor cells |
| - Tumor specific truncation mutations that retain tumor suppressor targeting domains |
| - Signature mutations that eliminate replicative capacity of virus T antigen |
| - T antigen knockdown kills MCV +ve MCC cells |
| - Small T antigen transduction induces focus formation and soft agar colony formation in Rat-1 cells |

## 6.2 MODEL: MOLECULAR EVOLUTION OF MCC

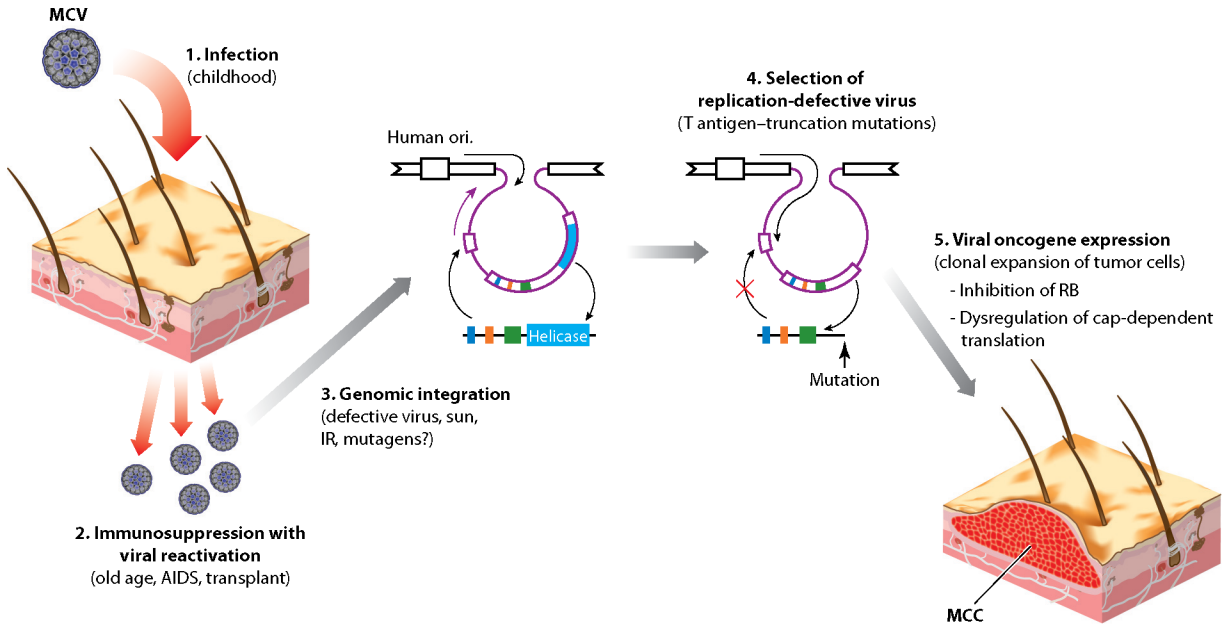



**Figure 36 Steps in molecular evolution of Merkel cell carcinoma.**
(Adpated from Chang and Moore, Ann Rev Pathol, 2011)

A model for the molecular evolution of MCC thus involves (Figure 36): MCV infection acquired in early childhood, followed by reactivation of virus in the context of immunosuppression or senescence of immune surveillance in elderly; genomic integration of MCV and natural selection pressure against replication-competent virus; expression of viral oncoprotein resulting in clonal expansion of transformed cells leading to MCC.

The discovery of MCV in ~80% MCC, has made a dramatic impact on the study of this cancer. Although MCV's importance in MCC is widely accepted, the precise mechanisms contributing to MCC tumor formation are still unknown. Future studies promise to elucidate signaling pathways targeted by MCV leading to new approaches to treating and preventing MCC.

## 6.3 SUMMARY

New knowledge of the viral etiology of a subset of MCC, has been successfully applied in both diagnosis and treatment of the disease. To assess MCV infection and its association with MCC and other human diseases, a monoclonal antibody (CM2B4) against an exon 2 epitope in MCV large T antigen was developed. We showed that this antibody recognizes both endogenous and overexpressed, transfected MCV LT and does not cross react with SV40, JCV and BKV T antigens. Using CM2B4, we showed that the expression of MCV T antigen localizes to the nuclei of MCC tumor cells, but is absent in adjacent non-tumor cells. The study was extended to two tissue microarrays. 74.2% (46/62) CK20 positive MCC tissues were found to be positive for CM2B4 staining. A quantitative PCR assay was also developed and MCV genome was found to be present at 1.6-48 copies per tumor cell.

Both these assays were used to survey a large set of human malignancies for their association with MCV. These malignancies included (325 +173 + 10) hematolymphoid tumors, (26) neuroendocrine tumors and (2) cases of concurrent CLL and MCC. Only 2.2% of 325 hematolymphoid malignancies tested showed evidence for MCV infection. However the viral copy numbers in these positive samples was 2-4 log lower than that seen in MCC.

In conclusion, these findings document that MCCs frequently express MCV T antigen, with good correlation between PCR-based and immunohistochemical detection methods. Immunostaining with mAb CM2B4 detects MCV LT in the majority of Merkel cell tumors, but not in other cancers. CM2B4 is a useful reagent for the diagnosis of MCV positive MCC.

To find therapeutic targets for this aggressive cancer, cellular genes dysregulated by MCV infection were examined. Digital transcriptome subtraction (DTS) profiles of MCV positive versus negative tumor tissue libraries were compared. The analysis found that survivin was upregulated seven-fold in virus positive as compared to virus negative tumors. Upon knockdown of MCV T antigen in MCV positive MCC cell lines, an associated decrease in survivin protein and mRNA was observed. Exogenously expressed MCV large T antigen increased survivin expression in non-MCC BJ primary cells. This effect was dependent on an intact

retinoblastoma family-binding domain in MCV LT. Survivin expression appears to be critical for MCV positive MCC cell survival because knockdown of survivin killed these cells. To follow this up, MCC cells were treated with a survivin targeting compound, YM155 (currently in Phase II trials for various cancers). YM155 initiates irreversible, non-apoptotic programmed cell death in MCV positive MCC cells, but not in MCV negative MCC cells.

An *in vitro* drug screening of 1360 other pharmacologically active compounds resulted in a single compound, bortezomib, having similar potency to YM155. However, although bortezomib was potent it was not selective for killing MCV infected MCC cells.

Both YM155 and bortezomib were then tested in MKL-1 cell xenograft models for preclinical evaluation. Bortezomib did not significantly improve mouse survival (33%) over saline (24%) during treatment. In contrast, all YM155-treated mice survived (100%, $p<0.001$) the 3-week treatment period. Tumors resumed growth once YM155 treatment was stopped, suggesting that YM155 is cytostatic rather than cytotoxic *in vivo*. YM155 showed no toxicity in mice, whereas bortezomib resulted in significant adverse effects.

## 6.4 GENERAL DISCUSSION

Detection of MCV large T antigen expression, determined using the CM2B4 antibody, further provided the mechanistic underpinning, which supports the notion that MCV causes a subset of MCC. Knockdown of MCV LT kills cells, indicating that MCV LT is critical for MCC survival. MCV LT induces survivin expression and this effect is dependent on the retinoblastoma family binding LFCDE domain of MCV LT. Many viruses including SV40 and papillomaviruses possess LXCXE motifs and target RB family members. In melanocytes, Rb and p53 seem to regulate the survivin promoter through its predicted E2F binding sites [475, 531]. However, the link between RB targeting by viruses and survivin expression has not yet been characterized.

Survivin inhibition has become an important and encouraging therapeutic approach to cancer. Virtually every human tumor expresses survivin and exploits one of its many functions. The presence of survivin in cancer is universally linked to resistance to apoptosis, evasion of cell cycle checkpoints, resistance to therapy, and metastasis [461, 476, 480]. Several survivin antagonists, including YM155 and Terameprecol have reached clinical trials (Table 18, Chapter 4). Valid concerns that survivin's essential role in stem cells [532] and some expression in normal tissues [533] would compromise these compounds *in vivo*, were resolved by the success of Phase I and II trials of these molecules [480, 496, 534]. This may reflect selective "survivin addiction" by the tumor, or "qualitative differences" in survivin network wiring in transformed cells versus normal healthy cells [476, 535].

Although survivin knockdown leads to apoptotic cell death, YM155 mediated cell death appears non-apoptotic. Autophagy was observed in cells treated with YM155, however I believe that in this context autophagy constitutes a futile cytoprotective attempt of dying cells to adapt to lethal stress rather than defining a mechanism that is executing a cell death program. In support of this notion, studies have shown that knockdown of essential autophagy genes (such as ATG5 and ATG7), which inhibit autophagosome formation, failed to prevent and at times even accelerated chemotherapy-induced cell death [528, 536]. Cells appear to be dying with autophagy but not by autophagy. YM155 treated MCV positive MCC cells probably undergo a quick, sudden form of necroptotic cell death. Due to the lack of direct assays that can determine necrosis and non-apoptotic cell death the exact mechanism of YM155-mediated cellular death is difficult to determine.

Single agent YM155 treatment was cytostatic but not cytotoxic for MKL-1 cell MCC xenografts in immunodeficient NSG mice. During the YM155 IP dosing over three weeks, most tumors regressed and none of the mouse xenograft tumors (n=21) reached the euthanasia end-point (2cmX2cm tumors). However, tumors resumed growth once treatment was stopped. No toxicity was noted during treatment (e.g. weight loss, ruffled fur, hair loss, hunched posture etc). Although a xenograft mouse system is an imperfect model of human cancer, it offers an *in vivo* approach to measuring therapeutic modalities that is different from cell culture systems.

Although the genetics and cellular complexity of these *in vivo* tumors are not representative of the original tumor in its native state, there are advantages of using mouse models for preclinical evaluation of therapeutic responses to drugs. They are easy to use, relatively inexpensive and reproducible. Treatment can be initiated when the tumor reaches an optimal size and progression of a number of these synchronized, easily observable tumors can be followed accordingly in an *in vivo* system. In addition, only a few mice are required for analytical drug efficacy studies and xenografts form tumors in shorter times with a high degree of predictability as compared to other genetically engineered mouse models.

For the evaluation of YM155 in MCC *in vivo*, we used a mouse xenograft model of a MCV positive MCC human cell line (MKL-1). This model closely resembled and maintained certain features (CK20 positivity, MCV LT and sT expression etc) of MCC tumors. Whereas both bortezomib and YM155 were equally potent in cell culture and showed similar $EC_{50}$ concentrations, their effect on tumor cells differed in the mouse and resulted in different outcomes. The discovery of MCV as a cause of MCC is impetus to the use of viral oncogenes for the creation of transgenic mouse models, similar to the SV40 and MPyV models. In the future of MCV research, the development and characterization of such MCC models, in conjunction with xenograft models such as those used in this study they will be valuable for the preclinical evaluation of compounds such as YM155.

Survivin is a downstream target gene of multiple oncogenic signaling circuits such as STAT3, Akt, EGFR, Notch, Wnt, VEGF and Ang-1 [476, 537-541]. Monotherapeutic, small molecule inhibitors of these pathways, have produced modest but promising results. Preliminary work in our laboratory supports the importance of the Akt pathway and other common oncogenic pathways in MCV positive MCC cells. Combined inhibition of such pathways and survivin, is likely to enhance efficacy for treatment of MCC and other cancers, without compromising safety and toxicity.

Survivin antagonists such as YM155, act more broadly as “pathway antagonists” despite being single target agents since they disable multiple survivin networks by inhibiting the nodal/central protein. Several clinical trials with YM155 have been completed, with more

underway. In patients with advanced non-small cell lung cancer, YM155 produced a disease control rate of 43% in phase II studies [542]. The treatment was well tolerated and produced two partial repsonses and disease stabilization in 14 out of 37 patients. Phase II studies of YM155 in melanoma however were less promising. They produced only one partial response in patients with stage III and stage IV disease [543]. The Eastern Oncology Cooperative Group (ECOG) clinical trial (E1611) for YM155 and bortezomib in MCC patinets will begin in the fourth quarter of 2012, and I sincerely hope that YM155 lives its promise for MCC.

Using genomic technologies, the primary viral cause for ~75% of MCC cases was discovered, new diagnostic tests developed and a promising rational drug candidate was identified. Moving from discovery to the clinic in less than four years is a resounding success in MCC oncology. The approaches described above for diagnosis and therapy remain promising and bode well for their continued evaluation in the clinic.

### 6.4.1 FUTURE DIRECTIONS

To optimize YM155 in treating MCC, experiments determining the outcome of prolonged YM155 treatment and increased YM155 dosing are underway. Combination studies of YM155 with cytotoxics and/or radiation therapy for MCC may prove more effective. Sequential inhibition of survivin in tumor cells pretreated with taxanes (such as docetaxol), which disrupt microtubule function, produces an increased anticancer activity with no toxicity in preclinical models [513, 544-546]. Similar approaches that evaluate combination chemotherapies with YM155 and low dose topoisomerase inhibitors and/or alkylating agents (such as etoposide, carboplatin, currently used for MCC in the clinic[547]), and even YM155 and bortezomib, have potential for MCC treatment and will be considered.

My findings will direct future work in the laboratory to resolve the following unanswered questions: What is the mechanism behind upregulation of survivin by MCV LT? How does MCV LT increase survivin expression through its LFCDE domain? If the Rb/E2F pathway is involved in the phenotype shown, then it would be interesting to identify and

narrow down the specific RB family members (pRb, p107 and p130) and specific E2F transcription factors involved. Chromatin immunoprecipitation experiments to check for E2F binding to the survivin promoter, in the presence and absence of MCV LT and LFCDE mutants, may be an approach to address some of these questions. Assessing survivin upregulation by MCV LT after the knockdown of Rb and E2F family proteins, or in cells lacking expression of these proteins is another experimental approach to this puzzle. Besides determining how YM155 treatement kills MCV positive MCC cells, gene expression microarray analysis and structural studies may be informative in identifying other targets and effects of YM155.

## APPENDIX A

**Table 21 Polyomavirus Information**

| Virus | Abbreviation | Host | GenBank ID | | | |
|---|---|---|---|---|---|---|
| | | | Full genome | Large T antigen | Major capsid protein VP1 | Genome size |
| *Orthopolyomaviruses* (type species *Simian virus 40* ) | | | | | | |
| Baboon polyomavirus 1 | SA12 | Monkey | NC_007611 | YP_406555 | YP_406554 | 5230 bp |
| Bat polyomavirus | BatPyV | Bat | NC_011310 | YP_002261489 | YP_002261488 | 5081 bp |
| B-lymphotropic polyomavirus | LPyV | Monkey | NC_004763 | NP_848008 | NP_848007 | 5270 bp |
| BK polyomavirus | BKV | Human | NC_001538 | YP_717940 | YP_717939 | 5153 bp |
| Bornean orang-utan polyomavirus | OraPyV1 | Orang-utan | NC_013439 | YP_003264534 | YP_003264533 | 5168 bp |
| Bovine polyomavirus | BPyV | Cattle | NC_001442 | NP_040788 | NP_040787 | 4697 bp |
| California sea lion polyomavirus | SLPyV | Sea Lion | NC_013796 | YP_003429323 | YP_003429322 | 5112 bp |
| Hamster polyomavirus | HaPyV | Hamster | NC_001663 | NP_056730 | NP_0567331 | 5366 bp |
| Human Polyomavirus 9 | HPyV9 | Human | NC_015150 | YP_04243706 | YP_004243705 | 5026 bp |
| JC Polyomavirus | JCV | Human | NC_001699 | NP_043512 | NP_043511 | 5130 bp |
| Merkel cell polyomavirus | MCV | Human | NC_010277 | AC125312* | YP_001651048 | 5387 bp |
| Murine pneumotropic virus | MPtV | Mouse | NC_001505 | NP_041232 | NP_041234 | 4754 bp |
| Murine polyomavirus | MPyV | Mouse | NC_001515 | NP_041264 | NP_041267 | 5297 bp |
| Simian virus 40 | SV40 | Monkey | NC_001669 | YP_003708382 | YP_003708381 | 5243 bp |

| | | | | | | |
|---|---|---|---|---|---|---|
| Squirrel monkey polyomavirus | SqPyV | Monkey | NC_009951 | YP_001531349 | YP_001531348 | 5075 bp |
| Sumatran orang-utan polyomavirus | OraPyV2 | Orang-utan | FN356901 | CAX87759 | CAX87757 | 5358 bp |
| Trichodysplasia spinulosa-associated polyomavirus | TSPyV | Human | NC_014361 | YP_003800007 | YP_003800006 | 5232 bp |
| Chimpanzee polyomavirus | ChPyV | Chimpanzee | NC_014743 | YP_004046683 | YP_004046682 | 5086 bp |
| *Wukipolyomavirus* (type species *KI polyomavirus*) | | | | | | |
| Human polyomavirus 6 | HPyV6 | Human | NC_014406 | YP_003848919 | YP_003848918 | 4926 bp |
| Human polyomavirus 7 | HPyV7 | Human | NC_014407 | YP_003848924 | YP_003848923 | 4952 bp |
| KI Polyomavirus | KIPyV | Human | NC_009238 | YP_001111259 | YP_001111258 | 5040 bp |
| WU Polyomavirus | WUPyV | Human | NC_009539 | YP_001285488 | YP_001285487 | 5229 bp |
| *Avipolyomavirus* (type species *Avian polyomavirus*) | | | | | | |
| Avian polyomavirus | APyV | Birds | NC_004764 | YP_004061429 | YP_004061428 | 4981 bp |
| Canary polyomavirus | CaPyV | Canary | GU345044 | ADM88652 | ADM88650 | 5421 bp |
| Crow polyomavirus | CPyV | Jackdaw | NC_007922 | YP_529828 | YP_529827 | 5079 bp |
| Finch polyomavirus | FPyV | Bullfinch | NC_007923 | YP_529834 | YP_529833 | 5278 bp |
| Goose polyomavirus | GHPyV | Goose | NC_004800 | NP_849170 | NP_849169 | 5256 bp |

* MCV full length large T- 206 isolate was used in this study

The MCV Truncated T - 339 isolate commonly used is YP_001651046

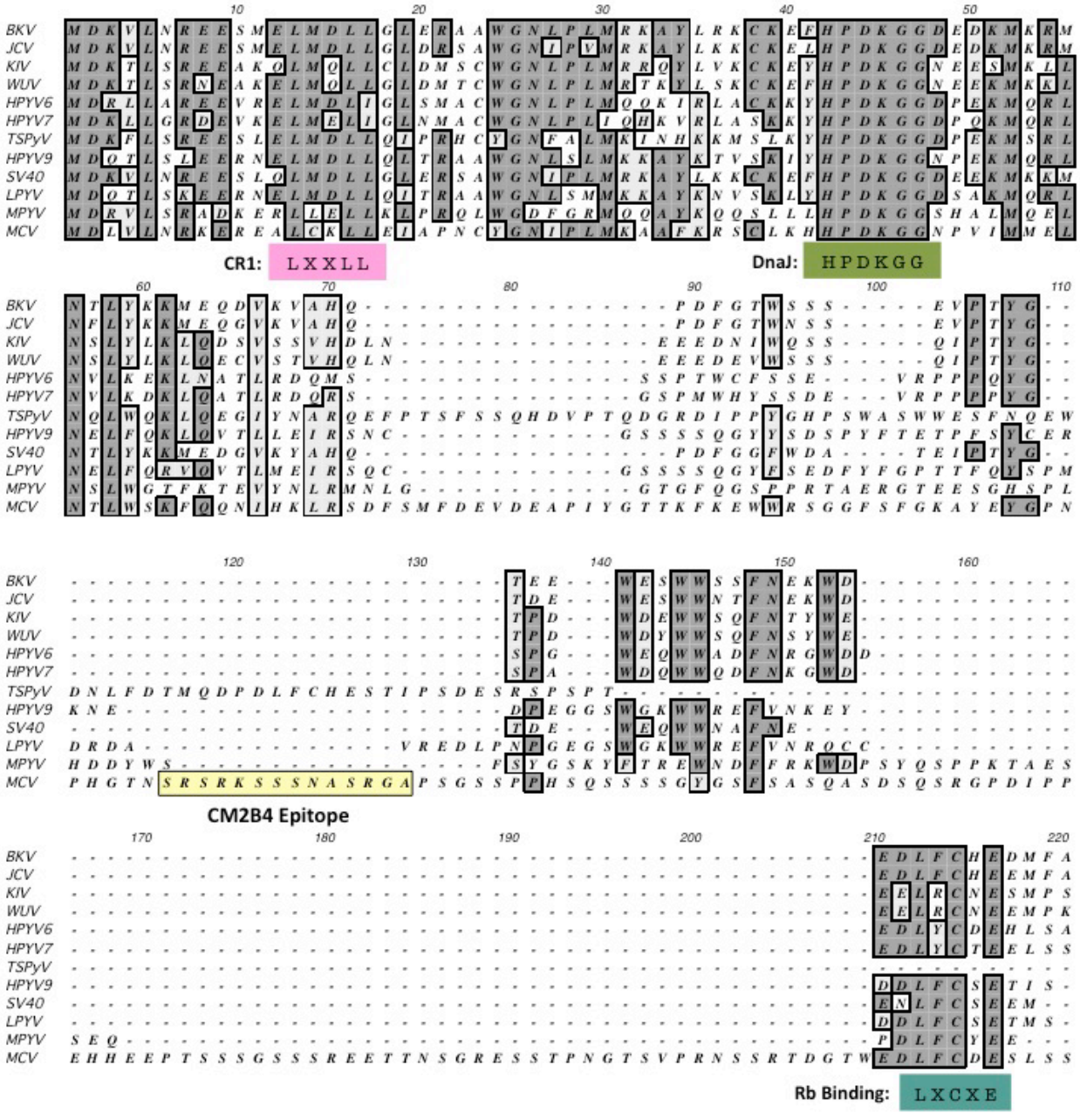



**Figure 37 Updated amino acid alignment of MCV large T antigen with other human polyomavirus, SV40, LPyV and MPyV large T antigens.**

## A.1 DETAILS OF DISEASE CATEGORIES OF TABLE 15

**Lymphomas and Myeloid disorders --** Acute Lymphoblastic Leukemia (1/136), Acute myeloid leukemia (0/11), Angiocentric T cell lymphoma (nasal type) (0/1), Angioimmunoblastic T-cell lymphoma (1/2), Burkitt lymphoma/Burkitt cell leukemia (0/2), Chronic Lymphocytic Leukemia (32/168), Chronic myelogenous leukemia (0/5), Classical Hodgkin lymphoma (3/41), Cutaneous gamma/delta T cell lymphoma (0/2), Diffuse follicular center lymphoma (0/1), Diffuse large B cell lymphoma (2/118), B-cell lymphoma (9/260), Extranodal marginal zone B-cell lymphoma (0/2), Extranodal NK/T-cell lymphoma, nasal type (0/2), Feces of Leukemic children (0/75), Follicular lymphoma (2/49), Hodgkin lymphoma (4/76), Large granular lymphocyte leukemia (0/1), Lymphoblastic leukemia/lymphoma (0/5), Lymphomas (1/11), Lymphoplasmacytic lymphoma (0/4), Mantle cell lymphoma (0/15), Marginal zone lymphoma (1/9), Mucosa-associated lymphoid tissue lymphoma (0/5), Mycosis Fungoides (0/25), Myelodysplastic syndrome (0/3), Myeloid disorder (0/1), Myeloid hyperplasia (0/1), Nasal Swabs from Leukemic children (2/106), Necrotizing lymphadenitis (0/2), NK/T-cell lymphoma (2/18), Nodular lymphocyte predominant Hodgkin lymphoma (0/3), Hodgkins lymphoma --Nodular sclerosis (1/29), Non-Hodgkin lymphoma (11/166), Pagetoid reticulosis/ cutaneous T cell lymphoma (0/1), Peripheral T-cell lymphoma (0/1), Plasmacytoid dendritic cell tumor (0/1), Post-transplant lymphoproliferative disorder (0/13), Primary cutaneous peripheral T cell lymphoma (1/4), Primary effusion lymphoma (0/6), Pseudolymphomas (2/13), Reactive Hyperplasia Lymphoid Samples (0/6), Splenic marginal zone lymphoma (0/1), T cell lymphomas (0/17), T-cell lymphoma, unspecified (1/82), Unclassified lymphomas (1/6)

**Neuroendocrine cancers --** Cervical neuroendocrine carcinomas (0/4), Combined squamous and neuroendocrine carcinomas (0/7), High grade mucosal neuroendocrine carcinoma (0/8), High grade neuroendocrine carcinoma of the cervix (0/1), Large cell neuroendocrine carcinoma (0/2), Neuroendocrine small cell carcinoma (0/4), Neuroendocrine tumor of GI tract (0/15), Neuroendocrine tumors (0/40), Pulmonary neuroendocrine carcinomas (0/26), Salivary gland high-grade neuroendocrine carcinoma (0/7), Small cell lung neuroendocrine carcinoma (0/5), Visceral high grade neuroendocrine tumors (32 from lung, 16 from gastrointestinal tract,20 female reproductive system, 3 soft tissue, 2 from head and neck and 1 from bladder) (1/74), Well differentiated intestinal neuroendocrine carcinoma (0/3)

**Skin cancers** – Actinic Keratosis (12/92), Epidermodysplasia-Verruciformis skin lesions (16/19), Atypical Fibroxanthoma (4/23), Basosquamous carcinomas (0/3), Immunosuppressed non melanoma skin cancer (NMSC) (2/45), Inflammatory skin diseases (2/25), Keratoacanthoma (16/87), Kaposi's Sarcoma (6/63), Lentigo Maligna Melanoma (0/10), Malignant Melanoma (2/28), Melanoma (0/95), Metastatic melanoma in the liver (0/94), Naevoid basal cell carcinoma syndrome (5/26), Porokeratosis (0/1), Psoriasis (1/44), Serborrheic keratosis (19/81), Skin cancers (nonspecified) (0/2), Skin with proliferative, inflammatory or mucosal lesions (0/15), Squamous cell carcinoma (155/737), Trichoblastoma (12/42), Urine from Psoriasis patient (0/6), Whole blood from Psoriasis patient (0/6), Basal cell carcinoma (77/381).

**Central Nervous System diseases --** Astrocytoma (0/11), Cerebrospinal fluid from Hematopoietic stem cell transplantation patients with neurological symptoms (0/20), CNS Tumors (0/25), Encephalitis (0/12), Ependymoma (0/3), Ewing Sarcoma/ Primitive Neuroectodermal tumors (1/7), Gliomas (0/2), Ewing tumor (0/30), Medulloblastoma/PNET (0/5), Meningioma (0/1), Multiple sclerosis (0/115), Neuroblastomas (0/57), neurological syndromes (0/2), Unspecified intracranial intraspinal neoplasm (0/1)

**Other viral related disorders --** BKV positive nephritis (0/1), Benign common warts in immunosuppressed patients (5/20), Common warts from healthy individuals (4/60), Papilloma wart (0/4), HPV +ve Invasive carcinoma of uterine cervix (0/26), Progressive Multifocal Leukoencephalopathy (PML) (0/35)

**Other cancers and diseases** -- Adenocarcinoma (large bowel) (0/38), Biliary cirrhosis (2/2), Cancerous Prostrate epithelia (0/28), Carcinomas in situ, immunocompetent patients (2/24), FDC Sarcoma (0/1), Fibromatosis (0/4), Follicular lesions of lung (0/10), Fulminant hepatitis (0/9), Gastric cancer (1/20), Mixed cellularity tumors (2/10), Bladder cancer (6/8), Bowen's disease (23/126), Breast Cancer (0/718), Colon and Colorectal Cancers (11/190), Desmoplastic tumor (0/24), Esophageal cancer (3/5), HPV -ve invasive carcinoma of uterine cervix (0/18), Inflammatory tissues (0/7), Large bowel cancer (0/1), Lipoma (0/1), Liver cancer (10/20), Lower respiratory tract (bronchioalveolar and bronchioaspirate) samples from patients with Respiratory syndromes (15/87), Lung cancer

(10/29), Lymph nodes with non-MCC metastatic cancer (0/27), Mesothelioma (0/45) , Metastatic breast carcinoma in the liver (0/16), Metastatic carcinoma in the ovary (0/39), Metastatic colon adenocarcinoma (liver) (3/4), Non small cell lung cancer (5/51), Other immunosuppressed samples (nonspecified) (1/36), Other neoplastic non-MCC tissues (0/13), Ovarian cancer (0/143), Primary Pulmonary Hypertension (PPH) (0/10), Non cutaneous small cell undifferentiated carcinoma (1/75), Prostrate adenocarcinoma (4/22), Pulmonary small cell carcinomas (0/12), Renal clear cell carcinoma (3/16), Respiratory tract samples (immunocompetent individuals) (6/250), Respiratory tract samples (immunosuppressed individuals) (4/55), Rhabdomyosarcoma (0/25), Seminoma (1/9), Small cell carcinomas (9/24), Small cell lung cancer (10/76), WILD Syndrome (27 swabs, whole blood and cellular pellet) (1/1), Whole blood from colon cancer patients (0/64), Other unspecified neoplasms (1/13)

## APPENDIX B

**Table 22 High throughput compound library screen results**

| LOPAC[1280] Library Screen Results | | |
|---|---|---|
| **Compound Name** | **Class** | **% Survival Index** |
| 7-Chloro-4-hydroxy-2-phenyl-1, 8-naphthyridine | Adenosine | 64.1 |
| 3-Isobutyl-1-methylxanthine | Adenosine | 70.9 |
| 8-(3-Chlorostyryl) caffeine | Adenosine | 81.3 |
| 8-(p-Sulfophenyl)theophylline | Adenosine | 84.1 |
| 2-Chloroadenosine | Adenosine | 84.3 |
| 1,3-Dimethyl-8-phenylxanthine | Adenosine | 88.5 |
| FSCPX | Adenosine | 88.9 |
| MRS 1754 | Adenosine | 89.0 |
| 5 -N-Methyl carboxamidoadenosine | Adenosine | 90.0 |
| 1,3-Diethyl-8-phenylxanthine | Adenosine | 90.1 |
| Metrifudil | Adenosine | 90.2 |
| 2-Phenylaminoadenosine | Adenosine | 92.5 |
| S- (4-Nitrobenzyl)-6-thioguanosine | Adenosine | 93.3 |
| 1,3-Dipropyl-7-methylxanthine | Adenosine | 93.4 |
| 8-Cyclopentyl-1,3-dipropylxanthine | Adenosine | 94.6 |
| MRS 1523 | Adenosine | 94.9 |
| N6-Cyclohexyladenosine | Adenosine | 95.2 |
| Alloxazine | Adenosine | 97.0 |
| 1,3-Dipropyl-8-p-sulfophenylxanthine | Adenosine | 99.0 |
| Chloro-IB-MECA | Adenosine | 99.5 |
| N6-Methyladenosine | Adenosine | 100.6 |
| Theophylline | Adenosine | 100.7 |
| Xanthine amine congener | Adenosine | 101.3 |
| 1,7-Dimethylxanthine | Adenosine | 102.9 |
| N6-Benzyl-5 -N-ethylcarboxamidoadenosine | Adenosine | 103.1 |
| erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride | Adenosine | 104.0 |
| Theobromine | Adenosine | 104.1 |
| VUF 5574 | Adenosine | 104.9 |
| HE-NECA | Adenosine | 105.6 |
| 3-n-Propylxanthine | Adenosine | 105.7 |
| 1-Allyl-3,7-dimethyl-8-p-sulfophenylxanthine | Adenosine | 106.0 |
| CGS-21680 hydrochloride | Adenosine | 106.4 |

| | | |
|---|---|---|
| N6-Cyclopentyladenosine | Adenosine | 106.4 |
| 5 -(N-Cyclopropyl)carboxamidoadenosine | Adenosine | 107.8 |
| 8-Cyclopentyl-1,3-dimethylxanthine | Adenosine | 108.3 |
| 3,7-Dimethyl-I-propargylxanthine | Adenosine | 109.5 |
| (S)-ENBA | Adenosine | 109.7 |
| S-(4-Nitrobenzyl)-6-thioinosine | Adenosine | 110.0 |
| Dipyridamole | Adenosine | 110.3 |
| N6-Phenyladenosine | Adenosine | 111.2 |
| IB-MECA | Adenosine | 111.9 |
| R(-)-N6-(2-Phenylisopropyl)adenosine | Adenosine | 113.1 |
| N6-Cyclopentyl-9-methyladenine | Adenosine | 113.2 |
| CGS-15943 | Adenosine | 114.3 |
| Adenosine | Adenosine | 114.5 |
| N6-2-Phenylethyladenosine | Adenosine | 116.4 |
| Dilazep hydrochloride | Adenosine | 117.4 |
| 5 -N-Ethylcarboxamidoadenosine | Adenosine | 118.0 |
| Propentofylline | Adenosine | 119.7 |
| AB-MECA | Adenosine | 120.4 |
| Aminophylline ethylenediamine | Adenosine | 121.6 |
| Caffeine | Adenosine | 127.1 |
| Etazolate hydrochloride | Adenosine | 131.5 |
| N6-2-(4-Aminophenyl)ethyladenosine | Adenosine | 133.6 |
| S(-)-Pindolol | Adrenergic | 94.4 |
| Isotharine mesylate | Adrenoceptor | 78.1 |
| Naphazoline hydrochloride | Adrenoceptor | 80.4 |
| p-Iodoclonidine hydrochloride | Adrenoceptor | 81.5 |
| Doxazosin mesylate | Adrenoceptor | 81.6 |
| S(+)-Isoproterenol (+)-bitartrate | Adrenoceptor | 82.3 |
| Chloroethylclonidine dihydrochloride | Adrenoceptor | 85.6 |
| Nortriptyline hydrochloride | Adrenoceptor | 86.2 |
| UK 14,304 | Adrenoceptor | 87.6 |
| Ritodrine hydrochloride | Adrenoceptor | 90.1 |
| Terazosin hydrochloride | Adrenoceptor | 90.7 |
| WB-4101 hydrochloride | Adrenoceptor | 92.8 |
| p-Aminoclonidine hydrochloride | Adrenoceptor | 92.9 |
| (-)-Isoproterenol hydrochloride | Adrenoceptor | 93.6 |
| (-)-alpha-Methylnorepinephrine | Adrenoceptor | 94.3 |
| (±)-Isoproterenol hydrochloride | Adrenoceptor | 94.6 |
| Moxisylyte hydrochloride | Adrenoceptor | 94.8 |
| Naftopidil dihydrochloride | Adrenoceptor | 95.0 |
| MHPG sulfate potassium | Adrenoceptor | 95.0 |
| Oxymetazoline hydrochloride | Adrenoceptor | 95.0 |
| R(-)-Isoproterenol (+)-bitartrate | Adrenoceptor | 95.5 |
| Amitriptyline hydrochloride | Adrenoceptor | 96.3 |
| DSP-4 hydrochloride | Adrenoceptor | 96.8 |
| Methoxamine hydrochloride | Adrenoceptor | 97.0 |
| Pindolol | Adrenoceptor | 97.1 |
| Betaxolol hydrochloride | Adrenoceptor | 97.3 |
| (-)-Epinephrine bitartrate | Adrenoceptor | 97.4 |

| | | |
|---|---|---|
| Terbutaline hemisulfate | Adrenoceptor | 97.6 |
| Fenspiride hydrochloride | Adrenoceptor | 98.5 |
| Moxonidine hydrochloride | Adrenoceptor | 99.0 |
| (S)-(-)-propafenone hydrochloride | Adrenoceptor | 99.3 |
| Salbutamol | Adrenoceptor | 99.4 |
| A-315456 | Adrenoceptor | 99.5 |
| (±)-Sotalol hydrochloride | Adrenoceptor | 99.7 |
| Xylazine hydrochloride | Adrenoceptor | 100.6 |
| Tetrahydrozoline hydrochloride | Adrenoceptor | 100.6 |
| Imiloxan hydrochloride | Adrenoceptor | 101.0 |
| Bretylium tosylate | Adrenoceptor | 101.1 |
| Doxepin hydrochloride | Adrenoceptor | 101.3 |
| Dobutamine hydrochloride | Adrenoceptor | 101.8 |
| Phenoxybenzamine hydrochloride | Adrenoceptor | 101.8 |
| S(-)-Timolol maleate | Adrenoceptor | 102.3 |
| R(+)-Atenolol | Adrenoceptor | 102.6 |
| (±)-Normetanephrine hydrochloride | Adrenoceptor | 102.6 |
| (±)-CGP-12177A hydrochloride | Adrenoceptor | 102.7 |
| Yohimbine hydrochloride | Adrenoceptor | 103.3 |
| Benoxathian hydrochloride | Adrenoceptor | 103.5 |
| Nylidrin hydrochloride | Adrenoceptor | 103.8 |
| Nisoxetine hydrochloride | Adrenoceptor | 104.0 |
| (±)-Metoprolol (+)-tartrate | Adrenoceptor | 104.4 |
| Metaproterenol hemisulfate | Adrenoceptor | 104.4 |
| (-)-Ephedrine hemisulfate | Adrenoceptor | 105.1 |
| (±)-Atenolol | Adrenoceptor | 105.3 |
| Fiduxosin hydrochloride | Adrenoceptor | 105.4 |
| Rauwolscine hydrochloride | Adrenoceptor | 105.5 |
| Prazosin hydrochloride | Adrenoceptor | 105.6 |
| ICI 118,551 hydrochloride | Adrenoceptor | 106.8 |
| (±)-Norepinephrine (+)bitartrate | Adrenoceptor | 107.0 |
| L(-)-Norepinephrine bitartrate | Adrenoceptor | 107.4 |
| Formoterol | Adrenoceptor | 107.5 |
| L-765,314 | Adrenoceptor | 108.5 |
| 6-Hydroxy-DL-DOPA | Adrenoceptor | 108.6 |
| MK-912 | Adrenoceptor | 108.7 |
| Maprotiline hydrochloride | Adrenoceptor | 108.8 |
| MHPG piperazine | Adrenoceptor | 109.1 |
| Salmeterol xinafoate | Adrenoceptor | 109.4 |
| (±)-Propranolol hydrochloride | Adrenoceptor | 110.1 |
| Guanabenz acetate | Adrenoceptor | 110.1 |
| B-HT 933 dihydrochloride | Adrenoceptor | 110.4 |
| (±)-Epinephrine hydrochloride | Adrenoceptor | 110.5 |
| Fenoterol hydrobromide | Adrenoceptor | 110.8 |
| R(-)-Denopamine | Adrenoceptor | 112.8 |
| BRL 37344 sodium | Adrenoceptor | 113.2 |
| Bromoacetyl alprenolol menthane | Adrenoceptor | 113.9 |
| Amiodarone hydrochloride | Adrenoceptor | 114.2 |
| SKF 86466 | Adrenoceptor | 114.2 |

| | | |
|---|---|---|
| (±)-Octopamine hydrochloride | Adrenoceptor | 114.3 |
| RX 821002 hydrochloride | Adrenoceptor | 114.4 |
| 6-Fluoronorepinephrine hydrochloride | Adrenoceptor | 114.4 |
| Tulobuterol hydrochloride | Adrenoceptor | 114.4 |
| Labetalol hydrochloride | Adrenoceptor | 114.6 |
| (±)-Vanillylmandelic acid | Adrenoceptor | 114.7 |
| Phentolamine mesylate | Adrenoceptor | 115.1 |
| Xamoterol hemifumarate | Adrenoceptor | 115.2 |
| Cirazoline hydrochloride | Adrenoceptor | 115.3 |
| Urapidil, 5-Methyl- | Adrenoceptor | 115.4 |
| CL 316,243 | Adrenoceptor | 115.7 |
| (±)-Synephrine | Adrenoceptor | 115.8 |
| Desipramine hydrochloride | Adrenoceptor | 116.9 |
| (S)-Propranolol hydrochloride | Adrenoceptor | 117.2 |
| Protriptyline hydrochloride | Adrenoceptor | 117.2 |
| Alprenolol hydrochloride | Adrenoceptor | 117.4 |
| S(-)-Atenolol | Adrenoceptor | 117.5 |
| Phenylephrine hydrochloride | Adrenoceptor | 117.9 |
| Urapidil hydrochloride | Adrenoceptor | 118.3 |
| Xylometazoline hydrochloride | Adrenoceptor | 119.3 |
| SR 59230A oxalate | Adrenoceptor | 119.8 |
| Guanfacine hydrochloride | Adrenoceptor | 120.0 |
| CGP 20712A methanesulfonate | Adrenoceptor | 121.9 |
| Amoxapine | Adrenoceptor | 122.3 |
| Clonidine hydrochloride | Adrenoceptor | 123.1 |
| Tomoxetine | Adrenoceptor | 124.4 |
| Albuterol hemisulfate | Adrenoceptor | 129.8 |
| (±)-Pindobind | Adrenoceptors | 26.4 |
| DL-alpha-Difluoromethylornithine hydrochloride | Angiogenesis | 83.2 |
| Niclosamide | Antibiotic | 29.2 |
| Demeclocycline hydrochloride | Antibiotic | 87.7 |
| Cinoxacin | Antibiotic | 90.1 |
| Phosphomycin disodium | Antibiotic | 92.8 |
| Praziquantel | Antibiotic | 93.2 |
| Thiolactomycin | Antibiotic | 95.7 |
| Cefsulodin sodium salt hydrate | Antibiotic | 97.0 |
| Vancomycin hydrochloride from Streptomyces orientalis | Antibiotic | 97.3 |
| Doxycycline hydrochloride | Antibiotic | 97.6 |
| Cephapirin sodium | Antibiotic | 97.9 |
| Ofloxacin | Antibiotic | 99.5 |
| Nalidixic acid sodium | Antibiotic | 100.5 |
| Cefmetazole sodium | Antibiotic | 100.6 |
| Pyrazinecarboxamide | Antibiotic | 102.1 |
| Aminopterin | Antibiotic | 102.7 |
| Oxolinic acid | Antibiotic | 103.7 |
| Cephradine | Antibiotic | 104.0 |
| Cephalexin hydrate | Antibiotic | 105.0 |
| Cefaclor | Antibiotic | 109.1 |
| Lomefloxacin hydrochloride | Antibiotic | 111.3 |

| | | |
|---|---|---|
| Paromomycin sulfate | Antibiotic | 111.4 |
| Cefazolin sodium | Antibiotic | 112.6 |
| Mevastatin | Antibiotic | 112.6 |
| Cefotaxime sodium | Antibiotic | 113.9 |
| Cephalothin sodium | Antibiotic | 115.8 |
| Ceftriaxone sodium | Antibiotic | 116.1 |
| Trimethoprim | Antibiotic | 118.2 |
| Cephalosporin C zinc salt | Antibiotic | 122.3 |
| Foliosidine | Anticonvulsant | 86.8 |
| Phenytoin sodium | Anticonvulsant | 88.5 |
| Valproic acid sodium | Anticonvulsant | 89.5 |
| 5,5-Diphenylhydantoin | Anticonvulsant | 93.9 |
| Ethosuximide | Anticonvulsant | 94.6 |
| Lamotrigine | Anticonvulsant | 97.7 |
| N-(4-Amino-2-chlorophenyl)phthalimide | Anticonvulsant | 102.7 |
| Primidone | Anticonvulsant | 105.4 |
| Dubinidine | Anticonvulsant | 106.7 |
| Zonisamide sodium | Anticonvulsant | 109.6 |
| Gabapentin | Anticonvulsant | 116.9 |
| Carbamazepine | Anticonvulsant | 122.7 |
| (S)-(+)-Camptothecin | Apoptosis | 17.2 |
| Etoposide | Apoptosis | 21.7 |
| beta-Lapachone | Apoptosis | 51.5 |
| Retinoic acid | Apoptosis | 53.2 |
| Emetine dihydrochloride hydrate | Apoptosis | 73.5 |
| XK469 | Apoptosis | 87.6 |
| Benzamide | Apoptosis | 103.6 |
| 1,5-Isoquinolinediol | Apoptosis | 105.5 |
| Aurintricarboxylic acid | Apoptosis | 108.5 |
| 4-Amino-1,8-naphthalimide | Apoptosis | 110.2 |
| m-Iodobenzylguanidine hemisulfate | Apoptosis | 112.7 |
| 3-aminobenzamide | Apoptosis | 120.7 |
| Flumazenil | Benzodiazepine | 79.0 |
| Methyl beta-carboline-3-carboxylate | Benzodiazepine | 97.3 |
| N,N-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide | Benzodiazepine | 98.2 |
| Methyl 6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate | Benzodiazepine | 109.0 |
| Zopiclone | Benzodiazepine | 109.5 |
| L-655,708 | Benzodiazepine | 111.2 |
| CB34 | Benzodiazepine | 124.8 |
| 3-Methyl-6-(3-[trifluoromethyl]phenyl)-1,2,4-triazolo[4,3-b]pyridazine | Benzodiazepine | 105.0 |
| Iodoacetamide | Biochemistry | 1.4 |
| Tetraethylthiuram disulfide | Biochemistry | 3.7 |
| Z-L-Phe chloromethyl ketone | Biochemistry | 37.8 |
| Acetamide | Biochemistry | 71.8 |
| L-Leucinethiol, oxidized dihydrochloride | Biochemistry | 75.5 |
| 4-Chloromercuribenzoic acid | Biochemistry | 78.8 |
| 1-Deoxynojirimycin hydrochloride | Biochemistry | 85.0 |

| | | |
|---|---|---|
| 4-Methylpyrazole hydrochloride | Biochemistry | 85.4 |
| Benserazide hydrochloride | Biochemistry | 88.9 |
| Actinonin | Biochemistry | 89.0 |
| Bestatin hydrochloride | Biochemistry | 91.5 |
| PD 404,182 | Biochemistry | 92.1 |
| L-azetidine-2-carboxylic acid | Biochemistry | 92.6 |
| 1,10-Phenanthroline monohydrate | Biochemistry | 93.3 |
| 3-Hydroxybenzylhydrazine dihydrochloride | Biochemistry | 94.7 |
| 10-(alpha-Diethylaminopropionyl)-phenothiazine hydrochloride | Biochemistry | 97.8 |
| 2,2 -Bipyridyl | Biochemistry | 97.9 |
| E-64 | Biochemistry | 98.0 |
| N-Methyl-1-deoxynojirimycin | Biochemistry | 98.3 |
| 4-Hydroxybenzhydrazide | Biochemistry | 98.6 |
| Chlorothiazide | Biochemistry | 99.5 |
| N-p-Tosyl-L-phenylalanine chloromethyl ketone | Biochemistry | 100.8 |
| Acetazolamide | Biochemistry | 101.3 |
| N-Ethylmaleimide | Biochemistry | 105.6 |
| Tetraisopropyl pyrophosphoramide | Biochemistry | 107.6 |
| Furafylline | Biochemistry | 107.8 |
| L-alpha-Methyl DOPA | Biochemistry | 109.6 |
| Hydrochlorothiazide | Biochemistry | 109.8 |
| 4-Aminobenzamidine dihydrochloride | Biochemistry | 110.1 |
| L-allylglycine | Biochemistry | 110.6 |
| 2-Chloro-2-deoxy-D-glucose | Biochemistry | 110.8 |
| Diethylenetriaminepentaacetic acid | Biochemistry | 111.6 |
| Sodium Oxamate | Biochemistry | 112.4 |
| (±)-p-Aminoglutethimide | Biochemistry | 112.4 |
| 3,4-Dichloroisocoumarin | Biochemistry | 112.6 |
| Betaine hydrochloride | Biochemistry | 113.4 |
| P1,P4-Di(adenosine-5 )tetraphosphate triammonium | Biochemistry | 117.0 |
| O-(Carboxymethyl)hydroxylamine hemihydrochloride | Biochemistry | 117.0 |
| S-(-)-Carbidopa | Biochemistry | 118.0 |
| Ethylene glycol-bis(2-aminoethylether)-N,N,N ,N - tetraacetic acid | Biochemistry | 118.4 |
| beta-Chloro-L-alanine hydrochloride | Biochemistry | 119.5 |
| 2,4-Dinitrophenyl 2-fluoro-2-deoxy-beta-D-glucopyranoside | Biochemistry | 119.8 |
| Epibestatin hydrochloride | Biochemistry | 120.5 |
| 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride | Biochemistry | 121.9 |
| Phosphoramidon disodium | Biochemistry | 127.4 |
| Benzamidine hydrochloride | Biochemistry | 129.1 |
| SKF 96365 | Ca2+ Channel | 56.1 |
| NNC 55-0396 | Ca2+ Channel | 70.5 |
| Diltiazem hydrochloride | Ca2+ Channel | 94.6 |
| Mibefradil dihydrochloride | Ca2+ Channel | 96.1 |
| Nitrendipine | Ca2+ Channel | 98.1 |
| Nifedipine | Ca2+ Channel | 98.2 |
| Nicardipine hydrochloride | Ca2+ Channel | 98.3 |

| | | |
|---|---|---|
| (±)-Verapamil hydrochloride | Ca2+ Channel | 98.8 |
| YS-035 hydrochloride | Ca2+ Channel | 100.7 |
| (±)-Methoxyverapamil hydrochloride | Ca2+ Channel | 102.0 |
| FPL 64176 | Ca2+ Channel | 103.2 |
| Cinnarizine | Ca2+ Channel | 104.9 |
| MRS 1845 | Ca2+ Channel | 105.8 |
| Phloretin | Ca2+ Channel | 108.2 |
| Nimodipine | Ca2+ Channel | 113.6 |
| (±)-Bay K 8644 | Ca2+ Channel | 116.1 |
| Bepridil hydrochloride | Ca2+ Channel | 116.3 |
| Felodipine | Ca2+ Channel | 128.3 |
| SKF 94836 | Calcium Signaling | 95.1 |
| ( R)-(+)-WIN 55,212-2 mesylate | Cannabinoid | 90.8 |
| Palmitoylethanolamide | Cannabinoid | 92.9 |
| CP55940 | Cannabinoid | 105.8 |
| N-arachidonylglycine | Cannabinoid | 108.5 |
| JWH-015 | Cannabinoid | 110.4 |
| Indomethacin morpholinylamide | Cannabinoid | 102.7 |
| Bay 11-7085 | Cell Cycle | 3.8 |
| Ellipticine | Cell Cycle | 5.9 |
| Caffeic acid phenethyl ester | Cell Cycle | 40.4 |
| Pyrocatechol | Cell Cycle | 83.4 |
| Apigenin | Cell Cycle | 87.8 |
| Se-(methyl)selenocysteine hydrochloride | Cell Cycle | 89.2 |
| Daidzein | Cell Cycle | 90.8 |
| MDL 28170 | Cell Cycle | 93.3 |
| Retinoic acid p-hydroxyanilide | Cell Cycle | 99.3 |
| TG003 | Cell Cycle | 101.7 |
| 5-Fluorouracil | Cell Cycle | 102.8 |
| Minocycline hydrochloride | Cell Cycle | 103.5 |
| Ganciclovir | Cell Cycle | 105.6 |
| Ribavirin | Cell Cycle | 109.1 |
| Fusidic acid sodium | Cell Cycle | 114.8 |
| Rotenone | Cell Stress | 36.3 |
| U-83836 dihydrochloride | Cell Stress | 49.5 |
| N,N,N ,N -Tetramethylazodicarboxamide | Cell Stress | 82.5 |
| 3-Nitropropionic acid | Cell Stress | 84.3 |
| 2,3-Dimethoxy-1,4-naphthoquinone | Cell Stress | 86.8 |
| (±)-Taxifolin | Cell Stress | 91.2 |
| (+)-Catechin Hydrate | Cell Stress | 95.5 |
| Morin | Cell Stress | 96.0 |
| Isoxanthopterin | Cell Stress | 99.0 |
| Astaxanthin | Cell Stress | 101.5 |
| (±)-alpha-Lipoic Acid | Cell Stress | 101.8 |
| U-74389G maleate | Cell Stress | 102.0 |
| Lonidamine | Cell Stress | 102.1 |
| Carcinine dihydrochloride | Cell Stress | 106.9 |
| Hypotaurine | Cell Stress | 107.5 |
| 5,5-Dimethyl-1-pyrroline-N-oxide | Cell Stress | 109.1 |

| | | |
|---|---|---|
| Allopurinol | Cell Stress | 115.9 |
| Amifostine | Cell Stress | 124.7 |
| Caffeic Acid | Cell Stress | 130.0 |
| Proglumide | Cholecystokinin | 94.6 |
| CR 2945 | Cholecystokinin | 100.0 |
| Lorglumide sodium | Cholecystokinin | 113.3 |
| 2-(alpha-Naphthoyl)ethyltrimethylammonium iodide | Cholinergic | 20.5 |
| MG 624 | Cholinergic | 38.7 |
| (-)-Eseroline fumarate | Cholinergic | 72.9 |
| N,N,N-trimethyl-1-(4-trans-stilbenoxy)-2-propylammonium iodide | Cholinergic | 78.6 |
| Pentolinium di[L(+)-tartrate] | Cholinergic | 79.5 |
| Atropine methyl bromide | Cholinergic | 82.9 |
| Telenzepine dihydrochloride | Cholinergic | 85.2 |
| L-Hyoscyamine | Cholinergic | 86.7 |
| (±)-Vesamicol hydrochloride | Cholinergic | 87.2 |
| Oxybutynin Chloride | Cholinergic | 87.5 |
| Decamethonium dibromide | Cholinergic | 90.5 |
| BW 284c51 | Cholinergic | 91.0 |
| Gallamine triethiodide | Cholinergic | 91.1 |
| Linopirdine | Cholinergic | 91.1 |
| Carbachol | Cholinergic | 91.7 |
| Neostigmine bromide | Cholinergic | 92.0 |
| Hemicholinium-3 | Cholinergic | 92.1 |
| (-)-Nicotine hydrogen tartrate salt | Cholinergic | 92.8 |
| Acetyl-beta-methylcholine chloride | Cholinergic | 93.7 |
| WB 64 | Cholinergic | 94.3 |
| Ivermectin | Cholinergic | 94.4 |
| (-)-Physostigmine | Cholinergic | 95.3 |
| (-)-Scopolamine,n-Butyl-, bromide | Cholinergic | 98.2 |
| Karakoline | Cholinergic | 98.3 |
| Pancuronium bromide | Cholinergic | 98.9 |
| Bethanechol chloride | Cholinergic | 98.9 |
| Mecamylamine hydrochloride | Cholinergic | 100.1 |
| Methylcarbamylcholine chloride | Cholinergic | 100.4 |
| Pilocarpine nitrate | Cholinergic | 100.4 |
| 4-Diphenylacetoxy-N-(2-chloroethyl)piperidine hydrochloride | Cholinergic | 100.7 |
| Propantheline bromide | Cholinergic | 100.8 |
| (-)-Scopolamine methyl bromide | Cholinergic | 100.9 |
| Benztropine mesylate | Cholinergic | 101.4 |
| Tropicamide | Cholinergic | 101.8 |
| (+)-cis-Dioxolane iodide | Cholinergic | 102.0 |
| Dicyclomine hydrochloride | Cholinergic | 102.7 |
| Edrophonium chloride | Cholinergic | 103.1 |
| Pempidine tartrate | Cholinergic | 103.7 |
| 4-DAMP methiodide | Cholinergic | 103.8 |
| Tetraethylammonium chloride | Cholinergic | 104.2 |
| Trihexyphenidyl hydrochloride | Cholinergic | 104.5 |

| | | |
|---|---|---|
| Pirenzepine dihydrochloride | Cholinergic | 105.6 |
| Methoctramine tetrahydrochloride | Cholinergic | 106.3 |
| Oxotremorine methiodide | Cholinergic | 106.3 |
| Oxotremorine sesquifumarate salt | Cholinergic | 106.8 |
| McN-A-343 | Cholinergic | 107.0 |
| Hydroxytacrine maleate | Cholinergic | 107.1 |
| Ipratropium bromide | Cholinergic | 107.3 |
| 1,1-Dimethyl-4-phenyl-piperazinium iodide | Cholinergic | 107.9 |
| DBO-83 | Cholinergic | 108.5 |
| 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride | Cholinergic | 108.8 |
| (+)-Pilocarpine hydrochloride | Cholinergic | 109.4 |
| Orphenadrine hydrochloride | Cholinergic | 109.7 |
| Choline bromide | Cholinergic | 110.7 |
| Arecaidine propargyl ester hydrobromide | Cholinergic | 110.9 |
| alpha-Lobeline hydrochloride | Cholinergic | 111.0 |
| Propofol | Cholinergic | 111.2 |
| Arecoline hydrobromide | Cholinergic | 112.3 |
| Acetylthiocholine chloride | Cholinergic | 112.5 |
| Bromoacetylcholine bromide | Cholinergic | 113.1 |
| Hexamethonium dichloride | Cholinergic | 114.2 |
| (+)-Nicotine (+)-di-p-toluoyl tartrate | Cholinergic | 114.3 |
| Hexahydro-sila-difenidol hydrochloride, p-fluoro analog | Cholinergic | 115.1 |
| Dihydro-beta-erythroidine hydrobromide | Cholinergic | 116.1 |
| Aminobenztropine | Cholinergic | 116.2 |
| (-)-Scopolamine methyl nitrate | Cholinergic | 116.9 |
| Hexamethonium bromide | Cholinergic | 117.7 |
| (-)-Scopolamine hydrobromide | Cholinergic | 118.2 |
| OXA-22 iodide | Cholinergic | 119.4 |
| DL-Homatropine hydrobromide | Cholinergic | 120.5 |
| Pyridostigmine bromide | Cholinergic | 120.5 |
| (±)-Muscarine chloride | Cholinergic | 121.6 |
| (-)-Cotinine | Cholinergic | 126.4 |
| Betaine aldehyde chloride | Cholinergic | 127.4 |
| Succinylcholine chloride | Cholinergic | 135.5 |
| Atropine sulfate | Cholinergic | 136.5 |
| Atropine methyl nitrate | Cholinergic | 143.8 |
| 5-Nitro-2-(3-phenylpropylamino)benzoic acid | Cl- Channel | 115.0 |
| N-Phenylanthranilic acid | Cl- Channel | 124.8 |
| R(+)-IAA-94 | Cl- Channel | 139.8 |
| Papaverine hydrochloride | Cyclic Nucleotides | 80.7 |
| Isoliquiritigenin | Cyclic Nucleotides | 81.1 |
| Milrinone | Cyclic Nucleotides | 87.4 |
| Protoporphyrin IX disodium | Cyclic Nucleotides | 88.5 |
| YC-1 | Cyclic Nucleotides | 91.9 |
| Forskolin | Cyclic Nucleotides | 93.7 |
| 8-Bromo-cAMP sodium | Cyclic Nucleotides | 95.3 |
| Quercetin dihydrate | Cyclic Nucleotides | 96.0 |
| Trequinsin hydrochloride | Cyclic Nucleotides | 97.1 |
| SQ 22536 | Cyclic Nucleotides | 98.8 |

| | | |
|---|---|---|
| Na-p-Tosyl-L-lysine chloromethyl ketone hydrochloride | Cyclic Nucleotides | 99.3 |
| NS 2028 | Cyclic Nucleotides | 101.5 |
| ODQ | Cyclic Nucleotides | 101.7 |
| Ro 20-1724 | Cyclic Nucleotides | 104.0 |
| T-1032 | Cyclic Nucleotides | 104.1 |
| Pentoxifylline | Cyclic Nucleotides | 106.0 |
| Enoximone | Cyclic Nucleotides | 106.4 |
| Quazinone | Cyclic Nucleotides | 107.5 |
| T-0156 | Cyclic Nucleotides | 107.8 |
| Imazodan | Cyclic Nucleotides | 109.9 |
| Zardaverine | Cyclic Nucleotides | 110.8 |
| Cilostazol | Cyclic Nucleotides | 112.2 |
| Rolipram | Cyclic Nucleotides | 112.8 |
| Vinpocetine | Cyclic Nucleotides | 113.0 |
| 8-(4-Chlorophenylthio)-cAMP sodium | Cyclic Nucleotides | 116.0 |
| Zaprinast | Cyclic Nucleotides | 117.0 |
| 8-Methoxymethyl-3-isobutyl-1-methylxanthine | Cyclic Nucleotides | 119.7 |
| 8-Bromo-cGMP sodium | Cyclic Nucleotides | 120.3 |
| Cilostamide | Cyclic Nucleotides | 128.1 |
| 9-cyclopentyladenine | Cyclic Nucleotides | 130.0 |
| Ibudilast | Cyclic Nucleotides | 151.9 |
| CV-3988 | Cytokines & Growth Factors | 113.4 |
| Brefeldin A from Penicillium brefeldianum | Cytoskeleton and ECM | 22.3 |
| Vincristine sulfate | Cytoskeleton and ECM | 41.1 |
| Podophyllotoxin | Cytoskeleton and ECM | 42.5 |
| Colchicine | Cytoskeleton and ECM | 46.8 |
| Vinblastine sulfate salt | Cytoskeleton and ECM | 48.2 |
| Taxol | Cytoskeleton and ECM | 63.5 |
| (±)-Thalidomide | Cytoskeleton and ECM | 81.7 |
| Nocodazole | Cytoskeleton and ECM | 82.3 |
| 2,3-Butanedione | Cytoskeleton and ECM | 104.9 |
| Clodronic acid | Cytoskeleton and ECM | 122.3 |
| Chlorambucil | DNA | 89.9 |
| Nimustine hydrochloride | DNA | 92.9 |
| Cisplatin | DNA | 97.1 |
| Phosphonoacetic acid | DNA | 101.5 |
| Carmustine | DNA | 108.1 |
| CB 1954 | DNA | 116.8 |
| Chloroquine diphosphate | DNA | 117.3 |
| Cyclophosphamide monohydrate | DNA | 122.0 |
| Carboplatin | DNA | 139.4 |
| Melphalan | DNA Metabolism | 41.3 |
| Mitoxantrone | DNA Metabolism | 43.0 |
| Idarubicin | DNA Metabolism | 49.8 |
| Cytosine-1-beta-D-arabinofuranoside hydrochloride | DNA Metabolism | 65.9 |
| 5-azacytidine | DNA Metabolism | 72.0 |
| (-)Amethopterin | DNA Metabolism | 84.8 |
| Ancitabine hydrochloride | DNA Metabolism | 86.2 |
| Methotrexate | DNA Metabolism | 87.2 |

| | | |
|---|---|---|
| Hydroxyurea | DNA Metabolism | 91.5 |
| 5-fluoro-5 -deoxyuridine | DNA Metabolism | 94.3 |
| Altretamine | DNA Metabolism | 94.9 |
| Mizoribine | DNA Metabolism | 110.0 |
| 5-Bromo-2 -deoxyuridine | DNA Metabolism | 111.9 |
| Azelaic acid | DNA Metabolism | 119.7 |
| Amsacrine hydrochloride | DNA Repair | 7.0 |
| p-Benzoquinone | DNA Repair | 100.4 |
| O6-benzylguanine | DNA Repair | 108.0 |
| Apomorphine hydrochloride hemihydrate | Dopamine | 42.3 |
| 3-Phenylpropargylamine hydrochloride | Dopamine | 44.5 |
| R(-)-Propylnorapomorphine hydrochloride | Dopamine | 52.2 |
| SKF 89626 | Dopamine | 61.3 |
| L-750,667 trihydrochloride | Dopamine | 73.2 |
| LE 300 | Dopamine | 73.8 |
| R(-)-N-Allylnorapomorphine hydrobromide | Dopamine | 74.9 |
| Dopamine hydrochloride | Dopamine | 75.2 |
| Indatraline hydrochloride | Dopamine | 77.8 |
| N-(p-Isothiocyanatophenethyl)spiperone hydrochloride | Dopamine | 78.1 |
| Loxapine succinate | Dopamine | 79.0 |
| Dihydrexidine hydrochloride | Dopamine | 79.3 |
| Thiothixene hydrochloride | Dopamine | 80.3 |
| Tiapride hydrochloride | Dopamine | 80.5 |
| Methylergonovine maleate | Dopamine | 82.0 |
| R(-)-2,10,11-Trihydroxyaporphine hybrobromide | Dopamine | 82.2 |
| Pimozide | Dopamine | 84.6 |
| Promazine hydrochloride | Dopamine | 86.0 |
| L-741,626 | Dopamine | 87.6 |
| 4-Hydroxy-3-methoxyphenylacetic acid | Dopamine | 87.6 |
| Dihydroergocristine methanesulfonate | Dopamine | 87.6 |
| 3-Methoxy-4-hydroxyphenethylamine hydrochloride | Dopamine | 87.8 |
| SKF 83565 hydrobromide | Dopamine | 88.0 |
| (±)-Chloro-APB hydrobromide | Dopamine | 88.2 |
| Metoclopramide hydrochloride | Dopamine | 89.9 |
| GBR-12935 dihydrochloride | Dopamine | 90.6 |
| JL-18 | Dopamine | 91.3 |
| A-77636 hydrochloride | Dopamine | 91.9 |
| R(+)-Terguride | Dopamine | 92.5 |
| R(-)-2,10,11-Trihydroxy-N-propylnoraporphine hydrobromide | Dopamine | 92.6 |
| 1-Phenyl-3-(2-thiazolyl)-2-thiourea | Dopamine | 94.4 |
| 4 -Chloro-3-alpha-(diphenylmethoxy)tropane hydrochloride | Dopamine | 94.8 |
| Fluphenazine dihydrochloride | Dopamine | 95.1 |
| Haloperidol | Dopamine | 95.2 |
| 1-(4-Hydroxybenzyl)imidazole-2-thiol | Dopamine | 95.4 |
| BP 897 | Dopamine | 96.0 |
| (±)-Sulpiride | Dopamine | 96.2 |
| SKF 75670 hydrobromide | Dopamine | 96.3 |

| | | |
|---|---|---|
| (±)-Quinpirole dihydrochloride | Dopamine | 96.4 |
| Perphenazine | Dopamine | 96.7 |
| R(-)-Apocodeine hydrochloride | Dopamine | 97.0 |
| L-745,870 hydrochloride | Dopamine | 97.8 |
| cis-(±)-8-OH-PBZI hydrobromide | Dopamine | 97.9 |
| Fusaric acid | Dopamine | 98.7 |
| (+)-Butaclamol hydrochloride | Dopamine | 99.0 |
| Domperidone | Dopamine | 99.1 |
| R(+)-6-Bromo-APB hydrobromide | Dopamine | 99.3 |
| N-Methyldopamine hydrochloride | Dopamine | 99.3 |
| R-(+)-7-Hydroxy-DPAT hydrobromide | Dopamine | 99.4 |
| Propionylpromazine hydrochloride | Dopamine | 99.5 |
| Chlorpromazine hydrochloride | Dopamine | 99.7 |
| 4-Hydroxyphenethylamine hydrochloride | Dopamine | 100.1 |
| Fenoldopam bromide | Dopamine | 101.5 |
| Prochlorperazine dimaleate | Dopamine | 101.5 |
| 4-Methoxy-3-hydroxyphenethylamine hydrochloride | Dopamine | 101.7 |
| BTCP hydrochloride | Dopamine | 101.9 |
| Triflupromazine hydrochloride | Dopamine | 102.5 |
| S-(-)-Eticlopride hydrochloride | Dopamine | 102.7 |
| Thioridazine hydrochloride | Dopamine | 103.2 |
| R(+)-SCH-23390 hydrochloride | Dopamine | 103.5 |
| (-)-Sulpiride | Dopamine | 103.8 |
| cis-(Z)-Flupenthixol dihydrochloride | Dopamine | 104.4 |
| Amantadine hydrochloride | Dopamine | 104.6 |
| GYKI 52895 | Dopamine | 104.8 |
| L-3,4-Dihydroxyphenylalanine | Dopamine | 105.1 |
| Ropinirole hydrochloride | Dopamine | 105.1 |
| Spiperone hydrochloride | Dopamine | 105.3 |
| (±)-SKF 38393, N-allyl-, hydrobromide | Dopamine | 105.3 |
| S(-)-IBZM | Dopamine | 105.6 |
| SKF 83959 hydrobromide | Dopamine | 105.8 |
| Fluspirilene | Dopamine | 106.0 |
| S(-)-DS 121 hydrochloride | Dopamine | 106.4 |
| PD 168,077 maleate | Dopamine | 107.0 |
| U-99194A maleate | Dopamine | 107.1 |
| R(+)-3PPP hydrochloride | Dopamine | 107.3 |
| (+)-Bromocriptine methanesulfonate | Dopamine | 107.7 |
| S(+)-Raclopride L-tartrate | Dopamine | 108.0 |
| Cortexolone maleate | Dopamine | 108.4 |
| L-3,4-Dihydroxyphenylalanine methyl ester hydrochloride | Dopamine | 108.9 |
| N-Acetyldopamine monohydrate | Dopamine | 109.6 |
| S(-)-3PPP hydrochloride | Dopamine | 110.1 |
| Piribedil maleate | Dopamine | 110.3 |
| R(+)-Lisuride hydrogen maleate | Dopamine | 110.3 |
| Chlorprothixene hydrochloride | Dopamine | 110.4 |
| S-(-)-Lisuride | Dopamine | 110.9 |
| N-(2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl)-3-methoxybenzamide | Dopamine | 111.1 |

| | | |
|---|---|---|
| (±)-7-Hydroxy-DPAT hydrobromide | Dopamine | 111.4 |
| (±)-PD 128,907 hydrochloride | Dopamine | 111.4 |
| Trifluoperazine dihydrochloride | Dopamine | 111.7 |
| R(-)-SCH-12679 maleate | Dopamine | 112.4 |
| (±)-6-Chloro-PB hydrobromide | Dopamine | 112.9 |
| Risperidone | Dopamine | 112.9 |
| Mesulergine hydrochloride | Dopamine | 113.1 |
| Bupropion hydrochloride | Dopamine | 113.5 |
| Quinelorane dihydrochloride | Dopamine | 113.6 |
| Droperidol | Dopamine | 113.7 |
| (±)-Octoclothepin maleate | Dopamine | 114.0 |
| (±)-PPHT hydrochloride | Dopamine | 114.3 |
| 6,7-ADTN hydrobromide | Dopamine | 115.6 |
| Amfonelic acid | Dopamine | 116.6 |
| Ergocristine | Dopamine | 116.7 |
| U-101958 maleate | Dopamine | 116.8 |
| (±)-SKF-38393 hydrochloride | Dopamine | 117.9 |
| GBR-12909 dihydrochloride | Dopamine | 118.9 |
| Nomifensine maleate | Dopamine | 120.1 |
| Pergolide methanesulfonate | Dopamine | 121.2 |
| (±)-Butaclamol hydrochloride | Dopamine | 121.3 |
| S(+)-PD 128,907 hydrochloride | Dopamine | 121.4 |
| (-)-Quinpirole hydrochloride | Dopamine | 121.4 |
| Dipropyldopamine hydrobromide | Dopamine | 121.8 |
| Trifluperidol hydrochloride | Dopamine | 124.7 |
| 3,4-Dihydroxyphenylacetic acid | Dopamine | 127.0 |
| Clozapine | Dopamine | 128.2 |
| Agroclavine | Dopamine | 130.6 |
| Farnesylthiosalicylic acid | G protein | 77.9 |
| (-)-Perillic acid | G protein | 100.8 |
| SCH-202676 hydrobromide | G protein | 107.9 |
| NF449 octasodium salt | G protein | 126.7 |
| TPMPA | GABA | 74.2 |
| GABA | GABA | 86.3 |
| Picrotoxin | GABA | 89.8 |
| Isoguvacine hydrochloride | GABA | 90.4 |
| (±)-Baclofen | GABA | 92.1 |
| CGP-13501 | GABA | 93.0 |
| 3-Amino-1-propanesulfonic acid sodium | GABA | 93.7 |
| Gabaculine hydrochloride | GABA | 93.9 |
| NCS-356 | GABA | 94.2 |
| 3-alpha,21-Dihydroxy-5-alpha-pregnan-20-one | GABA | 94.5 |
| NCS-382 | GABA | 94.9 |
| (+)-Hydrastine | GABA | 95.8 |
| 3-Aminopropylphosphonic acid | GABA | 96.9 |
| THIP hydrochloride | GABA | 97.5 |
| Dehydroisoandrosterone 3-sulfate sodium | GABA | 98.5 |
| Muscimol hydrobromide | GABA | 99.2 |
| NO-711 hydrochloride | GABA | 99.3 |

| | | |
|---|---|---|
| (-)-Bicuculline methbromide, 1(S), 9(R) | GABA | 99.9 |
| SB 205384 | GABA | 101.1 |
| Guvacine hydrochloride | GABA | 101.9 |
| 5alpha-Pregnan-3alpha-ol-20-one | GABA | 103.0 |
| (±)-gamma-Vinyl GABA | GABA | 103.5 |
| (E)-4-amino-2-butenoic acid | GABA | 105.9 |
| Pregnenolone sulfate sodium | GABA | 106.3 |
| N-Methyl-beta-carboline-3-carboxamide | GABA | 107.0 |
| Piperidine-4-sulphonic acid | GABA | 110.7 |
| SKF 89976A hydrochloride | GABA | 111.3 |
| Isonipecotic acid | GABA | 112.8 |
| CGP-7930 | GABA | 114.0 |
| 5alpha-Pregnan-3alpha-ol-11,20-dione | GABA | 116.7 |
| 5-Aminovaleric acid hydrochloride | GABA | 117.9 |
| 2-Hydroxysaclofen | GABA | 119.9 |
| Phaclofen | GABA | 120.7 |
| Tracazolate | GABA | 122.4 |
| gamma-Acetylinic GABA | GABA | 122.6 |
| Imidazole-4-acetic acid hydrochloride | GABA | 123.3 |
| (±)-Nipecotic acid | GABA | 124.6 |
| SR-95531 | GABA | 127.0 |
| cis-4-Aminocrotonic acid | GABA | 132.4 |
| PK 11195 | GABA | 132.7 |
| SKF 97541 hydrochloride | GABA | 135.6 |
| Sobuzoxane | Gene Regulation | 96.2 |
| Spermidine trihydrochloride | Glutamate | 24.5 |
| SIB 1757 | Glutamate | 72.2 |
| Kainic acid | Glutamate | 73.0 |
| Eliprodil | Glutamate | 80.0 |
| Aniracetam | Glutamate | 83.9 |
| DNQX | Glutamate | 85.4 |
| Cyclothiazide | Glutamate | 87.8 |
| Ifenprodil tartrate | Glutamate | 88.1 |
| (±)-2-Amino-3-phosphonopropionic acid | Glutamate | 91.4 |
| Pentamidine isethionate | Glutamate | 92.3 |
| (±)-HA-966 | Glutamate | 94.2 |
| Dihydrokainic acid | Glutamate | 94.5 |
| D-Cycloserine | Glutamate | 95.4 |
| (±)-CPP | Glutamate | 96.2 |
| trans-(±)-ACPD | Glutamate | 97.0 |
| 2,6-Difluoro-4-[2-(phenylsulfonylamino)ethylthio]phenoxyacetamide | Glutamate | 97.1 |
| L-Glutamic acid, N-phthaloyl- | Glutamate | 97.4 |
| (2S,1 S,2 S)-2-(carboxycyclopropyl)glycine | Glutamate | 98.0 |
| CR 2249 | Glutamate | 98.3 |
| GYKI 52466 hydrochloride | Glutamate | 98.3 |
| (-)-MK-801 hydrogen maleate | Glutamate | 98.3 |
| CX 546 | Glutamate | 98.6 |
| (±)-Ibotenic acid | Glutamate | 100.3 |

| | | |
|---|---|---|
| Piracetam | Glutamate | 100.4 |
| 6,7-Dichloroquinoxaline-2,3-dione | Glutamate | 100.8 |
| L-701,324 | Glutamate | 100.8 |
| SIB 1893 | Glutamate | 101.8 |
| NS 521 oxalate | Glutamate | 101.8 |
| NBQX disodium | Glutamate | 102.8 |
| (+)-Quisqualic acid | Glutamate | 102.8 |
| Flupirtine maleate | Glutamate | 103.0 |
| (S)-3,5-Dihydroxyphenylglycine | Glutamate | 103.6 |
| N-Methyl-D-aspartic acid | Glutamate | 103.8 |
| (±)-AMPA hydrobromide | Glutamate | 103.9 |
| trans-Azetidine-2,4-dicarboxylic acid | Glutamate | 104.1 |
| Quinolinic acid | Glutamate | 104.1 |
| Dextromethorphan hydrobromide monohydrate | Glutamate | 104.3 |
| (±)-2-Amino-4-phosphonobutyric acid | Glutamate | 104.3 |
| S(-)-Willardiine | Glutamate | 104.4 |
| 3-Methoxy-morphanin hydrochloride | Glutamate | 105.2 |
| MDL 105,519 | Glutamate | 105.9 |
| Kynurenic acid | Glutamate | 106.0 |
| CNQX disodium | Glutamate | 106.4 |
| ATPO | Glutamate | 106.9 |
| S-5-Iodowillardiine | Glutamate | 107.3 |
| IEM-1460 | Glutamate | 107.3 |
| gamma-D-Glutamylaminomethylsulfonic acid | Glutamate | 107.5 |
| N-(3,3-Diphenylpropyl)glycinamide | Glutamate | 108.1 |
| 6-Methyl-2-(phenylethynyl)pyridine hydrochloride | Glutamate | 108.1 |
| O-Phospho-L-serine | Glutamate | 108.3 |
| DL-threo-beta-hydroxyaspartic acid | Glutamate | 108.7 |
| Spermine tetrahydrochloride | Glutamate | 108.7 |
| L-Methionine sulfoximine | Glutamate | 110.6 |
| 5-Fluoroindole-2-carboxylic acid | Glutamate | 110.6 |
| (+)-MK-801 hydrogen maleate | Glutamate | 110.6 |
| Phthalamoyl-L-glutamic acid trisodium | Glutamate | 111.1 |
| L-Glutamine | Glutamate | 111.4 |
| Memantine hydrochloride | Glutamate | 111.6 |
| (±)-2-Amino-5-phosphonopentanoic acid | Glutamate | 112.0 |
| 7-Chlorokynurenic acid | Glutamate | 112.1 |
| (S)-MAP4 hydrochloride | Glutamate | 112.7 |
| Cystamine dihydrochloride | Glutamate | 113.0 |
| N-Acetyl-L-Cysteine | Glutamate | 113.2 |
| Ro 8-4304 | Glutamate | 114.3 |
| L-2-aminoadipic acid | Glutamate | 114.6 |
| MDL 26,630 trihydrochloride | Glutamate | 115.9 |
| D(-)-2-Amino-7-phosphonoheptanoic acid | Glutamate | 116.0 |
| L-Cysteinesulfinic Acid | Glutamate | 116.3 |
| L-Aspartic acid | Glutamate | 118.0 |
| (±)-cis-Piperidine-2,3-dicarboxylic acid | Glutamate | 118.2 |
| (±)-2-Amino-7-phosphonoheptanoic acid | Glutamate | 118.5 |
| (±)-alpha-Methyl-4-carboxyphenylglycine | Glutamate | 118.6 |

| | | |
|---|---|---|
| Felbamate | Glutamate | 119.8 |
| CNS-1102 | Glutamate | 120.1 |
| AIDA | Glutamate | 121.0 |
| ATPA | Glutamate | 121.3 |
| Dextrorphan D-tartrate | Glutamate | 122.3 |
| 5,7-Dichlorokynurenic acid | Glutamate | 122.9 |
| Putrescine dihydrochloride | Glutamate | 126.0 |
| 1-Aminocyclopropanecarboxylic acid hydrochloride | Glutamate | 127.7 |
| L-Glutamic acid hydrochloride | Glutamate | 128.6 |
| CPCCOEt | Glutamate | 131.2 |
| Riluzole | Glutamate | 131.5 |
| 1,10-Diaminodecane | Glutamate | 132.3 |
| D-Serine | Glutamate | 134.8 |
| Chelidamic acid | Glutamate | 136.8 |
| Arcaine sulfate | Glutamate | 141.8 |
| Ro 25-6981 hydrochloride | Glutamate | 151.6 |
| cis-Azetidine-2,4-dicarboxylic acid | Glutamate | 152.6 |
| Taurine | Glycine | 87.7 |
| Phenylbenzene-omega-phosphono-alpha-amino acid | Glycine | 147.5 |
| Clemastine fumarate | Histamine | 77.4 |
| Terfenadine | Histamine | 86.1 |
| Fexofenadine hydrochloride | Histamine | 87.9 |
| Pheniramine maleate | Histamine | 88.6 |
| Doxylamine succinate | Histamine | 90.0 |
| Promethazine hydrochloride | Histamine | 90.8 |
| (+)-Brompheniramine maleate | Histamine | 92.7 |
| Diphenhydramine hydrochloride | Histamine | 98.3 |
| L-Histidine hydrochloride | Histamine | 98.4 |
| Cimetidine | Histamine | 98.6 |
| Famotidine | Histamine | 99.0 |
| Ketotifen fumarate | Histamine | 99.8 |
| Clemizole hydrochloride | Histamine | 100.6 |
| Methapyrilene hydrochloride | Histamine | 100.8 |
| 1-Methylhistamine dihydrochloride | Histamine | 101.4 |
| 4-Imidazolemethanol hydrochloride | Histamine | 101.8 |
| (±)-Chlorpheniramine maleate | Histamine | 102.6 |
| Histamine dihydrochloride | Histamine | 103.1 |
| SKF 95282 dimaleate | Histamine | 103.2 |
| Pyrilamine maleate | Histamine | 104.0 |
| Loratadine | Histamine | 105.3 |
| 4-Imidazoleacrylic acid | Histamine | 107.0 |
| 3-(1H-Imidazol-4-yl)propyl di(p-fluorophenyl)methyl ether hydrochloride | Histamine | 107.0 |
| (+)-Chlorpheniramine maleate | Histamine | 107.1 |
| Thioperamide maleate | Histamine | 107.4 |
| Imetit dihydrobromide | Histamine | 108.3 |
| Triprolidine hydrochloride | Histamine | 111.9 |
| (±)-Brompheniramine maleate | Histamine | 114.0 |
| SKF 91488 dihydrochloride | Histamine | 116.5 |

| | | |
|---|---|---|
| Histamine, R(-)-alpha-methyl-, dihydrochloride | Histamine | 127.8 |
| Ranitidine hydrochloride | Histamine | 149.3 |
| 2-methoxyestradiol | Hormone | 52.0 |
| Cortexolone | Hormone | 63.4 |
| Raloxifene hydrochloride | Hormone | 79.3 |
| Danazol | Hormone | 84.5 |
| Nilutamide | Hormone | 94.2 |
| Beclomethasone | Hormone | 95.1 |
| Hydrocortisone | Hormone | 95.1 |
| Flutamide | Hormone | 98.9 |
| 4-Androstene-3,17-dione | Hormone | 99.4 |
| (R,R)-cis-Diethyl tetrahydro-2,8-chrysenediol | Hormone | 99.6 |
| Progesterone | Hormone | 99.8 |
| Corticosterone | Hormone | 102.0 |
| 4-Androsten-4-ol-3,17-dione | Hormone | 102.8 |
| 5alpha-Androstane-3alpha,17beta-diol | Hormone | 103.2 |
| Androsterone | Hormone | 103.9 |
| Estrone | Hormone | 104.7 |
| 17alpha-hydroxyprogesterone | Hormone | 105.4 |
| Triamcinolone | Hormone | 106.2 |
| Cortisone 21-acetate | Hormone | 107.1 |
| trans-Dehydroandrosterone | Hormone | 107.6 |
| Cyproterone acetate | Hormone | 108.0 |
| Budesonide | Hormone | 108.7 |
| Mifepristone | Hormone | 108.9 |
| Tolazamide | Hormone | 109.8 |
| Acetohexamide | Hormone | 110.3 |
| Chlorpropamide | Hormone | 110.6 |
| beta-Estradiol | Hormone | 111.2 |
| Hydrocortisone 21-hemisuccinate sodium | Hormone | 112.5 |
| 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane | Hormone | 113.2 |
| Cortisone | Hormone | 113.4 |
| 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole | Hormone | 115.5 |
| Spironolactone | Hormone | 118.4 |
| Betamethasone | Hormone | 121.7 |
| Tolbutamide | Hormone | 122.9 |
| Idazoxan hydrochloride | Imidazoline | 67.9 |
| Antozoline hydrochloride | Imidazoline | 86.7 |
| BU99006 | Imidazoline | 95.9 |
| Metrazoline oxalate | Imidazoline | 100.5 |
| Benazoline oxalate | Imidazoline | 104.7 |
| Rilmenidine hemifumarate | Imidazoline | 109.1 |
| Agmatine sulfate | Imidazoline | 117.8 |
| Harmane | Imidazoline | 117.8 |
| AGN 192403 hydrochloride | Imidazoline | 133.9 |
| BU224 hydrochloride | Imidazoline | 138.3 |
| Efaroxan hydrochloride | Imidazoline | 107.3 |
| Leflunomide | Immune System | 77.3 |
| 3 -Azido-3 -deoxythymidine | Immune System | 79.4 |

| | | |
|---|---|---|
| Pirfenidone | Immune System | 90.1 |
| 6-Aminohexanoic acid | Immune System | 102.4 |
| Oxatomide | Immune System | 104.8 |
| Amiprilose hydrochloride | Immune System | 105.6 |
| 3-deazaadenosine | Immune System | 106.3 |
| 2 ,3 -didehydro-3 -deoxythymidine | Immune System | 106.9 |
| (E)-5-(2-Bromovinyl)-2 -deoxyuridine | Immune System | 111.0 |
| Acyclovir | Immune System | 131.3 |
| 2 ,3 -dideoxycytidine | Immune System | 133.5 |
| Calmidazolium chloride | Intracellular Calcium | 3.5 |
| Calcimycin | Intracellular Calcium | 35.9 |
| Thapsigargin | Intracellular Calcium | 45.8 |
| N-(4-Aminobutyl)-5-chloro-2-naphthalenesulfonamide hydrochloride | Intracellular Calcium | 95.0 |
| W-7 hydrochloride | Intracellular Calcium | 96.4 |
| Thio-NADP sodium | Intracellular Calcium | 98.8 |
| Dantrolene sodium | Intracellular Calcium | 113.0 |
| TMB-8 hydrochloride | Intracellular Calcium | 135.4 |
| SCH-28080 | Ion Channels | 116.6 |
| Sanguinarine chloride | Ion Pump | 3.2 |
| Dihydroouabain | Ion Pump | 38.0 |
| Ouabain | Ion Pump | 46.1 |
| 5-(N-Ethyl-N-isopropyl)amiloride | Ion Pump | 69.2 |
| 5-(N,N-Dimethyl)amiloride hydrochloride | Ion Pump | 80.0 |
| Ruthenium red | Ion Pump | 90.0 |
| Benzamil hydrochloride | Ion Pump | 97.4 |
| Metolazone | Ion Pump | 98.4 |
| 3 ,4 -Dichlorobenzamil | Ion Pump | 98.6 |
| Lansoprazole | Ion Pump | 98.7 |
| Furosemide | Ion Pump | 98.8 |
| R(+)-Butylindazone | Ion Pump | 108.5 |
| Bumetanide | Ion Pump | 111.3 |
| 5-(N,N-hexamethylene)amiloride | Ion Pump | 114.5 |
| 5-(N-Methyl-N-isobutyl)amiloride | Ion Pump | 128.5 |
| Flunarizine dihydrochloride | Ion Pump | 132.3 |
| Disopyramide phosphate | K+ Channel | 20.9 |
| Dequalinium dichloride | K+ Channel | 39.1 |
| Rutaecarpine | K+ Channel | 81.4 |
| IMID-4F hydrochloride | K+ Channel | 81.9 |
| Propafenone hydrochloride | K+ Channel | 86.2 |
| 2,3-Butanedione monoxime | K+ Channel | 87.4 |
| Quinine sulfate | K+ Channel | 99.0 |
| NS-1619 | K+ Channel | 99.2 |
| PNU-37887A | K+ Channel | 101.8 |
| Clotrimazole | K+ Channel | 104.2 |
| 4-Aminopyridine | K+ Channel | 105.3 |
| Glipizide | K+ Channel | 105.5 |
| Pinacidil | K+ Channel | 106.4 |
| 5-hydroxydecanoic acid sodium | K+ Channel | 109.9 |

| | | |
|---|---|---|
| Minoxidil | K+ Channel | 110.9 |
| Glibenclamide | K+ Channel | 112.4 |
| DCEBIO | K+ Channel | 113.0 |
| Psora-4 | K+ Channel | 118.0 |
| Diazoxide | K+ Channel | 126.3 |
| Hydroquinone | Leukotriene | 73.6 |
| Nordihydroguaiaretic acid from Larrea divaricata (creosote bush) | Leukotriene | 81.0 |
| Ebselen | Leukotriene | 84.7 |
| U-75302 | Leukotriene | 93.6 |
| SR 2640 | Leukotriene | 98.0 |
| AA-861 | Leukotriene | 99.8 |
| MK-886 | Leukotriene | 114.3 |
| Tranilast | Leukotriene | 115.1 |
| REV 5901 | Leukotriene | 119.9 |
| BWB70C | Leukotriene | 132.3 |
| U-73122 | Lipid | 73.1 |
| Sandoz 58-035 | Lipid | 87.2 |
| Clofibrate | Lipid | 99.6 |
| Bromoenol lactone | Lipid | 104.0 |
| MJ33 | Lipid | 106.3 |
| 7,7-Dimethyl-(5Z,8Z)-eicosadienoic acid | Lipid | 111.6 |
| D-609 potassium | Lipid | 112.7 |
| Citicoline sodium | Lipid | 114.1 |
| Ranolazine dihydrochloride | Lipid | 115.3 |
| ET-18-OCH3 | Lipid | 138.9 |
| L-165,041 | Lipid Signaling | 109.1 |
| (Z)-Gugglesterone | Lipid Signaling | 123.8 |
| Melatonin | Melatonin | 85.8 |
| 2-Iodomelatonin | Melatonin | 90.1 |
| 6-Chloromelatonin | Melatonin | 97.2 |
| IIK7 | Melatonin | 98.8 |
| N-Acetyltryptamine | Melatonin | 107.6 |
| N-Acetyl-5-hydroxytryptamine | Melatonin | 107.9 |
| K 185 | Melatonin | 110.3 |
| 6-Hydroxymelatonin | Melatonin | 124.7 |
| 1-(4-Chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid | Multi-Drug Resistance | 78.1 |
| Sulfaphenazole | Multi-Drug Resistance | 85.3 |
| 1-(4-Fluorobenzyl)-5-methoxy-2-methylindole-3-acetic acid | Multi-Drug Resistance | 95.0 |
| 3-Aminopropionitrile fumarate | Multi-Drug Resistance | 95.9 |
| SKF-525A hydrochloride | Multi-Drug Resistance | 96.1 |
| Sodium Taurocholate | Multi-Drug Resistance | 103.2 |
| L-Buthionine-sulfoximine | Multi-Drug Resistance | 109.9 |
| Ketoconazole | Multi-Drug Resistance | 110.3 |
| 1-benzoyl-5-methoxy-2-methylindole-3-acetic acid | Multi-Drug Resistance | 113.4 |
| S-(p-Azidophenacyl)glutathione | Multi-Drug Resistance | 113.7 |
| DL-Buthionine-[S,R]-sulfoximine | Multi-Drug Resistance | 116.2 |
| 1-Aminobenzotriazole | Multi-Drug Resistance | 126.9 |
| Lidocaine hydrochloride | Na+ Channel | 85.9 |

| | | |
|---|---|---|
| N-Acetylprocainamide hydrochloride | Na+ Channel | 86.0 |
| Lidocaine N-ethyl bromide quaternary salt | Na+ Channel | 93.5 |
| Mexiletene hydrochloride | Na+ Channel | 94.8 |
| Lidocaine N-methyl hydrochloride | Na+ Channel | 95.8 |
| Flecainide acetate | Na+ Channel | 95.9 |
| N-Bromoacetamide | Na+ Channel | 96.9 |
| Quinidine sulfate | Na+ Channel | 97.3 |
| Triamterene | Na+ Channel | 97.9 |
| Procainamide hydrochloride | Na+ Channel | 101.2 |
| Tetracaine hydrochloride | Na+ Channel | 101.6 |
| Procaine hydrochloride | Na+ Channel | 106.0 |
| Prilocaine hydrochloride | Na+ Channel | 110.3 |
| R(-)-Me5 | Na+ Channel | 112.7 |
| Phenamil methanesulfonate | Na+ Channel | 113.8 |
| Disopyramide | Na+ Channel | 114.3 |
| Amiloride hydrochloride | Na+ Channel | 128.3 |
| Quinacrine dihydrochloride | Neurotransmission | 5.0 |
| N-Oleoyldopamine | Neurotransmission | 30.4 |
| 1-Amino-1-cyclohexanecarboxylic acid hydrochloride | Neurotransmission | 82.2 |
| Phenelzine sulfate | Neurotransmission | 86.3 |
| Clorgyline hydrochloride | Neurotransmission | 87.5 |
| L-162,313 | Neurotransmission | 88.4 |
| Carisoprodol | Neurotransmission | 90.1 |
| NBI 27914 | Neurotransmission | 93.1 |
| 3-Iodo-L-tyrosine | Neurotransmission | 93.8 |
| R-(-)-Desmethyldeprenyl hydrochloride | Neurotransmission | 96.9 |
| DL-alpha-Methyl-p-tyrosine | Neurotransmission | 97.6 |
| Iproniazid phosphate | Neurotransmission | 98.5 |
| Tranylcypromine hydrochloride | Neurotransmission | 101.1 |
| DL-Thiorphan | Neurotransmission | 102.5 |
| 6-Methoxy-1,2,3,4-tetrahydro-9H-pyrido[3,4b] indole | Neurotransmission | 103.1 |
| DL-p-Chlorophenylalanine methyl ester hydrochloride | Neurotransmission | 103.6 |
| Nialamide | Neurotransmission | 104.0 |
| (-)-cis-(1S,2R)-U-50488 tartrate | Neurotransmission | 104.3 |
| Icilin | Neurotransmission | 104.9 |
| Ro 16-6491 hydrochloride | Neurotransmission | 104.9 |
| (±)-3-(3,4-dihydroxyphenyl)-2-methyl-DL-alanine | Neurotransmission | 105.9 |
| Chlormezanone | Neurotransmission | 107.1 |
| 3,5-Dinitrocatechol | Neurotransmission | 107.2 |
| Pentylenetetrazole | Neurotransmission | 107.3 |
| R-(-)-Deprenyl hydrochloride | Neurotransmission | 107.7 |
| (±)-2,3-Dichloro-alpha-methylbenzylamine hydrochloride | Neurotransmission | 109.1 |
| N-Succinyl-L-proline | Neurotransmission | 109.2 |
| SB 218795 | Neurotransmission | 110.3 |
| alpha-Methyl-DL-tyrosine methyl ester hydrochloride | Neurotransmission | 110.6 |
| Ro 41-1049 hydrochloride | Neurotransmission | 110.7 |
| L-368,899 | Neurotransmission | 110.9 |
| (6R)-5,6,7,8-Tetrahydro-L-biopterin hydrochloride | Neurotransmission | 111.3 |
| Hydroxylamine hydrochloride | Neurotransmission | 111.6 |

| | | |
|---|---|---|
| Hydralazine hydrochloride | Neurotransmission | 111.9 |
| Captopril | Neurotransmission | 113.3 |
| Semicarbazide hydrochloride | Neurotransmission | 113.7 |
| Lithium Chloride | Neurotransmission | 115.1 |
| p-Fluoro-L-phenylalanine | Neurotransmission | 115.5 |
| Pargyline hydrochloride | Neurotransmission | 117.4 |
| Ro 41-0960 | Neurotransmission | 118.5 |
| L-alpha-Methyl-p-tyrosine | Neurotransmission | 121.1 |
| Iofetamine hydrochloride | Neurotransmission | 121.6 |
| BRL 52537 hydrochloride | Neurotransmission | 125.6 |
| Debrisoquin sulfate | Neurotransmission | 126.0 |
| 2-Cyclooctyl-2-hydroxyethylamine hydrochloride | Neurotransmission | 126.7 |
| (±)-p-Chlorophenylalanine | Neurotransmission | 133.8 |
| Diphenyleneiodonium chloride | Nitric Oxide | 23.4 |
| Ammonium pyrrolidinedithiocarbamate | Nitric Oxide | 46.7 |
| 4-Phenyl-3-furoxancarbonitrile | Nitric Oxide | 59.5 |
| Thiocitrulline | Nitric Oxide | 80.3 |
| NG-Nitro-L-arginine | Nitric Oxide | 83.6 |
| S-Nitrosoglutathione | Nitric Oxide | 87.4 |
| L-N5-(1-Iminoethyl)ornithine hydrochloride | Nitric Oxide | 92.0 |
| L-Canavanine sulfate | Nitric Oxide | 94.7 |
| 1400W dihydrochloride | Nitric Oxide | 95.3 |
| Chlorzoxazone | Nitric Oxide | 97.5 |
| L-Arginine | Nitric Oxide | 99.6 |
| alpha-Guanidinoglutaric acid | Nitric Oxide | 101.2 |
| NG-Nitro-L-arginine methyl ester hydrochloride | Nitric Oxide | 102.3 |
| 1,4-PBIT dihydrobromide | Nitric Oxide | 102.6 |
| 1-[2-(Trifluoromethyl)phenyl]imidazole | Nitric Oxide | 102.8 |
| Aminoguanidine hydrochloride | Nitric Oxide | 104.0 |
| 7-Nitroindazole | Nitric Oxide | 104.2 |
| (±)-AMT hydrochloride | Nitric Oxide | 104.4 |
| N^G,N^G-Dimethylarginine hydrochloride | Nitric Oxide | 104.6 |
| 1,3-PBIT dihydrobromide | Nitric Oxide | 105.0 |
| L-N6-(1-Iminoethyl)lysine hydrochloride | Nitric Oxide | 105.8 |
| Sepiapterin | Nitric Oxide | 106.2 |
| TFPI hydrochloride | Nitric Oxide | 106.4 |
| 3-Bromo-7-nitroindazole | Nitric Oxide | 106.7 |
| NG-Monomethyl-L-arginine acetate | Nitric Oxide | 107.8 |
| Sodium nitroprusside dihydrate | Nitric Oxide | 109.5 |
| S-Nitroso-N-acetylpenicillamine | Nitric Oxide | 109.7 |
| S-Methyl-L-thiocitrulline acetate | Nitric Oxide | 109.9 |
| NG-Hydroxy-L-arginine acetate | Nitric Oxide | 110.9 |
| NADPH tetrasodium | Nitric Oxide | 112.2 |
| Aminoguanidine hemisulfate | Nitric Oxide | 112.8 |
| Molsidomine | Nitric Oxide | 114.4 |
| S-Ethylisothiourea hydrobromide | Nitric Oxide | 114.5 |
| 3-Morpholinosydnonimine hydrochloride | Nitric Oxide | 117.7 |
| Guanidinoethyl disulfide dihydrobromide | Nitric Oxide | 119.2 |
| 2-(2-Aminoethyl)isothiourea dihydrobromide | Nitric Oxide | 125.5 |

| | | |
|---|---|---|
| S-Methylisothiourea hemisulfate | Nitric Oxide | 129.5 |
| DM 235 | Nootropic | 102.2 |
| Oxiracetam | Nootropic | 108.5 |
| Centrophenoxine hydrochloride | Nootropic | 133.0 |
| L-687,384 hydrochloride | Opioid | 85.6 |
| Loperamide hydrochloride | Opioid | 88.5 |
| Naloxone hydrochloride | Opioid | 89.5 |
| Guanidinyl-naltrindole di-trifluoroacetate | Opioid | 92.7 |
| (-)-trans-(1S,2S)-U-50488 hydrochloride | Opioid | 94.2 |
| Nalbuphine hydrochloride | Opioid | 94.4 |
| Levallorphan tartrate | Opioid | 94.7 |
| Naloxonazine dihydrochloride | Opioid | 95.0 |
| (+)-Cyclazocine | Opioid | 96.6 |
| (±)-N-Allylnormetazocine hydrochloride | Opioid | 96.8 |
| (+)-N-Allylnormetazocine hydrochloride | Opioid | 96.9 |
| Naloxone benzoylhydrazone | Opioid | 97.6 |
| U-69593 | Opioid | 97.7 |
| Naltriben methanesulfonate | Opioid | 98.3 |
| AC 915 oxalate | Opioid | 98.5 |
| nor-Binaltorphimine dihydrochloride | Opioid | 98.9 |
| (+)-trans-(1R,2R)-U-50488 hydrochloride | Opioid | 99.3 |
| PRE-084 | Opioid | 102.4 |
| ICI 204,448 hydrochloride | Opioid | 103.8 |
| (±) trans-U-50488 methanesulfonate | Opioid | 105.8 |
| Naltrexone hydrochloride | Opioid | 106.1 |
| SNC80 | Opioid | 107.9 |
| Naltrindole hydrochloride | Opioid | 112.3 |
| Carbetapentane citrate | Opioid | 113.5 |
| Metaphit methanesulfonate | Opioid | 113.6 |
| U-62066 | Opioid | 113.7 |
| Noscapine hydrchloride | Opioid | 117.3 |
| (-)-3-Methoxynaltrexone hydrochloride | Opioid | 117.9 |
| GR-89696 fumarate | Opioid | 118.1 |
| 2-Chloroadenosine triphosphate tetrasodium | P2 Receptor | 88.5 |
| PPADS | P2 Receptor | 97.3 |
| MRS 2159 | P2 Receptor | 97.4 |
| Azathioprine | P2 Receptor | 99.8 |
| PPNDS tetrasodium | P2 Receptor | 100.0 |
| MRS 2179 | P2 Receptor | 102.8 |
| Uridine 5 -diphosphate sodium | P2 Receptor | 104.7 |
| Reactive Blue 2 | P2 Receptor | 106.4 |
| alpha,beta-Methylene adenosine 5 -triphosphate dilithium | P2 Receptor | 107.7 |
| NF 023 | P2 Receptor | 107.8 |
| 2-Methylthioadenosine triphosphate tetrasodium | P2 Receptor | 108.1 |
| 2-Methylthioadenosine diphosphate trisodium | P2 Receptor | 115.2 |
| ARL 67156 trisodium salt | P2 Receptor | 116.0 |
| Suramin hexasodium | P2 Receptor | 119.1 |
| BRL 50481 | Phosphodiesterase | 100.2 |
| Dequalinium analog, C-14 linker | Phosphorylation | 3.2 |

| | | |
|---|---|---|
| NSC 95397 | Phosphorylation | 3.4 |
| Chelerythrine chloride | Phosphorylation | 3.5 |
| ZM 39923 hydrochloride | Phosphorylation | 13.0 |
| CGP-74514A hydrochloride | Phosphorylation | 23.8 |
| Tyrphostin A9 | Phosphorylation | 27.9 |
| Rottlerin | Phosphorylation | 32.0 |
| Tyrphostin AG 879 | Phosphorylation | 34.8 |
| Cantharidin | Phosphorylation | 40.3 |
| Cantharidic Acid | Phosphorylation | 42.3 |
| Myricetin | Phosphorylation | 50.5 |
| Tyrphostin AG 808 | Phosphorylation | 62.4 |
| Tyrphostin AG 555 | Phosphorylation | 62.6 |
| IC 261 | Phosphorylation | 66.0 |
| rac-2-Ethoxy-3-octadecanamido-1-propylphosphocholine | Phosphorylation | 66.2 |
| SU 6656 | Phosphorylation | 66.7 |
| Tyrphostin AG 835 | Phosphorylation | 73.0 |
| Purvalanol A | Phosphorylation | 74.4 |
| Kenpaullone | Phosphorylation | 75.7 |
| Emodin | Phosphorylation | 84.2 |
| U0126 | Phosphorylation | 84.6 |
| Tyrphostin 47 | Phosphorylation | 85.6 |
| 2,6-Diamino-4-pyrimidinone | Phosphorylation | 86.0 |
| Piceatannol | Phosphorylation | 86.6 |
| Palmitoyl-DL-Carnitine chloride | Phosphorylation | 86.7 |
| rac-2-Ethoxy-3-hexadecanamido-1-propylphosphocholine | Phosphorylation | 86.9 |
| Tyrphostin 25 | Phosphorylation | 87.0 |
| Tyrphostin AG 528 | Phosphorylation | 87.2 |
| SB 202190 | Phosphorylation | 87.7 |
| 1-(5-Isoquinolinylsulfonyl)-3-methylpiperazine dihydrochloride | Phosphorylation | 88.3 |
| Daphnetin | Phosphorylation | 89.0 |
| Tyrphostin AG 34 | Phosphorylation | 90.0 |
| Sphingosine | Phosphorylation | 90.2 |
| Tyrphostin AG 494 | Phosphorylation | 90.4 |
| ML-7 | Phosphorylation | 90.5 |
| Diacylglycerol kinase inhibitor I | Phosphorylation | 90.8 |
| 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride | Phosphorylation | 91.0 |
| Tyrphostin 23 | Phosphorylation | 91.6 |
| Tyrphostin AG 490 | Phosphorylation | 92.8 |
| H-89 | Phosphorylation | 93.0 |
| Cyclosporin A | Phosphorylation | 93.4 |
| H-7 dihydrochloride | Phosphorylation | 93.5 |
| Genistein | Phosphorylation | 94.4 |
| Indirubin-3 -oxime | Phosphorylation | 94.5 |
| HA-1004 hydrochloride | Phosphorylation | 94.8 |
| H-8 dihydrochloride | Phosphorylation | 95.1 |
| SB 415286 | Phosphorylation | 95.3 |
| Phorbol 12-myristate 13-acetate | Phosphorylation | 95.5 |

| | | |
|---|---|---|
| Tyrphostin AG 698 | Phosphorylation | 95.8 |
| Y-27632 dihydrochloride | Phosphorylation | 96.2 |
| Hispidin | Phosphorylation | 96.2 |
| LY-294,002 hydrochloride | Phosphorylation | 96.9 |
| 1,4-Dideoxy-1,4-imino-D-arabinitol | Phosphorylation | 97.2 |
| LFM-A13 | Phosphorylation | 97.2 |
| Me-3,4-dephostatin | Phosphorylation | 98.4 |
| Tetramisole hydrochloride | Phosphorylation | 98.8 |
| ML-9 | Phosphorylation | 98.9 |
| SP600125 | Phosphorylation | 99.7 |
| H-9 dihydrochloride | Phosphorylation | 101.1 |
| DL-Stearoylcarnitine chloride | Phosphorylation | 101.5 |
| SU 4312 | Phosphorylation | 102.0 |
| PD 98,059 | Phosphorylation | 102.1 |
| Tamoxifen citrate | Phosphorylation | 102.7 |
| Dephostatin | Phosphorylation | 102.9 |
| Tyrphostin AG 538 | Phosphorylation | 104.3 |
| Endothall | Phosphorylation | 106.6 |
| Tyrphostin 51 | Phosphorylation | 107.3 |
| Tyrphostin AG 537 | Phosphorylation | 107.6 |
| DL-erythro-Dihydrosphingosine | Phosphorylation | 107.9 |
| Tyrphostin AG 1478 | Phosphorylation | 107.9 |
| Olomoucine | Phosphorylation | 107.9 |
| Oleic Acid | Phosphorylation | 108.5 |
| I-OMe-Tyrphostin AG 538 | Phosphorylation | 108.7 |
| GW2974 | Phosphorylation | 109.1 |
| Furegrelate sodium | Phosphorylation | 109.2 |
| GW5074 | Phosphorylation | 109.3 |
| HA-100 | Phosphorylation | 109.8 |
| Tyrphostin 1 | Phosphorylation | 109.8 |
| S(-)-p-Bromotetramisole oxalate | Phosphorylation | 109.9 |
| Adenosine 3 ,5 -cyclic monophosphate | Phosphorylation | 110.0 |
| SU 5416 | Phosphorylation | 112.6 |
| Ceramide | Phosphorylation | 113.8 |
| (-)-Tetramisole hydrochloride | Phosphorylation | 114.1 |
| Tyrphostin AG 126 | Phosphorylation | 115.3 |
| Wortmannin from Penicillium funiculosum | Phosphorylation | 115.4 |
| Norcantharidin | Phosphorylation | 117.9 |
| Tyrphostin AG 112 | Phosphorylation | 119.6 |
| SB 216763 | Phosphorylation | 119.7 |
| Tyrphostin AG 527 | Phosphorylation | 120.2 |
| Roscovitine | Phosphorylation | 123.9 |
| Diacylglycerol Kinase Inhibitor II | Phosphorylation | 125.1 |
| 7-Cyclopentyl-5-(4-phenoxy)phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine | Phosphorylation | 126.5 |
| Meclofenamic acid sodium | Prostaglandin | 82.8 |
| loxoprofen | Prostaglandin | 83.7 |
| (-)-Naproxen sodium | Prostaglandin | 85.6 |
| Ketoprofen | Prostaglandin | 95.6 |

| | | |
|---|---|---|
| Niflumic acid | Prostaglandin | 96.5 |
| S(+)-Ibuprofen | Prostaglandin | 101.0 |
| Resveratrol | Prostaglandin | 101.2 |
| (±)-Ibuprofen | Prostaglandin | 103.6 |
| Indomethacin | Prostaglandin | 104.1 |
| Sulindac sulfone | Prostaglandin | 104.6 |
| Etodolac | Prostaglandin | 104.8 |
| Diclofenac sodium | Prostaglandin | 105.1 |
| Piroxicam | Prostaglandin | 106.1 |
| Ketorolac tris salt | Prostaglandin | 106.2 |
| Nimesulide | Prostaglandin | 114.3 |
| Meloxicam sodium | Prostaglandin | 114.4 |
| SC 19220 | Prostaglandin | 114.6 |
| 1-Methylimidazole | Prostaglandin | 114.7 |
| Sulindac | Prostaglandin | 117.9 |
| Acetylsalicylic acid | Prostaglandin | 122.6 |
| SC-560 | Prostaglandin | 124.9 |
| Oxaprozin | Prostaglandin | 125.4 |
| AL-8810 | Prostaglandin | 134.7 |
| Phenylbutazone | Prostaglandin | 137.9 |
| BW 245C | Prostanoids | 115.2 |
| LY-367,265 | Serotonin | 25.2 |
| Parthenolide | Serotonin | 41.8 |
| Zimelidine dihydrochloride | Serotonin | 46.3 |
| WAY-100635 maleate | Serotonin | 61.8 |
| Fluoxetine hydrochloride | Serotonin | 75.5 |
| LY-278,584 maleate | Serotonin | 77.5 |
| SB 224289 hydrochloride | Serotonin | 84.6 |
| GR 127935 hydrochloride | Serotonin | 85.0 |
| (±)-8-Hydroxy-DPAT hydrobromide | Serotonin | 85.8 |
| N-omega-Methyl-5-hydroxytryptamine oxalate salt | Serotonin | 86.9 |
| SB 228357 | Serotonin | 89.6 |
| alpha-Methyl-5-hydroxytryptamine maleate | Serotonin | 89.7 |
| Mianserin hydrochloride | Serotonin | 90.2 |
| p-MPPI hydrochloride | Serotonin | 90.7 |
| Citalopram hydrobromide | Serotonin | 91.5 |
| S(-)-UH-301 hydrochloride | Serotonin | 91.8 |
| Ritanserin | Serotonin | 92.3 |
| SB-215505 | Serotonin | 92.7 |
| VER-3323 hemifumarate salt | Serotonin | 92.8 |
| p-MPPF dihydrochloride | Serotonin | 93.2 |
| ( R)-(-)-DOI hydrochloride | Serotonin | 93.6 |
| Dihydroergotamine methanesulfonate | Serotonin | 93.8 |
| 5-Hydroxyindolacetic acid | Serotonin | 93.8 |
| LY-310,762 hydrochloride | Serotonin | 94.0 |
| Clomipramine hydrochloride | Serotonin | 94.9 |
| 5-Methoxy DMT oxalate | Serotonin | 95.5 |
| S-(+)-Fluoxetine hydrochloride | Serotonin | 96.8 |
| Methysergide maleate | Serotonin | 97.2 |

| | | |
|---|---|---|
| LY-53,857 maleate | Serotonin | 97.4 |
| BRL 15572 | Serotonin | 98.5 |
| O-Methylserotonin hydrochloride | Serotonin | 98.6 |
| SB 204741 | Serotonin | 98.9 |
| 5-Hydroxy-L-tryptophan | Serotonin | 99.4 |
| R-(+)-8-Hydroxy-DPAT hydrobromide | Serotonin | 99.7 |
| N,N-Dipropyl-5-carboxamidotryptamine maleate | Serotonin | 100.2 |
| Serotonin hydrochloride | Serotonin | 100.3 |
| Trazodone hydrochloride | Serotonin | 101.0 |
| Imipramine hydrochloride | Serotonin | 101.5 |
| Quipazine dimaleate | Serotonin | 102.2 |
| Metergoline | Serotonin | 103.8 |
| Methiothepin mesylate | Serotonin | 104.2 |
| 3-Tropanyl-3,5-dichlorobenzoate | Serotonin | 104.3 |
| S(-)-Pindolol | Serotonin | 104.6 |
| BW 723C86 | Serotonin | 104.9 |
| NAN-190 hydrobromide | Serotonin | 105.0 |
| 1-Phenylbiguanide | Serotonin | 106.4 |
| Amperozide hydrochloride | Serotonin | 106.5 |
| (±)-DOI hydrochloride | Serotonin | 107.2 |
| GR 4661 | Serotonin | 107.6 |
| Buspirone hydrochloride | Serotonin | 108.0 |
| SR 57227A | Serotonin | 108.3 |
| 1-(m-Chlorophenyl)-biguanide hydrochloride | Serotonin | 108.4 |
| GR 125487 sulfamate salt | Serotonin | 109.2 |
| R(+)-UH-301 hydrochloride | Serotonin | 109.8 |
| 1-(3-Chlorophenyl)piperazine dihydrochloride | Serotonin | 109.9 |
| Quipazine, N-methyl-, dimaleate | Serotonin | 110.2 |
| Cyproheptadine hydrochloride | Serotonin | 110.4 |
| 1-(1-Naphthyl)piperazine hydrochloride | Serotonin | 110.5 |
| SB 206553 hydrochloride | Serotonin | 111.3 |
| SB 203186 | Serotonin | 111.6 |
| Reserpine | Serotonin | 111.6 |
| 3-Tropanylindole-3-carboxylate methiodide | Serotonin | 111.9 |
| Quipazine, 6-nitro-, maleate | Serotonin | 112.9 |
| Spiroxatrine | Serotonin | 113.3 |
| Cyclobenzaprine hydrochloride | Serotonin | 113.3 |
| CGS-12066A maleate | Serotonin | 113.7 |
| Tryptamine hydrochloride | Serotonin | 114.6 |
| SB 204070 hydrochloride | Serotonin | 114.8 |
| Pirenperone | Serotonin | 115.2 |
| Trimipramine maleate | Serotonin | 115.3 |
| PAPP | Serotonin | 116.2 |
| 3-Tropanyl-indole-3-carboxylate hydrochloride | Serotonin | 116.3 |
| BRL 54443 maleate | Serotonin | 118.6 |
| SB 269970 hydrochloride | Serotonin | 119.4 |
| Fluvoxamine maleate | Serotonin | 120.0 |
| Ro 04-6790 dihydrochloride | Serotonin | 121.0 |
| R-(-)-Fluoxetine hydrochloride | Serotonin | 121.7 |

| | | |
|---|---|---|
| L-Tryptophan | Serotonin | 123.8 |
| 2-Methyl-5-hydroxytryptamine maleate | Serotonin | 124.9 |
| 5-Carboxamidotryptamine maleate | Serotonin | 128.7 |
| Alaproclate hydrochloride | Serotonin | 130.2 |
| SB 200646 hydrochloride | Serotonin | 130.5 |
| 1-(2-Methoxyphenyl)piperazine hydrochloride | Serotonin | 131.8 |
| GR 113808 | Serotonin | 132.7 |
| Ketanserin tartrate | Serotonin | 134.5 |
| BMY 7378 dihydrochloride | Serotonin | 135.2 |
| SDZ-205,557 hydrochloride | Serotonin | 139.2 |
| Cysteamine hydrochloride | Somatostatin | 95.6 |
| Seglitide | Somatostatin | 103.2 |
| (±)-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride | Sphingolipid | 77.3 |
| N-Oleoylethanolamine | Sphingolipid | 99.6 |
| L-Cycloserine | Sphingolipid | 101.7 |
| DL-Cycloserine | Sphingolipid | 115.9 |
| L-703,606 oxalate | Tachykinin | 81.3 |
| WIN 62,577 | Tachykinin | 88.2 |
| L-733,060 hydrochloride | Tachykinin | 100.7 |
| SB 222200 | Tachykinin | 105.9 |
| L-732,138 | Tachykinin | 123.4 |
| L-655,240 | Thromboxane | 101.8 |
| Picotamide | Thromboxane | 149.2 |
| 13-cis-retinoic acid | Transcription | 62.4 |
| 6-Nitroso-1,2-benzopyrone | Transcription | 66.6 |
| GW9662 | Transcription | 77.0 |
| TTNPB | Transcription | 92.2 |
| Ciprofibrate | Transcription | 96.2 |
| 6(5H)-Phenanthridinone | Transcription | 103.0 |
| TCPOBOP | Transcription | 104.1 |
| Fenofibrate | Transcription | 109.2 |
| D-ribofuranosylbenzimidazole | Transcription | 113.8 |
| Tetradecylthioacetic acid | Transcription | 116.5 |
| GW1929 | Transcription | 118.7 |
| GW7647 | Transcription | 143.2 |
| Dihydrocapsaicin | Vanilloid | 71.5 |
| Capsazepine | Vanilloid | 97.7 |
| N-Vanillylnonanamide | Vanilloid | 102.5 |
| SB-366791 | Vanilloid | 103.9 |
| Vanillic acid diethylamide | Vanilloid | 106.0 |
| **NCI Approved Oncology Drug Set II Screening Results** | | |
| Pentostatin | Adenosine | 140.0 |
| Etoposide | Apoptosis | 19.7 |
| Arsenic trioxide | Apoptosis | 122.8 |
| Zolendronic acid | Cell Biology | 101.3 |
| Mitotane | Cell Biology/Adrenal gland function | 130.4 |
| Capecitabine | Cell Cycle | 105.2 |

| | | |
|---|---|---|
| Fluorouracil | Cell Cycle | 107.3 |
| Amifostine | Cell Stress | 102.8 |
| Allopurinol | Cell Stress | 115.9 |
| Dexrazoxone | Cell Stress | 131.8 |
| Vinblastine sulfate | Cytoskeleton and ECM | 36.8 |
| Vincristine sulfate | Cytoskeleton and ECM | 38.6 |
| Ixabepilone | Cytoskeleton and ECM | 43.3 |
| Paclitaxel | Cytoskeleton and ECM | 47.1 |
| Vinorelbine tartrate | Cytoskeleton and ECM | 54.7 |
| Docetaxel | Cytoskeleton and ECM | 62.6 |
| Estramustine disodium phosphate | Cytoskeleton and ECM | 111.7 |
| Thalidomide | Cytoskeleton and ECM | 112.4 |
| Daunorubicin HCl | DNA | 1.6 |
| Valrubicin | DNA | 2.3 |
| Topotecan HCl | DNA | 3.8 |
| Plicamycin | DNA | 12.3 |
| Dactinomycin | DNA | 41.3 |
| Cladribine | DNA | 49.6 |
| Gemcitabine HCl | DNA | 64.4 |
| Nitrogen mustard | DNA | 70.8 |
| Mitomycin C | DNA | 78.3 |
| Fludarabine | DNA | 92.1 |
| Decitabine | DNA | 98.4 |
| Nelarabine | DNA | 99.0 |
| Oxaliplatin | DNA | 101.8 |
| Cytarabine hydrochloride | DNA | 104.0 |
| Irinotecan HCl | DNA | 104.7 |
| Azacitidine | DNA | 105.0 |
| Thiotepa | DNA | 107.3 |
| Pipobroman | DNA | 108.0 |
| Lomustine, CCNU | DNA | 109.7 |
| Mercaptopurine | DNA | 110.1 |
| Thioguanine | DNA | 110.7 |
| Floxuridine | DNA | 111.1 |
| Busulfan | DNA | 111.4 |
| Streptozocin | DNA | 113.1 |
| Procarbazine hydrochloride | DNA | 114.0 |
| Methoxsalen | DNA | 115.1 |
| Temozolomide | DNA | 117.1 |
| Carboplatin | DNA | 117.5 |
| Cyclophosphamide | DNA | 120.2 |
| Pemetrexed | DNA | 120.7 |
| Uracil mustard | DNA | 123.6 |
| Acrichine | DNA | 123.7 |
| Carmustine | DNA | 125.6 |
| Dacarbazine | DNA | 126.3 |
| Triethylenemelamine | DNA | 126.9 |
| Chlorambucil | DNA | 127.0 |
| Cisplatin | DNA | 127.8 |

| | | |
|---|---|---|
| Ifosfamide | DNA | 129.2 |
| Doxorubicin HCl | DNA | 2.2 |
| Teniposide | DNA Repair | 7.4 |
| Mitoxantrone | DNA Metabolism | 1.3 |
| Melphalan | DNA Metabolism | 94.3 |
| Hydroxyurea | DNA Metabolism | 105.7 |
| Methotrexate | DNA Metabolism | 106.3 |
| Altretamine | DNA Metabolism | 110.0 |
| Clofarabine | DNA Repair | 33.1 |
| Bleomycin | DNA Repair | 104.3 |
| Vorinostat | Gene Regulation and Epigenetics | 38.4 |
| Exemestane | Hormone | 96.1 |
| Fulvestrant | Hormone | 99.0 |
| Letrozole | Hormone | 100.5 |
| Raloxifene HCl | Hormone | 103.5 |
| Megestrol acetate | Hormone | 104.2 |
| Anastrozole | Hormone | 105.0 |
| Imiquimod | Immune System | 98.7 |
| Lenalidomide | Immune system | 100.1 |
| Aminolevulinic acid | Light sensitivity | 114.7 |
| Everolimus | mTOR | 80.1 |
| Rapamycin | mTOR | 115.8 |
| Lapatinib | Phosphorylation | 96.7 |
| Nilotinib | Phosphorylation | 98.0 |
| Tamoxifen citrate | Phosphorylation | 102.5 |
| Dasatinib | Phosphorylation | 104.3 |
| Sunitinib | Phosphorylation | 105.7 |
| Erlotinib HCl | Phosphorylation | 106.3 |
| Gefitinib | Phosphorylation | 109.7 |
| Imatinib | Phosphorylation | 110.4 |
| Sorafenib | Phosphorylation | 119.1 |
| Celecoxib | Prostaglandin | 113.8 |
| Bortezomib | Proteasome | 8.4 |
| Tretinoin | Transcription | 95.7 |

Reference : The NCI/DTP Open Chemical Repository, http://dtp.cancer.gov

| ATPase Inhibitors | | |
|---|---|---|
| Bithionol | Antihelmintic | 120.7 |
| Hexachlorophene | Antiseptic | 81.3 |
| MAL2-11B | ATPase | 92 |
| MAL3-101 | ATPase | 87 |
| DMT3084 | ATPase | 99 |
| MAL2-51 | ATPase | 93 |

References :

- Wright CM, Seguin SP, Fewell SW, Zhang H, Ishwad C, Vats A, Lingwood CA, Wipf P, Fanning E, Pipas JM, Brodsky JL.Inhibition of Simian Virus 40 replication by targeting the molecular chaperone function and ATPase activity of T antigen.Virus Res. 2009 Apr;141(1):71-80.
- S. P. Seguin *et al*., High-Throughput Screening Identifies a Bisphenol Inhibitor of SV40 Large T Antigen ATPase Activity. Journal of biomolecular screening, (Sep 23, 2011).

| p53-MDM2 Inhibitors | | |
|---|---|---|
| Nutlin-3 | p53 | 12.6 |
| YH264A | p53 | 92.7 |
| YH265A | p53 | 99.1 |
| KK_NW_16A | p53 | 99.6 |

References :

- Czarna, A., *et al*. Robust generation of lead compounds for protein-protein interactions by computational and MCR chemistry: p53/Hdm2 antagonists. Angewandte Chemie 49, 5352-5356 (2010).
- Popowicz, G.M., *et al*. Structures of low molecular weight inhibitors bound to MDMX and MDM2 reveal new approaches for p53-MDMX/MDM2 antagonist drug discovery. Cell cycle 9, 1104-1111 (2010).

**Bayesian Hierarchical Changepoint Analysis of the Effect of YM155 on Bortezomib on Tumor Xenograft Volume**

(Report by Dr Normolle on August 28, 2011, published in Arora *et al*, STM, 2012)

# 1 Model Setup

The hierarchical Bayes piecewise linear model [1] previously presented, with a change of parameterization to enhance sharing of information, was employed, where the groups are indexed as follows:

| $i$ | Cell Line | Treatment | # Animals |
|---|---|---|---|
| 1 | MKL-1 | Bortezomib | 21 |
| 2 | MKL-1 | Saline | 31 |
| 3 | MKL-1 | YM155 | 23 |
| 4 | MS-1 | Saline | 5 |
| 5 | MS-1 | YM155 | 6 |
| 6 | UISO | Saline | 5 |
| 7 | UISO | YM155 | 5 |

*Table 1* Experimental group label definitions and sample sizes.

This is a hierarchical Bayesian model, where animal $ij$ has four model parameters: the nadir log-volume ($a_{ij}$), growth delay time ($e^{r_{ij}}$), pre-nadir shrinkage rate ($e^{b_{1ij}}$) and post-nadir growth rate ($e^{b_{2ij}}$). The values of $r_{ij}$, $b_{1ij}$ and $b_{2ij}$ are exponentiated to ensure they are positive. The animal-level parameters are distributed around a corresponding treatment group-level parameter, as described in Block 2, below. The treatment group-level parameters are centered around a mean meta-parameter (Block 4, below), which allows for sharing of information between treatment groups. This is of particular value in this model, where, in some treatment groups (e.g., Saline), there is no tumor shrinkage, so the growth delay is pushed towards zero, and there is then no information to estimate the rate of tumor shrinkage (because there is no shrinkage), but the sharing of information enhances numerical stability by defaulting the vanishingly short tumor shrinkage period to have a tumor shrinkage rate with a sensible, if irrelevant value.

The model accommodates volumes below the limit of quantitation, which are generally recorded as zero. We take volumes coded as zero to be values below the limit of quantization (LQ), where the limit of quantitation is defined to be the smallest non-zero value; for the present data set, that volume equals $4\text{mm}^3$ ($\log_2(4) = 2$). BLQ volumes are utilized in the estimation process, considered left-censored at LQ.

The complete model parameterization is presented in the following blocks, where $\mathcal{N}(\mu, \tau)$ is a normal distribution with mean $\mu$ and precision $\tau$, and $\mathcal{G}(\sigma, \eta)$ is a gamma distribution with scale $\sigma$ and shape $\eta$ (mean $\sigma/\eta$ and variance $\sigma/\eta^2$):

**1. Piecewise linear model for log-volume**

$$\begin{aligned}
\mu_{ij}(t) &= a_{ijt} + e^{b_{1ij}}\max(e^{r_{ij}} - t, 0) + e^{b_{2ij}}\max(t - e^{r_{ij}}, 0) \\
\omega_{ij}(t) &= e^{\theta\mu_{ij}(t)} \\
\log_2(v_{ij})(t) &\sim \mathcal{N}(\mu_{ij}(t), \omega_{ij}(t))
\end{aligned}$$

**2. Animal-Level regression parameters**

$$\begin{aligned}
a_{ij} &\sim \mathcal{N}(\alpha_i, \tau_a) \\
b_{1ij} &\sim \mathcal{N}(\beta_{1i}, \tau_{b_1}) \\
b_{2ij} &\sim \mathcal{N}(\beta_{2i}, \tau_{b_2}) \\
r_{ij} &\sim \mathcal{N}(\rho_i, \tau_a)
\end{aligned}$$

**3. Experimental group regression parameters**

$$\begin{aligned}
\alpha_i &\sim \mathcal{N}(\alpha, \tau_\alpha) \\
\beta_{1i} &\sim \mathcal{N}(\beta_1, \tau_{\beta_1}) \\
\beta_{2i} &\sim \mathcal{N}(\beta_2, \tau_{\beta_2}) \\
\rho_i &\sim \mathcal{N}(\rho, \tau_\rho)
\end{aligned}$$

**4. Mean meta-parameters**

$$\begin{aligned}
\alpha &\sim \mathcal{N}(0, 0.01) \\
\beta_1 &\sim \mathcal{N}(0, 0.01) \\
\beta_2 &\sim \mathcal{N}(0, 0.01) \\
\rho &\sim \mathcal{N}(0, 0.01) \\
\theta &\sim \mathcal{N}(0, 0.01)
\end{aligned}$$

**5. Dispersion meta-parameters**

$$\begin{aligned}
\tau_a &\sim \mathcal{G}(0.1, 0.1) & \tau_\alpha &\sim \mathcal{G}(0.1, 0.1) \\
\tau_{b_1} &\sim \mathcal{G}(0.1, 0.1) & \tau_{\beta_1} &\sim \mathcal{G}(0.1, 0.1) \\
\tau_{b_2} &\sim \mathcal{G}(0.1, 0.1) & \tau_{\beta_2} &\sim \mathcal{G}(0.1, 0.1) \\
\tau_r &\sim \mathcal{G}(0.1, 0.1) & \tau_\rho &\sim \mathcal{G}(0.1, 0.1)
\end{aligned}$$

The most interpretable parameters are in Block 3, Experimental group regression parameters. Differences in these parameters will represent differences between, for instance, growth delay rates between treatments.

# 2 Parameter Estimates

Parameters as described in the Model Setup section were estimated using JAGS (Just Another Gibbs Sampler) with 20,000 MCMC iterations. Traces of the estimated parameters were checked visually for convergence. The relationship of all the estimated parameters to the data can be checked visually in the section Animal-Level Model; the numerical estimates of the treatment-level parameters are presented here with 95% credible intervals.

| | $\alpha_i$ | $e^{\beta_{1i}}$ | $e^{\beta_{2i}}$ | $e^{\rho_i}$ |
|---|---|---|---|---|
| MKL-1 Bortezomib | 5.5(5,6) | 0.00069(6.1e-12,1.4) | 0.32(0.28,0.35) | 1.3e-05(7.7e-21,0.053) |
| MKL-1 Saline | 5.3(4.9,5.7) | 0.00037(5.5e-10,0.1) | 0.4(0.37,0.44) | 1.1e-05(1.3e-10,0.041) |
| MKL-1 YM155 | 4.1(3.6,4.6) | 0.0014(3.5e-08,0.025) | 0.37(0.33,0.41) | 5.5(2.6,9.5) |
| MS-1 Saline | 4.4(3.4,5.3) | 0.00053(2.2e-10,0.61) | 0.21(0.17,0.26) | 2.2e-05(3.3e-19,0.19) |
| MS-1 YM155 | 4.4(3.5,5.2) | 0.00049(2.2e-11,0.35) | 0.19(0.16,0.23) | 5.6e-06(5.6e-20,0.17) |
| UISO Saline | 2.4(1.5,3.5) | 0.00055(3.3e-10,0.26) | 0.19(0.16,0.23) | 1.7e-05(5.2e-16,1.5) |
| UISO YM155 | 2.6(1.5,3.6) | 0.00025(1.6e-11,0.017) | 0.23(0.19,0.28) | 11(3.1,36) |

*Table 2* Estimated values and 95% credible intervals for nadir volume ($\alpha_i$), shrinkage rate ($e^{\beta_{1i}}$), growth rate ($e^{\beta_{2i}}$) and time to nadir ($e^{\rho_i}$).

The most notable feature of the estimates is the increased delay in growth in animals with MKL-1 and UISO tumors treated with YM155 (5.5 and 11 days, respectively). Animals with MS-1 tumors so treated experienced no such apparent delay in growth. Tumors with delay in growth did not shrink prior to the resumption of growth ($e^{\beta_{1i}} \approx 0$). The post-nadir growth rates of all the animals with MKL-1 tumors were similar, and faster than MS-1 and UISO tumors. The estimated nadir ($\alpha_i$) appears smaller for the UISO tumors, but the nadir value may be confounded with the baseline tumor volumes, which are significantly smaller in the UISO tumors than the others, as displayed in Figure 1.

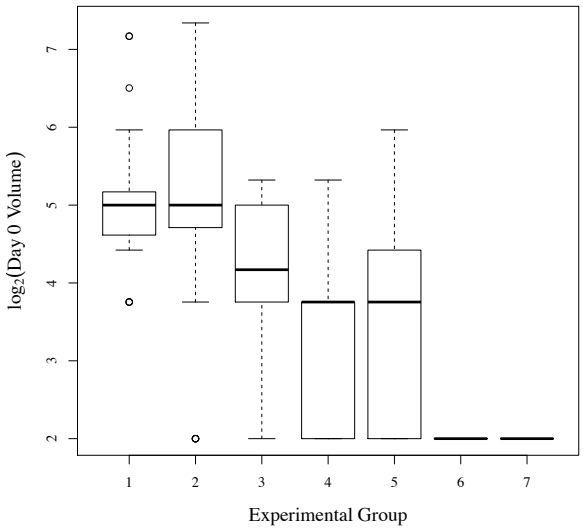



*Figure 1* Day 0 volumes (base 2 log-transformed). The experimental groups are defined in Table 1; groups 6 and 7 are UISO Saline and YM155, respectively.

# 3 Plots by Treatment and Cell Line

The panels of curves in Figures 2 and 3 represent the summary of the population model for the seven experimental groups. The red lines indicate the central estimates, while the shaded areas 95% credible regions (Bayesian confidence regions) for the growth curves. The credible regions are wider for the experimental groups with fewer animals. It is seen that there is evidence of growth delay for the YM155-treated animals with MKL-1 and UISO tumors, but not for the other experimental groups. It is also seen that the YM155 does not uniformly suppress tumor growth; it appears to have no effect on some tumors, while delaying growth a great deal more than average in others. Figure 2 displays the population estimates against the observed tumor volume profiles, demonstrating the correspondence between the population estimates and the raw data, while Figure 3 displays the population- and animal-level models together, to demonstrate the correspondence between the animal-level and population-level models.

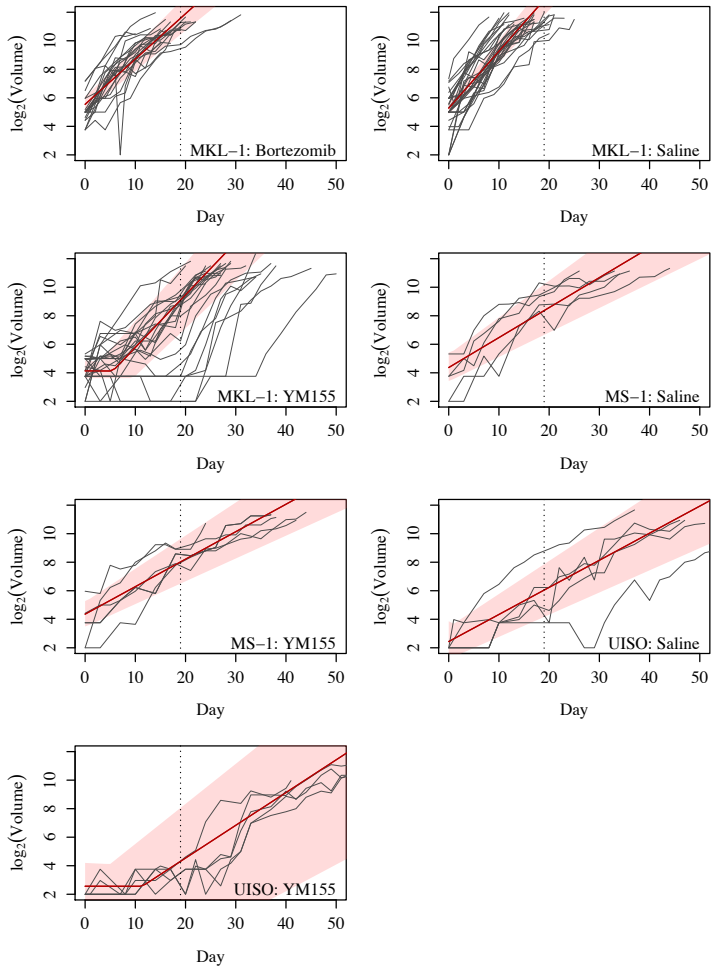



*Figure 2* Population tumor volume growth curves for the seven experimental groups. The bold red lines are the central estimates, while the shaded regions are 95% credible intervals. Individual animal growth curves are represented by grey lines. The limit of quantitation is 2 on this scale; 0 volumes are represented as 2.

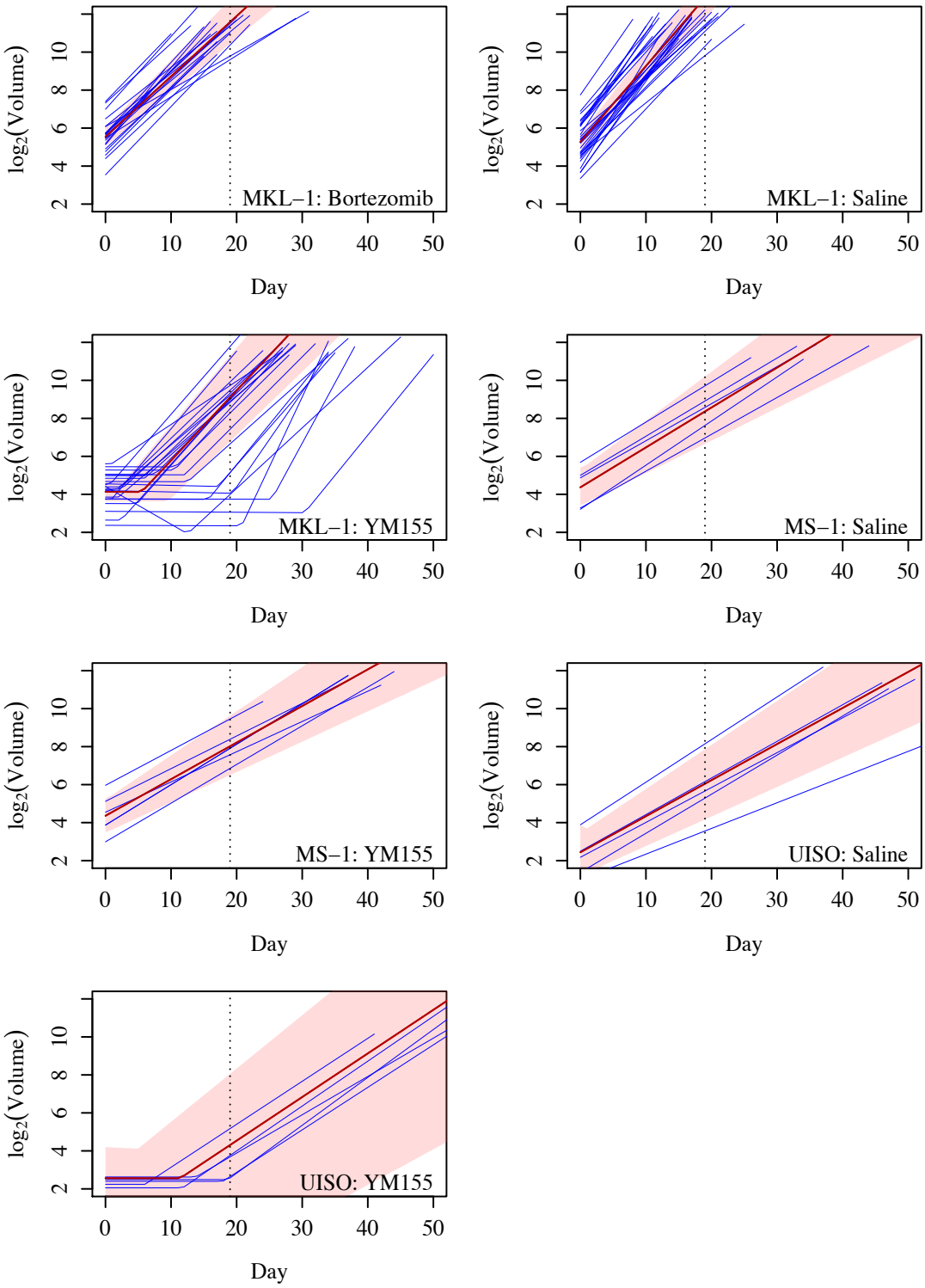



*Figure 3* Population tumor volume growth curves for the seven experimental groups. The bold red lines are the central estimates, while the shaded regions are 95% credible intervals. Modeled individual animal growth curves are represented by blue lines.

# 4 Animal-Level Model Fit

The Figures 4.1-4.12 confirm the fit of the model to the data. Circles are the observed data, blue lines are the animal-level models (based on $a_{ij}, b_{1ij}, b_{2ij}$ and $r_{ij}$, for the $i^{\text{th}}$ animal in the $j^{\text{th}}$ group), and the red lines are the experimental group-level model (based on $\alpha_j, \beta_{1j}, \beta_{2j}$ and $\rho_j$, for the $i^{\text{th}}$ animal in the $j^{\text{th}}$ group)). The animals are sorted according to the experimental group in Table 1.

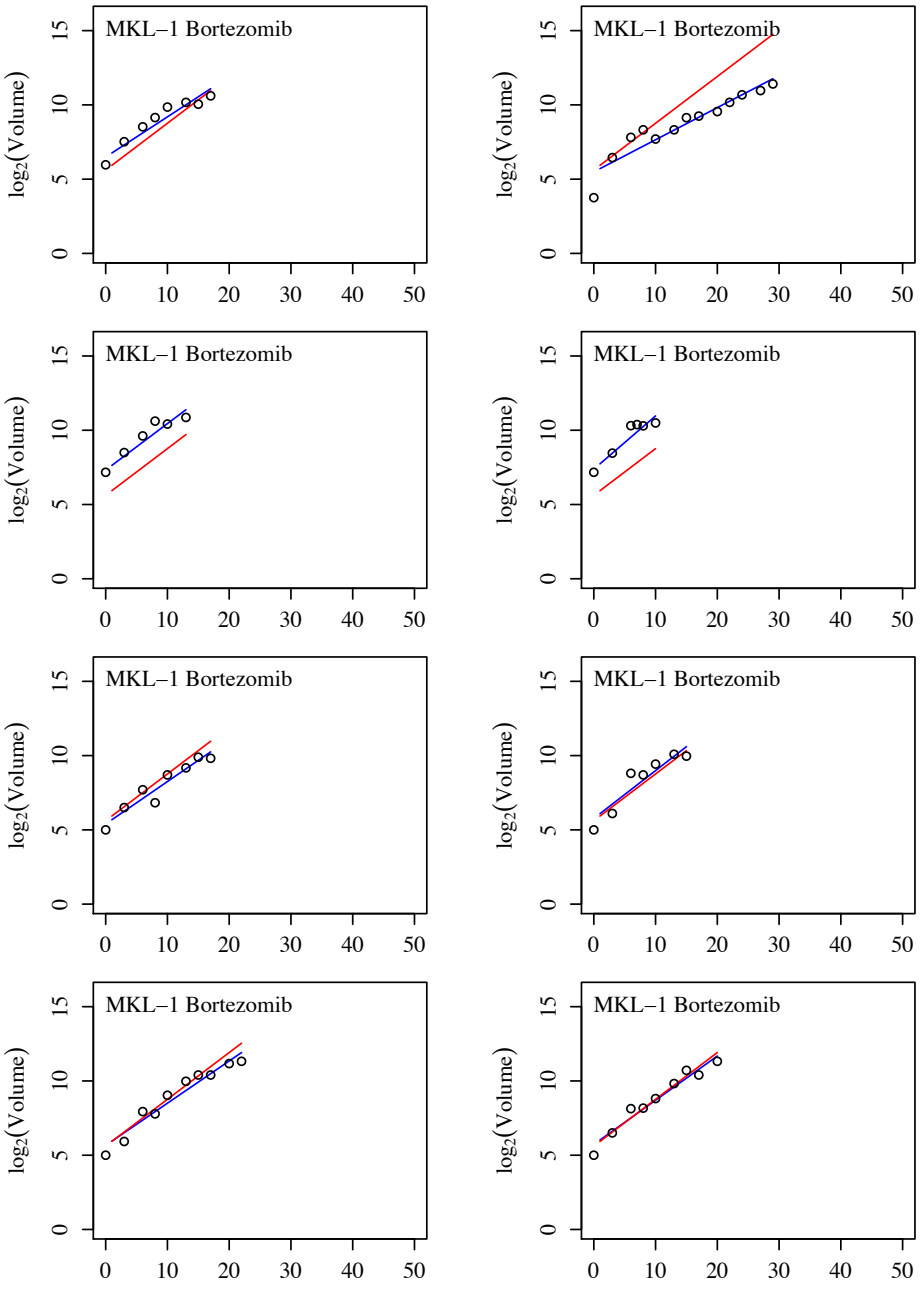



*Figure 4.1* Animal-level plots. Circles are data, blue lines animal-level model, red lines experimental group-level model.

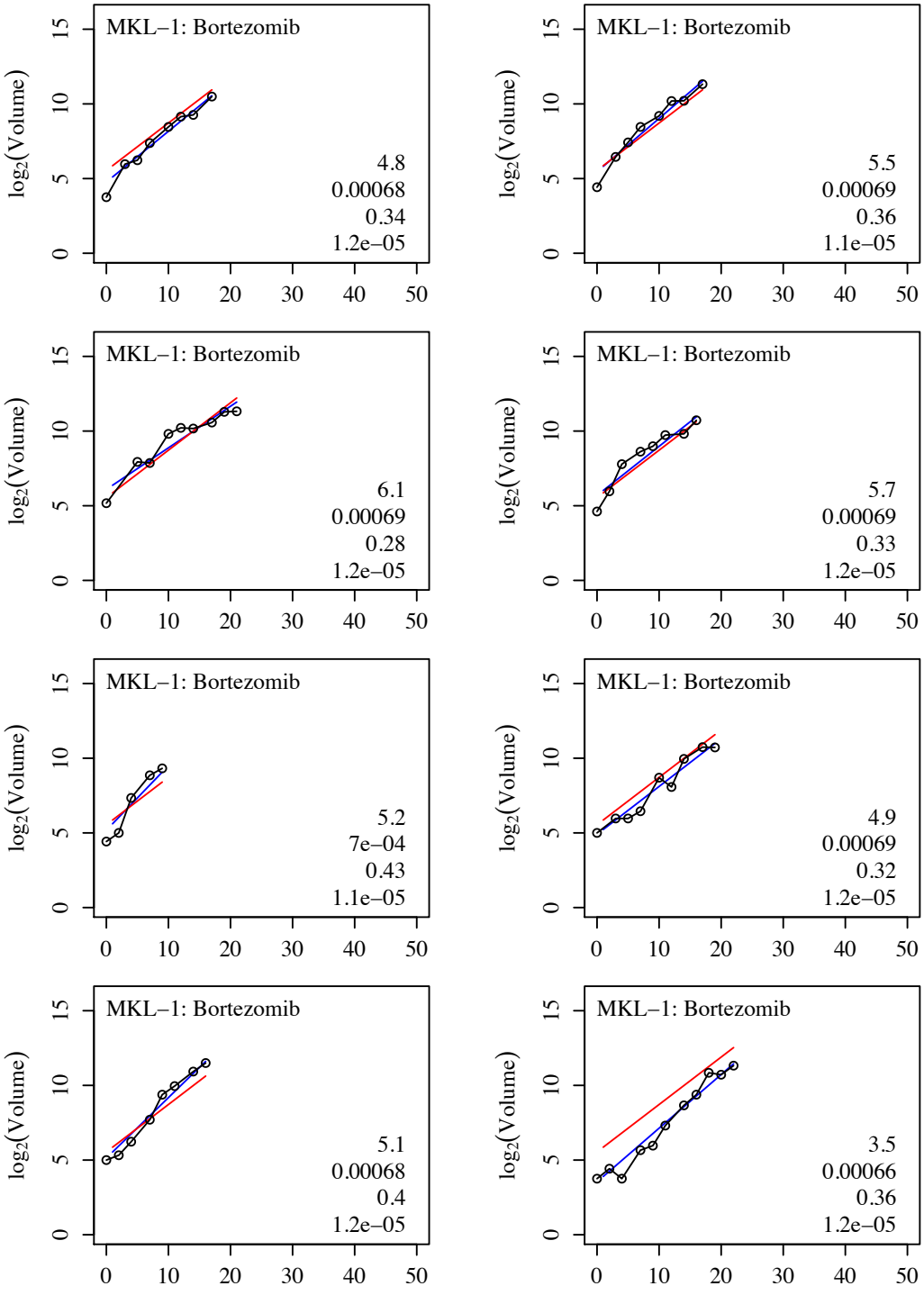



*Figure 4.2* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

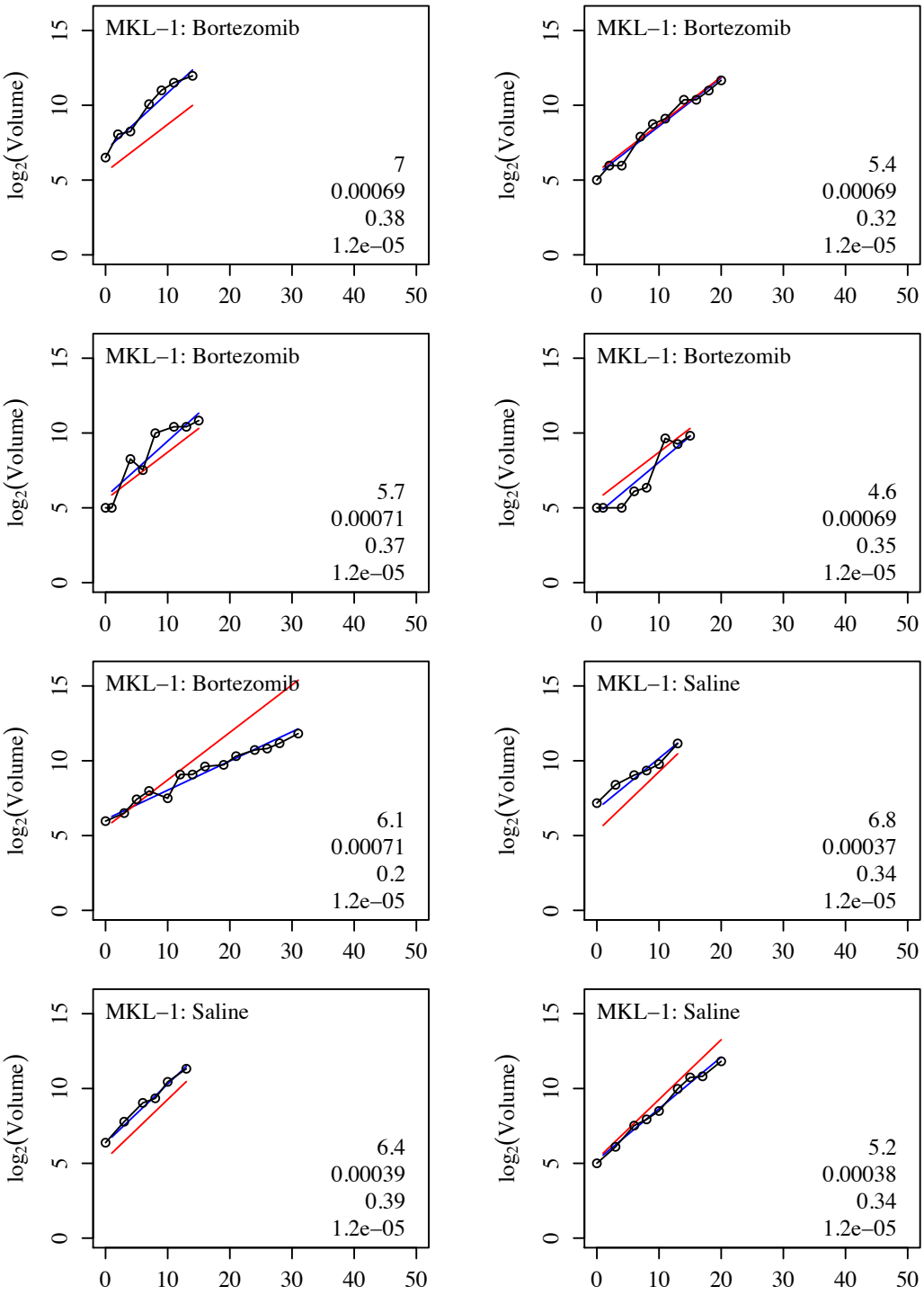



*Figure 4.3* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

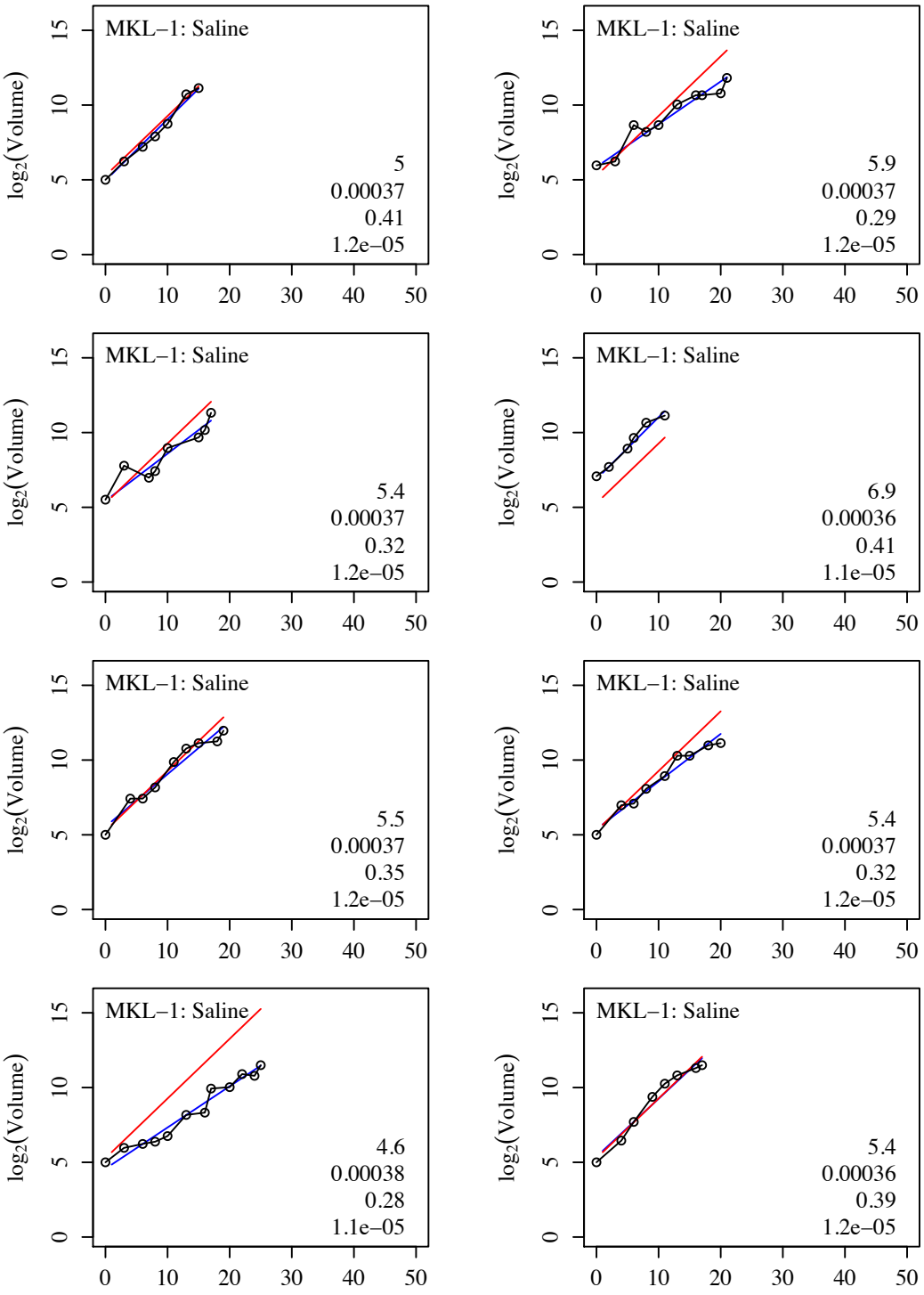



*Figure 4.4* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

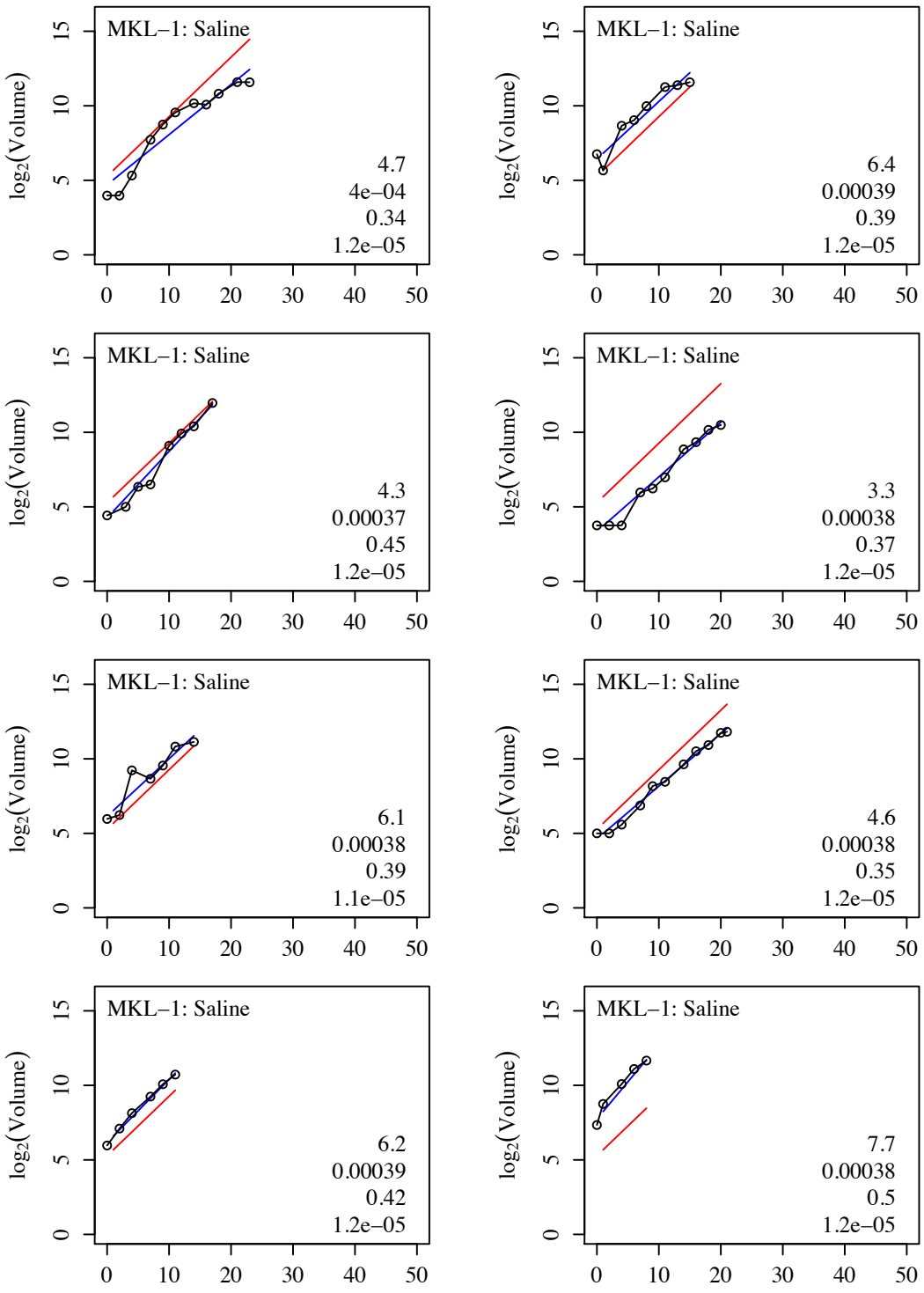



*Figure 4.5* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

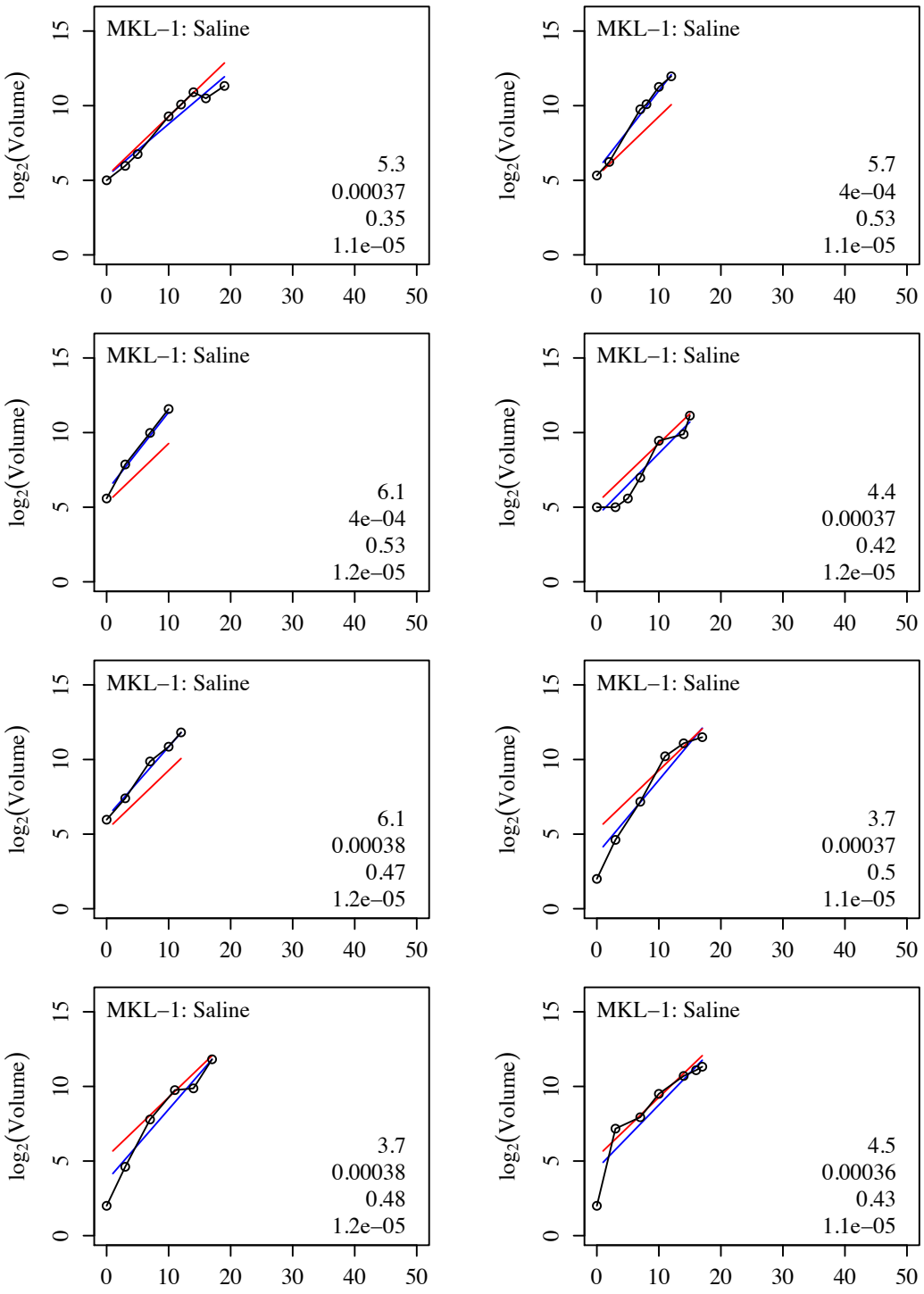



*Figure 4.6* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

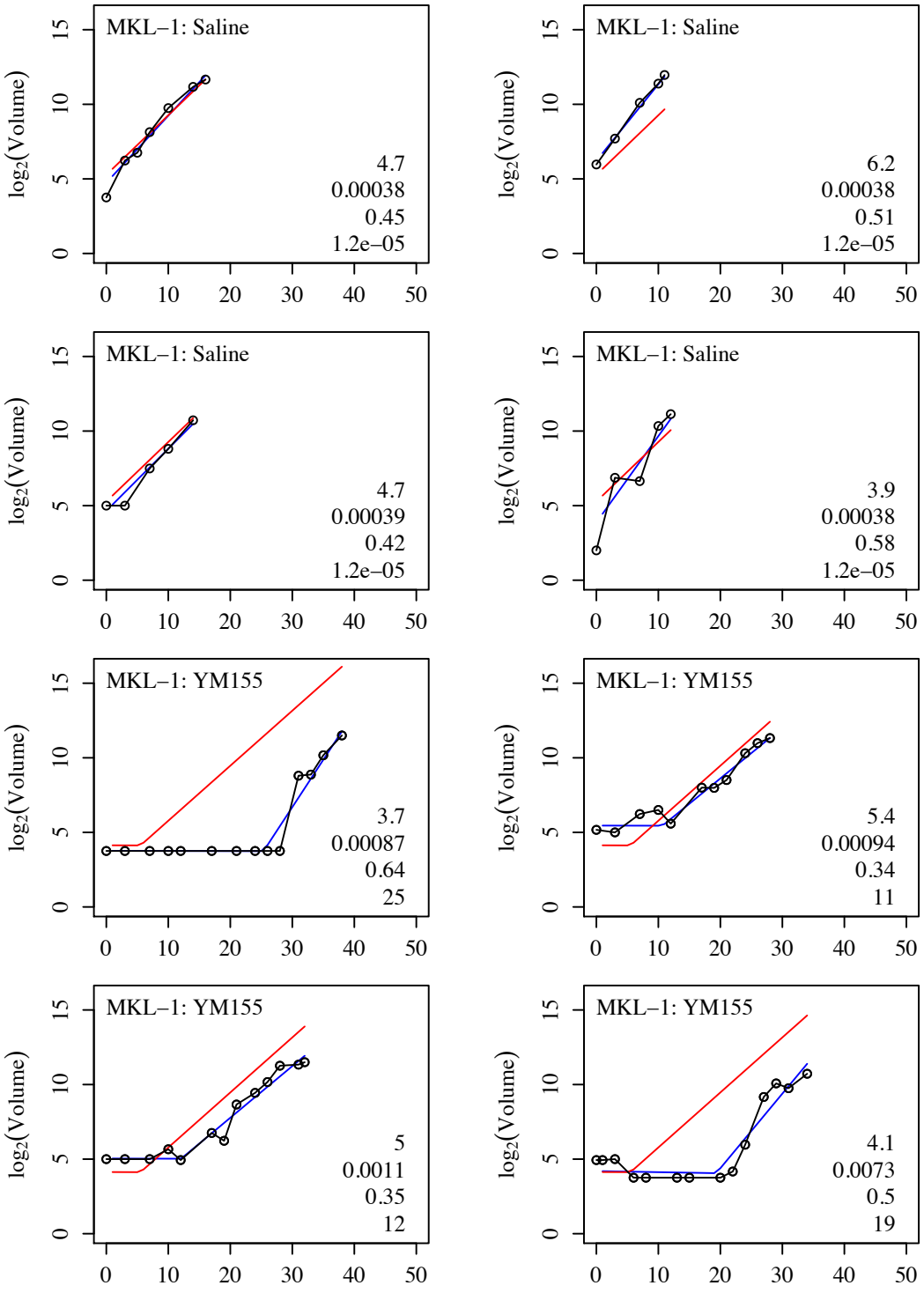



*Figure 4.7* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

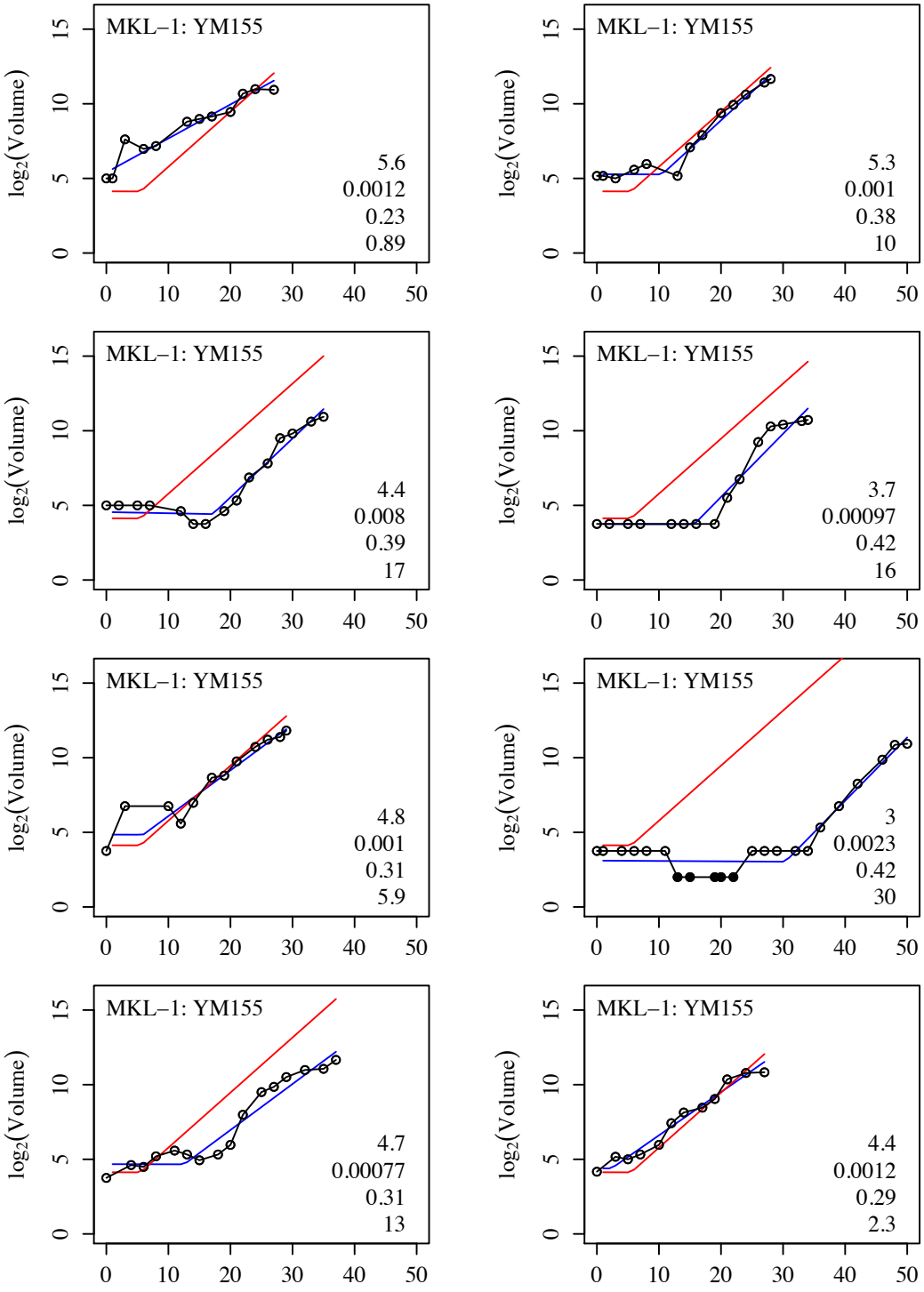



*Figure 4.8* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

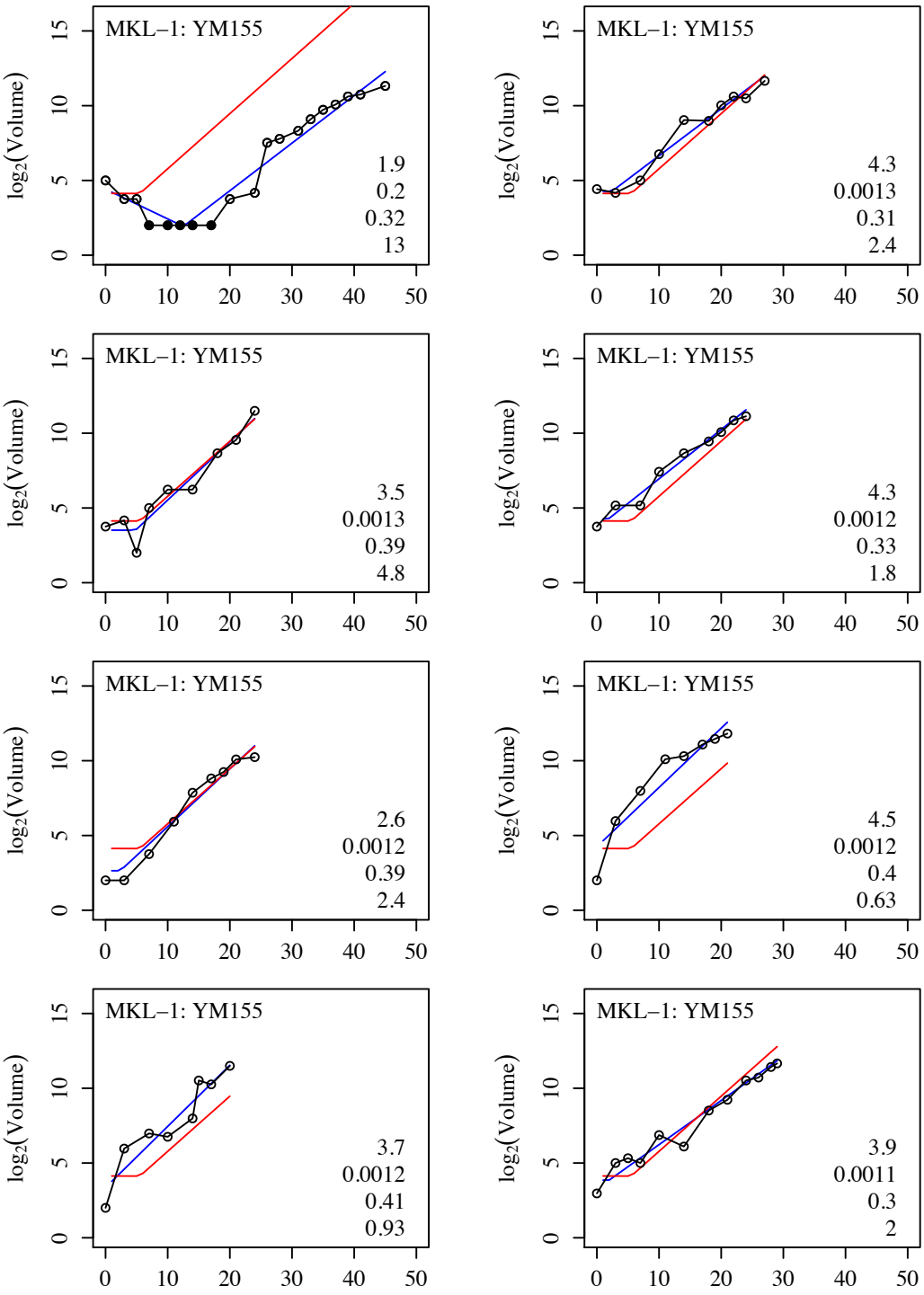



*Figure 4.9* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

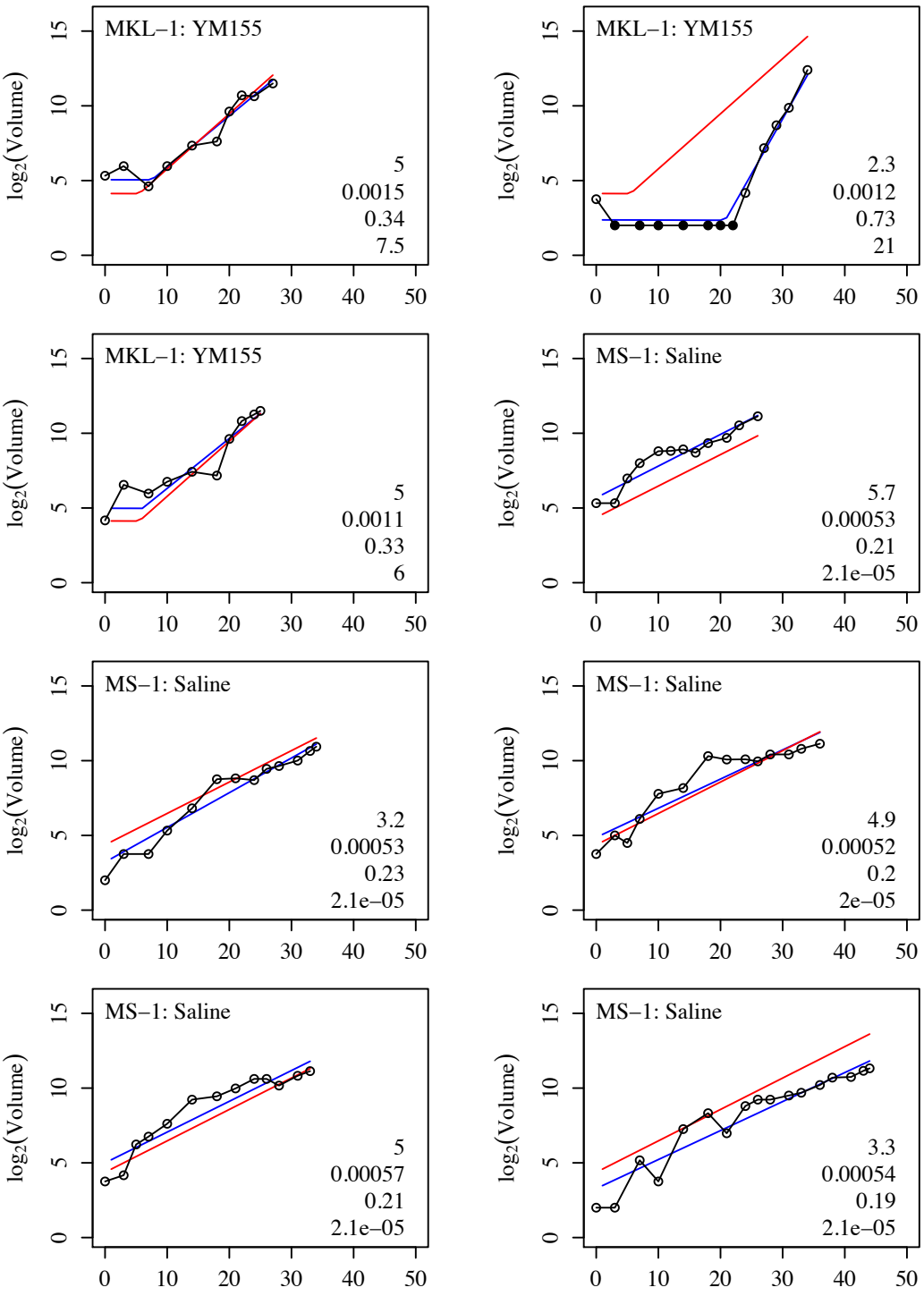



*Figure 4.10* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

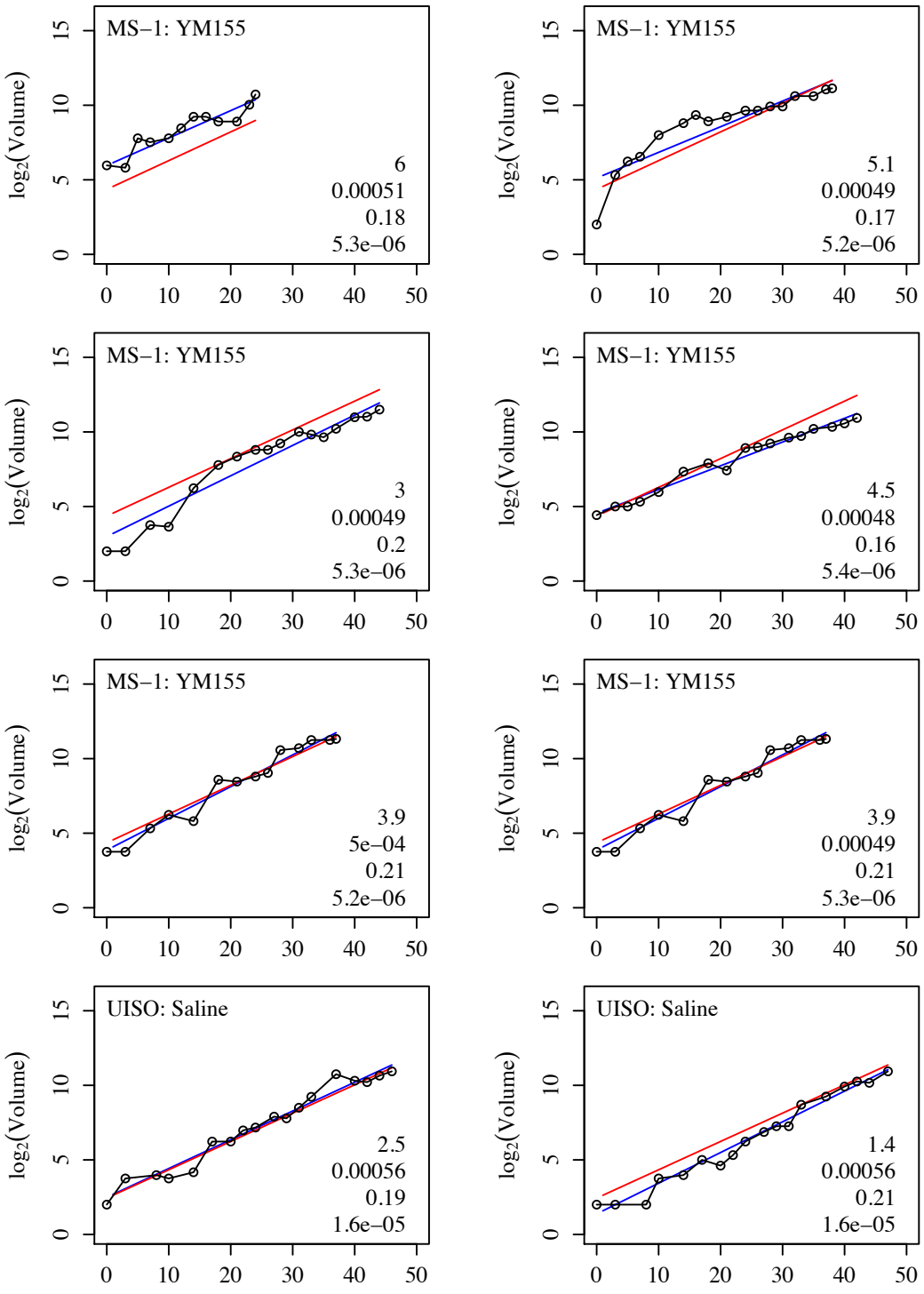



*Figure 4.11* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

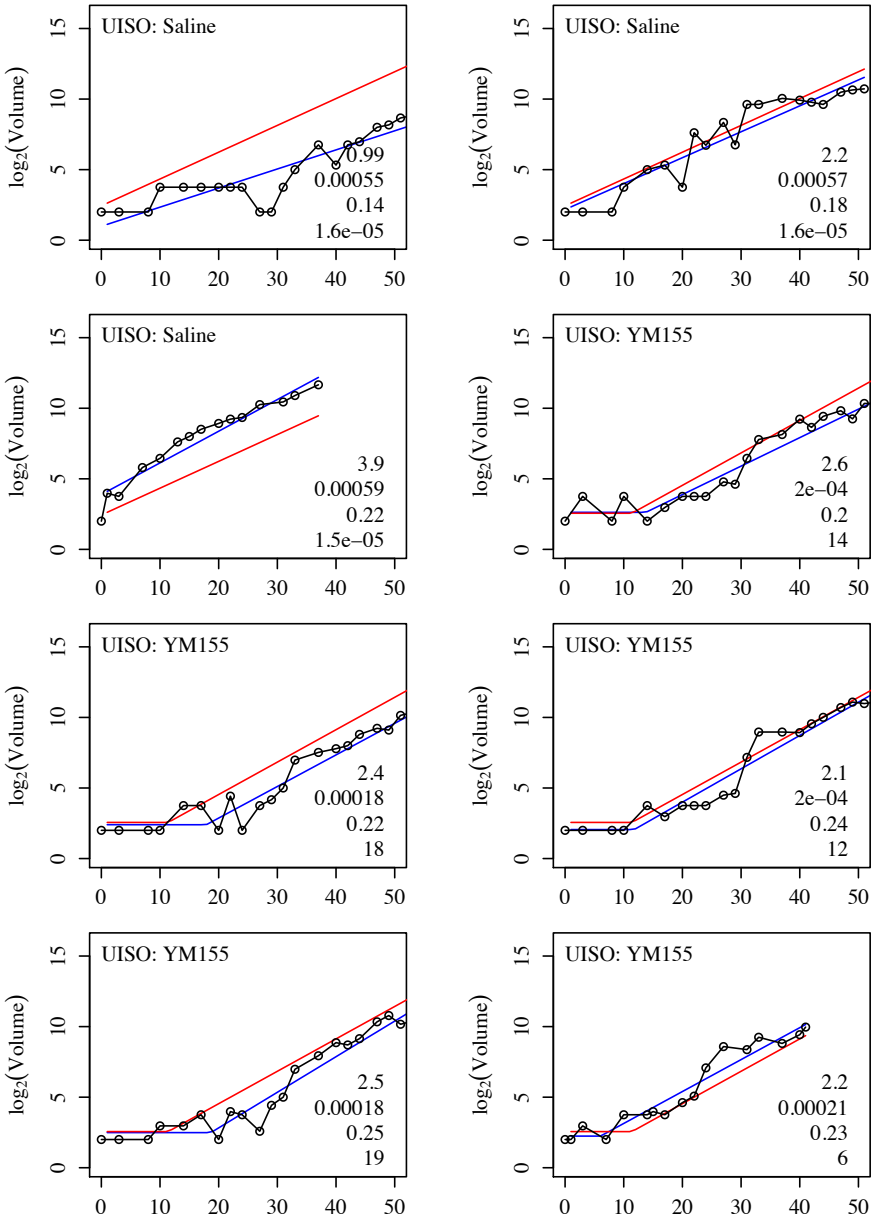



*Figure 4.12* Animal-level plots. Open circles are data, filled circles are BLQ, blue lines animal-level model, red lines experimental group-level model. Values are (top to bottom) $a_i, e^{b_{1i}}, e^{b_{2i}}, e^{r_i}$.

# References

[1] Zhao L, Morgan M, Parsels L, Maybaum J, Lawrence T, Normolle D. (2011) Bayesian Hierarchical Changepoint Methods in Modeling the Tumor Growth Profiles in Xenograft Experiments. *Clinical Cancer Research* **17(5):** 1057-64. PMID: 21131555.

## BIBLIOGRAPHY


1. Parkin, D.M., *Global cancer statistics in the year 2000.* Lancet Oncol, 2001. 2(9): p. 533-43.

2. Parkin, D.M., *The global health burden of infection-associated cancers in the year 2002.* Int J Cancer, 2006. 118(12): p. 3030-44.

3. Rous, P., *A transmissible avian neoplasm. (Sarcoma of the common fowl) by Peyton Rous, M.D., Experimental Medicine for Sept. 1, 1910, vol. 12, pp.696-705.* The Journal of experimental medicine, 1979. 150(4): p. 738-53.

4. Goldie, S.J., et al., *Projected clinical benefits and cost-effectiveness of a human papillomavirus 16/18 vaccine.* Journal of the National Cancer Institute, 2004. 96(8): p. 604-15.

5. Lavanchy, D., *Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures.* Journal of viral hepatitis, 2004. 11(2): p. 97-107.

6. Rous, P., *Landmark article (JAMA 1911;56:198). Transmission of a malignant new growth by means of a cell-free filtrate. By Peyton Rous.* JAMA : the journal of the American Medical Association, 1983. 250(11): p. 1445-9.

7. Gross, L., *A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice.* Proc Soc Exp Biol Med, 1953. 83(2): p. 414-21.

8. Marx, J.L., *Cancer gene research wins medicine Nobel.* Science, 1989. 246(4928): p. 326-7.

9. Talalay, P., G.W. Ashman, and W.R. Bryan, *1966 nobel laureates in medicine or physiology.* Science, 1966. 154(3747): p. 362-5.

10. Temin, H.M. and S. Mizutani, *RNA-dependent DNA polymerase in virions of Rous sarcoma virus.* Nature, 1970. 226(5252): p. 1211-3.

11. Baltimore, D., *RNA-dependent DNA polymerase in virions of RNA tumour viruses.* Nature, 1970. 226(5252): p. 1209-11.

12. Epstein, M.A., B.G. Achong, and Y.M. Barr, *Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma.* Lancet, 1964. 15: p. 702-3.

13. Masumi, A., et al., *The histone acetylase PCAF is a phorbol-ester-inducible coactivator of the IRF family that confers enhanced interferon responsiveness.* Mol Cell Biol, 1999. 19(3): p. 1810-20.

14. *Proceedings of the IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Epstein-Barr Virus and Kaposi's Sarcoma Herpesvirus/Human Herpesvirus 8. Lyon, France, 17-24 June 1997.* IARC Monogr Eval Carcinog Risks Hum, 1997. 70: p. 1-492.

15. Griffin, B.E., *Epstein-Barr virus (EBV) and human disease: facts, opinions and problems.* Mutation research, 2000. 462(2-3): p. 395-405.

16. Kelly, G.L. and A.B. Rickinson, *Burkitt lymphoma: revisiting the pathogenesis of a virus-associated malignancy.* Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program, 2007: p. 277-84.

17. Knudson, A.G., Jr., *Mutation and cancer: statistical study of retinoblastoma.* Proceedings of the National Academy of Sciences of the United States of America, 1971. 68(4): p. 820-3.

18. Javier, R.T. and J.S. Butel, *The history of tumor virology.* Cancer research, 2008. 68(19): p. 7693-706.

19. Moore, P.S. and Y. Chang, *Why do viruses cause cancer? Highlights of the first century of human tumour virology.* Nat Rev Cancer, 2010. 10(12): p. 878-89.

20. Blumberg, B.S., H.J. Alter, and S. Visnich, *A "New" Antigen in Leukemia Sera.* JAMA : the journal of the American Medical Association, 1965. 191: p. 541-6.

21. Blumberg, B.S., et al., *The relation of infection with the hepatitis B agent to primary hepatic carcinoma.* The American journal of pathology, 1975. 81(3): p. 669-82.

22. Beasley, R.P., et al., *Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan.* Lancet, 1981. 2(8256): p. 1129-33.

23. Blumberg, B.S. and W.T. London, *Hepatitis B virus and the prevention of primary hepatocellular carcinoma.* N Engl J Med, 1981. 304(13): p. 782-4.

24. Pagano, J.S., et al., *Infectious agents and cancer: criteria for a causal relation.* Seminars in cancer biology, 2004. 14(6): p. 453-71.

25. Buynak, E.B., et al., *Vaccine against human hepatitis B.* JAMA : the journal of the American Medical Association, 1976. 235(26): p. 2832-4.

26. Hilleman, M.R., *Critical overview and outlook: pathogenesis, prevention, and treatment of hepatitis and hepatocarcinoma caused by hepatitis B virus.* Vaccine, 2003. 21(32): p. 4626-49.

27. Choo, Q.L., et al., *Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome.* Science, 1989. 244(4902): p. 359-62.

28. Alter, H.J., *The unexpected outcomes of medical research: serendipity and the Australia antigen. Blumberg BS, Alter HJ, Visnich S. A new antigen in leukemia sera [J Am Med Assoc 1965;191:541-546].* Journal of hepatology, 2003. 39(2): p. 149-52.

29. Alter, H.J., et al., *Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis.* The New England journal of medicine, 1989. 321(22): p. 1494-500.

30. Tan, A., et al., *Viral hepatocarcinogenesis: from infection to cancer.* Liver international : official journal of the International Association for the Study of the Liver, 2008. 28(2): p. 175-88.

31. Wakita, T., et al., *Production of infectious hepatitis C virus in tissue culture from a cloned viral genome.* Nature medicine, 2005. 11(7): p. 791-6.

32. Poiesz, B.J., et al., *Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma.* Proceedings of the National Academy of Sciences of the United States of America, 1980. 77(12): p. 7415-9.

33. Yoshida, M., *Discovery of HTLV-1, the first human retrovirus, its unique regulatory mechanisms, and insights into pathogenesis.* Oncogene, 2005. 24(39): p. 5931-7.

34. Miyoshi, I., et al., *Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells.* Nature, 1981. 294(5843): p. 770-1.

35. Gallo, R.C., *History of the discoveries of the first human retroviruses: HTLV-1 and HTLV-2.* Oncogene, 2005. 24(39): p. 5926-30.

36. Hinuma, Y., et al., *Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera.* Proceedings of the National Academy of Sciences of the United States of America, 1981. 78(10): p. 6476-80.

37. Matsuoka, M. and K.T. Jeang, *Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation.* Nature reviews. Cancer, 2007. 7(4): p. 270-80.

38. zur Hausen, H., *Condylomata acuminata and human genital cancer.* Cancer Res, 1976. 36(2 pt 2): p. 794.

39. Boshart, M., et al., *A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer.* Embo J, 1984. 3(5): p. 1151-7.

40. Durst, M., et al., *A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions.* Proc Natl Acad Sci U S A, 1983. 80(12): p. 3812-5.

41. zur Hausen, H., *Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis.* J Natl Cancer Inst, 2000. 92(9): p. 690-8.

42. zur Hausen, H., *Cervical carcinoma and human papillomavirus: on the road to preventing a major human cancer.* J Natl Cancer Inst, 2001. 93(4): p. 252-3.

43. zur Hausen, H., *Oncogenic DNA viruses.* Oncogene, 2001. 20(54): p. 7820-3.

44. zur Hausen, H., *Papillomaviruses and cancer: from basic studies to clinical application.* Nat Rev Cancer, 2002. 2(5): p. 342-50.

45. Frazer, I.H., D.R. Lowy, and J.T. Schiller, *Prevention of cancer through immunization: Prospects and challenges for the 21st century.* European journal of immunology, 2007. 37 Suppl 1: p. S148-55.

46. Kirnbauer, R., et al., *Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic.* Proceedings of the National Academy of Sciences of the United States of America, 1992. 89(24): p. 12180-4.

47. Rose, R.C., et al., *Expression of human papillomavirus type 11 L1 protein in insect cells: in vivo and in vitro assembly of viruslike particles.* Journal of virology, 1993. 67(4): p. 1936-44.

48. Schiller, J.T. and D.R. Lowy, *Prospects for cervical cancer prevention by human papillomavirus vaccination.* Cancer research, 2006. 66(21): p. 10229-32.

49. Koutsky, L.A., et al., *A controlled trial of a human papillomavirus type 16 vaccine.* The New England journal of medicine, 2002. 347(21): p. 1645-51.

50. Munoz, N., et al., *Impact of human papillomavirus (HPV)-6/11/16/18 vaccine on all HPV-associated genital diseases in young women.* Journal of the National Cancer Institute, 2010. 102(5): p. 325-39.

51. Noad, R. and P. Roy, *Virus-like particles as immunogens.* Trends in microbiology, 2003. 11(9): p. 438-44.

52. Chang, Y., et al., *Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma.* Science, 1994. 265: p. 1865-69.

53. Engels, E.A., et al., *Trends in cancer risk among people with AIDS in the United States 1980-2002.* AIDS, 2006. 20(12): p. 1645-54.

54. Martro, E., et al., *Risk factors for human Herpesvirus 8 infection and AIDS-associated Kaposi's sarcoma among men who have sex with men in a European multicentre study.* International journal of cancer. Journal international du cancer, 2007. 120(5): p. 1129-35.

55. Casper, C., et al., *HIV serodiscordant sex partners and the prevalence of human herpesvirus 8 infection among HIV negative men who have sex with men: baseline data from the EXPLORE Study.* Sexually transmitted infections, 2006. 82(3): p. 229-35.

56. Moore, P.S. and Y. Chang, *Kaposi's sarcoma-associated herpesvirus*, in *Fields Virology*, D. Knipe, et al., Editors. 2001, Lippincott, Williams & Wilkins: Philadelphia. p. 2803-2833.

57. Moore, P.S. and Y. Chang, *Kaposi's sarcoma-associated herpesvirus immunoevasion and tumorigenesis: two sides of the same coin?* Annu Rev Microbiol, 2003. 57: p. 609-39.

58. Agelli, M. and L.X. Clegg, *Epidemiology of primary Merkel cell carcinoma in the United States.* Journal of the American Academy of Dermatology, 2003. 49(5): p. 832-41.

59. Hodgson, N.C., *Merkel cell carcinoma: changing incidence trends.* J Surg Oncol, 2005. 89(1): p. 1-4.

60. Engels, E.A., et al., *Merkel cell carcinoma and HIV infection.* Lancet, 2002. 359(9305): p. 497-8.

61. Buell, J.F., et al., *Immunosuppression and Merkel cell cancer.* Transplantation proceedings, 2002. 34(5): p. 1780-1.

62. Heath, M., et al., *Clinical characteristics of Merkel cell carcinoma at diagnosis in 195 patients: the AEIOU features.* J Am Acad Dermatol, 2008. 58(3): p. 375-81.

63. Penn, I. and M.R. First, *Merkel's cell carcinoma in organ recipients: report of 41 cases.* Transplantation, 1999. 68(11): p. 1717-21.

64. Takabayashi, M., et al., *Merkel cell carcinoma developing after antithymocyte globulin and cyclosporine therapy for aplastic anemia.* Anti-cancer drugs, 2003. 14(3): p. 251-3.

65. Howard, R.A., et al., *Merkel cell carcinoma and multiple primary cancers.* Cancer Epidemiol Biomarkers Prev, 2006. 15(8): p. 1545-9.

66. Kaae, J., et al., *Merkel cell carcinoma: incidence, mortality, and risk of other cancers.* Journal of the National Cancer Institute, 2010. 102(11): p. 793-801.

67. Vlad, R. and T.J. Woodlock, *Merkel cell carcinoma after chronic lymphocytic leukemia: case report and literature review.* Am J Clin Oncol, 2003. 26(6): p. 531-4.

68. Shuda, M., et al., *Human Merkel cell polyomavirus infection I. MCV T antigen expression in Merkel cell carcinoma, lymphoid tissues and lymphoid tumors.* Int J Cancer, 2009. 125(6): p. 1243-9.

69. Tolstov, Y.L., et al., *Lack of evidence for direct involvement of Merkel cell polyomavirus (MCV) in chronic lymphocytic leukemia (CLL).* Blood, 2010. 115(23): p. 4973-4.

70. Toracchio, S., et al., *Lymphotropism of Merkel cell polyomavirus infection, Nova Scotia, Canada.* Emerging infectious diseases, 2010. 16(11): p. 1702-9.

71. Andres, C., et al., *Prevalence of MCPyV in Merkel cell carcinoma and non-MCC tumors.* Journal of cutaneous pathology, 2010. 37(1): p. 28-34.

72. Murakami, M., et al., *Presence of Merkel cell polyomavirus in Japanese cutaneous squamous cell carcinoma.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2011. 50(1): p. 37-41.

73. Reisinger, D.M., et al., *Lack of evidence for basal or squamous cell carcinoma infection with Merkel cell polyomavirus in immunocompetent patients with Merkel cell carcinoma.* Journal of the American Academy of Dermatology, 2010. 63(3): p. 400-3.

74. Feng, H., et al., *Clonal integration of a polyomavirus in human Merkel cell carcinoma.* Science, 2008. 319(5866): p. 1096-100.

75. Feng, H., et al., *Human transcriptome subtraction by using short sequence tags to search for tumor viruses in conjunctival carcinoma.* J Virol, 2007. 81(20): p. 11332-40.

76. Popovic, M., et al., *Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS.* Science, 1984. 224(4648): p. 497-500.

77. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. 220(4599): p. 868-71.

78. Salahuddin, S.Z., et al., *Isolation of infectious human T-cell leukemia/lymphotropic virus type III (HTLV-III) from patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC) and from healthy carriers: a study of risk groups and tissue sources.* Proceedings of the National Academy of Sciences of the United States of America, 1985. 82(16): p. 5530-4.

79. *Proceedings of the IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Human Immunodeficiency Viruses and Human T cell Lymphotropic Viruses. Lyon, France, 11-18 June 1996.* IARC Monogr Eval Carcinog Risks Hum, 1996. 67: p. 1-424.

80. Montagnier, L., *25 years after HIV discovery: prospects for cure and vaccine (Nobel lecture).* Angewandte Chemie, 2009. 48(32): p. 5815-26.

81. Gallo, R.C. and L. Montagnier, *The discovery of HIV as the cause of AIDS.* The New England journal of medicine, 2003. 349(24): p. 2283-5.

82. Weiss, R.A., *On viruses, discovery, and recognition.* Cell, 2008. 135(6): p. 983-6.

83. Johne, R., et al., *Taxonomical developments in the family Polyomaviridae.* Archives of virology, 2011. 156(9): p. 1627-34.

84. Dilworth, S.M., *Polyoma virus middle T antigen and its role in identifying cancer-related molecules.* Nat Rev Cancer, 2002. 2(12): p. 951-6.

85. Pipas, J.M., *Common and unique features of T antigens encoded by the polyomavirus group.* J Virol, 1992. 66(7): p. 3979-85.

86. Poulin, D.L. and J.A. DeCaprio, *Is there a role for SV40 in human cancer?* J Clin Oncol, 2006. 24(26): p. 4356-65.

87. Stewart, S.E., B.E. Eddy, and N. Borgese, *Neoplasms in mice inoculated with a tumor agent carried in tissue culture.* Journal of the National Cancer Institute, 1958. 20(6): p. 1223-43.

88. Eddy, B.E., et al., *Identification of the oncogenic substance in rhesus monkey kidney cell culture as simian virus 40.* Virology, 1962. 17: p. 65-75.

89. Sweet, B.H. and M.R. Hilleman, *The vacuolating virus, S.V. 40.* Proc Soc Exp Biol Med, 1960. 105: p. 420-7.

90. Shah, K. and N. Nathanson, *Human exposure to SV40: review and comment.* American journal of epidemiology, 1976. 103(1): p. 1-12.

91. Sherwood, R.W., et al., *Effects of adenovirus vaccine on acute respiratory disease in U. S. Army recruits.* JAMA : the journal of the American Medical Association, 1961. 178: p. 1125-7.

92. Lopez-Rios, F., et al., *Evidence against a role for SV40 infection in human mesotheliomas and high risk of false-positive PCR results owing to presence of SV40 sequences in common laboratory plasmids.* Lancet, 2004. 364(9440): p. 1157-66.

93. Carter, J.J., et al., *Lack of serologic evidence for prevalent simian virus 40 infection in humans.* Journal of the National Cancer Institute, 2003. 95(20): p. 1522-30.

94. Rollison, D.E., et al., *Markers of past infection with simian virus 40 (SV40) and risk of incident non-Hodgkin lymphoma in a Maryland cohort.* Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 2005. 14(6): p. 1448-52.

95. Strickler, H.D. and J.J. Goedert, *Exposure to SV40-contaminated poliovirus vaccine and the risk of cancer--a review of the epidemiological evidence.* Developments in biological standardization, 1998. 94: p. 235-44.

96. Carbone, M., et al., *Simian virus 40-like DNA sequences in human pleural mesothelioma.* Oncogene, 1994. 9(6): p. 1781-90.

97. Shah, K.V., *SV40 and human cancer: a review of recent data.* International journal of cancer. Journal international du cancer, 2007. 120(2): p. 215-23.

98. zur Hausen, H., *SV40 in human cancers--an endless tale?* Int J Cancer, 2003. 107(5): p. 687.

99. Ahuja, D., M.T. Saenz-Robles, and J.M. Pipas, *SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation.* Oncogene, 2005. 24(52): p. 7729-45.

100. Bullock, P.A., *The initiation of simian virus 40 DNA replication in vitro.* Critical reviews in biochemistry and molecular biology, 1997. 32(6): p. 503-68.

101. Bullock, P.A., Y.S. Seo, and J. Hurwitz, *Initiation of simian virus 40 DNA replication in vitro: pulse-chase experiments identify the first labeled species as topologically unwound.* Proceedings of the National Academy of Sciences of the United States of America, 1989. 86(11): p. 3944-8.

102. Simmons, D.T., *SV40 large T antigen functions in DNA replication and transformation.* Advances in virus research, 2000. 55: p. 75-134.

103. Sullivan, C.S. and J.M. Pipas, *T antigens of simian virus 40: molecular chaperones for viral replication and tumorigenesis.* Microbiology and molecular biology reviews : MMBR, 2002. 66(2): p. 179-202.

104. zur Hausen, H. and L. Gissmann, *Lymphotropic papovaviruses isolated from African green monkey and human cells.* Med Microbiol Immunol (Berl), 1979. 167(3): p. 137-53.

105. Viscidi, R.P. and B. Clayman, *Serological cross reactivity between polyomavirus capsids.* Adv Exp Med Biol, 2006. 577: p. 73-84.

106. White, M.K., M. Safak, and K. Khalili, *Regulation of gene expression in primate polyomaviruses.* Journal of virology, 2009. 83(21): p. 10846-56.

107. Zerrahn, J., et al., *Independent expression of the transforming amino-terminal domain of SV40 large I antigen from an alternatively spliced third SV40 early mRNA.* The EMBO journal, 1993. 12(12): p. 4739-46.

108. Ito, Y., N. Spurr, and B.E. Griffin, *Middle T antigen as primary inducer of full expression of the phenotype of transformation by polyoma virus.* Journal of virology, 1980. 35(1): p. 219-32.

109. Riley, M.I., et al., *Tiny T antigen: an autonomous polyomavirus T antigen amino-terminal domain.* Journal of virology, 1997. 71(8): p. 6068-74.

110. Trowbridge, P.W. and R.J. Frisque, *Identification of three new JC virus proteins generated by alternative splicing of the early viral mRNA.* Journal of neurovirology, 1995. 1(2): p. 195-206.

111. Abend, J.R., M. Jiang, and M.J. Imperiale, *BK virus and human cancer: innocent until proven guilty.* Seminars in cancer biology, 2009. 19(4): p. 252-60.

112. Shuda, M., et al., *T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus.* Proc Natl Acad Sci U S A, 2008. 105(42): p. 16272-16277.

113. Schaffhausen, B.S. and T.M. Roberts, *Lessons from polyoma middle T antigen on signaling and transformation: A DNA tumor virus contribution to the war on cancer.* Virology, 2009. 384(2): p. 304-16.

114. Oliveira, M.L., S.M. Brochado, and M.C. Sogayar, *Mechanisms of cell transformation induced by polyomavirus.* Braz J Med Biol Res, 1999. 32(7): p. 861-5.

115. Prins, C. and R.J. Frisque, *JC virus T' proteins encoded by alternatively spliced early mRNAs enhance T antigen-mediated viral DNA replication in human cells.* Journal of neurovirology, 2001. 7(3): p. 250-64.

116. Tolstov, Y.L., et al., *Human Merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays.* Int J Cancer, 2009. 125(6): p. 1250-6.

117. Daniels, R., D. Sadowicz, and D.N. Hebert, *A very late viral protein triggers the lytic release of SV40.* PLoS pathogens, 2007. 3(7): p. e98.

118. Butin-Israeli, V., N. Drayman, and A. Oppenheim, *Simian virus 40 infection triggers a balanced network that includes apoptotic, survival, and stress pathways.* Journal of virology, 2010. 84(7): p. 3431-42.

119. Zullo, J., C.D. Stiles, and R.L. Garcea, *Regulation of c-myc and c-fos mRNA levels by polyomavirus: distinct roles for the capsid protein VP1 and the viral early proteins.* Proceedings of the National Academy of Sciences of the United States of America, 1987. 84(5): p. 1210-4.

120. Sullivan, C.S., et al., *SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells.* Nature, 2005. 435(7042): p. 682-6.

121. Seo, G.J., et al., *Evolutionarily conserved function of a viral microRNA.* Journal of virology, 2008. 82(20): p. 9823-8.

122. Sullivan, C.S., et al., *Murine Polyomavirus encodes a microRNA that cleaves early RNA transcripts but is not essential for experimental infection.* Virology, 2009. 387(1): p. 157-67.

123. Tsai, B., et al., *Gangliosides are receptors for murine polyoma virus and SV40.* The EMBO journal, 2003. 22(17): p. 4346-55.

124. Low, J.A., et al., *Identification of gangliosides GD1b and GT1b as receptors for BK virus.* Journal of virology, 2006. 80(3): p. 1361-6.

125. Elphick, G.F., et al., *The human polyomavirus, JCV, uses serotonin receptors to infect cells.* Science, 2004. 306(5700): p. 1380-3.

126. Erickson, K.D., R.L. Garcea, and B. Tsai, *Ganglioside GT1b is a putative host cell receptor for the Merkel cell polyomavirus.* Journal of virology, 2009. 83(19): p. 10275-9.

127. Eash, S., et al., *The human polyomaviruses.* Cellular and molecular life sciences : CMLS, 2006. 63(7-8): p. 865-76.

128. Fanning, E. and K. Zhao, *SV40 DNA replication: from the A gene to a nanomachine.* Virology, 2009. 384(2): p. 352-9.

129. Pipas, J.M., *SV40: Cell transformation and tumorigenesis.* Virology, 2009. 384(2): p. 294-303.

130. Gjoerup, O. and Y. Chang, *Update on human polyomaviruses and cancer.* Advances in cancer research, 2010. 106: p. 1-51.

131. Horvath, C.J., et al., *Simian virus 40-induced disease in rhesus monkeys with simian acquired immunodeficiency syndrome.* The American journal of pathology, 1992. 140(6): p. 1431-40.

132. Knipe, D.M., P.M. Howley, and B.N. Fields, eds. *Fields' Virology*. 2001, Lippincott Williams & Wilkins: Philadelphia. 3087.

133. *DNA Tumor Viruses*, ed. B.D.a.J.M. Pipas2009: Springer.

134. Small, M.B., Y. Gluzman, and H.L. Ozer, *Enhanced transformation of human fibroblasts by origin-defective simian virus 40.* Nature, 1982. 296(5858): p. 671-2.

135. Gardner, S.D., et al., *New human papovavirus (B.K.) isolated from urine after renal transplantation.* Lancet, 1971. 1(7712): p. 1253-7.

136. Padgett, B.L., et al., *Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy.* Lancet, 1971. 1(7712): p. 1257-60.

137. Maginnis, M.S. and W.J. Atwood, *JC virus: an oncogenic virus in animals and humans?* Seminars in cancer biology, 2009. 19(4): p. 261-9.

138. Houff, S.A., et al., *Neuroradiological studies of JCV-induced astrocytomas in nonhuman primates.* Progress in clinical and biological research, 1983. 105: p. 253-9.

139. London, W.T., et al., *Brain tumors in owl monkeys inoculated with a human polyomavirus (JC virus).* Science, 1978. 201(4362): p. 1246-9.

140. Ohsumi, S., M. Motoi, and K. Ogawa, *Induction of undifferentiated tumors by JC virus in the cerebrum of rats.* Acta pathologica japonica, 1986. 36(6): p. 815-25.

141. Ohsumi, S., et al., *Induction of undifferentiated brain tumors in rats by a human polyomavirus (JC virus).* Japanese journal of cancer research : Gann, 1985. 76(6): p. 429-31.

142. Walker, D.L., et al., *Human papovavirus (JC): induction of brain tumors in hamsters.* Science, 1973. 181(4100): p. 674-6.

143. Horie, Y., M. Motoi, and K. Ogawa, *Early stages of development of rat brain tumors induced by JC virus: a sequential histological and immunohistochemical study.* Acta medica Okayama, 1989. 43(5): p. 271-9.

144. Grossi, M.P., et al., *Transformation of human embryonic fibroblasts by BK virus, BK virus DNA and a subgenomic BK virus DNA fragment.* The Journal of general virology, 1982. 63(2): p. 393-403.

145. Grossi, M.P., et al., *Transformation of hamster kidney cells by fragments of BK virus DNA.* Journal of virology, 1982. 41(1): p. 319-25.

146. Major, E.O. and G. Di Mayorca, *Malignant transformation of BHK21 clone 13 cells by BK virus--a human papovavirus.* Proceedings of the National Academy of Sciences of the United States of America, 1973. 70(11): p. 3210-2.

147. Portolani, M. and M. Borgatti, *Stable transformation of mouse, rabbit and monkey cells and abortive transformation of human cells by BK virus, a human papovavirus.* The Journal of general virology, 1978. 38(2): p. 369-74.

148. Tognon, M., et al., *Oncogenic transformation by BK virus and association with human tumors.* Oncogene, 2003. 22(33): p. 5192-200.

149. Corallini, A., et al., *Oncogenity of BK virus for immunosuppressed hamsters.* Archives of virology, 1982. 73(3-4): p. 243-53.

150. Imperiale, M.J., *The human polyomaviruses, BKV and JCV: molecular pathogenesis of acute disease and potential role in cancer.* Virology, 2000. 267(1): p. 1-7.

151. Jiang, M., et al., *The role of polyomaviruses in human disease.* Virology, 2009. 384(2): p. 266-73.

152. Caldarelli-Stefano, R., et al., *JC virus in human glial-derived tumors.* Human pathology, 2000. 31(3): p. 394-5.

153. Jung, W.T., et al., *JC virus T-antigen expression in sporadic adenomatous polyps of the colon.* Cancer, 2008. 112(5): p. 1028-36.

154. Ricciardiello, L., et al., *JC virus DNA sequences are frequently present in the human upper and lower gastrointestinal tract.* Gastroenterology, 2000. 119(5): p. 1228-35.

155. Laghi, L., et al., *JC virus DNA is present in the mucosa of the human colon and in colorectal cancers.* Proceedings of the National Academy of Sciences of the United States of America, 1999. 96(13): p. 7484-9.

156. Ricciardiello, L., et al., *Mad-1 is the exclusive JC virus strain present in the human colon, and its transcriptional control region has a deleted 98-base-pair sequence in colon cancer tissues.* Journal of virology, 2001. 75(4): p. 1996-2001.

157. Murai, Y., et al., *High JC virus load in gastric cancer and adjacent non-cancerous mucosa.* Cancer science, 2007. 98(1): p. 25-31.

158. Rencic, A., et al., *Detection of JC virus DNA sequence and expression of the viral oncoprotein, tumor antigen, in brain of immunocompetent patient with oligoastrocytoma.* Proceedings of the National Academy of Sciences of the United States of America, 1996. 93(14): p. 7352-7.

159. Shin, S.K., et al., *Oncogenic T-antigen of JC virus is present frequently in human gastric cancers.* Cancer, 2006. 107(3): p. 481-8.

160. Kean, J.M., et al., *Seroepidemiology of human polyomaviruses.* PLoS pathogens, 2009. 5(3): p. e1000363.

161. Knowles, W.A., et al., *Population-based study of antibody to the human polyomaviruses BKV and JCV and the simian polyomavirus SV40.* Journal of medical virology, 2003. 71(1): p. 115-23.

162. Egli, A., et al., *Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors.* The Journal of infectious diseases, 2009. 199(6): p. 837-46.

163. Allander, T., et al., *Identification of a third human polyomavirus.* Journal of virology, 2007. 81(8): p. 4130-6.

164. Gaynor, A.M., et al., *Identification of a novel polyomavirus from patients with acute respiratory tract infections.* PLoS pathogens, 2007. 3(5): p. e64.

165. Wattier, R.L., et al., *Role of human polyomaviruses in respiratory tract disease in young children.* Emerging infectious diseases, 2008. 14(11): p. 1766-8.

166. Zhuang, W.L., et al., *[Detection and clinical characterization of WU polyomavirus in acute respiratory tract infection in children].* Zhonghua er ke za zhi. Chinese journal of pediatrics, 2010. 48(2): p. 90-4.

167. Ren, L., et al., *WU and KI polyomavirus present in the respiratory tract of children, but not in immunocompetent adults.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2008. 43(3): p. 330-3.

168. Jartti, T., et al., *New respiratory viral infections.* Current opinion in pulmonary medicine, 2012.

169. Venter, M., A. Visser, and R. Lassauniere, *Human polyomaviruses, WU and KI in HIV exposed children with acute lower respiratory tract infections in hospitals in South Africa.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2009. 44(3): p. 230-4.

170. Bialasiewicz, S., et al., *Whole-genome characterization and genotyping of global WU polyomavirus strains.* Journal of virology, 2010. 84(12): p. 6229-34.

171. Bialasiewicz, S., et al., *A newly reported human polyomavirus, KI virus, is present in the respiratory tract of Australian children.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2007. 40(1): p. 15-8.

172. Dang, X., et al., *Infrequent detection of KI, WU and MC polyomaviruses in immunosuppressed individuals with or without progressive multifocal leukoencephalopathy.* PloS one, 2011. 6(3): p. e16736.

173. Furuse, Y., et al., *Detection of novel respiratory viruses from influenza-like illness in the Philippines.* Journal of medical virology, 2010. 82(6): p. 1071-4.

174. Zhao, L., et al., *Identification of WU polyomavirus from pediatric patients with acute respiratory infections in Beijing, China.* Archives of virology, 2010. 155(2): p. 181-6.

175. Johne, R., et al., *Rolling-circle amplification of viral DNA genomes using phi29 polymerase.* Trends in microbiology, 2009. 17(5): p. 205-11.

176. Schrama, D., et al., *No evidence for association of HPyV6 or HPyV7 with different skin cancers.* The Journal of investigative dermatology, 2012. 132(1): p. 239-41.

177. Wyatt, A.J., et al., *Virus-associated trichodysplasia spinulosa.* The American journal of surgical pathology, 2005. 29(2): p. 241-6.

178. van der Meijden, E., et al., *Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient.* PLoS pathogens, 2010. 6(7): p. e1001024.

179. Tan, B.H. and K.J. Busam, *Virus-associated Trichodysplasia spinulosa.* Advances in anatomic pathology, 2011. 18(6): p. 450-3.

180. Kazem, S., et al., *Trichodysplasia spinulosa is characterized by active polyomavirus infection.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2012. 53(3): p. 225-30.

181. Scuda, N., et al., *A novel human polyomavirus closely related to the african green monkey-derived lymphotropic polyomavirus.* Journal of virology, 2011. 85(9): p. 4586-90.

182. Viscidi, R.P. and B. Clayman, *Serological cross reactivity between polyomavirus capsids.* Advances in experimental medicine and biology, 2006. 577: p. 73-84.

183. Trusch, F., et al., *Seroprevalence of the human polyomavirus 9 (HPyV9) and cross-reactivity to the African green monkey-derived lymphotropic polyomavirus (LPV).* The Journal of general virology, 2012.

184. Raptis, L. and A. Vultur, *Neoplastic transformation assays.* Methods Mol Biol, 2001. 165: p. 151-64.

185. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. 144(5): p. 646-74.

186. Ahuja, D., et al., *A structure-guided mutational analysis of simian virus 40 large T antigen: identification of surface residues required for viral replication and transformation.* Journal of virology, 2009. 83(17): p. 8781-8.

187. Ali, S.H., et al., *Cul7/p185/p193 binding to simian virus 40 large T antigen has a role in cellular transformation.* Journal of virology, 2004. 78(6): p. 2749-57.

188. Cotsiki, M., et al., *Simian virus 40 large T antigen targets the spindle assembly checkpoint protein Bub1.* Proceedings of the National Academy of Sciences of the United States of America, 2004. 101(4): p. 947-52.

189. Fei, Z.L., et al., *Association of insulin receptor substrate 1 with simian virus 40 large T antigen.* Molecular and cellular biology, 1995. 15(8): p. 4232-39.

190. Srinivasan, A., et al., *The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain.* Molecular and cellular biology, 1997. 17(8): p. 4761-73.

191. Stubdal, H., et al., *Inactivation of pRB-related proteins p130 and p107 mediated by the J domain of simian virus 40 large T antigen.* Molecular and cellular biology, 1997. 17(9): p. 4979-90.

192. Stubdal, H., J. Zalvide, and J.A. DeCaprio, *Simian virus 40 large T antigen alters the phosphorylation state of the RB-related proteins p130 and p107.* Journal of virology, 1996. 70(5): p. 2781-8.

193. Yaciuk, P., et al., *Simian virus 40 large-T antigen expresses a biological activity complementary to the p300-associated transforming function of the adenovirus E1A gene products.* Molecular and cellular biology, 1991. 11(4): p. 2116-24.

194. Lill, N.L., et al., *p300 family members associate with the carboxyl terminus of simian virus 40 large tumor antigen.* Journal of virology, 1997. 71(1): p. 129-37.

195. DeCaprio, J.A., et al., *SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene.* Cell, 1988. 54(2): p. 275-83.

196. Lane, D.P. and L.V. Crawford, *T antigen is bound to a host protein in SV40-transformed cells.* Nature, 1979. 278(5701): p. 261-3.

197. Linzer, D.I. and A.J. Levine, *Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells.* Cell, 1979. 17(1): p. 43-52.

198. Butel, J.S. and J.A. Lednicky, *Cell and molecular biology of simian virus 40: implications for human infections and disease.* J Natl Cancer Inst, 1999. 91(2): p. 119-34.

199. DeCaprio, J.A., *How the Rb tumor suppressor structure and function was revealed by the study of Adenovirus and SV40.* Virology, 2009. 384(2): p. 274-84.

200. Chen, S. and E. Paucha, *Identification of a region of simian virus 40 large T antigen required for cell transformation.* Journal of virology, 1990. 64(7): p. 3350-7.

201. Dyson, N., et al., *The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product.* Science, 1989. 243(4893): p. 934-7.

202. Munger, K., et al., *Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product.* The EMBO journal, 1989. 8(13): p. 4099-105.

203. Whyte, P., et al., *Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product.* Nature, 1988. 334(6178): p. 124-9.

204. Burkhart, D.L. and J. Sage, *Cellular mechanisms of tumour suppression by the retinoblastoma gene.* Nature reviews. Cancer, 2008. 8(9): p. 671-82.

205. Godbout, R., et al., *Somatic inactivation of genes on chromosome 13 is a common event in retinoblastoma.* Nature, 1983. 304(5925): p. 451-3.

206. Comings, D.E., *A general theory of carcinogenesis.* Proceedings of the National Academy of Sciences of the United States of America, 1973. 70(12): p. 3324-8.

207. Lee, W.H., et al., *Human retinoblastoma susceptibility gene: cloning, identification, and sequence.* Science, 1987. 235(4794): p. 1394-9.

208. Morris, E.J. and N.J. Dyson, *Retinoblastoma protein partners.* Advances in cancer research, 2001. 82: p. 1-54.

209. Weinberg, R.A., *the biology of cancer*2007: Garland Science.

210. Dyson, N., et al., *The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and JC virus.* Cell, 1989. 58(2): p. 249-55.

211. Ewen, M.E., et al., *An N-terminal transformation-governing sequence of SV40 large T antigen contributes to the binding of both p110Rb and a second cellular protein, p120.* Cell, 1989. 58(2): p. 257-67.

212. Hannon, G.J., D. Demetrick, and D. Beach, *Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins.* Genes & development, 1993. 7(12A): p. 2378-91.

213. Mayol, X., et al., *Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain.* Oncogene, 1993. 8(9): p. 2561-6.

214. Kim, H.Y., B.Y. Ahn, and Y. Cho, *Structural basis for the inactivation of retinoblastoma tumor suppressor by SV40 large T antigen.* EMBO J, 2001. 20(1-2): p. 295-304.

215. Zalvide, J., H. Stubdal, and J.A. DeCaprio, *The J domain of simian virus 40 large T antigen is required to functionally inactivate RB family proteins.* Mol Cell Biol, 1998. 18(3): p. 1408-15.

216. Sullivan, C.S., P. Cantalupo, and J.M. Pipas, *The molecular chaperone activity of simian virus 40 large T antigen is required to disrupt Rb-E2F family complexes by an ATP-dependent mechanism.* Molecular and cellular biology, 2000. 20(17): p. 6233-43.

217. Zalvide, J., H. Stubdal, and J.A. DeCaprio, *The J domain of simian virus 40 large T antigen is required to functionally inactivate RB family proteins.* Molecular and cellular biology, 1998. 18(3): p. 1408-15.

218. Litovchick, L., et al., *Evolutionarily conserved multisubunit RBL2/p130 and E2F4 protein complex represses human cell cycle-dependent genes in quiescence.* Molecular cell, 2007. 26(4): p. 539-51.

219. Hollstein, M., et al., *Database of p53 gene somatic mutations in human tumors and cell lines.* Nucleic acids research, 1994. 22(17): p. 3551-5.

220. Lane, D.P., *Cancer. p53, guardian of the genome.* Nature, 1992. 358(6381): p. 15-6.

221. Yew, P.R. and A.J. Berk, *Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein.* Nature, 1992. 357(6373): p. 82-5.

222. Scheffner, M., et al., *The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53.* Cell, 1990. 63(6): p. 1129-36.

223. Kierstead, T.D. and M.J. Tevethia, *Association of p53 binding and immortalization of primary C57BL/6 mouse embryo fibroblasts by using simian virus 40 T-antigen mutants bearing internal overlapping deletion mutations.* Journal of virology, 1993. 67(4): p. 1817-29.

224. Lilyestrom, W., et al., *Crystal structure of SV40 large T-antigen bound to p53: interplay between a viral oncoprotein and a cellular tumor suppressor.* Genes & development, 2006. 20(17): p. 2373-82.

225. Bargonetti, J., et al., *Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53.* Genes & development, 1992. 6(10): p. 1886-98.

226. Conzen, S.D. and C.N. Cole, *The three transforming regions of SV40 T antigen are required for immortalization of primary mouse embryo fibroblasts.* Oncogene, 1995. 11(11): p. 2295-302.

227. Zhao, R., et al., *The transcriptional program following p53 activation.* Cold Spring Harbor symposia on quantitative biology, 2000. 65: p. 475-82.

228. Rathi, A.V., et al., *Simian virus 40 T-antigen-mediated gene regulation in enterocytes is controlled primarily by the Rb-E2F pathway.* Journal of virology, 2009. 83(18): p. 9521-31.

229. Hermannstadter, A., et al., *Wild-type p53 enhances efficiency of simian virus 40 large-T-antigen-induced cellular transformation.* Journal of virology, 2009. 83(19): p. 10106-18.

230. Tiemann, F. and W. Deppert, *Stabilization of the tumor suppressor p53 during cellular transformation by simian virus 40: influence of viral and cellular factors and biological consequences.* Journal of virology, 1994. 68(5): p. 2869-78.

231. Deppert, W., T. Steinmayer, and W. Richter, *Cooperation of SV40 large T antigen and the cellular protein p53 in maintenance of cell transformation.* Oncogene, 1989. 4(9): p. 1103-10.

232. Herzig, M., M. Novatchkova, and G. Christofori, *An unexpected role for p53 in augmenting SV40 large T antigen-mediated tumorigenesis.* Biological chemistry, 1999. 380(2): p. 203-11.

233. Borger, D.R. and J.A. DeCaprio, *Targeting of p300/CREB binding protein coactivators by simian virus 40 is mediated through p53.* Journal of virology, 2006. 80(9): p. 4292-303.

234. Brosh, R. and V. Rotter, *When mutants gain new powers: news from the mutant p53 field.* Nature reviews. Cancer, 2009. 9(10): p. 701-13.

235. Dittmer, D., et al., *Gain of function mutations in p53.* Nature genetics, 1993. 4(1): p. 42-6.

236. Campbell, K.S., et al., *DnaJ/hsp40 chaperone domain of SV40 large T antigen promotes efficient viral DNA replication.* Genes & development, 1997. 11(9): p. 1098-110.

237. Stubdal, H., J. Zalvide, and J.A. DeCaprio, *Simian virus 40 large T antigen alters the phosphorylation state of the RB-related proteins p130 and p107.* J Virol, 1996. 70(5): p. 2781-8.

238. Berk, A.J., *Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus.* Oncogene, 2005. 24(52): p. 7673-85.

239. Iyer, N.G., H. Ozdag, and C. Caldas, *p300/CBP and cancer.* Oncogene, 2004. 23(24): p. 4225-31.

240. Howe, J.A., et al., *Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis.* Proceedings of the National Academy of Sciences of the United States of America, 1990. 87(15): p. 5883-7.

241. Wang, H.G., et al., *Identification of specific adenovirus E1A N-terminal residues critical to the binding of cellular proteins and to the control of cell growth.* Journal of virology, 1993. 67(1): p. 476-88.

242. Yaciuk, P., et al., *Simian virus 40 large-T antigen expresses a biological activity complementary to the p300-associated transforming function of the adenovirus E1A gene products.* Mol Cell Biol, 1991. 11(4): p. 2116-24.

243. Eckner, R., et al., *Association of p300 and CBP with simian virus 40 large T antigen.* Molecular and cellular biology, 1996. 16(7): p. 3454-64.

244. Poulin, D.L., A.L. Kung, and J.A. DeCaprio, *p53 targets simian virus 40 large T antigen for acetylation by CBP.* Journal of virology, 2004. 78(15): p. 8245-53.

245. Barbeau, D., et al., *Functional interactions within adenovirus E1A protein complexes.* Oncogene, 1994. 9(2): p. 359-73.

246. Fuchs, M., et al., *The p400 complex is an essential E1A transformation target.* Cell, 2001. 106(3): p. 297-307.

247. Samuelson, A.V., et al., *p400 is required for E1A to promote apoptosis.* The Journal of biological chemistry, 2005. 280(23): p. 21915-23.

248. Chan, H.M., et al., *The p400 E1A-associated protein is a novel component of the p53 --> p21 senescence pathway.* Genes & development, 2005. 19(2): p. 196-201.

249. Sarikas, A., et al., *The cullin7 E3 ubiquitin ligase: a novel player in growth control.* Cell cycle, 2008. 7(20): p. 3154-61.

250. Kasper, J.S., et al., *Simian virus 40 large T antigen's association with the CUL7 SCF complex contributes to cellular transformation.* Journal of virology, 2005. 79(18): p. 11685-92.

251. Zhao, X., et al., *Ataxia telangiectasia-mutated damage-signaling kinase- and proteasome-dependent destruction of Mre11-Rad50-Nbs1 subunits in Simian virus 40-infected primate cells.* Journal of virology, 2008. 82(11): p. 5316-28.

252. Xu, X., et al., *The CUL7 E3 ubiquitin ligase targets insulin receptor substrate 1 for ubiquitin-dependent degradation.* Molecular cell, 2008. 30(4): p. 403-14.

253. Meraldi, P. and P.K. Sorger, *A dual role for Bub1 in the spindle checkpoint and chromosome congression.* The EMBO journal, 2005. 24(8): p. 1621-33.

254. Schliekelman, M., et al., *Impaired Bub1 function in vivo compromises tension-dependent checkpoint function leading to aneuploidy and tumorigenesis.* Cancer research, 2009. 69(1): p. 45-54.

255. Cahill, D.P., et al., *Mutations of mitotic checkpoint genes in human cancers.* Nature, 1998. 392(6673): p. 300-3.

256. Keller, S.R. and G.E. Lienhard, *Insulin signalling: the role of insulin receptor substrate 1.* Trends in cell biology, 1994. 4(4): p. 115-9.

257. D'Ambrosio, C., et al., *Transforming potential of the insulin receptor substrate 1.* Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research, 1995. 6(5): p. 557-62.

258. Prisco, M., et al., *Nuclear translocation of insulin receptor substrate-1 by the simian virus 40 T antigen and the activated type 1 insulin-like growth factor receptor.* The Journal of biological chemistry, 2002. 277(35): p. 32078-85.

259. Lassak, A., et al., *Insulin receptor substrate 1 translocation to the nucleus by the human JC virus T-antigen.* The Journal of biological chemistry, 2002. 277(19): p. 17231-8.

260. Yu, Y. and J.C. Alwine, *Interaction between simian virus 40 large T antigen and insulin receptor substrate 1 is disrupted by the K1 mutation, resulting in the loss of large T antigen-mediated phosphorylation of Akt.* Journal of virology, 2008. 82(9): p. 4521-6.

261. Lee, J.H. and T.T. Paull, *ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex.* Science, 2005. 308(5721): p. 551-4.

262. Wu, X., et al., *SV40 T antigen interacts with Nbs1 to disrupt DNA replication control.* Genes & development, 2004. 18(11): p. 1305-16.

263. Welcker, M. and B.E. Clurman, *The SV40 large T antigen contains a decoy phosphodegron that mediates its interactions with Fbw7/hCdc4.* The Journal of biological chemistry, 2005. 280(9): p. 7654-8.

264. Pipas, J.M., *Mutations near the carboxyl terminus of the simian virus 40 large tumor antigen alter viral host range.* Journal of virology, 1985. 54(2): p. 569-75.

265. Poulin, D.L. and J.A. DeCaprio, *The carboxyl-terminal domain of large T antigen rescues SV40 host range activity in trans independent of acetylation.* Virology, 2006. 349(1): p. 212-21.

266. Berger, L.C., et al., *Interaction between T antigen and TEA domain of the factor TEF-1 derepresses simian virus 40 late promoter in vitro: identification of T-antigen domains important for transcription control.* Journal of virology, 1996. 70(2): p. 1203-12.

267. Dickmanns, A., et al., *The kinetics of simian virus 40-induced progression of quiescent cells into S phase depend on four independent functions of large T antigen.* Journal of virology, 1994. 68(9): p. 5496-508.

268. Cicala, C., et al., *Simian virus 40 small-t antigen stimulates viral DNA replication in permissive monkey cells.* Journal of virology, 1994. 68(5): p. 3138-44.

269. Sleigh, M.J., et al., *Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells.* Cell, 1978. 14(1): p. 79-88.

270. Mungre, S., et al., *Mutations which affect the inhibition of protein phosphatase 2A by simian virus 40 small-t antigen in vitro decrease viral transformation.* Journal of virology, 1994. 68(3): p. 1675-81.

271. Gjoerup, O. and Y. Chang, *Update on human polyomaviruses and cancer.* Adv Cancer Res, 2010. 106: p. 1-51.

272. Hahn, W.C., et al., *Creation of human tumour cells with defined genetic elements.* Nature, 1999. 400(6743): p. 464-8.

273. Turk, B., et al., *Simian virus 40 small-t antigen binds two zinc ions.* Journal of virology, 1993. 67(6): p. 3671-3.

274. Sablina, A.A. and W.C. Hahn, *SV40 small T antigen and PP2A phosphatase in cell transformation.* Cancer metastasis reviews, 2008. 27(2): p. 137-46.

275. Sontag, E., *Protein phosphatase 2A: the Trojan Horse of cellular signaling.* Cellular signalling, 2001. 13(1): p. 7-16.

276. Pallas, D.C., et al., *Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A.* Cell, 1990. 60(1): p. 167-76.

277. Hahn, W.C., et al., *Enumeration of the simian virus 40 early region elements necessary for human cell transformation.* Mol Cell Biol, 2002. 22(7): p. 2111-23.

278. Chen, W., et al., *Identification of specific PP2A complexes involved in human cell transformation.* Cancer cell, 2004. 5(2): p. 127-36.

279. Zhao, J.J., et al., *Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase.* Cancer cell, 2003. 3(5): p. 483-95.

280. Millward, T.A., S. Zolnierowicz, and B.A. Hemmings, *Regulation of protein kinase cascades by protein phosphatase 2A.* Trends in biochemical sciences, 1999. 24(5): p. 186-91.

281. Saenz Robles, M.T. and J.M. Pipas, *T antigen transgenic mouse models.* Seminars in cancer biology, 2009. 19(4): p. 229-35.

282. Saenz-Robles, M.T., C.S. Sullivan, and J.M. Pipas, *Transforming functions of Simian Virus 40.* Oncogene, 2001. 20(54): p. 7899-907.

283. Brinster, R.L., et al., *Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors.* Cell, 1984. 37(2): p. 367-79.

284. Chen, J., et al., *T-antigen mutant activities in vivo: roles of p53 and pRB binding in tumorigenesis of the choroid plexus.* Oncogene, 1992. 7(6): p. 1167-75.

285. Chen, J.D. and T. Van Dyke, *Uniform cell-autonomous tumorigenesis of the choroid plexus by papovavirus large T antigens.* Molecular and cellular biology, 1991. 11(12): p. 5968-76.

286. Saenz Robles, M.T., et al., *Induction versus progression of brain tumor development: differential functions for the pRB- and p53-targeting domains of simian virus 40 T antigen.* Molecular and cellular biology, 1994. 14(4): p. 2686-98.

287. Symonds, H., et al., *p53-dependent apoptosis suppresses tumor growth and progression in vivo.* Cell, 1994. 78(4): p. 703-11.

288. Simin, K., et al., *pRb inactivation in mammary cells reveals common mechanisms for tumor initiation and progression in divergent epithelia.* PLoS biology, 2004. 2(2): p. E22.

289. Xiao, A., et al., *Astrocyte inactivation of the pRb pathway predisposes mice to malignant astrocytoma development that is accelerated by PTEN mutation.* Cancer cell, 2002. 1(2): p. 157-68.

290. Bennoun, M., et al., *The amino-terminal region of SV40 large T antigen is sufficient to induce hepatic tumours in mice.* Oncogene, 1998. 17(10): p. 1253-9.

291. Kim, S.H., et al., *Expression of wild-type and mutant simian virus 40 large tumor antigens in villus-associated enterocytes of transgenic mice.* Proceedings of the National Academy of Sciences of the United States of America, 1994. 91(15): p. 6914-8.

292. Hauft, S.M., et al., *Expression of SV-40 T antigen in the small intestinal epithelium of transgenic mice results in proliferative changes in the crypt and reentry of villus-associated enterocytes into the cell cycle but has no apparent effect on cellular differentiation programs and does not cause neoplastic transformation.* The Journal of cell biology, 1992. 117(4): p. 825-39.

293. Markovics, J.A., et al., *Intestinal dysplasia induced by simian virus 40 T antigen is independent of p53.* Journal of virology, 2005. 79(12): p. 7492-502.

294. Rathi, A.V., M.T. Saenz Robles, and J.M. Pipas, *Enterocyte proliferation and intestinal hyperplasia induced by simian virus 40 T antigen require a functional J domain.* Journal of virology, 2007. 81(17): p. 9481-9.

295. Hill, R., et al., *Heterogeneous tumor evolution initiated by loss of pRb function in a preclinical prostate cancer model.* Cancer research, 2005. 65(22): p. 10243-54.

296. Masumori, N., et al., *A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential.* Cancer research, 2001. 61(5): p. 2239-49.

297. Tevethia, M.J., et al., *A simian virus 40 large T-antigen segment containing amino acids 1 to 127 and expressed under the control of the rat elastase-1 promoter produces pancreatic acinar carcinomas in transgenic mice.* Journal of virology, 1997. 71(11): p. 8157-66.

298. Toker, C., *Trabecular carcinoma of the skin.* Archives of dermatology, 1972. 105(1): p. 107-10.

299. Pearse, A.G., *The neuroendocrine (APUD) cells of the skin.* The American Journal of dermatopathology, 1980. 2(2): p. 121-3.

300. Halata, Z., M. Grim, and K.I. Bauman, *Friedrich Sigmund Merkel and his "Merkel cell", morphology, development, and physiology: review and new results.* The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology, 2003. 271(1): p. 225-39.

301. Lucarz, A. and G. Brand, *Current considerations about Merkel cells.* European journal of cell biology, 2007. 86(5): p. 243-51.

302. Van Keymeulen, A., et al., *Epidermal progenitors give rise to Merkel cells during embryonic development and adult homeostasis.* The Journal of cell biology, 2009. 187(1): p. 91-100.

303. Haeberle, H., et al., *Molecular profiling reveals synaptic release machinery in Merkel cells.* Proceedings of the National Academy of Sciences of the United States of America, 2004. 101(40): p. 14503-8.

304. Maricich, S.M., et al., *Merkel cells are essential for light-touch responses.* Science, 2009. 324(5934): p. 1580-2.

305. Sibley, R.K., L.P. Dehner, and J. Rosai, *Primary neuroendocrine (Merkel cell?) carcinoma of the skin. I. A clinicopathologic and ultrastructural study of 43 cases.* The American journal of surgical pathology, 1985. 9(2): p. 95-108.

306. Tang, C.K. and C. Toker, *Trabecular carcinoma of the skin: an ultrastructural study.* Cancer, 1978. 42(5): p. 2311-21.

307. Van Gele, M., et al., *Gene-expression profiling reveals distinct expression patterns for Classic versus Variant Merkel cell phenotypes and new classifier genes to distinguish Merkel cell from small-cell lung carcinoma.* Oncogene, 2004. 23(15): p. 2732-42.

308. Ball, N.J. and G. Tanhuanco-Kho, *Merkel cell carcinoma frequently shows histologic features of basal cell carcinoma: a study of 30 cases.* Journal of cutaneous pathology, 2007. 34(8): p. 612-9.

309. Shipkov, C.D., et al., *Merkel cell carcinoma vs. basal cell carcinoma: histopathologic challenges.* Journal of cutaneous pathology, 2008. 35(8): p. 789-90.

310. Bobos, M., et al., *Immunohistochemical distinction between merkel cell carcinoma and small cell carcinoma of the lung.* The American Journal of dermatopathology, 2006. 28(2): p. 99-104.

311. Van Gele, M., et al., *Combined karyotyping, CGH and M-FISH analysis allows detailed characterization of unidentified chromosomal rearrangements in Merkel cell carcinoma.* International journal of cancer. Journal international du cancer, 2002. 101(2): p. 137-45.

312. Busam, K.J., et al., *Small cell sweat gland carcinoma in childhood.* The American journal of surgical pathology, 1998. 22(2): p. 215-20.

313. Chan, J.K., et al., *Cytokeratin 20 immunoreactivity distinguishes Merkel cell (primary cutaneous neuroendocrine) carcinomas and salivary gland small cell carcinomas from small cell carcinomas of various sites.* The American journal of surgical pathology, 1997. 21(2): p. 226-34.

314. Jensen, K., S. Kohler, and R.V. Rouse, *Cytokeratin staining in Merkel cell carcinoma: an immunohistochemical study of cytokeratins 5/6, 7, 17, and 20.* Applied immunohistochemistry & molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry, 2000. 8(4): p. 310-5.

315. Hanly, A.J., et al., *Analysis of thyroid transcription factor-1 and cytokeratin 20 separates merkel cell carcinoma from small cell carcinoma of lung.* Journal of cutaneous pathology, 2000. 27(3): p. 118-20.

316. Battifora, H. and E.G. Silva, *The use of antikeratin antibodies in the immunohistochemical distinction between neuroendocrine (Merkel cell) carcinoma of the skin, lymphoma, and oat cell carcinoma.* Cancer, 1986. 58(5): p. 1040-6.

317. Calder, K.B., et al., *A case series and immunophenotypic analysis of CK20-/CK7+ primary neuroendocrine carcinoma of the skin.* Journal of cutaneous pathology, 2007. 34(12): p. 918-23.

318. Cheuk, W., et al., *Immunostaining for thyroid transcription factor 1 and cytokeratin 20 aids the distinction of small cell carcinoma from Merkel cell carcinoma, but not pulmonary from extrapulmonary small cell carcinomas.* Archives of pathology & laboratory medicine, 2001. 125(2): p. 228-31.

319. Haneke, E., H.J. Schulze, and G. Mahrle, *Immunohistochemical and immunoelectron microscopic demonstration of chromogranin A in formalin-fixed tissue of Merkel cell carcinoma.* Journal of the American Academy of Dermatology, 1993. 28(2 Pt 1): p. 222-6.

320. Shah, I.A., et al., *Neurofilament immunoreactivity in Merkel-cell tumors: a differentiating feature from small-cell carcinoma.* Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc, 1993. 6(1): p. 3-9.

321. Goessling, W., P.H. McKee, and R.J. Mayer, *Merkel cell carcinoma.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2002. 20(2): p. 588-98.

322. Koljonen, V., *Merkel cell carcinoma.* World journal of surgical oncology, 2006. 4: p. 7.

323. Miller, R.W. and C.S. Rabkin, *Merkel cell carcinoma and melanoma: etiological similarities and differences.* Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 1999. 8(2): p. 153-8.

324. Allen, P.J., et al., *Merkel cell carcinoma: prognosis and treatment of patients from a single institution.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2005. 23(10): p. 2300-9.

325. Edge, S.B., Byrd, D.R.,Compton, C.C., Fritz, A.G., Greene, F.L., Trotti, A., *AJCC Cancer Staging Manual,* 2010, Springer.

326. Lemos, B.D., et al., *Pathologic nodal evaluation improves prognostic accuracy in Merkel cell carcinoma: analysis of 5823 cases as the basis of the first consensus staging system.* Journal of the American Academy of Dermatology, 2010. 63(5): p. 751-61.

327. Albores-Saavedra, J., et al., *Merkel cell carcinoma demographics, morphology, and survival based on 3870 cases: a population based study.* Journal of cutaneous pathology, 2010. 37(1): p. 20-7.

328. Sihto, H., et al., *Clinical factors associated with Merkel cell polyomavirus infection in Merkel cell carcinoma.* Journal of the National Cancer Institute, 2009. 101(13): p. 938-45.

329. Reichgelt, B.A. and O. Visser, *Epidemiology and survival of Merkel cell carcinoma in the Netherlands. A population-based study of 808 cases in 1993-2007.* European journal of cancer, 2011. 47(4): p. 579-85.

330. Lyhne, D., et al., *Rising incidence of Merkel cell carcinoma.* Journal of plastic surgery and hand surgery, 2011. 45(6): p. 274-80.

331. Bichakjian, C.K., et al., *Merkel cell carcinoma: critical review with guidelines for multidisciplinary management.* Cancer, 2007. 110(1): p. 1-12.

332. Paulson, K.G., et al., *Array-CGH reveals recurrent genomic changes in Merkel cell carcinoma including amplification of L-Myc.* The Journal of investigative dermatology, 2009. 129(6): p. 1547-55.

333. Lemos, B. and P. Nghiem, *Merkel cell carcinoma: more deaths but still no pathway to blame.* J Invest Dermatol, 2007. 127(9): p. 2100-3.

334. Swick, B.L., et al., *Merkel cell carcinoma: evaluation of KIT (CD117) expression and failure to demonstrate activating mutations in the C-KIT proto-oncogene - implications for treatment with imatinib mesylate.* Journal of cutaneous pathology, 2007. 34(4): p. 324-9.

335. Kartha, R.V. and U.N. Sundram, *Silent mutations in KIT and PDGFRA and coexpression of receptors with SCF and PDGFA in Merkel cell carcinoma: implications for tyrosine kinase-based tumorigenesis.* Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc, 2008. 21(2): p. 96-104.

336. Brunner, M., et al., *Expression of VEGF-A/C, VEGF-R2, PDGF-alpha/beta, c-kit, EGFR, Her-2/Neu, Mcl-1 and Bmi-1 in Merkel cell carcinoma.* Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc, 2008. 21(7): p. 876-84.

337. Namikawa, K., et al., *Is the MAPK pathway involved in the aggressivity of Merkel cell carcinoma?* European journal of dermatology : EJD, 2007. 17(6): p. 556.

338. Worda, M., et al., *T1796A BRAF mutation is absent in Merkel cell carcinoma.* The British journal of dermatology, 2005. 153(1): p. 229-32.

339. Houben, R., et al., *Absence of classical MAP kinase pathway signalling in Merkel cell carcinoma.* The Journal of investigative dermatology, 2006. 126(5): p. 1135-42.

340. Pulitzer, M.P., B.D. Amin, and K.J. Busam, *Merkel cell carcinoma: review.* Adv Anat Pathol, 2009. 16(3): p. 135-44.

341. Sarnaik, A.A., et al., *Clinical recognition, diagnosis, and staging of merkel cell carcinoma, and the role of the multidisciplinary management team.* Current problems in cancer, 2010. 34(1): p. 38-46.

342. Kearsley, J.H., T. Hurst, and S.K. Khoo, *Chemosensitivity testing of primary cultures of Merkel cell cancer.* Anticancer Drugs, 1993. 4(5): p. 571-5.

343. Voog, E., et al., *Chemotherapy for patients with locally advanced or metastatic Merkel cell carcinoma.* Cancer, 1999. 85(12): p. 2589-95.

344. Jouary, T., et al., *Neoadjuvant polychemotherapy in locally advanced Merkel cell carcinoma.* Nat Rev Clin Oncol, 2009. 6(9): p. 544-8.

345. Krasagakis, K., et al., *Co-expression of KIT receptor and its ligand stem cell factor in Merkel cell carcinoma.* Dermatology, 2009. 218(1): p. 37-43.

346. Yang, Q., et al., *Merkel cell carcinoma: lack of KIT positivity and implications for the use of imatinib mesylate.* Appl Immunohistochem Mol Morphol, 2009. 17(4): p. 276-81.

347. Samlowski, W.E., et al., *A Phase II Trial of Imatinib Mesylate in Merkel Cell Carcinoma (Neuroendocrine Carcinoma of the Skin): A Southwest Oncology Group Study (S0331).* Am J Clin Oncol, 2009.

348. Ely, H. and A. Pascucci, *Merkel cell carcinoma: treatment with bleomycin.* Dermatol Online J, 2008. 14(9): p. 3.

349. Fields, R.C., et al., *Recurrence after complete resection and selective use of adjuvant therapy for stage I through III Merkel cell carcinoma.* Cancer, 2011.

350. Georgiou, N.A., et al., *Bleomycin has antiviral properties against drug-resistant HIV strains and sensitises virus to currently used antiviral agents.* Int J Antimicrob Agents, 2006. 27(1): p. 63-8.

351. Shah, M.H., et al., *G3139 (Genasense) in patients with advanced merkel cell carcinoma.* American journal of clinical oncology, 2009. 32(2): p. 174-9.

352. Willmes, C., et al., *Type I and II interferons inhibit Merkel cell carcinoma via modulation of the Merkel cell polyomavirus T-antigens.* Cancer research, 2012.

353. Fiers, W., et al., *Complete nucleotide sequence of SV40 DNA.* Nature, 1978. 273(5658): p. 113-20.

354. Cheng, J., et al., *Cellular transformation by Simian Virus 40 and Murine Polyoma Virus T antigens.* Seminars in cancer biology, 2009. 19(4): p. 218-28.

355. Pawlita, M., A. Clad, and H. zur Hausen, *Complete DNA sequence of lymphotropic papovavirus: prototype of a new species of the polyomavirus genus.* Virology, 1985. 143(1): p. 196-211.

356. Pastrana, D.V., et al., *Quantitation of human seroresponsiveness to Merkel cell polyomavirus.* PLoS Pathog, 2009. 5(9): p. e1000578.

357. Sariyer, I.K., et al., *Infection by agnoprotein-negative mutants of polyomavirus JC and SV40 results in the release of virions that are mostly deficient in DNA content.* Virology journal, 2011. 8: p. 255.

358. Jay, G., et al., *Identification of the SV40 agnogene product: a DNA binding protein.* Nature, 1981. 291(5813): p. 346-9.

359. Fischer, H. and G. Sauer, *Identification of virus-induced proteins in cells productively infected with simian virus 40.* Journal of virology, 1972. 9(1): p. 1-9.

360. Feng, H., et al., *Cellular and viral factors regulating merkel cell polyomavirus replication.* PloS one, 2011. 6(7): p. e22468.

361. Kwun, H.J., et al., *The minimum replication origin of merkel cell polyomavirus has a unique large T-antigen loading architecture and requires small T-antigen expression for optimal replication.* Journal of virology, 2009. 83(23): p. 12118-28.

362. Kwun, H.J., et al., *The minimum replication origin of merkel cell polyomavirus has a unique large T-antigen loading architecture and requires small T-antigen expression for optimal replication.* J Virol, 2009. 83(23): p. 12118-28.

363. Seo, G.J., C.J. Chen, and C.S. Sullivan, *Merkel cell polyomavirus encodes a microRNA with the ability to autoregulate viral gene expression.* Virology, 2009. 383(2): p. 183-7.

364. Lee, S., et al., *Identification and validation of a novel mature microRNA encoded by the Merkel cell polyomavirus in human Merkel cell carcinomas.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2011. 52(3): p. 272-5.

365. Liu, X., et al., *Merkel cell polyomavirus large T antigen disrupts lysosome clustering by translocating human vam6p from the cytoplasm to the nucleus.* J Biol Chem, 2011. 286(19): p. 17079-90.

366. Shuda, M., et al., *T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus.* Proc Natl Acad Sci U S A, 2008. 105(42): p. 16272-7.

367. Martel-Jantin, C., et al., *Genetic variability and integration of Merkel cell polyomavirus in Merkel cell carcinoma.* Virology, 2012. 426(2): p. 134-42.

368. Shuda, M., et al., *T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus.* Proceedings of the National Academy of Sciences of the United States of America, 2008. 105(42): p. 16272-7.

369. Nakamura, T., et al., *Nuclear localization of Merkel cell polyomavirus large T antigen in Merkel cell carcinoma.* Virology, 2010. 398(2): p. 273-9.

370. Kalderon, D., et al., *A short amino acid sequence able to specify nuclear location.* Cell, 1984. 39(3 Pt 2): p. 499-509.

371. Houben, R., et al., *Merkel cell polyomavirus-infected Merkel cell carcinoma cells require expression of viral T antigens.* J Virol, 2010. 84(14): p. 7064-72.

372. Shuda, M., et al., *Human Merkel cell polyomavirus small T antigen is an oncoprotein targeting the 4E-BP1 translation regulator.* The Journal of clinical investigation, 2011.

373. Houben, R., et al., *An intact retinoblastoma protein-binding site in Merkel cell polyomavirus large T antigen is required for promoting growth of Merkel cell carcinoma cells.* International journal of cancer. Journal international du cancer, 2012. 130(4): p. 847-56.

374. Griffin, B.E., et al., *Early mutants of polyoma virus (dl8 and dl23) with altered transformation properties: is polyoma virus middle T antigen a transforming gene product?* Cold Spring Harbor symposia on quantitative biology, 1980. 44 Pt 1: p. 271-83.

375. Hahn, W.C., et al., *Enumeration of the simian virus 40 early region elements necessary for human cell transformation.* Molecular and cellular biology, 2002. 22(7): p. 2111-23.

376. Treisman, R., et al., *Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein.* Nature, 1981. 292(5824): p. 595-600.

377. Carter, J.J., et al., *Association of Merkel cell polyomavirus-specific antibodies with Merkel cell carcinoma.* Journal of the National Cancer Institute, 2009. 101(21): p. 1510-22.

378. Chen, T., et al., *Serological evidence of Merkel cell polyomavirus primary infections in childhood.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2011. 50(2): p. 125-9.

379. Touze, A., et al., *Generation of Merkel cell polyomavirus (MCV)-like particles and their application to detection of MCV antibodies.* Journal of clinical microbiology, 2010. 48(5): p. 1767-70.

380. Tolstov, Y.L., et al., *Human Merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays.* International journal of cancer. Journal international du cancer, 2009. 125(6): p. 1250-6.

381. Viscidi, R.P., et al., *Age-specific seroprevalence of Merkel cell polyomavirus, BK virus, and JC virus.* Clinical and vaccine immunology : CVI, 2011. 18(10): p. 1737-43.

382. Faust, H., et al., *Antibodies to Merkel cell polyomavirus correlate to presence of viral DNA in the skin.* The Journal of infectious diseases, 2011. 203(8): p. 1096-100.

383. Pastrana, D.V., et al., *Positive correlation between Merkel cell polyomavirus viral load and capsid-specific antibody titer.* Medical microbiology and immunology, 2012. 201(1): p. 17-23.

384. Touze, A., et al., *High levels of antibodies against merkel cell polyomavirus identify a subset of patients with merkel cell carcinoma with better clinical outcome.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2011. 29(12): p. 1612-9.

385. Pastrana, D.V., et al., *Quantitation of human seroresponsiveness to Merkel cell polyomavirus.* PLoS pathogens, 2009. 5(9): p. e1000578.

386. Tolstov, Y.L., et al., *Asymptomatic Primary Merkel Cell Polyomavirus Infection among Adults.* Emerging infectious diseases, 2011. 17(8): p. 1371-80.

387. Paulson, K.G., et al., *Antibodies to merkel cell polyomavirus T antigen oncoproteins reflect tumor burden in merkel cell carcinoma patients.* Cancer research, 2010. 70(21): p. 8388-97.

388. Paulson, K.G., et al., *Transcriptome-wide studies of merkel cell carcinoma and validation of intratumoral CD8+ lymphocyte invasion as an independent predictor of survival.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2011. 29(12): p. 1539-46.

389. Iyer, J.G., et al., *Merkel cell polyomavirus-specific CD8 and CD4 T-cell responses identified in Merkel cell carcinomas and blood.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2011. 17(21): p. 6671-80.

390. Kumar, A., et al., *T-helper cell-mediated proliferation and cytokine responses against recombinant Merkel cell polyomavirus-like particles.* PloS one, 2011. 6(10): p. e25751.

391. Bialasiewicz, S., et al., *Merkel cell polyomavirus DNA in respiratory specimens from children and adults.* Emerging infectious diseases, 2009. 15(3): p. 492-4.

392. Bofill-Mas, S. and R. Girones, *Role of the environment in the transmission of JC virus.* Journal of neurovirology, 2003. 9 Suppl 1: p. 54-8.

393. Bofill-Mas, S., et al., *Newly described human polyomaviruses Merkel cell, KI and WU are present in urban sewage and may represent potential environmental contaminants.* Virology journal, 2010. 7: p. 141.

394. Loyo, M., et al., *Quantitative detection of Merkel cell virus in human tissues and possible mode of transmission.* International journal of cancer. Journal international du cancer, 2010. 126(12): p. 2991-6.

395. McQuaig, S.M., et al., *Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay.* Applied and environmental microbiology, 2006. 72(12): p. 7567-74.

396. Babakir-Mina, M., et al., *Identification of the novel KI and WU polyomaviruses in human tonsils.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2009. 46(1): p. 75-9.

397. Tan, C.S., et al., *Detection of JC virus DNA and proteins in the bone marrow of HIV-positive and HIV-negative patients: implications for viral latency and neurotropic transformation.* The Journal of infectious diseases, 2009. 199(6): p. 881-8.

398. Lopez, V., et al., *Does JC polyomavirus cause nephropathy in renal transplant patients?* Transplantation proceedings, 2010. 42(8): p. 2889-91.

399. Taheri, S., et al., *Comparison of polyomavirus (BK virus and JC viruses) viruria in renal transplant recipients with and without kidney dysfunction.* Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences, 2011. 16(7): p. 916-22.

400. Neumann, F., et al., *Replication, gene expression and particle production by a consensus Merkel Cell Polyomavirus (MCPyV) genome.* PloS one, 2011. 6(12): p. e29112.

401. Schowalter, R.M., D.V. Pastrana, and C.B. Buck, *Glycosaminoglycans and sialylated glycans sequentially facilitate Merkel cell polyomavirus infectious entry.* PLoS pathogens, 2011. 7(7): p. e1002161.

402. Wetzels, C.T., et al., *Ultrastructural proof of polyomavirus in Merkel cell carcinoma tumour cells and its absence in small cell carcinoma of the lung.* PloS one, 2009. 4(3): p. e4958.

403. Chang, Y. and P.S. Moore, *Merkel cell carcinoma: a virus-induced human cancer.* Annual review of pathology, 2012. 7: p. 123-44.

404. Leonard, J.H., et al., *Characterisation of four Merkel cell carcinoma adherent cell lines.* International journal of cancer. Journal international du cancer, 1995. 60(1): p. 100-7.

405. Rosen, S.T., et al., *Establishment and characterization of a neuroendocrine skin carcinoma cell line.* Lab Invest, 1987. 56(3): p. 302-12.

406. Fischer, N., et al., *Detection of Merkel cell polyomavirus (MCPyV) in Merkel cell carcinoma cell lines: cell morphology and growth phenotype do not reflect presence of the virus.* International journal of cancer. Journal international du cancer, 2010. 126(9): p. 2133-42.

407. Thirunarayanan, N., et al., *Enhanced tumorigenicity of fibroblasts transformed with human herpesvirus 8 chemokine receptor vGPCR by successive passage in nude and immunocompetent mice.* Oncogene, 2007.

408. Fernandez-Figueras, M.T., et al., *Expression profiles associated with aggressive behavior in Merkel cell carcinoma.* Mod Pathol, 2007. 20(1): p. 90-101.

409. Moore, P.S., et al., *Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma.* AIDS, 1996. 10(2): p. 175-80.

410. Poulin, D.L., A.L. Kung, and J.A. DeCaprio, *p53 targets simian virus 40 large T antigen for acetylation by CBP.* J Virol, 2004. 78(15): p. 8245-53.

411. Campbell, K.S., et al., *DnaJ/hsp40 chaperone domain of SV40 large T antigen promotes efficient viral DNA replication.* Genes Dev, 1997. 11(9): p. 1098-110.

412. Al-Uzri, A., D.M. Stablein, and A.C. R, *Posttransplant diabetes mellitus in pediatric renal transplant recipients: a report of the North American Pediatric Renal Transplant Cooperative Study (NAPRTCS).* Transplantation, 2001. 72(6): p. 1020-4.

413. Chan, J.K., et al., *Cytokeratin 20 immunoreactivity distinguishes Merkel cell (primary cutaneous neuroendocrine) carcinomas and salivary gland small cell carcinomas from small cell carcinomas of various sites.* Am J Surg Pathol, 1997. 21(2): p. 226-34.

414. Moll, R., et al., *Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies.* Am J Pathol, 1992. 140(2): p. 427-47.

415. Moll, R., D.L. Schiller, and W.W. Franke, *Identification of protein IT of the intestinal cytoskeleton as a novel type I cytokeratin with unusual properties and expression patterns.* J Cell Biol, 1990. 111(2): p. 567-80.

416. Kassem, A., et al., *Frequent Detection of Merkel Cell Polyomavirus in Human Merkel Cell Carcinomas and Identification of a Unique Deletion in the VP1 Gene.* Cancer Res, 2008. 68(13): p. 5009 - 5013.

417. Dorries, K., et al., *Infection of human polyomaviruses JC and BK in peripheral blood leukocytes from immunocompetent individuals.* Virology, 1994. 198(1): p. 59-70.

418. Tornatore, C., et al., *Detection of JC virus DNA in peripheral lymphocytes from patients with and without progressive multifocal leukoencephalopathy.* Ann Neurol, 1992. 31(4): p. 454-62.

419. Koljonen, V., et al., *Chronic lymphocytic leukaemia patients have a high risk of Merkel-cell polyomavirus DNA-positive Merkel-cell carcinoma.* British journal of cancer, 2009. 101(8): p. 1444-7.

420. Craig, P.J., et al., *Incidental chronic lymphocytic leukaemia in a biopsy of Merkel cell carcinoma.* Journal of cutaneous pathology, 2009. 36(6): p. 706-10.

421. Papageorgiou, K.I. and M.G. Kaniorou-Larai, *A case report of Merkel cell carcinoma on chronic lymphocytic leukemia: differential diagnosis of coexisting lymphadenopathy and indications for early aggressive treatment.* BMC cancer, 2005. 5: p. 106.

422. Becker, J.C., et al., *MC Polyomavirus Is Frequently Present in Merkel Cell Carcinoma of European Patients.* J Invest Dermatol, 2008.

423. Foulongne, V., et al., *Merkel cell polyomavirus and Merkel cell carcinoma, France.* Emerg Infect Dis, 2008. 14(9): p. 1491-3.

424. Diamandopoulos, G.T., *Leukemia, lymphoma, and osteosarcoma induced in the Syrian golden hamster by simian virus 40.* Science, 1972. 176(31): p. 173-5.

425. Pope, J.H. and W.P. Rowe, *Detection of Specific Antigen in Sv40-Transformed Cells by Immunofluorescence.* J Exp Med, 1964. 120: p. 121-8.

426. Allen, P.J., et al., *Merkel cell carcinoma: prognosis and treatment of patients from a single institution.* J Clin Oncol, 2005. 23(10): p. 2300-9.

427. Leong, A.S., et al., *Criteria for the diagnosis of primary endocrine carcinoma of the skin (Merkel cell carcinoma). A histological, immunohistochemical and ultrastructural study of 13 cases.* Pathology, 1986. 18(4): p. 393-9.

428. Plaza, J.A. and S. Suster, *The Toker tumor: spectrum of morphologic features in primary neuroendocrine carcinomas of the skin (Merkel cell carcinoma).* Annals of diagnostic pathology, 2006. 10(6): p. 376-85.

429. Ralston, J., L. Chiriboga, and D. Nonaka, *MASH1: a useful marker in differentiating pulmonary small cell carcinoma from Merkel cell carcinoma.* Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc, 2008. 21(11): p. 1357-62.

430. Walsh, N.M., *Primary neuroendocrine (Merkel cell) carcinoma of the skin: morphologic diversity and implications thereof.* Human pathology, 2001. 32(7): p. 680-9.

431. Aydin, A., et al., *Cutaneous undifferentiated small (Merkel) cell carcinoma, that developed synchronously with multiple actinic keratoses, squamous cell carcinomas and basal cell carcinoma.* The Journal of dermatology, 2003. 30(3): p. 241-4.

432. Iacocca, M.V., et al., *Mixed Merkel cell carcinoma and squamous cell carcinoma of the skin.* Journal of the American Academy of Dermatology, 1998. 39(5 Pt 2): p. 882-7.

433. Tang, C.K., et al., *Unusual cutaneous carcinoma with features of small cell (oat cell-like) and squamous cell carcinomas. A variant of malignant Merkel cell neoplasm.* The American Journal of dermatopathology, 1982. 4(6): p. 537-48.

434. Whitby, D., et al., *Detection of Kaposi's sarcoma-associated herpesvirus (KSHV) in peripheral blood of HIV-infected individuals predicts progression to Kaposi's sarcoma.* Lancet, 1995. 364: p. 799-802.

435. Sastre-Garau, X., et al., *Merkel cell carcinoma of the skin: pathological and molecular evidence for a causative role of MCV in oncogenesis.* J Pathol, 2009. 218(1): p. 48-56.

436. Laude, H.C., et al., *Distinct merkel cell polyomavirus molecular features in tumour and non tumour specimens from patients with merkel cell carcinoma.* PLoS pathogens, 2010. 6(8): p. e1001076.

437. Pastrana, D.V., et al., *Characterization of monoclonal antibodies specific for the Merkel cell polyomavirus capsid.* Virology, 2010. 405(1): p. 20-5.

438. Becker, J.C., et al., *MC polyomavirus is frequently present in Merkel cell carcinoma of European patients.* The Journal of investigative dermatology, 2009. 129(1): p. 248-50.

439. Dworkin, A.M., et al., *Merkel cell polyomavirus in cutaneous squamous cell carcinoma of immunocompetent individuals.* The Journal of investigative dermatology, 2009. 129(12): p. 2868-74.

440. Foulongne, V., et al., *Merkel cell polyomavirus DNA detection in lesional and nonlesional skin from patients with Merkel cell carcinoma or other skin diseases.* The British journal of dermatology, 2010. 162(1): p. 59-63.

441. Garneski, K.M., et al., *Merkel cell polyomavirus is more frequently present in North American than Australian Merkel cell carcinoma tumors.* The Journal of investigative dermatology, 2009. 129(1): p. 246-8.

442. Kassem, A., et al., *Merkel cell polyomavirus sequences are frequently detected in nonmelanoma skin cancer of immunosuppressed patients.* International journal of cancer. Journal international du cancer, 2009. 125(2): p. 356-61.

443. Mangana, J., et al., *Prevalence of Merkel cell polyomavirus among Swiss Merkel cell carcinoma patients.* Dermatology, 2010. 221(2): p. 184-8.

444. Mertz, K.D., et al., *Inflammatory monocytes are a reservoir for Merkel cell polyomavirus.* The Journal of investigative dermatology, 2010. 130(4): p. 1146-51.

445. Ridd, K., S. Yu, and B.C. Bastian, *The presence of polyomavirus in non-melanoma skin cancer in organ transplant recipients is rare.* The Journal of investigative dermatology, 2009. 129(1): p. 250-2.

446. Varga, E., et al., *Detection of Merkel cell polyomavirus DNA in Merkel cell carcinomas.* The British journal of dermatology, 2009. 161(4): p. 930-2.

447. Wieland, U., et al., *Merkel cell polyomavirus DNA in persons without merkel cell carcinoma.* Emerging infectious diseases, 2009. 15(9): p. 1496-8.

448. Foulongne, V., et al., *Merkel cell polyomavirus in cutaneous swabs.* Emerging infectious diseases, 2010. 16(4): p. 685-7.

449. Schowalter, R.M., et al., *Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin.* Cell Host Microbe, 2010. 7(6): p. 509-15.

450. Goh, S., et al., *Merkel cell polyomavirus in respiratory tract secretions.* Emerging infectious diseases, 2009. 15(3): p. 489-91.

451. Kantola, K., et al., *Merkel cell polyomavirus DNA in tumor-free tonsillar tissues and upper respiratory tract samples: implications for respiratory transmission and latency.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2009. 45(4): p. 292-5.

452. Sharp, C.P., et al., *Reactivation and mutation of newly discovered WU, KI, and Merkel cell carcinoma polyomaviruses in immunosuppressed individuals.* The Journal of infectious diseases, 2009. 199(3): p. 398-404.

453. Campello, C., et al., *A molecular case-control study of the Merkel cell polyomavirus in colon cancer.* Journal of medical virology, 2011. 83(4): p. 721-4.

454. Husseiny, M.I., et al., *A comparative study of Merkel cell, BK and JC polyomavirus infections in renal transplant recipients and healthy subjects.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2010. 49(2): p. 137-40.

455. Helmbold, P., et al., *Frequent occurrence of RASSF1A promoter hypermethylation and Merkel cell polyomavirus in Merkel cell carcinoma.* Molecular carcinogenesis, 2009. 48(10): p. 903-9.

456. Helmbold, P., et al., *Frequent hypermethylation of RASSF1A tumour suppressor gene promoter and presence of Merkel cell polyomavirus in small cell lung cancer.* European journal of cancer, 2009. 45(12): p. 2207-11.

457. Pantulu, N.D., et al., *Detection of a novel truncating Merkel cell polyomavirus large T antigen deletion in chronic lymphocytic leukemia cells.* Blood, 2010. 116(24): p. 5280-4.

458. Bhatia, K., et al., *Immunological detection of viral large T antigen identifies a subset of Merkel cell carcinoma tumors with higher viral abundance and better clinical outcome.* International journal of cancer. Journal international du cancer, 2010. 127(6): p. 1493-6.

459. Schrama, D., et al., *Merkel cell polyomavirus status is not associated with clinical course of merkel cell carcinoma.* The Journal of investigative dermatology, 2011. 131(8): p. 1631-8.

460. Dallaglio, K., A. Marconi, and C. Pincelli, *Survivin: a dual player in healthy and diseased skin.* The Journal of investigative dermatology, 2012. 132(1): p. 18-27.

461. Altieri, D.C., *Survivin and IAP proteins in cell-death mechanisms.* The Biochemical journal, 2010. 430(2): p. 199-205.

462. Lippens, S., et al., *Cell death in the skin.* Apoptosis : an international journal on programmed cell death, 2009. 14(4): p. 549-69.

463. Barrett, R.M., R. Colnaghi, and S.P. Wheatley, *Threonine 48 in the BIR domain of survivin is critical to its mitotic and anti-apoptotic activities and can be phosphorylated by CK2 in vitro.* Cell cycle, 2011. 10(3): p. 538-48.

464. Dohi, T., et al., *An IAP-IAP complex inhibits apoptosis.* The Journal of biological chemistry, 2004. 279(33): p. 34087-90.

465. Dohi, T., et al., *Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis.* The Journal of clinical investigation, 2004. 114(8): p. 1117-27.

466. Song, Z., X. Yao, and M. Wu, *Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis.* The Journal of biological chemistry, 2003. 278(25): p. 23130-40.

467. Bongiovanni, L., E.J. Muller, and L. Della Salda, *Survivin in skin pathologies.* Experimental dermatology, 2011. 20(6): p. 457-63.

468. Adams, R.R., M. Carmena, and W.C. Earnshaw, *Chromosomal passengers and the (aurora) ABCs of mitosis.* Trends in cell biology, 2001. 11(2): p. 49-54.

469. Rosa, J., et al., *Survivin modulates microtubule dynamics and nucleation throughout the cell cycle.* Molecular biology of the cell, 2006. 17(3): p. 1483-93.

470. O'Connor, D.S., et al., *Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin.* Proceedings of the National Academy of Sciences of the United States of America, 2000. 97(24): p. 13103-7.

471. Colnaghi, R., et al., *Separating the anti-apoptotic and mitotic roles of survivin.* The Journal of biological chemistry, 2006. 281(44): p. 33450-6.

472. Fortugno, P., et al., *Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function.* Journal of cell science, 2002. 115(Pt 3): p. 575-85.

473. Khan, S., et al., *Extracellular, cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential.* British journal of cancer, 2009. 100(7): p. 1073-86.

474. Ahn, J.K., et al., *Increased extracellular survivin in the synovial fluid of rheumatoid arthritis patients: fibroblast-like synoviocytes as a potential source of extracellular survivin.* Inflammation, 2010. 33(6): p. 381-8.

475. Raj, D., et al., *Survivin repression by p53, Rb and E2F2 in normal human melanocytes.* Carcinogenesis, 2008. 29(1): p. 194-201.

476. Altieri, D.C., *Survivin, cancer networks and pathway-directed drug discovery.* Nature reviews. Cancer, 2008. 8(1): p. 61-70.

477. Mirza, A., et al., *Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway.* Oncogene, 2002. 21(17): p. 2613-22.

478. Fortugno, P., et al., *Regulation of survivin function by Hsp90.* Proceedings of the National Academy of Sciences of the United States of America, 2003. 100(24): p. 13791-6.

479. Adida, C., et al., *Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation.* The American journal of pathology, 1998. 152(1): p. 43-9.

480. Altieri, D.C., *Validating survivin as a cancer therapeutic target.* Nat Rev Cancer, 2003. 3(1): p. 46-54.

481. Grossman, D., et al., *Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma.* The Journal of investigative dermatology, 1999. 113(6): p. 1076-81.

482. Kawasaki, H., et al., *Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer.* Cancer research, 1998. 58(22): p. 5071-4.

483. Lu, C.D., D.C. Altieri, and N. Tanigawa, *Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas.* Cancer research, 1998. 58(9): p. 1808-12.

484. Monzo, M., et al., *A novel anti-apoptosis gene: Re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 1999. 17(7): p. 2100-4.

485. Saitoh, Y., Y. Yaginuma, and M. Ishikawa, *Analysis of Bcl-2, Bax and Survivin genes in uterine cancer.* International journal of oncology, 1999. 15(1): p. 137-41.

486. Satoh, K., et al., *Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors.* Cancer, 2001. 92(2): p. 271-8.

487. Swana, H.S., et al., *Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer.* The New England journal of medicine, 1999. 341(6): p. 452-3.

488. Tanaka, K., et al., *Expression of survivin and its relationship to loss of apoptosis in breast carcinomas.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2000. 6(1): p. 127-34.

489. Yoshida, H., et al., *Survivin, bcl-2 and matrix metalloproteinase-2 enhance progression of clear cell- and serous-type ovarian carcinomas.* International journal of oncology, 2001. 19(3): p. 537-42.

490. Marusawa, H., et al., *HBXIP functions as a cofactor of survivin in apoptosis suppression.* The EMBO journal, 2003. 22(11): p. 2729-40.

491. Borbely, A.A., et al., *Effects of human papillomavirus type 16 oncoproteins on survivin gene expression.* The Journal of general virology, 2006. 87(Pt 2): p. 287-94.

492. Bowen, A.R., et al., *Proliferation, apoptosis, and survivin expression in keratinocytic neoplasms and hyperplasias.* The American Journal of dermatopathology, 2004. 26(3): p. 177-81.

493. Gualco, E., et al., *IGF-IR-dependent expression of Survivin is required for T-antigen-mediated protection from apoptosis and proliferation of neural progenitors.* Cell death and differentiation, 2010. 17(3): p. 439-51.

494. Kim, J. and J.M. McNiff, *Nuclear expression of survivin portends a poor prognosis in Merkel cell carcinoma.* Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc, 2008. 21(6): p. 764-9.

495. Tucci, M.G., et al., *Immunohistochemical study of apoptosis markers and involvement of chemokine CXCR4 in skin Merkel cell carcinoma.* J Eur Acad Dermatol Venereol, 2006. 20(10): p. 1220-5.

496. Altieri, D.C., *Targeting Survivin In Cancer.* Cancer letters, 2012.

497. Ryan, B.M., N. O'Donovan, and M.J. Duffy, *Survivin: a new target for anti-cancer therapy.* Cancer treatment reviews, 2009. 35(7): p. 553-62.

498. Houben, R., et al., *Merkel cell polyomavirus-infected Merkel cell carcinoma cells require expression of viral T antigens.* Journal of virology, 2010. 84(14): p. 7064-72.

499. Ronan, S.G., et al., *Merkel cell carcinoma: in vitro and in vivo characteristics of a new cell line.* J Am Acad Dermatol, 1993. 29(5 Pt 1): p. 715-22.

500. Wright, C.M., et al., *Inhibition of Simian Virus 40 replication by targeting the molecular chaperone function and ATPase activity of T antigen.* Virus Res, 2009. 141(1): p. 71-80.

501. Seguin, S.P., et al., *High-Throughput Screening Identifies a Bisphenol Inhibitor of SV40 Large T Antigen ATPase Activity.* Journal of biomolecular screening, 2011.

502. Czarna, A., et al., *Robust generation of lead compounds for protein-protein interactions by computational and MCR chemistry: p53/Hdm2 antagonists.* Angewandte Chemie, 2010. 49(31): p. 5352-6.

503. Popowicz, G.M., et al., *Structures of low molecular weight inhibitors bound to MDMX and MDM2 reveal new approaches for p53-MDMX/MDM2 antagonist drug discovery.* Cell cycle, 2010. 9(6): p. 1104-11.

504. Shuda, M., et al., *Human Merkel cell polyomavirus small T antigen is an oncoprotein targeting the 4E-BP1 translation regulator.* The Journal of clinical investigation, 2011. 121(9): p. 3623-34.

505. Houben, R., et al., *Comparable expression and phosphorylation of the retinoblastoma protein in Merkel cell polyoma virus-positive and negative Merkel cell carcinoma.* Int J Cancer, 2010. 126(3): p. 796-8.

506. Shuda, M., et al., *Human Merkel cell polyomavirus infection I. MCV T antigen expression in Merkel cell carcinoma, lymphoid tissues and lymphoid tumors.* International journal of cancer. Journal international du cancer, 2009. 125(6): p. 1243-9.

507. Nakahara, T., et al., *YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts.* Cancer research, 2007. 67(17): p. 8014-21.

508. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic acids research, 2001. 29(9): p. e45.

509. Shen, W., et al., *Two novel heterozygous mutations of EVC2 cause a mild phenotype of Ellis-van Creveld syndrome in a Chinese family.* American journal of medical genetics. Part A, 2011.

510. Ashburner, M., et al., *Gene ontology: tool for the unification of biology. The Gene Ontology Consortium.* Nature genetics, 2000. 25(1): p. 25-9.

511. Ohtani, K., J. DeGregori, and J.R. Nevins, *Regulation of the cyclin E gene by transcription factor E2F1.* Proceedings of the National Academy of Sciences of the United States of America, 1995. 92(26): p. 12146-50.

512. Iwasa, T., et al., *Marked anti-tumour activity of the combination of YM155, a novel survivin suppressant, and platinum-based drugs.* Br J Cancer, 2010. 103(1): p. 36-42.

513. Tolcher, A.W., et al., *A phase II study of YM155, a novel small-molecule suppressor of survivin, in castration-resistant taxane-pretreated prostate cancer.* Annals of oncology : official journal of the European Society for Medical Oncology / ESMO, 2011.

514. Tu, S.P., et al., *Suppression of survivin expression inhibits in vivo tumorigenicity and angiogenesis in gastric cancer.* Cancer research, 2003. 63(22): p. 7724-32.

515. Zhang, R., et al., *Survivin knockdown by short hairpin RNA abrogates the growth of human hepatocellular carcinoma xenografts in nude mice.* Cancer gene therapy, 2010. 17(4): p. 275-88.

516. Klionsky, D.J., A.M. Cuervo, and P.O. Seglen, *Methods for monitoring autophagy from yeast to human.* Autophagy, 2007. 3(3): p. 181-206.

517. Sarek, G., et al., *Reactivation of the p53 pathway as a treatment modality for KSHV-induced lymphomas.* J Clin Invest, 2007. 117(4): p. 1019-28.

518. Petre, C.E., S.H. Sin, and D.P. Dittmer, *Functional p53 signaling in Kaposi's sarcoma-associated herpesvirus lymphomas: implications for therapy.* J Virol, 2007. 81(4): p. 1912-22.

519. Einsele, H., *Bortezomib.* Recent results in cancer research. Fortschritte der Krebsforschung. Progres dans les recherches sur le cancer, 2010. 184: p. 173-87.

520. LeBlanc, R., et al., *Proteasome inhibitor PS-341 inhibits human myeloma cell growth in vivo and prolongs survival in a murine model.* Cancer research, 2002. 62(17): p. 4996-5000.

521. Nakahara, T., et al., *Broad spectrum and potent antitumor activities of YM155, a novel small-molecule survivin suppressant, in a wide variety of human cancer cell lines and xenograft models.* Cancer science, 2011. 102(3): p. 614-21.

522. Nakahara, T., et al., *Broad spectrum and potent antitumor activities of YM155, a novel small-molecule survivin suppressant, in a wide variety of human cancer cell lines and xenograft models.* Cancer Sci, 2011. 102(3): p. 614-21.

523. Nakahara, T., et al., *YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts.* Cancer Res, 2007. 67(17): p. 8014-21.

524. Ambrosini, G., C. Adida, and D.C. Altieri, *A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma.* Nature medicine, 1997. 3(8): p. 917-21.

525. Takeuchi, H., et al., *Survivin expression by metastatic melanoma predicts poor disease outcome in patients receiving adjuvant polyvalent vaccine.* International journal of cancer. Journal international du cancer, 2005. 117(6): p. 1032-8.

526. Pina-Oviedo, S., et al., *Effects of JC virus infection on anti-apoptotic protein survivin in progressive multifocal leukoencephalopathy.* Am J Pathol, 2007. 170(4): p. 1291-304.

527. Jiang, Y., et al., *Aberrant regulation of survivin by the RB/E2F family of proteins.* The Journal of biological chemistry, 2004. 279(39): p. 40511-20.

528. Kroemer, G. and B. Levine, *Autophagic cell death: the story of a misnomer.* Nature reviews. Molecular cell biology, 2008. 9(12): p. 1004-10.

529. Santhanam, A.N., et al., *Role of 3'UTRs in the translation of mRNAs regulated by oncogenic eIF4E--a computational inference.* PloS one, 2009. 4(3): p. e4868.

530. Bouvard, V., et al., *Carcinogenicity of malaria and of some polyomaviruses.* The Lancet Oncology, 2012.

531. Jiang, Y., et al., *Aberrant regulation of survivin by the RB/E2F family of proteins.* J Biol Chem, 2004. 279(39): p. 40511-20.

532. Blum, B., et al., *The anti-apoptotic gene survivin contributes to teratoma formation by human embryonic stem cells.* Nature biotechnology, 2009. 27(3): p. 281-7.

533. Fukuda, S. and L.M. Pelus, *Survivin, a cancer target with an emerging role in normal adult tissues.* Molecular cancer therapeutics, 2006. 5(5): p. 1087-98.

534. Altieri, D.C., *Survivin, cancer networks and pathway-directed drug discovery.* Nat Rev Cancer, 2008. 8(1): p. 61-70.

535. Weinstein, I.B. and A.K. Joe, *Mechanisms of disease: Oncogene addiction--a rationale for molecular targeting in cancer therapy.* Nature clinical practice. Oncology, 2006. 3(8): p. 448-57.

536. Shen, S., O. Kepp, and G. Kroemer, *The end of autophagic cell death?* Autophagy, 2012. 8(1): p. 1-3.

537. Daly, C., et al., *Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1).* Genes & development, 2004. 18(9): p. 1060-71.

538. Kim, P.J., et al., *Survivin and molecular pathogenesis of colorectal cancer.* Lancet, 2003. 362(9379): p. 205-9.

539. Lee, C.W., et al., *Molecular dependence of estrogen receptor-negative breast cancer on a notch-survivin signaling axis.* Cancer research, 2008. 68(13): p. 5273-81.

540. O'Connor, D.S., et al., *Control of apoptosis during angiogenesis by survivin expression in endothelial cells.* The American journal of pathology, 2000. 156(2): p. 393-8.

541. Tran, J., et al., *A role for survivin in chemoresistance of endothelial cells mediated by VEGF.* Proceedings of the National Academy of Sciences of the United States of America, 2002. 99(7): p. 4349-54.

542. Giaccone, G., et al., *Multicenter phase II trial of YM155, a small-molecule suppressor of survivin, in patients with advanced, refractory, non-small-cell lung cancer.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2009. 27(27): p. 4481-6.

543. Lewis, K.D., et al., *A multi-center phase II evaluation of the small molecule survivin suppressor YM155 in patients with unresectable stage III or IV melanoma.* Investigational new drugs, 2011. 29(1): p. 161-6.

544. O'Connor, D.S., et al., *A p34(cdc2) survival checkpoint in cancer.* Cancer cell, 2002. 2(1): p. 43-54.

545. Yamanaka, K., et al., *Antitumor activity of YM155, a selective small-molecule survivin suppressant, alone and in combination with docetaxel in human malignant melanoma models.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2011. 17(16): p. 5423-31.

546. Nakahara, T., et al., *YM155, a novel survivin suppressant, enhances taxane-induced apoptosis and tumor regression in a human Calu 6 lung cancer xenograft model.* Anti-cancer drugs, 2011. 22(5): p. 454-62.

547. Fields, R.C., et al., *Five hundred patients with Merkel cell carcinoma evaluated at a single institution.* Annals of surgery, 2011. 254(3): p. 465-73; discussion 473-5.