MIRNA PROFILING OF TUMOR-DERIVED EXOSOMES

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Cancers and infectious diseases are becoming a growing public health problem in the world today. The importance of my work for public health is in detecting these diseases earlier and more accurately, potentially leading to better therapies and higher survival rates for patients. Current diagnostic techniques focus on detecting antibodies from serum, gene expression and miRNA profiles of tumor tissues and, more recently, in the bodily fluids of patients.

This dissertation shows a novel technique that makes use of small microvesicles called exosomes and the microRNAs (miRNAs) they carry for the potential diagnosis of cancer. Exosomes are small (40-100 nm) membrane-bound vesicles that are created from the inverse budding of the multivesicular endosome and originate from a variety of tumor types. Exosomes can be easily purified from cell cultures and serum of patients and have recently been shown to carry small non-coding RNAs called miRNAs. In the first part of this study, I developed techniques that enabled us to increase the amount of our exosome and total RNA starting material before proceeding to use these as potential diagnostics for head and neck cancers.

Although the use of exosomes to diagnose diseases is not novel, the use of miRNAs present in tumor-derived exosomes is a new approach. In the final two chapters, I discuss the use of exosomes to diagnose KSHV viral infections as well as head and neck cancer. Increasing the accuracy and reducing the amount of starting material needed for these studies would provide a non-invasive technique to detect viral infections and cancers. This would help in providing earlier therapeutic treatments and help to increase the longevity and quality of life of patients.

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NOMENCLATURE

KSHV: Kaposi Sarcoma associated virus.

HCMV: Human cytomegalovirus

EBV: Epstein Barr Virus

DC: Dendritic Cells

DMEM: Dulbecco's modified eagle medium

RPMI: Roswell Park Memorial Institute

PBS: phosphate buffered saline

Rpm: revolutions per minute

qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction

miRNA: microRNA

mRNA: messenger RNA

DNA: deoxyribonucleic acid

RNA: ribonucleic acid

MHC: major histocompatibility complex

TEM: transmission electron microscopy

HNC: head and neck cancer

PREFACE

I would like to dedicate this thesis to a dear friend of mine, Michel-André Cardin, who unfortunately could not be with us for this moment. He would have cherished being present for my defense. I would like to express my thanks to my advisor, Dr. Paul Robbins, for his guidance and patience throughout this process. I have matured as a scientist and a person thanks to his efforts. I would also like to thank the members of my committee, Eleanor Feingold, Robert Ferrell, and Saleem Khan, for their support throughout this process.

Thanks to the members of the Robbins lab for their friendship, guidance, and help with keeping my cells alive when I was sick, in particular Ja'Nean, Daniel, Jeremey, Melanie, Maliha, Joan, Dan, Chenjie, Khaleel, Nicole, and Vaughn. And finally, to my late puppy Bear, for always putting a smile on my face, no matter what the circumstances; you are missed dearly.

1.0 SCOPE OF PROBLEM

Cancers and other diseases caused by viruses comprise a large number of the health issues that are plaguing our society today. The number of deaths resulting from cancers is second only to heart disease in the United States, with 600,000 new deaths each year (1). Current research avenues dedicated to increasing the survival rates of patients approach the disease problem in two ways.

The first approach is perfecting the diagnostic procedure because it has been shown that the earlier that cancers are detected, the better the chance of treating them and the better the quality of life of the patient. Diagnostic tests are also being perfected to increase accuracy and to lower the rate of false positives. Current diagnostic approaches use analysis of rare, circulating tumor cells in serum, sequencing of serum-circulating nucleic acids, antibody testing, and genetic testing for predisposition to diseases.

The second avenue is to increase the number and efficacies of therapeutic agents against cancers. Many different approaches are being pursued, such as antibody targeting, viral targeting, and vaccines. Chemotherapy and surgery are still the two most commonly used treatments. Following a combination of early detection and new therapies is helping to extend the current lifespan of patients and to bring us in the direction of finding a cure for the most common and deadly cancers.

The research discussed in this document is based on the study of microvesicles called exosomes, which have the potential of being used as both cancer diagnostic and therapeutic tools. Most of this document focuses on the diagnostic potential of exosomes in detecting certain viral infections or cancers early enough to be able to intervene with meaningful therapeutic agents and save lives.

1.1 EXOSOMES: BACKGROUND AND POTENTIAL.

Exosomes are microvesicles that play a large part in how the immune system perceives and reacts to immunological stress, such as cancers and viral infections. Exosomes were first discovered during the differentiation of reticulocytes in sheep and thought to be a mechanism for the cell to rid itself of harmful or excess proteins (2). The exosome field has matured significantly since that initial discovery and now recognizes exosomes as a means for cell-to-cell contact and information transfer. Also important is the fact that patients diagnosed with cancers and infections also appear to have higher levels of exosomes in their serum, although the source of the exosomes is still unclear.

Exosomes are 40-100 nm long microvesicles derived from most hematopoietic cells and many types of cancers (3). They are formed from the fusion of multivesicular bodies (MVB) with the plasma membrane. Sorting of membrane proteins into this pathway usually occurs by the ubiquitination of the cytoplasmic domains and recognition of this sequence by the endosomal sorting complex required for transport machinery (ESCRT) (4). The ESCRT complex is then recycled to aid more cargo to reach its destination. In addition, some proteins are found in ILVs that are not ubiquitinated, such as transferrin receptors that are sorted due to their physical

structures and preferences to raft-like microdomains. Much of the focus on exosomes has been on their composition, which is comprised of lipid rafts, cytoskeleton proteins, membrane trafficking proteins, transmembrane molecules, T cell-stimulating molecules, signaling molecules, and recently, mRNA and miRNAs. Exosomes can be either immunostimulatory or immunosuppressive, depending on the cells they originate from, since they retain much of the protein composition of their parent cells.

Immunostimulatory exosomes are known to interact directly with CD4+ or CD8+ T cells, which can lead to activation in a major histocompatibility complex (MHC) peptide-restricted manner (5). Both MHC I and MHC II molecules have been found on the surface of exosomes derived from antigen presenting cells (APC). Several reports show that exosomes require dendritic cells (DC) to prime naïve CD8+ T cells in vivo. Following the discovery of immunostimulatory exosome function, researchers attempted to create exosome vaccines (6-8). This was done in two ways: the first was to engineer DCs to produce antigen-presenting exosomes (6, 9), and the second method was to pulse peptides specific to an antigen onto exosomes in order to trigger a cytotoxic T lymphocyte response (10-12). These findings have led to several clinical trials. The first vaccine trial was conducted in 1998 with melanoma patients (13, 14), followed with another clinical trial reported in 2005 in collaboration between the Institut Curie and the Institut Gustave-Roussey (15). In this trial, exosomes were processed from in vitro pulsed tumor antigens in order to trigger tumor rejection. The melanoma antigen MAGE3 was pulsed onto DC and then the DC-derived exosomes were administered to patients. No toxicity was seen, while a positive regression of skin tumors and a reduction in metastases occurred in several patients.

Tumor cells also produce high levels of exosomes that are able to regulate the immune response and modulate the tumor microenvironment. Tumor-derived exosomes are one type of immunosuppressive exosomes that are known and are suggested to play a large role in shaping the suppression of immune responses towards their progenitor tumors. For example, exosomes were shown to carry Fas ligand (FasL) (16) and NKG2D (17-20), which promote T cell apoptosis, natural killer cell activation and proliferation, respectively. In fact, a filter has recently been developed, termed the Hemopurifier^{TM} (21), which replaces a filter in a dialysis machine. The idea behind the Hemopurifier was to remove immunosuppressive exosomes from the bloodstream in order to cause a marked improvement in the immune system's ability to detect and reject tumors. In the initial clinical trial, six out of the 16 patients had a reduction of their lesions by 50% or more. While the study was meant as a proof of concept, it suggests that by using a combination of an exosome vaccine and the Hemopurifier^{TM} for therapy, patients could see large improvements in the long-term survival from certain cancers and possibly other exosome-related diseases. A few examples of both immunostimulatory and immunosuppressive exosomes can be seen in Figure 1.

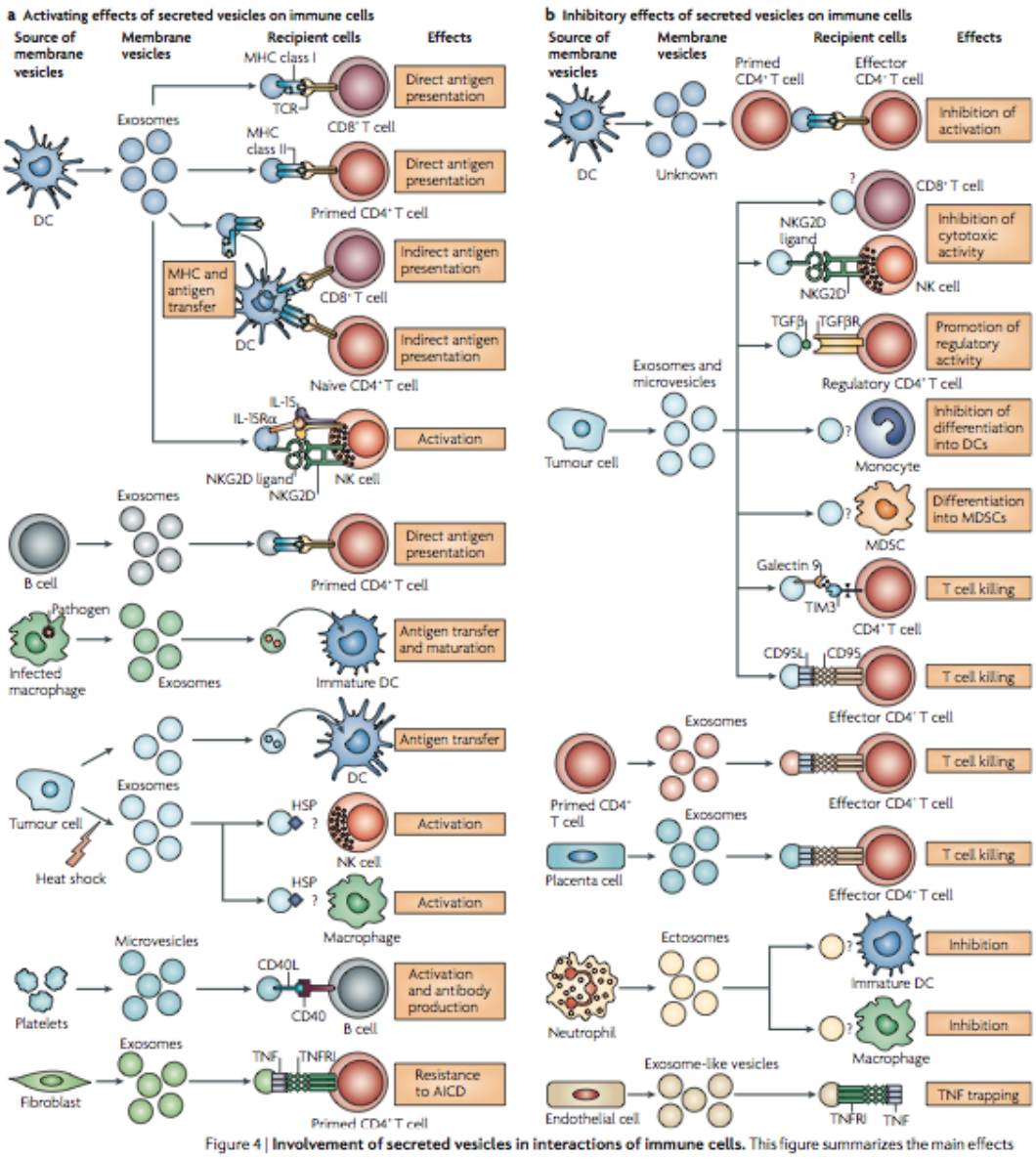


Figure 4 | **Involvement of secreted vesicles in interactions of immune cells.** This figure summarizes the main effects

Figure 1: Involvement of secreted vesicles in interactions of immune cells.(22)

The recent discovery of genetic material in exosomes in the form of messenger RNA and miRNA (23) strengthens the argument that exosomes are important for cell-to-cell signaling, both in the form of protein transfers but also now in the form of a genetic transfer between the cells of the immune system. Cell-to-cell communication is central to how exosomes function and how pathogens or cancers use them to evade the immune system since microvesicles are known to transfer biologically active cargo between cells(24). Exosomes exert their effect on cells in two different ways. First, they can interact with a target cell surface, resulting in downstream signaling. This has been observed with DC-derived exosomes containing MHC-bound peptides that are able to stimulate T cells(25-28). Alternatively, exosomes are endocytosed by their target cells followed by the release of their protein and RNA cargo, which can directly affect cellular gene expression. An example of this would be Nef-1, which is secreted by HIV-1 infected T cell-derived exosomes and internalized by bystander CD4+T cells, thus causing apoptosis(29). Another example of an exchange of protein is gliomas that often express a truncated form of EGFRvIII that is not produced by all of the tumor cells. Microvesicles containing this receptor are released into the surroundings and can then merge with the plasma membrane of the surrounding tumors that are lacking this receptor (30). This can transfer oncogenic activity into the receiving cells by activating MAPK and morphological changes. This is but one example of how exosomes can transfer proteins and change the morphology of the target cells to better suit their environment. DC-derived exosomes contain many important regulatory molecules, such as MHC class I and II as well as adhesion molecules that may target exosomes to their specific cells. MHC Class II-dependent exosomes containing Fas Ligand (FasL) are capable of reducing paw swelling in the DTH model (31). While no mechanistic information is available on how RNA gets into exosomes, much of the literature in this field is currently dedicated to creating

RNA profiles of tumor-derived exosomes. As stated previously, better cancer diagnostic scientists would lead to earlier detection of disease as well as increased information on the stage of disease. As evidenced by miRNA profiling of exosomes, cancer diagnosis using this approach may be on the horizon. By pulling down exosomes with disease-specific antibodies and coupling this with RNA profiling, the field of diagnosis of various diseases is expected to become more accurate in the coming years.

1.1.1 Exosomes and cancer

Much of the research performed on exosomes from cancer patients has dealt with their protein and RNA components. It is becoming increasingly clear that microvesicles can modulate target cells through cell-to-cell interactions, with the transfer of both proteins and RNA, including mRNA and miRNA, to neighboring cells. This information transfer can change the pattern of gene expression of the target cells to induce cell signaling (32-34). A growing body of evidence points to the fact that exosomes play a role in the spread of cancers, and in many cases, in the suppression of immune system-triggered responses to cancer. One cause of this is the cellular stress response to exosomes. It was recently shown that a p53-regulated gene product, TSAP6, was capable of enhancing exosome production during a p53 response to stress (35). This could be one of many reasons why tumors produce higher levels of exosomes when compared to non-tumorgenic cells.

Tumor-derived exosomes can also change the phenotype of the cells they are targeted to and can change the differentiation pathway of T cells that favors tumor growth. An example of how tumor exosomes affect their environment is myeloid-derived suppressor cells (MDSCs). Tumor-derived exosomes are in part taken up by bone marrow myeloid cells and have now been

shown to switch the differentiation pathway of these cells towards the MDSC pathway (CD11b+ Gr-1+) (36). It was further shown that these MDSC-mediated tumor-promoting effects were specific to tumor-derived exosomes and lend to the belief that specific factors (in particular PGE2 and TGF-β) are enriched in these exosomes. Figure 2 shows several roles for microvesicles in cancer.

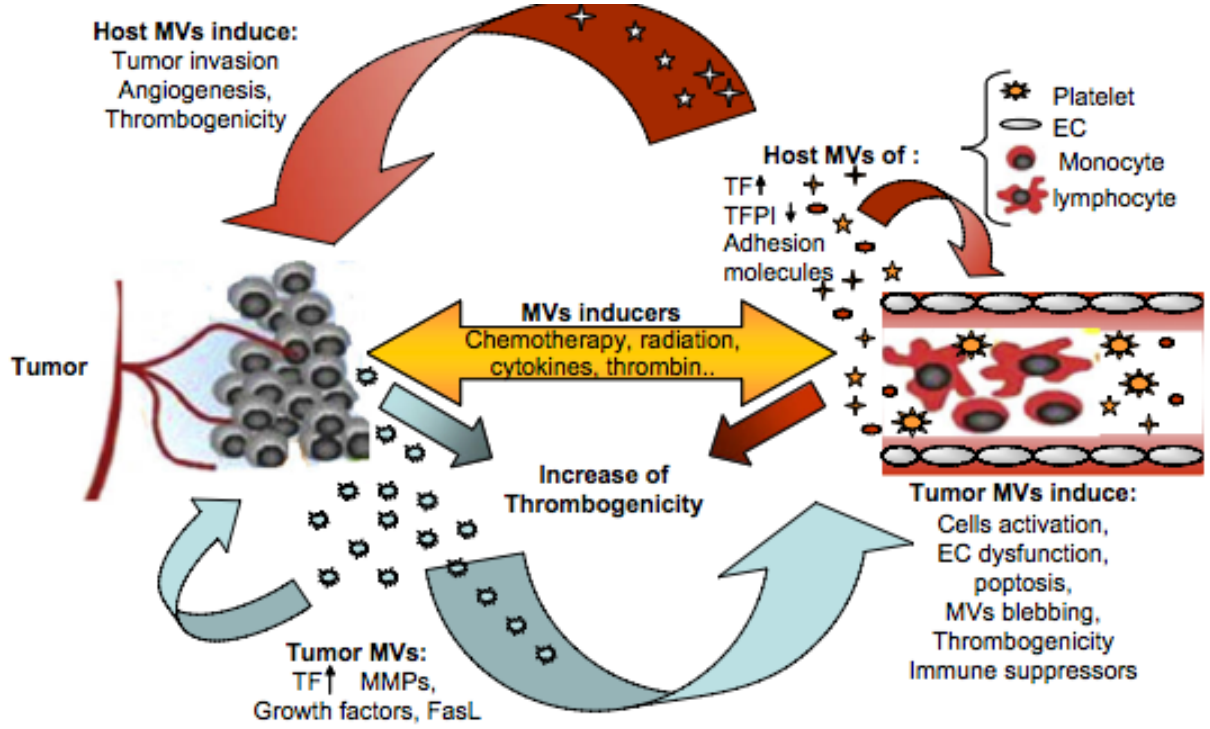


Figure 2: The role of microvesicles (MVs) in cancer (37).

Tumors usually form microenvironments that help to further their survival; for example, by altering the pH surrounding the tumors, which plays a key factor in exosome trafficking (38). Exosomes from cancer cells have been shown to influence angiogenesis by transferring growth factors such as the truncated epidermal growth factor receptor (EGFRvIII), which is then circulated and transferred to cancer cells that are lacking this receptor (30, 39-41). This transfer promotes the increased vascularization of the tumor, thus helping tumors to become more metastatic. It is generally thought that exosomes derived from cancers exhibit many of the same

cellular characteristics of their parent cells and help to further tumor survival, growth and metastasis.

Another example of how exosomes affect their environments is through the presence of pro-apoptotic proteins on the surface of exosomes that can induce T cell death. For example, certain tumor-derived exosomes express FasL, and upon encountering activated (Fas-Positive) T cells, they can trigger the apoptotic death of those cells. In exosomes derived from patients with oral cancer, a high level of FasL was correlated with the apoptosis of T cells in these patients (16). MHC Class II+ exosomes in plasma can also suppress inflammation in a Fas Ligand/Fas-dependent manner. These CD11b+, CD11c-, FasL+ exosomes were capable of reducing swelling in a delayed-type hypersensitivity (DTH) model in mice (31). Furthermore, genetically engineered bone marrow-derived DCs that express IL-4+ exosomes were able to decrease the severity of collagen-induced arthritis and inhibit DTH in mice.

Human placental-derived exosomes have been shown to be important for blocking the immune response to allogeneic fetus. These exosomes originated from human placenta and were shown to contain NKG2D, which serves to down-regulate the NKG2D receptor on NK, CD8+, and αδ T cells to reduce in-vitro cytotoxicity(19). This process is thought to play a major role in the fetal immune escape mechanism and enforces the view that placenta, through the release of exosomes, have an immunosuppressive role. Human tumor-derived exosomes have also been found to down-modulate NKG2D expression (17). Exosomes produced by several cancer cell lines triggered down-regulation of surface NKG2D expression by NK and CD8+ T cells, which resulted from sustained interaction with the target cells. Placental-derived exosomes also induce T cells apoptosis through the expression of FasL and PD-L1. The recent discovery of RNA in exosomes suggests that they may play a role in mediating the biological effects of exosomes.

1.2 CELLULAR FUNCTIONS OF MIRNA

MicroRNAs (miRNA) are part of a growing family of small non-coding RNAs, which include snoRNAs, siRNAs, piRNAs, qiRNAs, and tasi-RNAs. MiRNAs were discovered in 1993 by Victor Ambros et al. when a small RNA encoded by the lin-4 gene was shown to play an important role in the developmental timing of Caenorhabditis elegans(42). This small RNA was thought to modulate the lin-14 protein. It was not until several years later that researchers discovered the presence of miRNAs in a variety of other species, including Homo Sapiens, and their importance in biological regulatory systems became apparent. MiRNAs are highly conserved across many species and play an important role in the biological viability of many organisms from plants to mammals. The machinery involved in the maturation of miRNAs is highly conserved, which led many to believe that miRNAs play a significant evolutionary role. Interestingly, while mature miRNA sequences may be conserved, the targets may not necessarily be conserved in nature. MiRNA genes represent only 1% of the genome in some species, but it is widely thought that 30% of the genome is regulated by at least one miRNA. They have also been implicated in a wide variety of functions, including but not limited to development, proliferation, differentiation, apoptosis, stress response, and T cell maturation, which will be described later.

MiRNAs are small noncoding RNAs that are approximately 22 nt in length. The majority of miRNAs are transcribed by the Polymerase II machinery into a primary RNA transcript called pri-miRNA. These pri-miRNA transcripts can be several kilobases long, are 5’ capped and contain a poly-A tail. Transcription can occur either from an intronic or an exonic region and can result either in a single pri-miRNA or a long polycistronic transcript encoding several miRNAs. This polycistronic transcription has been well documented in the case of the miR-17-92 miRNA cluster (43-45). Once the pri-miRNAs are synthesized, they are cleaved by a combination of an

RNase III-like enzyme named Drosha and the RNA-binding protein DGCR8 into a smaller transcript called pre-miRNA. The pre-miRNAs are between 70-100 nt in size and contain the mature miRNA hairpin. The complete pre-miRNA is transported out of the nucleus to the cytoplasm by exportin-5, involving the RAN-GTP pathway. Once in the cytoplasm, the RNase II enzyme dicer cleaves the pre-miRNA, leaving a mature miRNA that is approximately 22 nt long, which is incorporated into the protein complex RISC which contains Argonaute 2 (AGO2) as well as several other proteins. At this stage, one RNA strand is degraded, leaving only one strand to guide the RISC complex to its target mRNA. MiRNAs have been shown to either repress translation or degrade the mRNA by binding to their 3' UTR. With complete base pair complementarity, the target is usually degraded, while imperfect pairing results in the inhibition of mRNA translation. Over 90% of miRNA so far have been shown to repress translation of mRNA rather than degrade it, and new research indicates that repression happens predominantly in organelles called GW bodies or P-bodies (46). Figure 3 shows an example of an miRNA maturation pathway.

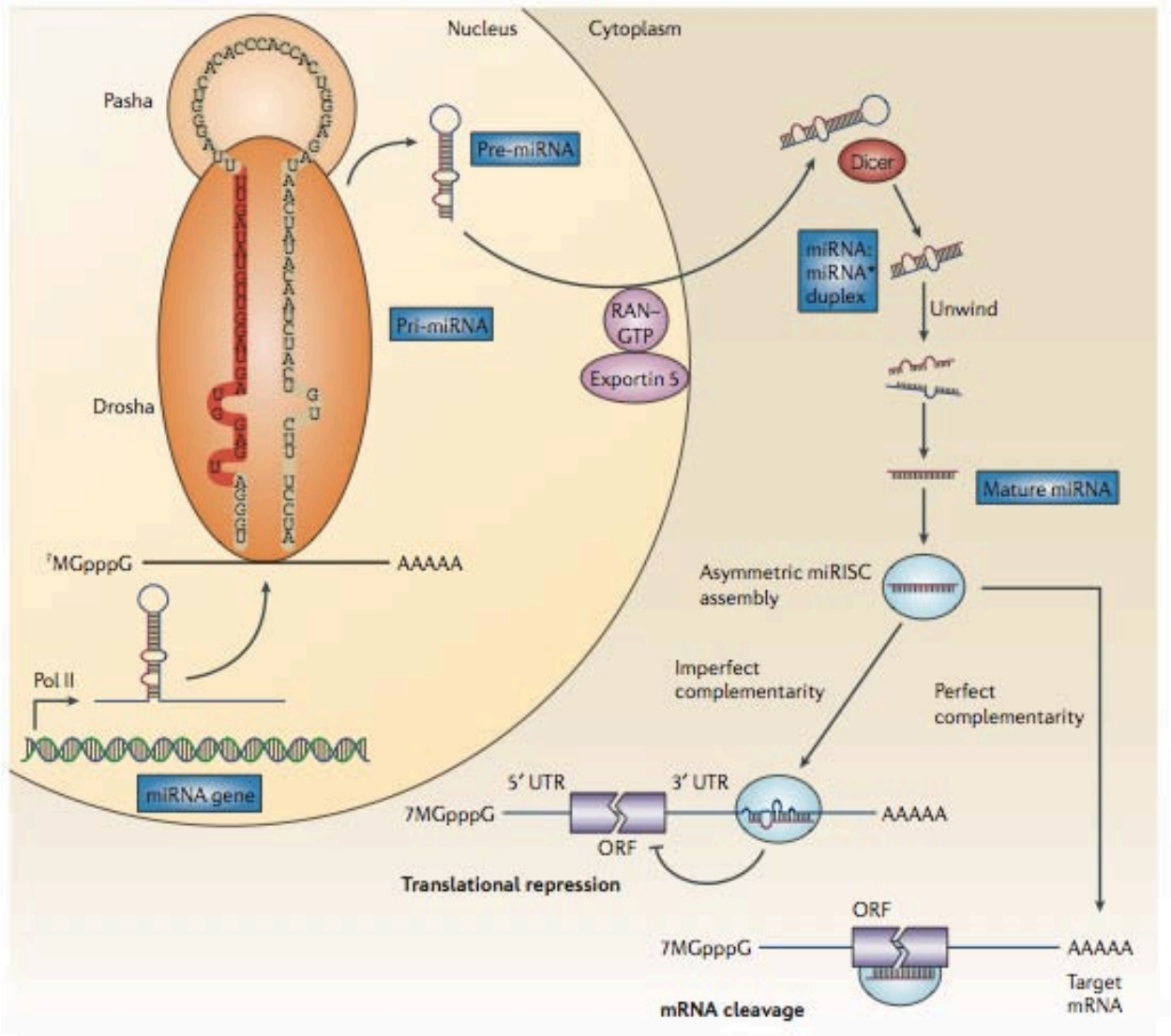


Figure 3: MiRNA maturation pathway (47).

Profiling of cancers using miRNA profiling is becoming a more widely used method, with the advent of more precise and sensitive RNA detection techniques. Microarray techniques have been modified to work with the smaller miRNAs and are continually updated when new versions of the Sagner miRNA or miRBase library are released (48). The use of locked nucleic acids (LNA) is another new method that has been developed by Exiqon. LNAs allow for the detection of small RNAs by improving the bonds between the RNA of interest and the target probe without needing a cDNA conversion step (49). All of the above techniques have allowed researchers to use smaller starting quantities of total RNA than was previously possible with Northern blot analysis and has allowed the detection of lower levels of expressed miRNAs.

MiRNAs have been implicated in a number of regulatory pathways causing dysregulation in a range of diseases, from developmental and metabolic diseases to cancers, by affecting pathways in development and differentiation, transcriptional and post-transcriptional gene silencing, and stem cell and germ line maintenance. MiRNAs have been linked to many human diseases by being aberrantly expressed in prostate (50), breast (51-55), brain (56-58), colon (59), stomach (60) (61), head, and neck cancers (62-64) and a variety of central nervous system disorders, including schizophrenia (65, 66) and Alzheimer's disease (67).

Several miRNAs, including miR-221, let-7a, miR-21, miR-34a, miR-24 and miR-376, and the miR-17-92 cluster, have all been implicated in the developmental process (44, 68). The chromatin-silencing pathway has been well reviewed by several papers (69, 70). MiR-208 over-expression is one of the causes of heart diseases (71). MiR-205 has been shown to play a role in epithelial cell differentiation (72). Down-regulation of miR-205 appears to promote the progression of head and neck (62, 73) and breast cancers since its normal function is thought to inhibit tumor cell migration and metastasis (74).

1.2.1 MiRNA profiling in cancers

MiRNA expression profiling has become an important tool to study disease pathogenesis and prognosis. Some miRNAs act as tumor suppressors by inhibiting the expression of oncogenes, while other miRNAs can act as oncogenes by regulating the expression of tumor suppressor genes.

In chronic lymphocytic leukemia (CLL), researchers discovered 13 miRNAs that were distinguishable between indolent and aggressive forms of the disease (75). In particular, mirR-155 was over-expressed in different lymphomas. The miR-29 family and miR-181 were found to be under-expressed, which coincides with the fact that these miRNAs regulate the TCL1 oncogene, which is over-expressed in an aggressive form of CLL. In addition to these reports, by profiling several different CLL forms, it was determined that a mutation in the precursor of miR-15/miR16 caused these miRNAs to not be made. MIR-15/16 regulates BCL2, a known oncogene that protects cells from apoptosis, in effect making these miRNAs act like tumor suppressors in CLL.

In breast cancer, miR-21 is over-expressed and can mediate cell survival and proliferation by targeting well-known tumor suppressor genes PTEN, PDCD4 and TPM1. MIR-21 has been suggested to be over-expressed in many other types of cancers, including lung, ovarian, and pancreatic cancers, just to name a few. MiR-21 targets the 3'UTR of the mRNA encoding for programmed cell death 4 (PDCD4) in breast cancer cells and is thought to play a role in apoptosis in many different cell lines (76, 77). Post-transcriptional down-regulation of PDCD4 stimulates invasion, intravasation and metastasis in colorectal cancer (78). In addition, miR-21 regulates the expression of the phosphatase and tensin homolog (PTEN) tumor suppressor gene in human hepatocellular cancer and, in general, the tropomyosin 1 gene (TPM1). Interestingly,

miR-125b and miR-205 are down-regulated in breast cancer and are found to regulate oncogenes HER-2 and HER-3. MiR-206 may play a role in the regulation of estrogen receptor (ER) and progesterone receptor expression in breast cancer as well (79). This information can help to classify subtypes of breast cancers in addition to the stage and prognosis.

MiRNAs are also involved in the processes leading to metastatic tumors, such as cell adhesion, migration, and invasion, as well as angiogenesis. In a recent study, miR-10b was found to be an initiator of tumor invasion and metastasis in breast cancer (52). It is highly expressed in metastatic breast cancer cell lines, and the over-expression of miR-10b in a non-metastatic cell line causes invasion and metastasis. The expression of the transcription factor Twist, which binds the miR-10b promoter, causes the inhibition of homeobox D10 RNA, which in turn causes the over-expression of the pro-metastatic gene RHOC. In another study, miR-373 was identified through a functional screen to also have metastatic potential in breast cancer cells (51).

Understanding the miRNA machinery and identifying miRNAs that are disregulated can lead to improved miRNA-targeted therapies. Our increased knowledge of how miRNAs function has resulted in the development of a new class of small RNAs that disrupt the function of mature miRNAs. These small RNAs are called antagomirs and bind directly to the miRNA of interest to help repress their effects (80, 81). Much still needs to be learned in terms of how best to use these antagomirs as therapeutic agents. The first challenge is to find safe and efficient ways to deliver them into target cells. The second is how to regulate the dosage of antagomirs in order to lower the in-vivo levels of miRNAs without completely eliminating them. Future work in this field should reveal the potential of antagomirs as therapeutic agents.

In 2007, an important advance was made linking microvesicles as carriers of RNAs, including mRNAs and miRNAs (23). A subsequent paper suggested that exosomes were a means

of shuttling RNA information between cells (82). Exosomes are now being perceived as important modulators of the immune system, and the question arose as to the possibility of exosomes affecting their environment with more than just proteins by being efficient cell-to-cell communicators and by being able to transfer genetic material to a variety of cell types. Soon after this discovery, several laboratories investigated the types of RNAs present in exosomes and their potential as therapeutic or diagnostic tools. Since the initial discovery of exosomes, a pilot study was done to determine if ovarian cancer could be diagnosed by profiling miRNAs from tumor-derived exosomes(83). MiRNAs isolated from exosomes in patients with non-small-cell lung cancer were used to create a signature that could help with early diagnosis of this disease(84). Exosomes have also been hypothesized to transfer both protein and RNA at synapses in the nervous system, which is a rapid process(85). Finally, placenta-specific miRNAs were found in exosomes from pregnant mothers (86). The release of exosomes from the placenta is thought to keep the fetus from being rejected and could also be a source of important transfer of immunity between the mother and the child.

My research deals with the miRNA profiling of exosomes from virally-infected cells and in head and neck cancer patients. I initially began this quest by developing the techniques needed to do this research in a highly sensitive and accurate fashion. This led to the development of a method for large-scale purification of exosomes and the discovery of new techniques to minimize the amount of total RNA needed for analysis by the various miRNA detection assays. I followed this by testing several samples from head and neck cancer patients for the presence of specific miRNA profiles. I also looked at the simpler question of whether exosomes could be carriers of virally-encoded miRNAs. This study was done in KSHV infected cell lines and some background on herpes virus and KSHV follows.

1.2.2 Herpes Virus Background

The herpesvirus family (*herpesviridae*) is of particular interest in this chapter as KSHV is a member of this family. The herpesvirus family consists of large, enveloped viruses that contain 120-240 kb of double-stranded DNA. Depending on the host system and tissue specificity, they are divided into three subfamilies encompassing eight known human herpesviruses. In humans, there are three α-, three β-, and two γ-herpesviruses. One of the major identifiers of herpesvirus infection is its ability to become latent and maintain latency when the genome circularizes and remains as an episome. In this stage, there is very little gene expression and the infections can persist, in some cases for the lifetime of the host. This very complex balance is played out between the immune surveillance system and viral immune evasion mechanisms.

Latency does not cause any immediate problems for the infected cells, although in two human γ-herpesviruses, it has been linked to tumorgenesis. Epstein-Barr Virus (EBV) can cause several diseases, including Burkitt's lymphomas (BL), non-Hodgkin's disease and nasopharyngeal carcinoma. Kaposi's sarcoma-associated herpesvirus (KSHV) is implicated in Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD). This is in contrast to the α- and β-herpesviruses, which are not tumorgenic but cause diseases when they enter the lytic phase with uncontrolled virus replication. One particular example is encephalitis that is caused by herpes simplex virus 1 (HSV-1).

1.2.3 Virally encoded miRNAs and their targets

The first virally encoded miRNAs were discovered in EBV in 2004 (87) from Burkitt's lymphoma (BL) cells. This finding ignited a new field that has lead to the identification of over

140 herpesvirus miRNAs. A standard herpesvirus genome is separated into two sets of genes: latent genes and lytic genes. Depending upon the time at which they are expressed during the infection cycle, herpesvirus genes are divided into three classes: immediate-early (IE) genes that regulate viral latency, early genes which are responsible for DNA replication, and late genes that encode viral capsid proteins. It is believed that at least 25% of the herpesvirus genome is dedicated to hijacking host cell responses for viral infection purposes, but that number may even be higher due to the discovery of virally-encoded miRNAs (88). The role of viral miRNAs in replication and latent life cycle is not yet well-understood, although several lines of evidence show the complexity of this pathway. The viral adaptation to the miRNA machinery is also consistent with the long co-evolution of viruses with their hosts, as it is much simpler to create pre-miRNAs capable of repressing a transcript than to create proteins for this purpose. In the following paragraphs, I will describe how several herpesviruses have evolved an miRNA machinery to deal with latency and the lytic viral production pathways. Figure 4 is an example of several viral miRNAs, their parent family, and potential viral and cellular targets.

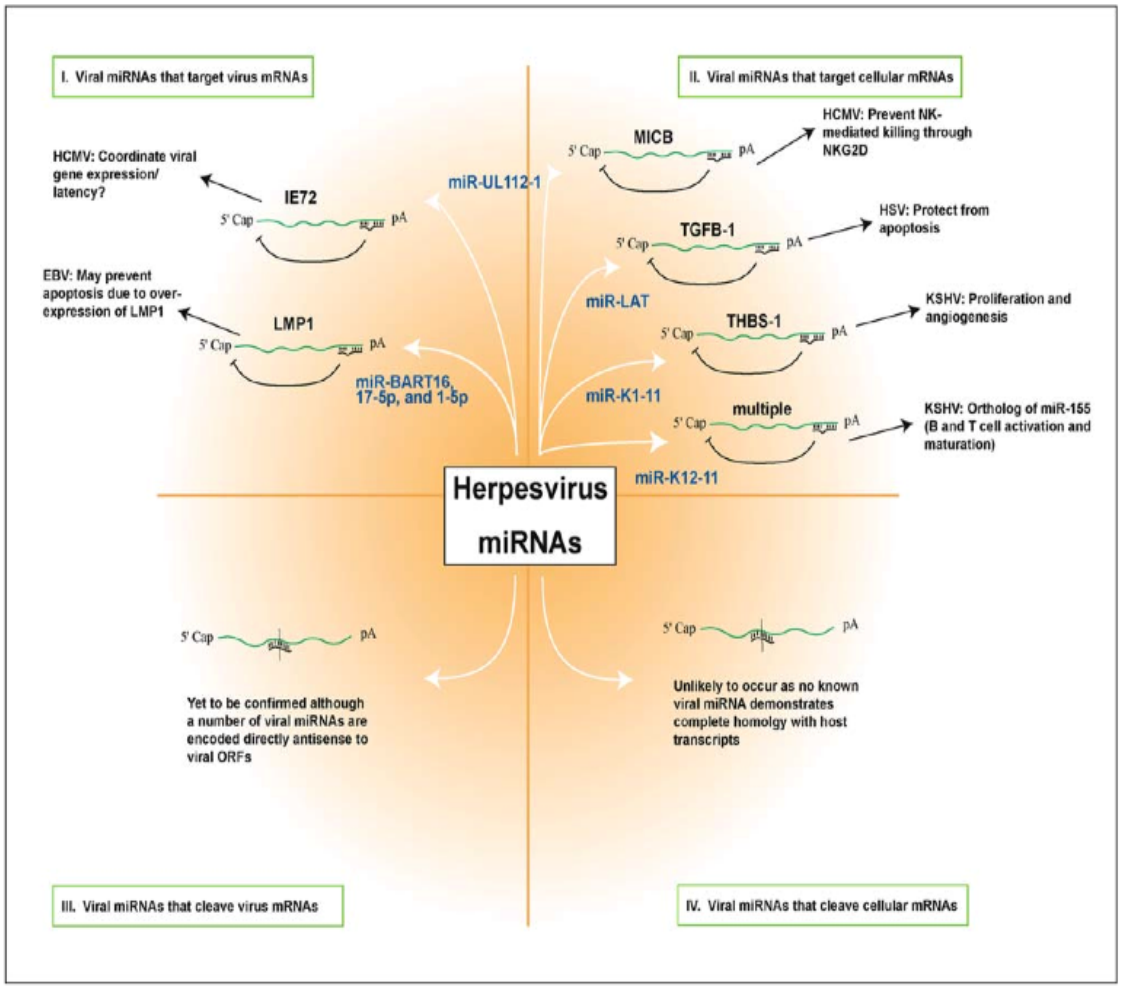


Figure 4: Herpesvirus miRNAS and their functions (89).

1.2.4 α-herpesviruses and miRNAs

Of the different herpesviridae, the α-herpesviruses are known for their faster reproduction cycles. This family includes herpes simplex viruses 1 and 2 (HSV-1 and -2), Marek's disease viruses 1 and 2 (MDV-1 and -2), and Herpes B. All of the above mentioned viruses encode miRNAs. *Ceropithecine herpesvirus* 1 (Herpes B) was recently shown to encode three virally-encoded miRNAs, named miRNAs 2RC, miRNA 4 and miRNA 20 (90). Not much else is known about these particular miRNAs. HSV-1 is the prototypic member of the α-herpesvirus subfamily and induces lytic infections in epithelial cells. For viruses to produce the proper environment in the host cell, they have evolved several functions to help to ensure their efficient reproduction. These functions include the promotion of viral gene transcription, inhibition of host mRNA synthesis, and the shutoff of host protein synthesis. HSV-1 is known to encode several miRNAs named miR-(H1-H6), with some of these capable of blocking apoptosis through the targeting of translation of TGF-β1 and SMAD3 mRNA during latent infection (91-93). Even more recently, miR-H7 and -H8 were discovered, adding to the miRNA list (94). HSV-2 encodes several miRNAs in its latency-associated transcript (LAT), namely miR-I, miR-II, and miR-III. miR-II encodes two mature miRNAs, miR-II-5p and miR-II-3p. Homology studies between HSV-1 and HSV-2 have distinguished a few similar miRNAs and thus their potential targets. In particular, it was shown that HSV-1 and HSV-2 encode three miRNAs within their LAT genes that are positionally conserved (95, 96). Functionally homologous miRNA, miR-H2, in both HSV-1 and HSV-2 has been shown to down-regulate ICP0 (92) that may help in establishing a latent infection, since ICP0 is similar to IE1 in HCMV.

1.2.5 β-herpesviruses and miRNAs

β-herpesviruses are known for their slower replication cycle, longer latency, and the fact that they usually target leukocytes as compared to neurons for β-herpesviruses and B-cells for β-herpesviruses. In this family, one virus, the human cytomegalovirus (HCMV), is of particular interest because it remains the only β-herpesviruses known to encode miRNAs. In HCMV, 11 pre-miRNAs yielding 14 mature miRNAs were discovered (97). In contrast to the other herpesviruses where the miRNA genes are usually clustered together, in HCMV, they are scattered and expressed early in the lytic process. In HCMV, miR-UL112-1 targets the viral gene IE1, which encodes an immediate-early gene product required for activating transcription of early genes in HCMV (98, 99). MIR-UL112-1 in HCMV is also known to down-regulate MHC class I polypeptide-related sequence B (MICB) expression by targeting the 3' UTR of its mRNA (100, 101). Interestingly, MICB surface expression is also targeted by the viral UL16 protein, showing MICB to be an important molecule to target for immune evasion during viral infection (102, 103). The fact that HCMV encodes an miRNA that targets both the major immediate-early region of its genome and human MICB transcripts in conjunction with proteins capable of regulating the MICB genes shows that miRNAs play a very important role in the establishment of latency and preserving immune evasion in targeted cells.

1.2.6 γ-herpesviruses and miRNAs

The γ-herpesvirus family is more known for its variable rate of reproduction. This family encodes two viruses of particular interest, EBV and KSHV. Viral targets were the first to be discovered since these particular miRNAs are usually transcribed antisense to the viral mRNA

target, making them easier to discover. The first known target of a virally-encoded miRNA was the mRNA encoding for an EBV DNA polymerase, BALF5, which is down-regulated by EBV miR-BART2 (104). The role of this down-regulation is still unclear but appears to play a role in the stabilization of viral latency. In addition to the auto-regulation of viral targets, viral miRNAs also target cellular mRNAs (92).

Since these initial discoveries, a few cellular targets of viral miRNAs have also been discovered. In EBV, miR-BART5 targets a pro-apoptotic factor called PUMA (105). PUMA is a downstream target of p53, and it is thought that the down-regulation of PUMA by miRNA-BART5 may protect EBV-infected cells from virally-induced apoptosis (106). MICA and MICB are stress-induced ligands that are recognized by NKG2D receptors. Lowering the level of both MICA and MICB reduced the susceptibility to NKG2D-dependent killing of these cells by Natural Killer (NK) cells. This result suggests a new type of mechanism for how viruses and virally-induced tumors might evade the immune system. In a subsequent study, EBV miR-BART2-5p was discovered to target the 3' UTR of the MICB mRNA as well (107). In that same study, KSHV miR-K12-7 was also shown to target the 3' UTR of the MICB mRNA. An important observation is that few of the miRNAs in the herpesvirus family that have been found to date are conserved, suggesting that these miRNAs evolved separately to target the same mRNAs in order to evade the immune system during infection.

KSHV is the other γ-herpesviruses of interest due to the growing amount of information on its pathogenesis. It is thought that at least one KSHV miRNA also targets the IE proteins to enhance the latency of the virus (99). In KSHV, miRK12-5, miR-K12-9, and miR-k12-10 were discovered to target BCLAF1 (108). BCLAF1 can act both as a pro- or anti-apoptotic factor, depending on the context. It was experimentally shown that levels of BCLAF1 were elevated

after infection with KSHV-expressing mutant miRNAs. BCALF1 is therefore seen as a stabilizer of KSHV latency, as its higher levels decreased viral production. The seed sequence of another KSHV miRNA, miR-K12-11, was an exact match to cellular miRNA miR-155, suggesting a similar role (109). In the same study, miR-K12-11 was shown to target BACH-1 3'UTR. BACH-1 is a known transcription factor that down-regulates heme oxygenase-1 (110). The over-expression of miR-155 is associated with a number of B cell lymphomas and solid organ tumors and is also a significant marker of tumorgenesis, which is important for the profiling of several types of cancers (111). KSHV miR-K12-2, miR-K12-2, miR-K12-3, miR-K12-4, and miR-K12-5 all target thrombospondin 1 (THBS-1) (112), whose down-regulation leads to angiogenesis and proliferation of the infected cells.

The above findings show an important role for virally-encoded miRNAs in the reproduction cycle of viruses and in the immune system evasion of tumors. The targeting of MICB by HCMV and KSHV shows a method by which infected cells evade NK cell activity and thus evade the immune system. The discovery of an miR-155 ortholog in KSHV hints at the evolution of viral miRNAs that mimic their cellular counterparts. Of particular interest in this evolutionary adaptation to the regulation of MICB is the fact that both KSHV and HCMV encode the proteins K5 (KSHV) (113) and UL16 (HCMV) (102) that mediate the sequestration of not only MICB but also other NKG2D ligands.

The next section discusses important exosome biological activities in tumor escape and viral pathogenesis in several models, including how virally-encoded miRNAs play a major role in making exosomes efficient at immune regulation.

1.3 THE ROLE OF EXOSOMES IN INFECTIOUS DISEASES

To explain the rationale for looking for virally-encoded miRNAs in exosomes in this study, it is important to first look at the important role that exosomes play in the viral life cycle. As stated in Chapter 2, exosomes can be both tumor-suppressors and tumor-activators. Of particular note are the recent discoveries of how exosomes play a role in several infectious diseases, including those caused by the human immunodeficiency virus (HIV), EBV, HCMV, and prions.

1.3.1 HIV hijacks the miRNA and exosome pathways

It was reported that exosomes can act as "Trojan" exosomes in 2003 (114). Retroviruses are enveloped positive strand RNA viruses that replicate through a DNA intermediate that is inserted into the cellular genome. This Trojan exosome hypothesis states that retroviruses can use the pre-existing exosome biogenesis pathway for the formation of infectious particles as a method of both receptor- and envelope-independent mode of infection. There are many similarities in the host cells and retrovirus lipid compositions, including higher levels of cholesterol and glycosphingolipids as well as several enriched protein components, such as tetraspannins, GPI proteins, and membrane proteins. The pathway of release for both exosomes and retroviruses are shared through the common intralumenal vesicles (ILV), which form by inward budding of the endosome membrane in primary macrophages (115). Multivesicular bodies consist of endosomes enriched in ILVs. It was also shown that exosomes secreted from CD8+ T cells suppress the transcription of the HIV-1 genome (116). These findings, in addition to the fact that APC derived exosomes contain MHC molecules, indicate that HIV is using exosomes for its own propagation

and immune evasion. This knowledge might be applicable to anti-HIV vaccines in the future or, as stated in the above study on CD8+ T cell-derived exosomes, possibly repression of transcription. Another recent study describes the capture of HIV-1 particles by mature dendritic cells for mediating trans-infection of T lymphocytes by using the exosomal pathway (117). HIV-1 was also shown to encode its own miRNA, HIV1-miR-H1. This miRNA was shown in the same study to reduce cellular responses to infection, in particular, by targeting the apoptosis antagonizing transcription factor (AATF) (118). In turn, the miRNA-induced AATF knockdown was responsible for the suppression of the BCL-2, C-MYC, Par-4, and Dicer genes. The added knowledge that HIV encodes a viral miRNA that has a profound effect in the virus' ability to cripple cellular responses to infection makes HIV a good example of how exosomes can be used as Trojans.

1.3.2 EBV proteins found in exosomes

HIV is not the only virus to make use of exosomes for immune escape and viral propagation. EBV's latent membrane protein (LMP 1) was discovered in exosomes infected by the virus (119). Preparations of these LMP1-containing exosomes derived from lymphoblastoid cell lines were shown to inhibit the proliferation of peripheral blood mononuclear cells, suggesting that this protein might be involved in immune regulation.

A further study discovered both the presence of LMP1 and galectin 9 (C15) or galectin 9 only (C17) in nasopharyngeal carcinoma-derived exosomes (NPC), which are often associated with EBV (120). They confirmed LMP1's ability to inhibit T cell activity. By use of recombinant LMP1, they were also able to inhibit T cells, suggesting a strong role for exosomes containing LMP1 proteins and possibly other factors in the aid of EBV immune evasion. Another study

found FGF-2, a potent angiogenic factor that is important in immune evasion, to also be localized with LMP1 (121).

1.3.3 Exosomes as a Trojan horse as it pertains to HIV and Prions

Viruses are not the only infectious agents to utilize exosomes to spread and evade the immune system. Prion diseases are infectious neurodegenerative disorders linked to the increase of improperly folded proteins called scrapie (122). These prions are associated with exosomes located on the cell membrane, as seen by immunoelectron microscopy. In addition to this, the p53 protein has been shown to respond to stress signals by regulating the transcription of several genes. In particular, a p53-regulated gene product called TSAP6 was shown to enhance exosome production during stress response (35, 123). This could be a way by which viral infections increase the number of exosomes released in order to help escape from host immune response.

The above research presents a strong case for the natural evolution of exosomes as pathogen-evasion tools. Viruses, tumors, and prions use a variety of mechanisms to evade the immune system and shape their local environment to favor propagation. While exosomes have been shown to help viruses propagate themselves, no virally-encoded miRNAs have yet to be detected in the exosomes purified from virally infected cell lines. In chapter 3, I will discuss the discovery of KSHV-encoded miRNAs in exosomes derived from a KSHV-infected B-cell line and how this discovery has the potential of increasing the accuracy of detecting KSHV infections.

1.4 CURRENT METHODS OF DIAGNOSING CANCER AND VIRAL INFECTIONS

A common way to diagnose most cancers and viral infections is by collecting serum from patients and testing for a panel of antibodies present. These techniques can be very accurate in the case of some cancers or can have varied results, as is seen with KSHV antibody tests. Some progress has been made with gene expression and miRNA profiling of cancers. However, this usually occurs after a tumor has been biopsied and thus is not desirable for the early detection of cancers.

Recently, the detection of circulating apoptotic factors in patients of acute cerebral infarction has led to some success (124). Serum-circulating nucleic acids have also been detected and could be a predictor of disease (125). This was tested with patients that have invasive ductal breast cancer and patients with multiple sclerosis (126). Genetic tests can be indicators for the development of disease at a later stage of life but can only be used as predictors at this time.

As can be seen by the abundant material in the literature, exosomes can be produced at a constitutive or higher level based on stress response. EBV and HIV have both been shown to stimulate exosome production, and it has been previously reported that cancer patients produce a higher-than-normal quantity of exosomes. The evidence that miRNAs are detected in exosomes supports the hypothesis that exosomes could be very important cell-to-cell communicators as well as being capable of promoting tumor and immune evasion in humans. By detecting cancer-specific profiles of miRNA isolated from exosomes and looking at specific virally-encoded miRNAs, we can increase the diagnostic potential of exosomses.

2.0 EXOSOMES AND HEAD AND NECK CANCER

2.1 ABSTRACT

The prevalence of head and neck cancer (HNC) is growing in America, where 48,000 individuals have developed HNC in 2009 and an estimated 11,000 will die in the USA alone (1). Current diagnostic methods rely on antibodies and biopsies as the primary means of early detection of cancers. Once a person is diagnosed, they may go through years of antibody therapy or chemotherapy, sometimes in combination with surgery, both of which are tiring and taxing on patients. In this chapter, I discuss a new approach of diagnosing cancers by purifying microvesicles from patient sera and isolating miRNAs from these vesicles. I will describe steps for the large-scale purification of exosomes and the isolation and quantification of miRNAs from these exosomes. In particular, I will describe the above approach using samples from head and neck cancer patients. The ability to isolate miRNA from exosomes and profile these from small amounts of serum could provide more sensitive and accurate tests that could lead to earlier diagnosis and thus better preventive care for these patients. In addition, this research applies as a proof of concept for the diagnosis of other diseases.

2.2 INTRODUCTION TO HEAD AND NECK CANCER

Cancers are a growing problem in America, and the early diagnosis and treatment of them is becoming an important part of most pharmaceutical company pipelines. The basis of most treatments and therapies lies in the molecular understanding of how cancers develop and progress through the disease state. Head and neck cancer is described as one of the most painful types of cancers, capable of affecting speech, causing disfigurement, and sometimes even affecting basic functions such as breathing and swallowing. Much of the research in this field is focused on improving the molecular markers of the disease in order to increase detection rates and thus better the long-term survival of these patients. Early diagnosis of this disease can be useful for various therapeutic approaches. Currently, one option for patients with early detection of HNC involves tumor dissection followed by radiotherapy. Patients with later stage operable tumors could benefit from resection and sometimes reconstructive surgery, or if the stage is more advanced, a more conservative approach for therapy.

In my research, I examined several different oral cancer cell lines as potential model systems and tools for the profiling of miRNAs from exosomes. I started with exosomes purified from cell culture supernatant in order to have a more reliable and reproducible model system. Later, I studied exosomes purified from the serum of patients with head and neck cancer. Cancers have a profound effect on the cells in their immediate surroundings and on the immune system in general. Many cancers evade the immune response and go undetected and un-rejected. Exosomes are heralded as one of the main mechanisms by which cancers evade the immune system, and recently, they have been described as capable of transferring both genetic material and proteins as a wider form of cell-to-cell signaling.

The exact nature of the genetic alterations is still unclear, but the pattern appears to be similar to the pattern for colorectal cancer. Preliminary work was done to describe the genetic progression from the benign to the invasive form of HNC in 1996 (127). The current view states that three mutations are required for the progression of the disease. The first is an alteration in the p53 tumor suppressor protein (128), the second is an inactivation of the cyclin dependent kinase inhibitor p16 (129), and finally, the over-expression of epidermal growth factor receptor (EGFR) in a large percentage of oral cancers(130). More recently, a combination of radiotherapy and EGFR antagonists has been used as therapy for patients with minimal success (131).

Head and neck cancer was also profiled for miRNA expression patterns in several studies. MIR-21, miR-18a, and miR-221 were identified in one study to show significant up-regulation in tumors, whereas miR-375 was down-regulated (132). This study was performed by comparing several tumors with several non-cancerous head and neck cancer cell lines and later was expanded to a larger sample of HNC tumors. Tumors from five patients and their corresponding adjacent normal tissues were compared for the presence of any miRNA differences (132). Several more miRNAs were identified that were either over-expressed or under-expressed, including let-7i, miR-103/107, miR-155, miR-21, and miR-125a,b. In later studies, miRNAs were correlated with highly metastatic HNC cancer (36, 61, 76, 133-141). MIR-138 was identified in this study to be expressed at a lower level in the highly metastatic cells. The knockdown of this miRNA seemed to enhance cell invasion and suppress apoptosis. In another study done with HNC cell lines, miR-205 was described as exclusively over-expressed in the HNC-positive cell lines vs. the non-cancer cell lines (62). This group also identified let-7a, miR-16, and miR-21 as being highly expressed, while miR-342, miR-246, and miR-373 as expressed at a low level in all of the cell lines. There are several discrepancies between the

literature on over-expressed and under-expressed miRNAs based on the different comparison sets of cells and the different types of HNC tested. Due to these discrepancies, I chose a small subset of miRNAs that were consistently over-expressed in all of the samples as well as a few that were found to be up-regulated in individual studies. Information on the dysregulation of some miRNAs was not available at the time I started these experiments and thus could not be incorporated due to the very limited starting material.

2.3 MATERIALS AND METHODS

2.3.1 Transmission Electron Microscopy

All transmission electron microscopy (TEM) work was done in the Center for Biologic Imaging (CBI) laboratory at the University of Pittsburgh with the aid of Nicole Bianco, PhD in our laboratory. Exosomes were placed on specially created Formvar/carbon-coated grids made by the CBI and incubated for 1 minute before removing the excess, followed by a 1 minute incubation with 1% uranylacetate in PBS. The excess uranyl acetate was blotted off with filter paper and the samples read for analysis. The transmission electron microscope used was a JEOL-1210 computer-controlled high-contrast 120 kv, and the final analysis was done either by Nicole Bianco, PhD or ChenJe- Yang.

2.3.2 Cell Culture

Cell lines were cultured, expanded, and maintained throughout the exosome purification process. The majority of cell lines were obtained from ATCC and maintained according to their protocols. The cells used were TA3 and MCF-7 breast cancer lines, as well as EG7 and the corresponding EL4 cell line. The HNC cell lines were PCI-13, PCI-30, SCC-47, and SCC-90. The PCI-13 and PCI-30 cells were thawed, split, and expanded every two days. SCC-47 and SCC-90 grew slower and produced a large amount of debris, thus the supernatant was replaced every two days. The cells were cultured until 80% confluent and then split at a high density. When cell factories were used, the cell medium was left for three days without change. Placental exosome samples were collected and purified by the Douglas Taylor laboratory. All cell cultures and exosome purifications were done according to the protocol below. All HNC cell lines were grown in RPMI with 10% FBS and an addition of 1% HEPES and 1% PENSTREP provided by GIBCO. MCF-7 and TA3 were grown in the same manner as the HNC cell lines but with DMEM instead of RPMI.

2.3.3 Exosome Purification

Cells were expanded to create 12 T-175 flasks at 80% confluence. An extra T-175 flask was maintained for cell collection and propagation. This produced around 100 million cells for the seeding of the cell factory. The speed of growth needed to be adjusted to maintain the proper confluence levels of the cells. Cells from cell factories were harvested every 3-4 days.

Cells were collected and 100 million cells were seeded in a 10-chamber cell factory from Nunc (CAT#164327). The simplest method of loading the cell factory was to collect all of the

cells by centrifugation and to dilute the pellet with 1.8 L of media in the glass-loading container, making sure to mix well for an even distribution. Once the factory was loaded, cells were harvested after 3-4 days until they were 80% confluent.

Once the cells were confluent, the cultures were poured into 1 L 0.22 μM media sterilization filters. The larger surface area of these filters minimizes the clogging from any debris in the supernatant. Optionally, if the cells being used were non-adherent or produced substantial debris, the supernatant was first spun down in larger conical 500 ml viral prep flasks for 30 min at 2000Xg. Control cells were also collected at this time. This was done by one of two ways. The first was to collect cells from a T-175 flask growing alongside the cell factory. The second approach was to pour enough trypsin into the cell factory to cover ¼th of a layer, mix hard, and pour. The cells were then washed three times with PBS and spun down at 12000Xg for 1 minute, with the final pellet collected in 300 μl of Ambion's RNAqueous lysis buffer and stored at -80^{o}C until RNA purification.

The large volume of filtered supernatant was then concentrated down by using Satorius's vivacell concentrators with a 100-kDa cutoff. Each concentrator was pre-washed with 50 ml of distilled water and then with 50 ml of PBS to remove any ethanol and glycerol still present on the filter membrane. The supernatant was then loaded into the vivacells and spun for 20 min at 3000Xg. The flow-through was discarded and the remaining volume was then collected. This process was repeated until no more supernatant remained. The resulting volume was between 50-150 ml of concentrated supernatant.

The resultant supernatant was loaded and balanced into a SW-31ti rotor and spun at 100,000Xg for 2 hours. The supernatant was poured off and any remaining fluid was delicately removed with a pipette. The pellets were combined into one tube, unless the downstream

application was protein work in addition to RNA work, in which case the pellets were separated into two aliquots. The pellet was re-suspended with 30 ml of sterile PBS and spun again at 100,000Xg for 2 hours. Again, any remaining supernatant was removed from the resulting pellet followed by final re-suspension. If a separate pellet was saved for protein work, it was suspended in 100 µl of PBS and stored at 4˚C. Th e pellet to be used for RNA analysis was re-suspended in 100 µl of Ambion's lysis buffer and stored at -80˚C. Figure 4 shows the experimental flow of the large-scale exosome purification protocol. Figure 5 is the example purification protocol.

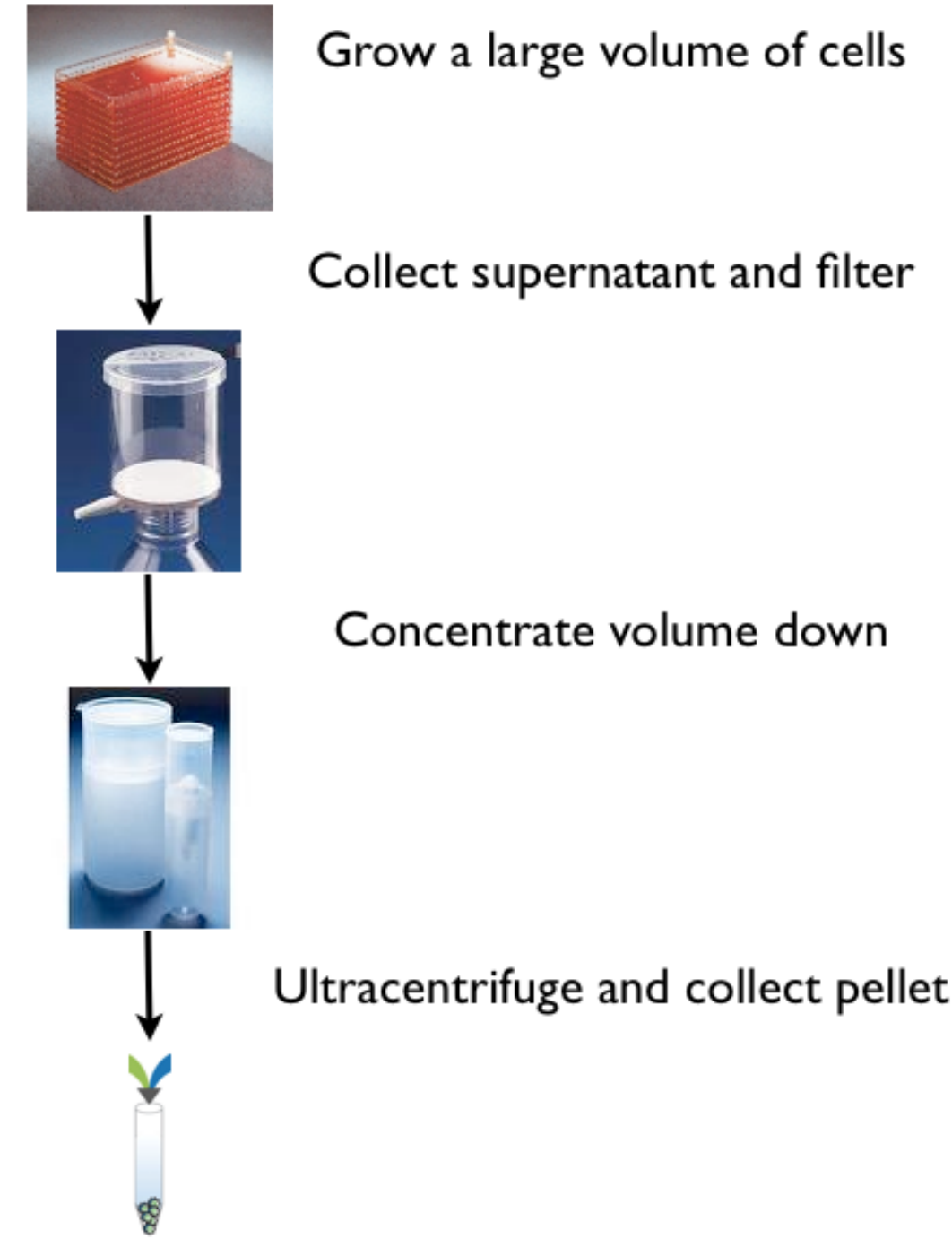


Figure 5: Large scale exosome purification protocol

2.3.4 Head and neck tumor-derived exosomes

HNC-derived exosomes were obtained from Theresa Whiteside's laboratory, and her student, Ben Hilldorfer, did the preparations based on a protocol provided by Douglas Taylor. In short, patient serum was collected and pre-cleared at low speeds in a centrifuge. The samples were spun for 10 minutes at 1500Xg, 15 minutes at 3500Xg, and then 20 minutes at 10,000Xg. The sample was then placed in a pre-washed Sepharose 2B loaded column, where large molecules flowed through while small molecules were retained in the column matrix. The flow-through was collected in 1 ml aliquots and the protein concentration determined by a Bradford assay kit from Bio-Rad. The first protein fraction, which contains exosomes, was collected and spun on a Beckman SW-34 rotor twice, for one hour each time, at 100,000xg. The supernatant was carefully removed and the pellet re-suspended in 100 µl Ambion's RNAqueious lysis buffer. The samples were then frozen at -80˚C until RNA extraction and analysis.

2.3.5 RNA Extraction

I began by using Invitrogen's micro-midi kit with several small modifications but eventually used Ambion's RNAqueous RNA extraction kit. This kit provided the cleanest total RNA for all future downstream applications. The samples collected from the exosome preparations were thawed on ice, vortexed vigorously, and then spun at 1200Xg for 5 minutes to collect any large debris. The supernatant was slowly removed and placed into a new tube. At this point, 1.25X volume of cold 200-proof ethanol was added to each sample (in this case 125 µl). If any precipitate appeared, another quick spin at 1200Xg for 1 minute was performed. The supernatant was then loaded on the supplied filter cartridge and the rest of the Ambion protocol was followed

as per the instructions. Elutions were done with 2 x 10 µl of elution buffer and then the samples were either frozen or characterized immediately. The RNA was eluted in 20 µl of Ambion's elution buffer and stored at -80˚C until quantification by nanodrop.

2.3.6 Quantification and Characterization of RNA derived from exosomes

The RNA from the previous step was characterized before processing it for any downstream applications. Initially, I used ribogreen to determine the mass of the RNA collected, but I soon switched to using a nanodrop. The choice was guided by the fact that a nanodrop is significantly more accurate and requires only 1 µl of sample for quantification. To determine the RNA population, I used Bioagilent's RNA 6000 Pico Chip because an electrogram is produced that can distinguish between small and large RNAs, as can be seen in Figure 6.

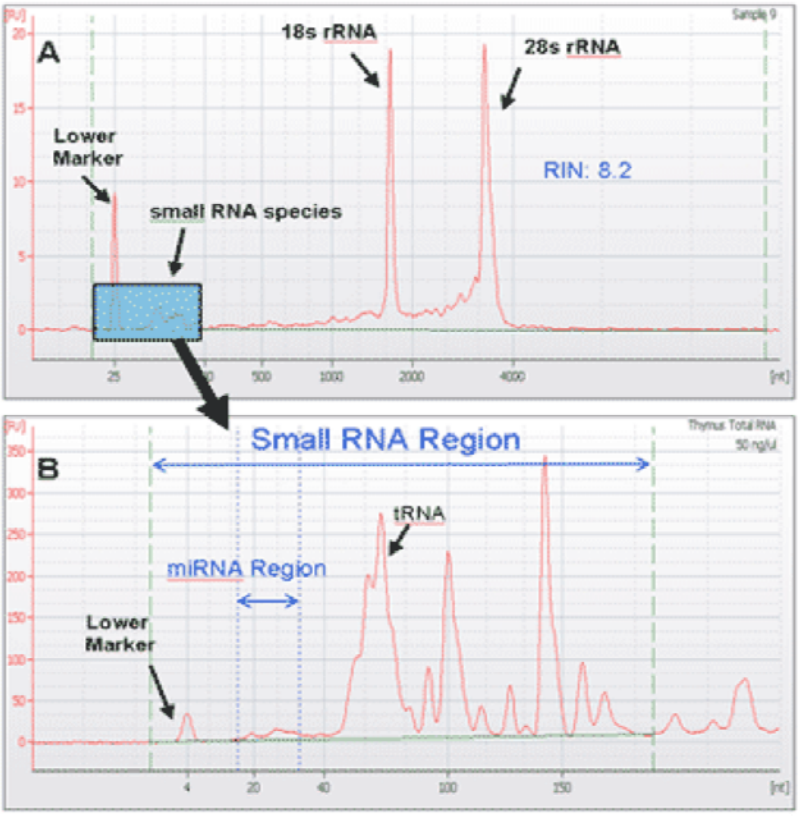


Figure 6: a) A typical RNA 6000 picochip from Bioagilent. Note the separation of small RNAs that are seen at 25 seconds compared to the larger rRNAs. b) The newer model of Bioagilent chips that can distinguish smaller RNA molecules

2.3.7 Quantitative RT-PCR

Quantitative RT-PCR was performed by using Invitrogen's MIRQ-ER500 first strand synthesis kit coupled with the SybrGreenER kit. This kit was specifically formulated for the Bio-Rad IQ5 iCycler machine. We used the Bio-Rad IQ5 machine and the Sybr greenER kit with a few modifications. The primers were ordered from Invitrogen's validated primer catalog, which kept most of the annealing temperatures around 60˚C. An example workflow taken from the Invitrogen manual is shown below. Figure 7 shows how qRT-PCR is performed normally.

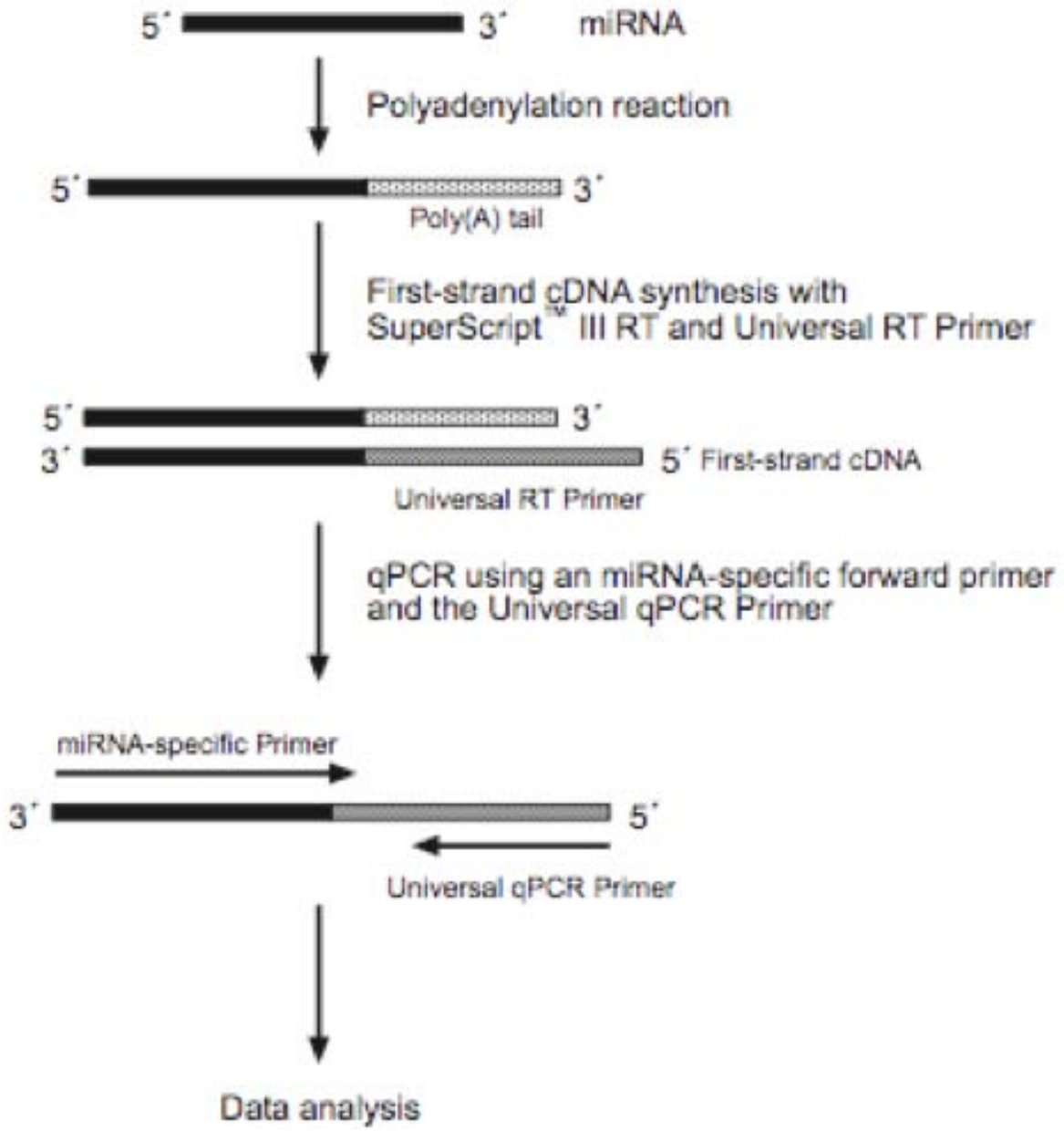


Figure 7: Workflow of quantitative real-time PCR taken from Invitrogen's MIRQ-ER manual.

See Figure 8 for a list of primers. A few additional steps were added to the standard Invitrogen protocol.

mir Name	Sequence
hsa-mir-21	TAGCTTATCAGACTGATGTTGA
hsa-mir-205	TCCTTCATTCCACCGGAGTCTG
let-7a	TGAGGTAGTAGGTTGTATAGTT
let-7i	AGAGGTAGTAGGTTGCATAGTT
let-7f-1	TGAGGTAGTAGATTGTATAGTT
hsa-mir-103-1	AGCAGCATTGTACAGGGCTATGA
hsa-mir-155	TTAATGCTAATCGTGATAGGGGT
hsa-mir-181a-1	AACATTCAACGCTGTCGGTGAGT
hsa-mir-142-5p	CATAAAGTAGAAAGCACTACT
hsa-mir-146b-5p	TGAGAACTGAATTCCATAGGCT
hsa-mir-17	CAAAGTGCTTACAGTGCAGGTAG
hsa-mir-31	AGGCAAGATGCTGGCATAGCT
hsa-mir-145	GGATTCCTGGAAATACTGTTCT
hsa-mir-98	TGAGGTAGTAAGTTGTATTGTT

Figure 8: Primers used for HNC profiling

In short, the RNA was collected by the process stated above and quantified by nanodrop or Bio-Agilent picochip, and a varying amount of sample was used depending on the RNA yield. HNC samples were determined to have a maximum useable quantity of 50 ng per sample, and this is the amount I used. A master mix was created with 50 ng of total RNA per sample, 5 µl of miRNA reaction Buffer, 2.5 mM MnCl2, 1 µl of diluted ATP, 0.5 µl of poly A polymerase, and the remaining volume was made up to 25 ul. The ATP was previously diluted at a 100x in 1mM Tris, pH 8.0 to match the protocol, and the tubes were mixed, centrifuged, and then incubated on a PCR machine for 30 minutes at 37˚C.

After 15 minutes, the samples were collected and used for the first strand synthesis steps. Due to the low quantities of RNA available, I decided to maximize the yield by using as much of the available sample as possible. Thus, 20 µl of the preceding poly A reaction was collected and placed in 5 µl of supplied annealing buffer with 15 µl of the Universal RT Primer, for a final volume of 40 ul. This mixture was then incubated on a heat block for 5 minutes at 65°C after which the samples were removed and placed on ice for 1 minute.

In the next step, the first strand synthesis reaction, 50 ul of 2x First-Strand Reaction Mix was added to the previous reaction, along with 10 ul of SuperScript III RT / RNaseOUT Enzyme mix. The tubes were spun down and heated on a thermal cycler preheated to 50°C for 50 minutes. The reaction was then incubated at 85˚C for 5 minutes to stop the reaction. Each sample was diluted 1:10, and aliquots of 40 ul of each sample were created to avoid freeze-thaw cycles and the samples were stored at -20˚C. The volumes stated in the manual were halved due to the 25 ul total volume limit we had on the Bio-Rad iCycler. The reaction was as follows: 12.5 ul of the SYBR GreenER qPCR SuperMix for iCycler was combined with 0.5 ul of forward primer, 0.5 ul of universal qPCR primer, and 2.5 ul of diluted template to reach a final volume of 25 ul. All primers were stored at -20˚C at a 100 uM stock and diluted in DEPC water immediately prior to the runs. Each run was made with a master mix and a volume multiplied to 1.1 to cover for pipetting errors. The annealing temperatures were experimentally determined to be 56˚C. The runs were setup with an initial 10 minute run at 95˚C to activate the polymerase and then cycled for 65 cycles at 95˚C for 15 seconds and at 57˚C for 60 seconds. The completed reaction was kept at 4˚C prior to analysis.

Primers were first confirmed to be appropriate for amplification by using the RNA samples from cell lines as templates, with a starting concentration of 100 ng of total RNA per

sample. These initial primer tests were carried out on cellular RNA from 4 HNC cell lines separated into two groups. Two cell lines were HPV-16+ and two were HPV-16-. Samples were collected in-house and compared to an RNA extraction carried out by Abigail Wald, a member of Saleem Khan's laboratory. This analysis was carried out in the same fashion as the other samples.

Analysis of samples was done using the ΔΔCt method using a standardized template provided by the Khan laboratory. Lack of any known or characterized normal controls forced us to use miR-103 as a normalization miRNA on each plate in order to account for different variations in the runs. In addition, PCI-13 exosomes were used as a normal tissue sample for comparison to all of the HNC samples. Each plate had at least one normal control (NC) sample present, in addition to the active disease (AD) and not expressing disease (NED) samples.

2.3.8 Microarray analysis:

Following the expanded purification protocols and improved RNA purification protocols, we sent several samples for analysis to our National Institute of Health collaborator Dr. Ena Wang, PhD. I sent quantified RNA samples from an MCF-7 breast cancer cell line and the corresponding purified exosomes. The arrays were loaded with 2 ug of total RNA, labeled, and hybridized overnight and the results statistically analyzed by Dr. Ena Wang.

2.3.9 Sucrose gradients of exosome preparations

A sucrose gradient was used to determine if the recovered exosome fractions corresponded with the RNA extracted from the purifications. A continuous sucrose gradient, between 30%- 70%

sucrose, was created using a gradient machine. The total volume of the gradient was 12 ml. Exosomes collected from the above expanded purification protocol were collected and re-suspended in 200 ul of PBS and then loaded slowly on the top of the sucrose cushion. The tubes were balanced and centrifuged in a Beckman-Sorvall ultracentrifuge at 100,000Xg for 16 hours. The centrifuge tube containing the sucrose gradient was very slowly removed from the rotor to reduce the disturbance of the layers. The gradients were then collected using the traditional method of removing 1 ml aliquots and placing them in pre-weighed Eppendorf tubes. By subtracting the weight of the empty tube from the weight of the tube with the fraction, each fraction's density was determined. Exosomes traditionally appear between fractions of 1.12 – 1.15g/ml (142). The individual fractions were then diluted in 11 ml of PBS and spun at 100,000Xg for 2 hours. This process was repeated one more time to remove any lingering sucrose from the fractions. Before the second wash, each fraction was split into two groups, with one group going directly to RNA isolation following the above protocols for RNA purification and picochip analysis. The second portion of the sample was used for Western blot analysis.

2.3.10 Western Blot Analysis

Western blot analysis was used to determine which fractions contained exosomes and how pure the yield was after the sucrose gradient. Due to the interference of sucrose with the protein assay, I loaded a fixed volume in each sample well. Two 10% polyacrylamide gels were cast with large 10-well combs following our laboratory's standard protocol. Each sample was mixed with 5x loading dye at a final volume of 20 ul and placed on a 100˚C heat block for 5 minutes. Each well was then loaded with 20 ul of corresponding samples and run at 0.01A for each 10cm^2 gel. The electrophoresis portion continued until the ladders were well differentiated and the HSC70

antibody from Abcam (CAT#ab1427) was near the middle of the gel at the 73 kDa position. The proteins were then transferred to a membrane by a semi-dry method for 50 minutes at room temperature using polyvinylidene fluoride (PVDF) as the transfer medium. The blots were blocked overnight in a 1% nonfat dry milk and PBS-T solution. The following day, the blots were rinsed with PBS-T 4 times for 5 minutes to remove any excess blocking reagent and then incubated for 1 hour at room temperature with HSC70 antibody, rinsed as above, and placed in a new container. The next step was to incubate the blots with the corresponding secondary antibody, and rinse them in the same way as the above two times. The HRP was then activated using the Western Lightening Products. The blots were exposed for 5 minutes to produce the appropriate exposure levels.

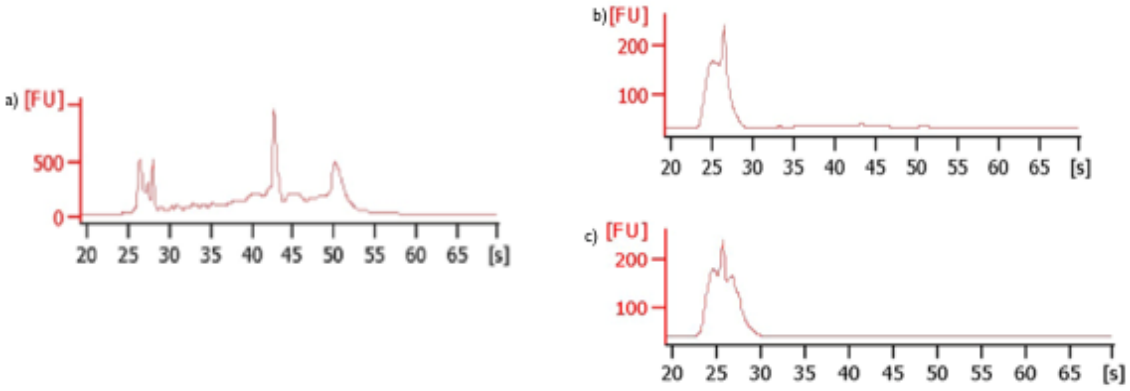
2.4 RESULTS

When we began to investigate the presence of RNAs in exosomes, it became clear that standard cell culture protocols would not provide sufficient material for analysis. Typical volumes of supernatant used for purifications were in the range of a few milliliters for mice and human serum up to 180 ml for supernatant from cells. These volumes gave relatively low yields of exosomes and even lower yields of total RNA. In order to alleviate these problems, we had to scale up the number of cells used and the volume of supernatant collected. I faced two technical hurdles. The first was to find an appropriate growth chamber to expand the number of cells producing supernatant. The second hurdle was to find an efficient method of reducing the volume of supernatant by concentration to make the future ultracentrifugation spins more

manageable. Below are the results from these experiments in exosome expansion and in the RNA purification methods. In addition, all the results from the patient samples are shown.

2.4.1 Large scale purification of exosomes

In order to extract enough RNA for analysis and all further downstream applications, I had to expand the exosome preparation and modify the RNA purification as well as the RNA detection and analysis techniques. Figure 8 shows the results of a large-scale sample preparation. This experiment was done by expanding the basic 180 ml of supernatant collected to a full 2 liters of supernatant. The RNA was collected using the RNAqueous kit from Ambion and the samples were run on a Bioagilent picochip and are shown in Figure 8. Figure 8a shows MCF-7 cells that were collected and analyzed as per above and a fixed volume loaded on all the picochips. Figure 8b shows an example of a simple 180 ml exosome preparation from the same cell line, while Figure 8c shows the results of a full 2 liter extraction. Figure 8d is a table comparing both smaller and expanded purifications and the corresponding mass of total RNA extracted from each. As can be seen, there is a substantive increase in RNA yield from this process, and some refinements have recently been made that can increase this yield even further.



d)

Volume Supernatant (ml)	MCF-7 Cells (ng Total RNA)	MCF-7 Exosomes (ng Total RNA)
180	2260	63
2000	2508	460

Figure 9: Sample electrograms from a large scale MCF-7 exosome purification compared to a normal purification. a) Shows a sample RNA electrogram for MCF-7 Cells. b&c) Electrograms of a normal MCF-7 exosome purification and an expanded purification respectively. d) An example of the difference in total RNA levels from a normal purification compared to an expanded one.

2.4.2 Sucrose gradients

Following the expansion of the exosome purification protocols and RNA extraction techniques, I wanted to show that the isolation of exosomes did indeed correlate with the presence of RNA. To do this, I prepared a sucrose gradient and removed individual fractions for analysis by use of scanning electron microscope, Western blot, and electrogram. Figure 10 shows the different fractions after analysis. Figure 10a shows the fraction number and the corresponding gradient weight. The literature states that exosomes separate in gradients 1.13g/ml – 1.17g/ml. Figure 10b shows each fraction run on a Western blot. The Western blot was probed with HSC70 antibody, since the protein is found commonly and predominately on exosomes. Finally, Figure 8c shows electrograms for fractions 7-10, which were hypothesized to contain the exosomes.

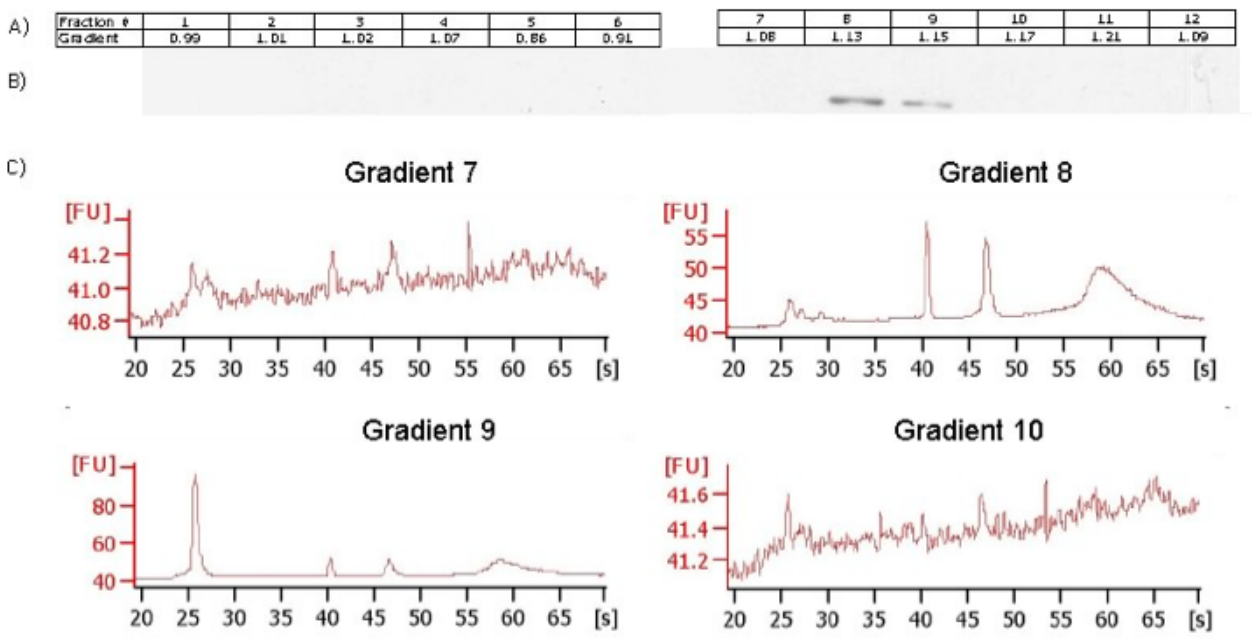


Figure 10: Different fractions isolated from a sucrose gradient of TA/3 cells

Figure 11(a-d) shows the sucrose fractions 7-10 respectively analyzed by scanning electron microscope. The fractions in Figure 9b,c correspond to the presence of HSC70 on the Western blot and the presence of RNA on the electrograms, while Figure 9a,d do not show very many exosomes, if any.

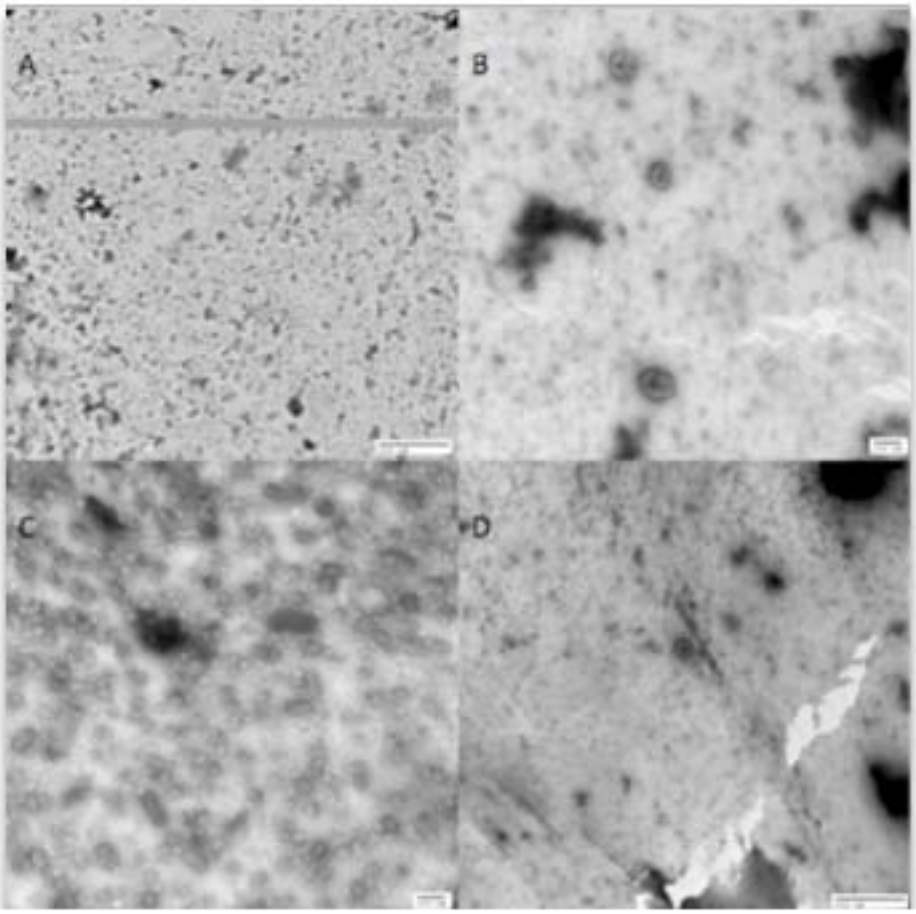


Figure 11: Sample purification of TA/3 derived exosomes viewed by SEM. a-d) correspond to sucrose gradients 7-10 respectively

2.4.3 Messenger RNA and miRNA arrays of MCF-7 cells and exosomes

To determine what mRNAs and miRNAs were present in the exosomes, I decided to test the total RNA from MCF-7 cells and exosomes on microarrays. These cells were chosen due to the fact that they grow a large number of exosomes when compared to other cell lines tested. Total RNA was isolated as per the protocols above, and the RNA from the cells and corresponding exosomes was sent to Ena Wang at the NIH Clinical Center. Two different arrays were run. The first was an mRNA array where 2 ug of total RNA from each sample was loaded on the array and the

analysis was done by Ena Wang PhD. Figure 12 shows transcripts that are over-expressed in MCF-7 exosomes compared to the cells on an mRNA array. The top eight most highly detected mRNAs in exosomes are also shown in this figure. It is interesting to note that BCL-2-like 11 as well as TGF-β transcripts were present. Both of these genes are known to play a role in breast cancer.

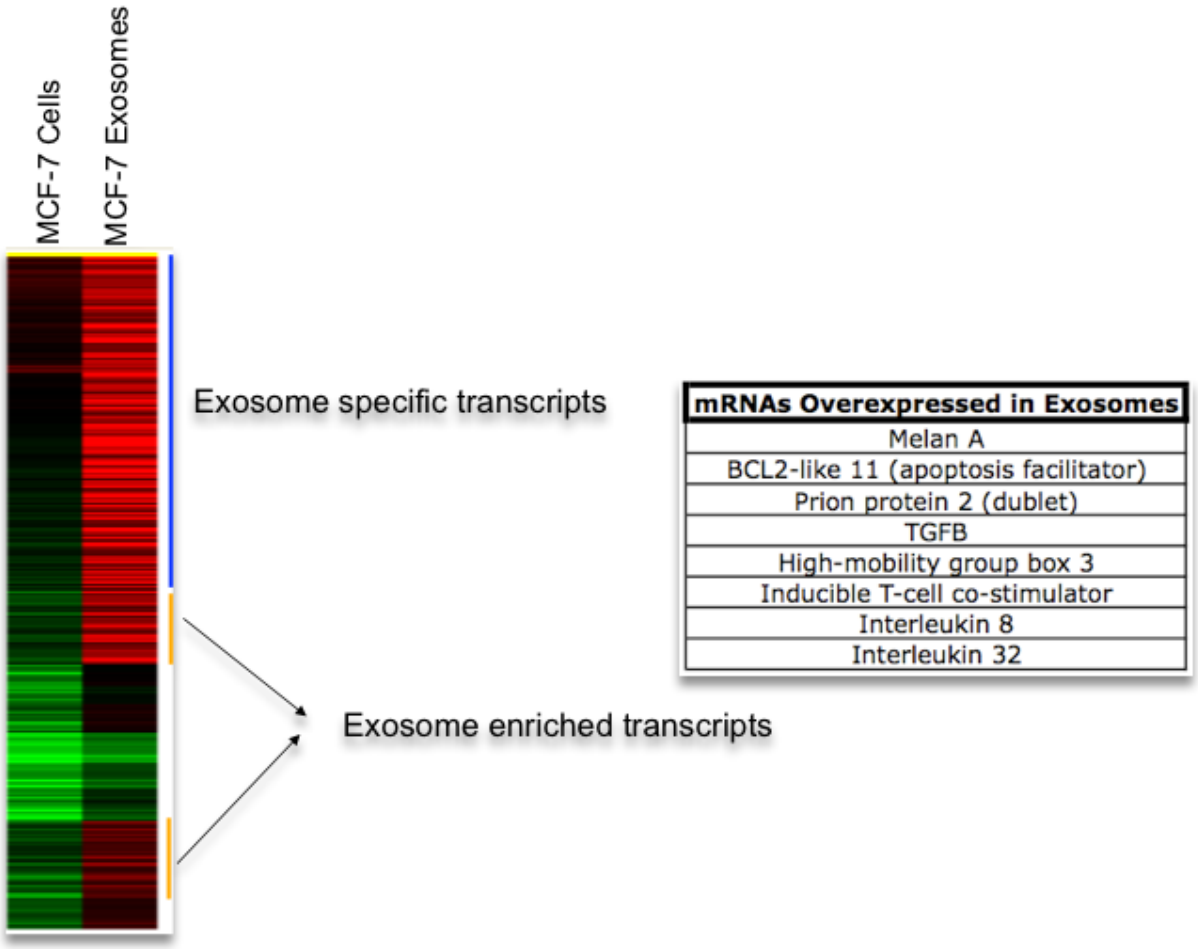


Figure 12: Array results for an mRNA array run on MCF-7 cells and exosomes.

Figure 13 below is from an miRNA array run on both MCF-7 cells and their corresponding exosomes. The array was also loaded with 2 ug of total RNA per sample and hybridized overnight. Red in that figure signifies 2-fold or higher levels of miRNA.

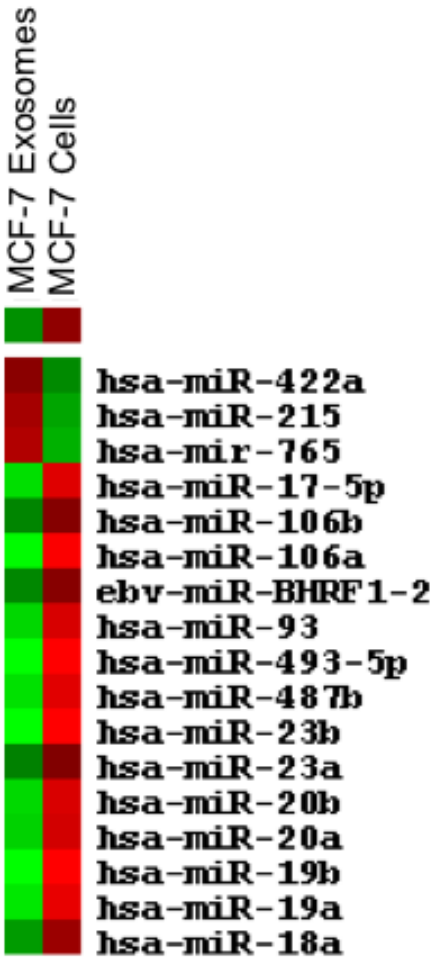


Figure 13: miRNA array of total RNA from MCF-7 cells compared to exosomes.

2.4.4 QRT-PCR analysis of head and neck cancer samples

Given the limited sample material, I decided to use qRT-PCR as the profiling method, focusing on miRNA known to be involved in HNC. This came in the form of profiles from HNC cancer cell lines and patients with several different types of HNC. Since there are multiple types of head and neck cancers, I obtained primers for several miRNAs that appeared to be present at high levels in different cell lines, as well as a few that were commonly present at low levels (shown in section 2.4.6). Several primers were also used for miRNAs that were cell line specific as well as cancer specific. The primers were ordered from Invitrogen's website and were previously validated by them and had known annealing temperatures.

Initially, the primers needed to be tested on known RNA samples. Total RNA was extracted from PCI-13 and PCI-30 cells, both of which are HPV16+ cell lines, and compared to the HPV16- cell lines SCC-47 and SCC-90. Figure 14 shows the results of this experiment. All data were normalized to miR-103. The results of this experiment were confirmed by Abigail Wald, who worked with these cells to determine if any patterns were similar in her experiments. MIR-181a, let-7i, let-7a, and miR-31 had previously been confirmed to work using her methods. In addition, a pattern of HPV16+/- differences could be detected for several miRNAs. I did not pursue this line of experiments any further.

The next step was to test these same primers on total RNA from a cell and exosome pair. This was done using the PCI-13 cells since, unlike the other three cell lines, they produced a measureable number of exosomes and a high enough level of RNA for analysis. PCI-13 cells were grown in a cell factory and 2 liters of supernatant was collected for exosome purification. Total RNA was extracted from the cells and their corresponding exosomes. The results are

shown in the second graph of Figure 14. All miRNAs were normalized to miR-103, and PCI-13 cells were used as a control. As can be seen in Figure 14, there were several miRNAs that were found at a higher level in exosomes than in the corresponding cells. All of the data in Figure 14 was from a starting point of 50 ng of total RNA.

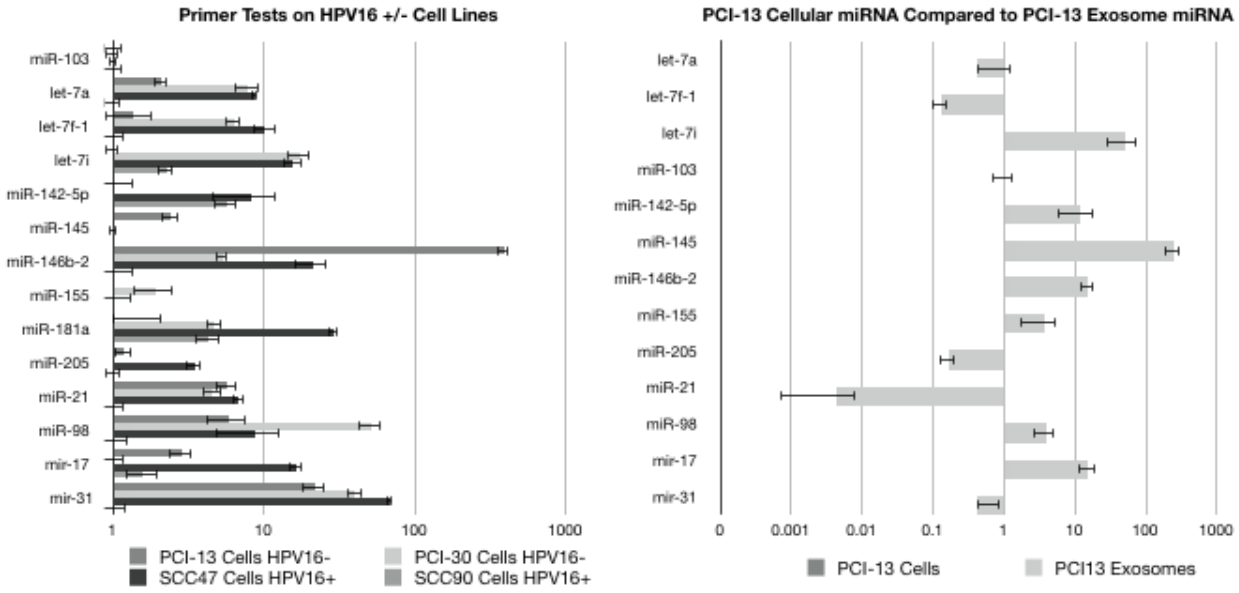


Figure 14: Testing of primers by qRT-PCR on head and neck cell lines.

The next step was to run the qRT-PCR miRNA profile on all of the HNC cancer samples acquired from Theresa Whiteside's laboratory. One criterion used for inclusion was the presence of more than 50 ng of total RNA after nanodrop analysis, as anything less would not have produced any reliable quantitative results. The samples were organized into three groups, four normal control (NC) samples, seven active disease (AD) samples, and finally, four not expressing disease (NED) samples. Normal control samples came from volunteers that did not have any known cancers or infections. Exosomes present in individuals with no known disease are found at much lower levels than in individuals with cancer. Active disease samples were

taken from patients with HNC. Which type of cancer they had was not privy to me, and thus these exosomes could be isolated from any number of cancers in the broad category of HNC.

No normalization controls are known for miRNA work in exosomes, and even less information is known about how miRNA find their way into exosomes and which miRNA are consistently present in these microvesicles. I chose to use miR-103 to normalize all other miRNA, based on a paper stating that the levels of this miRNA were kept constant in a variety of different cell lines tested (143). Total RNA for all the samples was analyzed by nanodrop, and 50 ng of total RNA was used in all qRT-PCR assays. Samples were run in triplicate. As there were no normal tissue samples in this case, all samples were analyzed in a relative fashion. Figure 15 below shows the results of qRT-PCR analysis of exosomes extracted from four NC patients.

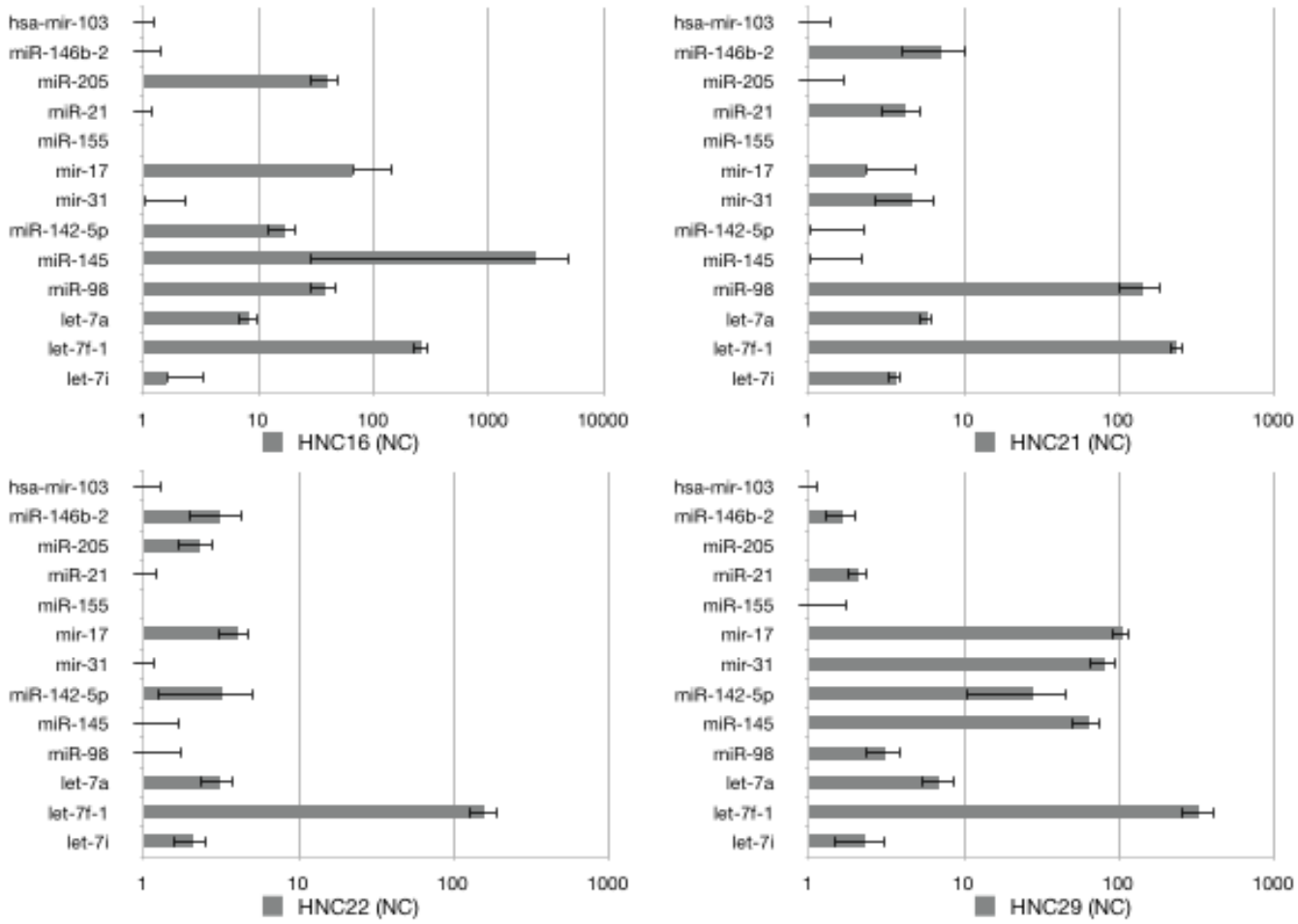


Figure 15: Exosomes from normal control individuals compared to a panel of miRNAs

Following the analysis of NC samples, I looked at the results from patients with AD. Figure 14 shows the results from seven AD patients. Statistical analysis was performed by biostatistician, Mark Friedgan, to determine if there was any correlation between AD samples and NC samples. Due to the very low number of samples and the low levels of RNA used for the experiments, any results determined could not be statistically significant.

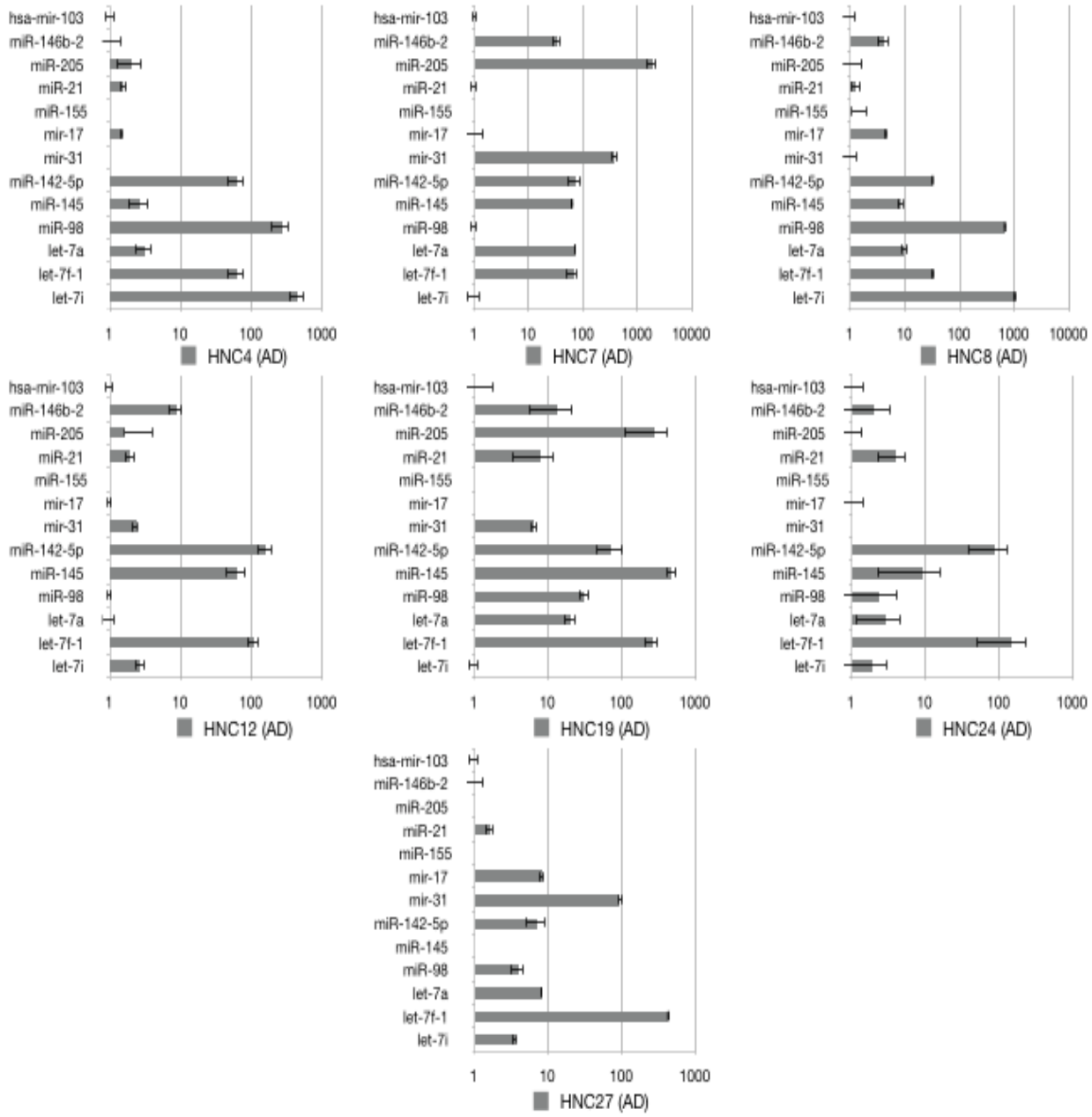


Figure 16: Exosomes from patients diagnosed with head and neck cancer compared to a panel of miRNAs.

The final group of samples was from four patients not expressing disease, or NED. It was not clear whether these samples were from patients in remission, who had been cured, or individuals that had biopsies of benign tumors. Figure 17 shows the results of all four NED patient exosomes.

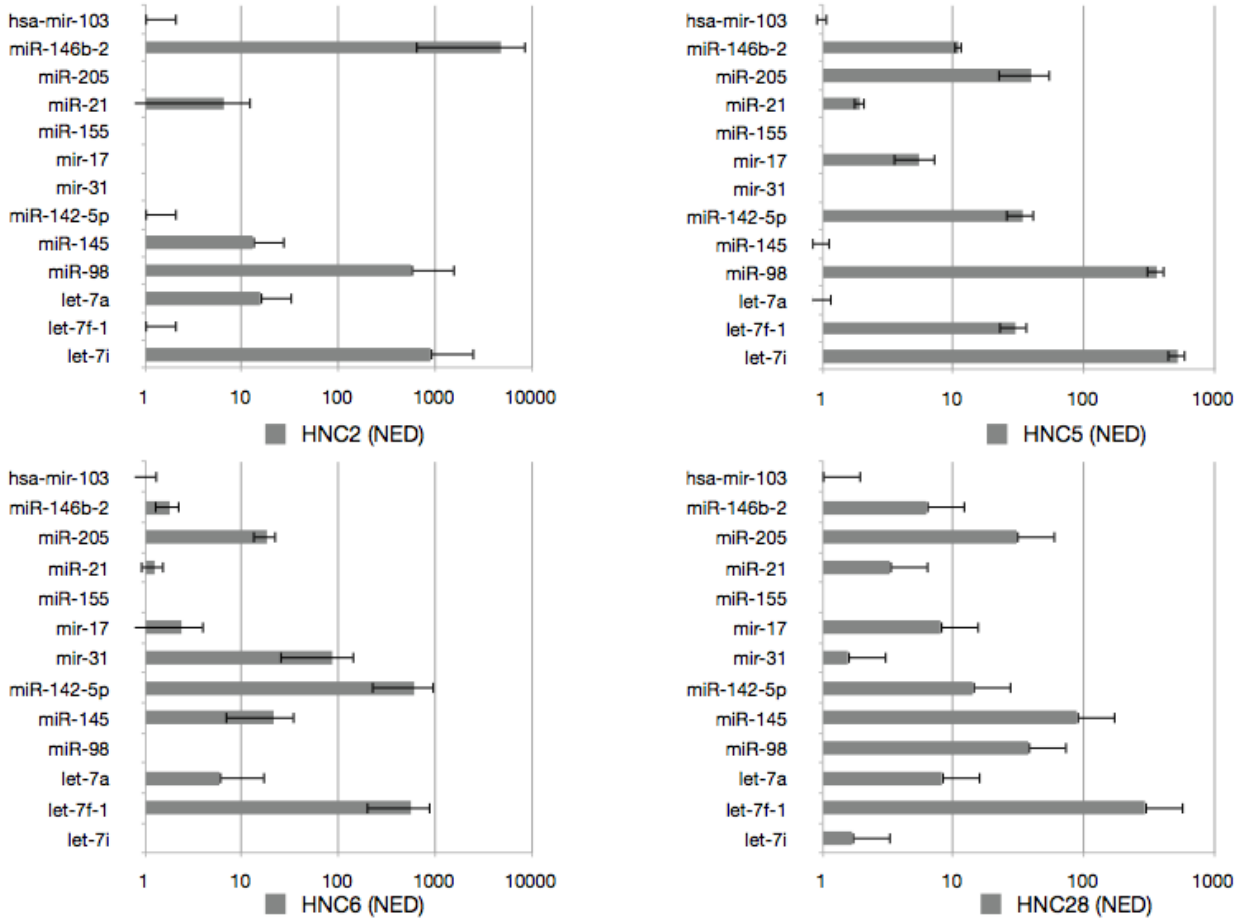


Figure 17: Exosomes from patients diagnosed not expressing disease compared to a panel of miRNAs.

Figure 18 lists the results from a correlation study done on each sample group compared to all of the miRNAs present. The sample number was too small to have any accurate correlations, but these values are listed as they could support a trend in the data. More samples, higher levels of starting RNA, and samples run in higher then triplicates are all factors in

increasing the correlation values for this experiment. A more positive value indicates that the higher concentrations of miRNAs correlate with a higher likelihood of being associated with that group. A more negative value means that lower concentrations of miR correlate with a higher likelihood of being in that group. The trends for this correlation data suggest that miR-205 is positively correlated to AD, while miR-17 is negatively correlated with that same group. The trends for the NC group suggest that miR-17 is positively correlated with that group, while let-7i was negatively correlated with the NC control group.

	miR-146b-2	miR-205	miR-21	miR-155	mir-17	mir-31
AD Correlation	-0.248	**0.312**	0.017	0.026	**-0.372**	0.245
NC Correlation	-0.163	-0.184	-0.188	0.207	**0.628**	-0.139
	miR-142-5p	**miR-145**	**miR-98**	**let-7a**	**let-7f-1**	**let-7i**
AD Correlation	-0.060	-0.201	-0.005	0.293	-0.251	0.052
NC Correlation	-0.278	0.410	-0.269	-0.194	0.187	**-0.338**

Figure 18: Correlation results of AD and NC samples to determine any trends.

2.5 DISCUSSION

Exosomes have long been known to play an important role in the immune system. It was not until recently that our group and others detected the presence of RNA in exosomes and started exploring the possibility that exosomes may play a larger role than previously expected in cell-to-cell communication. It was previously shown that tumor-derived exosomes can transfer EGFRvIII to glioma cells (30). Then in 2007, while we were analyzing RNA from exosomes, another group showed that mast cells carried both mRNA and miRNA and that the mRNA was functional and could be transcribed (23). This form of RNA transfer raised the possibility of

using exosomes as both a therapeutic and diagnostic tool for cancers and other diseases. The mechanism of RNA sorting into exosomes is still largely unknown and thus hard to harness as a therapeutic delivery tool, but it has much potential in the future. Therefore, we initially focused on the use of these tumor-derived exosomes as a diagnostic tool for a variety of diseases. Before initiating these studies, there was much to be understood about the quantity and quality of RNA extracted from exosomes, as well as a having a need to develop optimal techniques for analyzing small quantities of RNA cheaply and accurately.

In this chapter, I describe a set of protocols to isolate larger quantities of exosomes from cell culture to extract larger amounts of total RNA. The RNA from these large-scale isolation procedures was then used in downstream applications such as qRT-PCR and miRNA microarrays to possibly identify miRNA profiles that could be correlated with the disease state. The final goal of these expanded protocols was to use exosomes as cancer diagnostic tools, in particular for head and neck cancer. In the time it took to design and conduct these experiments and test it in our model of HNC, others had also tested similar tools in several disease models.

The first necessary step was to find ways to detect and quantify small amounts of RNA since the types and quantities of RNA present in the exosomes was unknown. I started by using Bio-Agilent's picochips that yielded important information on the sizes and quality of RNA. Representative electrograms of RNA can be seen in Figures 9 and 10. Once I confirmed the presence of RNA in exosomes, I scaled up the tissue culture protocols in order to collect larger quantities of RNA for downstream applications such as miRNA and qRT-PCR analyses. The expanded protocols yielded sufficient quantities of RNA with the same quality as before, which permitted me to do both quality control and detection of miRNAs on an array. The first arrays run were on MCF-7 cells, both for the detection of mRNA and miRNA, and can be seen in

Figures 12 and 13. Both of these arrays yielded interesting results. The mRNA array demonstrated an enrichment of exosomes with transcripts BCL2-like 11 and TGF-β, both of which are known to play a role in breast cancer. The miRNA array led to a list of miRNAs possibly implicated in breast cancer, but future experiments will be needed to investigate this issue.

2.5.1 Head and neck exosomes and miRNA correlation

Due to our established interactions with the laboratory of Dr. Theresa Whiteside, I was able to receive 28 exosome samples from patients with head and neck cancer. I had limited knowledge of the samples, other than that they came from three groups. The first was active disease, the second was not expressing disease, and the final group was normal controls. Due to the low sample number and limited resources, I decided to approach this problem by gathering a list of miRNAs profiled in HNC from the literature. The list was a composite of miRNA profiles in HNC cell lines combined with any profiles from tissue biopsies from HNC patients. This yielded several possible miRNA candidates that were either over- or under-expressed in HNC, and I obtained primers to analyze these miRNAs (Figure 9).

After isolating the RNA and confirming that the primers amplified the appropriate miRNA using RNA isolated from oral cancer cell lines, I then carried out qRT-PCR analysis of RNA isolated from all of the above exosomes samples using these primers. This was followed by statistical analysis to detect any possible patterns or profiles in the exosome samples. Due to the very low level of starting RNA per sample and the small sample set, I could only get a trend for our data when we excluded the group not expressing the disease. Our correlation data shows that higher levels of miR-205 are positively correlated with AD, while lower levels of let-7i are

present in the normal control group. A more positive value (Figure 19) indicates that higher concentrations of a particular miRNA correlate with a higher likelihood of being associated with that group. A more negative value means that lower concentrations of a particular miRNA correlate with a higher likelihood of being in that group. The literature from cell lines and tissues does support the let-7i trend to appear at higher levels in AD samples (or express at a lower level in NC groups) (144). On the other hand, MiR-205 has been shown to be either up- or down-regulated, depending on the type of HNC cancer and cell lines. One study showed that over-expression of miR-205 correlated with the disease state (62, 145), while another study showed an under-expression of miR-205 in cancer (73, 146). In my study, I found lower levels of miR-17 in HNC samples compared to the normal controls (Figure 17). MiR-17 has been shown to be over-expressed in squamous cell carcinoma of the tongue (147), but it is down-regulated in nasopharyngeal carcinoma.

2.5.2 Limitations and strengths of this approach and possible improvements

The strengths of the approach that I took are the ability to analyze a select number of miRNAs on a small sample group and to be able to use very little starting RNA material. The future of exosome diagnostics will lay with more sensitive RNA detection techniques and a lower level of false positives. While these are some of the strengths of using qRT-PCR and the nanodrop to analyze miRNAs, there are a few items that will be needed to expand on this work in the future as well as in other models.

The first item that needs to change will be to increase the sample size. A larger sample size will yield more statistically significant results and increase the accuracy of the work.

Another way to improve the accuracy would be to couple the exosome purification steps with the use of an antibody to pull down exosomes that are specific to HNC in general. An example would be using EpCam in Ovarian cancer (83). The current approach to diagnostics requires considerable resources and time. The most widely used method is to extract total RNA and run it on miRNA arrays and then to validate by qRT-PCR. This requires a great deal of sample material and regular access to patients. Future work will most likely use a form of qRT-PCR array on known miRNAs that are differentially expressed in HNC cancer.

In summary, a larger number of samples, more knowledge about the type of HNC and increased access to patients with HNC would yield more significant results. In addition, coupling the exosome purification process with HNC-specific antibody pull-downs would most likely increase the accuracy of the profiling. As it stands, I show a trend of miRNAs available in the AD group compared to the NC group. I hope that future work on this will yield a viable and reproducible approach to the problem of HNC diagnostics by using exosomes as the diagnostic tool.

3.0 EXOSOMES AS A DIAGNOSTIC TOOL FOR DETECTING VIRAL INFECTIONS

3.1 ABSTRACT

Infectious diseases are the highest cause of mortality in the world today. These diseases comprise four main groups: bacterial, viral, fungal, and Protozoan. Viral diseases will be the subject of this chapter and, in particular, Kaposi's sarcoma-associated herpesvirus (KSHV), a member of the herpes family of viruses. Kaposi sarcoma (KS) is a cancer that occurs in cells lining the lymph nodes or blood vessels, forming lesions of abnormal purple, red, or brown colors and is caused by an infection of KSHV in people. The most common type of Kaposi's sarcoma in the USA is the AIDS-related KS.

KS has been a growing problem in the USA since the AIDS epidemic started. Prior to this epidemic, according to the CDC, there were two cases per one million people, but after this epidemic started, the number rose to 47 cases in 1 million in the early 1990s. With the advent of new HIV therapies, the rate of KS has been going down, but is still a problem for transplant groups, with an incidence of one per 200 transplants. The early diagnosis of KS can help to quickly put patients on a therapeutic path by implementing the use of highly active antiretroviral therapy.

This chapter is based on the isolation of exosomes from cells infected with KSHV and the detection of virally encoded miRNAs in them. As most herpesviruses encode their own unique miRNAs, they are an ideal model for RNA-based diagnostic testing. In addition, KSHV was chosen as the initial model due to collaborators providing access to a KSHV-infected cell line as well as access to a bank of patient samples for future experiments.

3.2 IMPORTANCE OF DETECTING VIRAL INFECTIONS

Viral infections are a large problem in the world today. Viral diseases range from genital herpes, to HIV, to influenza and many others that have afflicted the world's population. With advances in understanding how viruses replicate and spread, as well as increased sensitivity in detection technologies, we are entering an age where the diagnostics of many viral diseases will be done at earlier stages of the viral infection cycle. These technological advances could lead to many antiviral therapies and better control of viral reproduction and spread. The advent of better sequencing and more sensitive RNA detection technologies has helped the research community detect virally-encoded miRNAs in a few virus types and, in particular, the herpesvirus family. The discovery of virally-encoded miRNAs has lead to a slew of research into the potential targets of these miRNAs, the overall miRNA functions, and the evolutionary roles of these small RNAs. This chapter deals with using virally encoded miRNAs as potential diagnostic markers of disease, in particular when combined with microvesicles as the delivery method.

3.3 EXOSOMES AND INFECTIOUS DISEASES

Prior to discussing virally-encoded miRNAs, it is important to understand how viruses and prions use exosomes in their lifecycles. Research studies have recently shown that prions, HIV, EBV, and possibly other viruses use the exosome machinery to further their goals. In the next few paragraphs, I will discuss how infectious agents utilize the exosome machinery.

Prions were the first infectious agents discovered to utilize exosomes for their spread. Prion diseases are infectious neurodegenerative disorders linked to the increase of improperly folded proteins called scrapie (122). These prions are associated with exosomes on the cell membrane, as seen by immunoelectron microscopy, but the first report that exosomes can act as "Trojan" exosomes for the spread of viruses was published in 2003 (114).

This Trojan exosome hypothesis states that retroviruses use the pre-existing exosome biogenesis pathway for the release of both exosomes and retroviruses through the common intralumenal vesicles (ILV), which form by inward budding of the endosome membrane in primary macrophages (115). It was also shown that exosomes secreted from CD8+ T cells noncytotoxicly suppress the transcription of HIV-1 (116). Another recent study describes the capture of HIV-1 particles by mature dendtritic cells for mediating trans-infection of T lymphocytes by using the exosomal pathway (117). The added knowledge that HIV encodes a viral miRNA, HIV1-miR-H1, has a profound effect on the virus' ability to cripple cellular responses to infection. This makes HIV a good example of how exosomes can be used as Trojans (118).

HIV is not the only virus to make use of exosomes for immune escape and viral propagation. EBV's latent membrane protein (LMP 1) was discovered in exosomes of cells infected by the virus (119). Preparations of LMP1 containing exosomes derived from

lymphoblastoid cell lines were described to inhibit the proliferation of peripheral blood mononuclear cells, suggesting that this protein might be involved in immune regulation.

A further study discovered both the presence of LMP1 and galectin 9 (C15) or galectin 9 only (C17) in nasopharyngeal carcinoma-derived exosomes (NPC), which are often associated with EBV (120). By using recombinant LMP1, the authors were able to demonstrate the inhibition of T cells, suggesting a strong role for exosomes containing LMP1 proteins in aiding immune evasion by EBV. Another study found that FGF-2, a potent angiogenic factor important in immune evasion, also co-localizes with LMP1 (121). In addition to this, p53 protein has been shown to respond to stress signals by regulating the transcription of several genes. In particular, a p53-regulated gene product called TSAP6 was shown to enhance exosome production during stress response (35, 123). This could be a way that viral infections increase the number of exosomes released to helping to escape viral immune response.

3.4 THE HERPESVIRUS FAMILY AND KAPOSI SARCOMA ASSOCIATED VIRUS

Kaposi Sarcoma-associated Herpes Virus (KSHV) is a member of the herpesvirus family. The herpesvirus family (*herpesviridae*) family consists of large enveloped viruses that contain 120-240 kb of double-stranded DNA. Depending on the host system and tissue specificity, they are split up into three subfamilies encompassing eight known human herpesviruses. In humans, there are three α-, three β-, and two γ-herpesviruses. One of the major identifiers of herpesvirus infections is their ability to become latent and maintain latency when the genome circularizes and remains as an episome. In this stage, there is very little gene expression and the infections can persist in some cases for the lifetime of the host. This very complex balance is played out

between the immune surveillance system and viral immune evasion mechanisms. Of particular interest to us is KSHV, a γ-herpesviruses. Kaposi's sarcoma-associated herpes virus (KSHV) is implicated in Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD). This is in contrast to the α- and β-herpesviruses, which are not tumorgenic but cause diseases when they enter the lytic phase with uncontrolled virus replication. The seroprevalance of KSHV in western society is low, usually less than 5%, but can reach around 50% in some African populations.

3.5 DIAGNOSTICS OF KSHV

Current diagnostic tests for detecting KSHV infections are done using antibody assays. No definitive test exists and the rates of false positives are still high, making current detection methods unreliable. Some headway is being made in developing whole-virus KSHV Elisa and two immunofluorescence assays that could detect antibodies against lytic (IFA-lytic) and latency-associated nuclear antigens (IFA-LANA) (148). The typical KS immunohistochemical profile looks for a panel of antibodies against mesenchyme-derived cells and antibodies against proteins present in EC (CD31, PECAM-1, CD34, and von Willebrand factor.) Creating a more accurate and reliable method of detecting KSHV infection and the presence of KS would be very beneficial for the treatment of the disease. In particular, miRNA testing could be brought to the Brazilian Amazon, where the seroprevalance reaches levels higher than 50%.

3.6 VIRALLY ENCODED MIRNAS AND KSHV

The first targets of virally-encoded miRNAs to be discovered were viral ones because they are usually transcribed antisense to the viral mRNA target, making them easier to discover. The first known target of a virally-encoded miRNA was the mRNA encoding an EBV DNA polymerase, BALF5, which is downregulated by EBV miR-BART2 (104). The role of this down-regulation is still unclear but seems to play a role in the stabilization of viral latency. In addition to the autoregulation of viral targets, viral miRNAs also target cellular mRNA (92).

KSHV is the other γ-herpesviruses of interest due to the growing amount of information on its pathogenesis. It is thought that at least one KSHV miRNA targets the IE proteins to enhance the early latency of the virus (99). In KSHV, miRK12-5, miR-K12-9, and miR-k12-10 were discovered as targets of BCLAF1 (108). BCLAF1 can act both as a pro- or anti-apoptotic factor, depending upon the context. It was experimentally shown that levels of BCLAF1 were elevated after infection with KSHV containing mutations in the above miRNA genes. BCALF1 is therefore seen as a stabilizer of KSHV latency, as higher levels decreased viral production. The seed sequence of another KSHV miRNA, miR-K12-11, was an exact match to cellular miRNA miR-155, suggesting a similar role (109). In this same study, miR-K12-11 was shown to target the 3'UTR of the BACH-1 mRNA. BACH-1 is a known transcriptional factor that down-regulates heme oxygenase-1 (110). The over-expression of miR-155 is associated with a number of B cell lymphomas and solid organ tumors and is also a significant marker of tumorgenesis for several types of cancers (111). KSHV miR-K12-2, miR-K12-2, miR-K12-3, miR-K12-4, and miR-K12-5 all target thrombospondin 1 (THBS-1) (112), whose down-regulation leads to angiogenesis and proliferation of the infected cells.

These combined findings show an important role for virally-encoded miRNAs in the reproduction cycle of viruses and in the immune evasion of tumors. The targeting of MICB by HCMV and KSHV shows a method by which infected cells evade NK cell activity and thus evade the immune system. The discovery of a miR-155 ortholog in KSHV hints at the evolution of viral miRNAs that mimic cellular ones. Of particular interest in this evolutionary adaptation to the regulation of MICB is the fact that both KSHV and HCMV encode proteins K5 (KSHV) (113) and UL16 (HCMV) (102) that mediate the sequestration of not only MICB but also other NKG2D ligands.

While exosomes have been shown to help viruses propagate themselves, virally-encoded miRNAs have yet to be detected in exosomes purified from virally infected cell lines. In this chapter, I will discuss the discovery of KSHV-encoded miRNAs in exosomes derived from a KSHV-infected B-cell line and the potential uses of this discovery in improving diagnostic methods.

3.7 MATERIALS AND METHODS

3.7.1 Cell culture

B-cells and BCBL-1 cells were cultured in RPMI-1640 cell culture medium. The cell media was comprised of 10% 40 nm pre-cleared FBS, 1% HEPES, 1% L-Glutamine in RPMI from GIBCO. These cells were non-adherent cells and thus required extra care during growth and expansion. Cells were grown in NUNC 3-layer flasks filled with 100 ml of media for three to four days prior to splitting. Cell expansion and splitting was done by collecting the supernatant with the cells

and spinning them down at 300Xg for 15 minutes in a conical viral collection flask from Corning (CAT#431123). The supernatant was poured out and bleached and the cells re-suspended in an appropriate amount of media. For expansion, we used a dozen 3-layer flasks and re-suspended the cells in 2 L of media and loaded this cell mixture into a 10-layer cell factory. These cells were grown for three to four days or until the cell factory appeared cloudy or confluent.

3.7.2 Exosome Purification

Cells were expanded to create 12 T-175 flasks at 80% confluence. An extra T-175 flask was maintained for cell collection and propagation. This produced around 100 million cells for the seeding of the cell factory. The speed of growth needed to be adjusted to maintain the proper confluence levels of your cells. Cells from cell factories were harvested every three to four days.

Cells were collected and 100 million cells were seeded in a 10-chamber cell factory from Nunc (CAT#164327). The simplest method of loading the cell factory was to collect all of the cells by centrifugation and to dilute the pellet with 1.8 L of media in the glass-loading container, making sure to mix well for an even distribution. Once the factory was loaded, cells were harvested after three to four days until they were 80% confluent.

Once the cells were confluent, the cultures were poured into 1 L 0.22 μM media sterilization filters. The larger surface area of these filters minimizes the clogging from any debris in the supernatant. Optionally, if the cells being used were non-adherent or produced substantial debris, the supernatant was first spun down in larger conical 500 ml viral prep flasks for 30 minutes at 2000Xg. Control cells were also collected at this time. This was done by one of two ways. The first was to collect cells from a T-175 flask growing alongside the cell factory. The second approach was to pour enough trypsin into the cell factory to cover ¼th of a layer, mix

hard, and pour. The cells were then washed three times with PBS and spun down at 12000Xg for 1 minute, with the final pellet collected in 300 µl of Ambion's RNAqueous lysis buffer and stored at -80^{o}C until RNA purification.

The large volume of filtered supernatant was then concentrated down by using Satorius's vivacell concentrators with a 100-kDa cut off. Each concentrator was pre-washed with 50 ml of distilled water and then with 50 ml of PBS to remove any ethanol and glycerol still present on the filter membrane. The supernatant was then loaded into the vivacells and spun for 20 minutes at 3000Xg. The flow-through was discarded and the remaining volume was then collected. This process was repeated until no more supernatant remained. The resulting volume was between 50-150 ml of concentrated supernatant.

The resultant supernatant was loaded and balanced into a SW-31ti rotor and spun at 100,000Xg for 2 hours. The supernatant was poured off and any remaining fluid was delicately removed with a pipette. The pellets were combined into one tube unless the downstream application included protein work in addition to RNA work, in which case the pellets were separated into two aliquots. The pellet was re-suspended with 30 ml of sterile PBS and spun again at 100,000Xg for 2 hours. Again, any remaining supernatant was removed from the resulting pellet, followed by a final re-suspension. If a separate pellet was saved for protein work, it was suspended in 100 µl of PBS and stored at 4˚C. The pellet to be used for RNA analysis was resuspended in 100 µl of Ambion's lysis buffer and stored at -80˚C. Figure 4 shows the experimental flow of the large-scale exosome purification protocol.

3.7.3 Transmission Electron Microscopy

All transmission electron microscopy (TEM) work was done in the Center for Biologic Imaging (CBI) laboratory at the University of Pittsburgh with the aid of Nicole Bianco, PhD in our laboratory. Exosomes were placed on specially created Formvar/carbon coated grids made by the CBI and incubated for 1 minute before removing the excess, followed by a 1 minute incubation with 1% uranylacetate in PBS. The excess uranyl acetate was blotted off with filter paper and the samples read for analysis. The transmission electron microscope used was a JEOL-1210 computer-controlled high contrast 120 kv, and the final analysis was done either by Nicole Bianco, PhD or ChenJe- Yang.

3.7.4 Quantitative Real-Time PCR.

Quantitative real-time PCR was accomplished using the same Invitrogen kit as in chapter 2. The viral miRNA sequences were obtained from the Sanger Mirbase version 10 library and corroborated by the literature. Annealing temperatures were checked to be higher than 55^{o}C. Primers were tested on both BCBL-1 cells and non-infected B-Cell lines to determine specificity. Figure 19 shows a list of miRNA sequences and associated names.

Name	Sequence
kshv-miR-K12-1	ATTACAGGAAACTGGGTGTAAGC
kshv-miR-K12-2	AACTGTAGTCCGGGTCGATCTG
kshv-miR-K12-3	TCACATTCTGAGGACGGCAGCGA
kshv-miR-K12-4-5p	AGCTAAACCGCAGTACTCTAG(G)
kshv-miR-K12-4-3p	TAGAATACTGAGGCCTAGCTGA
kshv-miR-K12-5	TAGGATGCCTGGAACTTGCCGG
kshv-miR-K12-6-5p	CCAGCAGCACCTAATCCATCGG
kshv-miR-K12-6-3p	TGATGGTTTTCGGGCTGTTGAG
kshv-miR-K12-7	TGATCCCATGTTGCTGGCGCT(CA)
kshv-miR-K12-8-5p	CTCCCTCACTAACGCCCCGC
kshv-miR-K12-8-3p	CTAGGCGCGACTGAGAGAG(CA)
kshv-miR-K12-9-5p	ACCCAGCTGCGTAAACCCCGCT
kshv-miR-K12-9-3p	CTGGGTATACGCAGCTGCGTA
kshv-miR-K12-10	TAGTGTTGTCCCCCCGAGTGGC
kshv-miR-k12-11	TTAATGCTTAGCCTGTGTCCGA
kshv-miR-k12-12	ACCAGGCCACCATTCCTCTCCG

Figure 19: List of known KSHV-encoded miRNAs as of version 10 of the Sagner miRNA library.

3.8 RESULTS

3.8.1 Exosomes are present in supernatant from KSHV infected cell lines

I first began by growing cells from a KSHV-infected B cell line called BCBL-1. The idea was to detect exosomes first and then try to purify them from a large-scale preparation for future RNA work. In addition to this, I compared the BCBL-1 exosome purifications to those collected from a non-infected B cell line. Figure 20 shows two exosome samples visualized with TEM. The left image is a sample of B cell exosomes, while the right image is a sample of the BCBL-1 exosomes. Most of the B cell exosomes seem to be in the lower 40-60 nm range in size.

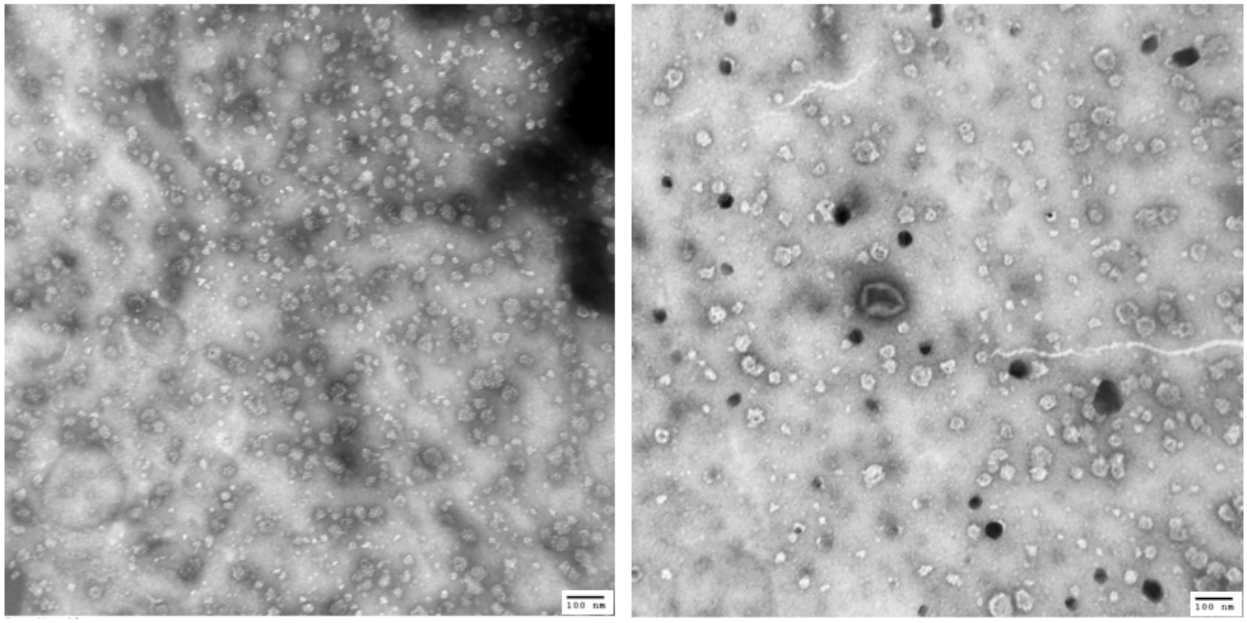


Figure 20: TEM of exosomes. The left panel shows TEM pictures of exosomes isolated from B cell supernatant while the right one was isolated from BCBL-1 supernatant

3.8.2 Microarray analysis of mIRNAs from BCBL-1 derived exosomes

In order to identify the presence of both specific viral and cellular miRNAs, I needed to analyze the RNA purified from BCBL-1-derived exosomes on microarrays. Thanks to our collaboration with Dr. Ena Wang at the NIH Clinical Center, all RNA isolated from BCBL-1 cells and the corresponding exosomes were shipped to her laboratory for miRNA detection and analysis. In short, 2 ug of total RNA was labeled and loaded on each miRNA array. The samples were hybridized overnight. The arrays were done in duplicate using total RNA isolated from BCBL-1 cells and from BCBL-1-derived exosomes, followed by statistical analysis of the results for a threefold or higher change. All samples with a greater than 16-fold expression and a cutoff of $p<0.01$ are shown in Figure 21. The left side represents miRNAs enriched in exosomes, while the right side represents miRNAs enriched in BCBL-1 cells. In order to compare more samples, the

arrays were compared together to show the results in Figure 21. All miRNAs that showed a threefold or higher difference between intracellular and exosomal miRNAs are shown in Figure 22. As can be seen from both Figures 21 and 22, there are certain miRNAs that are found in higher and/or lower levels in exosomes as compared to their cellular counterparts. The next step was to test the RNA from BCBL-1 derived exosomes using qRT-PCR.

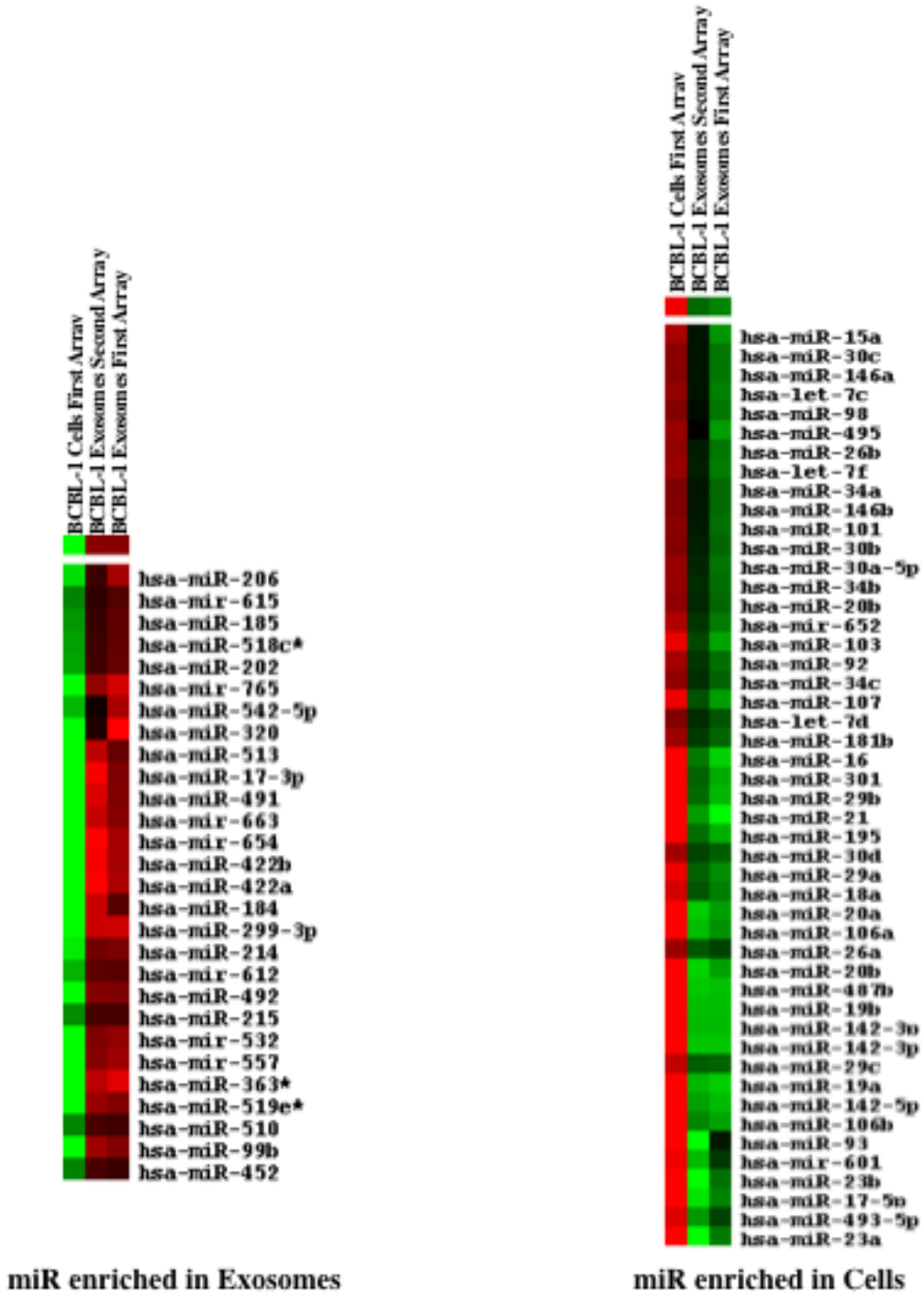


Figure 21: One sample pair of BCBL-1 cells and exosomes compared to on two different arrays. The values shown have a cutoff of p<0.01 and red signifies a greater than 16-fold expression. Each array used 2ug of labeled total RNA.

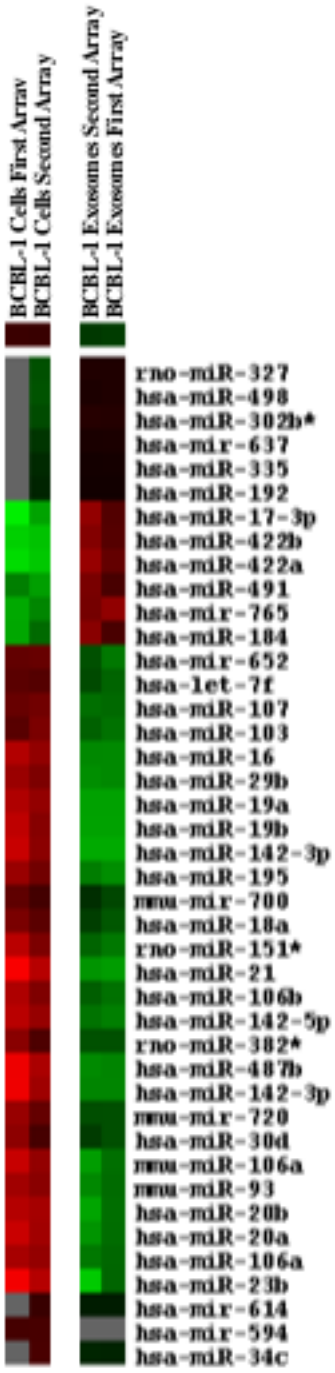


Figure 22: One sample of BCBL-1 cells compared to exosomes run on two arrays.

The values shown have a cutoff of p<0.01. Red signifies a greater than threefold difference.

Each array used 2ug of labeled total RNA.

3.8.3 qRT-PCR analysis of miRNAs isolated from KSHV infected cells and exosomes

In order to check for KSHV miRNAs specifically encoded by the virus, I decided to use the more sensitive approach of qRT-PCR coupled with the Invitrogen miRNA SyberGreen ER kit. In essence, 100 ng of total RNA was quantified and used to produce cDNA templates for the quantitative aspect of the PCR. Samples were then diluted 1:10 and each experiment was done in triplicate using the appropriate primers. B cell RNA only yielded miR-103, as was expected in cells not infected by KSHV. KSHV-infected cell lines were analyzed for the presence of KSHV-specific miRNAs. All 12 miRNAs were tested on two different cellular and exosome sample pairs. The results of the data are shown in Figure 23. Only four miRNAs (KSHV-K12-K9 thru K12-K-12) were found to be consistently present in higher levels in the exosomes as compared to the cells.

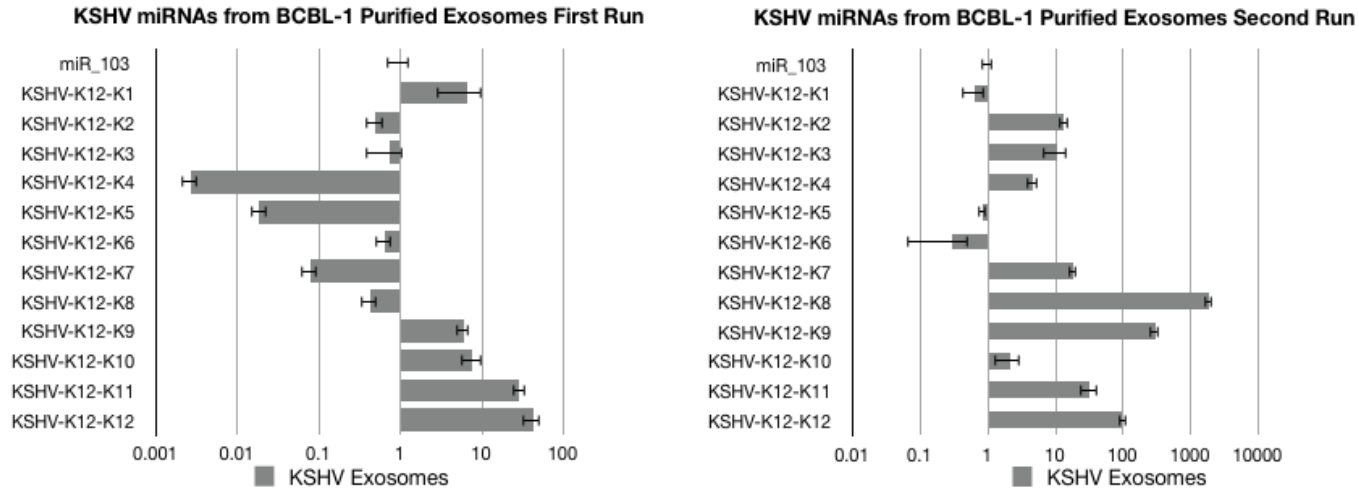


Figure 23: qRT-PCR results from RNA of BCBL-1 cells and exosomes shown for two different sample pairs.

3.9 DISCUSSION

The use of exosomes as a diagnostic tool is slowly becoming more and more accepted. Some of the preliminary work in this field was done in the diagnostics of cancers by specific miRNA profiles of RNA isolated from cancer-derived exosomes. The question of whether virally-encoded miRNAs are present in exosomes derived from KSHV-infected cells is an easier problem to solve than that of profiling cancers. In practice, the diagnostic results for KSHV miRNAs in the exosomes simply involve testing for the presence or absence of a particular miRNA rather than changes in the relative levels of such miRNAs.

The first thing I tested was whether any exosomes were produced by B cells and KSHV-infected B cells. Exosomes are produced in varying levels based on the cells they come from and thus, results may vary. As can be seen in Figure 20, both the B and BCBL-1 cells produced detectable amounts of exosomes. These exosomes seem to be on the lower end of the size spectrum, near the 40-60 nm range. The next step was to send RNA samples for miRNA array analysis. This yielded some interesting results, including several miRNAs found at a higher or lower level in exosomes as compared to their progenitor cells. While no detectable patterns were evident, this led me to test these samples for the presence of KSHV-encoded miRNAs.

Virally-encoded miRNAs for KSHV were selected from the Sanger library miRbase version 10. I compared results between infected and uninfected cell lines to check for specificity of miRNAs. Results for these experiments can be seen in Figure 23. B cells were found to contain miR-103 both intracellularly as well as in their exosomes (Figure 23). Furthermore, as expected, B cells did not contain any viral miRNAs. KSHV-infected BCBL-1 cells contained miR-103 as well as 12 virally-encoded miRNAs (Figure 23). These results clearly show that

exosomes isolated from the serum of KSHV-infected patients could be used as a non-invasive method for the detection of the virus.

In addition to this work on RNA from BCBL-1-derived exosomes, I attempted to detect KSHV-encoded miRNAs from exosomes isolated from KSHV-positive human serum samples. This work was done in collaboration with Dr Rinaldo's laboratory and Varsha Shridhar, a PhD student working in an infectious disease laboratory. None of the samples produced any noticeable exosome pellets or detectable levels of RNA. This was most likely due to the frozen nature of the starting samples and the need for added purification steps to produce cleaner samples.

A large number of miRNAs are either hypothetical in nature or have only been validated with no further information about their target or tissue specificity. At this stage, we have shown that KSHV can be used as a model system for isolating exosomes and detecting virally-encoded miRNAs. It is possible that as technology improves and RNA detection techniques become more sensitive, better diagnostic approaches will be developed using this model and applied to other viral infections.

Future work in this field will need to utilize a larger sample size with higher purity of exosome preparations from fresh samples. It is also possible that in vivo KSHV-infected cells do not produce many exosomes in their latent phase as compared to productive viral infection. Some work can be done in EBV-, HIV- and HCMV-specific cell lines and patients as well.

4.0 CONCLUSIONS AND FUTURE DIRECTIONS

4.1 SUMMARY OF FINDINGS

Head and neck cancer is a common cancer that affects many individuals worldwide. Creating a more sensitive diagnostic test for this disease would put patients on a therapeutic path at an earlier time, potentially averting the full-blown disease. I used miRNAs extracted from HNC cell-derived exosomes as well as exosomes purified from patient serum to establish a trend that is consistent with the literature. I suggest that higher levels of miR-205 and lower levels of miR-17 are associated with the active disease group. MiR-17 has been suggested to be up-regulated in HNC nasopharynx (145), while miR-205 was highly expressed in HNC cancer cell lines (62, 144). Thus, my data suggest that miR-17 and miR-205 in the exosomes may be useful as biomarkers for HNC.

In addition to analyzing miRNA profiles in HNC-derived exosomes, I looked at the simpler question of whether virally-encoded miRNAs could be detected in KSHV-derived exosomes. This was an easier problem because it was not dependent upon relative amounts or quantitative analysis, but solely based on detecting the KSHV miRNAs. Based on the literature, I created a set of primers for miRNAs that are specific to KSHV (109, 150). To ensure that these primers were specific for KSHV-encoded miRNAs, I used uninfected B-cells as my control. None of the controls showed the presence of virally-encoded miRNAs, while cellular miRNAs

could be detected. Armed with this knowledge, I tested several pairs of KSHV-infected cells and their exosomes for the presence of virally-encoded miRNAs. I found that all of the miRNAs tested were present. I believe that by using this approach, it will be possible to detect miRNAs in exosomes derived from other herpesviruses or possibly HIV-infected cells.

Taken together, my results show the potential of exosomes as a non-invasive diagnostic tool for the detection of infectious diseases and certain types of cancers. As more information is available on miRNA targets and more accurate miRNA profiles are created for cancer progression, the diagnostic potential of exosomes will increase. It is already possible to detect stages of cancer for ovarian cancer (83). While not discussed in this dissertation, the possible therapeutic uses of exosomes have also increased with the discovery that they contain miRNAs. Thus, it might be possible to use exosomes as a delivery mechanism for miRNAs.

4.2 FUTURE DIRECTIONS

There are a number of ways to improve on the results described in this dissertation. Below is an outline of several improvements that can be attempted. Since exosomes present in the serum of patients are generally a mixture of tumor-specific as well as non-specific exosomes, the first improvement would be to increase the purity of the specific exosomes by antibody pull-down approaches. Also, sensitivity of miRNA detection and analysis needs to be improved. Finally, increasing the sample size and specificity of miRNA primers would lead to more statistically significant results. Below are some examples of improvements that would facilitate the diagnostic potential of exosomes.

The first item to improve is the isolation of exosomes. Currently, exosomes from HNC patient serum are isolated with no preprocessing for selection of a specific exosome population. The human body releases exosomes naturally, and the levels only increase when the immune system is stressed. In addition, due to the broad category of HNC, there are many different subtypes of cancers associated with this disease. The additional step of isolating exosomes using antibodies to individual HNC types would add a level of specificity. Since many HNCs are associated with HPV16, it might also be possible to use an HPV16+ specific antibody as a way of sorting out cancer-specific exosomes and then profile the miRNA from these.

The second improvement would be to increase the sample size from four in each group to 10-15 per disease group. The larger number of samples would provide more data and thus statistically significant results. By combining this increased sample size with patient information about the specific type of cancer, miRNA profiles would become more robust.

Finally, if resources were limitless, all of my samples would have been analyzed by miRNA arrays prior to the use of qRT-PCR for a higher level of specificity and the screening of larger numbers of miRNAs. As technology improves, the costs of arrays should go down and the sensitivity should increase. Some companies have already developed qRT-PCR-based miRNA arrays by using picogram levels of total RNA and in greater than 5,000 well plate designs. It is possible that this technology will be affordable enough in the near future such that these experiments can be performed in a more cost-effective manner.

4.3 A NOTE ON BIOLOGICAL ACTIVITY AND THE POTENTIAL OF MIRNA IN EXOSOMES AS THERAPEUTIC TOOLS

Little is known about the biological activity of exosome-derived RNA once it enters the target cells. Even less is known about the molecular pathways involved in sorting miRNAs into exosomes. How can these ideas be tested, and what benefits could be gained from such knowledge? I take this opportunity to share some of these thoughts below.

The first question that arises is whether the RNA present in the exosomes is biologically active once it has reached its target. This was partially addressed with mRNA in the initial Nature paper describing the discovery of RNA in exosomes (23). One way that we tried to address this issue was by collecting exosomes from KSHV-infected cell lines and feeding these exosomes in varying concentrations to a DC cell line in order to see if we could detect KSHV-specific miRNAs in the target DC cell lines. Although this experiment was unsuccessful, it could have been due to several variables, including exosome concentration, timing, and what the appropriate target cells to use are.

Another method that I initiated but did not finish was to use a commercially available vector made by Invitrogen that over-expressed an miRNA against the luciferase gene. The hope was to use a cell line that produced an abundant quantity of exosomes and transfect it with either the miR-Luciferase or a control vector. Exosomes would then be isolated from transfected cells and tested for the presence of miR-LUC. If this transcript was detected in the exosomes, they could then be incubated with a target cell line that was expressing the luciferase gene. Luciferase expression could then be tested in an exosome dose-dependent manner. This experiment could reveal whether miRNAs carried by exosomes are biologically functional. Success of this approach can then be used to test the therapeutic potential of exosomes containing a chosen

miRNA. By combining the use of exosomes as a delivery mechanism and the loading of these exosomes with a therapeutic miRNA or antagomir, exosomes could become important therapeutic vehicles.

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