THE DNA DAMAGE RESPONSE PATHWAY IN ORAL SQUAMOUS CELL CARCINOMA

by

Rahul Atul Parikh

M.B.B.S., Seth G.S. Medical College and K.E.M. Hospital, Mumbai, India, 2001

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This dissertation was presented

by

Rahul A. Parikh

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and approved by

Dissertation Advisor:
Susanne M. Gollin, Ph.D.
Professor
Department of Human Genetics
Graduate School of Public Health
University of Pittsburgh

Committee Member:
Robert E. Ferrell, Ph.D.
Professor
Department of Human Genetics
Graduate School of Public Health
University of Pittsburgh

Committee Member:
Baskaran Rajasekaran, Ph.D.
Associate Professor
Department of Molecular Genetics and Biochemistry
School of Medicine
University of Pittsburgh

Committee Member:
William S. Saunders, Ph.D.
Associate Professor
Department of Biological Sciences
School of Arts and Sciences
University of Pittsburgh

Committee Member:
Lin Zhang, Ph.D.
Assistant Professor
Department of Pharmacology
School of Medicine
University of Pittsburgh

Susanne M. Gollin, Ph.D.

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Nearly 45% of oral squamous cell carcinomas (OSCC) are characterized by amplification of chromosomal band 11q13, which occurs by breakage-fusion-bridge (BFB) cycle mechanism. The first step in this cycle involves loss of distal 11q. Consequently, critical genes involved in the DNA damage response pathway, *MRE11A, ATM*, *H2AFX* and *CHEK1* are lost in the step preceding 11q13 amplification. We hypothesized that this loss on distal 11q may lead to a diminished DNA damage response in OSCC. Characterization of OSCC using fluorescence *in situ* hybridization revealed partial loss of *MRE11A, ATM, H2AFX* and *CHEK1* in all cell lines with 11q13 amplification and in additional cell lines without 11q13 amplification. Quantitative microsatellite analysis and loss of heterozygosity studies confirmed this loss. Quantitative PCR and immunoblotting revealed reductions in RNA and protein expression of MRE11A, ATM and H2AX. All cell lines with distal 11q loss exhibited aberrant gamma-H2AX foci and increased chromosomal instability to ionizing radiation. Surprisingly, distal 11q loss also correlated with reduced sensitivity to ionizing radiation. Although the literature attributes poor prognosis in OSCC to 11q13 gene amplification, our results suggest that distal 11q deletions may be equally if not more significant.

We observed an upregulation of the ATR–CHEK1 pathway in a subset of OSCC with loss of the G1 cell cycle checkpoint. We hypothesized that this upregulation protects

OSCC from premature chromatin condensation or mitotic catastrophe (cell death) by enhancing the S phase and G_2 phase checkpoints. In OSCC, we observed a gain in *ATR* gene copy number; whereas *CHEK1* is partially lost at the gene level. However, we observed that both ATR and CHEK1 are overexpressed in a subset of OSCC with loss of the G_1 cell cycle checkpoint. Inhibition of ATR or CHEK1 with caffeine or with the respective siRNAs results in an increased susceptibility of OSCC to DNA damaging agents. Thus, inhibition of the ATR–CHEK1 pathway in OSCC may aid the current therapeutic modalities used in the treatment of OSCC. The public health significance of our studies involves the development and use of distal 11q loss and ATR–CHEK1 upregulation as biomarkers for OSCC.

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PUBLIC HEALTH SIGNIFICANCE

Cancer is a growing health problem in the U.S. and worldwide. Cancer is a leading cause of morbidity and mortality in the U.S., second only to coronary heart disease. With significant improvements in the health care industry and longevity, the incidence of cancer is predicted to increase significantly over the next few decades (YANCIK 2005). Since carcinogenesis involves a stepwise accumulation of genetic insults over a period of time, these genetic defects can be used to differentiate tumor cells from normal cells. Such molecular biomarkers will also be useful in predicting response to conventional therapy and patient prognosis. Distal 11q loss and 3q gain are common genetic events that occur in the process of head and neck squamous cell carcinogenesis. Our studies demonstrate that 11q loss in OSCC is associated with defects in DNA damage response, increased chromosomal instability and resistance to ionizing radiation. Thus, distal 11q loss may be useful as a molecular biomarker to predict the behavior of OSCC. Although oral tumors represent less than 5% of all cancers in the U.S., they have a very low survival rate and very high rates of relapse, secondary tumor development and resistance to therapy. We observed that ATR and CHEK1 overexpression protect OSCC from DNA damaging agents and provide a growth and survival advantage. Thus, inhibition of the upregulated ATR–CHEK1 pathway can sensitize a subset of OSCC to DNA damaging agents and potentiate conventional forms of therapy. The results of this study will not only help us to understand the basic biology of OSCC, but also play a role in our choice of therapeutic modalities, and focus development of targeted therapy for a subset of OSCC.

1.0 INTRODUCTION

1.1 OVERVIEW

Cancer is a common term used to describe malignant tumors. Although most researchers and physicians can practically describe cancer, it is very difficult to define. Cancer or neoplasia include different conditions characterized by uncoordinated and unregulated growth of cells with a tendency to invade normal tissue and/or spread to distant sites (metastasis). Certain tumors, like Chronic Myelogenous Leukemia (CML) arise from a single cell which underwent a genetic change and thus, the cells are clonal in origin. In comparison, solid tumors acquire a number of genetic insults over time and are constantly evolving. The early genetic insults occur in a large area surrounding the tumor and thus, secondary tumors can develop from the primary tumor–adjacent "normal tissue" (SLAUGHTER *et al*. 1953). Certain solid tumors undergo a multi–step process of cancer progression, which was first described by Vogelstein and his colleagues in colon cancer (KINZLER *et al*. 1996). With improvements in the diagnosis and treatment of medical conditions and diseases, there is a significant increase in life expectancy. This leads to an aging population with a very high proportion of individuals in the 65+ year age group. Since cancer is the leading cause of morbidity and mortality in this age group, the incidence and prevalence of cancer is also expected to increase exponentially over the next decade (YANCIK 2005). Thus, innovative strategies for prevention, early diagnosis and effective treatment are needed to effectively manage the extremely large burden of cancer patients in the near future.

1.2 EPIDEMIOLOGY OF ORAL SQUAMOUS CELL CARCINOMA

Worldwide, head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer and the third most common cancer in developing nations. There were 404,575 new cases in 2002, constituting 3.7% of the total estimated cancer cases worldwide (PARKIN *et al*. 2002). According to a recent study by the American Cancer Society, in 2006, there are expected to be approximately 30,990 new cases of HNSCC and 7,430 deaths related to HNSCC (JEMAL *et al*. 2006). HNSCC encompass tumors of the tongue, oral cavity, pharynx and larynx. Of these, tumors of the oral cavity and tongue, that is oral squamous cell carcinomas (OSCC) account for more than 90% of all HNSCC (SCHANTZ and YU 2002). Although, the elderly population account for most of the cases of OSCC, recently there has been a significant increase in OSCC incidence in the younger age group (MARTIN-GRANIZO *et al.* 1997, SILVERMAN 2001). Leukoplakia, erythroplakia, nicotine stomatitis, tobacco pouch keratosis and oral submucosal fibrosis are common premalignant lesions which may progress to OSCC (NEVILLE and DAY 2002). Leukoplakia, which is a white discoloration in the lining of the oral cavity, is very common in the elderly population. Nearly 10-15% of leukoplakia lesions progress to OSCC over a period of time (PETTI 2003). In addition to tobacco and alcohol consumption, dietary habits, genetic predisposition, and unknown risk factors may play a role in the increased incidence of OSCC observed recently (LLEWELLYN *et al*. 2004). In spite of better methods of diagnosis and new procedures for treatment, there has not been a significant difference in the five year survival rate for HNSCC over the past 30 years; it was 59% during 1995-2000 and 54% during 1974-1976 (JEMAL *et al*. 2006). Thus, there is a need to develop new biomarkers for effective screening, and therapeutic strategies for treatment of leukoplakia and OSCC.

1.3 ETIOLOGY OF ORAL SQUAMOUS CELL CARCINOMA

The common risk factors for oral squamous cell carcinoma are tobacco chewing, smoking, alcohol consumption and human papillomavirus (HPV) infection (RHODUS 2005). Vitamins A and C, on account of their antioxidant activity are shown to be protective against the development of oral leukoplakia and squamous cell carcinoma (NAGAO *et al*. 2000). The combined effects of tobacco use, alcohol consumption and poor dietary habits account for over 90% of head and neck cancer cases (REICHART 2001).

1.3.1 Tobacco

In the U.S., cigarette smoking is a common form of tobacco consumption (TOMAR 2003). However, in the rest of the world, the use of smokeless tobacco either by chewing it or by inhaling it is fairly common. Cigarette smoke and smokeless tobacco contain a number of carcinogens, like tobacco-specific nitrosamines (TSNA), benzopyrene, aldehyde derivatives like formaldehyde, acetaldehyde and crotonaldehyde, arsenic, nickel and nicotine (REICHART 2001). Cultural habits and lifestyle difference may account for the disparity in OSCC incidence in the U.S. versus the rest of the world. For example, in India, where chewing tobacco with betel quid is very common, OSCC is the most common cancer (GOLDENBERG *et al*. 2004). However, in this day and age of globalization, these habits and lifestyles have disseminated throughout the world and may account for the increased incidence of OSCC worldwide (GOLDENBERG *et al*. 2004).

In the U.S., there have been a number of arguments in favor of promoting use of smokeless tobacco to reduce the use of cigarettes. Nearly 4.5% of males and 0.3% females use

smokeless forms of tobacco (TOMAR 2003). This form of tobacco consumption is predominantly seen among young adults between 18–25 years of age and is a growing concern (TOMAR 2003). Smokeless tobacco leads to the development of premalignant conditions like oral submucosal fibrosis (OSF), leukoplakia and erythroplakia (CHAKRABARTI *et al*. 1991, SHIU and CHEN 2004).

Table 1. Common forms of smokeless tobacco (adapted from GOLDENBERG *et al*. 2004)

Vernacular name	Ingredients	Population
Pan/betel quid	Areca nut, betel leaf, lime, condiments and sweeteners with or without tobacco	Indian subcontinent, Southeast Asia, Southern America
Zarda	Boiled tobacco	India and Middle East
Mishri	Burned tobacco	India
Shammah	Tobacco, ash and lime	Saudi Arabia, Yemen
Toombak	Tobacco and sodium bicarbonate	Sudan

At the cellular level, cigarette smoke condensate (CSC) induces DNA double strand breaks (DSB) by generating reactive oxygen species in normal human fibroblasts and keratinocytes. These DSB contribute to the formation of anaphase bridges, micronuclei and chromosomal imbalances in normal cells and cancer cell lines (LUO *et al.* 2004). Cigarette smoking has been correlated with increased expression of the fragile site, FRA3B and a deletion of the tumor suppressor gene, *FHIT* (STEIN *et al*. 2002). Cigarette smoking is also associated with increased fragile site expression and sister chromatid exchanges and thus, can induce chromosomal rearrangements (KAO-SHAN *et al*. 1987). The Areca nut, an important constituent of certain types of smokeless tobacco, contains a number of carcinogenic nitrosamines which induce cytogenetic abnormalities (DAVE *et al*. 1992). Thus, cigarette smoking or smokeless tobacco consumption can predispose normal cells to precancerous and cancerous genetic changes.

1.3.2 Alcohol

Chronic alcohol consumption is an important risk factor for developing cancer of the oral cavity, pharynx, larynx and esophagus (BROWN 2005). Alcohol can act in a number of ways to induce or facilitate carcinogenesis. Ethyl alcohol acts as a solvent and increases the permeability of the oral mucosa to a number of carcinogens like nitrosamines, asbestos, polycyclic hydrocarbons and carcinogens present in tobacco (SEITZ *et al.* 1998, 2004). It has been shown that chronic alcoholism is associated with poor oral hygiene and deficiency of anti–oxidants like reduced glutathione, zinc, magnesium and vitamins A and C (POSCHL *et al*. 2004). On absorption, ethyl alcohol is converted into acetaldehyde by the action of alcohol dehydrogensase (ADH) (SEITZ *et al.* 2004). Acetaldehyde has been shown to interfere with DNA synthesis, enhance cell injury, cause cell cycle defects and induce chromosomal aberrations. Thus, acetaldehyde generated from alcohol metabolism has a strong mutagenic and carcinogenic effect (SEITZ *et al.* 2004). Alcohol, by itself, can induce the formation of toxic oxygen radicals and reactive oxygen species, which may contribute to DNA damage in cells (SEITZ *et al.* 2004).

Alcohol consumption combined with the use of tobacco accounts for nearly 75% of upper aerodigestive tract tumors (OGDEN 2005). In addition to aerodigestive tract cancers, alcohol consumption leads to cancers of the liver, colon, rectum, breast, and pancreas (BOFFETTA and HASHIBE 2006). When compared with tobacco consumption, public awareness regarding alcohol consumption and its causal relationship to OSCC and other cancers is poor (FABIAN *et al*. 1996). Thus, there is a need to improve public awareness regarding the harmful effects of alcohol consumption and its causal relationship with a number of cancers.

1.3.3 Viral infections

Human papillomaviruses (HPV) are important etiological factors for cervical and other anogenital cancers. Although there are over seventy HPV strains, only HPV 16, 18, 31, 33, 35 and 45 are associated with cancers (DE VILLIERS 1994). A strong association between oral cancer and the high–risk HPVs 16 and 18 has been demonstrated (McKAIG *et al*. 1998, GILLISON *et al*. 2000, MORK *et al*. 2001). Most of the dysplastic and carcinomatous changes induced by pathogenic HPVs are mediated by the viral oncoproteins E6 and E7. Both E6 and E7 target critical tumor suppressor genes involved in cell cycle regulation (MUNGER *et al*. 1992). The p53 protein is targeted for degradation by the oncoprotein E6 (WERNESS *et al*. 1990). E7 acts on pRB and disrupts the pRB–E2F complex; this causes changes in transcription and activation of a number of genes involved in cell proliferation (PHELPS *et al*. 1998). Recently, members of our laboratory demonstrated HPV integration at three distinct chromosomal fragile sites in an OSCC cell line (RAGIN *et al*. 2003). Thus, HPV integration at fragile sites may enhance chromosomal instability in OSCC. HPV may contribute to carcinogenesis by interfering with activation of the NFκB signaling pathway (SPITKOVSKY *et al*. 2002) and activation of telomerase (KLINGELHUTZ *et al*. 1996)

The Epstein Barr virus (EBV) commonly causes nasopharyngeal carcinoma, Burkitt lymphoma, Hodgkin lymphoma and other B–cell lymphomas (YOUNG and RICKINSON 2004). The EBV–encoded latent membrane protein 1 (LMP1) is the central transforming factor and is essential for transforming B lymphocytes *in vitro* (KAYE *et al*. 1993). EBV has been associated with OSCC, especially in immunocompromised patients. Investigators have isolated EBV-derived LMP1 from OSCC. Thus, EBV infection may play a role in OSCC pathogenesis (GONZALEZ–MOLES *et al*. 2002).

1.3.4 Other risk factors

Even though environmental factors or personal habits like alcohol and tobacco consumption and HPV infection account for 85–90% of OSCC, nearly 15% of patients who develop OSCC do not have any risk factors. Fanconi anemia patients have a 500–fold risk of developing OSCC, supporting the possibility of genetic susceptibility to head and neck cancer. A number of disorders, which involve mutations in DNA damage response genes, including xeroderma pigmentosum, Fanconi anemia, Bloom syndrome, and ataxia telangiectasia demonstrate an increased susceptibility to cancer development (PRIME *et al*. 2001). It has been observed that specific haplotypes at the X-ray repair complementing defective repair in Chinese hamster cells 1 (*XRCC1*) and glutathione S-transferase M3 (*GSTM3*) loci may be associated with an increased risk of progression from leukoplakia to OSCC (MAJUMDER *et al*. 2005). In addition, polymorphisms of the glutathione S-transferase T1 (GSST1) are associated with altered susceptibility to OSCC (EVANS *et al*. 2004). Thus, a genetic component and gene–environment interactions may play a role in the susceptibility to develop OSCC.

It has been shown that reactive oxygen species are important mediators in the carcinogenic action of tobacco and ethyl alcohol in OSCC. Thus, anti–oxidants like vitamins A and C are protective against the formation OSCC. People with poor oral hygiene and nutritional defects (common in chronic alcoholics) have an increased incidence of OSCC (POSCHL *et al*. 2004). Red and processed meat consumption leads to a 2-3 fold increase in risk of developing OSCC, while consumption of fruits and vegetables reduces the risk of developing OSCC by half (LEVI *et al*. 1998). Thus, a healthy lifestyle and dietary habits may confer protection against a number of tumors, including OSCC.

1.4 CHARACTERISTICS OF ORAL SQUAMOUS CELL CARCINOMA

1.4.1 Chromosomal instability

One hallmark of solid tumors is chromosomal instability, which helps drive cancer growth and progression. As the tumor progresses, its genotype evolves into one that is optimized for proliferation, spread and invasion into surrounding tissues. Thus, over a period of time, genetic alterations that confer a growth advantage are selected (ALBERTSON *et al*. 2003). Chromosomal instability is a state of continuous, dynamic, propagated changes in chromosome structure and/or number, which is usually seen in solid tumors. OSCC exhibit a high level of chromosomal instability with near-triploid or tetraploid karyotypes composed of multiple clonal numerical and structural chromosomal abnormalities (VAN DYKE *et al*. 1994, GOLLIN 2001, JIN *et al*. 2002). Chromosomal segregational defects involving multipolar spindles are a common cause of numerical chromosomal instability in OSCC (SAUNDERS *et al*. 2000, GISSELSSON *et al*. 2002). The anaphase bridges and micronuclei observed in OSCC may be caused by defects in the DNA damage response and/or dysfunctional telomeres (GOLLIN 2001, GISSELSSON 2003). Anaphase bridges are intermediates in the process of gene amplification (SHUSTER *et al*. 2000, SAUNDERS *et al*. 2000).

Changes in DNA ploidy and chromosomal abnormalities can be detected in tumor–adjacent normal tissue and dysplastic tissue and also in premalignant lesions of the oral cavity (SUDBO *et al*. 2001, SUDBO *et al*. 2002). Thus, chromosomal instability is a relatively early event in head and neck tumor development. A high degree of chromosomal aneuploidy and changes in DNA ploidy in OSCC are correlated with lymph node metastasis, poor response to treatment and poor prognosis (HOLM 1982, STELL 1991). Even though OSCC exhibit a high

degree of cytogenetic heterogeneity, certain chromosomal changes like 11q13 amplification, gains involving 3q21–q29, 5p, 8q, 18q and 22q and losses involving 3p, 8p, 9p, 11q, 13q and 21q occur regularly and may play a role in OSCC development and progression (BOCKMUHL and PETERSEN 2002).

1.4.2 11q13 amplification

Amplification of chromosomal band 11q13, one form of chromosomal instability, is present in nearly 45% of OSCC (GOLLIN 2001). 11q13 amplification is an independent prognostic factor which correlates with higher stage disease, lymph node involvement, shorter time to recurrence, and reduced overall survival (AKERVALL *et al*. 1997, FRACCHIOLLA *et al*. 1997, MICHALIDES *et al.* 1997, MIYAMOTO *et al*. 2003). Gene amplification, the generation of extra copies of a gene or genes, is a common genetic defect in human tumors, including OSCC (SCHWAB 1998). Amplification and subsequent overexpression of critical genes has been shown to lead to dysregulation of the cell cycle, resulting in cellular proliferation and tumor formation and/or progression (LUNDBERG *et al.* 1999). Chromosomal band 11q13, which harbors the locus for a key cell cycle regulatory gene, cyclin D1 gene (*CCND1*) and other neighboring genes, is the most frequently amplified genetic segment in OSCC (AKERVALL *et al*. 1997, GOLLIN 2001, HUANG *et al*. 2002). 11q13 amplification is also present in a smaller percentage of other carcinomas (SCHRAML *et al*. 1999). Several genes have been shown to be amplified in this region, including *CCND1*, *EMS1* which encodes human cortactin, an actin binding protein possibly involved in the organization of the cytoskeleton and cell adhesion structures, *TAOS1* and *TAOS2*, two genes that we have identified with unknown function (HUANG *et al*. 2002 and unpublished data), *FADD*, the FAS-associating protein with death

domain gene, and *FGF3* and *FGF4* (fibroblast growth factors 3 and 4, also called *INT2* and *HSTF1*). Cyclin D1 is a critical cell cycle regulatory protein that drives the cell from the G1 to the S phase of the cell cycle. Cyclin D1 binds a cyclin-dependent kinase, CDK4 or CDK6. This is followed by phosphorylation and inactivation of the retinoblastoma protein pRB, resulting in release of the bound E2F transcription factors and cell cycle progression (WEINBERG 1995). Overexpression of *CCND1* may lead to shortening of the G_1 phase of the cell cycle and thus, premature transition to the S phase of the cell cycle, resulting in propagation of unrepaired DNA damage, accumulation of genetic alterations, and a growth advantage for the cells. Hittelman and colleagues report that 11q13 amplification occurs early in the pathogenesis of OSCC, in premalignant lesions prior to development of invasive carcinoma (IZZO *et al.* 1998). 11q13 amplification with cyclin D1 overexpression is a critical event in the pathogenesis of OSCC and is associated with a poor prognosis (AKERVALL *et al*. 1997).

11q13 amplification in OSCC occurs by the breakage–fusion–bridge (BFB) mechanism, first described in maize by geneticist Barbara McClintock in 1938 (McCLINTOCK 1938, McCLINTOCK 1939). As depicted in Figure 1, the first step in the BFB model of 11q13 amplification is loss of a distal portion of chromosome 11, resulting in an unprotected chromosome end which fuses with its sister chromatid to form a dicentric chromosome. During chromosomal segregation, the two centromeres are pulled to different poles, leading to additional breaks and the cycle continues until a derivative chromosome 11 with 11q13 amplification is formed (SHUSTER *et al*. 2000). During the initial step of 11q13 amplification, a segment of distal 11q harboring important genes involved in DNA damage repair like *MRE11A* (11q21), *ATM* (11q22.3), *H2AFX* (11q23.2) and *CHEK1* (11q24) is lost.

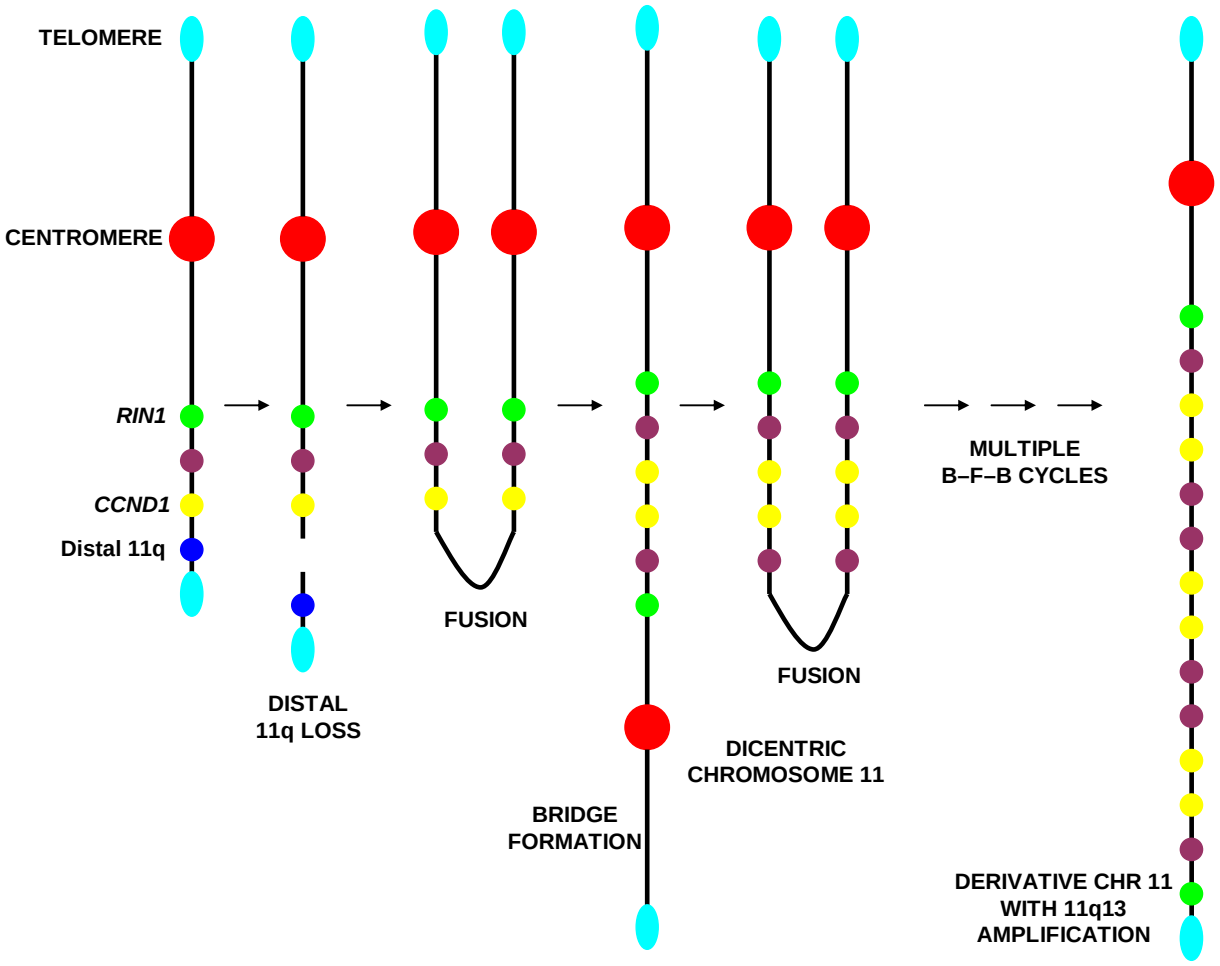


Figure 1. 11q13 amplification in OSCC by the BFB mechanism

The initiating event in 11q13 amplification is a break at the distal end of chromosome 11 resulting in a sticky end which fuses with its sister chromatid to give rise to a dicentric chromosome. The two centromeres of this dicentric chromosome are pulled to opposite poles, forming a bridge between the two daughter cells. Breakage of the dicentric chromosome yields more sticky ends and the cycle continues until a derivative chromosome 11 with 11q13 amplification is capped and stabilized by a chromosomal segment bearing a telomere.

Most of the clinical correlations with 11q13 amplification have been attributed to amplification and overexpression of *CCND1* and genes present in the 11q13 amplicon. However, the loss of distal 11q, with a number of important genes including *ATM, H2AFX, MRE11A* and *CHEK1* may play an important role in the development and progression of OSCC.

1.4.3 Genetic progression models

The concept of field cancerization was first described in oral tumors in 1953 (SLAUGHTER *et al*. 1953). They proposed that dysplastic changes occur simultaneously in multiple areas or over a widespread area. Thus, a tumor is surrounded by dysplastic tissue which is precancerous. This model explains how local recurrences, multiple tumors or second primary tumors may develop in the vicinity of or at the site of the primary tumor. In terms of genetic changes, it has been demonstrated that a number of 'genetic insults' accumulate and contribute to chromosomal instability and eventually lead to the formation of a tumor (KINZLER *et al*. 1996). The chronological order of the genetic insults and their relative contribution to tumor formation can be evaluated by studying the dysplastic and premalignant tissue surrounding the primary tumor. A number of such genetic insults and their chronological order have been described in OSCC (CALIFANO *et al*. 1996, FORASTIERE *et al*. 2001). Common events described in the progression to OSCC are loss of heterozygosity at 9p21 which involves loss of p16, loss of heterozygosity at 3p14 (*FHIT* gene), 17p13 (*TP53* gene) and amplification of 11q13 (HA and CALIFANO 2003). 3q gain is frequently seen in frank carcinoma of the head and neck and is associated with poor prognosis (ASHMAN *et al*. 2003). In OSCC, deletion or mutation of the *TP53* gene and the p16 INK4A/ARF (*CDKN2A*) locus have been observed in the 'normal tissue' surrounding the tumors, which suggest that these are early changes. Such alterations have also been observed in breast, cervical and lung cancer. Amplification of chromosomal band 11q13 in the form of a homogenously staining region (HSR) leads to the amplification and overexpression of a number of genes like *cyclin D1* (*CCND1*), *Cortactin (EMS1), TAOS1* and *2,* and *FGF3* and *4* (HUANG *et al*. 2002). This event may trigger and drive tumor formation in nearly half of all oral tumors.

1.5 THE DNA DAMAGE RESPONSE PATHWAYS

1.5.1 Important players in the DNA damage response

The DNA damage response involves the sensing of DNA damage followed by transduction and amplification of the damage signal through a very complex network of cellular pathways. The initial DNA damage is detected by sensors which attract a number of proteins involved in DNA damage response to the site of damage. The transducers, in turn activate multiple effectors which initiate and regulate different cellular activities like cell cycle checkpoints, DNA repair, telomere maintenance and apoptosis (KHANNA and JACKSON 2001, JACKSON 2002).

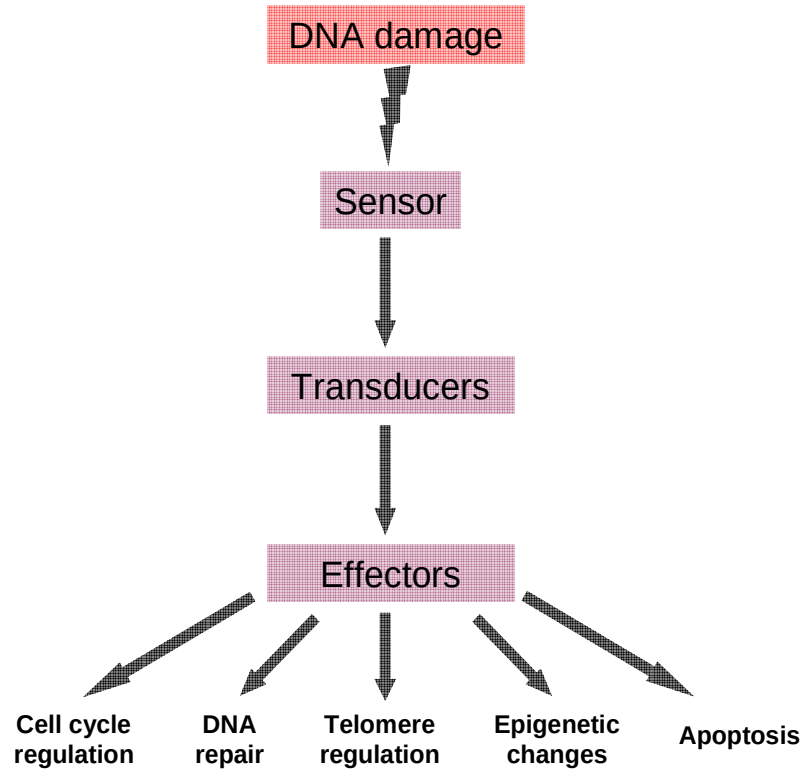


Figure 2. A schematic representation of DNA damage signal transduction

The DNA damage is initially detected by sensors like the MRN and 9-1-1 complexes. The damage signal is then modulated, transduced and amplified by the protein kinases ATM and ATR. A number of downstream effectors, like CHEK2, CHEK1, BRCA2 and p53 are activated

and trigger a number of cellular responses to DNA damage (Figure 2 adapted from JACKSON 2002).

The MRE11A, RAD50 and NBS1 (MRN) complex and the RAD9, HUS1 and RAD1 (9–1–1) complex act as sensors for DNA damage. Both of these complexes play an important role in the detection and modulation of the DNA damage response. The MRE11A protein has intrinsic DNA binding and nuclease activity and forms a complex with RAD50 and NBS1 (TRUJILLO *et al*. 1998). Under normal conditions, the components of the MRN complex are diffusely scattered in the nucleus. However, in response to DSB, these components assemble and bind to the area of damage, forming visible foci (STRACKER *et al*. 2004). Following phosphorylation of NBS1 by ATM on Ser-343, the MRN complex increases the efficiency of ATM activity by improving ATM binding to its substrates (LEE *et al*. 2004). Inherited mutations in *MRE11A* lead to ataxia telangiectasia-like disorder, characterized by chromosomal instability and a defective S-phase checkpoint (STEWART *et al*. 1999).

The 9–1–1 complex is a heterotrimeric complex with a sliding clamp–like structure which resembles the structure of proliferating nuclear antigen (PCNA) (THELEN *et al*. 1999). The 9–1–1 complex is loaded to areas of damaged DNA with the help of RAD17 which acts as a clamp loader. The clamp–like action of the 9–1–1 complex at sites of DNA damage helps to recruit, activate and modulate ATR and its downstream effectors like CHEK1, BRCA1 and the Fanconi proteins. Thus, the 9–1–1 complex coordinates cell cycle progression and DNA repair at sites of DNA damage (PARRILLA–CASTELLAR *et al.* 2004). Inhibition or depletion of the 9–1–1 complex leads to impaired ATR–dependent CHEK1 phosphorylation, cell cycle checkpoint defects and chromosomal abnormalities (BAO *et al.* 2004). Recently, amplification and

overexpression of RAD9A on 11q13 have been reported in breast and lung cancer (CHENG *et al*. 2005, MANIVA *et al*. 2005).

The phosphatidyl inositol 3–kinase (PI3K) like protein kinases, ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) act as transducers of the DNA damage signal (SHILOH 2001). In response to DNA damage, both ATM and ATR phosphorylate a number of substrates on serine (SQ) or threonine (TQ) residues and activate them (SHILOH 2001a). The *ATR* gene is located in chromosomal band 3q24, while *ATM* is located at 11q22.3. ATM responds mainly to DNA double-strand breaks (DSBs) caused by ionizing radiation, while ATR is activated in response to stalled replication forks and DNA damage induced by UV radiation or certain chemotherapeutic drugs (HELT *et al*. 2005). After DNA damage, ATM undergoes autophosphorylation at Ser-1981. This causes a conformational change and activation of the ATM protein (BAKKENIST *et al*. 2004). The activation of ATM or ATR initiates a signaling cascade that involves the phosphorylation of substrates like CHEK2, p53, MDM2, NBS1, SMC1, BRCA1, and H2AX (SHILOH 2001a, SHILOH 2001b). Thus, following DNA damage, ATM and/or ATR orchestrate a coordinated cascade of events that culminate in cell cycle arrest and DNA repair or apoptosis (ABRAHAM 2001, SHILOH 2001a, SHILOH 2001b). At the cellular level, ATM loss is associated with chromosomal instability, radioresistant DNA synthesis, as well as a loss of the G_1 checkpoint in response to DNA damage (MEYN 1999). An ATM–deficient (AT) cell line has been shown to have an overactivated ATR-CHEK1 pathway which results in a prolonged G_2 arrest after ionizing radiation (WANG *et al.* 2003). Inhibition of this upregulated ATR-CHEK1 pathway sensitizes AT cells to ionizing radiation (WANG *et al.* 2003). Homozygous deletions of the *ATR* gene in mice are embryonic lethal (DE KLEIN *et al*. 2000). In humans, partial loss of ATR activity causes the autosomal

recessive disorder, Seckel syndrome (O'DRISCOLL *et al*. 2003). ATR plays an important in replication surveillance and repair along with its partners, the ATR interacting protein (ATRIP) and replication protein A (RPA) (CORTEZ *et al*. 2001, ZOU and ELLEDGE 2003, BYUN *et al*. 2005). ATR has also been shown to play a very important role in regulating chromosomal fragile sites (CASPER *et al*. 2002). It has been shown that ATM regulates loading of ATR to sites of DNA damage (CUADRADO *et al*. 2006). Thus, even though ATM and ATR are activated in response to different types of DNA damage, there is overlap between the two pathways.

It has been shown that the histone variant, H2AX is phosphorylated on its Serine-139 residue by ATM or ATR in response to DNA damage (BURMA *et al*. 2001, WARD and CHEN 2001). The phosphorylated H2AX (γ-H2AX) forms visible complexes at sites of DNA double strand breaks that help in recruitment and localization of numerous DNA repair factors like ATM, ATR, BRCA1, the MRN complex, CHEK1 and CHEK2 (SHILOH 2001). The phosphorylation of H2AX persists until DNA repair is completed or the cell is forced to undergo apoptosis. Haploinsufficiency of an H2AX allele on a p53-deficient background has been shown to cause clonal nonreciprocal translocations, amplifications, and increased genetic instability and also increases cancer susceptibility in mice (BASSING *et al.* 2003, CELESTE *et al*. 2003).

CHEK1 and CHEK2 are highly conserved effector kinases which transmit signals from ATM and ATR to downstream proteins. This in turn leads to cell cycle checkpoint activation, and DNA repair (BARTEK and LUCAS 2003). CHEK1 mainly acts during the G2 and S phases of the cell cycle to initiate cell cycle checkpoints in response to DNA damage (LIU *et al*. 2000). The early lethality of CHEK1-deficient embryonic cells (TAKAI *et al*. 2000) and early embryonic lethality of CHEK1-deficient mice (LIU *et al*. 2000) suggests that CHEK1 plays a very important role during early development and viability in mammals (KALOGEROPOULOS

et al. 2004). CHEK1-induced G_2M arrested is mediated through phosphorylation and inactivation of the Cdc25 phosphatases (SANCHEZ et al. 1997). In addition to CHEK1 and CHEK2, a number of other proteins like p53, MDM2, ABL1, SMC1, BRCA1 and BRCA2, the Fanconi proteins and are phosphorylated and activated by ATM and ATR (SHILOH *et al*. 2001a, SHILOH *et al*. 2001b). Thus, perturbation of either ATM or ATR may have a ripple effect and affect the regulation and control of numerous proteins and pathways involved in the DNA damage response.

1.5.2 Cell cycle checkpoints and cancer

The cell cycle is a series of predetermined events every cell passes through prior to division into two daughter cells. The cell cycle is divided into four main phases, G_1 phase which is the first gap or growth phase, S phase, during which histones and DNA are synthesized, G_2 or the second gap or growth phase and the mitotic (M) phase during which the cell divides. The major purpose of the cell cycle is to assure high fidelity duplication of the genetic material and equal distribution to the two daughter cells. In response to stress or damage to the cell, there are three major cell cycle checkpoints: one at the G_1-S transition, the second within the S phase and one at the G_2-M transition. Every cell has an option to stop or proceed further during the G_1 and the G_2 phases of the cell cycle in response to pro– and anti-proliferative signals (LUKAS *et al.* 2004). The G_1-S phase and the G_2-M phase checkpoints are placed just before the critical steps of DNA replication and chromosome segregation, cell division, respectively (LUKAS *et al*. 2004). It has been suggested that defects in cell cycle checkpoints may contribute to the genomic instability seen in various cancers (KASTAN *et al.* 2004). The fact that p53, a very important

regulator of the G_1 phase checkpoint is mutated in more than 50% of all cancers provides proof that cell cycle defects are linked to development of cancer (LEVINE 1997, MASSAGUE 2004).

The dominant checkpoint in mammalian cells traversing through the G_1 phase into the S phase is a p53–dependent pathway which is initiated mainly by ATM/ATR and CHEK2/CHEK1, respectively in response to DNA damage (LUKAS *et al*. 2004, LEVINE 1997, MASSAGUE 2004). Factors promoting the entry of cells into S phase involve expression of the cyclins D, E and A, whereas those which inhibit entry into the S phase are activated p53, p21 and p27, and dephosphorylated Rb. The G_1 cell cycle checkpoint is frequently dysregulated in OSCC (MICHALIDES *et al*. 2002).

The mammalian S phase response to DNA damage is a combination of three separate responses (BARTEK *et al*. 2004). A replication checkpoint is activated in response to stalled replication forks during DNA synthesis (BARTEK *et al*. 2004). A separate S phase checkpoint prevents cells from dividing before faithful replication is complete. A loss of this checkpoint results in apoptosis of the cells with incomplete DNA replication. In response to DNA double strand breaks outside replicating areas, an independent intra-S phase checkpoint is activated. All three S phase checkpoints are p53 independent, but rely on the ATR/ATM kinases and CHK1/CHK2 proteins for activation (BARTEK *et al*. 2004). Abrogation of the S phase DNA damage checkpoint by caffeine leads to premature mitosis, premature chromatin condensation (PCC) and apoptosis in response to DNA damage (NGHIEM *et al.* 2001).

The aim of the G_2 (G_2M) checkpoint is to prevent cells with improper DNA replication or DNA damage from entering mitosis. The critical regulator of the G_2 checkpoint is the pro-mitotic cyclin B/CDK 1 kinase complex which in turn is regulated by the ATR, CHEK1 and CDC25 phosphatase family of proteins (O'CONNELL *et al*. 2005). The G_2 checkpoint partly relies on

p53-dependent mechanisms. However, it has been shown that p53-independent mechanisms are sufficient to sustain G_2 arrest. Entry of cells with DNA damage into mitosis results in activation of an M phase–specific, p53-independent pathway that results in mitotic catastrophe and apoptosis (O'CONNELL *et al*. 2005). It has been shown that knockout of either CHEK1 or ATR, results in mitotic catastrophe during embryogenesis or loss of replication checkpoint control in cycling *Xenopus* extracts (HEKMAT-NEJAD *et al.* 2000).

Since the G_1 phase checkpoint is lost in a large number of tumors, there is growing interest in S and G_2 phase checkpoint abrogating agents which can sensitize these tumors to chemotherapy and radiotherapy (ZHOU *et al*. 2003).

1.5.3 DNA damage repair machinery

Endogenous or exogenous agents continuously inflict normal cells with DNA damage. The most common form of DNA damage is in the form of double strand breaks (DSB) (JACKSON 2002). In response to DNA damage, different repair pathways are activated depending on the type of DNA damage and the cell cycle phase during which the damage is induced. *In vivo*, hypoxia, replicative stress and deprivation from nutrients commonly lead to the formation of reactive oxygen species which induce DSB. Exogenous factors which cause DSB are ionizing radiation and certain chemicals (KHANNA and JACKSON 2001). Thus, normal cells continuously undergo a basal or background level of DNA damage which needs to be repaired.

The common DNA damage repair pathways include homologous recombination repair (HRR), non-homologous end-joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) (WOOD *et al*. 2001). HRR is the predominant form of repair during the S and the G_2M phases of the cell cycle. In HRR, the DNA segment lost is

accurately reproduced using the intact DNA sequence on the sister chromatid. Genes involved in this repair mechanism include: *RAD51, RAD54, XRCC2, XRCC3, BRCA1*, *ATM*, *ATR, BLM*, *Tip60* and *p53* (BERNSTEIN *et al*. 2002). NHEJ which predominantly occurs during the G_1 phase of the cell cycle involves direct ligation of broken DNA ends. Thus, NHEJ is a form of salvage repair which results in a considerable loss of genetic material in certain cases (CHU *et al*. 1997). Ligase IV, XRCC1, and the DNA–PK complex including the Ku70, Ku80 proteins and catalytic unit of DNA–PK (DNA–PK_{CS}) play an important role in NHEJ (SMITH and JACKSON 1999, HEFFERIN and TOMKINSON 2003). Since NHEJ often results in loss of genetic material. it is only used when there is no template to guide the formation of the same DNA material. During the S, G_2 and M phases, when a sister chromatid is available, conservative HR occurs preferentially (SALEH-GOHARI *et al.* 2001). DNA damage caused by UV radiation, certain chemotherapeutic agents like cisplatinum, and bulky adducts which distort the normal configuration of the DNA helices are repaired by NER. NER can occur in non-transcribed areas and is called global genomic repair (GGR) or it can occur during transcription, which is called transcription coupled repair (TCR) (WOOD 1997). Defects in genes involved in NER lead to an increased predisposition to cancer, especially skin cancer (HOEIJMAKERS 1994, DE BOER and HOEIJMAKERS 2000). BER protects mammalian cells from mutations or biochemical changes like methylation, oxidation or reduction, which affect individual bases (SWEASY *et al.* 2006, BERNSTEIN *et al*. 2002). Usually only the affected nucleotide is excised, i.e., short–patch BER. In contrast, up to eight nucleotides around the affected nucleotide can be excised in long–patch BER (KROKAN *et al*. 2000). The MMR pathway corrects nucleotide mismatches and insertion or deletion loops (IDL) (KUNKEL and ERIE 2005). In humans, MSH2, MSH3 and MSH6 form heterodimers which recognize and repair base–base mismatches and IDL (JIRICNY

2006). Mutations of genes involved in MMR lead to a very high rate of background mutations, microsatellite instability, and predisposition to cancer (PELTOMAKI 2003). Defects in the MMR pathway lead to development of hereditary non-polyposis colorectal cancer (HNPCC) syndrome and other tumors (AALTONEN *et al*. 1993, ESHLEMAN and MARKOWITZ 1996). In addition to mismatch repair, MMR proteins are also involved in sensing DNA damage, regulation of homologous recombination and DNA recombination (MOHINDRA *et al*. 2002, JIRICNY 2006).

Thus, there are a number of DNA damage repair pathways which are activated in response to the nature, cell cycle time–point and duration of the DNA damage. Defects in one or more of these pathways are a common feature of different tumors.

1.5.4 Telomeres and telomerase activity in cancer

Telomeres are highly specialized "TTAGGG" repeats which protect the ends of chromosomes. A loss of telomere integrity results in end–to–end chromosomal fusions and misrecognition of chromosomal ends as DSB (ARTANDI *et al*. 2000). Telomerase is a ribonucleoprotein complex with enzymatic activity which maintains telomeres. A catalytic unit, telomerase reverse transcriptase (TERT) and telomerase RNA (TR) are important components of telomerase (NUGENT and LUNDBLAD 1998). Telomerase adds the TTAGGG repeats to the ends of chromosomes (BLACKBURN 1991). Telomerase is normally active only in germline cells and embryonic stem cells. In somatic cells, the telomere length shortens after every cycle of cell division until it reaches a "critical length." This signals the senescent cell to undergo programmed cell death. It has been demonstrated that enhancing telomerase activity causes telomere lengthening and increases the life span of normal human cells (BODNAR *et al*. 1998,

VAZIRI and BENCHIMOL 1998). In contrast, most cancer cells activate telomerase or rely on other pathways, collectively called alternative lengthening of telomeres (ALT), to prevent telomere attrition (KIM *et al*. 1994, BRYAN *et al*. 1997). Thus, a high level of telomerase activity in advanced cancers allows the tumor cells to proliferate continuously and indefinitely (MASER and DEPINHO 2002). Thus, telomere length serves as a surveillance system to monitor the number of cell divisions and prevent genomic instability and progression to cancer. p53 deficiency protects cells with critically short telomeres from cell death (CHIN *et al.* 1999). Telomere loss in cells is associated with end to end chromosome fusions, resulting in chromosomal translocations and formation of dicentric chromosomes and anaphase bridges (O'HAGAN *et al*. 2002, SABATIER *et al*. 2005). Thus, telomere dysfunction on a p53 deficient background can promote chromosomal instability and initiate pro–carcinogenic changes.

1.5.5 Cell death and apoptosis in cancer

Growth and progression of any tumor can be achieved either by increased proliferation or reduction in cell death. Thus, inactivating mutations or deletions of pro–apoptotic genes or translocation or amplification of anti–apoptotic genes may confer tumor cells with a survival advantage. The *TP53* gene and its family members, *TP63* and *TP73* are master regulators of the delicate balance between cell proliferation and cell death. p53 and Rb mediate a form of programmed cell death called apoptosis. A number of genes involved in apoptotic pathways are highly conserved evolutionarily. Since cells with excessive DNA damage or telomere attrition are subjected to apoptosis, apoptosis can be considered as a mechanism which protects cells from carcinogenic changes. Loss of p53 and Rb is commonly seen in tumors and causes uncoupling of damage/stress signals, cell growth and cell proliferation (SHERR and McCORMICK 2002).

Loss of apoptotic pathways, a common feature of tumors, confers the cancer cells with a growth advantage (HANAHAN and WEINBERG 2000). Apoptosis is mediated by a family of protease enzymes called caspases (ALNEMRI *et al*. 1996). All caspases are tightly regulated and activated only when a cell is committed to undergo apoptosis (RIEDL and SHI 2004). Defects in apoptotic pathways due to loss of p53 function or by other mechanisms is a common feature of cancer (IGNEY and KRAMMER 2002). There is an increased interest in enhancing apoptosis in cancer cells (FESIK 2005).

In addition to apoptosis, there are a number of other mechanisms that lead to cell death, including premature chromatin condensation (PCC), mitotic catastrophe (MC), necrosis and autophagy (OKADA and MAK 2004, BROKER *et al*. 2005). MC is triggered in cells which undergo premature mitosis with DNA damage. Morphologically, cells that undergo mitosis appear as giant cells with micronucleus formation and nuclear fragmentation (CASTEDO *et al*. 2004). Survivin, a regulator of the M phase checkpoint plays an important role in protecting cells from undergoing mitotic catastrophe. PCC is fragmentation of chromatin following premature entry of the cell with DNA damage through the replicative S phase checkpoint. Morphologically, S phase PCC resemble mitotic catastrophe. Necrosis usually follows an inflammatory reaction and involves swelling of the cell followed by release of enzymes and electrolyte and pH imbalances. Autophagy is a lysosomal mediated degradation of cellular components and presents as vacuolation and bleb formation within the cells (BURSCH *et al*. 2000, KIM *et al*. 2000). Since, a number of tumors have defects in the p53–mediated apoptotic pathways, there is growing interest in inducing cell death in these tumors through other mechanisms (BROKER *et al*. 2005).

2.0 MATERIALS AND METHODS

2.1 SUBJECTS AND SAMPLE COLLECTION

OSCC cell lines were established from tumors surgically removed from anonymous consenting, previously untreated patients (GOLLIN *et al*. unpublished data). Normal human keratinocytes (NHOK) were established from uvulopalatopharyngeal tissue obtained from University of Pittsburgh Medical Center. The tissue was collected from anonymous consenting patients with IRB approval. Peripheral blood for Fluorescence in situ Hybridization (FISH) was collected from normal anonymous donors. GM09607, an AT fibroblast cell line, was purchased from Coriell Cell Repositories. The hTERT cell line was obtained from Dr. Jim Rheinwald.

2.2 CELL CULTURE

We selected eleven OSCC cell lines for the distal 11q loss study and twenty OSCC cell lines for ATR and CHEK1 studies from the OSCC cell lines established in our laboratory. The ATM–deficient, AT cell line (GM09607) was used as a positive control, since it is documented to have an upregulated ATR–CHEK1 pathway, and normal human oral keratinocytes (NHOK) or human keratinocytes transfected with *TERT* and HEK293 cell line were used as negative controls.

2.2.1 OSCC cell lines

OSCC were cultured in M10 medium composed of Minimal Essential Medium (Gibco Invitrogen, Grand Island, NY), supplemented with 1% non-essential amino acids, 1% L-glutamine, 0.05mg/ml gentamicin and 10% fetal bovine serum (FBS) (Gibco Invitrogen). For subculturing OSCC, adherent cells were detached from the flask surface by trypsinizing with 0.05% trypsin and 0.02% EDTA (Irvine Scientific) for 3–5 min at 37^{o}C in 5% CO_2 incubator. An equal amount of M10 medium was used to inhibit trypsin activity following detachment and cells were replated.

2.2.2 AT null cell line

GM09607 (Coriell Cell Repositories, Camden, NJ) was cultured using Dulbecco's Modified Eagles Medium (DMEM) (Gibco Invitrogen), supplemented with 1% non-essential amino acids, 0.05mg/ml penicillin-streptomycin-L-glutamine, and 10% FBS. Subculturing was performed as described for OSCC.

2.2.3 Controls

We used anonymous NHOK cells established in our laboratory from uvulopalatopharyngoplasty specimens as controls. In brief, NHOK cells were cultured in serum-free KGM-2 medium (Clonetics, Walkersville, MD), supplemented with bovine pituitary extract (BPE), hEGF, insulin (bovine), hydrocortisone, GA-1000 (Gentamicin, Amphotericin B), epinephrine and transferrin as per the manufacturer's instructions (supplements supplied in the KGM-2 BulletKit™,

Clonetics). The hTERT cells were cultured in Keratinocyte-SFM supplemented with 25µg/ml BPE, 0.2ng/ml epidermal growth factor, 0.3mM $CaCl_2$ and penicillin-streptomycin (Gibco Invitrogen). These keratinocytes were expanded to high density in a 1:1 mixture of Keratinocyte-SFM and DMEM-F12. The DMEM-F12 was a 1:1 mixture of calcium-free and glutamine-free DMEM and Ham's F-12 supplemented with 25µg/ml bovine pituitary extract, 0.2ng/ml epidermal growth factor, 1.5mM L-glutamine and penicillin-streptomycin (Gibco Invitrogen).

2.3 PREPARATION OF DNA PROBES FOR FISH

A single colony of *E. coli* carrying the individual BAC (mapping to *ATR, ATM, CHEK1, CCND1 H2AFX, MRE11A* and *TP53*) (Individual BAC numbers are listed in APPENDIX A) was incubated overnight at 37°C in 5 ml of Luria–Bertani (LB) medium with 50 µg/ml Chloramphenicol. The bacteria were centrifuged at 10,000 x g for 30s. The bacteria were resuspended in 100 µl of STET (8% sucrose, 5% Triton X100, 50mM EDTA, 50 mM Tris pH 8.0). Freshly prepared alkaline SDS (0.2 NaOH, 1% SDS) was added to lyse the bacteria and the solution was incubated at 24°C for 2 min. Cold ammonium acetate (4°C) was added and the solution was incubated for 5 min on ice. Following this step, the bacteria were centrifuged at 4°C for 15 min at 16,000 x g. Equal amounts of phenol and chloroform were added to the supernatant to extract the DNA. The top layer of the mixture was treated with 0.6 x volume of isopropanol and centrifuged at 4°C for 15 min at 16,000 x g. The supernatant was drained and the pellet washed with 70% ethyl alcohol and air dried. The DNA was resuspended in 100– 200 µl of Tris–EDTA (TE) buffer (QIAGEN, Valencia, CA) and stored at 4°C.

2.4 FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

In order to prepare mitotic cells for FISH analyses, HNSCC cells were harvested following 5 h of 0.1μg/ml Colcemid™ (Irvine Scientific, Santa Ana, CA) treatment, hypotonic KCl (0.075M) treatment for 16 min and fixation in 3:1 methanol:glacial acetic acid. All other cells were harvested using the same method, except that 1 h of Colcemid™ was preferred for non-tumor cells.

FISH analysis was used to detect copy number changes for the respective genes in the OSCC cell lines. For FISH analysis, cells were harvested, dropped onto slides, treated with RNase/2XSSC, and dehydrated using serial treatments with 70%, 80% and 100% of ethyl alcohol. Chromatin was denatured with 70% formamide and dehydrated in 70%, 80% and 100% of ethyl alcohol. The BAC probes for FISH, described in detail in Appendix A, were obtained from Children's Hospital of Oakland Research Institute (CHORI, San Francisco, CA). Using a nick translation kit from Vysis, Inc. (Downers Grove, IL), extracted DNA was precipitated with ethyl alcohol, resuspended in hybridization buffer, and allowed to pre-anneal for 1-2 h at 37^{o}C. Each probe was hybridized for 16 h at 37^{o}C, after which slides were washed with SSC/Tween-20. Slides were counterstained with DAPI and mounted with antifade prior to analysis. All FISH analyses were carried out using an Olympus BX-51 epiflorescence microscope (Olympus Microscopes, Melville, NY). An Applied Imaging CytoVision workstation with Genus v3.6 software was used for image capture and analysis (Applied Imaging, San Jose, CA).

2.5 PARAFFIN FISH

A 4-5 μM thick slice of paraffin embedded tissue was mounted on a positively charged microscope slide. The slides were aged overnight at 60^{o}C, following which they were deparaffinized with Xylene for 5 min at room temperature. The slides were dehydrated with a series of 70%, 80% and 100% ethyl alcohol washes, each wash lasting 2 min at room temperature. The slides were then treated with 0.5 X SKIP Dewax solution at 80^{o}C for 15 min followed by treatment with pepsin containing protease solution for 15 min at 37^{o}C. After two washes with 2XSSC, each lasting 5 min, the slides were fixed in 10% Formalin for 10 min at room temperature. After 2XSSC washes, the slides were dehydrated with a series of 70%, 80% and 100% ethyl alcohol washes and allowed to dry on slide warmer. The probes for paraffin FISH were prepared as described for regular FISH. The hybridization of the FISH probes and post hybridization treatment of the paraffin slides was carried out as described for regular FISH. Unless otherwise specified, 100 nuclei from tumor tissue and 100 nuclei from normal tissue were analyzed for copy number changes of different genes.

2.6 ANAPHASE BRIDGE FORMATION ASSAY

To check for the presence of *ATR* gene in anaphase bridges, each OSCC cell line was plated in chamber slides and allowed to grow until the cell lines reached 80–90% confluence. 5 ml of Colcemid™ was added to each chamber slide and the slides were incubated at 37^{0}C in 5% CO_2 incubator for a period of 24 h. At the end of 24 h, the medium was aspirated and cells were fixed with 3:1 methanol to acetic acid fixative for a period of 45 min. FISH using BAC probes to the

ATR gene labeled with Spectrum Green™, CEP3 labeled with Spectrum Orange™ and CEP11 labeled with Spectrum Aqua™ was performed as described above. Fifty anaphase bridges per OSCC or GM09607 were evaluated for the presence of *ATR*, CEP3 or CEP11.

2.7 CLONOGENIC CELL SURVIVAL ASSAY

To assess cell survival in response to ionizing radiation, we performed a clonogenic survival assay**.** Two thousand cells were seeded in 60 mm Petri dishes and allowed to adhere overnight. Cells were then treated with increasing doses of γ-irradiation at 1, 2.5, 5, and 10 Gy using a Gammacell 1000 Elite irradiator (Nordion International, Inc., Ottawa, Canada) with a ^{137}Cs source at a dose rate of 4.42 Gy/min. The culture medium was replaced at the end of 7 days. Untreated cells cultured in parallel were used to determine relative plating efficiency. After 12 days, the cells were fixed with 70% ethyl alcohol, and stained with Giemsa (Sigma, St. Louis, MO), and the number of colonies was counted. A colony was defined as a cluster of ≥50 cells, having formed from a single cell. All experiments were performed in triplicate, and the error was reported as one standard deviation from the mean.

2.8 RNA EXTRACTION AND REAL TIME PCR

RNA extraction for real time PCR was performed using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The extracted RNA was purified using the RNeasy Mini kit (QIAGEN) and resuspended in 100 µl RNase free water. The RNA samples were

purified of unwanted DNA with the DNA–free DNase kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA concentrations were determined using the SmartSpec 3000 (Bio-Rad Laboratories) and normalized to 40ng/µl. Reverse transcription was carried with three inputs for each sample: 400ng of total RNA, 100ng of total RNA and a negative control with no reverse transcriptase. The RT set-up is described in the Table below:

Table 2. QRT–PCR reagents

Reagent	Company	Input		
		400 ng	100 ng	No reverse transcriptase
10 x PCR Buffer II	Applied Biosystems	10 µl	10 µl	10 µl
$MgCl_2$ (25 mM)	Applied Biosystems	30 µl	30 µl	30 µl
dNTP (25 µM)	Roche Molecular Biochemicals	4 µl	4 µl	4 µl
MMLV 10 U/µl	Ambion	1 µl	1 µl	0 µl
RNase Inhibitor (40 U/µl)	Applied Biosystems	1 µl	1 µl	1 µl
Hex Primer (500 µM)	Applied Biosystems	2.5 µl	2.5 µl	2.5 µl
Nuclease free Water	Ambion	41.5 µl	49 µl	42.5 µl
RNA (amount)		10 µl	2.5 µl	10 µl

The thermocycler conditions were set up as: 25 ^{o}C for 10 min, 48 ^{o}C for 40 min, 95 ^{o}C for 5 min and hold at 10 ^{o}C. The cDNA was diluted 2.5 times to yield working concentrations of 1.6ng/µl and 0.4ng/µl.

For quantitative PCR (qRT–PCR), 5µM of each primer, 10 µM of probe, 25 mM dNTPs, 25mM $MgCl_2$, AmpliTaq Gold enzyme (Applied Biosystems) were used. The Taqman primers and probes for *ATR*, *CHEK1* and the control, 18S rRNA were obtained from Applied Biosystems. qRT–PCR was carried out at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60s using the 7300 Real-Time PCR System (Applied Biosystems). Each sample was run in triplicate along with the no reverse transcriptase control. For each plate, at least three wells were set up with the master mix but without any cDNA template (no template

control). The RNA expression levels were quantified relative to the Universal Reference cDNA obtained from Clontech (Mountain View, CA).

2.9 IMMUNOBLOTTING

Immunoblotting was utilized to detect protein expression of MRE11A, ATM and H2AX in HNSCC cell lines, and also to assess the phosphorylation levels of H2AX following exposure to 2.5 Gy IR. Flasks of each cell line were trypsinized, washed with ice cold 1x phosphate–buffered saline (PBS) and lysed on ice with a solution containing 50 mM Tris, 1% Triton X-100 (Sigma), 0.1% sodium dodecyl sulfate (Bio-Rad Laboratories, Hercules, CA), 150 mM NaCl (Fisher Chemicals, Fairlawn, NJ), 1mM dithiothretol (DTT) (Fisher Scientific, Inc., Hampton, NH), 10 μg/ml leupeptin (Roche Applied Science, Indianapolis, IN), 10 μg/ml pepstatin (Roche Applied Science), and 1 nM phenyl methyl sulfonyl fluoride (PMSF) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The soluble cell lysate was centrifuged at 2000 x g for 15 min and transferred to a clean microcentifuge tube. Histone H2AX was extracted from the remaining pellet containing insoluble protein and chromatin. The pellet was treated with 0.1% HCl for 20 min on ice, and then centrifuged at 10,000 x g for 10 min. The supernatant was again transferred to a clean microfuge tube.

Protein concentrations resulting from the standard and acid lysis procedures were determined using the Bio-Rad Quick Start Bradford Protein Assay Kit using a SmartSpec 3000 (Bio-Rad Laboratories). The acid lysate was neutralized with Tris-EDTA (TE) pH 8.0 prior to normalization. Normalized lysates with a protein concentration of 1μg/μl were resolved by

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon-P membrane (Millipore Corporation, Billerica, MA). After blocking with 5% non-fat dry milk (NFDM) for 1 h, the membrane was incubated overnight with the desired primary antibody (APPENDIX C) at room temperature. Following three 5 min washes in 1xTBST (Tris-buffered Saline Tween-20), the membrane was incubated with the appropriate secondary antibody (1:3000) for 2 h. Target proteins were visualized using the Western Lighting™ Chemiluminescence Reagent Plus kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instructions. To verify equal protein loading in the gels, membranes were stripped and re-probed with antibodies against β-tubulin (Santa Cruz), actin (Sigma Immunochemicals, St. Louis, MO). Individual antibodies, their concentrations and characteristics are listed in APPENDIX C.

2.10 CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

Following the relevant treatments, mock, IR or aphidicolin, and with or without caffeine or respective siRNAs, floating and adherent cells were collected at the end of 24 h. These cells were washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol. The cells were then treated with 80 µg/ml RNase A and 50 µg/ml propidium iodide (Invitrogen-Molecular Probes, Carlsbad, CA) for 45 min at 37^{o}C. The stained cells were analyzed using a Coulter Epics XL Flow Cytometer in the UPCI Flow Cytometry Facility.

2.11 CHROMOSOME BREAKAGE STUDIES

To check for chromosomal damage in OSCC in response to ionizing radiation, the total weighted aberrations per cell were determined for UPCI:SCC084, 104 and 116. Briefly, UPCI:SCC084, 104 and 116 were subjected to 2.5 Gy of IR. The cells were re-seeded and allowed to repair for 48 h prior to being harvested after treatment with 18 nM Calyculin A for 30 min (Calbiochem, San Diego, CA). Harvested cells were subjected to a hypotonic treatment before being fixed in Carnoy's fixative. Slides were then prepared from the cell pellets, solid stained for 8 min in 4% Giemsa/PBS solution, and rinsed with distilled water. 50 cells per case (case includes control and IR-treated cells) were scored. Chromosome aberrations documented include: chromosome breaks, chromatid gaps or breaks, radials, giants, rings, minutes, dicentrics, fragments and dots. Chromosome breaks, radials, giants, rings and dicentrics were assigned twice the weight of the other aberrations since they involve two chromatid events. The total weighted aberrations were summed, and determined per chromosome and per cell for each treatment. The standard error of the mean was used as the estimate of error in the sample. A Student's t-test was used to compare the raw distributions of total weighted aberrations between the control and IR-treated samples.

2.12 PCC INDUCTION IN OSCC

To determine whether inhibition of the ATR–CHEK1 pathway can sensitize OSCC to DNA damaging agents, we treated OSCC cell lines with aphidicolin (Sigma), a DNA polymerase-α inhibitor to induce DNA damage, and caffeine (Sigma), a nonspecific kinase inhibitor to inhibit ATR activity. We used an AT cell line (GM09607) with a deficient G_1 phase checkpoint as our

positive control and NHOK cells as negative controls. Briefly 75-80% confluent flasks of cells were pretreated with 1 mM caffeine for 30 min following which 0.4 μM aphidicolin was added. The cell lines were harvested for metaphases 24 h after aphidicolin treatment. A fluorescence microscope was used to count mitotic cells that had characteristic features of either normal mitosis or PCC. Criteria for distinguishing PCC from normal metaphase were adapted from a previous report (NGHEIM *et al*. 2001). Briefly, interphase cells and cells that with intermediate morphology between normal and PCC were not included in the analysis. Partial metaphases with PCC were also not included in the analysis. The following criteria were used to identify mitoses as PCC or normal: PCC characteristics include well-defined particles by DAPI staining that were round, not oblong, particles with no hazy chromatin material; no chromatid-like pairs present; and borders of the cellular chromatin were irregular and composed of speckles, not smooth or with a hazy appearance (all characteristics must be met). Characteristics of normal mitoses include well-defined chromosomes with a primary constriction; at least 40 such chromosomes should be found in each metaphase spread.

2.13 siRNA TRANSFECTION

ATR or CHEK1 inhibitions were carried out using the respective siRNAs for a specific knockout. RNA interference of ATR and CHEK1 was performed using Smartpool ATR and CHEK1 duplexes respectively, obtained from Dharmacon (Lafayette, CO). Nonspecific (scrambled) control duplexes (Dharmacon) were used for nonspecific siRNA treatment. The duplexes were reconstituted in 1 X DNA–free RNA re-suspension buffer provided by Dharmacon and aliquoted according to the manufacturer's instructions. For transfection, the

OSCC cell lines were seeded in 60 mm dishes or T25 flasks and transfected with siRNA duplexes using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The individual siRNA duplex sequences are enumerated in Appendix B. The final working siRNA concentration achieved was between 90-100 nM. We used cells treated with no vector (untreated), empty vector (mock–treated), cells transfected with a nonspecific siRNA, and a specific ATR or CHEK1 siRNA for all of our experiments. At the end of 48 h post-transfection, appropriate treatments (ionizing radiation or aphidicolin treatment) were carried out as described earlier.

3.0 RESULTS

3.1 DISTAL 11Q LOSS IN A SUBSET OF OSCC

3.1.1 A segment of distal 11q is partially lost in a subset of OSCC

According to the BFB model, the first step in 11q13 amplification is loss of a segment of distal 11q. To determine whether genes located on distal 11q and involved in the DNA damage response are lost in OSCC, we carried out dual-color FISH with BAC probes to *MRE11A, ATM, H2AFX* and *CHEK1* along with a centromere 11 enumeration probe (CEP 11; D11Z1) (Vysis, Downers Grove, IL). Table 3 summarizes our FISH results in terms of copy number loss in relation to the ploidy of each cell line, derived from chromosome 11 centromere enumeration and consensus karyotypes. The copy number ratios of the distal 11q genes were normal or lost with respect to CEP 11. The eleven cell lines were grouped based on FISH assessment of 11q13 amplification and distal 11q loss as follows: most cell lines with 11q13 amplification demonstrated partial loss of copies of all four genes, including UPCI:SCC078, 084, 131 and 136 ("11q13 amplified with distal loss"). The OSCC cell lines, UPCI:SCC104, 142 and 122 had loss of one or more genes in the absence of 11q13 amplification ("Distal 11q loss but no 11q13 amplification"). UPCI:SCC099, 116 and 182 did not have 11q13 amplification or distal 11q loss ("no 11q13 amplification, no distal loss"). Interestingly, UPCI:SCC078 and 104 did not

demonstrate loss of the *CHEK1* gene. UPCI:SCC125 is comprised of a highly heterogeneous cell population, making its analysis less straightforward than the other cell lines. There is no evidence of 11q13 amplification in this cell line. While the copy number ratios seem to show that UPCI:SCC125 is relatively normal in copy number for each of the four distal genes, FISH results indicated that results for *MRE11A* (31% loss, 44% gain), *ATM* (52% loss, 1% gain), *H2AFX* (43% loss, 25% gain) and *CHEK1* (45% loss, 30% gain) are more a function of the average than the norm.

Table 3. Summary of FISH copy number changes for *MRE11A ATM, H2AFX* and *CHEK1* in OSCC cell lines.

Cell Line UPCI:	Ploidy	11q13 amp (+/-)	Distal 11q loss (+/-)	FISH Results			
				MRE11A[1]	*ATM*[1]	*H2AFX*[1]	*CHK*[1]
SCC099	2-4	-	-	1.00	0.98	1.02	1.00
SCC116	3-4	-	-	0.96	0.97	0.96	0.94
SCC182	3	-	-	1.01	0.97	0.98	1.01
SCC078	3-4	+	+	0.38	0.54	0.84	1.05
SCC084	2	+	+	0.51	0.52	0.52	0.52
SCC131	4	+	+	0.56	0.59	0.53	0.44
SCC136	4	+	+	0.57	0.60	0.56	0.53
SCC104	4	-	+	0.98	0.51	0.50	0.96
SCC122	3	-	+	0.69	0.68	0.71	0.71
SCC142	3	-	+	0.93	0.67	0.79	0.67
SCC125[2]	3	-	+/-	1.08	0.79	0.97	0.93

[1]Shading indicates partial loss or haploinsufficiency.

[2]UPCI:SCC125 is a highly heterogeneous cell line with respect to copy number showing comparable number of cells with gain, loss or normal copy number for any given gene. (Parikh, White *et al*. unpublished data).

Confirmation of physical loss on distal 11q was carried out by Dr. Xin Huang, using quantitative microsatellite analysis or QuMA. QuMA was used to map the DNA copy number of

segmental microsatellite loci along 11q (Figure 3). The microsatellites, D11S1358, D11S917 and D11S1893 are the most relevant as they map near the genes of interest on chromosome 11. The results, which demonstrate a loss in copy number of each of these microsatellites confirm the findings of our FISH experiments. A number of the tumor cell lines have near-triploid or near-tetraploid karyotypes, thus DNA copy number of 1 or 2 on the polyploid background reflects a loss of copy number.

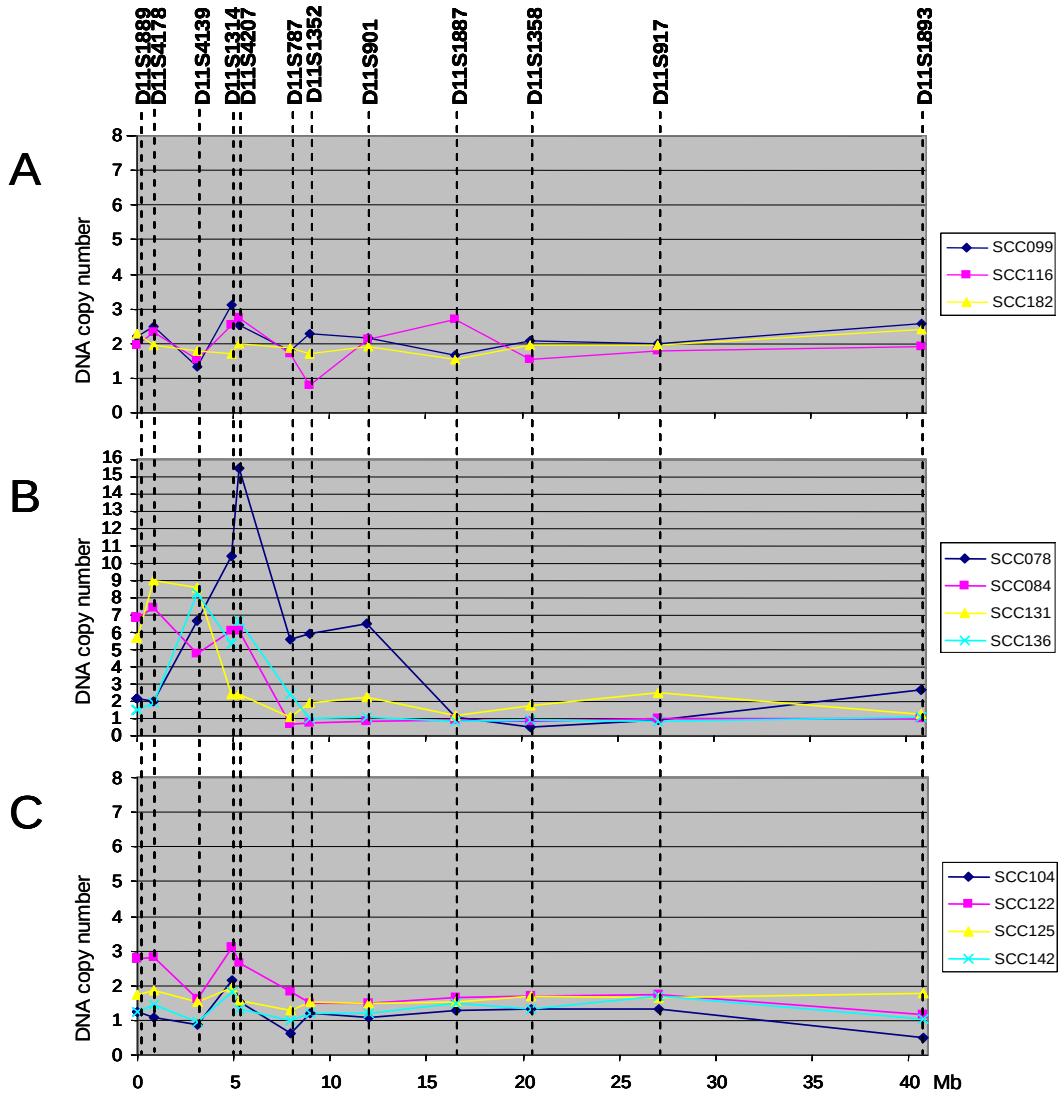
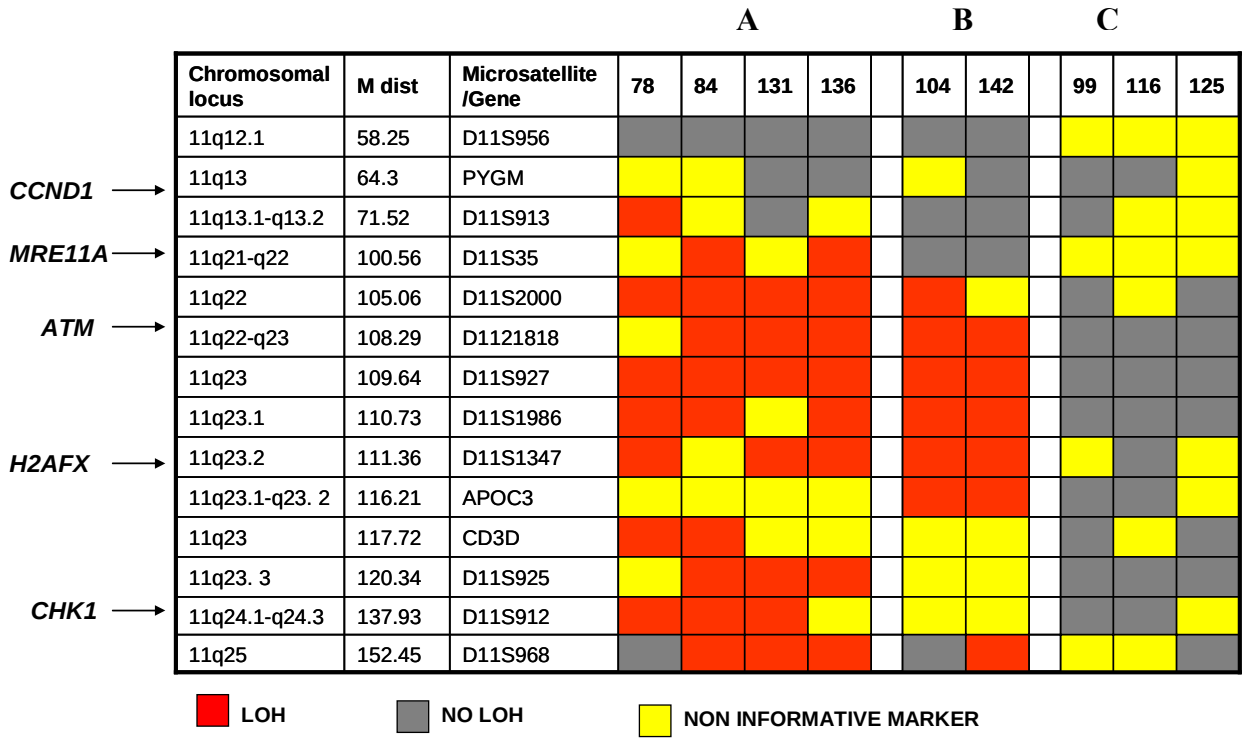


Figure 3. QuMA studies to map segmental loss of microsatellite loci on distal 11q.

QuMA was performed for each of the OSCC cell lines in the three groups by Dr. Xin Huang. The cell lines were grouped as **(A)** "no 11q13 amplification, no distal loss", **(B)** "11q13 amplification with distal 11q loss" and **(C)** "distal 11q loss but no 11q13 amplification".

LOH analysis was performed by Dr. Bora Baysal as an independent test to validate our FISH and QuMA results. Results were available for nine of the eleven OSCC cell lines studied by FISH and QuMA. The results of LOH analysis, summarized in Figure 4, substantiate the loss patterns we observed for distal 11q using FISH and QuMA, as extended segments of 11q were shown to have complete or partial LOH in the "distal 11q loss without 11q13 amplification" and "11q13 amplified with distal loss" OSCC cell line groupings. The cell lines with "no 11q13 amplification, no distal loss" do not demonstrate LOH along 11q.



Chromosomal locus	M dist	Microsatellite /Gene	78	84	131	136		104	142		99	116	125
11q12.1	58.25	D11S956											
11q13	64.3	PYGM											
11q13.1-q13.2	71.52	D11S913											
11q21-q22	100.56	D11S35											
11q22	105.06	D11S2000											
11q22-q23	108.29	D1121818											
11q23	109.64	D11S927											
11q23.1	110.73	D11S1986											
11q23.2	111.36	D11S1347											
11q23.1-q23. 2	116.21	APOC3											
11q23	117.72	CD3D											
11q23. 3	120.34	D11S925											
11q24.1-q24.3	137.93	D11S912											
11q25	152.45	D11S968											

Figure 4. Loss of heterozygosity studies performed on OSCC for multiple loci on distal 11q. LOH studies performed by Dr. Bora Baysal for multiple markers on chromosome 11q demonstrate loss of distal loci or genes in cell lines from each of the three groups: **A** "11q13

amplification with distal 11q loss", **B** "distal 11q loss but no 11q13 amplification" and **C** "no 11q13 amplification, no distal loss".

3.1.2 Distal 11q loss results in changes in expression of MRE11A, ATM, H2AX and CHEK1

Dr. Xin Huang carried out Taqman quantitative reverse transcriptase PCR (qRT-PCR) and immunoblotting to assess whether loss of one or more copies of the *MRE11A, ATM, H2AFX* and *CHEK1* genes translates into a reduction in their expression (Figure 5). UP3_344, 348 and 700 were the NHOK controls used for the study. Overall, we observed that cell lines with distal 11q loss generally exhibit a reduction on ATM and H2AFX expression relative to control NHOK cell line and cell lines with no distal 11q loss.

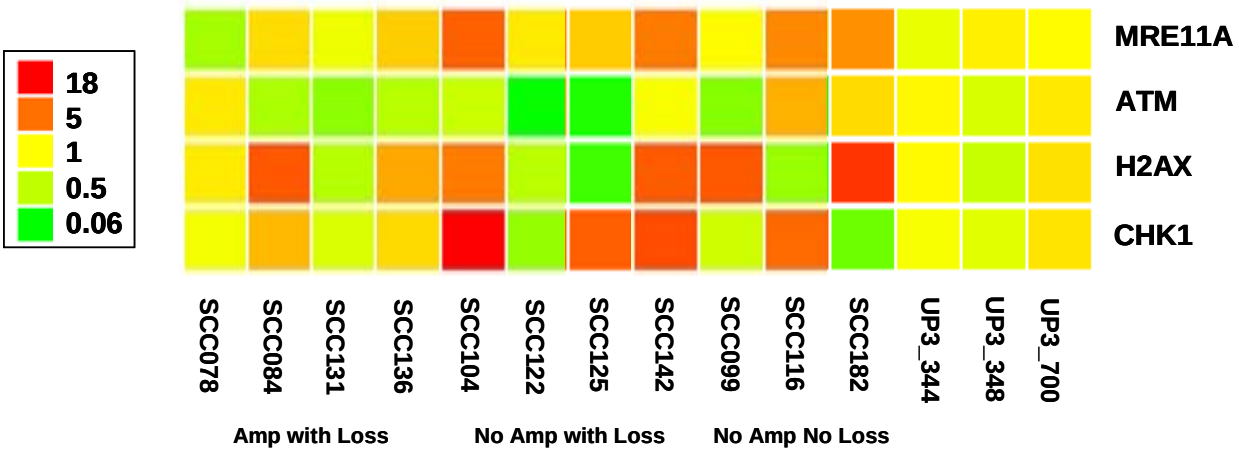


Figure 5. RNA expression changes for MRE11A, ATM, H2AX and CHEK1 in OSCC

qRT–PCR was performed for the four genes *MRE11A*, *ATM*, *H2AX* and *CHEK1* on distal 11q. Overall genetic loss of *ATM* and *H2AX* correlated well with a reduced expression.

UPCI:SCC084, 136, 142 and 125 show increased CHEK1 expression in spite of loss at the gene level. UP_344, 348 and 700 are normal human oral keratinocytes (control cell lines).

Protein expression in OSCC cell lines and various controls was assessed by immunoblotting (Figure 6). Cell lines with distal 11q loss tend to have an overall lower expression level of the MRE11A, ATM and H2AX proteins compared to those without distal 11q loss. Further, the relative trends in protein expression correlated with the quantitative RT-PCR analysis.

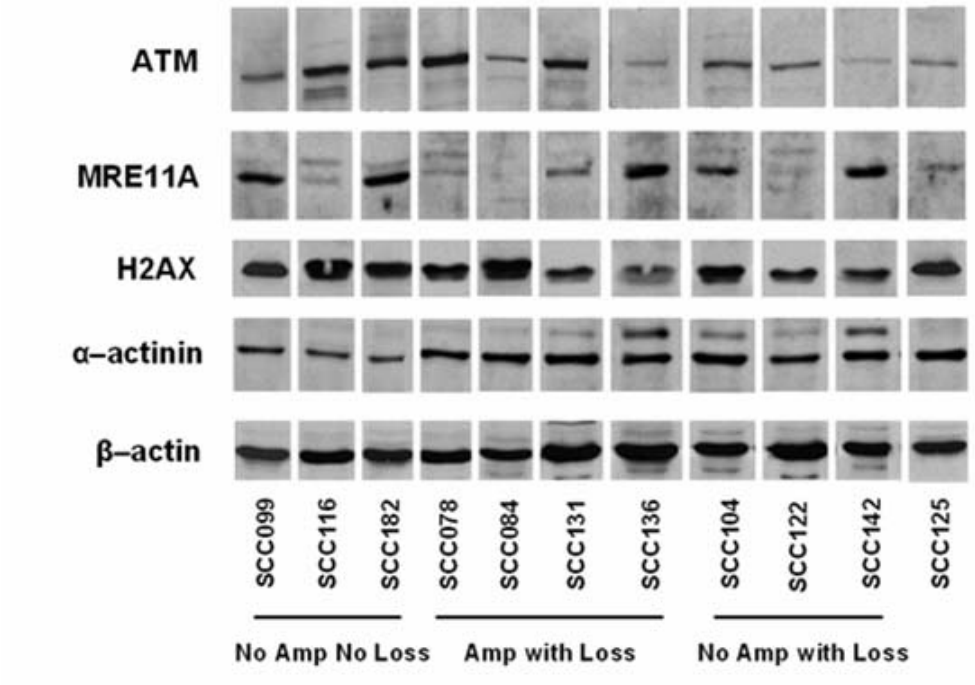


Figure 6. Protein expression changes for MRE11A, ATM and H2AX in OSCC
Immunoblotting was performed by Dr. Jason White to detect changes in protein expression for MRE11A, ATM and H2AX. We observed reduction in MRE11A, ATM and H2AX protein in most cell lines with loss irrespective of their amplification status.

Thus, a genetic loss of distal 11q leads to reduction on the RNA and protein expression for ATM, MRE11A and H2AX in a subset of OSCC.

3.2 CONSEQUENCES OF DISTAL 11Q LOSS IN OSCC

3.2.1 Distal 11q loss is associated with aberrant γ–H2AX focus formation

Using phosphorylation of H2AX as a surrogate marker for a proficient DNA damage response, Dr. Jason White evaluated the competency of OSCC cell lines to detect double strand breaks and initiate repair by formation of γ-H2AX foci after treatment with 2.5 Gy IR. Constitutive phosphorylation patterns were observed in some of the cell lines (UPCI:SCC099 and 116) in the absence of any apparent overt or exogenous genetic insult. Hence, deficiencies in focus formation were assayed by several measurements, including the mean number of foci per cell, the distribution of foci in 200 cells, and the percent of focus-positive cells. Fewer focus-positive cells, coupled with a reduced mean number of foci per cell relative to control, are indicative of a deficient DNA damage response. The distribution of foci also shows a deficient response when the distribution is skewed toward fewer signals as shown in Figure 7. The highest mean numbers of foci were seen in the cells with "no 11q13 amplification, no distal loss" (min = 5.40; max = 9.45), and these were similar to those of the control fibroblast cell line. The cell lines that are "11q13 amplified with distal loss" showed the lowest mean levels of H2AX foci. When treated with IR, the mean number of foci was more than two-fold lower than cell lines without 11q alteration (min = 3.02; max = 4.64). Cell lines with "distal 11q loss without 11q13 amplification" showed low to intermediate levels of γ-H2AX focus formation after IR (min = 3.09; max = 6.83). While there was focus formation in the untreated AT cell line (GM09607), there was essentially no difference between the untreated and treated cells one hour following treatment, showing that the cells do not respond normally to IR.

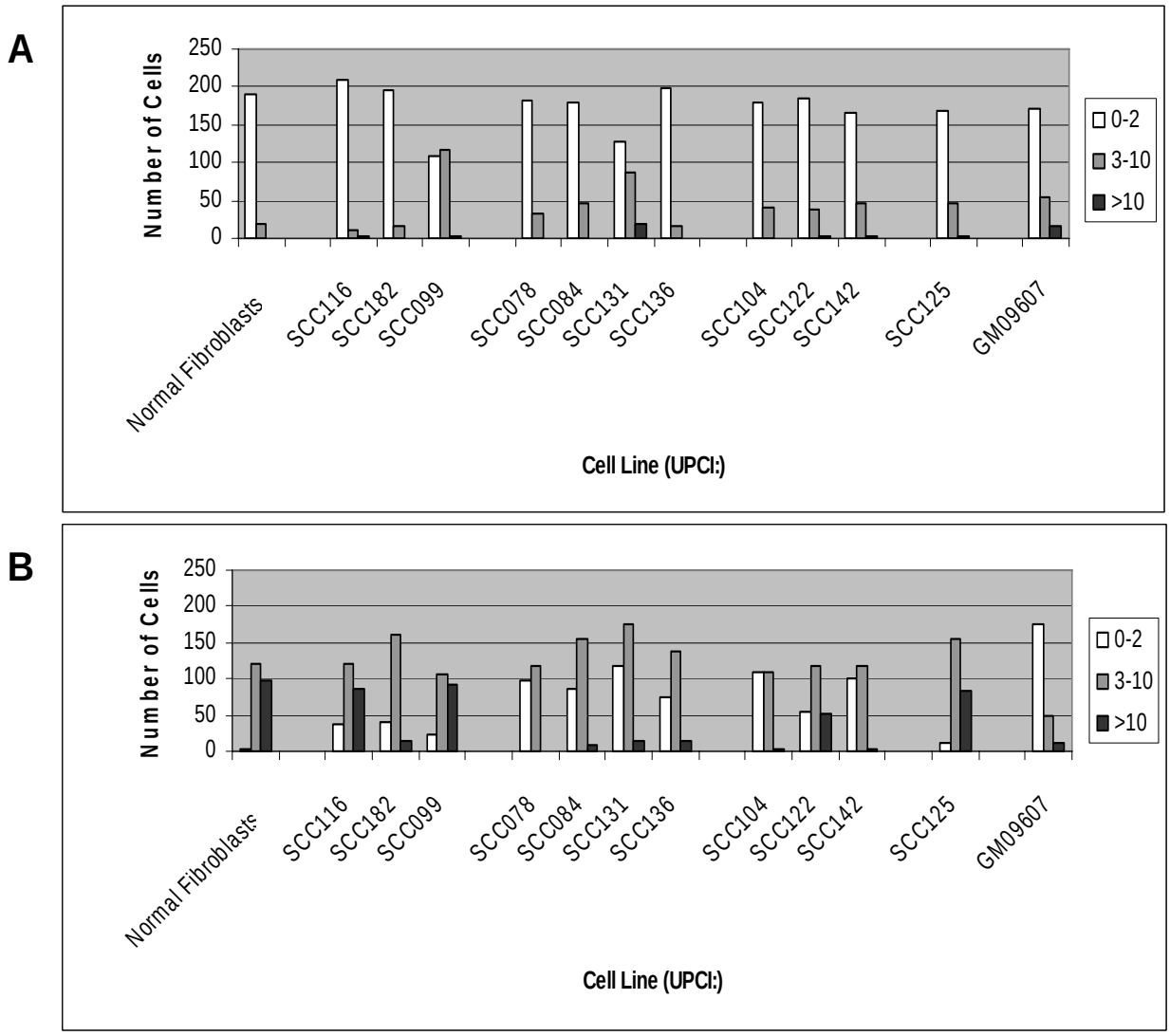


Figure 7. γ-H2AX focus formation at the end of 1 h following 2.5 Gy IR treatment.

The distribution of γ-H2AX focus formation was evaluated by Dr. Jason White in (**A)** untreated OSCC cell lines, NHOK and AT cell line (GM09607) and (**B)** OSCC cell lines, NHOK and AT cell line treated with 2.5 Gy IR at the end of 1 h.

3.2.2 Distal 11q loss is associated with chromosomal instability

The total number of weighted aberrations per chromosome were determined for UPCI:SCC084, 104 and 116. Examples of metaphase spreads from each of these cell lines are shown in Figure 9. Breakage was assessed 48 h post-IR, and breaks were evident in the cell lines with loss of distal 11q, irrespective of 11q13 status. Complete results are summarized in Table 4. We determined the 95% confidence intervals for each of the cell lines evaluated for IR–induced breakage. There was no significant difference in the total number of weighted aberrations per cell between the control (2.52 ± 0.97%) and treated (2.94 ± 0.62%) populations of UPCI:SCC116, which represents the "no 11q13 amplification, no distal loss" group. There is substantial agreement in the results for UPCI:SCC084 (C = 1.43 ± 0.51%; IR = 6.68 ± 1.40%; $p < 0.001$) and UPCI:SCC104 (C = 3.15 ± 0.76%; IR = 6.68 ± 1.05%, $p < 0.001$), both of which have distal 11q loss. An insignificant increase in chromosomal breaks in UPCI:SCC116 in response to ionizing radiation could be caused due to a highly efficient DNA damage repair pathway or may be as a result of cells with increased breaks undergoing apoptosis. Thus, UPCI:SCC084 (11q13 amplified with distal loss) and UPCI:SCC104 (distal 11q loss without 11q13 amplification) demonstrated elevated levels of chromosomal aberrations in response to ionizing radiation when compared to UPCI:SCC116 (no 11q13 amplification, no distal loss).

Table 4. Summary of chromosomal aberrations in OSCC in response to IR.

Shaded areas indicate statistically significant results with 'p' value ≤ 0.001.

Cell Line UPCI:	11q13 Amp	Distal 11q Loss	Treatment	Total Weighted Aberrations per Chromosome ± 95% CI	Total Weighted Aberrations per Cell ± 95% CI
SCC116	–	–	Control	0.05± 0.02	2.52 ± 0.97
			2.5 Gy IR	0.06± 0.01	2.94 ± 0.62
SCC084	+	+	Control	0.03± 0.01	1.43 ± 0.51
			2.5 Gy IR	0.15± 0.03	6.68 ± 1.40
SCC104	–	+	Control	0.07± 0.02	3.15 ± 0.76
			2.5 Gy IR	0.15± 0.02	6.68 ± 1.05

The frequency of anaphase bridges in five hundred cells was determined by Dr. Jason White and used an indicator of baseline chromosomal instability in OSCC. The frequencies of anaphase bridges in each cell line are summarized as percentages in Table 5. Overall, the lowest frequency was seen in normal male lymphocytes (0.7%). The lowest frequency among all OSCC cell lines was seen in UPCI:SCC116 (0.96%), and the highest frequency was seen in UPCI:SCC142 (5.63%). In cell lines with no 11q13 amplification and no distal loss, the maximum frequency (1.31%; UPCI:SCC099) was lower than any of the frequencies observed in the "11q13 amplified with distal loss" and "distal 11q loss without 11q13 amplification" groups. In a comparison of proportions, most cell lines with distal 11q loss, irrespective of 11q13 amplification, have significantly elevated levels of anaphase bridges ($p<0.05$). The comparison was made relative to normal male lymphocytes. For two of the cell lines, UPCI:SCC078 and 122, the p-value is less than 0.1. Since all anaphase bridge frequencies were measured in untreated cell populations, the intrinsic level of chromosomal instability appears to be higher in cell lines with 11q loss.

Table 5. Baseline anaphase bridge formation in OSCC.

Anaphase bridge formation was determined in eleven OSCC by Dr. Jason White. All OSCC cell lines with significantly high (p value ≤ 0.05) anaphase bridge formation at rest are highlighted in grey.

Cell Line	11q13 Amplification	Distal 11q Loss	Anaphase bridge frequency
SCC099	–	–	0.96
SCC116	–	–	1.27
SCC182	–	–	1.31
SCC078	+	+	1.86
SCC084	+	+	2.65
SCC131	+	+	3.08
SCC136	+	+	3.58
SCC104	–	+	4.14
SCC122	–	+	1.87
SCC142	–	+	5.63
SCC125	–	+	3.1

3.2.3 Distal 11q loss is associated with radioresistance

We used clonogenic survival assays to detect sensitivity of OSCC to survival after DNA damage induced by ionizing radiation (Figure 8 and Table 6). Results for the assay in triplicate, were grouped as "no 11q13 amplification, no distal loss" (UPCI:SCC099, 116 and 182), "11q13 amplified with distal loss" (UPCI:SCC078, 084, 131 and 136), and "distal 11q loss without 11q13 amplification" (UPCI:SCC104, 122 and 142). Also included were normal human oral keratinocyte (NHOK) cells as normal controls and UPCI:SCC125 (separated due to its heterogeneity in copy number by FISH). In the grouped analysis, nearly 60% of NHOKs

survived a single unfractionated dose of 1 Gy, and none survived a dose of 10 Gy. Similarly, the OSCC cells with "no 11q13 amplification, no distal loss" showed 56% survival at 1 Gy, and no survival at 10 Gy IR. Conversely, the OSCC cell lines grouped as "11q13 amplified with distal loss" had nearly 83% survival at 1 Gy, and 9% survival at 10 Gy IR. The "distal 11q loss without 11q13 amplification" cells had 80% survival at 1 Gy and 7% survival after exposure to 10 Gy IR. The UPCI:SCC125 cells had a high surviving fraction after 1 Gy (83.3 ± 5.0%), and a small, but visible surviving fraction at 10 Gy (0.7 ± 0.6%). Thus, cell lines with 11q13 amplification and distal 11q loss (UPCI:SCC078, 084, 131 and 136) and cell lines with distal 11q loss without 11q13 amplification (UPCI:SCC104, 122 and 142) demonstrate similar resistance to ionizing radiation. In contrast, normal human oral keratinocytes (NHOK) and cell lines without distal 11q loss are sensitive to ionizing radiation.

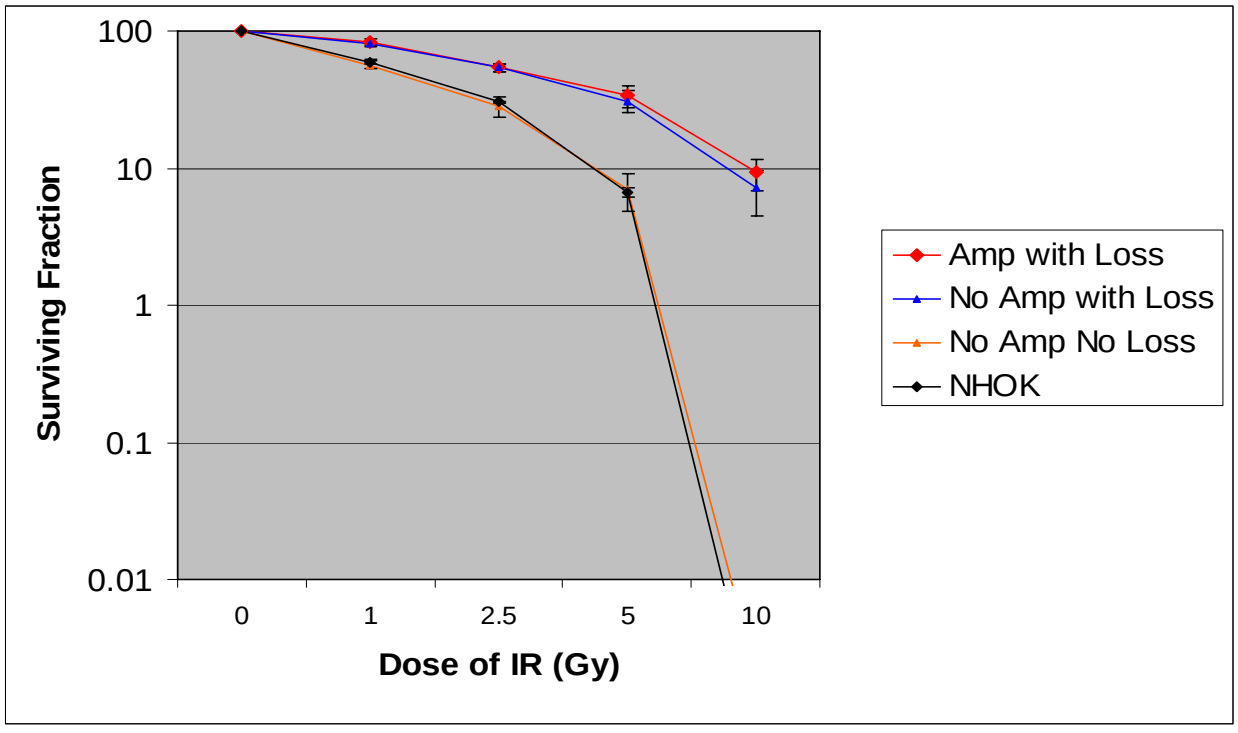


Figure 8. Clonogenic cell survival of OSCC to IR compared with control NHOK.

Grouped analysis for clonogenic cell survival demonstrates that the cell lines with no amplification but with loss (red), and cell lines with amplification and loss (blue) show a similar survival, while cell lines with no amplification and no loss (orange) show survival comparable to the control NHOK. At a very high IR dose of 10 Gy, cell lines with 11q loss, irrespective of their amplification status, have an average of 9% cells surviving.

Table 6. Results of clonogenic cell survival in OSCC in response to ionizing radiation.

Cell line	11q13 Amp	Distal 11q loss	Mock treated	1Gy	2.5Gy	5Gy	10Gy
NHOK	–	–	100±0.0	59.7±2.1	30.3±0.6	6.7±0.6	0.0±0.0
SCC099	–	–	100±0.0	58.0±1.0	29.7±1.5	5.7±1.2	0.0±0.0
SCC116	–	–	100±0.0	55.0±4.4	23.0±2.6	6.0±1.0	0.0±0.0
SCC182	–	–	100±0.0	56.0±5.2	32.7±4.0	9.3±2.1	0.0±0.0
SCC104	–	+	100±0.0	82.3±4.7	55.3±2.1	33.7±1.2	7.7±0.6
SCC122	–	+	100±0.0	77.0±1.0	54.7±0.6	24.0±1.7	4.0±1.0
SCC142	–	+	100±0.0	81.0±4.0	51.7±4.6	35.3±1.5	9.7±0.6
SCC078	+	+	100±0.0	83.0±2.6	56.7±0.6	38.0±1.7	7.0±0.0
SCC084	+	+	100±0.0	80.7±4.6	52.3±5.0	24.7±2.1	7.7±0.6
SCC131	+	+	100±0.0	86.7±2.5	51.7±3.8	37.3±2.1	11.3±2.5
SCC136	+	+	100±0.0	81.7±3.5	55.0±4.6	34.7±2.5	11.0±1.0
SCC125	–	+	100±0.0	83.3±5.0	40.3±3.2	27.7±0.6	0.7±0.6

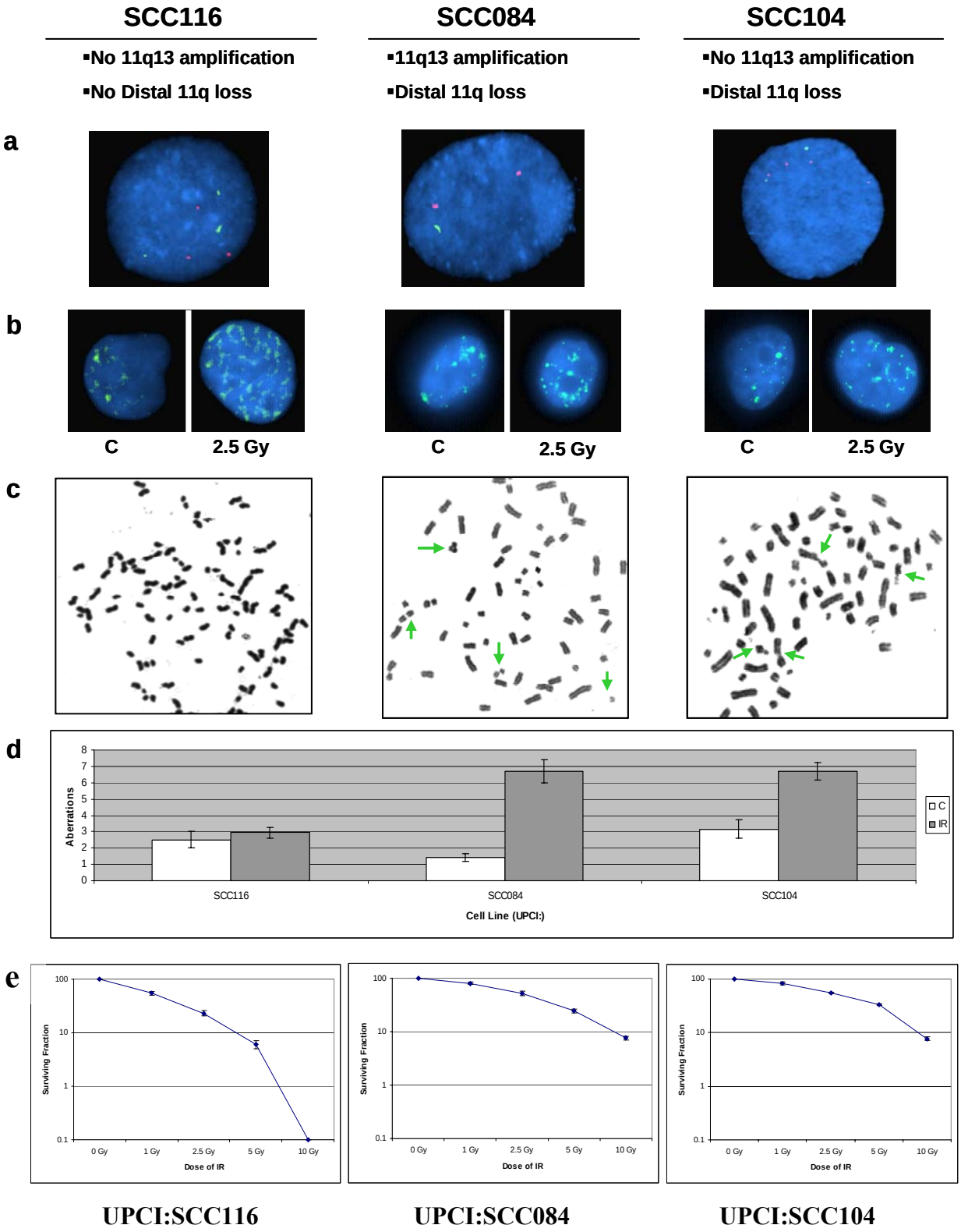

SCC116
SCC084
SCC104
▪No 11q13 amplification
▪No Distal 11q loss
▪11q13 amplification
▪Distal 11q loss
▪No 11q13 amplification
▪Distal 11q loss
a
b
C
2.5 Gy
C
2.5 Gy
C
2.5 Gy
c
d
Aberrations
SCC116
SCC084
SCC104
Cell Line (UPCI:)
C
IR
e
Surviving Fraction
Dose of IR
0 Gy
1 Gy
2.5 Gy
5 Gy
10 Gy
UPCI:SCC116
UPCI:SCC084
UPCI:SCC104

Figure 9. Summary of results for distal 11q loss and its effects in representative OSCC

Results from representative OSCC cell lines: UPCI:SCC116 with no amplification of 11q13 and no loss of distal 11q. UPCI:SCC084 with 11q13 amplification and loss of distal 11q and UPCI:SCC104 with loss of distal 11q, but no 11q13 amplification. In **(a)**, FISH images showing copy number in *H2AFX* (green) and CEP11 (red) in each cell lines. UPCI:SCC116, which is triploid, shows three CEP11 and three *H2AFX* signals. UPCI:SCC084, has two CEP11 signals and only one *H2AFX* signal. UPCI:SCC104 has four copies of the CEP11 and only two copies of *H2AFX*. In **(b)**, γ -H2AX focus formation in control and treated (2.5 Gy IR with 1 h repair) cells. After treatment, UPCI:SCC116 develops a large number of foci, while UPCI:SCC084 and UPCI:SCC104 have higher baseline levels of focus formation, but markedly fewer foci in response to IR. Examples of common breakage events are shown for each cell line in **(c)**, as indicated with a green arrow. A summary of the total weighted aberrations per cell for each representative cell line is shown in **(d)**. The cell survival of each representative cell line, UPCI: SCC116, 084 and 104 is depicted in **(e).** (Parikh, White *et al*. unpublished data).

3.3 LOSS OF THE G_1 PHASE CHECKPOINT IN OSCC

3.3.1 Loss of the G_1 checkpoint in a subset of OSCC

We treated OSCC with ionizing radiation and performed flow cytometry in order to study the cell cycle profiles of OSCC in response to DNA damaging agents. The cell cycle profiles of different cell lines to IR are summarized in Table 7. Figure 10 demonstrates the cell cycle profiles of two cell lines, UPCI:SCC066 and 104 in response to 5 Gy IR. We observed that even in untreated cells, UPCI:SCC104 has a considerably high percentage of cells in the S and G_2M phases. Following IR, SCC066 shows accumulation of cells in both the G_1 and G_2M phases while, UPCI:SCC104 shows a predominant accumulation of cells in the S and G_2M phases. Thus, UPCI:SCC104 demonstrates a loss of G_1 phase cell cycle checkpoint in response to IR.

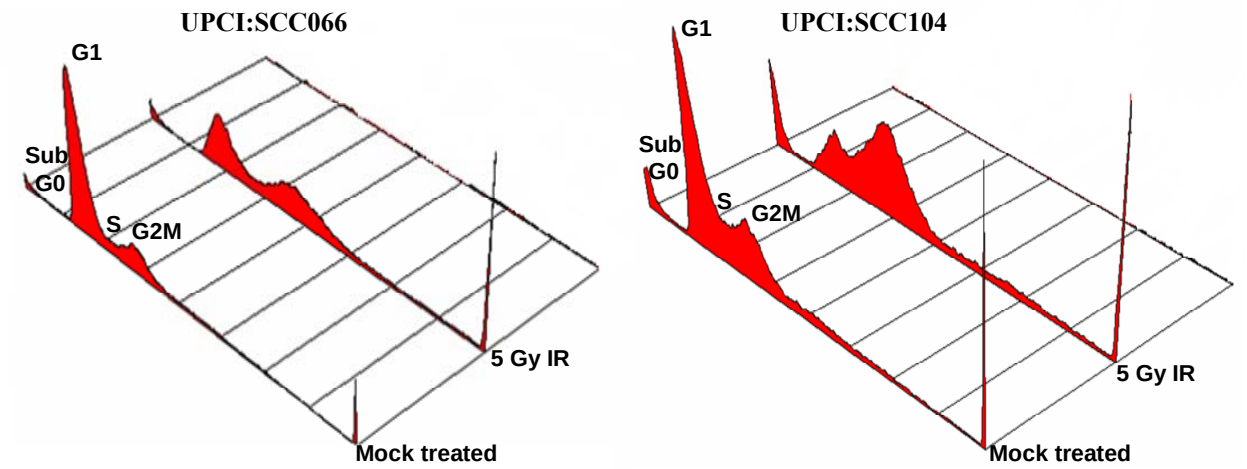


Figure 10. Comparison of cell cycle profiles of UPCI:SCC066 and 104 in response to IR. UPCI:SCC066 and 104 were either mock treated or treated with 5 Gy IR and allowed to recover for 24 h. At the end of 24 h, flow cytometric analysis show complete loss of the G_1 cell cycle checkpoint in UPCI:SCC104 with most cells accumulating in the S and G_2M phases, UPCI:SCC066 cells accumulate at both G_1 and G_2M cell cycle checkpoints.

Table 7. Results of cell cycle analysis in OSCC in response to ionizing radiation.

UPCI: Cell Line	Untreated				5 Gy IR (24 h)			
	Sub G_0	G_1	S	G_2M	Sub G_0	G_1	S	G_2M
NHOK	0.1%	71%	13%	15.9%	3%	75%	2%	20%
GM09607	2%	52.2%	6.8%	39%	6%	34%	8.9%	51.1%
SCC066	1.2%	70%	13.3%	15.5%	1.9%	50.3%	16.1%	31.7%
SCC084	0.7%	60%	10%	29..3%	2.4%	32%	12%	53.6%
SCC104	0.9%	53%	16%	30.1%	2.8%	22%	18%	57.2%
SCC105	0.7%	67%	13%	19.3%	4%	51%	15%	30%
SCC116	1.1%	61.9%	15%	22%	4%	49%	15%	32%
SCC131	1%	66.5%	15.5%	15%	2.5%	32%	11%	55.5%
SCC136	1%	59.9%	10%	29.1%	1.9%	31.6	25.5	41%
SCC142	1.3%	62.2%	14.2%	22.3%	2.6%	31%	16%	40.4%

Thus, we observed a loss of G_1 checkpoint in response to IR in 5 out of 8 OSCC studied. Even UPCI:SCC066, 105 and 116 with an intact G_1 checkpoint, had an increased accumulation of cells in the G_2M phase compared to NHOK. This may be either due to higher number of cells in the G_2M checkpoint at the time of DNA damage or due to cell line/tumor heterogeneity, wherein a fraction of cells in UPCI:SCC066, 105 and 116 lack p53 and are deficient in their G_1 checkpoint. A loss of the G_1 checkpoint in OSCC leads to an increased number of cells with unrepaired DNA damage entering the S and the G_2M phases of the cell cycle. Thus, OSCC with enhanced S and G_2M phase cell cycle checkpoints are able to avoid p53-independent cell death (PCC/MC) and have a growth advantage. Since the commonest cause of a dysregulated G_1 checkpoint is loss of p53 function, we decided to study the p53 pathway in OSCC.

3.3.2 The p53 pathway in OSCC

We studied activation of p53 in response to ionizing radiation in a subset of OSCC. In UPCI:SCC105 in our preliminary studies, we observed peak phosphorylation of p53 at the end of 1 h following treatment with 5 Gy of IR, so we decided to use the 1 h time point for our p53 phosphorylation studies. Following treatment with 5 Gy IR, we observed a loss of ser-15 phosphorylation in UPCI:SCC084 and 104. On further evaluation, we observed a complete loss of p53 expression in UPCI:SCC084 and 104. Interestingly, the loss of p53 expression correlated well with overexpression of ATR protein in UPCI:SCC084 and SCC104.

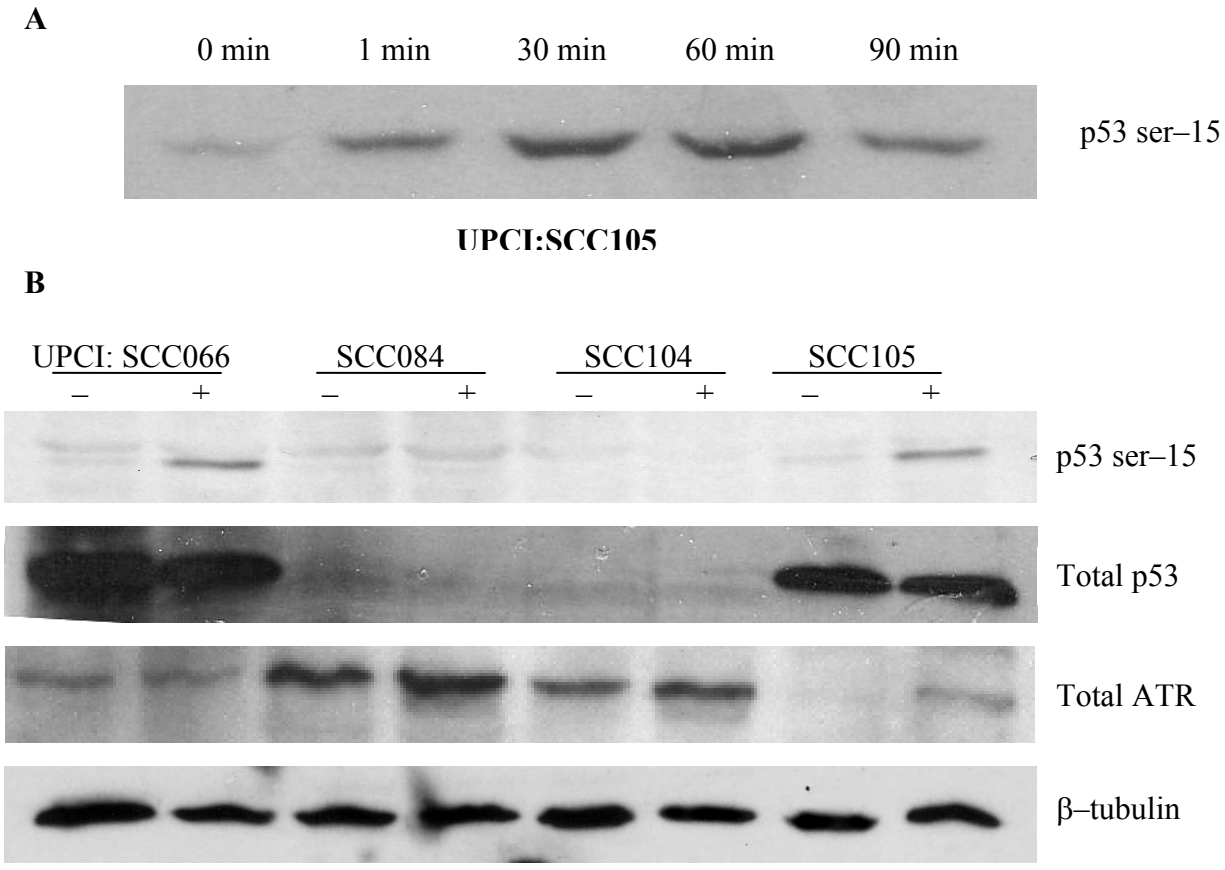


Figure 11. p53 activation and expression in response to IR in a subset of OSCC.
(A) Represents a time course for p53 phosphorylation following treatment with 5 Gy IR. In **(B)** we observe loss of p53 expression and ATR overexpression in UPCI:SCC084 and 104.

We observed that loss of p53 protein expression is associated with overexpression of ATR in a larger panel of OSCC cell lines (Figure 12). Thus, it is possible that either loss of p53 leads to ATR upregulation, or overexpressed ATR in OSCC may affect p53 regulation and function.

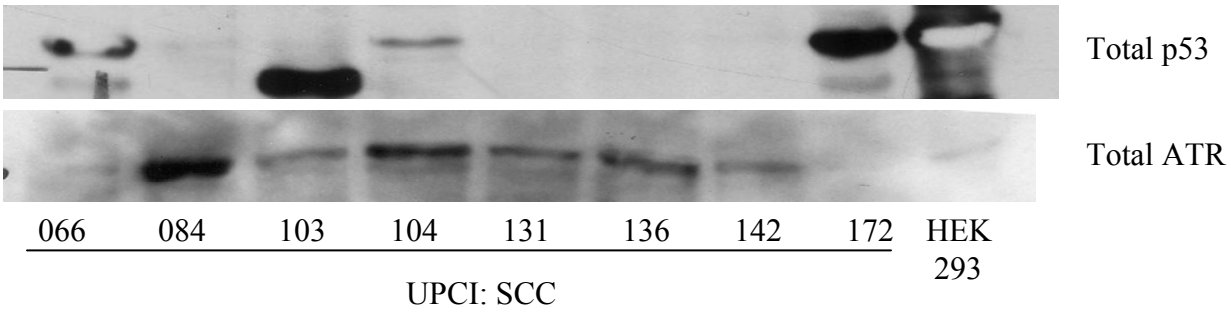


Figure 12. p53 and ATR expression in a subset of OSCC.

Loss of p53 expression correlates with an increased expression of ATR protein in UPCI:SCC084, 104, 131, 131, 136 and 142. UPCI:SCC103, which has a truncated p53 protein also has ATR overexpression. Expression in UPCI:SCC066 and 172 is comparable to that observed in HEK 293 cells.

To determine if loss of p53 expression in UPCI:SCC104 and 131 was due to deletion of the *TP53* gene on 17q13, we performed FISH using BAC probes to *TP53* and compared it to CEP17 labeled with Spectrum Orange™. We did not observe deletion of the *TP53* gene either in UPCI:SCC104 or SCC131 (Figure 13).

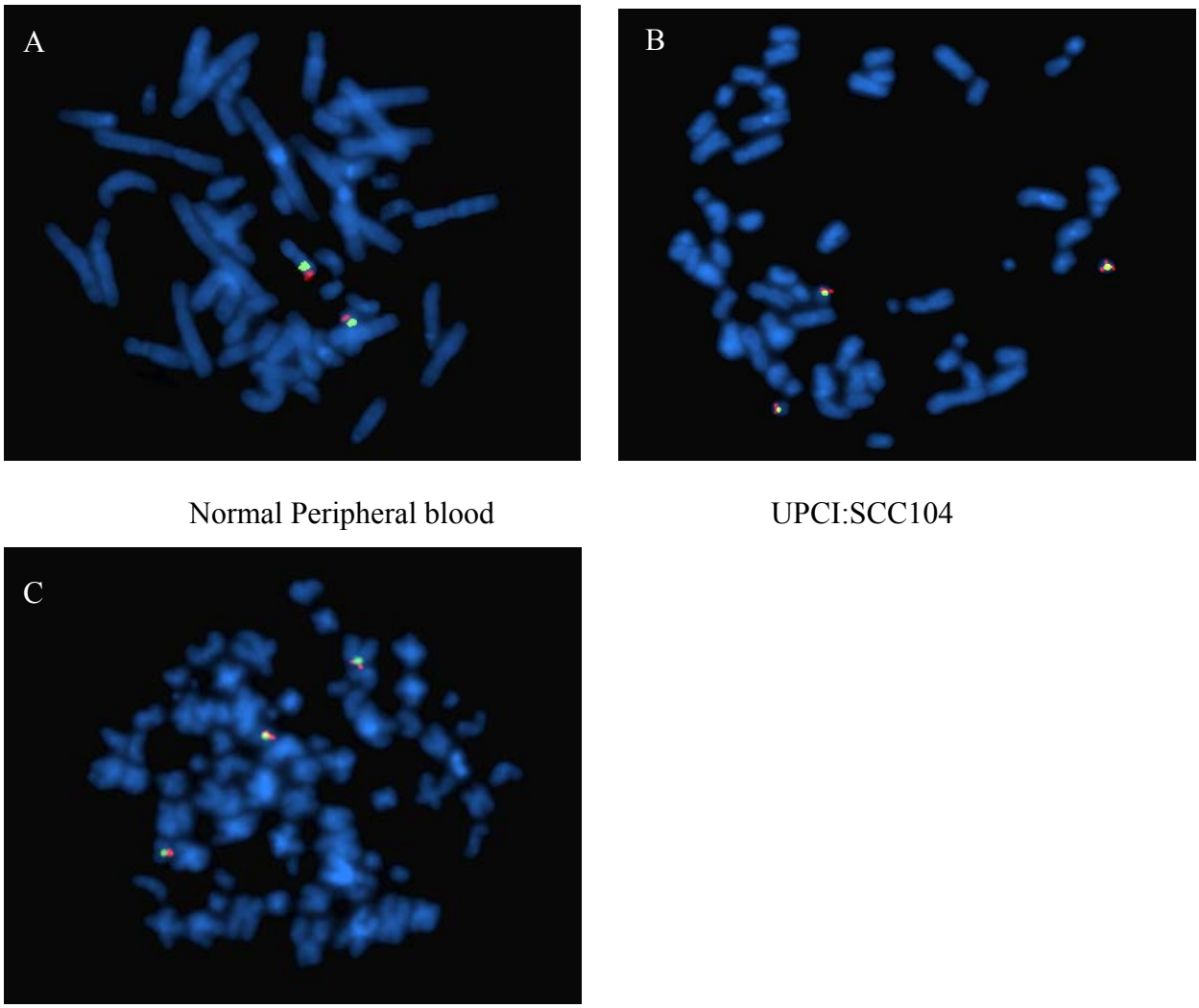


Figure 13. FISH results for the *TP53* gene (red) compared with CEP17 (green)

FISH using BAC probes mapping to the *TP53* locus and CEP 17 demonstrates that the *TP53* gene is not deleted in two cell lines UPCI:SCC104 **(B)** and UPCI:SCC131 **(C)**. Both SCC104 and 131 have wild-type *TP53* between exons 5–8, but complete loss of p53 protein expression.

TP53 gene sequencing from exons 5-8 was performed by Dr. Cheryl Telmer in a number of OSCC cell lines and the results are summarized in Table 8 (TELMER *et al*. 2003). We observed that a number of cell lines which were wild-type for the *TP53* gene between exons 5-8 showed a complete loss of p53 protein expression. This loss of expression could be due to loss of *TP53* transcription, deletion of the *TP53* gene or due to increased degradation of p53 protein by the proteasome.

Table 8. *TP53* mutations in OSCC.

'Wt' refers to wild-type *TP53* and 'Mut' to mutant *TP53*. Data for the Table obtained from (TELMER *et al*. 2003)

Cell line UPCI:	Dideoxy sequencing (Exons 5–8)	PMSG (Exons 5–9)	Mutation
SCC003	Wt	Wt	Wt
SCC036	Mut	Mut	I195F
SCC040	Wt	Wt	Wt
SCC056	Wt	Wt	Wt
SCC070	Wt	Mut	R248Q
SCC077	Wt	Mut	14711ins1
SCC084	Mut	Mut	13415del1
SCC099	Wt	Mut	P177R
SCC103	Mut	Mut	R306X
SCC104	Wt	Wt	Wt
SCC105	Mut	Mut	T155P
SCC131	Wt	Wt	Wt
SCC136	Mut	Mut	E224X
SCC142	Wt	Wt	Wt
SCC172	Mut	Mut	T155P
SCC182	Mut	Mut	E294X

MDM2 targets the p53 protein for proteasomal degradation and MDM2 amplification is common in a number of tumors (MOMAND *et al*. 1998). To check if enhanced proteasome mediated-p53 degradation is the cause for loss of p53 expression, we inhibited the proteasome using lactacystin and our results are shown in Figure 14. We found no increase in p53 levels,

which leads us to believe that the loss of expression is not caused by MDM2–mediated proteasomal degradation of p53.

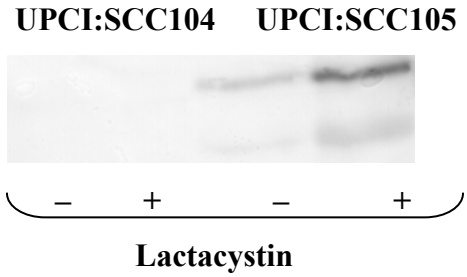


Figure 14. Changes in p53 expression following treatment with a proteosomal inhibitor. To check if MDM2 mediated proteasomal p53 degradation leads to loss of p53 expression in UPCI:SCC104, we inhibited the proteasome using Lactacystin.

Sequencing of the whole *TP53* gene in UPCI:SCC104, 131 and 142 was carried out by Dr. Cheryl Telmer who found early truncation mutations in these cell lines accounting for a loss of p53 protein expression. Thus, we hypothesized that loss of p53 expression causes a loss of the G_1 cell cycle checkpoint and leads to a compensatory increase in ATR and CHEK1 expression which regulate the S and G_2M phase cell cycle checkpoints.

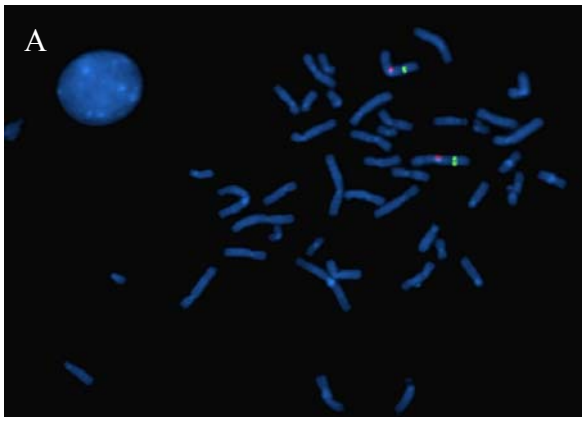
3.4 ATR–CHEK1 UPREGULATION IN OSCC

3.4.1 Copy number and structural changes involving the *ATR* and *CHEK1* genes

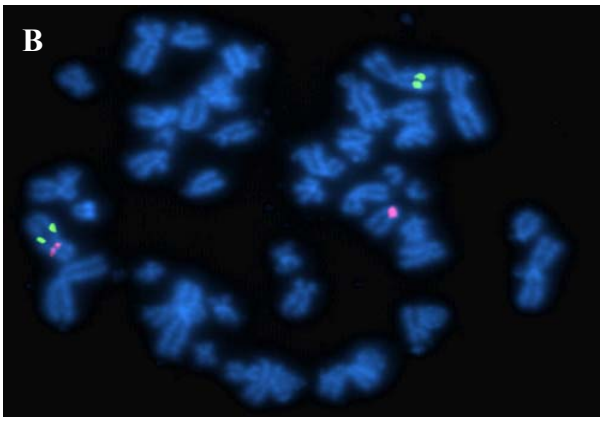
To determine if there are structural or numerical changes in the *ATR* and *CHEK1* genes in OSCC, we carried out dual-color FISH with BAC probes to *ATR* along with a centromere 3 enumeration probe (CEP 3) and BAC probes to *CHEK1* and compared it to centromere 11 enumeration probe (CEP 11; D11Z1) (Vysis, Downers Grove, IL). Table 9 summarizes our FISH results in terms of copy number gain in relation to the ploidy of each cell line, which was derived from chromosome 3 centromere enumeration and consensus karyotypes. We observed copy number gains in the *ATR* gene in all cell lines with distal 11q loss. In addition, we observed in a number of cell lines depicted in Figure 15 and Table 9 that copy number gains were associated with isochromosome 3q formation. In UPCI:SCC084, we did not observe copy number gain in the *ATR* gene. However, by metaphase FISH, we observed a translocation of one copy of the *ATR* gene. On further evaluation of UPCI:SCC084, the *ATR* gene was translocated to a derivative chromosome 11 with 11q13 amplification. In addition to OSCC, we observed low level amplification (8–10 copies per cell) in an ovarian tumor cell line, Ovcar–3. We observed that *CHEK1* was partially lost in all OSCC cell lines with 11q13 amplification. Loss of *CHEK1* was also seen in UPCI:SCC122 and 142 which did not demonstrate 11q13 amplification. Of the twenty OSCC cell lines studied, we observed *ATR* gain in ten OSCC and *CHEK1* loss in fifteen OSCC suggesting that both these events occur frequently in OSCC.

Table 9. Results of FISH analysis for the *ATR* and *CHEK1* genes.

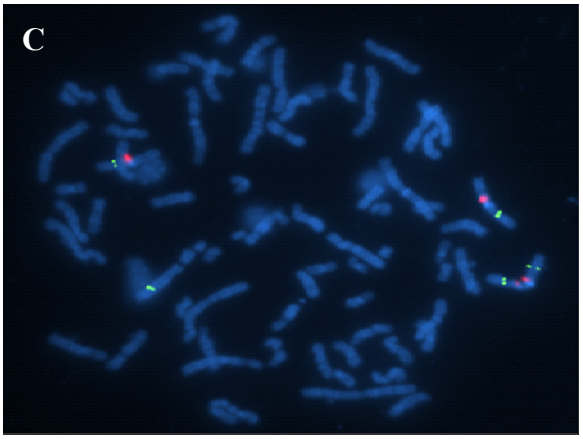
Cell line	11q13 Amp	*ATM* loss	*ATR*			*ATR*:CEP3 ratio	Iso 3q	*CHEK1*			*CHEK1*:CEP11 ratio
			% cells with gain	% cells with loss	% cells with no change			% cells with gain	% cells with loss	% cells with no change	
Control	–	–	–	–	100%	1.0	–	–	–	100%	1.0
SCC003	+	+	53%	1%	46%	1.6	–	1%	93%	6%	0.71
SCC029B	+	+	89%	2%	9%	1.4	–	–	99%	1%	0.61
SCC032	+	+	57%	–	43%	1.3	–	1%	99%	–	0.67
SCC040	+	+	53%	–	47%	1.6	–	5%	94%	1%	0.56
SCC066	–	–	2%	3%	95%	1.0	–	1%	1%	98%	1.01
SCC070	+	+	89%	1%	10%	2.1	+	–	98%	2%	0.57
SCC077	+	+	85%	1%	14%	2.2	+	2%	31%	67%	0.67
SCC078	+	+	9%	3%	88%	1.0	–	–	94%	6%	1.05
SCC084	+	+	7%	–	93%	1.0	–	2%	98%	–	0.52
SCC099	–	–	3%	6%	91%	1.0	–	–	100%	–	1.0
SCC103	+	+	91%	1%	8%	1.1	–	2%	94%	4%	0.74
SCC104	+	+	98%	–	2%	2.2	+	1%	11%	88%	0.97
SCC105	–	+	2.5%	0.5%	97%	1.1	–	12%	32%	56%	0.85
SCC116	–	–	3%	2%	95%	1.0	–	3%	10%	87%	0.94
SCC122	–	+	1%	9%	90%	1.0	–	1%	82%	17%	0.71
SCC131	+	+	92.5%	–	7.5%	2.1	+	–	98%	2%	0.44
SCC136	+	+	78.5%	–	21.5%	1.3	–	–	96%	4%	0.53
SCC142	–	+	98.5%	–	1.5%	2.1	+	–	91%	9%	0.67
SCC172	+	+	3%	5%	92%	1.1	–	5%	90%	5%	0.71
SCC182	–	–	1%	1%	98%	1.0	–	3%	–	97%	1.01
MDA231			6%	4%	90%	1.0	–	5%	1%	94%	1.1
MCF7			–	1%	99%	1.0	–	–	3%	97%	1.0
SK–OV3			4%	3%	93%	1.1	–	1%	–	99%	1.0
OVCAR3			99%	–	1%	9	–	8%	–	92%	1.1



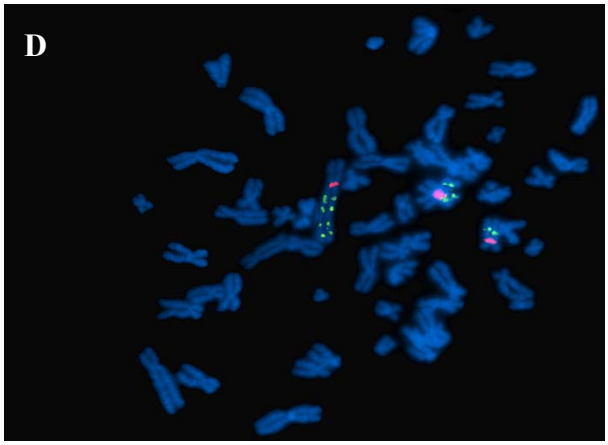
Peripheral blood



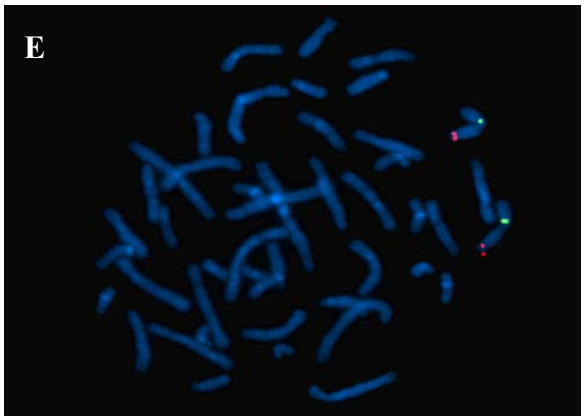
UPCI:SCC084



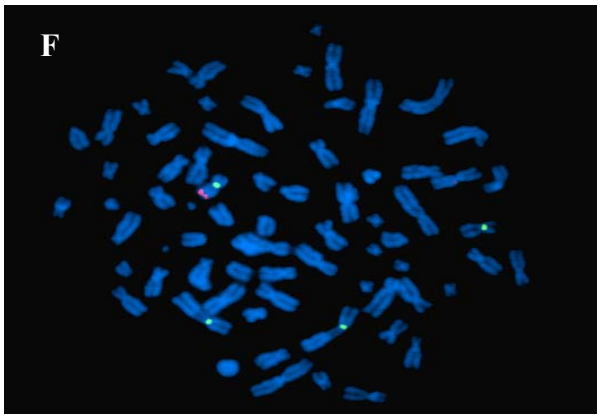
UPCI:SCC104



OVCAR–3



Peripheral blood



UPCI:SCC131

Figure 15. Structural and numerical changes in the *ATR* and *CHEK1* genes in tumor cell lines.

(A) Demonstrates two copies of *ATR* gene (green) and two CEP 3 (red) signals in a normal metaphase. **(B)** We observed a translocation of the *ATR* gene (green) in UPCI:SCC084. In UPCI:SCC104 **(C)** The *ATR* gene (green) is gained with isochromosome 3q formation. In an ovarian tumor cell line, OVCAR-3 **(D)**, we observe tandem copy number gain of the *ATR* gene (green) compared to CEP 3 (red). **(E)** Represents normal metaphase with two *CHEK1* (red) and two CEP 11(green) signals **(F)** UPCI:SCC131 with loss of the *CHEK1* gene (red) compared to CEP 11 (green).

We evaluated five primary squamous cell carcinomas of the oral cavity for *ATR* and *CHEK1* copy number alterations (Table 10, Figure 16). We observed a gain in copy number of the *ATR* gene in the tumor tissue in all five tumors, while the surrounding normal tissue exhibited normal copy number for the *ATR* gene and CEP 3. Similarly, *CHEK1* was partially lost in 35% to 65% of cells in the tumor tissue, while the adjacent normal tissue showed no loss of the gene. We observed high level *CCND1* amplification in 65% to 100% of the tumor cells, suggesting that 11q13 amplification may be an early change in tumor development. Thus, *ATR* gain and *CHEK1* loss are present not only in OSCC cell lines, but also in primary head and neck tumors.

Table 10. *ATR*, *CHEK1* and *CCND1* FISH in primary head and neck tumors.

'T' represents the head and neck primary tumor and 'N' represents surrounding normal tissue. Figures in each column represent % cells with gain, % cells with loss or % cells with no change.

Sample ID:		***ATR***			***CHEK1***			***CCND1***		
		Amp/ Gain	Normal	Loss	Amp/ Gain	Normal	Loss	Amp/ Gain	Normal	Loss
0402584	N	2%	98%	–	–	99%	1%	2%	98%	–
	T	54%	46%	–	–	35%	65%	100%	–	–
3L621340	N	1%	99%	–	–	98%	2%	1%	99%	–
	T	58%	42%	–	–	65%	35%	99%	1%	–
1H620781	N	2%	98%	–	–	98%	2%	3%	94%	3%
	T	32%	66%	2%	–	51%	49%	65%	34%	1%
3G040281	N	1%	98%	1%	1%	98%	1%	–	100%	–
	T	42%	58%	–	–	44%	56%	100%	–	–
1F620600	N	–	98%	2%	–	99%	1%	3%	95%	2%
	T	42%	57%	1%	–	37%	63%	68%	32%	–

Case # 3L621340: normal tissue

Case # 3L621340: tumor tissue

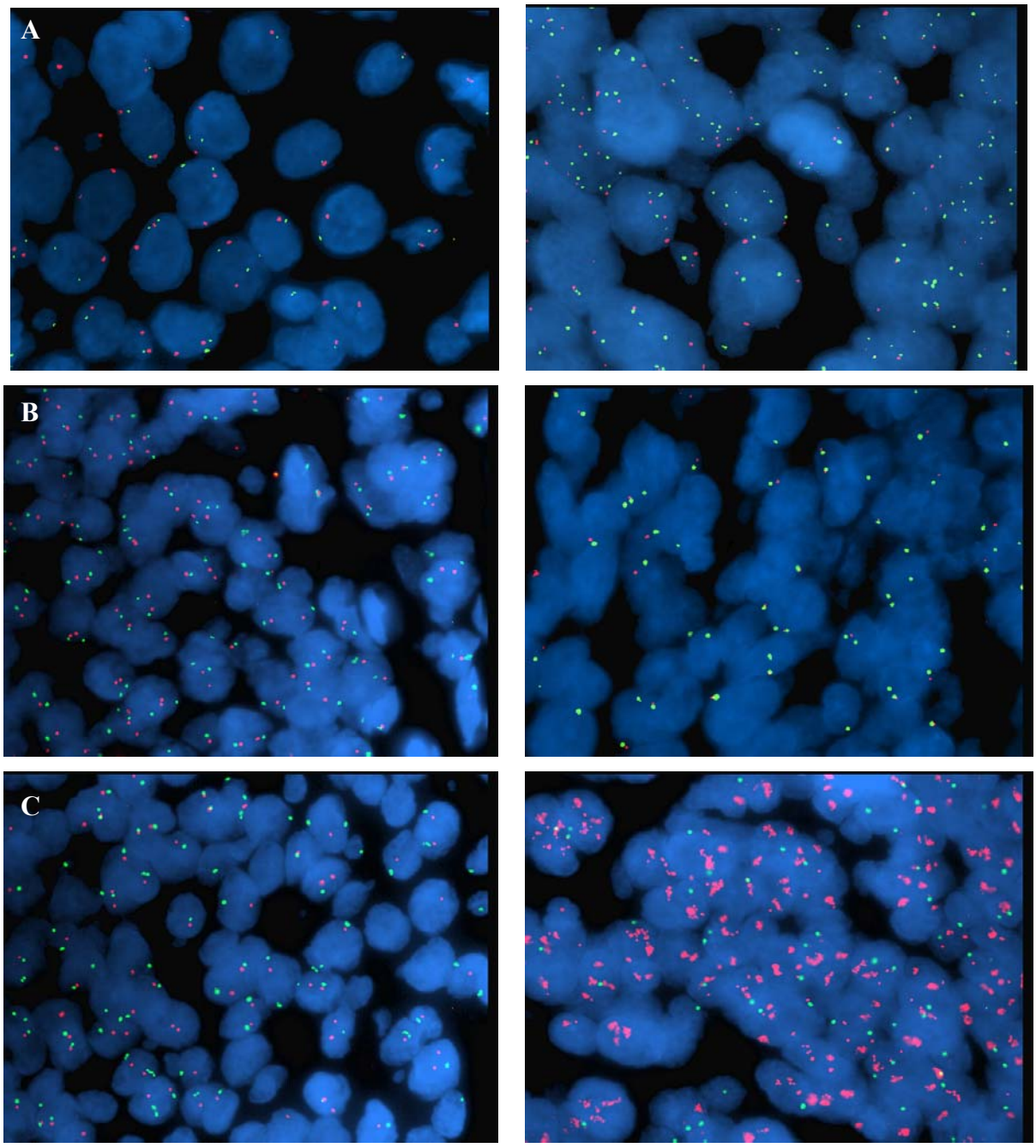


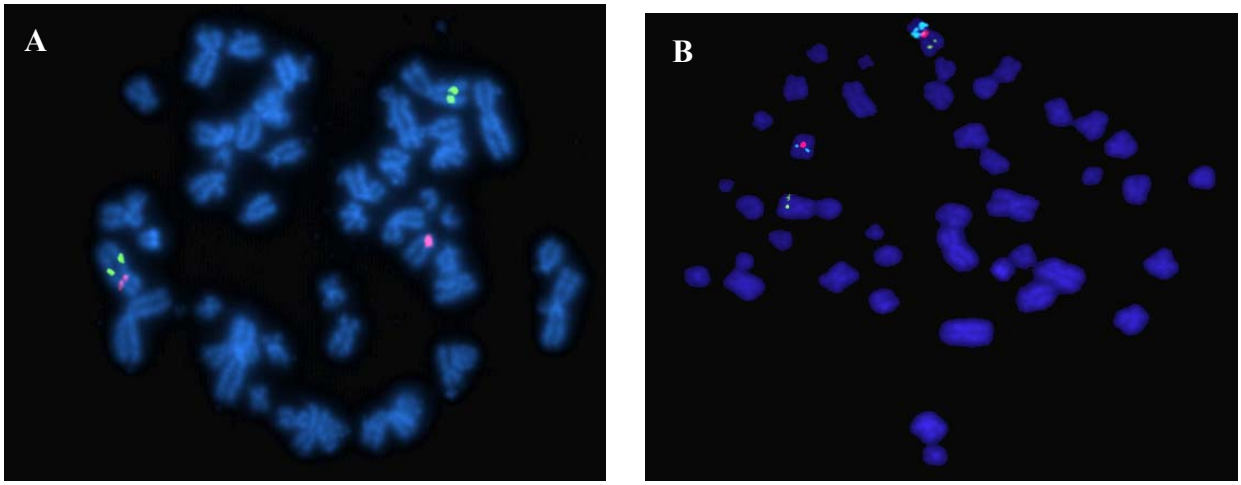
Figure 16. Results for *ATR*, *CHEK1* and *CCND1* FISH in primary head and neck tumors and adjacent normal tissue.

(A) demonstrates gain of *ATR* gene (green) copy number compared with CEP 3 (red), (**B**) shows loss of the *CHEK1* gene (red) compared to CEP 11 (green) and (**C**) demonstrates amplification of *CCND1* gene (red) compared to CEP 11 (green) in tumor tissue, but not in the adjacent normal tissue.

3.4.2 Mechanism of *ATR* gain and *ATR* translocations

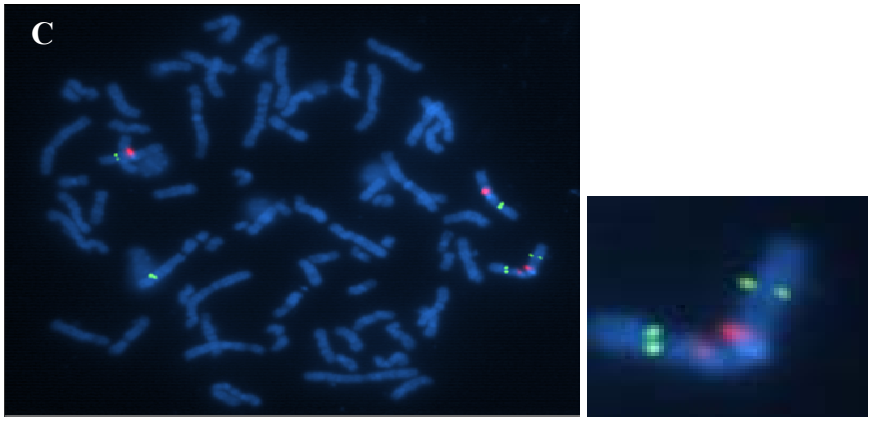
It has been previously reported that in OSCC, translocations of chromosome 3 are commonly associated with 11q13 amplification and frequently, chromosome 3 fragments cap the amplified chromosome 11. We observed that three of 20 OSCC cell lines studied (UPCI:SCC078, 084 and 172) had a translocation between the derivative chromosome 11 der(11) with 11q13 amplification and segments of 3q along with the *ATR* gene (Figure 17A, B). Similar translocations between chromosome 3 and der(11) with 11q13 amplification in OSCC were also reported by another group, suggesting that this event may be common in head and neck tumors (JIN *et al*. 2002). In all three OSCC cell lines, UPCI:SCC078, 084 and 172, this t(3;11) translocation was present in all twenty metaphases studied.

We observed isochromosome 3q formation in five of 20 OSCC cell lines (UPCI: SCC070, 077, 104, 131 and 142) studied. Isochromosome 3q formation is a common mechanism of 3q and *ATR* gain in OSCC. Isochromosome formation can occur either through centromere splitting or by fusion of two chromosomes 3 with breaks at 3p resulting in an isodicentric chromosome 3. We observed isodicentric chromosomes 3q in UPCI:SCC070, 077, 104, 131 and 142, suggesting that centromere splitting is not the mechanism by which isochromosome formation occurs in OSCC (Figure 17C).



UPCI:SCC084
(*ATR*: green, CEP3: red)

UPCI:SCC084
(*ATR*: green, CEP11: red, *CCND1*: aqua)



UPCI:SCC104 (*ATR*: green, CEP3: red)

Figure 17. *ATR* translocation in UPCI:SCC084 and isochromosome 3 formation in UPCI:SCC104

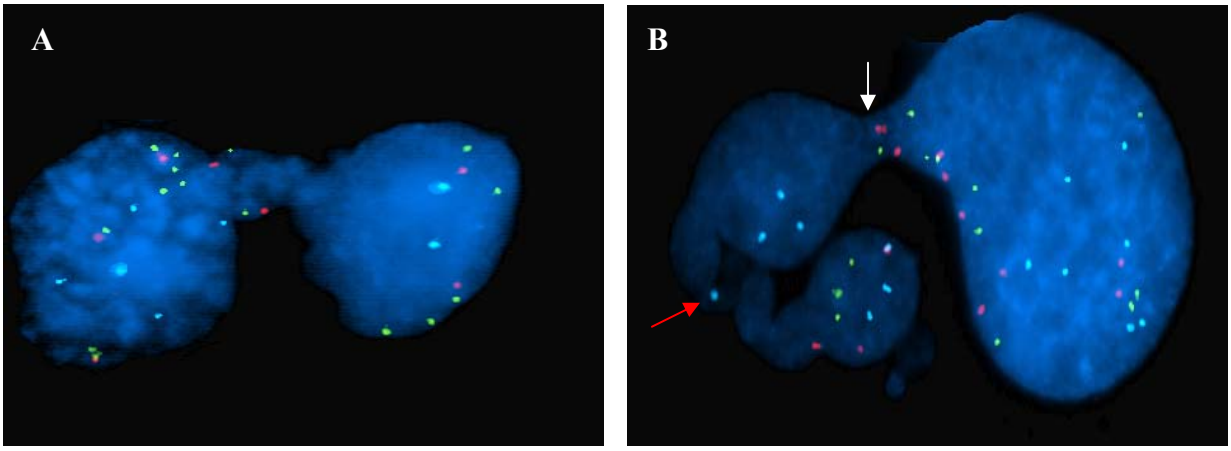
Panel A shows translocation of one copy of *ATR* gene (green) from chromosome 3 (red), further analysis of UPCI:SCC084 (Panel B) reveals that *ATR* (green) is translocated to the derivative chromosome 11 (red) with amplified *CCND1* (aqua). Panel C demonstrates UPCI:SCC104 with an isodicentric chromosome 3 and *ATR* gain (green).

Dicentric chromosomes are prone to being pulled to opposite poles during cell division, resulting in an anaphase bridge between the two daughter cells. The formation of a dicentric chromosome 11 is also an intermediate in the process of 11q13 amplification (Figure 1) and its

presence in anaphase bridges may indicate a stage of ongoing amplification and chromosomal instability (RESHMI *et al*. unpublished data). We determined the frequency of *ATR* gene and CEP 3 presence in anaphase bridges and compared it to the presence of CEP 11 in anaphase bridges in a subset of four OSCC cell lines and GM09607 (AT cell line). Our results for 50 anaphase bridges analyzed for each cell line are summarized in Table 11. We observed an increased frequency of *ATR* gene in anaphase bridges in UPCI:SCC104 and 131, both of which have gain of chromosome 3, but not in UPCI:SCC066 and 105 which have normal copy numbers for the *ATR* gene. Surprisingly, we observed an increase in the frequency of *ATR* gene present in anaphase bridges in GM09607, which does not have 3q and *ATR* gain. Since GM09607 relies heavily on the upregulated ATR–CHEK1 pathway for survival, anaphase bridges may serve as a mechanism for formation of isodicenteric 3q leading to *ATR* and 3q gain. Thus, the high frequency of *ATR* and CEP3 in anaphase bridges may represent ongoing selection for 3q gain. On the other hand, chromosome 3, on account of its large size may be prone to be present in anaphase bridges at an increased frequency.

Table 11. Frequency of *ATR*, CEP 3 and CEP11 in anaphase bridges in OSCC and GM09607.

Cell Line	11q13 amplification	*ATR* gain	Frequency of *ATR* in bridges	Frequency of CEP3 in bridges	Frequency of CEP11 in bridges
SCC066	–	–	4%	4%	6%
SCC104	–	+	26%	26%	14%
SCC105	–	–	6%	6%	22%
SCC131	+	+	28%	28%	24%
GM09607	–	–	16%	16%	10%



UPCI:SCC104 **GM09607 (AT cell line)**

Figure 18. Presence of *ATR* (green), CEP11 (aqua) and CEP3 (red) in anaphase bridge. UPCI:SCC104 (Panel A) demonstrates two *ATR* copies and two CEP 3 copies in the anaphase bridge. In GM09607 (Panel B) two *ATR* copies and two CEP 3 copies are present in the anaphase bridge (white arrow) while one CEP 11 copy is present in another anaphase bridge (red color).

3.4.3 ATR and CHEK1 are overexpressed in a subset of OSCC

To evaluate if changes in copy number of the *ATR* and *CHEK1* genes in OSCC lead to changes in expression, we studied ATR and CHEK1 RNA expression using qRT–PCR and protein expression by immunoblotting. Our qRT–PCR results are summarized in Figure 19. Since the RNA expression was measured relative to a universal reference with diploid chromosomal constitution, a relative expression < 1.5 in OSCC (with near–triploid to tetraploid karyotypes) can be considered as a reduction in relative RNA expression.

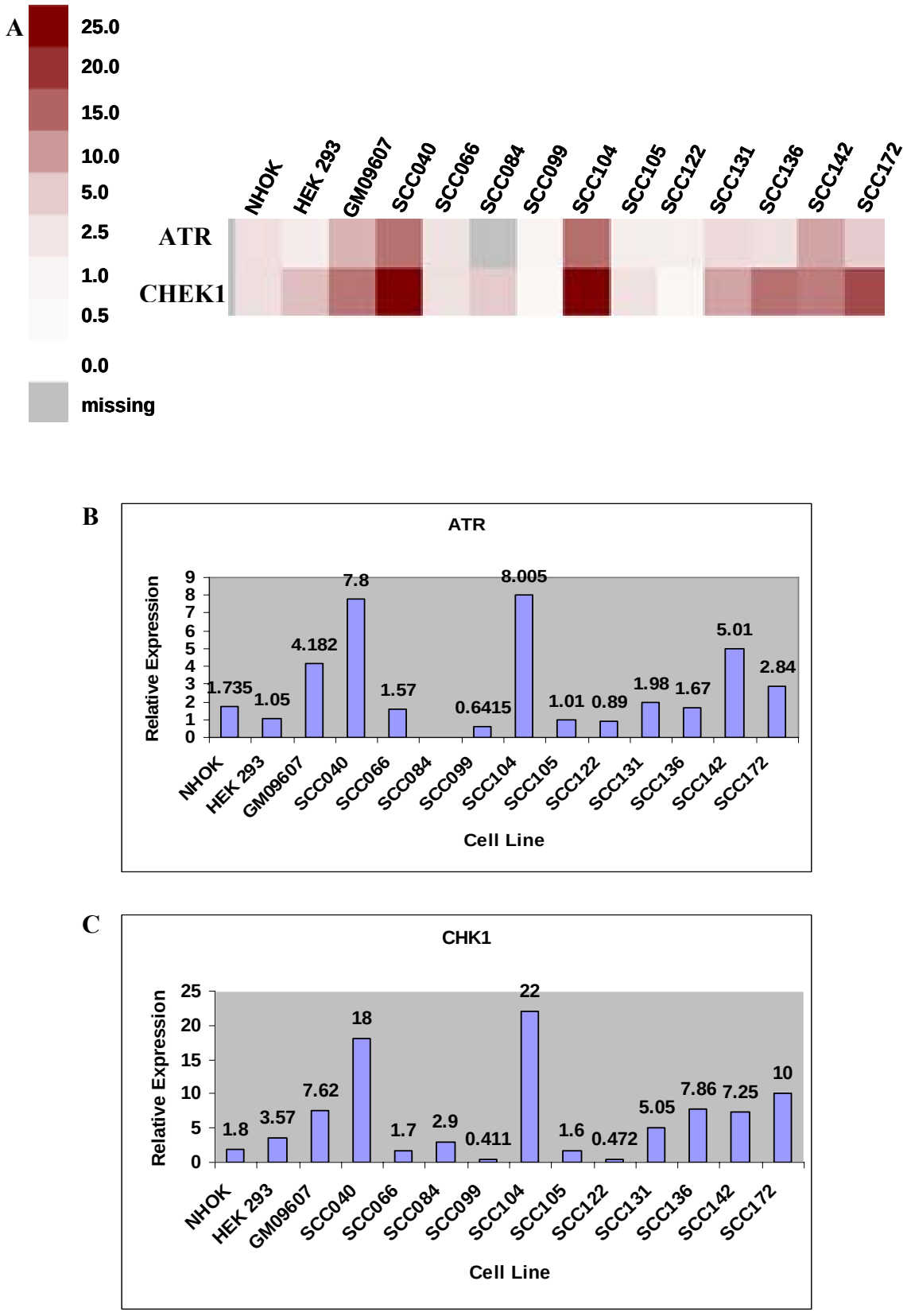


Figure 19. qRT–PCR analysis for ATR and CHEK1 RNA expression in a subset of OSCC.

(A) demonstrates a heat map of ATR (top row) and CHEK1 (bottom row) expression; (B) Bar graph depicting relative ATR expression; (C) Bar graph depicting relative CHEK1 expression.

We examined RNA expression by qRT–PCR in eleven OSCC cell lines and GM09607 (AT cell line). We used NHOK and HEK 293 cells as our control cell lines. UPCI:SCC066, 099, 105 and 122 showed ATR and CHEK1 expression equal to or lower than the NHOK and HEK293 control cell lines. GM09607 which has been shown to have an upregulated ATR–CHEK1 pathway, demonstrated a 4–fold increase in ATR expression and an 8–fold increase in CHEK1 expression. Six out of eleven cell lines, including UPCI: SCC040, 104, 131, 136, 142 and 172 demonstrated an increase in both ATR and CHEK1 expression. We observed around an 8–fold increase in ATR expression and an 18-20 fold increase in CHEK1 expression in two OSCC cell lines, UPCI:SCC040 and 104. It should be noted that all cell lines with *ATM* loss (UPCI:SCC 104, 131, 136 and 142) other than UPCI:SCC122 demonstrated a significant increase in ATR and CHEK1 RNA expression.

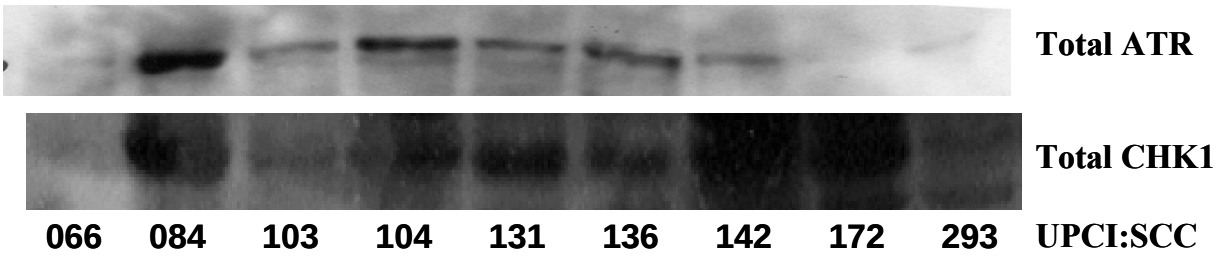


Figure 20. ATR and CHEK1 protein expression in OSCC detected by immunoblotting. Immunoblotting for ATR and CHEK1 demonstrates high ATR and CHEK1 expression in UPCI:SCC084, 104, 131, 136, 142 and 172.

Immunoblotting for ATR and CHEK1 proteins shown in Figure 20 confirmed our qRT-PCR results. We observed an increase in the expression of ATR in all the cell lines with copy number gain of the *ATR* gene. Although UPCI:SCC084, the cell line with the highest basal expression of ATR, did not have any gain of the ATR gene, it had a translocation of one of its copies of the *ATR* gene, which may account for the increased expression.

Earlier reports suggested that kinase-dead ATR can inhibit serine15–p53 phosphorylation in response to DNA damage and thus, block p53 activation in response to IR and ultraviolet (UV) radiation (TIBBETTS *et al*. 1999). To check whether the overexpressed ATR in UPCI:SCC084 and 104 was kinase-dead, we assayed CHEK1 phosphorylation on serine–345 following treatment with IR and UV radiation. Since CHEK1 s345 phosphorylation following UV radiation is preferentially mediated through ATR kinase, we conclude that ATR kinase activity is not lost in any of the four cell lines studied (Figure 21). On studying ATR-mediated phosphorylation of SMC1 on serine-957 in response to 20 J/m^2 UV radiation, we observed that UPCI:SCC084 and 104 had much higher levels of SMC1 phosphorylation. Since SMC1 phosphorylation occurs during the S and G_2M phases, our results confirm our earlier findings that the G_1 checkpoint is compromised in UPCI:SCC084 and 104. We observed increased phosphorylation of Cdc25C on serine–216 in response to 5 Gy IR in UPCI:SCC104 compared to UPCI:SCC066. Our observations suggest that an overactive ATR–CHEK1 response is present in UPCI:SCC084 and 104 and that a higher number of cells with DNA damage enter the S and G_2M phases in response to DNA damage.

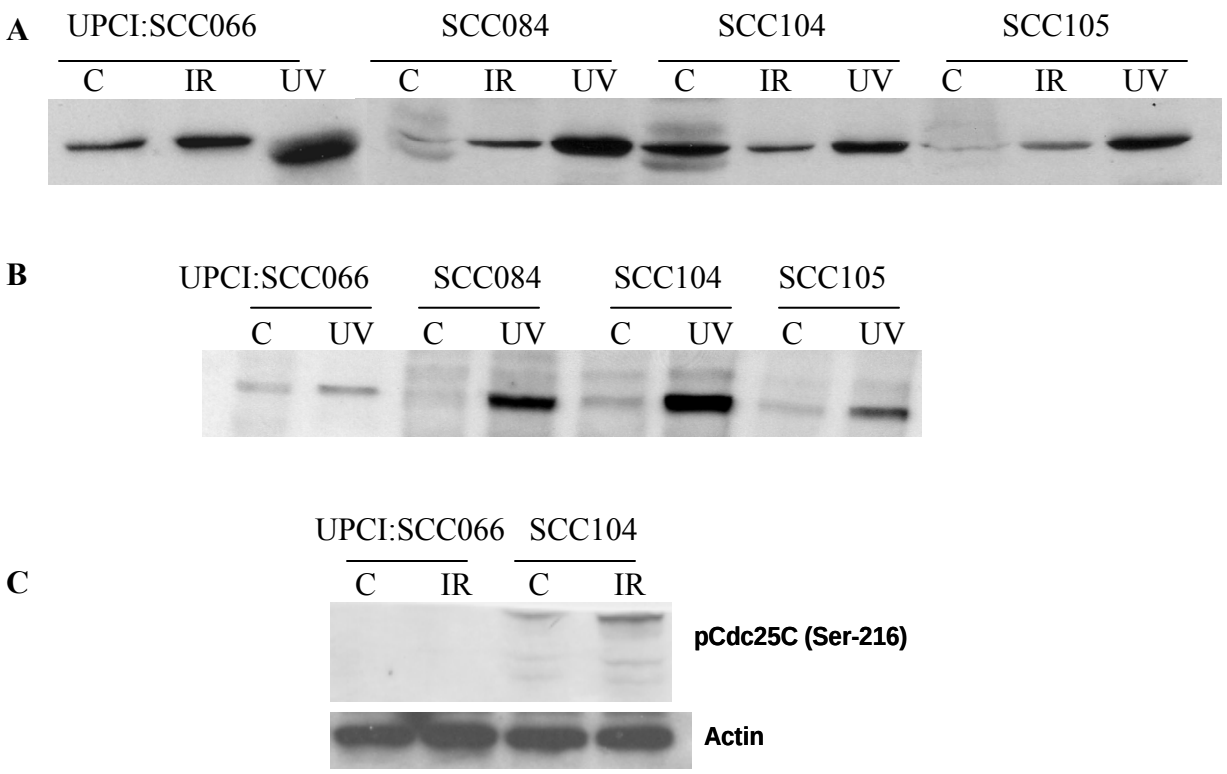


Figure 21. Activation of downstream effectors of ATR in response to IR and UV.

(A) Phosphorylation of CHEK1 on serine–345 was studied in response to 5 Gy IR and 20 J/m2 dose of UV radiation in UPCI:SCC066, 084, 104 and 105. **(B)** Phosphorylation of SMC1 on serine–957 was studied in response to 20 J/m2 dose of UV radiation in UPCI:SCC066, 084, 104 and 105. **(C)** Phosphorylation of Cdc25C serine–216 was studied in response to 5 Gy IR UPCI:SCC066 and 104.

3.4.4 An upregulated ATR–CHEK1 pathway is associated with radioresistance

We observed that all cell lines with an upregulated ATR–CHEK1 pathway demonstrate increased resistance to ionizing radiation. Since loss of p53 was also observed in these cell lines, the resistance to ionizing radiation could be as a result of both loss of p53–mediated apoptotic pathways and an upregulated ATR–CHEK1 pathway which promotes G_2M accumulation and HRR after DNA damage.

Table 12. Clonogenic cell survival of OSCC to different doses of IR

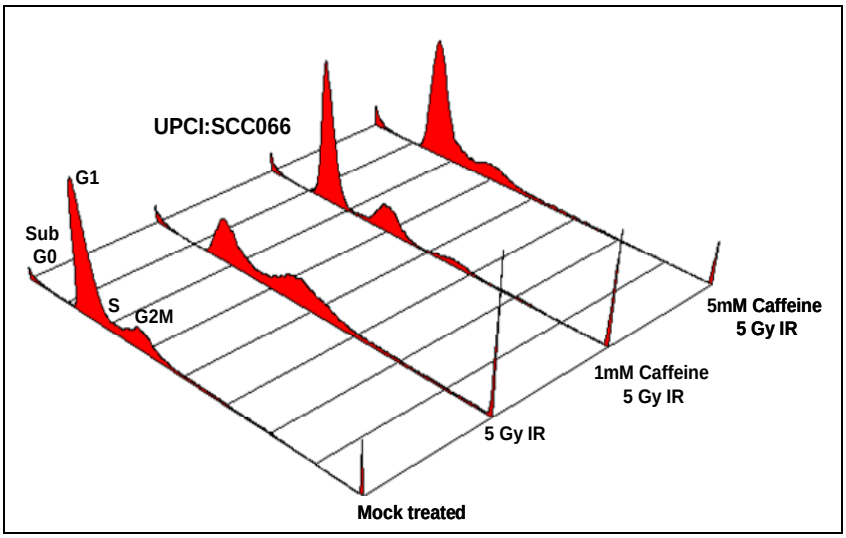
Cell line	ATR, CHEK1 overexpression	Mock treated	1Gy	2.5Gy	5Gy	10Gy
NHOK	–	100±0.0	59.7±2.1	30.3±0.6	6.7±0.6	0.0±0.0
SCC084	+	100±0.0	80.7±4.6	52.3±5.0	24.7±2.1	7.7±0.6
SCC104	+	100±0.0	82.3±4.7	55.3±2.1	33.7±1.2	7.7±0.6
SCC142	+	100±0.0	81.0±4.0	51.7±4.6	35.3±1.5	9.7±0.6
SCC131	+	100±0.0	86.7±2.5	51.7±3.8	37.3±2.1	11.3±2.5
SCC136	+	100±0.0	81.7±3.5	55.0±4.6	34.7±2.5	11.0±1.0
SCC066	–	100±0.0	56.7±1.1	29.5±2.2	5.5±1.0	0.0±0.0
SCC105	–	100±0.0	60.1±2.1	32.3±1.5	7.9±2.6	0.0±0.0

3.5 INHIBITION OF THE ATR–CHEK1 PATHWAY SENSITIZES OSCC TO DNA DAMAGING AGENTS

3.5.1 Caffeine, a non–specific kinase inhibitor sensitizes OSCC to IR-induced DNA damage

In order to study the relative contribution of ATR and CHEK1 overexpression to the radioresistance observed in a subset of OSCC, we determined the sensitivity of OSCC to caffeine, which is a non–specific inhibitor of ATM and ATR. OSCC were treated with caffeine in combination with a DNA damaging agent (IR or aphidicolin). The following endpoints were performed to estimate the sensitivity of OSCC to caffeine: the sub–G_0 population in flow cytometric studies, the surviving fraction using a clonogenic cell survival assay, and PCC/MC formation in response to caffeine.

We observed a prolonged S and G_2M accumulation in a subset of irradiated OSCC cells, which suggests that the S and G_2M checkpoint are enhanced in IR-treated OSCC. We hypothesized that the G_2M accumulation in irradiated OSCC was mediated through the ATR-CHEK1 pathway, as seen in irradiated AT cells (WANG *et al*. 2004). We treated OSCC with caffeine, a non-specific inhibitor of the ATM and ATR kinases and evaluated their cell cycle profiles following irradiation. We observed that caffeine clearly reduced the accumulation of cells in the S and G_2M phases and increased the sub-G_0 population (dead cells) in cell lines with an upregulated ATR-CHEK1 pathway, including GM09607 (AT cells). In comparison, cell lines with an intact G_1 checkpoint and normal ATR-CHEK1 expression did not demonstrate any sensitivity to caffeine.



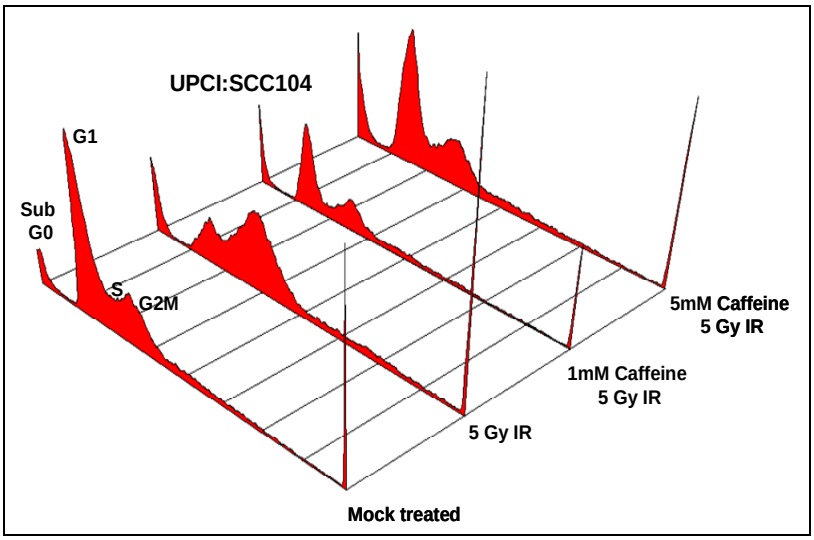


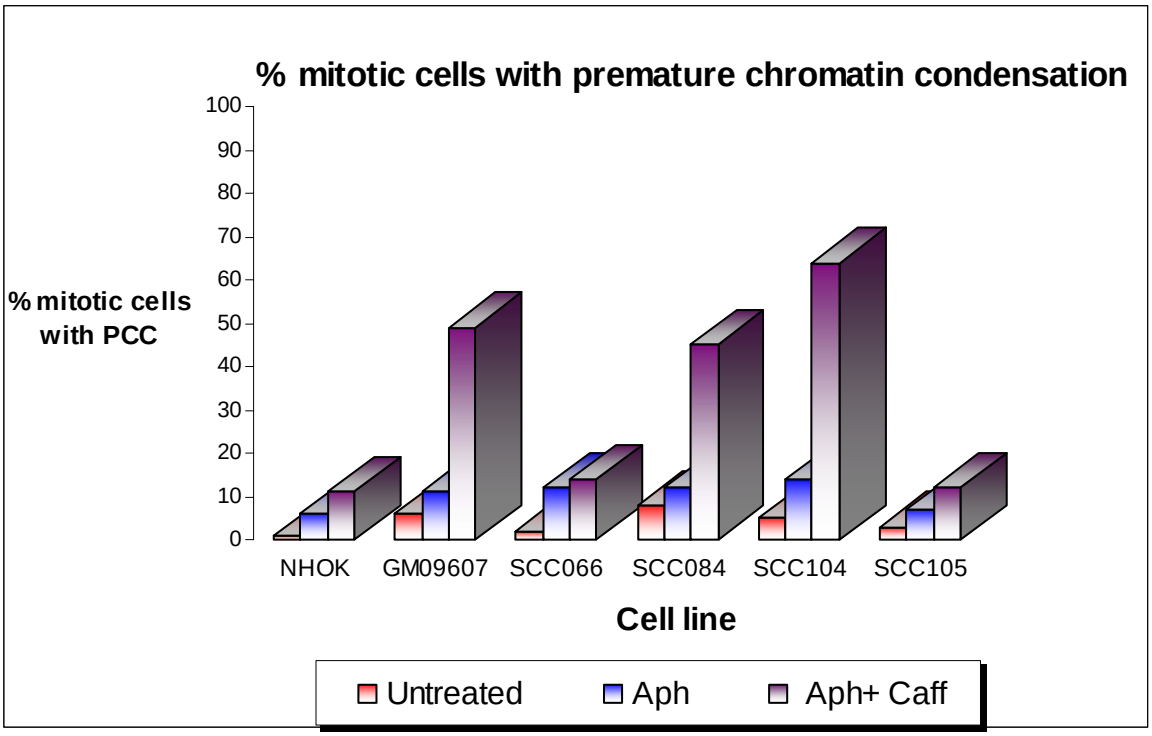
Figure 22. Cell cycle profiles of UPCI:SCC066 and 104 in response to IR and caffeine treatment

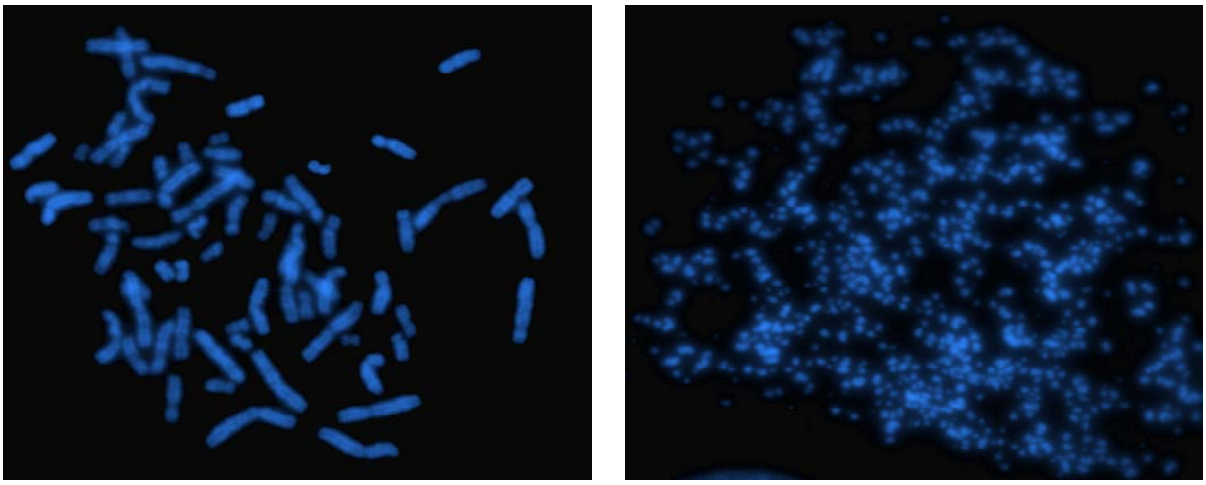
UPCI:SCC066 (Panel A) and UPCI:SCC104 (Panel B) were either treated with 5 Gy IR or pretreated with 1 mM caffeine 1 h prior to treatment with 5 Gy IR, or pretreated with 5 mM caffeine 1 h prior to 5 Gy IR and the cell cycle profiles were compared with untreated samples from the same cell line. Compared to UPCI:SCC066, SCC104 exhibited a loss of the G_1 checkpoint and predominant G_2M accumulation following treatment with IR. UPCI:SCC104 exhibited a caffeine dose-dependent reduction in G_2M accumulation and an increase in the sub

G_0 peak (dead cells), suggesting that caffeine sensitizes UPCI:SCC104 to IR mediated cell death.

To detect the mechanism of caffeine-induced cell death, we studied 'S' phase PCC and MC formation in response to only aphidicolin (0.4 μM) or pretreatment with 1 mM caffeine 1 h before aphidicolin treatment (Figure 23). Criteria for distinguishing PCC/MC from normal metaphase were adapted from a previous report (NGHEIM *et al*. 2001).We observed a very high percentage of mitotic cells with PCC/MC in UPCI:SCC084 and 104 if pretreated with 1 mM caffeine (Figure 23A). This high percentage was comparable with GM0607 (AT cells), which we used as our positive control. In comparison, UPCI:SCC066 and 105, with an intact G_1 checkpoint demonstrate low levels of PCC/MC formation, even after caffeine pretreatment. The morphology of PCC/MC is depicted in Figure 23B. Thus, caffeine-mediated elimination of the G_2M peak is caused due to increased death (by PCC/MC) of these cells by entering premature S phase or premature mitosis.

A





Untreated control **0.4μM Aph + 1mM Caffeine**

Figure 23. The frequency of PCC formation (cell death) in untreated cells, in response to aphidicolin with or without 1mM caffeine pretreatment.

(A) Demonstrates the frequency of mitotic cells undergoing PCC/MC in OSCC (UPCI:SCC cell lines), NHOK and GM09607. **(B)** Depicts PCC/MC formation in UPCI:SCC104.

We have observed that cell lines with an upregulated ATR-CHEK1 pathway had prolonged G_2M accumulation following irradiation and a reduced sensitivity to IR-induced cell death. However, the exact mechanism by which an enhanced G_2M checkpoint can promote radioresistance is not known. It is likely that prolonged G_2M accumulation may allow adequate time for repair and thus promote cell survival. Since caffeine eliminates the G_2M accumulation observed in OSCC, we studied the sensitivity of OSCC to different doses of caffeine by a clonogenic cell survival assay. We observed that caffeine enhances cell sensitivity to ionizing radiation, especially in OSCC with an upregulated ATR-CHEK1 pathway (Figures 24, 25). We observed that UPCI:SCC084 and 104 showed a significant (> 50%) reduction in colony survival at a dose of 0.5 mM caffeine and complete absence of survival at 1 mM caffeine. Thus, we conclude that OSCC with an upregulated ATR–CHEK1 pathway (UPCI:SCC084 and 104) exhibit increased sensitivity to caffeine in the absence of any DNA damaging agents.

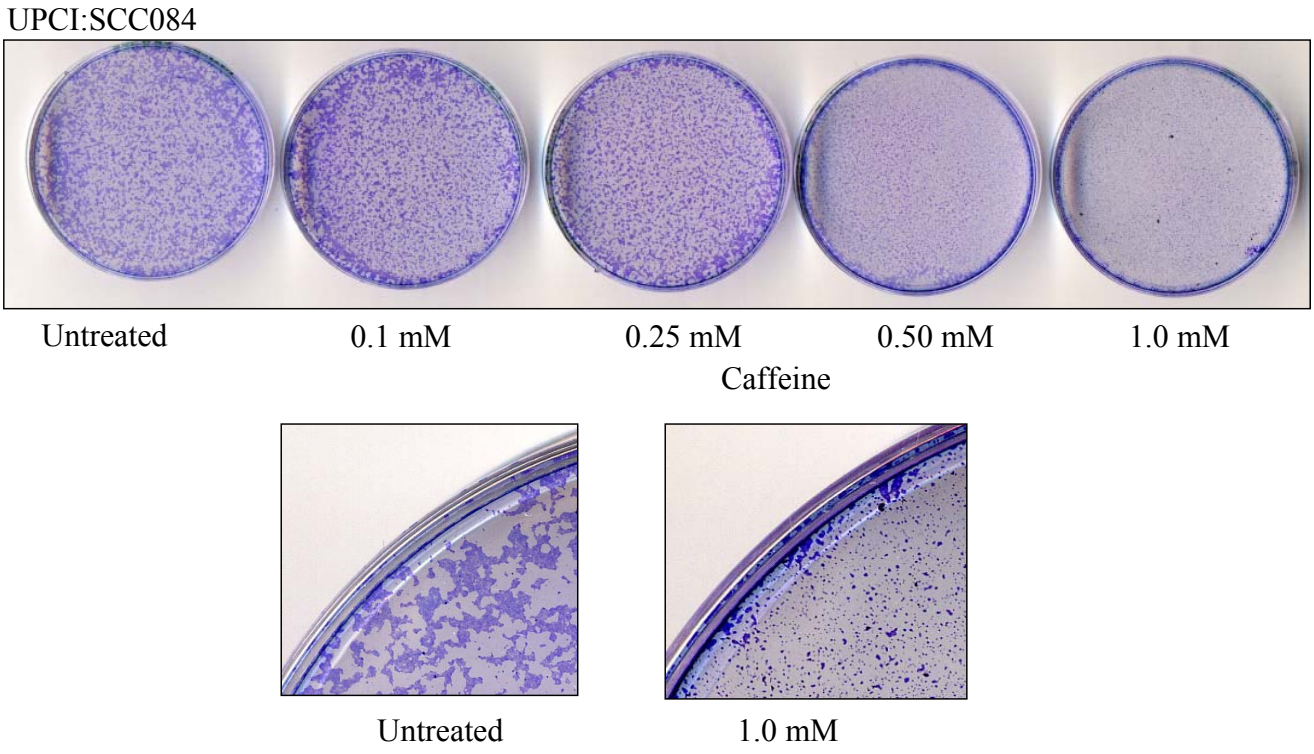


Figure 24. Clonogenic cell survival of UPCI:SCC084 to different doses of caffeine.

Complete inhibition of colony formation in UPCI:SCC084 at a dose of 1 mM caffeine.

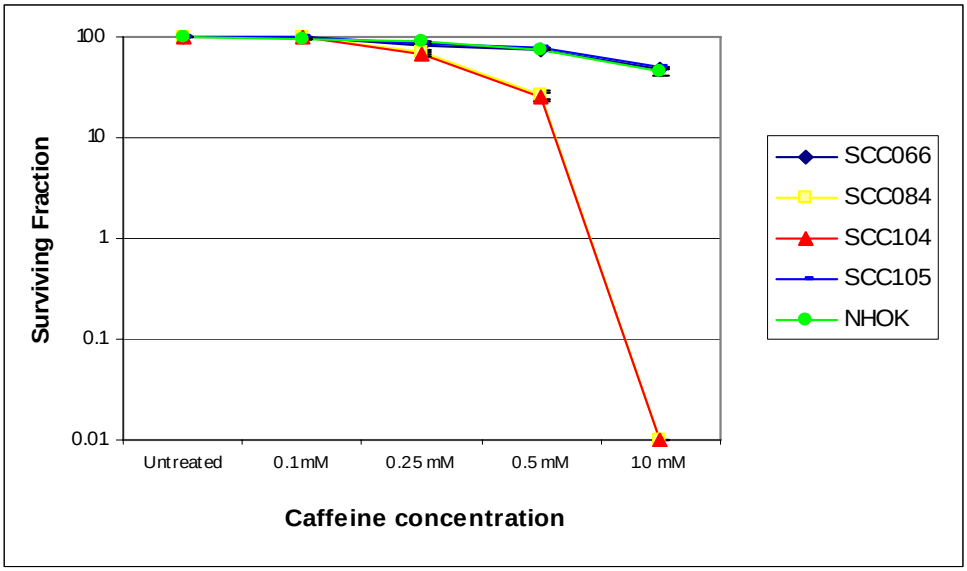


Figure 25. Clonogenic cell survival of OSCC to different doses of caffeine

UPCI:SCC084 and 104 exhibit increased sensitivity to caffeine compared to UPCI:SCC066, 105 and control NHOK.

3.5.2 ATR and CHEK1 siRNA sensitize a subset of OSCC to ionizing radiation and aphidicolin induced DNA damage

Small inhibitory RNAs (siRNA) are composed of 21–25 nucleotides which are complimentary to a known 'target' mRNA (ELBASHIR *et al*. 2001). Usually, a pool of two or more siRNA duplexes is used to specifically bind to and degrade the 'target' mRNA. Usually peak siRNA transfection is seen at the end of 48–72 hrs and the mRNA knockout lasts for 1–3 weeks, depending on the type and stability of the siRNAs. There is growing interest to use siRNA mediated gene targeting to inhibit specific genes involved in cancer and other diseases (WALL and SHI 2003). We used siRNA specific to ATR and CHEK1 to reduce their expression in UPCI:SCC066 and 104. Transfection efficiency was calculated using a nonspecific siRNA

labeled with a fluorescent tag called siGLO (Dharmacon). We confirmed a reduction in ATR and CHEK1 protein levels by immunoblotting at the end of 72 h. We observed a high level of ATR and CHEK1 knockout by their specific siRNAs, while a scrambled nonspecific siRNA did not inhibit ATR and CHEK1 protein expression (Figure 26).

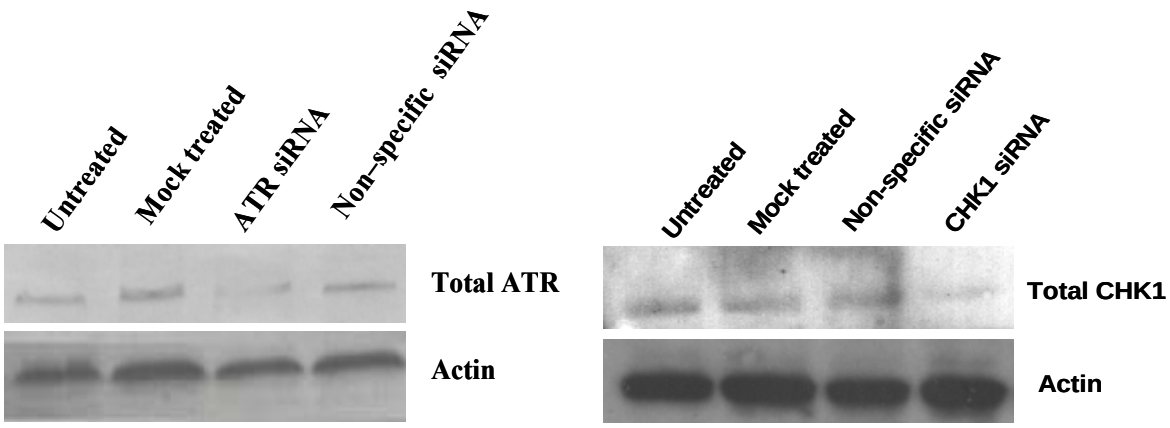
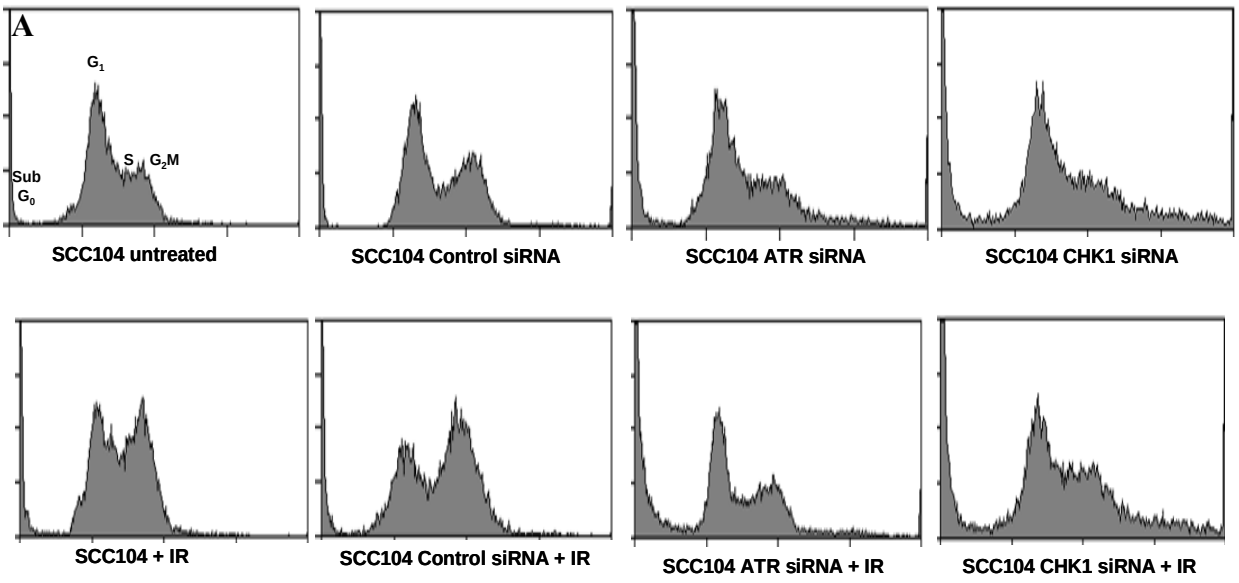


Figure 26. ATR and CHEK1 siRNA mediated protein knockout in UPCI:SCC104 .
We observed a nearly complete loss of ATR and CHEK1 protein expression at the end of 72 h following treatment with ATR and CHEK1 siRNAs, respectively.

We analyzed the cell cycle profiles of UPCI:SCC066 and 104 after ATR and CHEK1 knockout using the respective siRNAs (Figure 27). Even in the untreated sample, we observed a very high number of cells in the S (18%) and G_2M (29%) phases in UPCI:SCC104. Following treatment with 5 Gy IR, at the end of 24 h, we observed a very high percentage of cells (nearly 55%) accumulating in the G_2M phase in UPCI:SCC104 compared to 31% in UPCI:SCC066. Following treatment with ATR siRNA, in irradiated SCC104, we observed a significant reduction in the G_2M accumulation of cells from 55% to 18% and an increase in the sub–G_0 population (dead cells) from 1% to nearly 18%. Since we observed a corresponding increase in the sub–G_0 population, the reduction of the G_2M peak observed in SCC104 is due to cell death and not due to a reduction in the number of cells that enter G_2M. Even in unirradiated SCC104

cells, we observed a reduction in the G_2M accumulation and increased sub–G_0 population (15%). In comparison, unirradiated as well as irradiated SCC066 cells demonstrate a modest reduction in G_2M accumulation following treatment with ATR siRNA, but no corresponding increase in the dead (sub–G_0) cell population. We observed a similar sensitization of UPCI:SCC104, but not UPCI:SCC066 following treatment with CHEK1 siRNA. Following treatment with a non–specific scrambled siRNA, SCC104 does not exhibit any reduction in the G_2M accumulation or increase in the sub–G_0 population. This suggests that the effects observed were specific for ATR and CHEK1 inhibition.

Thus, UPCI:SCC104 was highly sensitized to IR following inhibition of the upregulated ATR–CHEK1 pathway with the respective siRNAs. Our results suggest that SCC104 with ATR and CHEK1 overexpression is highly susceptible to IR following treatment with ATR or CHEK1 siRNA.



B

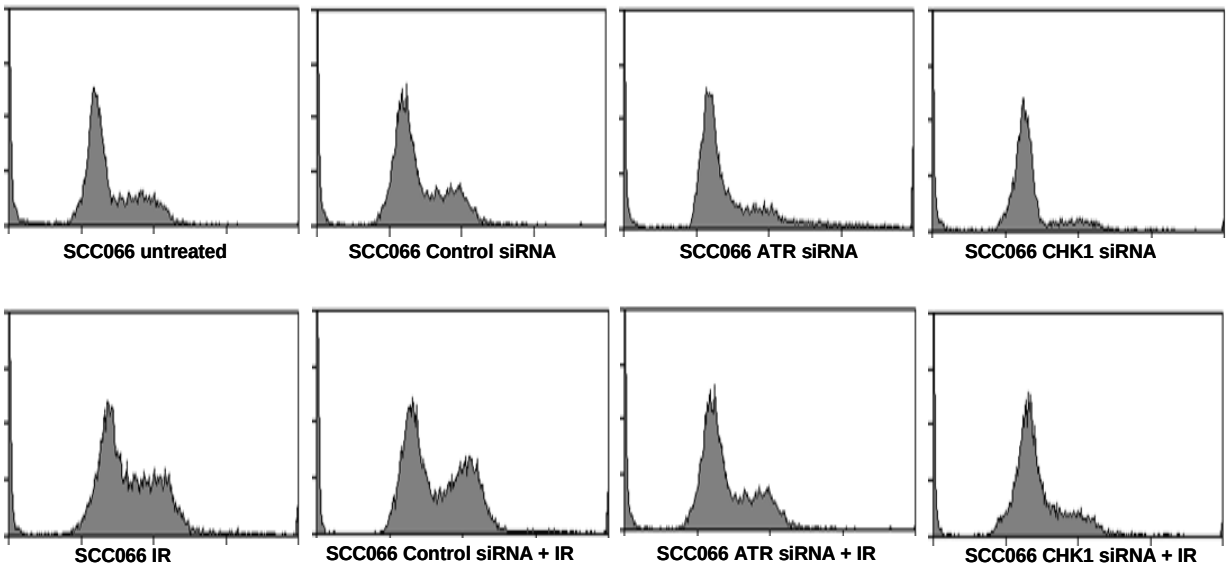


Figure 27. Flow cytometeric analysis following treatment of ATR and CHEK1 siRNA
Cell cycle profiles of **(A)** UPCI:SCC104 and **(B)** UPCI:SCC066 following treatment with non–specific siRNA, ATR siRNA and CHEK1 siRNA in nonirradiated cells or cells irradiated with 5 Gy IR are depicted. In comparison to SCC066, SCC104 shows increased accumulation of irradiated cells in the G_2M phase. On inhibition of ATR or CHEK1 with the respective siRNAs, we observed elimination of the G_2M accumulation of irradiated cells and an increase in the sub–G_0 dead cell population.

To determine if the PCC/MC formation we observed using caffeine was mediated through its inhibition of the ATR–CHEK1 pathway, we used ATR and CHEK1 siRNAs for a specific knockout. We observed very high level of PCC/MC in UPCI:SCC104 following treatment with either ATR or CHEK1 siRNA (Figure 28). In comparison, UPCI:SCC066 had a modest increase in the number of cells undergoing PCC/MC in response to inhibition of the ATR–CHEK1 pathway.

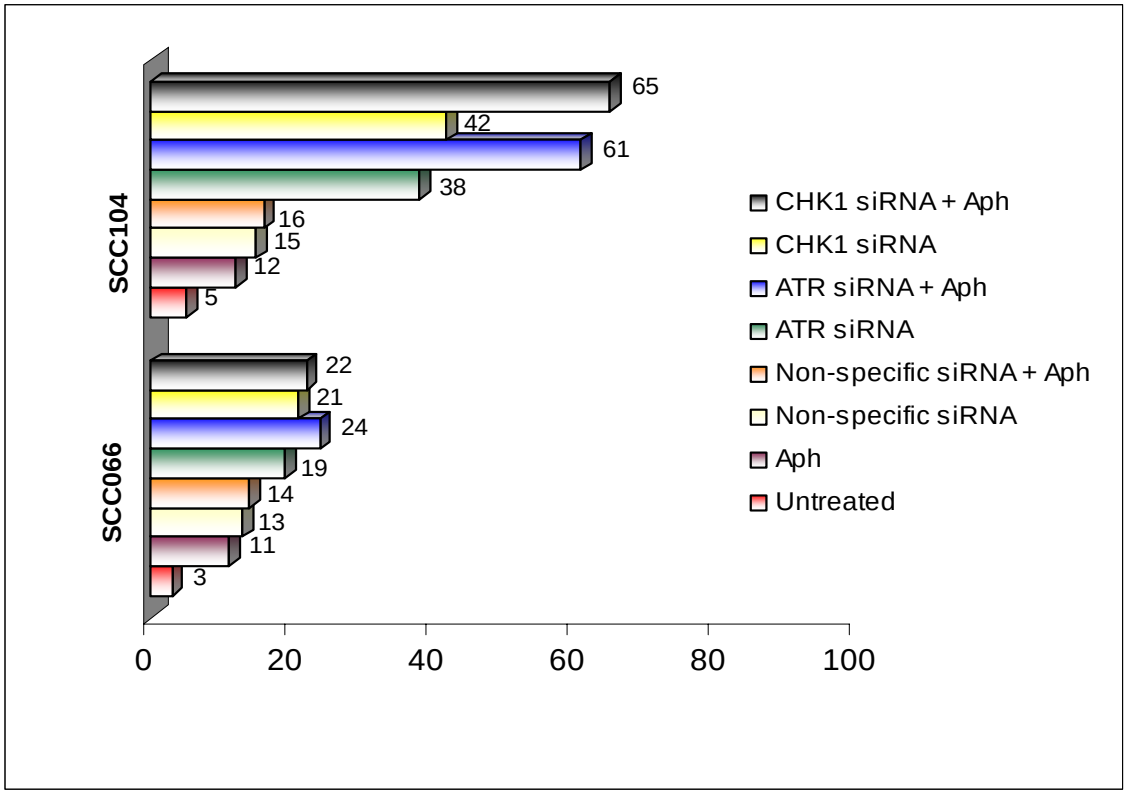
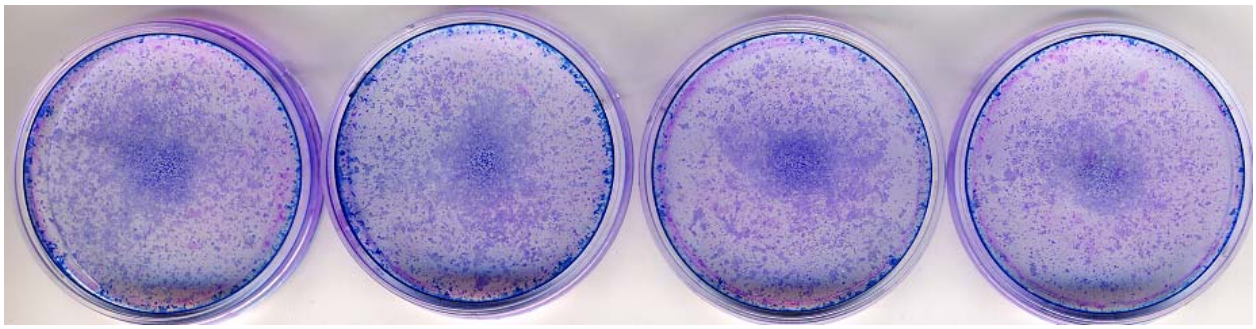


Figure 28. Induction of premature chromatin condensation and mitotic catastrophe following ATR and CHEK1 siRNA treatment.

The frequency of PCC/MC in UPCI:SCC066 and 104 in response to ATR and CHEK1 siRNA treatment with or without aphidicolin (Aph). The numbers next to the bars represent the percentage of mitotic cells undergoing PCC/MC.

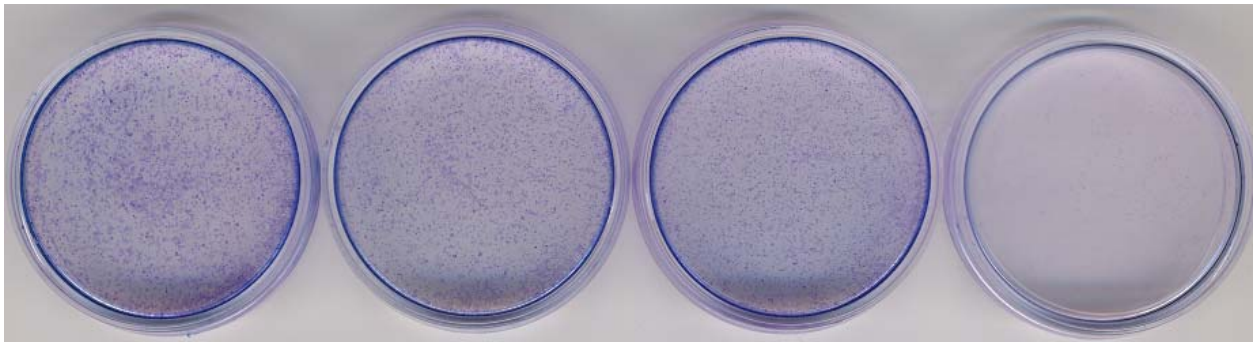
Finally, we observed cell survival of UPCI:SCC066 and 104 after treatment with ATR siRNA in the absence of any DNA damaging agents (Figure 29). We observed complete inhibition of colony formation in UPCI:SCC104 treated with ATR siRNA at the end of 12 days. In comparison, UPCI:SCC066 did not demonstrate a significant reduction in cell survival.

A UPCI:SCC066p21



Untreated Mock treated Non–specific siRNA ATR siRNA

B UPCI:SCC104p32



Untreated Mock treated Non–specific siRNA ATR siRNA

Figure 29. Clonogenic cell survival of UPCI:SCC066 and 104 to ATR siRNA treatment

UPCI:SCC066 (Panel A) and UPCI:SCC104 (Panel B) cells at 50% confluence were mock treated with empty Lipofectamine or treated with a nonspecific scrambled siRNA or treated with ATR siRNA and cell survival was compared to untreated tumor cells for each cell line. A modest reduction in cell survival was observed in UPCI:SCC066, while UPCI:SCC104 cells were highly sensitive to ATR inhibition.

Thus, our results suggest that the ATR–CHEK1 pathway is upregulated in a subset of OSCC and ATR and/or CHEK1 may be potential targets to sensitize a subset of OSCC to DNA damaging agents.

4.0 DISCUSSION

4.1 THE ROLE OF DISTAL 11Q LOSS IN OSCC CARCINOGENESIS

Loss of distal 11q has been shown by LOH to occur in a variety of tumors, including breast carcinoma, esophageal carcinoma, cervical carcinoma and prostate cancer, in addition to head and neck squamous cell carcinoma (WANG *et al*. 2004, MATSUMOTO *et al*. 2004; MIYAI *et al*. 2004, DAHIYA *et al*. 1997, JIN *et al.* 1998). We showed that distal 11q is lost in a large number of OSCC cell lines by FISH, QuMA and LOH studies (Table 3, Figure 3 and Figure 4, respectively). While LOH and QuMA show segmental loss from approximately 11q14.2 to 11qter, FISH targeted to specific genes (*MRE11A*, *ATM*, *H2AFX* and *CHEK1*) showed haploinsufficiency or partial loss of these genes in a subset of HNSCC cell lines. Confirmation of the physical loss along distal 11q allowed us to focus on the functional consequences of this event. We observed that loss of distal 11q in OSCC leads to partial loss of a number of genes involved in the DNA damage response and a reduction in expression of these genes at the RNA and protein level. We evaluated γ-H2AX focus formation in response to ionizing radiation as one measure of the competency of the DNA damage response in OSCC. We observed defective γ-H2AX focus formation in all OSCC with loss of distal 11q, irrespective of 11q13 amplification. In addition, OSCC with loss of distal 11q exhibited a high level of chromosomal breakage in response to ionizing radiation.

Baseline anaphase bridge formation was studied in untreated OSCC as a measure of the intrinsic level of chromosomal instability. Anaphase bridges were found to be increased in the subset of cell lines with loss of distal 11q, regardless of 11q13 amplification (Table 5). Cell lines without loss of distal 11q had frequencies similar to normal cells. In addition to being a hallmark of chromosomal instability, an increased incidence of anaphase bridges supports the BFB mechanism for gene amplification. It has been shown that *MRE11A* and *tefu (dATM)* knockout mutants in *Drosophila* exhibit a high frequency of telomere fusions and dicentric chromosomes (CIAPPONI *et al*. 2004, BI *et al*. 2004). These dicentric chromosomes break and initiate breakage-fusion-bridge cycles, similar to the anaphase bridges and BFB cycles seen in OSCC with distal 11q loss.

In addition to *MRE11A*, *ATM*, *H2AFX* and *CHEK1*, a number of important genes located on distal 11q may be lost in the process of 11q13 amplification. On searching the genes present on the long arm of chromosome 11 at the NCBI website (http://ncbi.nih.gov/mapview), we observed that important members belonging to the caspase family, several genes involved in the ubiquitination pathway, and genes involved in keratinocyte differentiation may be lost. Three members of the caspase family *CASP1*, *CASP4* and *CASP5* and important regulators of the caspase pathway, *CASP12P1, COP1, INCA* and *ICEBERG* map to a very small 0.5Mb segment in band 11q22.1–q22.3. The *BRCC2* gene at 11q24.1 encodes a protein with a BH3–like domain which induces caspase-mediated apoptosis (BROUSTAS *et al*. 2004). The high survival of OSCC with distal 11q loss after irradiation may be due to loss of critical members of the caspase pathway or *BRCC2* since caspases are the final effectors of apoptosis (COHEN 1997). *UBE4A*, *USP2* and *USP28*, on distal 11q are members of the ubiquitination pathway, which plays an important role in the stability and degradation of various proteins. Thus, loss of distal 11q can

confer changes in the ubiquitination pathway and alter the biochemical properties and half–life of different proteins. *POU2F3* at 11q23.3 is an important regulator of keratinocyte differentiation. Overexpression of POU2F3 induces expression of SPRR2A which is a marker for terminal keratinocyte differentiation (CABRAL *et al*. 2003). Expression of POU2F3 in cervical cancer cell lines induces differentiation of the cervical cancer cells and suppresses cancer cell replication and division (ENOMOTO *et al*. 2004). Thus, loss of *POU2F3* may result in loss of differentiation and dysplastic changes. Some of the important genes lost in the first step in the process of 11q amplification, along with the ones mentioned above are depicted in Table 13 and Figure 30.

Table 13. Important genes on distal 11q that may be lost in a subset of OSCC.

Gene name	Location	Distance from centromere	Function
PAK1	11q13.5	76.8 Mb	P21 activating kinase regulates cell motility and morphology
FAT3	11q14.3	91.9 Mb	Tumor suppressor homolog of Drosophila FAT
JRKL	11q21	95.8 Mb	Nuclear factor
DDI1	11q22.3	103.4 Mb	Induced in response to DNA damage
CASP1,4,5	11q22.1–22.3	104.3 Mb	Effectors of apoptosis
INCA	11q22.3	104.5 Mb	Regulates recruitment and activation of procaspase 1
ICEBERG	11q22.3	104.5 Mb	Caspase 1 inhibitor
NPAT	11q22.3	107.5 Mb	Nuclear
PPP2R1B	11q23	110.8 Mb	Protein phosphatase regulatory subunit
REXO2	11q23.1	113.2 Mb	3'–5' exonuclease involved in DNA repair
USP28	11q23.1	113.3 Mb	De–ubiquitinating enzyme
ZBTB16	11q23.1	113.4 Mb	Zinc finger transcription factor regulates histone deacetylase and cell cycle progression
UBEA	11q23.3	118 Mb	Conjugation factor required for poly– and multi ubiquitination
POU2F3	11q23.3	119.6 Mb	Induces terminal keratinocyte differentiation
BRCC2	11q24.1	121.5 Mb	BH3– like domain containing pro–apoptotic protein

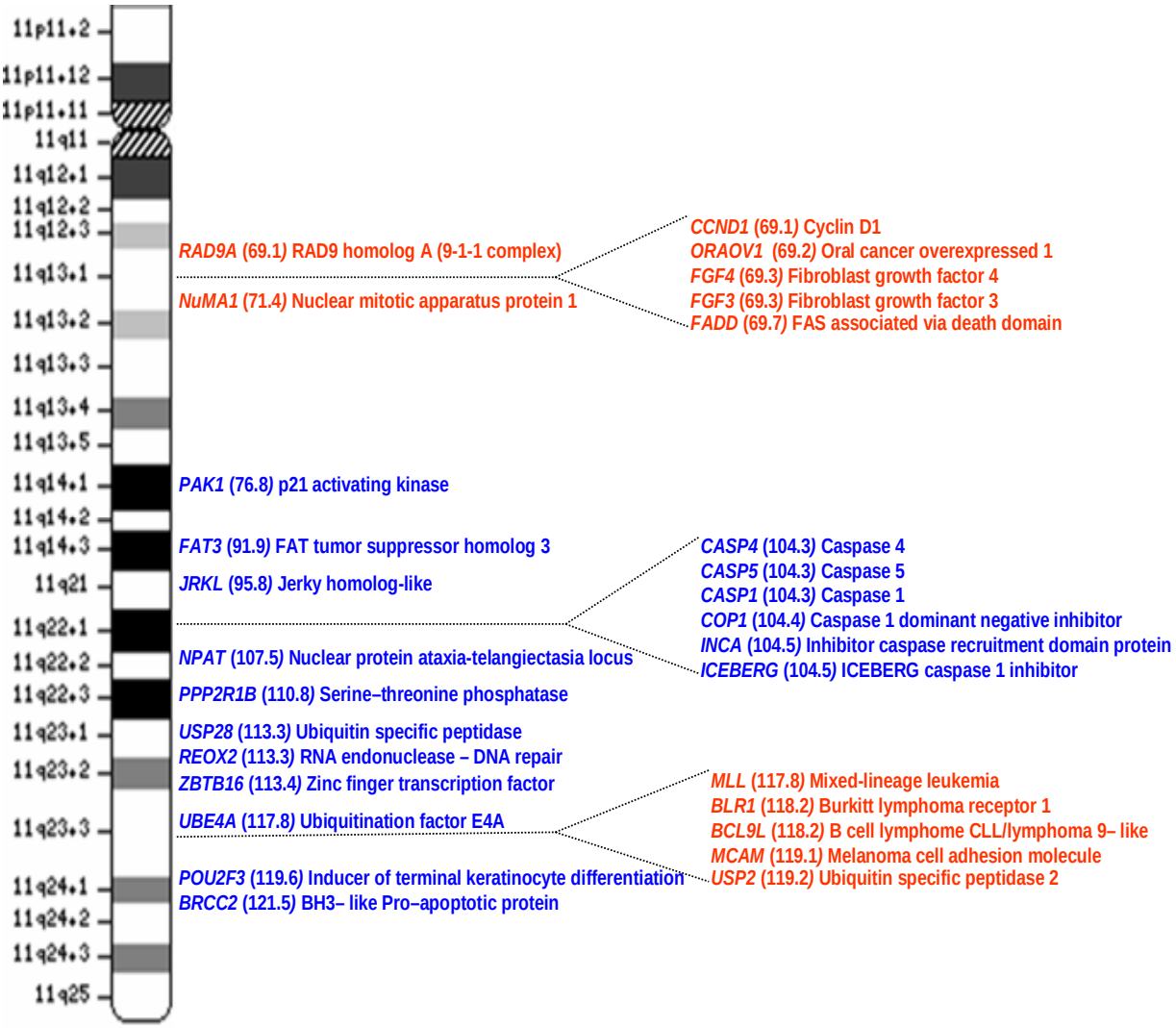


Figure 30. Important genes that can be amplified (red) or lost (blue) on distal 11q.

An ideogram of the distal arm of chromosome 11 with two areas where gene amplification (red) is common and genes which, if lost (blue) or haploinsufficient may contribute to the phenotype observed in OSCC. The figures in brackets represent approximate distance in Mb from the centromere. The ideogram was constructed with information obtained from the NCBI website (http://ncbi.nih.gov/mapview/).

Interestingly, we also observed that a number of proto–oncogenes including *MLL*, *BLR1*, *BCL1*, *BCL9* and *MCAM* are clustered in a short 1.5 Mb region in band 11q23.2–11q24. Amplification or translocation of this region is frequently seen in hematological malignancies (BERNARD and BERGER 1995). Co–amplification of 11q13 and 11q24 has also been reported in acute myeloid leukemia and myelodysplastic syndrome (ZATKOVA *et al*. 2004). The mixed–lineage leukemia gene (*MLL*) on 11q24 is believed to drive the 11q24 amplification in hematological tumors (POPPE *et al*. 2004, ZATKOVA *et al*. 2004). We observed amplification of chromosomal band 11q24 region in only UPCI:SCC070 (Figure 31) out of ten OSCC cell lines studied, suggesting that 11q24 amplification may also occur in solid tumors. On further evaluation, we observed that the *CCND1* gene on 11q13 formed the proximal and distal boundaries of the amplicon, in the form of an inverted duplication suggesting that amplification occurred by the BFB mechanism.

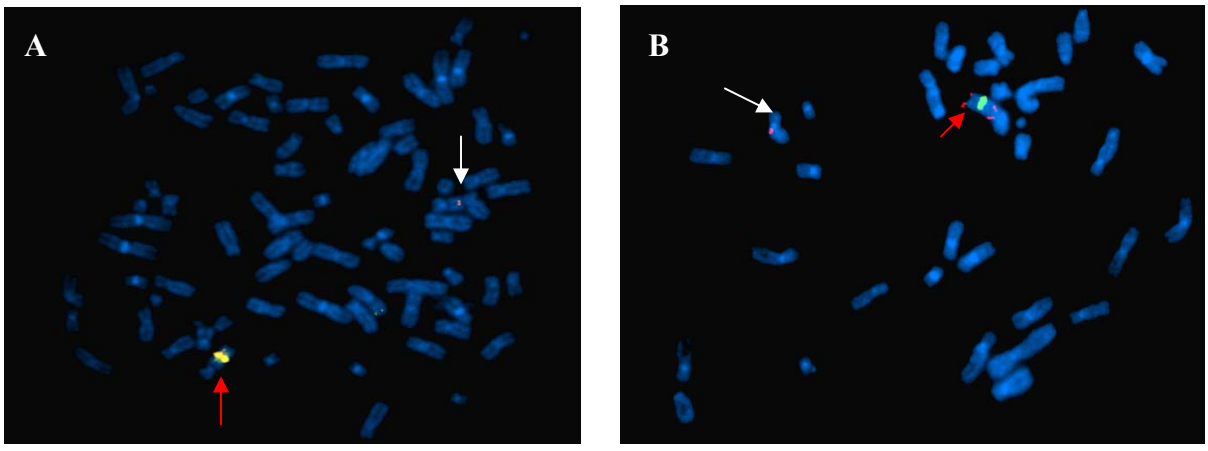


Figure 31. *MLL* (yellow) amplification in UPCI:SCC070.

Panel A shows *MLL* amplification in UPCI:SCC070 with a commercial breakapart probe labeled with Spectrum Green™ and Spectrum Orange™. If *MLL* is not broken or translocated, it appears yellow. Panel B shows a BAC mapping 1 Mb proximal to *MLL* labeled in green with *CCND1* in red which forms the boundaries of the 11q23-24 amplicon, suggesting that the amplification occurred by BFB mechanism.

Thus, distal 11q loss results in haploinsufficiency for a number of important genes present on distal 11q. The remaining normal allele of any of the genes on distal 11q can undergo mutation, deletion or hypermethylation leading to complete loss for the particular gene. The loss of genes on distal 11q including *MRE11A*, *ATM, H2AFX* and *CHEK1*, which precedes 11q13 amplification, may promote formation of anaphase bridges. Simultaneously, portions of distal 11q may be lost during each BFB cycle, leading to a propagated distal 11q loss–11q13 amplification cycle. Thus, both distal 11q loss and 11q13 amplification may act synergistically and provide growth and survival advantage to the cells undergoing these genetic alterations.

Loeb *et al*. proposed a "Mutator Phenotype Hypothesis" wherein cancer cells undergo changes which promote a very high level of background mutations and chromosomal abnormalities (LOEB *et al*. 2003). These mutations and chromosomal abnormalities accumulate to provide a growth advantage to cancer cells. The common genes which undergo mutations are those involved in DNA repair, DNA replication, telomere maintenance, apoptosis, checkpoint control and chromosomal segregation. It has been suggested that an intact DNA damage response acts as an efficient "anti-cancer barrier" (BARTKOVA *et al*. 2005, GORGOULIS *et al*. 2005). Loss of an intact DNA damage response promotes a mutator phenotype in somatic cells and leads to chromosomal instability. Thus, we observed that partial loss of distal 11q leads to defects in the DNA damage response which may contribute to a "mutator phenotype" effect in OSCC. We believe that loss of distal 11q leads to inadequate or inaccurate DNA repair and may promote mutation or chromosomal defects which contribute to the aneuploidy seen in OSCC.

4.2 ATR–CHEK1 UPREGULATION IN OSCC AND RADIOSENSITIVITY

A subset of all tumors, including oral squamous cell carcinomas, lack wild-type *TP53*, leading to a loss of the p53-dependent G_1 checkpoint (LEVINE 1997, MICHALIDES *et al*. 2002). Other factors which may contribute to a loss of G_1 cell cycle checkpoint in OSCC are cyclin D1 (*CCND1*) amplification and overexpression (SARTOR *et al*. 1999), deletion of the *RB1* gene (SARTOR *et al.* 1999), loss of the *CDKN2A* locus on 9p21 (SARTOR *et al.* 1999, SHINTANI *et al*. 2001) and haploinsufficiency or loss of the *ATM* gene on 11q22-q23. Loss of the G_1 phase checkpoint results in a large number of tumor cells entering the S and G_2 phases of the cell cycle with unrepaired DNA damage. Allowing cells with unrepaired DNA damage to pass through the S phase would trigger premature chromatin condensation. Similarly, allowing cells with unrepaired DNA damage to pass through the M phase would trigger mitotic catastrophe, both of which are p53–independent forms of cell death. Upregulation of ATR and CHEK1 would allow the cells to stop at the S or G_2M phase checkpoints, repair the DNA enabling the cells to resume progression through the cell cycle. This may exert a positive selection pressure for tumor cells that can upregulate ATR and CHEK1.

We observed that the *ATR* gene on 3q24 was translocated, gained or amplified in a subset of OSCC and also in an ovarian tumor cell line. In contrast, *CHEK1* was lost in a subset of OSCC. Our studies in primary head and neck tumors supported our results in OSCC that *ATR* is gained or amplified, while *CHEK1* is lost in the tumor cells compared to adjacent normal tissue. qRT–PCR and immunoblotting demonstrated that both ATR and CHEK1 are overexpressed in a subset of OSCC with loss of the G_1 checkpoint. Thus, our findings suggest that ATR–CHEK1 upregulation may confer a growth and survival advantage to tumor cells with loss of p53 protein.

Surprisingly, we observed that cell lines with an upregulated ATR–CHEK1 pathway are highly resistant to DNA damaging agents like IR and aphidicolin. Inhibition of the ATR–CHEK1 pathway nonspecifically using caffeine or specifically using the respective siRNA resulted in increased cell death by 'S' phase PCC formation or mitotic catastrophe, an increase in the sub–G_0 populations which represents dead cells by flow cytometry and reduced survival by a clonogenic cell survival assay. Thus, a subset of OSCC with a defective G_1 checkpoint may require an upregulated ATR–CHEK1 pathway for survival and inhibition of this pathway may sensitize OSCC to DNA damaging agents.

Loss of p53 function leads to loss of the G_1 checkpoint, loss of p53–dependent apoptotic pathways and allows cells to survive in spite of genetic instability (LEVINE 1997). It has been reported that the *TP53* gene, an important tumor suppressor gene, is mutated in nearly 50% of all tumors including OSCC. However, *TP53* in most cases is sequenced between exons 5–8 or exons 5–9, since this region is considered to be a "hotspot" for mutations. We detected a number of OSCC cell lines, UPCI:SCC104, 142 and 131 which had early truncation mutations leading to loss of p53 expression. It has been reported that sequencing between exons 5–9 may result in underestimating the frequency of *TP53* mutations by nearly 20% (BALZ *et al*. 2003). In addition to mutations, amplification or overexpression of MDM2, HPV infection and inhibition by E6 and promoter methylation may lead to loss of p53 function. If all mechanisms of loss of p53 function are taken into account, then the number of head and neck tumors with a nonfunctional p53 may be as high as 70–80%. If this is the case, then inhibition of the ATR–CHEK1 pathway may be advantageous in a very large subset of head and neck tumors.

ATR deficient mice are embryonic lethal and exhibit chromosomal fragmentation at a very early stage in development, suggesting that ATR plays an important role during normal

growth and development (BROWN and BALTIMORE 2000). In contrast, AT mice are viable but exhibit enhanced sensitivity to IR, accelerated ageing and develop multiple tumors suggesting that ATM is involved in DNA DSB processing and repair (SHILOH 2002). It has been suggested that both ATM and the MRN complex act upstream of ATR and CHEK1 and help to modulate the cellular response to ionizing radiation. If this is he case, then a compensatory increase in ATR and CHEK1 following loss of ATM or proteins of the MRN complex can be anticipated. In addition to the DNA damage response, ATR and its downstream effectors like CHEK1, BRCA1, and the Fanconi anemia group of proteins also play an important role fragile site maintenance and cellular responses to hypoxia. Thus, changes in ATR and CHEK1 expression may affect different cellular processes to achieve a survival benefit for the tumor cells.

In response to hypoxia, a number of stress pathways are activated. It has been shown that ATR kinase activity increases as the degree of hypoxia increases to a level which induces replication damage (HAMMOND *et al*. 2002, HAMMOND *et al*. 2003). This is associated with a rapid localization, activation and ATR focus formation at the sites of hypoxia induced–stalled replication forks (HAMMOND *et al*. 2002, HAMMOND *et al*. 2003). It is believed that hypoxia induces formation of single strand DNA (ssDNA) and stalled replication forks which lead to ATR–ATRIP dependent S phase arrest. Thus, the ATR–CHEK1 pathway is activated in response to hypoxic damage and this activation protects the cells from hypoxic DNA damage. Inhibition of ATR leads to sensitization of cells to hypoxic and reoxygenation insults (HAMMOND *et al*. 2004).

The tumor microenvironment plays an important role in the response of the cancer cells to radiotherapy and chemotherapy. Since radiotherapy and chemotherapy kill tumor cells by the

generation of toxic oxygen species, the effectiveness of these therapeutic modalities depends on adequate perfusion and oxygenation of the cancer cells. The tumor vasculature is highly disorganized and because cancer cells generally divide rapidly, hypoxia or a relative lack of oxygen is common in tumors. Since the hypoxia–sensitive cells are killed, the tumor is enriched for clones of cells resistant to radiotherapy and chemotherapy (VAUPEL *et al*. 2001). This is especially the case in head and neck tumors, where over a period of time, the highly aggressive and invasive cells predominate the tumor (JANSSEN *et al*. 2005). Hypoxia also selects for tumor cells which have a deficient p53-mediated apoptotic pathway (KINZLER, VOGELSTEIN 1996, GRAEBER *et al*. 1996). Hypoxia induces chromosomal breaks and promotes chromosomal rearrangements including deletions, translocations and chromosomal amplification (COQUELLE *et al*. 1998). Hypoxia, which is commonly seen in the tumor microenvironment, can select for cells with high level of chromosomal instability and cells which lack apoptotic pathways in response to DNA damaging agents. This phenomenon may account for the multiple secondary tumors, relapses and a poor response to conventional therapy in OSCC. Thus, pathways which regulate the cellular survival response to hypoxia may form important targets for treatment of OSCC. These findings assume a significant importance in light of our findings in OSCC, where the ATR–CHEK1 pathway is highly upregulated. Thus, the upregulated ATR–CHEK1 pathway in OSCC may serve to protect these cells from hypoxia and reoxygenation induced cell injury.

Generally, it has been demonstrated that cells which are radio– or chemoresistant exhibit an increased ability repair damaged DNA. This ability allows the cells to evade apoptotic pathways, which are activated in response to a specific threshold of DNA damage. The common mechanisms for DNA repair in tumor cells are homologous recombination and nonhomologous end joining. Both ATR and CHEK1 can increase homologous recombination repair and promote

DNA repair (BAOCHENG et al. 2005). In contrast, the FHIT protein decreases homologous recombination repair in cells (BAOCHENG et al. 2005). In OSCC, the formation of isochromosome 3q leads to two simultaneous genetic events which can promote increased homologous recombination repair in the tumor cells. Isochromosome 3q formation leads to an increase in copy number of the *ATR* gene, which can lead to increased ATR expression. Simultaneously, one or two copies of the *FHIT* gene located at (3p14.2) are lost in the process of isochromosome 3q formation. Since, FHIT downregulates HRR while ATR upregulates HRR, isochromosome 3q formation may improve lead to higher levels of HRR in the tumor cells conferring them with an increased ability to repair DNA and thus, radioresistance. Thus, upregulation of the ATR–CHEK1 pathway in OSCC can enhance the S and G_2M cell cycle checkpoints to DNA damage, protect cells from hypoxic injury and promote HRR.

4.3 RADIOSENSITIZING AGENTS FOR OSCC

Exposure to caffeine has been shown to sensitize cancer cells to DNA damaging agents including IR. It has been suggested that this sensitization may be related to overriding of the G_2M cell cycle checkpoint by caffeine. Robert Abraham and his colleagues demonstrated that the radiosensitizing effects of caffeine may be related to its inhibition of the ATM and ATR kinases (SARKARIA *et al*. 1999). The dose of caffeine required to inhibit 50% of kinase activity (IC_{50}) for different protein kinases is summarized in Table 14 (SARKARIA *et al*. 1999). Caffeine induces radiosensitization at doses between 1–2 mM, thus its sensitizing effect in tumors may be mediated through ATM or ATR. Since mTOR inhibition does not change the cellular response to IR, it is unlikely that the radiosensitizing effects of caffeine are mediated through mTOR inhibition (SARKARIA *et al*. 1999).

Table 14. IC_{50} for different kinases in response to caffeine

Kinase	IC_{50} for caffeine
ATM	0.2 mM
mTOR	0.4 mM
ATR	1.1 mM
CHEK1	5.0 mM
DNA–PK_{CS}	10.0 mM

In OSCC with an upregulated ATR–CHEK1 pathway, we observed a significant reduction in colony formation at a 0.5 mM dose of caffeine, and complete inhibition of cell survival at a 1 mM dose of caffeine. This sensitization effect of caffeine in OSCC could be due to inhibition of either ATM or the ATR kinase. GM09607, an ATM deficient cell line shows sensitivity to caffeine which is similar to the OSCC cell lines with an upregulated ATR–CHEK1 pathway. Thus, sensitivity of OSCC to caffeine is likely to be caused due to inhibition of ATR

activity. Since we observed significant inhibition of cell survival by 0.5 mM of caffeine, which is lower than the IC_{50} reported for caffeine–mediated ATR inhibition (SARKARIA *et al*. 1999), we believe that cell lines with an upregulated ATR–CHEK1 pathway may be hypersensitive to inhibition of this pathway. To confirm if ATR was the target of caffeine-mediated sensitization, we performed a highly specific siRNA-mediated ATR knockout in OSCC. We observed that ATR siRNA treatment completely inhibits colony survival in the absence of any extrinsic DNA damaging agents. An upregulated ATR–CHEK1 pathway can protect these cell lines with high levels of chromosomal breakage from PCC and MC, both p53–independent forms of cell death. Thus, it may be possible to use this characteristic of tumor cells by inhibiting the ATR–CHEK1 pathway and thus, sensitizing the tumor cells not only to extrinsic DNA damaging agents, but also to hypoxic damage, replicative stress and intrinsic or inherent chromosomal breakage.

Caffeine is a member of the methyl–xanthine family; other members of this family which are currently used in clinical practice are aminophylline, theophylline and pentoxyphylline. It has been demonstrated that these drugs at millimolar concentrations can inhibit DNA damage repair and override cell cycle checkpoints and induce cell death by premature chromatin condensation and mitotic catastrophe (DILLEHAY *et al*. 1988, MUSK, STEEL 1990, LI *et al*. 1998). In the past, caffeine pre-treatment has been used to assist chemotherapy or radiotherapy in the treatment of pancreatic cancer and osteosarcomas (TSUCHIYA *et al*. 1998)

Unlike caffeine, which inhibits ATM and ATR at lower concentrations, UCN–01 (7-hydroxystaurosporine) predominantly inhibits CHEK1 at very low (nanomolar) concentrations to induce radiosensitization (BUSBY *et al*. 2000, GRAVES *et al*. 2000). In addition to UCN–01, the indolocarbazole inhibitor SB-218078 also inhibits CHEK1 and sensitizes tumor cells to topoisomerase inhibitors (JACKSON et al. 2000). Both UCN–01 and SB-218078 bind to the

ATP–binding pocket of CHEK1 which interacts with cyclin dependant kinases and thus, inhibits CHEK1 activity (ZHAO *et al*. 2002). It has been demonstrated that the checkpoint abrogating and radiosensitizing properties of UCN–01 are predominantly seen in human tumor cells with defective p53 function (SHAO *et al*. 1997). However, inhibition of CHEK1 leads to increased initiation of DNA replication and increased DNA breaks in normal cells, which suggests that CHEK1 inhibition may not specifically target tumor cells (SYLJUASEN *et al*. 2005).

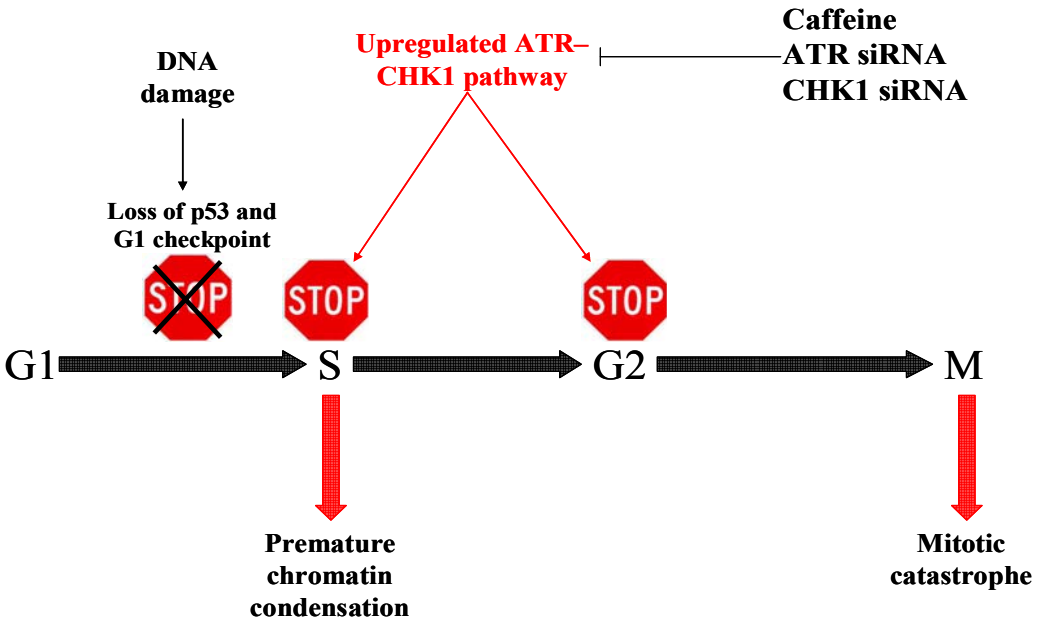


Figure 32. Model for OSCC sensitization to DNA damaging agents.

Loss of p53 function leads to loss of the G_1 cell cycle checkpoint in response to DNA damage in a subset of OSCC. An upregulated ATR–CHEK1 pathway enhances the S and G_2M checkpoints and protects cells with DNA damage from premature S phase entry and premature mitosis. Thus, there is a positive selection pressure for cells with an upregulated ATR–CHEK1 pathway. Inhibition of the ATR–CHEK1 pathway using caffeine or specific siRNA can increase the susceptibility of these cells to p53–independent forms of cell death by PCC or MC.

In addition to ATR and CHEK1, it is likely that other proteins in the pathway may also be upregulated. RAD9 overexpression has been reported in breast tumors and inhibition of the

overexpressed RAD9 leads to sensitization of the breast tumor cell line (CHENG et al. 2005). Since, RAD9 lies just within the 11q13 amplicon (Figure 30), it is likely that RAD9 is amplified and overexpressed in a subset of OSCC.

A subset of OSCC cells have an overactivated ATR–CHEK1 pathway, which leads to a prolonged G_2 accumulation in OSCC cells treated with IR. This prolonged G_2 accumulation may provide the irradiated OSCC cells with a chance to undergo HRR which occurs exclusively during the S and G_2M phases of the cell cycle. In addition to providing time for the OSCC cells to undergo HRR, both ATR and CHEK1 enhance HRR directly and promote cell survival (BAOCHENG *et al*. 2005). Our results suggest that a number of OSCC demonstrate a loss of the G_1 checkpoint and enhanced chromosomal breakage and cell survival following treatment with IR. Thus, agents like caffeine or UCN–01 which can inhibit the ATR–CHEK1 pathway may promote cell death in OSCC through p53–independent mechanisms.

4.4 3Q GAIN IN OSCC AND ITS RELATIONSHIP TO DISTAL 11Q LOSS

Chromosome 3q gain or amplification is a very common event in oropharyngeal squamous cell carcinoma, esophageal carcinoma, cervical carcinoma, prostate cancer, mantle cell lymphomas and carcinomas of the vulva (MONNI *et al*. 1998, SATTLER *et al*. 1999, YANG *et al*. 2001, PEI *et al*. 2001, NOGUCHI *et al*. 2003, STOLTZFUS et al. 2005). Past studies by karyotyping in our laboratory demonstrate that 3q gain is present in 40% of OSCC cell lines and is the most frequent chromosomal aberration after 11q13 amplification, which was seen in 45% of OSCC cell lines (LESE MARTIN *et al*. unpublished data). Others have reported the prevalence of 3q gain using comparative genome hybridization studies to be as high as 80-85% in primary head and neck tumors (BOCKMÜHL *et al*. 1998). A number of different genes on 3q are thought to drive this gain, though a single causal gene has not been confirmed. The genes implicated to drive 3q gain and amplification are phosphatidylinositol 3–kinase catalytic subunit (*PIK3CA*) (MA et al. 2000), eukaryotic initiation factor 5A2 (*EIF-5A2*) (GUAN *et al*. 2001, GUAN *et al*. 2004), *cyclin L* (*CCNL1*) (REDON *et al*. 2002), *TP73L* (REDON et al. 2001) and *TLOC1* (JUNG *et al*. 2006). While it is possible that one gene may be responsible for driving the 3q gain or amplification, it is also likely that, as seen in 11q13 amplification, a number of genes could be gained or amplified on 3q. However, 3q gain occurs over a much larger area compared to the small 1.5 Mb area amplified in OSCC (KETTUNEN *et al*. 2000, HUANG *et al*. 2002).

In cervical cancer, chromosome 3q gain is associated with the progression of severe dysplastic lesions to invasive cancer (HESELMEYER *et al*. 1996). Similarly, in head and neck cancer, 3q gain is associated with a poor prognosis, increased invasive ability and dissemination of the cancer to adjacent lymph nodes (BOCKMÜHL *et al*. 2000, BOCKMÜHL *et al*. 2002, SINGH *et al*. 2002). In head and neck cancer, it was observed that 3p deletion precedes 3q gain

and overrepresentation of 3q plays a role in the development of aneuploidy (HEMMER *et al*. 2006). Since a number of genes between 3q24–q28 are gained in tumors, it is difficult to pinpoint a single gene as being causal or necessary for the development of OSCC. The contribution of the gained/amplified genes in tumorigenesis can be evaluated by the degree they are overexpressed at the RNA and protein levels and studying the phenotype of tumors after siRNA–mediated inhibition of gene expression. In ovarian cancer, EIF-5A2 overexpression was correlated with an advanced tumor stage and inhibition of EIF-5A2 resulted in inhibition of cell growth (GUAN *et al*. 2004). In head and neck cancer, *CCNL1* gain is correlated with advanced clinical stage of the tumor and poor prognosis (STICHT *et al*. 2004). Our results suggest that the *ATR* gene on 3q24 may be one of the targets for 3q gain and this gain results in ATR overexpression at both the RNA and protein level. Reducing ATR expression through ATR siRNA mediated knockout of the mRNA promotes cell death in the tumors through PCC and MC and reduces cell survival to DNA damaging agents.

Along with 3q gain, we observed a number of cell lines, UPCI:SCC070, 077, 104, 131 and 142 with isochromosome 3q. Since isochromosome 3q formation involves deletion of 3p, important genes like *FHIT*, *FANCD2* and *ATRIP* located on 3p may be lost in these OSCC. ATRIP interacts with ATR and is known to facilitate ATR function (CORTEZ *et al*. 2001, ZOU, ELLEDGE 2003). Thus, loss of ATRIP may affect the efficiency of ATR functioning in OSCC. We believe that ATR function, although inefficient, may be adequate to protect the tumor cells from mitotic catastrophe and other p53–independent forms of cell death.

Jin and colleagues in 2002 reported a correlation between 11q13 amplification and 3q gain in HNSCC (JIN *et al*. 2002). The chromatin segment, 3q21–q29 was present on a derivative chromosome 11 with 11q13 amplification in four out of the eight cell lines they studied.

However, gain of 3q was seen in all cell lines with 11q13 amplification, which suggests a mechanistic link between amplification at band 11q13 and gain of distal 3q (JIN *et al*. 2002). It has been shown that upregulation of ATR occurs in ATM-deficient AT cell lines (GM09607) and the upregulated ATR can mitigate some of the effects of ATM loss (WANG *et al*. 2003). Since the first event during 11q13 amplification by BFB cycles is loss of distal 11q, including *ATM* (11q22.3), it is likely that a gain of 3q may be driven by a mechanism to gain *ATR* which can partly compensate for the loss of *ATM*. In addition to observing 3q gain in cell lines with 11q13 amplification, we observed 3q gain in two cell lines UPCI:SCC104 and 142 with *ATM* loss. However, UPCI:SCC122 with ATM loss did not demonstrate 3q or *ATR* gain. Thus, 3q gain could be related to loss of ATM on distal 11q. However, this relationship needs to be studied in a larger cohort of tumors.

4.5 CHRONOLOGY OF GENETIC EVENTS IN OSCC

Somatic cells undergo a series of genetic and epigenetic changes which accumulate over time to transform them into cancer cells (KINZLER *et al*. 1996). Cells that acquire pro–proliferative and pro–survival changes are selected for in tumors. Thus, over a period of time, tumors attain the ability to divide in an unregulated manner and later develop resistance to chemo- and radiotherapy (HANAHAN and WEINBERG. 2000). The common changes observed during carcinogenesis are overexpression of oncogenes and/or deletion or inactivation of tumor suppressor genes. Overexpression of oncogenes may be due to gene amplification (*CCND1* in OSCC, breast and esophageal cancer), gene translocation (*BCR–ABL1* in CML) or promoter hypomethylation (YABRO 1992). Loss of tumor suppressor gene function may be caused by gene deletion (FHIT on 3p in OSCC), gene mutation (*TP53* gene in different cancers), dominant negative mutant protein or promoter hypermethylation (MACLEOD 2000).

Hittelman and his colleagues observed a high frequency of microsatellite alterations at 9p21 and 3p14 (MAO *et al*. 1996). Losses of 3p, 8p, 13q 17p were shown to occur early during HNSCC progression (LI *et al*. 1994). Califano and colleagues proposed an initial multi–step model for head and neck squamous cell carcinoma formation and progression (CALIFANO *et al*. 1996). Deletions of 3p14, 9p21, 13q14.2 and 17p13.1 result in loss of the important tumor suppressor genes, *FHIT, CDKN2A/ARF, RB1* and *TP53* at these loci, respectively.

The *FHIT* gene at chromosomal band 3p14.2 encompasses a common fragile site FRA3B and is commonly deleted in a number of cancers (HUEBNER and CROCE 2001). Recent studies demonstrate that 80-90% of head and neck squamous cell carcinoma cell lines have alterations of atleast one allele of the *FHIT* gene, while 10-15% of the tumors have homozygous deletions (VIRGILIO *et al*. 1996). There is partial to complete loss of FHIT expression in a number of

tumors (CROCE *et al*. 1999) and exogenous FHIT expression inhibits the tumor potential of cancer cells (SIPRASHVILI *et al*. 1997). Even though FHIT protein is known to inhibit carcinogenesis in humans and mice, the exact mechanism of FHITs tumor suppressor role is not known. The *CDKN2A/INK4A* locus on 9p21, encodes multiple splice variants which vary in the structure of their first exon. The p19 alternate reading frame product ($p19^{ARF}$) can inhibit MDM2 and stabilize p53 in response to DNA damage (SHERR 1998). The $p16^{INK4A}$ splice variant regulates RB1 metabolism (SERRANO *et al*. 1996). Loss of $p16^{INK4A}$ and $p19^{ARF}$ leads to immortalization and cooperates with oncogene activation to promote tumorigenesis (CARNERO *et al*. 2000, VOORHOOVE and AGAMI 2003). Thus, deletion or promoter hypermethylation of the *CDKN2A/INK4A* locus on 9p21 results in simultaneous disruption of both the *RB1* and the p53 tumor suppressor pathways. Deletion of the *CDKN2A* gene or inactivation of $p16^{INK4A}$ and $p19^{ARF}$ is frequent in OSCC (SHINTANI *et al*. 2001) and is associated with 11q13 amplification in human tumor cells (LUKAS *et al*. 1995).

The retinoblastoma gene (*RB1*) on 13q14.2 is frequently inactivated in head and neck tumors (SARTOR *et al*. 1999). Loss of Rb protein results in unregulated cell proliferation through the G1 phase of the cell cycle and deregulated apoptosis (NEVINS 2001, HICKMAN *et al*. 2002). Along with Rb dysregulation, *TP53* mutations are also common in head and neck cancer (SARTOR *et al*. 1999). The p53 protein acts as a "molecular policeman" and maintains genome integrity, regulates cell cycle checkpoints and protects normal cells from transformation (LANE 1992, LEVINE 1997). Loss of p53 function in tumors can occur due to deletion or mutation of the *TP53* gene, promoter hypermethylation, or overexpression of MDM2 which targets p53 for degradation by the proteasome (CHANG *et al*. 1993). This loss of p53 function results in loss of the G1 checkpoint, defects in the DNA damage response, impairment in

telomere maintenance which results in sticky chromosomal ends, loss of a number of p53 dependent apoptotic pathways and fosters an environment which promotes genomic instability.

11q13 amplification seen in 50% of OSCC is believed to be an early event in the development of OSCC (GOLLIN 2001, GOLLIN 2004). During the process of BFB cycles, which lead to 11q13 amplification in OSCC, the preceding loss of distal 11q results in partial loss of genes in that region. Since, the break (11q loss) occurs prior to fusion and formation of anaphase bridges, distal 11q loss is also an early event which may follow loss of p53 function in OSCC. Since, distal 11q loss results in a defective DNA damage response, we believe that this may create a "permissive environment" to allow initiation and propagation of chromosomal imbalances and aneuploidy. Gain of 3q24–28 is seen in a very high proportion of head and neck tumors (BOCKMÜHL *et al*. 1998). 3q gain is thought to occur during the intermediate to late stages of cancer formation and may be associated with resistance to conventional therapy. Our results demonstrate that the *ATR* gene, on 3q24 is one of the important targets for gain/amplification and enables the tumor cells to survive DNA damage induced by hypoxia, replicative stress, IR and other DNA damaging agents. Thus, we propose a genetic progression model (Figure 33) incorporating our findings that distal 11q loss and 3q gain play an important role in development and progression of OSCC.

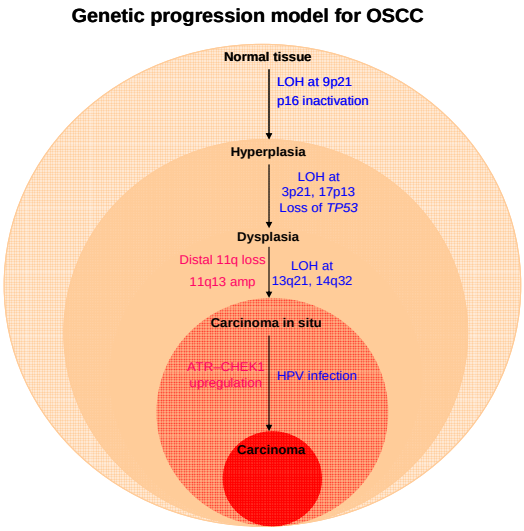


Figure 33. A genetic progression model for OSCC

LOH at 9p21, involving the *CDKN2A/ARF* locus and deletion of p16 are the earliest pre–neoplastic changes. This is followed by deletion of the *FHIT* gene at 3p21 and deletion or mutation of the *TP53* gene on 17p13. 11q13 amplification and distal 11q loss which precedes the amplification signal the progression to carcinoma *in situ*. Distal 11q loss can promote chromosomal instability and exert selection pressure for tumor cells with high malignant potential. 3q gain leads to upregulation of *ATR* and a number of other genes on 3q24-28 which promote development of cancer and confer resistance to DNA damaging agents.

APPENDIX A

LIST OF BACTERIAL ARTIFICIAL CHROMOSOMES (BAC) AND CENTROMERE ENUMERATION PROBES (CEP) USED FOR *FISH* ANALYSIS

Table 15: Different probes mapping to the respective genes used for *FISH* analysis

Gene name	BAC ID	Fluorescent tag
ATM	CTD2047A4	Spectrum Orange™
ATR	RP11-427D1	Spectrum Green™
	RP11-383G6	Spectrum Green™
CHEK1	RP11-712D22	Spectrum Orange™
CCND1	RP11-699M19	Spectrum Aqua™
H2AFX	RP11-892K21	Spectrum Green™
MRE11A	RP11-685N10	Spectrum Orange™
TP53	RP11-199F11	Spectrum Orange™

All BACs were purchased from the Children's Hospital Oakland Research Institute (C.H.O.R.I.), Oakland, CA. Two BACs RP11-427D1 and RP11-383G6 corresponding to the 5' and 3' ends of the *ATR* gene, respectively, were used simultaneously.

APPENDIX B

SEQUENCES FOR ATR AND CHEK1 siRNA

ATR and CHEK1 siRNAs were obtained from Dharmacon Inc. The individual sequences from the smartpool are outlined below:

ATR sequences:

GAACAACACUGCTGGUUUG	GAAGUCAUCUGUUCAUUAU
GAAAUAAGGUAGACUCAAU	CAACAUAAAUCCAAGAAGA

CHEK1 sequences:

UAAAGUACCACACAUCUUGUU	UAUUGGAUAUUGCCUUUCUU
AUAUGAUCAGGACAUGUGGUU	CCAUUGAUAGCCCAACUUCUU

APPENDIX C

LIST OF ANTIBODIES USED FOR IMMUNOBLOTTING

Table 16: List of antibodies used for immunoblotting with their relative concentrations

Antibody	Type	Company	Concentration
Total ATR	Rabbit polyclonal	Affinity Bioreagents, Golden, CO.	1:500–1:1000
Total p53 (D–01)	Mouse monoclonal	Santa Cruz	1:2000
Total ATM	Rabbit polyclonal	Cell Signaling	1:500
Total CHEK1	Rabbit polyclonal	Cell Signaling	1:1000
Total H2AX	Mouse monoclonal	Cell Signaling	1:1000
Total MRE11A	Goat polyclonal	Santa Cruz	1:1000
pATM (Ser 1981)	Rabbit polyclonal	Cell Signaling	1:500
pATR (Ser 428)	Mouse monoclonal	Cell Signaling	1:500
pBRCA1 (Ser 1497)	Goat polyclonal	Santa Cruz	1:1000
pCHEK1 (Ser 345)	Rabbit polyclonal	Cell Signaling	1:1000
pCHK2 (Thr 68)	Rabbit polyclonal	Cell Signaling	1:1000
pSMC1 (Ser 987)	Rabbit polyclonal	Cell Signaling	1:1000
pCDC25C(Ser 216)	Rabbit monoclonal	Cell Signaling	1:1000
γ–H2AX (Ser 139)	Mouse monoclonal	Upstate	1:1000
Actin	Mouse monoclonal	Sigma	1:2000
Tubulin	Rabbit polyclonal	Santa Cruz	1:2000

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