MECHANISMS LEADING TO CHROMOSOMAL INSTABILITY IN ORAL CANCER CELLS

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FORWARD

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MECHANISMS LEADING TO CHROMOSOMAL INSTABILITY
IN ORAL CANCER CELLS

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University of Pittsburgh, 2005

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer worldwide. In 2005, the American Cancer Society estimates that 29,370 new cases of oropharyngeal tumors will occur in the United States, accounting for 2.1 % of new cancer cases and 1.3% of cancer deaths. Squamous cell carcinoma of the head and neck and its subset, oral squamous cell carcinoma (OSCC), arise from a combination of environmental factors and genetic events. Although high-risk behaviors such as smoking cigarettes, using smokeless tobacco, and consuming excessive alcohol have been shown to play a role in OSCC development in many individuals, oral cancer has also been observed in people with no apparent history of tobacco or alcohol use. Unfortunately, more than 60% of patients identified as having SCCHN are diagnosed after reaching an advanced stage of the disease. As a result of late-stage tumor discovery, the overall survival rate for affected individuals is less than 50%, and remains one of the lowest when compared to other major cancer types.

One ongoing problem with treating OSCC is that tumors from the same site within the oral cavity respond differently to identical treatment regimens. This is due in part to OSCC expressing chromosomal instability (CIN), in which karyotypes vary from cell to cell. CIN may then promote survival of subpopulations within a given tumor. In an effort to determine a mechanism for the development and/or progression of OSCC, we first examined the chromosomal content of cloned cell populations isolated from two OSCC cell lines. We showed that no two cells from the original parent or subsequent daughter clones in either cell line expressed the identical karyotype, confirming the presence of CIN in OSCC.

Various ways in which CIN may arise in tumor cells include: 1) faulty mitotic apparatus machinery, 2) centrosome amplification, 3) telomere dysfunction, 4) defects in the DNA damage response, and 5) gene amplification. Previous studies from our laboratory identified 11q13 gene

amplification in the form of a homogeneously staining region (hsr) in approximately 45% of our OSCC cell lines. FISH analyses using probes for the *RIN1* and *CCND1* genes demonstrated an inverted duplication pattern within the 11q13 amplicon, suggesting a BFB cycle model for gene amplification. In order to confirm that BFB cycles lead to gene amplification, a form of CIN in our OSCC cells, we examined dividing cells from 29 OSCC cell lines for the presence of the inverted duplication pattern within anaphase bridges, which are known BFB cycle intermediates. We show that the inverted duplication pattern occurred in a higher frequency of anaphase bridges expressed by OSCC cell lines with 11q13 amplification compared to OSCC cell lines without 11q13 amplification. By demonstrating the inverted duplication pattern within anaphase bridges, we provide evidence that at least some cases of gene amplification expressed in the form of an hsr occur through the BFB cycle mechanism in OSCC cells. Furthermore, we show that hsrs within cells are heterogeneous, consistent with the suggestion that variations in the size of the amplicon are determined by the site of breakage (Toledo et al. 1992), a form of CIN that may also be explained by the BFB model of gene amplification.

We next set out to determine a mechanism by which gene amplification is initiated in OSCC cells. Recent reports have suggested that gene amplification in the form of a homogeneously staining region (hsr) may result from breaks in common fragile sites (CFS), sensitive regions of the genome which may form gaps or breaks in metaphase chromosomes when cells are grown under conditions that interfere with DNA replication or repair. Previous studies from our laboratory identified proximal and distal breakpoint regions relative to the 11q13 amplicon. We propose that common fragile sites in chromosome 11 may be located 1) between the *RIN1* and *CCND1* genes and 2) distal to the *CCND1* gene, and may play a role in chromosome breakage and subsequent gene amplification through BFB cycles. We mapped the CFS, *FRA11F*, to a 7.5 Mb region distal to the 11q13 amplicon at 11q14.2, and identified the DNA sequence that corresponds to it. OSCC cell lines expressing 11q13 amplification had loss of *FRA11F* sequences, while the *FRA11F* regions within cell lines without the 11q13 amplicon were not associated with distal chromosome loss or rearrangement. In OSCC cells containing more complex rearrangements of 11q, *FRA11F* sequences appeared to localize within the 11q13 amplicon. *FRA11F* was not only broken between its proximal and distal sequences in complex rearrangements, but also displayed the inverted duplication pattern we previously identified in the 11q13 amplicon using probes for *RIN1* and *CCND1*.

Combined, our findings demonstrate that 11q13 gene amplification in OSCC occurs through BFB cycles, and suggest that breakage or loss of *FRA11F* may play a key role in initiating 11q13 amplification. Further insight into the mechanisms initiating and promoting gene amplification will provide opportunities to identify new biomarkers to aid in diagnosis and prognosis of oral cancer and other cancers, and may be of use for developing novel therapeutic strategies for patients with SCCHN.

PUBLIC HEALTH RELEVANCE

In the United States, cancer is a leading cause of death, second only to heart disease (MOKDAD *et al.* 2004). A study in the *Journal of the American Medical Association* reported tobacco, poor diet, lack of physical activity, and alcohol consumption as the top four of risk factors associated with death in the United States (MCGINNIS and FOEGE 1993). Although it is estimated that in 2005, cancer of the oral cavity will account for only 2.1% of cancer cases and 1.3% of overall cancer deaths in the U. S. (JEMAL *et al.* 2005), high-risk behaviors such as smoking cigarettes, using smokeless tobacco, and consuming excessive alcohol have been shown to play a major role in OSCC development.

Exposure to environmental agents, including tobacco smoke, smokeless tobacco, alcoholic beverages, and/or viruses, such as human papillomavirus (FORASTIERE *et al.* 2001; HO and CALIFANO 2004; MORK *et al.* 2001) have a profound influence on cells within the oral cavity. These factors have been shown to induce genetic alterations including chromosomal alterations, DNA changes (eg. mutations, amplifications or deletions), and/or epigenetic alterations, such as changes in DNA methylation that affect genetic regulation. Genetic alterations in cells are useful biological markers that assist in early detection of cancer and response to therapy (SIDRANSKY 1995). Currently, however, there are no useful biomarkers to identify early changes involved in OSCC development. As a result, affected individuals are diagnosed late and have a survival rate of less than 50%, which remains one of the lowest when compared to other major cancer types.

One specific genetic alteration observed in 45% of OSCC is amplification of chromosomal band, 11q13. This event has been shown to follow dysplastic cellular changes, but occur prior to development of carcinoma *in situ* (FORASTIERE *et al.* 2001). Therefore, 11q13 amplification may be a useful biomarker for detecting OSCC. In addition, understanding the molecular mechanisms that promote 11q13 gene amplification may provide valuable information for devising novel prevention measures and therapies. In the current study, we show that the primary mechanism promoting 11q13 gene amplification is BFB cycles. Furthermore, we suggest that breakage at the common fragile site, *FRA11F*, may be responsible for initiating 11q13 gene amplification. Taken together, using the breakage frequency of *FRA11F* as a

molecular marker for early detection of OSCC may be useful for identifying individuals who may be at risk for disease progression through 11q13 gene amplification. By determining the primary mechanism that leads to 11q13 amplificaton in OSCC, additional investigations focusing on the biological basis of this process may provide important information for developing successful measures and treatments that will increase the survival rate for individuals afflicted with oral cancer.

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1. SPECIFIC AIMS

The purpose of this study is to explore the biological mechanisms that play a role in chromosomal instability in oral cancer. We and others have shown previously that karyotypes of OSCC cells contain near-triploid chromosome numbers and express various structural and numerical chromosome abnormalities (GOLLIN 2001; JIN *et al.* 2002). Of particular interest to our laboratory is the 11q13 hsr observed in 45% of OSCC cells. The hsr form of gene amplification has been shown in some cases to result from breakage within regions of the genome expressing late replication and AT-rich strands with high flexibility, namely, common fragile sites (CFS). Previous investigations from our laboratory revealed that genes within the 11q13 hsr of OSCC cells displayed an inverted duplication pattern of *CCND1* flanked by *RIN1*, supporting a BFB cycle mechanism for gene amplification. In addition, we recently identified proximal and distal breakpoint regions relative to the core region of the11q13 amplicon. We propose that common fragile sites in chromosome 11 may be located 1) between the *RIN1* and *CCND1* genes and 2) distal to the *CCND1* gene, and may play a role in chromosome breakage and subsequent gene amplification through BFB cycles. Therefore, **we hypothesize that 11q13 gene amplification in OSCC results from BFB cycles, and that breakage at one or more CFS may be the initial step in 11q13 gene amplification.** This study is designed to determine mechanisms by which 11q13 amplification occurs in OSCC cells by fulfilling the following Specific Aims:

1. Validate the BFB mechanism for 11q13 amplification in OSCC cells.
2. Identify and characterize common fragile sites (CFS) on the long arm of chromosome 11 in normal human cells.
3. Demonstrate a relationship between breakage "hotspots" identified as CFS and 11q13 gene amplification in OSCC cells.

The goal of this study is to define a mechanism for 11q13 gene amplification in oral cancer and to determine whether clustered breakpoint regions flanking the 11q13 amplicon

correspond to regions harboring CFS. Determining the major mechanism that promotes 11q13 amplification in OSCC may 1) identify a useful biomarker for early OSCC development and/or OSCC progression and 2) aid in developing strategies to prevent chromosome breakage which may lead to gene amplification.

2. INTRODUCTION AND BACKGROUND

2.1. ORAL CANCER EPIDEMIOLOGY

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer worldwide (PARKIN *et al.* 1999). In 2005, the American Cancer Society estimates that 29,370 new cases of oropharyngeal tumors will occur in the United States, accounting for 2.1% of new cancer cases and 1.3% of cancer deaths (JEMAL *et al.* 2005). Tumors of the oral cavity, commonly known as oral squamous cell carcinoma (OSCC), include all SCC of the alveolar ridge, retrimolar trigone, hard palate, floor of the mouth (FOM), buccal mucosa, gingiva, and tongue. The highest incidence of OSCC occurs in the FOM, followed by the alveolar ridge, retrimolar trigone, buccal mucosa, hard palate, and tongue. Of these, OSCC of the tongue shows the most predilection for lymph node metastasis, followed by the alveolar ridge and retromolar trigone, FOM, buccal mucosa, and hard palate.

The majority of oral cancer cases appear to occur in developing regions of the world, particularly in Central and South America, the Caribbean, Africa, Asia, and China (PISANI *et al.* 1999). As is the situation in the United States, persons of lower socioeconomic status show the highest incidence of OSCC. In addition, studies have shown that males are two to three times more likely to develop OSCC (FRANCESCHI *et al.* 2000; REICHART 2001), and that African American males have a higher propensity for oral cancer than do their Caucasian counterparts (JEMAL *et al.* 2005).

If identified early, the 5-year survival rates may be greater than 80%, however these rates decrease significantly for tumors identified at a later stage (FANG and FORASTIERE 2001). Unfortunately, more than 60% of patients diagnosed with OSCC are already at an advanced stage of the disease (KOWALSKI and SAN 1994; REPORT 2003). Due to the late stage at diagnosis of most patients, the overall survival rate is less than 50%, which remains one of the lowest of all

cancer types (FANG and FORASTIERE 2001; REPORT 2003). Therefore, it critical to devise more effective methods for improving the early detection of OSCC.

2.2. ETIOLOGY OF ORAL SQUAMOUS CELL CARCINOMA

To identify more effective biomarkers for the early detection of OSCC, it is important to understand the cellular processes and mechanisms by which normal oral mucosal cells become neoplastic. Both SCCHN and its subset, OSCC, arise through an accumulation of genetic alterations including chromosomal alterations, DNA changes (eg., mutations, amplifications or deletions), and/or epigenetic alterations, such as changes in DNA methylation that affect genetic regulation. These events are further influenced by exposure to environmental agents, including tobacco smoke, smokeless tobacco, alcoholic beverages, and/or viruses, such as human papillomavirus (FORASTIERE *et al.* 2001; HO and CALIFANO 2004; MORK *et al.* 2001).

2.2.1. Tobacco

The most prevalent risk factor for the majority of epithelial cancers is cigarette smoking. According to a study in the *British Medical Bulletin*, the global estimate for the number of individuals who smoke is greater than one billion (PETO *et al.* 1996). The International Agency for Research on Cancer reports that the carcinogenic effects of smoking have been found in major organs including: bone marrow, bladder, cervix, kidney, liver, lung, pancreas, stomach, ureter, and all areas of the oral cavity, nasal cavity, nasal sinuses, larynx, and esophagus (INTERNATIONAL AGENCY FOR RESEARCH ON CANCER 2004). Of particular note is that 95% of tumors arising within the oral cavity are attributed to smoking (REICHART 2001). Normal cells are influenced by cigarette smoke through various biological mechanisms, including the formation of DNA adducts, single- and double-strand DNA breakage, inefficient DNA repair, chromosome segregation defects, and the inability of cell cycle checkpoints to identify, arrest, repair, or target affected cells for apoptosis (BENDER *et al.* 1988; GU *et al.* 1992; HSU *et al.* 1991; NAKAYAMA *et al.* 1985). *In vitro* and *in vivo* cytogenetic effects of cigarette smoke condensate

(CSC) include the formation of micronuclei (BALANSKY *et al.* 1987; BALANSKY *et al.* 1988; MOHTASHAMIPUR *et al.* 1987; MOHTASHAMIPUR *et al.* 1988; SAUNDERS *et al.* 2000; STOICHEV *et al.* 1993), lagging chromosomes (SABHARWAL *et al.* 1975; SAUNDERS *et al.* 2000), as well as chromosomal aneuploidy and various structural chromosome rearrangements (LAFI and PARRY 1988; LUO *et al.* 2004). Micronuclei have also been observed in the oral mucosa of smokeless tobacco users (KAYAL *et al.* 1993; LIVINGSTON *et al.* 1990; STICH *et al.* 1992). In addition, sister chromatid exchanges (SCEs) are suggested to result from exposure to the alkylphenols and benzaldehydes in CSC (CURVALL *et al.* 1985; JANSSON *et al.* 1986; JANSSON *et al.* 1988). More recent studies have demonstrated that CSC induces anaphase bridges as well as DNA double-strand breaks (DSB) in normal human fibroblasts and normal oral kaeratinocytes, suggesting a direct association between cigarette smoking and genomic aberrations in human cells (LUO *et al.* 2004).

2.2.2. Alcohol

The World Cancer Report indicates that excessive alcohol consumption is causally associated cancers of the oral cavity and liver, and is also categorized as a risk factor for cancers of the breast and colon (REPORT 2003). Recent findings in chick embryos have suggested that alcohol exposure and increased expression of vascular endothelial growth factor (*VEGF*) in combination with induced angiogenesis may have an important role in cancer progression (GU *et al.* 2005). The carcinogenic effects of alcohol appear to be most potent in cancers of the head and neck, in which heavy drinkers are 5 to10 times more likely to be affected than nondrinkers (REPORT 2003). Although ethanol by itself has not been proven to be carcinogenic, it is suggested that ethanol may act as a solvent, promoting absorption of other carcinogens, such as those in tobacco smoke, into target organs (BLOT 1992). Alcohol has also been characterized as an enhancer of oxidizing agents within cells, which may lead to DNA damage, resulting in malignancy (SHAW *et al.* 1983). An alternative hypothesis suggests that genetic polymorphisms in alcohol metabolizing enzymes may result in various quantities of carcinogenic acetaldehyde (the primary metabolite of ethanol) within individuals consuming the same amount of alcohol (REPORT 2003). Interestingly, one study comparing dark colored spirits (such as Scotch whisky,

Cognac, brandy, and dark rum) to lighter colored spirits (such as gin and vodka) suggested that consumption of darker alcoholic beverages appears to carry a higher risk for hypopharyngeal cancer (ROTHMAN *et al.* 1989). In addition, an association between poor diet and increased alcohol uptake has been suggested as another risk factor for cancer development (BLOT 1992). When combined with cigarette smoking, the risk for OSCC increases profoundly. Studies have shown that while cigarette smoking alone may increase an individual's risk two to four-fold, heavy drinking individuals who also smoke elevate their risk for OSCC six to 15-fold (MAIER *et al.* 1992; MORSE *et al.* 1996; ZAVRAS *et al.* 2001)

2.2.3. Human papillomavirus (HPV)

A significant proportion of SCCHN (20-25% of oropharyngeal and 20-50% of tonsillar SCC) are associated with HPV (GILLISON *et al.* 2000; HA and CALIFANO 2004; MORK *et al.* 2001). Furthermore, HPV-positive individuals have a fifteen-fold increased risk of developing these cancers (MORK *et al.* 2001). However, this has not been proven in other subsets of SCCHN (GILLISON *et al.* 2000). In an OSCC cell line, we demonstrated integration of human papillomavirus (HPV) within three chromosomal fragile sites (CFS), including the recently cloned *FRA9E* (CALLAHAN *et al.* 2003), suggesting that there may be an association between breakage at CFS and chromosomal instability in OSCC (RAGIN *et al.* 2004). The mechanism by which genetic instability is promoted through HPV infection relies on expression of viral oncogenes, E6 and E7 (HA and CALIFANO 2004). While E6 expression may result in failed cytokinesis, E7 expression may promote uncoupling of the centrosome duplication cycle with the cell division cycle (DUENSING and MUNGER 2001). Despite distinct pathways, both mechanisms are associated with lagging chromosomes as well as anaphase bridges that result from chromosome breaks and rearrangements. Therefore, E6 and E7 may not be mutually exclusive in cancer cells, and may act in combination to drive the phenotype of CIN.

2.3. GENETIC INSTABILITY

An understanding of the neoplastic process requires discussion of multiple mechanisms involved in promoting changes within a cell that lead to inefficient repair of DNA damage and unregulated proliferation of the damaged cells. The overall term used to describe cumulative abnormalities present within a tumor is genomic instability. Genomic instability results from the processes by which a somatic cell may become transformed through intrinsic DNA alterations, epigenetic changes, structural chromosomal aberrations, and numerical chromosomal changes. Despite the ongoing debate regarding how cells progress to malignancy, there is a general consensus that aside from normal mutation rates and possible clonal expansion of these mutations, three main sources of error can affect genomic stability in human cancers: nucleotide-excision repair instability (NER), microsatellite instability (MIN), and CIN. Loeb and colleagues refer to these terms collectively as a "mutator phenotype," in which a mutation in any of the genes responsible for maintaining DNA fidelity through replication, repair, chromosome segregation, damage surveillance, or apoptosis may be responsible for human tumor formation and progression (LOEB *et al.* 2003). While NER and MIN are usually characteristic defects in rare or hereditary cancers containing mutations in DNA mismatch repair genes, CIN is prevalent in cancers that do not contain nucleotide or microsatellite instability (LENGAUER *et al.* 1998).

2.3.1. Nucleotide Excision Repair (NER)

Nucleotide excision repair refers to the process of removing covalent alterations of DNA bases formed by exposure to environmental agents such as chemicals, ultraviolet light, or ionizing radiation (LENGAUER *et al.* 1998; SANCAR *et al.* 2004). Six factors are involved in NER in humans. DNA damage is recognized by three proteins: XPA, RPA, and XPC-TFIIH. Their primary function is to recognize the site of damage by binding randomly to DNA. The XPG factor makes a 3' incision by replacing XPC at the damage site, followed by 5' incision by XPF-ERCC1, resulting in the release of the damaged DNA (reviewed in (SANCAR *et al.* 2004)).

Individuals unable to properly repair mutations through NER have a higher susceptibility to cancer. Deficiencies in NER are manifested through diseases such as ataxia telangiectasia (AT), Bloom syndrome, Cockayne syndrome, and xeroderma pigmentosum (XP). In addition, this DNA damage pathway is particularly important for eliminating DNA adducts formed by exposure to tobacco smoke and ultraviolet radiation. The majority of genes involved in the NER pathway appear to be polymorphic and have been studied in an effort to identify an association between genetic polymorphisms and cancer risk in smokers, including SCC. However, none of the polymorphisms observed in individuals at high-risk for developing cancer have shown an odds ratio greater than 2.1 (WU *et al.* 2004). Therefore, the role of NER polymorphisms in tobacco-related cancers remains to be elucidated.

2.3.2. Microsatellite Instability (MIN)

Similar to NER, microsatellite instability results from defective DNA repair. MIN is suggested to result from genetic mutations within a small number of genes (LENGAUER *et al.* 1997b) in which MIN is expressed as a result of unrepaired strand slippage during DNA replication. Microsatellites consist of simple repeats that range from di- or tri-nucleotide repeats to five or six nucleotide repeats found throughout the human genome (ELLEGREN 2004). In many epithelial cancers including OSCC, breast, endometrium, lung, prostate, and stomach, alterations in microsatellite repeats have been observed (IONOV *et al.* 1993; ISHWAD *et al.* 1995; THIBODEAU *et al.* 1993; WOOSTER *et al.* 1994). Interestingly, tumor cells with microsatellite instability (MIN) have been shown to express diploid karyotypes without chromosomal instability (CIN) (LENGAUER *et al.* 1998).

Studies of both sporadic and hereditary nonpolyposis colon cancer (HNPCC) suggest that many tumors contain defects in mismatch repair genes (Day et al. 1996; Lengauer et al. 1997b). To date, eight mismatch repair (MMR) genes have been identified in humans: *MSH2*, *MSH3*, *MSH5*, *MSH6*, *MLH1*, *MLH3*, *PMS1*, and *PMS2* (MACDONALD *et al.* 2004). Human tumors expressing MIN have been shown to have defects in all MMR genes, except *MSH3* and *MSH5*. Normally, MIN is identified through the protein MSH2, and both MSH3 and MSH2 assist in controlling the specificity of MMR. In order to repair the damaged DNA, a repair complex

consisting of MLH1, PMS2, MLH1/ MSH3, or MLH1/PMS1 as well as other factors is activated (MACDONALD *et al.* 2004). Studies of HNPCC, prostate tumors, and tumors of the small intestine expressing a high level of MIN have been shown to contain mutations in the *MLH1* and/or *MSH2* genes (Chen et al. 2003; Day et al. 1996; Lengauer et al. 1997b; Planck et al. 2003). Reduced expression levels of MLH1 and MSH2 have been associated with fewer recurrences and relapses for individuals with bladder cancer. In contrast, patients with tumor cells expressing normal protein levels of hRAD50 and hMuts carry a higher probability of relapse (CATTO *et al.* 2003). A similar phenotype has been shown in colorectal cell lines and sporadic colorectal tumors that are hypermethylated for *hMLH1* (ARNOLD *et al.* 2004; KOINUMA *et al.* 2004; KUISMANEN *et al.* 2000; WHEELER *et al.* 1999). In addition, higher mutation rates are observed in tumors expressing mutations in MMR genes and may be the result of altered tumor suppressor gene function (NICOLAIDES *et al.* 1994). MIN and loss of expression of MLH1 has also been observed in esophageal SCC, however the authors concluded that the MIN was due to random replication errors rather than MMR deficiency, since loss of heterozygosity for p53 was detected in 90% of the tumors studied (HAYASHI *et al.* 2003).

2.3.3. Chromosomal Instability

CIN is a common feature of human tumors, including oral cancer. Although the karyotype of a particular tumor may remain quite stable over time, CIN can lead to "variations on a theme" of a clonal cell population, often with each cell within a tumor expressing a different karyotype. Thus, CIN appears to be an important acquired feature of tumor cells, since propagation of diverse cells may aid certain cell subpopulations in evading standard therapies. Although there are several sources of CIN, the most prominent causes appear to be defects in chromosomal segregation, telomere stability, cell cycle checkpoint regulation, and the repair of DNA damage. Our understanding of the biological basis of chromosomal instability in cancer cells is increasing rapidly and we are finding that the seemingly unrelated origins of this phenomenon may be related through the complex network of cellular signaling pathways.

For nearly a century, neoplastic cells have been studied to investigate the origin of chromosomal aneuploidy and karyotypic instability (BOVERI 1914; LOEB *et al.* 2003; RASNICK

and DUESBERG 1999). Numerous reports have demonstrated that while aneuploidy most frequently results from chromosomal segregation defects and aberrant centrosome numbers (PIHAN and DOXSEY 1999; SAUNDERS *et al.* 2000), structural chromosomal abnormalities mainly result from telomere loss, anaphase bridging, and defects in the DNA damage response (BASSING *et al.* 2003; CELESTE *et al.* 2003; GISSELSSON *et al.* 2002; GISSELSSON *et al.* 2000; SHUSTER *et al.* 2000). Despite these observations, interactions between the biological and genetic events that induce "chromosomal chaos" in tumor cells remain unclear and the mechanism(s) by which factors individually and cooperatively promote the CIN phenomenon in cancer are only starting to become elucidated (Fig. 1).

To date, more than 100 genes have been identified in yeast that are responsible for promoting, inducing, and propagating CIN through defects in the mitotic apparatus, cell cycle regulation, and mitotic checkpoint control (SPENCER *et al.* 1990). Alterations in the expression of genes affecting mitotic spindle formation, such as β-tubulin (HUFFAKER *et al.* 1988) or mutations in the adenomatous polyposis coli (APC) gene, have been shown to alter chromosome segregation and promote CIN (PIHAN and DOXSEY 1999). In human leukemias and lymphomas, overexpression of the microtubule-associated protein, stathmin/Op18, has also been implicated in causing CIN (NYLANDER *et al.* 1995). We and others have demonstrated that gene amplification may contribute to CIN through recurrent BFB cycles in human tumors (CIULLO *et al.* 2002; HELLMAN *et al.* 2002; SHUSTER *et al.* 2000). Mitotic checkpoint control genes that identify and correct spindle assembly defects, DNA damage, and errors in DNA replication include the human homolog *BUB1* gene, *hBUB1*, which appears to play a direct role in causing CIN when mutated in colorectal cancer cell lines (CAHILL *et al.* 1998). Although defects in genes that regulate the DNA damage response have been shown to occur in the presence of CIN, the underlying cause and effect relationships are not well understood (GOLLIN 2004; GOLLIN 2005). In addition, previous studies have determined that centrosome amplification is an early event in

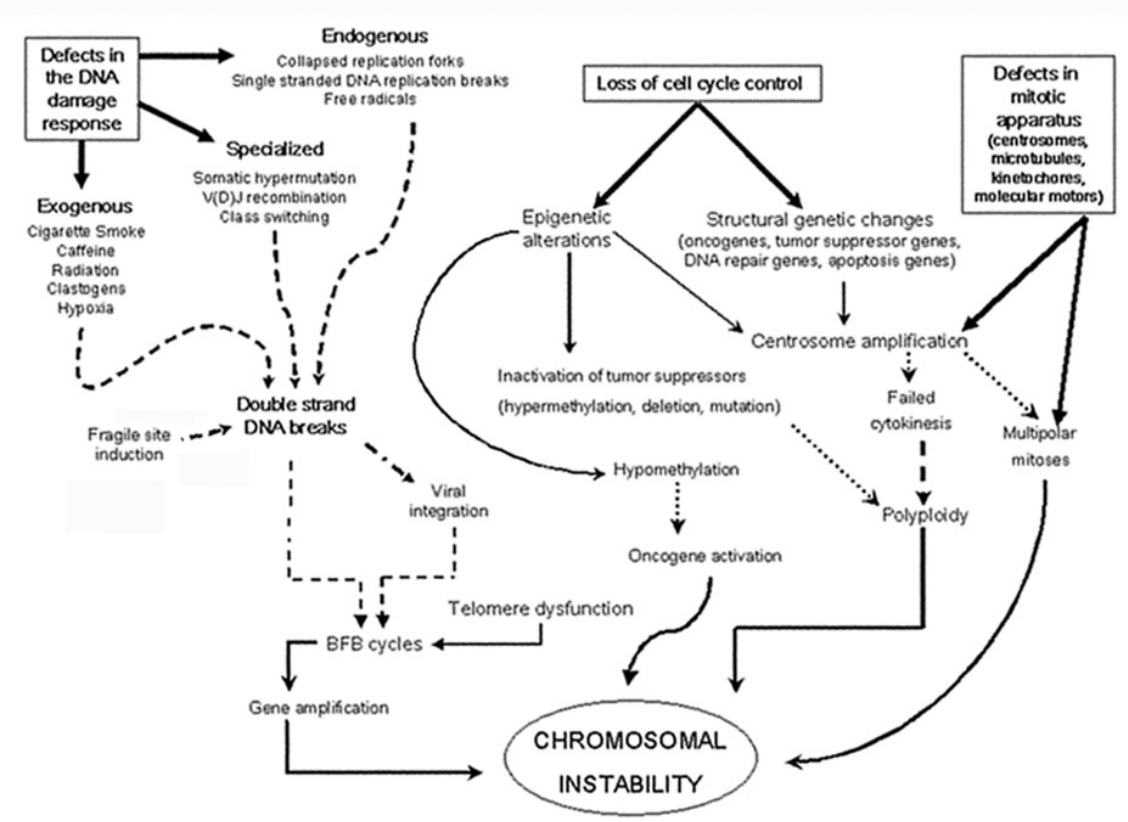

Figure 1. Origins of chromosomal instability[1].

tumor formation and CIN, although the link between the centrosome duplication cycle and the mitotic cycle has yet to be elucidated (BRINKLEY 2001; KRAMER *et al.* 2002). Despite the fact that the mechanism(s) responsible for alterations in centrosome number are not defined, centrosome status has become useful for monitoring neoplastic progression and for assessing patient prognosis for some tumor types (GISSELSSON *et al.* 2002; PIHAN *et al.* 1998). Thus, investigating the underlying causes of CIN may not only enhance diagnostic or prognostic information, but may also facilitate the development and administration of more effective treatment regimens for a wide variety of cancers.

The following sections review the general causes of chromosomal instability in human tumors. Specifically, they address the state of our knowledge regarding CIN in oral cancer and discuss various mechanisms responsible for the heterogeneous karyotypes seen in cancer cells within a tumor.

[1] In: Reshmi SC and Gollin SM (2005). J Dent Res 84(2): 107-17. Reprinted with permission.

2.3.3.1. Factors Leading to CIN: Loss of Cell Cycle Control

Alterations in genes governing cell cycle control provide a green light for continued proliferation of defective cells. Lengauer and colleagues (1998) described four main types of genes which, when altered, promote tumor progression: oncogenes, tumor suppressor genes, DNA repair genes, and genes regulating programmed cell death (apoptosis). Tumors displaying complex chromosomal aberrations often contain increased copy numbers of oncogenes known to promote cell differentiation and proliferation. At the same time, these tumors are also accumulating deletions or loss of genes responsible for detecting DNA damage, halting cell cycle progression, and/or mediating DNA repair (tumor suppressor genes) in cells prior to replication and/or cell division (KNUUTILA *et al.* 1999).

STRUCTURAL GENETIC ALTERATIONS

Previous studies have demonstrated that karyotypes of SCCHN and OSCC consist of near-triploid chromosome numbers and contain various patterns of cytogenetic aberrations, including structural and numerical chromosome abnormalities (GOLLIN 2001; JIN *et al.* 2002). These include, but are not limited to: aneuploidy (gains or losses of whole chromosomes resulting in a chromosome number altered from that of diploid cells, but which may or may not affect ploidy (AKERVALL *et al.* 1998); translocations (balanced or unbalanced rearrangement of chromosome segments or entire chromosome arms); insertions (breaks either within a chromosome or between two chromosomes resulting in the direct addition of chromosomal material or alternatively, the addition of material in the opposite, inverted direction); deletions (loss of small or large DNA segments); and amplifications (multiple additional copies of a specific gene or chromosomal region in the form of extrachromosomal double minutes (dmin) or intrachromosomal hsrs).

ONCOGENES

If unaltered, oncogenes (termed protooncogenes, or genes having the potential to be transformed into an oncogene as a result of mutation) refer to genes that direct cell growth through regulatory pathways. Oncogenes considered to play a role in SCCHN include growth factor receptors, such as *FGF3*, *EGFR* and *ERBB2*; intracellular signal transducers, such as *RAS* family members, *RAF1*, and *STAT3*; transcription factors, such as *MYC*, *FOS*, *JUN*, *MYB*; cell cycle regulators such as cyclin D1 (*CCND1*), and genes controlling apoptosis, such as *BCL2* and *BAX* (reviewed in (NAGPAL and DAS 2003). Alterations in any oncogene through chromosomal translocation, gene amplification, or viral insertion may provide an "on" switch for tumor development and/or progression.

TUMOR SUPPRESSOR GENES

Tumor suppressor genes (TSG) are negative growth regulators involved in cellular trafficking, regulation of the DNA damage response, and/or apoptosis (WEINBERG 1991). When tumor suppressor gene function or regulation is altered by mutation or hypermethylation (JAIN 2003), the ability to halt the proliferation of damaged cells is lost, allowing unrepaired cells to continue through the cell cycle. TSG including *FHIT*, *RB1*, *TP53,* and *CDKN2A* ($p16^{INK4A}$) have been shown to play key roles in SCCHN tumorigenesis (KOONTONGKAEW *et al.* 2000; NAKAHARA *et al.* 2000; VIRGILIO *et al.* 1996). Specifically, loss of *TP53* function has been shown to correlate with poor response to chemotherapeutic agents such as platinum drugs and fluorouracil, as well as with resistance to radiotherapy (HAMAKAWA *et al.* 1998; O'CONNOR *et al.* 1993; TEMAM *et al.* 2000). In addition, loss of 3p14 and/or 9p21 are considered to be early events in HNSCC, and along with *TP53* alterations, have been useful markers for monitoring increased recurrence risk (BRENNAN *et al.* 1995; CALIFANO *et al.* 1996; GOLLIN 2001; ISHWAD *et al.* 1996; ROSIN *et al.* 2002).

In the absence of a functional *TP53* gene, cells may become aneuploid (HARVEY *et al.* 1993). Studies of colorectal cancer cell lines with MIN have demonstrated that cells with *TP53* mutations do not exhibit CIN (BUNZ *et al.* 2002; ESHLEMAN *et al.* 1998; LENGAUER *et al.*

1997b). Therefore, it is plausible that while defects in cell cycle checkpoint genes such as *TP53* permit cells with CIN to continue through the cell cycle, mutations in genes affecting the mitotic apparatus are more likely to have a direct role in causing the observed CIN in various neoplastic tissues.

EPIGENETIC MODIFICATIONS

One of the most common, heritable mechanisms by which gene function can be altered without DNA sequence changes is the process of methylation. The presence of too little or too much methylation may have consequences in tumor cells. For example, undermethylated colorectal tumor cell lines (LENGAUER *et al.* 1997a), murine cells lacking DNA methyltransferase (*Dnmt1*) (CHEN *et al.* 1998; GAUDET *et al.* 2003) and methylation mutations in immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome (XU *et al.* 1999a) suggest that methylation defects contribute to genomic instability and CIN. In addition, retroviral integration and subsequent hypomethylation may encourage proto-oncogene activation (JAENISCH *et al.* 1985). However, while inhibitors of DNA methyltransferase have been shown to be effective in the treatment of some human cancers (KARPF and JONES 2002), they may actually cause an increased risk of genomic instability in others (GAUDET *et al.* 2003).

Hypermethylation has been shown to affect the fragile histidine triad (*FHIT*) gene in tumors of the breast and lung by silencing regions that may be important for suppression of tumor growth and proliferation under normal conditions (YANG *et al.* 2002; ZOCHBAUER-MULLER *et al.* 2001). Recent reports have also demonstrated hypermethylated regions in tumor suppressor genes, including *CDKN2B* (*p15*), *CDKN2A* (*p16*), and *TP53* in oral cancer (YEH *et al.* 2003) and hypomethylation of oncogenes in metastatic HNSCC (SMIRAGLIA *et al.* 2003). Investigators are finding increasing evidence that specific tumors carry signature methylation patterns (EADS *et al.* 2001; FENG *et al.* 2005; TSOU *et al.* 2002). Thus, various investigators have suggested that methylation patterns could be useful screening tools for identifying individuals who may be at an increased risk for cancer development (FEINBERG and TYCKO 2004; MOMPARLER 2003).

2.3.3.2. Factors Inducing CIN

DEFECTS IN DNA DAMAGE RESPONSE PATHWAYS

Recent studies have revealed that many cellular pathways appear to be interconnected and/or intertwined. The task of determining which DNA repair genes are involved in tumor progression and chromosomal instability remains challenging. This is partly due to the observation that the same gene may play a role in one or more DNA damage response pathways. In addition, if one repair pathway is unable to function, it has been demonstrated that another repair pathway may be activated in its place (CLINE and HANAWALT 2003). Examination of faulty DNA double strand break (DSB) repair caused by exposure to DNA damaging agents suggests that neoplasms may arise through factors influencing the ability of a cell to respond to alterations in DNA sequence. Such factors include gene mutation, chromosomal deletions or amplifications, and/or other chromosomal alterations. There are five major DNA repair pathways: homologous recombinational repair (HRR), non-homologous end joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) (BERNSTEIN *et al.* 2002). Each is comprised of proteins essential for detecting and repairing specific types of DNA damage, but may also promote apoptosis in irreparably damaged cells.

For the purpose of discussing chromosomal instability in oral cancer, there are key regulators that sense and respond to DSB, including the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) genes. Both have overlapping as well as unique responses to DNA damage and phosphorylate more than 15 known substrates, including p53, BRCA1, and the CHK1 and CHK2 kinases (SHILOH 2003). Several of these genes, including *ATM*, *TP53*, *BRCA1*, *CHEK2, FANC, BLM,* and *MRE11A* are involved in familial cancer syndromes and may influence CIN (BECKER-CATANIA and GATTI 2001; GOLLIN 2004; GOLLIN 2005). The majority of human tumors carry *TP53* mutations or are deficient in genes that initiate or respond to p53 function (NIGRO *et al.* 1989). The p53 protein plays a major role in directing proteins involved in homologous recombination DSB repair, including BRCA1 and BRCA2, as

well as RAD52. However, p53 may also negatively regulate proteins, including RAD51 (BUCCHOP *et al.* 1997), such that upregulation of RAD51 in tumors allows for a selective growth advantage for tumor cells in the absence of functional p53 (HENNING and STURZBECHER 2003).

Though not studied in the current investigation, ongoing research in our laboratory is pursuing the hypothesis that loss of distal 11q is a critical step for promoting subsequent events involved in OSCC progression. Chromosomal breakage at or near band 11q13 has been shown to result in loss of the distal segment of chromosome 11 (JIN *et al.* 1998b). Within the chromosomal segment distal to 11q13 are genes required for proper response to DNA damage. These include: *MRE11A* (*MRE11* meiotic recombination 11 homolog A) at 11q21, histone *H2AFX* (*H2A* histone family, member X) at 11q22.3, the ataxia-telangiactasia mutated (*ATM*) gene at 11q23.2-q23.3, and *CHEK1* (*CHK1* checkpoint homolog) at 11q24. Since localization of these genes is within the distal region of chromosome 11, which is lost prior to 11q13 gene amplification in almost half of OSCC, haploinsufficiency for these genes may be responsible for some of the observed CIN in oral cancer cells (Gollin and colleagues, unpublished data). Although the exact relationship between loss of function of these genes and CIN is currently unclear, the presence of haploinsufficiency for at least one of the DNA damage response genes, *H2AFX,* has been associated with the CIN phenotype in mice (BASSING *et al.* 2003; CELESTE *et al.* 2003). Fernandez-Capetillo et al. (2002) demonstrated that, under conditions of increasing DNA damage, *H2afx*$^{-/-}$ mice with functional Chk2 within the G_2-M checkpoint signaling complexes are able to activate DNA repair pathways that do not require *H2afx*. In addition, the authors showed that H2AX status affects phosphorylation of the DSB signaling protein, Trp5sbp, which has been recognized to affect phophorylation of Brca1 which is required for both S-phase and G_2-M checkpoints following IR-induction (XU *et al.* 2001). Somatic *MRE11A* mutations in colorectal cancer cell lines indicate the potential of a causal association between these mutations and CIN, but this remains to be determined (WANG *et al.* 2004b). In addition, mice with mutant alleles for *Mre11a* phenotypically express cell cycle defects and genomic instability through impaired ATM function, but only progress to malignancy when present in p53$^{+/-}$ mice (THEUNISSEN *et al.* 2003). The consequence of distal 11q loss in OSCC cells may be loss of multiple tumor suppressor genes, that when combined with subsequent 11q13 gene amplification, results in an aggressive malignant phenotype (JIN *et al.* 1998b).

CHROMOSOMAL ANEUPLOIDY

The term aneuploidy refers to the condition in which the chromosome number of the cells of an individual is not an exact multiple of the typical haploid set for that species (KING and STANSFIELD 2002). The presence of chromosomal aneuploidy in both early stage carcinomas and malignant neoplasms in general suggests its involvement, either directly or indirectly, in tumor progression (MITELMAN *et al.* 2004).

Inactivation of several chromosomal segregation-related genes in normal human fibroblasts has been shown to influence early chromosome changes in preneoplastic cells. For example, haploinsufficiency for the spindle assembly checkpoint gene, *MAD2*, results in elevated frequencies of CIN in human cancer cells and murine primary embryonic fibroblasts (MICHEL *et al.* 2004). Contrary to other inactivated checkpoint genes, only defects in *BUB1* provided cells with the ability evade apoptosis (MUSIO *et al.* 2003). Taken together, these studies suggest that the state of aneuploidy and/or chromosomal instability may be due to inefficient functioning of mitotic checkpoints, which may result in neoplastic progression.

Sporadic missegregation, polyploidization, and/or defects in the mitotic apparatus may also promote aneuploidy. Aborted mitosis (restitution) may result in failed formation of the cleavage furrow, leading to numerical doublings of chromosomes and centrosomes, termed tetraploidization (NIGG 2002). This has been observed in a variety of cancers and may give rise to chromosomal aneuploidy through subsequent chromosome loss (GALIPEAU *et al.* 1996; SHACKNEY *et al.* 1995; SOUTHERN *et al.* 1997). Oksala and Therman (1974) described the occurrence of tetraploidization particularly through disrupted cell division. In addition to mitotic arrest, a tetraploid chromosome number may result from failure of mitotic spindle formation (endomitosis), in which cells fall short of undergoing cytokinesis, resulting in a cell containing double the chromosome complement. Tetraploidization may also occur through C-mitosis (spindle arrest in which chromosomes fail to align, resulting in the formation of several micronuclei comprised of the equivalent number of chromosomes in a tetraploid nucleus) or endoreduplication (chromosome replication carried out two times without an interruption by mitosis). Still other cells may undergo amitosis (fusion of two nuclei from different stages of the

cell cycle) or tripolar mitoses, the latter of which can result in three daughter cells, two of which may have near-triploid karyotypes and one with a near-diploid karyotype, all exhibiting chromosomal aneuploidy and CIN.

The status of chromosomal ploidy in leukoplakia appears to be an important prognostic marker for oral carcinoma development and progression. Sudbø et al. (2004) carried out a retrospective study designed to assess cancer development in 150 patients with dysplastic oral leukoplakia. The data revealed that of the patients identified with aneuploid epithelial dysplasia, 96% (26/27) developed cancer compared to slightly fewer (80% or 16/20) of those with tetraploid lesions and only 5% (5/103) having diploid lesions. Forty-five of 47 (96%) patients had negative resection margins and postoperative radiotherapy. Of the five patients with a normal diploid chromosomal content, none developed a recurrence following tumor resection. However, more striking were the comparisons between patients with tetraploid lesions versus aneuploid lesions. The study revealed that 25% (4/16) of patients with tetraploid status developed a recurrence, but survived. This is in sharp contrast to the 26 patients with primary cancers due to aneuploid leukoplakia, in which 85% (22/26) showed evidence of recurrence and only one survived. Thus, there appears to be evidence suggesting that aneuploidy in the target tissue may be useful for predicting cancer development and recurrence, but has not yet proven to be useful for assessing response to intervention in OSCC.

MITOTIC APPARATUS DEFECTS

Karyotypic heterogeneity visualized by interphase cytogenetic analysis suggests that cytoskeletal defects may promote both clonal and nonclonal structural rearrangements in solid tumors, resulting in daughter cells that do not resemble each other or their mother cell (reviewed in (PIHAN and DOXSEY 1999; RESHMI *et al.* 2004). The mitotic machinery is comprised of microtubules, centrosomes, kinetochores, and molecular motors. Properly choreographed coordination of these structures is essential for accurate chromosome segregation during mitosis. Defective functioning of the mitotic apparatus may continuously influence differences in both chromosome number and structure through subsequent cell divisions.

Microtubules

Microtubules provide 'tracks' for chromosome movement and the microtubule motors carry the chromosome cargo along the tracks (PIHAN and DOXSEY 1999). Alterations in microtubules directly resulting in chromosomal aneuploidy have not been demonstrated. However, a study by Lingle et al. (1998) showed that in breast tumors, increased levels of the centrosomal proteins, centrin and γ-tubulin, corresponded with significantly larger sized centrosomes.

Centrosomes

David Hansemann (1890) was first to report the presence of abnormal mitoses in cancer cells. His findings provided the groundwork for Theodor Boveri's hypothesis that abnormal centrosome numbers influence chromosome segregation in cancer cells (BOVERI 1914). Subsequent studies have determined that centrosome amplification is indeed an early event in tumor formation (BRINKLEY 2001; LINGLE *et al.* 2002) and has been observed in cancers of the brain, bile duct, breast, colon, head and neck, lung, pancreas, and prostate and in human papillomavirus (HPV 16/18)- infected cervical cancers (NIGG 2002). Various proteins that associate with centrosomes have been shown to influence centrosome duplication in human cancer. Pericentrin levels were found to be elevated in breast and pancreas tumors (LINGLE *et al.* 2002; PIHAN *et al.* 1998; PIHAN *et al.* 2001; SATO *et al.* 2001; ZHOU *et al.* 1998). Overexpression of cyclin E is associated with centrosome hyperamplification in both cultured cells and p53 null, heterozygous, and wild-type mice (MUSSMAN *et al.* 2000). CIN has also resulted from alterations of the centrosomal protein, γ-tubulin, which may be driven by overexpression of DNA polymerase β (BERGOGLIO *et al.* 2002).

Overexpression of STK15/BTAK/aurora-A kinase in human pancreatic cancers (LI *et al.* 2003) and ovarian cancers (GRITSKO *et al.* 2003) also results in centrosome amplification. Katayama et al. (2004) recently demonstrated a role for aurora kinase A in p53 phosphorylation, subsequently leading to increased Mdm2 binding. Properly functioning Mdm2 targets p53 for ubiquitination and degradation. Thus, the presence of increased levels of aurora-A kinase protein combined with increased Mdm2 expression results in cells with abnormal centrosomes

and aberrant chromosome numbers to continue through the cell cycle by inactivating p53. Of particular interest in this study was the finding that MCF7 breast cancer cells overexpressing aurora-A kinase exposed to the DNA-damaging agent, cisplatin, were resistant to apoptosis. Thus, developing therapeutic agents targeting centrosomal proteins including aurora-A kinase are on the horizon (HARRINGTON *et al.* 2004).

Other genes that may influence centrosome amplification are key regulators of the DNA damage response pathway and include *ATR* (FUCHS and CLEVELAND 1998), *BRCA1* (SCHLIWA *et al.* 1999), *BRCA2* (PIEL *et al.* 2001), and *XRCC2/3* (GRIFFIN *et al.* 2000). Proper functioning of the CHEK2 DNA damage kinase is required for influencing centrosomes in the absence of CHEK1. The effect of losing the CHEK2 kinase appears to be epistatic, resulting in failed cytokinesis, increased centrosome number, and multipolar spindles (reviewed in Saunders, 2005). In addition, several genes necessary for protein degradation and mitosis have been shown to contribute to CIN by influencing centrosome numbers (reviewed in Nigg, 2002). Transactivation of *TP53*, *BRCA2*, and *GADD45A* (growth arrest and DNA-damage-inducible, alpha) by *BRCA1* has been implicated in centrosome amplification (reviewed in Deng, 2002). It has recently been shown that *NEK2* (never in mitosis gene a-related kinase) is critical for proper formation of bipolar spindles (FARAGHER and FRY 2003). *NEK2* appears to have an inverse relationship with *BRCA1*. Overexpression of NIMA-related kinase 2 in Brca1-specific small interfering RNA treated, wild-type and Gadd45a- null mouse cells blocks hyperamplification of centrosome (WANG *et al.* 2004a). Similar results have been observed between the interaction of BRCA1 with RB1 or CDK2 which may also give rise to cells without centrosome duplication (reviewed in Deng, 2002). These findings suggest that associations of BRCA1 with RB1 or CDK2 as well as proper functioning *NEK2* play essential roles in maintaining genome stability. Although defective genes within the DNA damage response pathway have individually been shown to affect chromosomal instability through improper DNA double strand break repair, the exact mechanism by which they act in centrosome amplification remains to be defined (GOLLIN 2004; GOLLIN 2005). However, centrosome amplification has enabled monitoring of neoplastic progression, and has been shown to aid in the prognostic assessment of certain tumor types (KUO *et al.* 2000; PIHAN *et al.* 2001), including those of the head and neck (GISSELSSON *et al.* 2002; GUSTAFSON *et al.* 2000).

Despite ongoing clinical trials in gene therapy, immunotherapy, and molecular-based agents for treatment of SCCHN, the heterogeneous nature of the cell populations within these tumors challenges the success of therapies targeted at specific genes. Due to the recently identified connection between the DNA damage checkpoints and the DNA damage response pathway, additional therapies targeting checkpoint kinases such as CHEK1 and CHEK2, may chemosensitize cancer cells defective in the G_2/M checkpoint and improve the overall prognosis for oral cancer patients (ZHOU and SAUSVILLE 2003). Other cancer therapies focusing on the mitotic apparatus have proven to be effective for some cancers (ECKHARDT 2002; WALCZAK and CARDUCCI 2002). For example, microtubule inhibitors, such as Taxol (paclitaxel), Taxotere (docetaxel), and *Vinca* alkaloids (Vindesine, Vinorelbine), promote mitotic arrest and induce apoptosis and are used to treat solid tumors of the breast, esophagus, prostate, and lung (MOLLINEDO and GAJATE 2003; WALCZAK and CARDUCCI 2002). Moreover, drugs such as VX-680 that inhibit Aurora kinases and consequently suppress tumor growth *in vivo* are on the horizon (HARRINGTON *et al.* 2004). However, it has been suggested that in tumors with improper functioning checkpoints, these drugs may actually promote further CIN (DRAVIAM *et al.* 2004). This has been shown in colorectal cancer cell lines harboring mutations in checkpoint genes, in which cell lines expressing CIN were unaffected by anti-cancer agents such as nocodazole (CAHILL *et al.* 1998). Thus, further insight into the mechanisms driving CIN will undoubtedly provide opportunities to identify new biomarkers to aid in diagnosis and prognosis, and achieve the ultimate goal of developing novel therapeutic strategies for patients with SCCHN.

TELOMERE DYSFUNCTION

Telomeres are small repetitive sequences located at the ends of chromosomes. Their biological function is to protect chromosome ends from being "sticky" and from shortening at each DNA replication (BLACKBURN and CHALLONER 1984). When telomeres become too short, programmed cell death is initiated through activation of *TP53*-mediated apoptosis (CHIN *et al.* 1999). In the absence of a functional *TP53* checkpoint, tumor cells with shortened telomeres may escape apoptosis. The resulting "sticky," uncapped chromosome ends are then free to associate with each other, causing end-to-end fusions which form dicentric chromosomes. We

and others have observed that migration of dicentric chromosomes during cell division may result in anaphase bridges (GISSELSSON *et al.* 2002; MCCLINTOCK 1938; MCCLINTOCK 1939). The pulling of two active centromeres to opposite poles creates an anaphase bridge, resulting in random, broken chromosome segments within each daughter cell. The free, uncapped ends of these broken chromosomes are then capable of joining with other chromosomes lacking telomeres, giving rise to CIN. If the new chromosome contains two active centromeres, anaphase bridge formation may again occur, promoting breakage-fusion-bridge (BFB) cycles (CHIN *et al.* 1999; MASER and DEPINHO 2002). However, some cancer cells may activate the telomere maintenance enzyme, telomerase, which provides a selective growth advantage through telomere stabilization (MEYERSON 1998; VAZIRI and BENCHIMOL 1998). Telomeres added to the ends of broken chromosomes resulting from BFB cycles may prevent chromosomes from further structural rearrangement and/or BFB cycles. Studies of breast cancer, colorectal cancer, and leukemias have demonstrated that low levels of telomerase are present in preneoplastic cells compared to significantly higher levels in advanced stage tumors (reviewed in Maser and DePinho, 2002). In addition, telomerase status and expression of its subunits, particularly telomerase reverse transcriptase, *hTERT*, has proven to be a useful prognostic indicator in patients with OSCC. Kannan et al. (1997) observed telomerase activity in normal, hyperplastic, well-differentiated, and moderately or poorly differentiated oral tumors and established a correlation between telomerase activity and OSCC tumor grade. Later, Lee and colleagues (2001) demonstrated that 35 of 46 (76%) oral tumors showed hTERT expression and telomerase activity compared to no activity in the control specimens. Due to the increased expression of telomerase in neoplastic cells, current research focusing on ways to reverse cellular immortality is actively being pursued. Studies have shown that oral dysplastic cells treated with 5-aza-2-deoxycytidine senesce resulting from the downregulation of telomerase (MCGREGOR *et al.* 2002). In addition, other therapies targeting telomerase have been suggested for a wide variety of tumor types (SHAY and RONINSON 2004).

2.3.3.3. Factors Propagating CIN

COMMON FRAGILE SITES (CFS)

Chromosomal fragile sites (CFS) are sensitive regions of the genome which may form gaps or breaks in metaphase chromosomes when cells are grown under conditions that interfere with DNA replication and/or repair, including treatment with the DNA polymerase-α inhibitor, aphidicolin (ARLT *et al.* 2003). When treated with the G_2M checkpoint inhibitor, caffeine, cells with unrepaired chromatin breaks enter mitosis and the incomplete repair may be visualized as breaks in chromosomes within metaphase cells (HECHT and GLOVER 1984).

In contrast to rare fragile sites which are usually associated with specific disease phenotypes, there appears to be no underlying basis for the existence of common fragile sites within all individuals. To date, nearly 90 common fragile sites have been identified (SUTHERLAND *et al.* 1998). Although the current understanding of the structural makeup and function of chromosomal fragile sites remains largely unknown, fragile sites are thought to be the sites of stalled replication forks that result in DSB (CASPER *et al.* 2002). Thus, a chromosomal rearrangement at a fragile site may be the result of an attempt to repair a cell exposed to DNA damaging agents such as cigarette smoke (GLOVER 1998; STEIN *et al.* 2002), caffeine, hypoxia, radiation, free radicals (RESHMI and GOLLIN 2005) and viruses such as HPV (RAGIN *et al.* 2004; THORLAND *et al.* 2003). Based on what is currently known regarding CFS, many cover fairly large regions of DNA and contain areas of high flexibility (ARLT *et al.* 2003). However, despite differences in chromatin structure that confer a late replication pattern (LE BEAU *et al.* 1998), the increasing evidence that CFS are highly conserved between species suggests that key genes may be located within them (GLOVER *et al.* 1998; KRUMMEL *et al.* 2002; ROZIER *et al.* 2004; SHIRAISHI *et al.* 2001).

A role for fragile sites in cancer was proposed two decades ago (LEBEAU and ROWLEY 1984; YUNIS and SORENG 1984). Both suggested that various cancer breakpoints including those in leukemias, lymphomas, and solid tumors may correspond to chromosomal fragile sites. Genes within this region may then be deleted, the function may be altered if translocated with another

chromosome, or alternatlvely involved in gene amplification. Several investigators have proposed that gene amplification may be the protective response of a cell to chromosomal breakage or DNA damage, treatment with cytotoxic drugs or to oxygen deprivation (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; KUO *et al.* 1998; PIPIRAS *et al.* 1998; SINGER *et al.* 2000; STARK 1993; TONNIES *et al.* 2003; WINDLE *et al.* 1991). To date, nine common fragile sites, *FRA3B, FRA4F, FRA6E, FRA7G, FRA7H, FRA8C, FRA9E, FRA16D,* and *FRAXB* have been characterized and shown to exhibit genomic instability through loss of heterozygosity (LOH), loss of expression, viral integration, or promoting gene amplification in a variety of human tumors (ARLT *et al.* 2002; CAHILL *et al.* 1998; CALLAHAN *et al.* 2003; DENISON *et al.* 2003; HUANG *et al.* 1998; HUEBNER and CROCE 2001; KRUMMEL *et al.* 2000; MORELLI *et al.* 2002; ROZIER *et al.* 2004). We previously reported LOH at 3p14.2 and aberrant transcript expression of *FHIT* in approximately one half of our OSCC cell lines (VIRGILIO *et al.* 1996). In addition, we demonstrated integration of human papillomavirus (HPV) within three CFS, including *FRA9E*, suggesting an association between breakage at a CFS and chromosomal instability in OSCC (RAGIN *et al.* 2004).

Direct evidence has been found to link gene amplification with BFB events involving fragile sites in humans. Ciullo and colleagues (2002) demonstrated that breakage at *FRA7I* induced amplification of the prolactin inducible protein (*PIP*) gene, which is overexpressed in tumors of the prostate and metastatic breast cancer (AUTIERO *et al.* 1999; CLARK *et al.* 1999). They determined the physical map location for *FRA7I*, and carried out fluorescence *in situ* hybridization (FISH) using a T47D breast carcinoma cell line. Dual-color FISH using a probe located proximally to *FRA7I* along with a probe for *FRA7I* revealed an inverted repeat pattern within T47D cells. Confirmation of the BFB mechanism was carried out by additional FISH studies using a probe for the *PIP* gene along with a region telomeric to *FRA7I,* in order to demonstrate that sequences distal to the amplicon were not duplicated. *In vivo* studies by Hellman et al. (2002) showed that amplification of the *MET* oncogene at *FRA7G* in gastric carcinomas resulted in clustering of recurrent breaks within the *FRA7G* site such that amplified segments displayed an inverted repeat pattern, as would be observed as a result of BFB cycles (HELLMAN *et al.* 2002; SHUSTER *et al.* 2000). Since subsequent breaks are considered to occur randomly as a result of anaphase bridging, BFB cycles may promote variations of amplicon length depending on the site of breakage (TOLEDO *et al.* 1992). Taken together, these

investigations suggest that CFS breakage may enable subsequent amplification of genes that are not necessarily located within the fragile region, but proximal to it. Intrachromosomal gene amplification through fusion of sister chromatids may ensue, leading to BFB cycles and further chromosomal instability.

Four fragile sites have been identified in and around chromosomal band 11q13: *FRA11A* at 11q13.3, *FRA11B* at 11q23.3, *FRA11F* at 11q14.2, and *FRA11H* at 11q13. Of these, increased breakage at *FRA11A* has been demonstrated in blood cells of smokers compared to nonsmokers (KAO-SHAN *et al.* 1987). In addition, chromosome breakage at a CFS was observed in *FRA11B*, a rare folate-sensitive fragile site implicated in Jacobsen syndrome through breakage and subsequent chromosomal deletion of the proto-oncogene, *CBL2* (JONES *et al.* 1994). Typical features of this disorder include psychomotor delay, trigonocephaly, facial dysmorphism, cardiac defects, and thrombocytopenia, though none appear to occur consistently (PENNY *et al.* 1995). However, genotypic manifestations of rare fragile sites have typically been found to occur as trinucleotide expansion repeat disorders, rather than loss at a single locus (JONES *et al.* 2000). Both *FRA11H* and *FRA11F* are common fragile sites flanking the region harboring *CCND1*. Therefore, it is possible that amplification of *CCND1* and other genes in band 11q13 may be due to their chromosomal location, since they appear to be surrounded by hotspots for chromosomal breakage. Previous investigations from our laboratory have uncovered proximal and distal breakpoint regions relative to the commonly amplified 11q13 segment (HUANG *et al.* 2002). Taken together, these findings suggest that two breakage events, perhaps at fragile sites, may occur 1) between the *RIN1* and *CCND1* genes and 2) distal to the *CCND1* gene, resulting in BFB cycles.

GENE AMPLIFICATION

Gene amplification is a common event in tumors and has been observed both *in vivo* and *in vitro* (STARK 1993; TLSTY *et al.* 1995). Models for initiating gene amplification propose that this event may be the protective response of a cell to treatment with cytotoxic drugs (KUO *et al.* 1998; SINGER *et al.* 2000; STARK 1993; TONNIES *et al.* 2003) or to oxygen deprivation (COQUELLE *et al.* 1998; RICE *et al.* 1986). Current studies have demonstrated that gene amplification may be the result of breakage at common fragile sites (CIULLO *et al.* 2002;

COQUELLE *et al.* 1997; PIPIRAS *et al.* 1998; STARK 1993; TONNIES *et al.* 2003). Collectively, these investigations propose that the initiating step for gene amplification is a double strand break.

Two basic forms of gene amplification have been observed in mammalian cells. Spriggs and colleagues (1962) first described the presence of dmin chromosomes in lung tumor cells. Dmin appear as extrachromosomal pairs of chromatin in metaphase cells, and have since been observed in a variety of human malignancies (SCHWAB 1999). A second form of gene amplification is present as a uniformly stained and expanded chromosomal band, termed an hsr. Hsrs were first described by Biedler and Spengler (1976) in metaphase cells from antifolate-resistant Chinese hamster lung cultures. The hsrs were later shown to correlate with resistance to methotrexate through increased levels of the enzyme, dihydrofolate reductase (MILBRANDT *et al.* 1981). Hsrs are early replicating (HAMLIN and BIEDLER 1981; MILBRANDT *et al.* 1981), suggesting that genes residing within the amplified region (amplicon) may have essential roles in regulating cellular functions (HOLMQUIST 1992). Regardless of the form of amplification, overexpression of genes in dmin or hsrs has been associated with tumor progression (BOCKMUHL *et al.* 2002; COQUELLE *et al.* 1998; GOLLIN 2001; MICHALIDES *et al.* 1995). In addition, the presence of gene amplification has proven to be a useful prognostic indicator for overall survival (AKERVALL *et al.* 1997; BRODEUR 2003; BRUCKERT *et al.* 2000; FIELD 1992) and response to therapy (PEGRAM *et al.* 2000).

In OSCC, amplification of chromosomal band 11q13 occurs in the form of an hsr (BARTKOVA *et al.* 1995; LESE *et al.* 1995; MICHALIDES *et al.* 1995). Amplification and overexpression of genes within 11q13 has been observed in approximately 45% of OSCC (AKERVALL *et al.* 1997; BARTKOVA *et al.* 1995; GOLLIN 2001; JIN *et al.* 1998b; LESE *et al.* 1995), and in smaller percentages of other carcinomas such as those of the breast, bladder, liver, pancreas, ovary, and aerodigestive tract (SCHRAML *et al.* 1999). Genes contained in this region include cyclin D1 (*CCND1*), cortactin (*EMS1*), fibroblast growth factors 3, 4, and 19 (*FGF3* and *FGF4* also known as *INT2* or *HSTF1*, respectively), fas associated via death domain (*FADD*), and tumor amplified and overexpressed sequence 1 and 2 (*TAOS1*and *TAOS2*)(BEKRI *et al.* 1997; HUANG *et al.* 2002; KATOH and KATOH 2003; SCHUURING *et al.* 1998). In particular, *CCND1* gene function is critical for regulating cell division (JEANNON and WILSON 1998), and has been shown to play a key role in the pathogenesis of OSCC (AKERVALL *et al.* 1997; CHAMPEME *et al.*

1995; MICHALIDES *et al.* 1995) as well as in local recurrence of breast cancer (CHAMPEME *et al.* 1995).

Previous investigations by our laboratory and others suggested a BFB cycle model for gene amplification (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; MCCLINTOCK 1938; MCCLINTOCK 1939; SHUSTER *et al.* 2000), in which gene amplification occurs through anaphase bridges resulting from dicentric chromosomes formed by sister chromatid fusion of broken chromosome ends. However, four other models for gene amplification have been observed in mammalian cells. These include, 1) unequal sister chromatid exchange; 2) re-replication and integration; 3) episome-excision, and 4) conservative transposition. The unequal sister chromatid exchange model is based on DNA damaging agents and/or chemicals in which cells overcome the insult through amplification of a protooncogene (PALL 1991). This requires recombination of sequences flanking the amplified locus, in which the one cell receives two copies of the locus, and the other cell is then deficient for that locus. The cell carrying the deletion would be unable to compensate for the lost locus (HAMLIN *et al.* 1984). In addition, gene amplification other models for gene amplification are not commonly assocaiated with an inverted duplication pattern of gene amplification. The surviving amplified cell may then continue through subsequent cell cycles, which may result in further unequal sister chromatid exchanges and amplicons with varying length arranged in tandem[2]. The overreplication and reintegration model suggests formation of: a) rereplicated DNA within loop structures that result in exrachromosomal DNA; b) sister chromatid breakage with varying degrees of fragmentation; c) inversion of the entire recombined sequence[3], d) a dicentric chromosome and loss of material distal to the recombination site; e) endoreduplication or the entire chromosome or partial duplication of a chromosome (reviewed in (SCHIMKE *et al.* 1986)). Both deletion/episome and conservative transposition models suggest a rolling circle replication intermediate (YOUNG and CULLUM 1987). The rolling circle replication intermediate within these models suggests that a double-stranded break occurring prior to replication will repair itself by non-homologous recombination with either similar sequence ends or non-homologous ends. Recombination of the chromosome ends may initiate replication fork formation, in which a chromosome loop would form into a rolling circle. The rolling circle would continue to promote replication within a

[2] Tandem duplication would resemble a sequence expressing: a-bcd-bcd-bcd-bcd-e.

[3] Suggests that an original sequence expressing –abcdefgh would be expressed as –hgfedcba within the overreplicated sequence.

single replication cycle until stalling of the replication fork occurs. Replication fork stalling may then lead to another recombination event, which integrates the tandem sequence back into the chromosome and is observed as an hsr (reviewed in (HAMLIN *et al.* 1991; HASTINGS and ROSENBERG 2002; SCHIMKE 1984; STARK 1993; STARK and WAHL 1984; TRASK and HAMLIN 1989; WINDLE and WAHL 1992). If the sequence does not reintegrate, extrachromosomal dmin would then be expressed within the cell. The deletion/episome model suggests the amplified gene locus results from a chromosomal deletion in which the deleted sequence remains in the form of an episome. The episome would then undergo rolling circle replication (WAHL 1989), and eventually manifest itself in the form of a dmin. This model requires the episome to re-integrate into its original locus in order to be observed as an hsr (TRASK and HAMLIN 1989). Lastly, the conservative transposition model for gene amplification suggests the presence of amplicon clusters on the same chromosome arm as the original locus, but distal to the original locus. Our current study will investigate the primary mechanism by which 11q13 gene amplification occurs in OSCC cells.

BREAKAGE-FUSION-BRIDGE (BFB) CYCLES

One consequence of chromosomal breakage (and or telomere loss) is thought to involve fusion of sister chromatids following replication, giving rise to a dicentric chromosome. At anaphase, the dicentric chromosome may be pulled to opposite poles, resulting in breakage, termed breakage-fusion-bridge cycles (MCCLINTOCK 1938; MCCLINTOCK 1939). The formation of dicentric chromosomes (ARTANDI *et al.* 2000; RIBONI *et al.* 1997; SAWYER *et al.* 2000) and anaphase bridging (GISSELSSON *et al.* 2002; SAUNDERS *et al.* 2000) then results in CIN. We and others have shown that BFB cycles may lead to gene amplification (SHUSTER *et al.* 2000; SINGER *et al.* 2000) which most likely occurs through sister chromatid fusion (MA *et al.* 1993) leading to the formation of inverted duplications of the amplified segment in which the size of the amplicon determined by the site(s) of breakage (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; TOLEDO *et al.* 1992). In addition, amplification due to BFB cycles may contain imperfect head-to-head symmetries as well as unequal distances between the amplicon and the telomere in clonal cell populations (TOLEDO *et al.* 1992). We and others have also identified anaphase bridges and

lagging chromosomes in OSCC cells (GISSELSSON *et al.* 2002; SAUNDERS *et al.* 2000). These observations strongly suggest the presence of BFB cycles as one possible mechanism for 11q13 amplification in OSCC.

SUMMARY

The major focus of the research presented herein addresses the question of how amplification of band 11q13 occurs and promotes CIN in OSCC. To summarize our previous findings, we: 1) identified 11q13 gene amplification in the form of an hsr (LESE *et al.* 1995); 2) determined the presence of an inverted duplication pattern within the 11q13 amplicon (SHUSTER *et al.* 2000); and 3) physically mapped the 11q13 amplicon and demonstrated breakpoint cluster regions flanking the amplified region (HUANG *et al.* 2002). The current study investigates three patterns of CIN observed in OSCC: numerical chromosomal aberrations, structural chromosomal alterations, and gene amplification. The following sections provide an overview of the rationale behind developing and testing our current hypothesis that **11q13 gene amplification in OSCC results from BFB cycles, and that breakage at a CFS may be the initial step in 11q13 gene amplification.**

3. MATERIALS AND METHODS

This investigation utilized twenty-eight human oral squamous cell carcinoma (OSCC) cell lines which were developed from outgrowths derived from primary tumors of consenting patients who were not previously treated with radiation therapy or chemotherapy (S.M. Gollin, J.K. Reddy, S. Comsa, K.M. Rossie, C.M. Lese, B.N. Appel, R. Wagner, E.N. Myers, and J.T. Johnson, unpublished, Table 1). Peripheral blood samples were collected from anonymous, karyotypically normal, healthy male and female donors.

3.1. CELL CULTURE

3.1.1. OSCC cell lines

All OSCC cell lines were obtained from cultures that were cryopreserved in the vapor phase of liquid nitrogen in 10% dimethyl sulfoxide (DMSO, Fisher Scientific, Pittsburgh, PA) in MEM medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA) and gentamicin (Irvine Scientific) (M10 medium). Each cell line was thawed using a 37°C waterbath followed by the addition of 1 mL of fresh M10 medium and centrifugation at 220 x g for 5 min. The medium was aspirated and cells were resuspended in 5 mL of fresh M10 medium. Cells were transferred to a T25 flask and maintained at 37°C in a humidified incubator with 5% CO_2 in air. Once optimal growth was achieved, cells were subcultured into multiple flasks with appropriate corresponding passage numbers (Appendix A). Some of these cells were then studied and others cryopreserved for future studies.

Table 1. Site of origin and features of patients from whom the OSCC cell lines were derived[4].

CELL LINE	SITE	GENDER	AGE	SMOKER
UPCI:SCC003	Tonsil	Female	65	Yes
UPCI:SCC016	Tongue	Female	81	No
UPCI:SCC029B	Buccal Mucosa	Male	85	No
UPCI:SCC032	RMT¶	Male	60	Yes
UPCI:SCC036	Tonsil	Male	56	Yes
UPCI:SCC040	Tongue	Male	51	No
UPCI:SCC056	Tongue	Male	76	Yes
UPCI:SCC066	Mandible	Female	75	Yes
UPCI:SCC070	RMT	Female	35	Yes
UPCI:SCC075	Tongue	Male	68	Yes
UPCI:SCC077	FOM	Male	57	Yes
UPCI:SCC078	FOM	Male	60	No
UPCI:SCC084	RMT	Male	53	Yes
UPCI:SCC099	FOM	Male	52	Yes
UPCI:SCC103	Tongue	Female	28	Yes
UPCI:SCC104	FOM	Male	58	Yes
UPCI:SCC105	Alveolar Ridge	Male	68	Yes
UPCI:SCC111	FOM	Female	69	Yes
UPCI:SCC114	FOM	Male	72	Yes
UPCI:SCC116	Alveolar Ridge	Male	58	No
UPCI:SCC122	Alveolar Ridge	Male	64	Yes
UPCI:SCC125	Alveolar Ridge	Female	78	Yes
UPCI:SCC131	FOM	Male	73	Yes
UPCI:SCC136	RMT	Female	64	Yes
UPCI:SCC142	FOM	Male	58	Yes
UPCI:SCC154	Base of Tongue	Male	53	Yes
UPCI:SCC172	Mandible	Male	69	Yes
UPCI:SCC182	Tonsil	Male	71	Yes

[4] RMT, Retrimolar Trigone; FOM, Floor of Mouth.

3.1.2. OSCC clones

Clones from two OSCC cell lines (UPCI:SCC040 and UPCI:SCC131) were developed according to the procedure described by Lengauer et al. (1997b). Briefly, single cell dilutions were carried out in one, 64-well plate for each cell line. Individual cells from the two OSCC cell lines were observed in four wells each. However, clones A and B from UPCI:SCC131 were lost due to contamination. The remaining clones were transferred to T25 flasks and cultured as usual (section 3.1.1).

To obtain sufficient cells for multiple experiments, two 75 mm^2 flasks were harvested for each clone. Briefly, cells were passaged approximately 12 times then grown to 70% confluency. To each flask, 0.1µg/ml Colcemid™ (Irvine Scientific) was added for 5 hr. Cells were removed using trypsin/EDTA (Irvine Scientific) and centrifuged at 320 x g for 7 min. Cell pellets were the subjected to a 0.075 M hypotonic KCl solution for 20 min at 37°C followed by the addition of 0.5 mL cold fixative solution (3:1 methanol:acetic acid) and centrifugation. The supernatant was removed by aspiration. Cells were resuspended and 7 ml of cold, fresh fixative was added to each pellet. Cells were centrifuged at 320 x g for 15 min, and washed two more times with fresh cold fixative, then stored in pellet form at -20°C until use.

3.1.3. Peripheral blood cells

3.1.3.1. Controls

Blood samples were obtained from anonymous, karyotypically normal, healthy male and female donors. Blood samples were collected in green top blood collection vials containing sodium heparin in order to prevent blood clot formation. Approximately 200 µl of blood was inoculated into 15 mL conical tubes containing 5 mL of PB Max™ (GIBCO) peripheral blood medium including 10% fetal bovine serum (FBS), gentamicin sulfate (35 mcg/mL), L-glutamine, and phytohemagglutinin and cultured for 72 hours at 37°C in a slant rack.

Cells were harvested 72 h after culture initiation. Briefly, 0.1 µg/ml Colcemid™ was added to each normal peripheral blood cell culture for 25 min (see Appendix B for detailed protocol). Cells were then centrifuged at 320 x g for 7 min. The supernatant was removed by aspiration and the cell pellets were resuspended by tapping. Cell pellets were then subjected to a 0.075 M hypotonic KCl solution for 15 min at 37°C followed by the addition of cold fixative solution (3:1 methanol:acetic acid), inverted, then centrifuged. The supernatant was removed by aspiration and 7 ml of cold fixative was added to resuspend each pellet. Cells were pelleted by centrifugation at 320 x g for 15 min, and washed multiple times until clean, opaque pellet was visualized. All fixed cell pellets were stored at -20°C until use.

3.1.3.2. Inducing common fragile site breakage in blood cells

To induce chromosome breakage that reveals the presence of common fragile sites, peripheral blood cells were cultured in peripheral blood medium for 48 h. Next, 0.4 µM aphidicolin was added to each culture for 21 h, followed by 6 h exposure to 5 mM caffeine and then a 3.5 h Colcemid™ arrest prior to harvest. Cells were then harvested according to the procedure described previously (section 3.1.3.1).

3.1.4. OSCC chamber slides

Following recovery from cryopreservation, one flask at 50% confluency from each OSCC cell line was plated in a chamber slide. To do this, the supernatant was removed from each flask. Cells were rinsed with 1XHBSS (Irvine Scientific). Cells were removed from flask using trypsin/EDTA (Irvine Scientific) followed by the addition of an equal volume of M10 medium with 10% FBS. For each chamber slide, 0.5 mL of cells was transferred to each of three chamber slides per cell line. Chamber slides were placed at 37°C in a humidified incubator with 5% CO_2 for one hour to promote adherence of cells to the slide and then flooded with 1.5 mL complete M10 medium. OSCC cells were maintained until each chamber slide reached 70-80%

confluence. Media was then removed by aspiration, and 2 mL fresh M10 was added to each chamber slide. Next, 5 µL of Colcemid™ was added to each chamber slide and the slides were placed at 37°C in a humidified incubator with 5% CO_2 for 21 h to maximize the number of cells in metaphase. Slides were then washed twice with M10 medium which was removed by aspiration. To each chamber slide, 2 ml of fresh M10 was added for 2 h. The M10 medium was then removed by aspiration and the cells on chamber slides were fixed in 3:1 methanol:acetic acid for 30 min, air dried, and stored with dessicant at -20°C until use.

3.2. CLASSICAL CYTOGENETIC ANALYSIS OF OSCC CLONES

Slides with cells from OSCC parental lines (UPCI:SCC131 and UPCI:SCC040) as well as daughter clones were trypsin-Giemsa banded using standard classical cytogenetic procedures (HEO *et al.* 1989). Briefly, slides were subjected to a 8.3% trypsin solution (Irvine Scientific, Santa Ana, CA) in 1XPBS followed by inactivation in 3.33% fetal bovine serum (Irvine Scientific) in 1XPBS, rinsed in 1XPBS, then stained with 6.7% Giemsa (Sigma) in Gurr's buffer followed by removal of excess stain with Gurr's buffer (Appendix C). Five cells from each of two clones, UPCI:SCC040 and UPCI:SCC131, were analyzed. Individual cells were captured digitally and karyotyped using the CytoVision Ultra System (Applied Imaging, Santa Clara, CA). A composite karyotype was generated in accordance with the ISCN nomenclature (ISCN 1995).

3.3. SPECTRAL KARYOTYPE (SKY™) ANALYSIS OF OSCC CLONES

In order to carry out 24-color FISH to further identify structural abnormalities observed in the G-banded karyotypes of the OSCC clones, fresh slides were prepared from each parental cell line and the respective daughter clones. The SkyPaint kit (Applied Spectral Imaging, Carlsbad, CA) was used according to published methods (VELDMAN *et al.* 1997). Ten metaphase

cells were captured using the SkyVision I System (Applied Spectral Imaging). Five cells from each parental cell line and each clone were selected for analysis.

3.4. PREPARATION OF DNA PROBES FOR FISH

3.4.1. Preparation of alpha-satellite probes

Alpha-satellite plasmids specific for chromosomes 4, 6, 7, 9, 17, 20, and X were provided by Dr. Mariano Rocchi of the University of Bari, Italy (http://www.biologia.uniba.it/rmc/). All plasmids were plated onto LB agar supplemented with ampicillin (50 μg/ml) and placed in a warm room for 16 h to grow individual colonies. Individual colonies were then picked and cultured in LB Broth supplemented with ampicillin (50μg/ml) and placed in a warm room for 16 h to maximize growth. Each culture was then extracted using the Qiagen midiprep kit procedure (Valencia, CA). Following verification of DNA concentration on a 1% agarose gel, extractions were stored at -20ºC until use.

Alpha-satellite plasmids specific for chromosomes 4, 6, 7, 9, 17, 20, and X were directly labeled with either Spectrum Green™-dUTP or Spectrum Orange™-dUTP as instructed by the Vysis nick translation kit protocol (Vysis, Downers Grove, IL)(see Appendix D). A commercial, CEP 11 probe directly labeled with Spectrum Green™ (Vysis) was also used for these analyses.

3.4.2. Preparation of BAC probes

Human BAC clones were identified from the RP11 library and ordered from the BACPAC Resources website (http://www.chori.org/bacpac). All clones used in the current study were obtained from Children's Hospital Oakland Research Institute (CHORI, Oakland, CA). BACs were cultured in LB Broth supplemented with chloramphenicol (50μg/ml) and extracted using the standard laboratory protocol for phenol/chloroform extraction (Appendix D).

For studying anaphase bridges in OSCC cell lines, human BAC clone (RP11-699M19) corresponding to the *CCND1* locus was used along with a cosmid contig for *RIN1*. The *RIN1* probe was a gift from Dr. John Colicelli of the University of California, Los Angeles and was originally obtained from a human glioblastoma cDNA library (COLICELLI *et al.* 1991). The 5-kb cDNA probe corresponded to two cosmids (LA11NCO-9C6, LA11NC01-41C9) which were used in combination to cover the *RIN1* region. For FISH, the *CCND1* BAC was labeled in Spectrum-Green™ (Vysis) and both cosmids spanning *RIN1* were labeled in Spectrum-Orange™ as instructed by the Vysis nick translation kit protocol (Vysis).

With the exception of the fluorescent tag, all other reagents were present in the Vysis nick translation kit (Vysis). A total of 1 µg of DNA was labeled in one of three fluorescent tags: Spectrum-Orange™ (Vysis), Spectrum-Green™ (Vysis), or diethylaminocoumarin-5-dUTP 213 (DEAC213; PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). For each slide, approximately 10 µl of each labeled probe was precipitated using human Cot-1 DNA, nuclease-free water, placental DNA, sodium acetate, and 100% ethanol (see Appendix D for detailed procedure). Probes were then dissolved in 7 µl hybridization buffer (50% formamide/2XSSC/10% dextran sulfate) and 3 µl water for a total of 10 µl per 22mm^2 coverslip area. Probes were agitated at 37°C for 30-60 min, pulse centrifuged in an Eppendorf centrifuge (Model 5415 D, Fisher Scientific) to ensure collection of the entire FISH probe, then denatured at 75°C for 5 min and pre-annealed at 37°C for 15-30 min.

3.5. FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

The general FISH procedure has been described elsewhere (LICHTER and RIED 1994). For the studies described, identical FISH procedures were carried out (for detailed procedure see Appendix E). Briefly, all slides for FISH were pretreated with 100 µg/ml RNase (Sigma-Aldrich Corp., St. Louis, MO) in 2XSSC for 30-60 min at 37°C to remove excess cytoplasm. Slides were then washed three times in 2XSSC at room temperature and dehydrated with a 70, 80, and 100% ethanol series. The slides were then denatured in a 70% formamide/2XSSC solution (pH 7.0) at 75°C for 2 min, followed by another ethanol dehydration series. Slides were placed on a 55°C slide warmer while probe was applied, coverslipped, and sealed with rubber cement.

Slides were incubated overnight in a 37°C humidified chamber. To remove excess unhybridized probe, slides were washed in 0.4XSSC/0.3%NP-40 Tween 20 at 72°C followed by a 2 min wash with 2XSSC/0.1% Tween 20 at room temperature. To visualize the DNA for analysis, slides were counterstained with 4',6-Diamidino-2-phenylindole (DAPI, Sigma) and antifade solution (Fisher Scientific, Pittsburgh, PA) was applied prior to sealing with a glass coverslip. All slides were analyzed using an Olympus BX61 fluorescence microscope. Images were captured using CytoVision Ultra with version 3.5 Genus software (Applied Imaging, Santa Clara, CA).

3.6. ANALYSIS OF FISH HYBRIDIZATIONS

3.6.1. OSCC clone comparison

Dual-color FISH with alpha-satellite probes was carried out on individual slides containing metaphase cells from the UPCI:SCC040 and UPCI:SCC131 parental cell lines and two daughter clones, each. Following FISH (see Section 3.5), slides were stored at –20°C until viewing. Approximately 200 nuclei were evaluated for each alpha-satellite probe. Analysis was carried out using an Olympus (New Hyde Park, NY) BHS fluorescence microscope and images were captured using the CytoVision Ultra (Applied Imaging, Santa Clara, CA).

3.6.2. Analysis of anaphase bridges

In order to identify and quantify the number of anaphase bridges expressing the inverted duplication pattern characteristic of the BFB model for gene amplification, we hybridized differentially labeled *CCND1* and *RIN1* probes to chamber slides from 29 OSCC cell lines. Of these, four, SCC cell lines were obtained from Thomas Carey, Ph.D. (University of Michigan Cancer Center) for our investigation. The *RIN1* probe was directly labeled in Spectrum Orange™ (Vysis) and the RP11-699M19 *CCND1* BAC was directly labeled in Spectrum Green™ (Vysis) and hybridized to cells on chamber slides (see Section 3.4.2). Approximately 40

anaphase bridges were analyzed from each cell line for the presence of *RIN1* and *CCND1* FISH sequences.

The amplification status of *CCND1* was also determined by examining interphase nuclei on the same chamber slide. We defined an OSCC cell line as amplified if at least 2% of interphase nuclei had >10 copies of the *CCND1* gene. In order to determine statistical significance, we used a t-test to compare the percentage of cells with amplification expressing the inverted duplication pattern of *CCND1* and/or *RIN1* in anaphase bridges versus those without amplification (Appendix I). In addition, the Spearman correlation test was carried out to determine if differences were present between the percentage of amplified cells and the percentage of anaphase bridges containing the inverted duplication sequences.

3.6.3. Identification of *FRA11F*

Previous studies by our laboratory identified two breakpoint cluster regions that flank the11q13 amplicon in our OSCC cell lines (Fig. 5, Huang et al., 2002). In order to determine the physical map location of *FRA11F,* BACs were selected from the RP11- library using the NCBI database as previously described (Section 3.4.2) and hybridized to cells treated to express common fragile sites (Section 3.1.3.2)(Table 2). To be considered a region within a fragile site, an individual BAC must be observed as having three distinct patterns of hybridization: 1) binding only to the proximal side of the chromosome break; 2) binding to both sides of the chromosome break, crossing the fragile site; and 3) binding only to the distal side of the chromosome break.

To localize *FRA11F*, BACs were labeled in Spectrum Orange™ or Spectrum Green™ as decribed (Section 3.4.2.) and hybridized in pairs to aphidicolin and caffeine (APCC) treated metaphase blood cells and cultured OSCC cells using the standard laboratory FISH protocol (Section 3.5).

Table 2. BAC clones used to identify *FRA11F* in peripheral blood cells treated to induce CFS with APCC.

BAC NAME
RP11-672A2
RP11-483P13
RP11-98G24
RP11-O31F2
RP11-79B7
RP11-118L16
RP11-89M14
RP11-281H14
RP11-613J18
RP11-19P3
RP11-131C11
RP11-89H11
RP11-141H6
RP11-313I2
RP11-30C9
RP11-18G9
RP11-325I16
RP11-208P3
RP11-841F15
RP11-372E19

3.6.4. Determination of *FRA11F* status in OSCC cells

To test our hypothesis that breakage at a common fragile site may initiate 11q13 gene amplification, we carried out dual-color FISH using *FRA11F* BACs RP11- 208P3, 303I2, 841F15, and 281H14 along with RP11-699M19 for *CCND1* on OSCC cell lines. For cell lines expressing complex hybridization patterns, we used three-color FISH for *CCND1*, proximal *FRA11F*, and distal *FRA11F* to further clarify the status of *FRA11F*. BACs for proximal *FRA11F* (RP11- 281H14, 303I2) were labeled in Spectrum Green™, distal *FRA11F* (RP11-

208P38) in Spectrum Orange™, and *CCND1* (RP11-699M19) directly labeled in diethylaminocoumarin- 5-dUTP 213 (DEAC 213) for aqua.

3.7. HELIX FLEXIBILITY ANALYSIS OF *FRA11F*

Previous studies have demonstrated that CFS contain regions of high flexibility (MISHMAR *et al.* 1998; MORELLI *et al.* 2002), although the structural chromatin element conferring this fragility remains to be elucidated. To determine the flexibility status of *FRA11F*, we used the FlexStab computer program downloaded from the Hebrew University of Jerusalem website, (http://leonardo.ls.huji.ac.il/departments/genesite/faculty/bkerem.htm), to analyze the 7.5 Mb sequence (see Appendix F for program). The purpose of this program is to measure variation in the DNA structure which is expressed as TWIST angle fluctuations, defined as rotation of base pair relative to the perpendicular base plane (SARAI *et al.* 1989). The program measures this by summing up dinucleotide values in a given window size, in which AT appears to express high fliexibility versus homonucleotide sequences (GG/CC and AA/TT). Similar output is obtained when measuring helix stability, however the values are based on a helix-to-coil transition which is expressed in Kcal/mol. Stability is assessed as a function of base sequence and its neighbor bases in which interactions may be: AA/TT, AT/TA, TA/AT, CA/GT, GT/CA, CT/GA, GA/CT, CG/GC, GC/CG, GG/CC (BRESLAUER *et al.* 1986). Using these interactions, the authors demonstrated that calculating values in terms of base sequence versus base composition results in different thermodynamic profiles.

Due to the extremely large size of the *FRA11F* sequence, it was divided into several segments of approximately 330 kb per run for flexibility analysis (Appendix G). The mean and standard deviation values were then calculated for each sequence using a program designed by Dr. Xin Huang of our laboratory group at the University of Pittsburgh based on previously described methods (MISHMAR *et al.* 1998). To determine the percentage of AT versus GC composition within the *FRA11F* sequence, we ran the FlexStab computer program again, substituting AT/GC content in the sequence file.

4. RESULTS

4.1. CIN IN OSCC CELLS

Our current investigation focuses on determining the origins of CIN in OSCC cells. Three characteristic patterns of CIN include: numerical chromosomal aberrations, structural chromosomal alterations, and gene amplification. The purpose of this study is to determine whether or not OSCC cells express heterogenous karyotypes despite a clonal background of chromosome aberrations. To investigate chromosomal instability in oral squamous cell carcinoma, two OSCC cell lines, UPCI:SCC040 and UPCI:SCC131, were cloned, harvested, and analyzed using FISH probes specific for the alpha-satellite region of eight different chromosomes. Cells from both OSCC cell lines showed variations in the modal chromosome number between clones of each cell line (Table 3). For example, clone A from UPCI:SCC040 showed a modal number of two for chromosome 4, however clone B contained a modal number of four for the same chromosome (Fig. 2, top left). Similarly, Clone B appeared to express a higher percentage of cells with an extra signal for the centromere of chromosome 9 compared to Clone A, whereas Clone A showed extra copies for the centromere of chromosome 7 in a higher percentage of cells than Clone B. However, signals for all other centromere probes appeared to be stable between the SCC040 clones (Fig. 2, bottom left). Comparison of clones from UPCI:SCC131 revealed similar variability to those of UPCI:SCC040. Interestingly, the modal number of chromosomes appeared to be distinct for each clone for the centromere probes of chromosomes 4 (Fig. 2, top right) and 7 in clones C and D, respectively. Although the modal chromosome number varied between clones from each cell line, one or two chromosomes from the panel remained relatively constant. UPCI:SCC040 expressed four copies of chromosome 17 and two copies of the X chromosome in each of the four clones, whereas UPCI:SCC131 had four copies of chromosome 20 (Fig. 2, bottom right) and two copies of the X chromosome in both clones. Among the chromosomes studied, chromosomes 4 and 9 appeared to vary the most between clones of each cell line.

Table 3. Chromosome instability in cloned UPCI: oral squamous cell carcinoma cell lines[5].

UPCI:Cell Line	Chrom[6] 4	Chrom 6	Chrom 7	Chrom 9	Chrom 11	Chrom 17	Chrom 20	Chrom X	Average
SCC040 Clone A	2 40%	4 23%	5 27%	4 24%	4 5%	4 11%	4 22%	2 9%	3.6 20%
SCC040 Clone B	4 15%	ND[7] ND	4 9%	5 22%	4 10%	4 4%	4 9%	2 4%	3.9 10%
SCC131 Clone C	2[c] 10%	ND ND	4 40%	6 39%	3/5 26%	3 26%	4[†] 16%	2 14%	3.6 21%
SCC131 Clone D	3 12%	ND ND	4 13%	7 46%	3 9%	4 18%	4 13%	2 4%	3.9 16%
Normal Lymphocytes	2 0%	2 5%	2 0%	2 1%	2 2%	2 3%	2 0%	2 5%	2 2%

Of particular interest were metaphase cells observed during the FISH analysis of each clone. Despite similar modal chromosome numbers obtained by interphase and metaphase FISH analyses, we found that the chromosome copy number did not necessarily represent the same chromosome in each cell analyzed. For example, UPCI:SCC131 Clone D showed the presence of three copies of chromosome 17 and four copies of chromosome 11. Although the findings for chromosome 17 remained the same (two normal chromosome 17s and one derivative chromosome 17), the chromosome 11 copy number signals represented two normal chromosomes 11 and two derivative chromosomes 11 with a homogeneously staining region (hsr) at band 11q13 in one cell (Fig. 3A, left). Another cell expressed two normal 11s, one derivative 11 with the same hsr, and one dicentric, translocated chromosome 11 with the hsr (Fig. 3A, right).

To characterize the variation in chromosome number and structure between individual cells within a clone compared to other clones and the parental cell lines, G-banding and SKY were carried out. Approximately five cells from each parental cell line and two daughter clones

[5] In: Reshmi et al. (2003). Genes Chromosomes Cancer 41:38-46. Reprinted with permission.
For each chromosome tested, the modal chromosome number was determined, and the percentage of cells that differed from the modal number is shown.
[6] Chromosome number.
[7] ND, not determined.
[†] Different cell passage used for analysis.

each were examined by both methods. All cell lines expressed gains, losses, and clonal structural abnormalities. However, despite a common background of abnormalities, no two cells were alike within a cell line or between the cell line clones (Fig. 4).

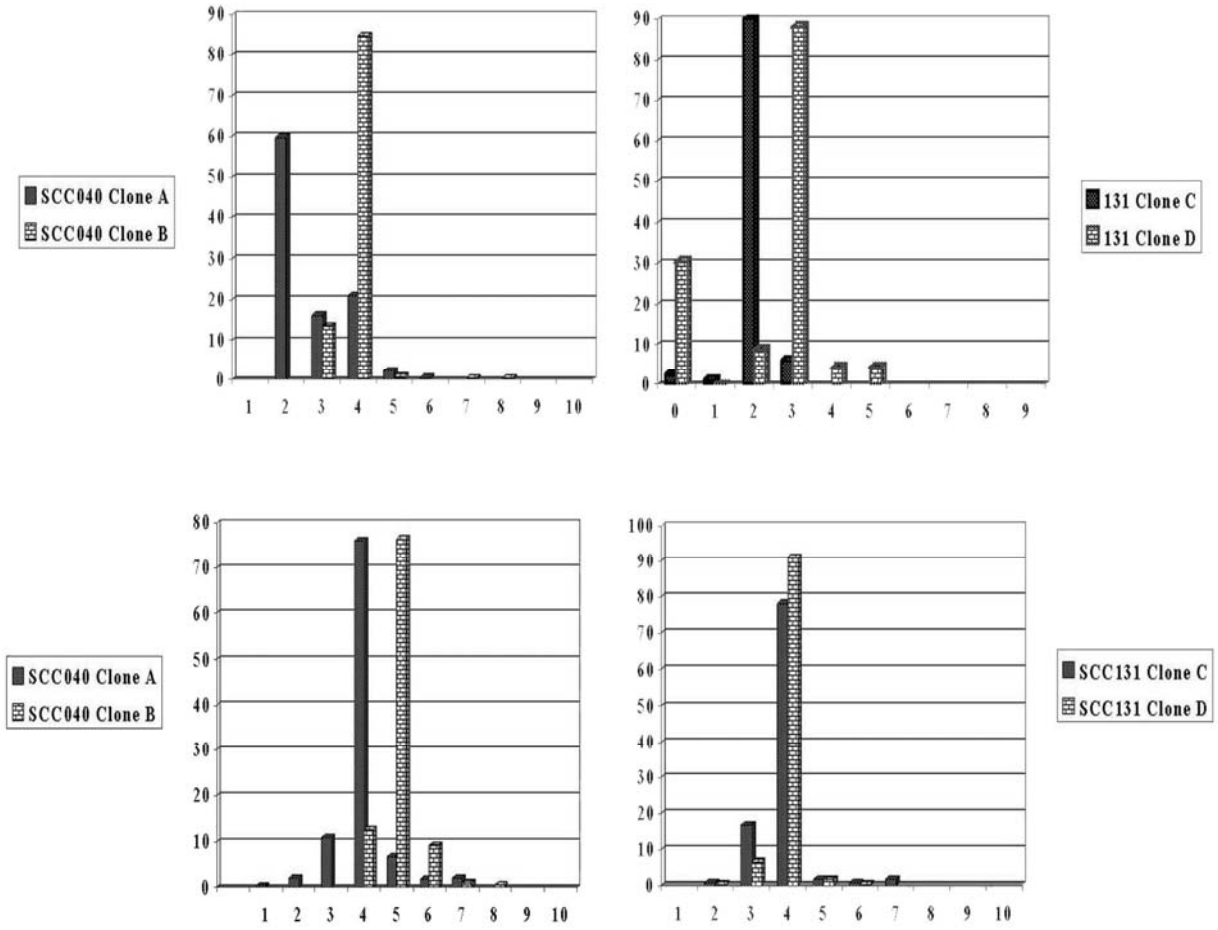

Figure 2. Alpha-satellite FISH results showing chromosomal variation between clones.[8]

[8] In: Reshmi et al. (2003). Genes Chromosomes Cancer 41:38-46. Reprinted with permission. UPCI:SCC040 Clones A and B (top, left) and UPCI:SCC131 Clones C and D (top, right) results for the centromere of chromosome 4; Chromosomal similarities from UPCI:SCC040 Clones A and B (bottom, left) for the centromere of chromosome 17; Chromosomal similarities from UPCI:SCC Clones C and D (bottom, right) for the centromere of chromosome 20. The X-axis represents the number of alpha-satellite signals present; the Y-axis represents the percentage of cells expressing each signal.

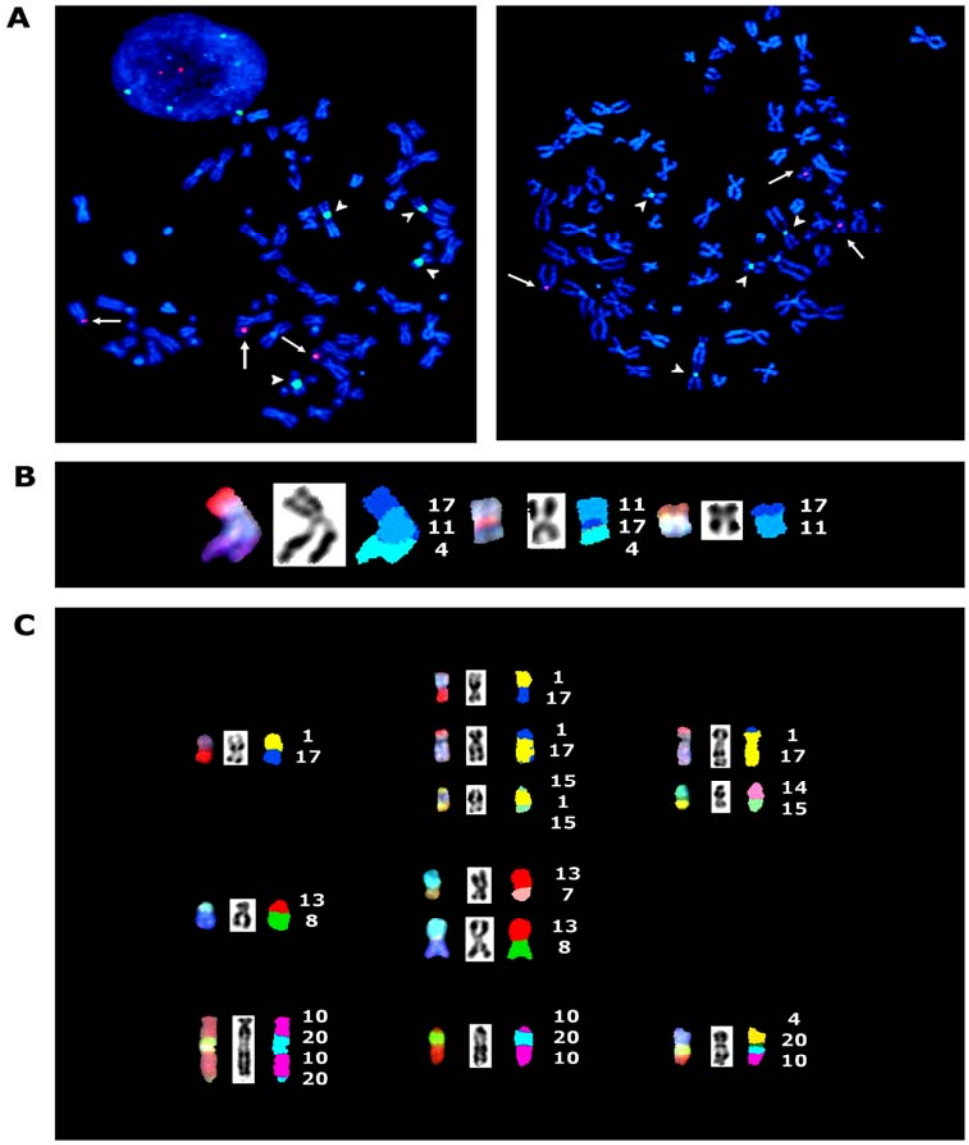

Figure 3. FISH and SKY marker chromosome evolution in OSCC cells[9].
A) Metaphase fluorescence *in situ* hybridization images from UPCI:SCC131 Clone D using centromere probes 11 (green, arrowheads) and 17 (orange, arrows). Note that the four copies of chromosome 11 represent structurally different chromosomes in each cell; B) Marker chromosome evolution in UPCI:SCC131. Note that markers contain material of similar chromosomal origin, however vary in segment size; C) Marker chromosome evolution in UPCI:SCC040. Note the novel translocations from segments of the parental cell line (far left) that have combined with broken chromosome segments of other chromosomes (Clones A and B, respectively).

[9] In: Reshmi et al. (2003). Genes Chromosomes Cancer 41:38-46. Reprinted with permission.

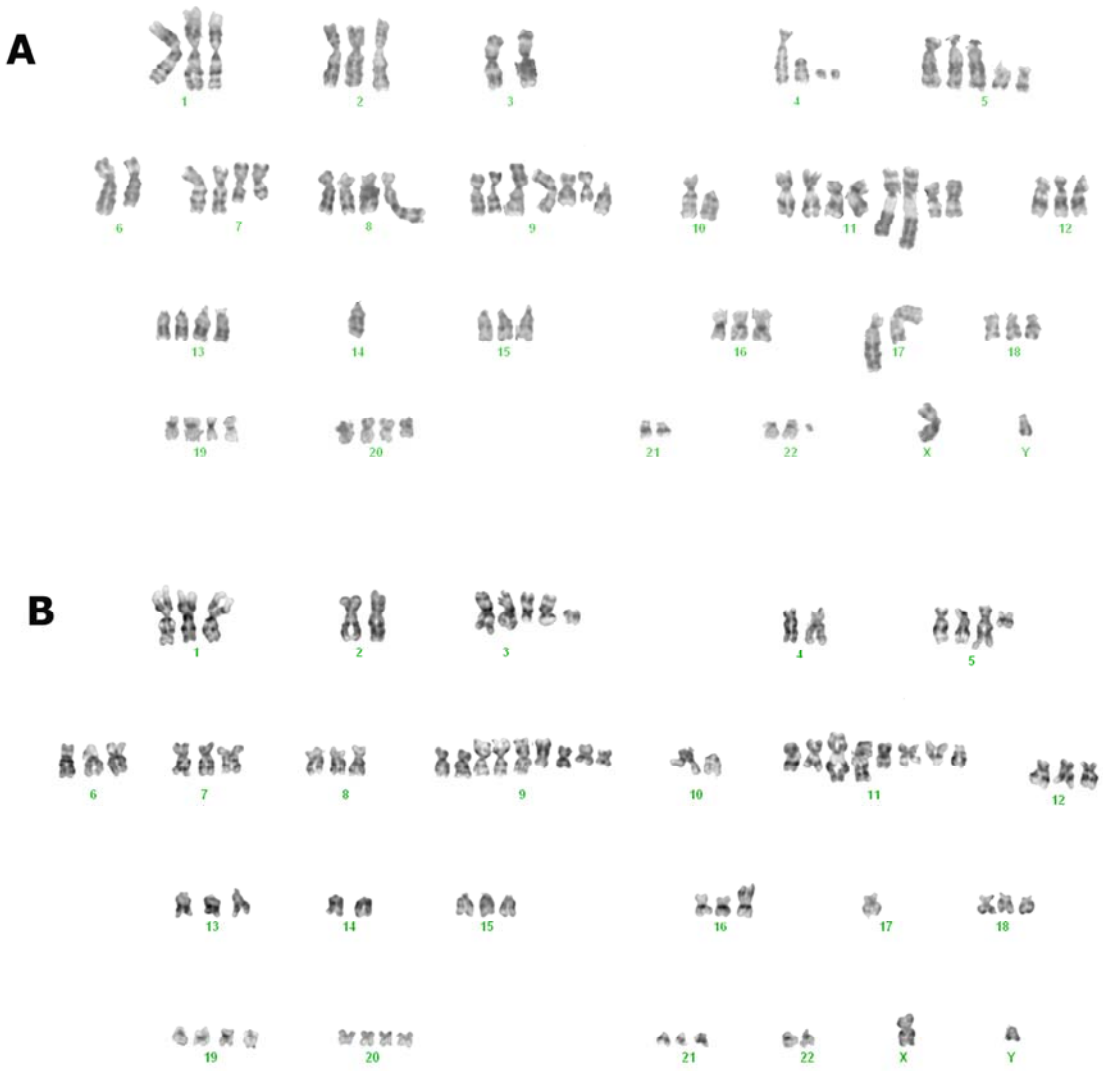

Figure 4. Representative G-banded karyotypes from UPCI:SCC131 parental cell line and Clones C and D[10].

[10] In: Reshmi et al. (2003). Genes Chromosomes Cancer 41:38-46. Reprinted with permission. UPCI:SCC131, passage p18: 78,XY,-X,-3,del(4)(?)x2,+del(4)(q?),+der(5)t(5;7)(?;?)x2[2],der(7)t(X;7)(p11.2;p11.2),+der(7)t(X;7)(p11.2;p11.2),+der(8)t(3;8;13)(?;?;?),del(9)(q12),+der(9)t(9;14)(p21;q12)x2,+der(9)t(9;14)(?;q12),+der(9)t(9;14)(?;?),-10,del(10)(p10),del(11)(p13),+del(11)(q13),hsr(11)(q13),+der(11)t(4;11;17)(?;q13;?)x2,+der(?)t(4;11;17)(?;?;?)x2,+13,-14,-14,-17,der(17)t(2;17)(p13;p11.2),der(17)t(2;17)(q21;q21),+19,+del(20)(q11.2),-21,del(22)(q?); B) UPCI:SCC131, passage 33. Clone C: 78,XY,-X, -2,del(3)(?),+der(3)t(3;16)(q21;p13.1)x2,4,+der(5)t(5;7)(?;?),der(9)t(9;14)(p21;q12),+der(9)t(9;14)(p21;q12)x2,+der(9)t(9;14)(p21;?),+der(9)t(9;14)(?;?)x3,-10,der(10)t(2;10)(?;p10),del(11)(p13),+der(?)t(4;11)(?;?)x2,+der(11)t(4;11;17)(?;q13;?)x2,+der(?)t(11;17)(?;?),-14,der(16)t(3;16)(?;?),-17,-17,+19,+20,-22.

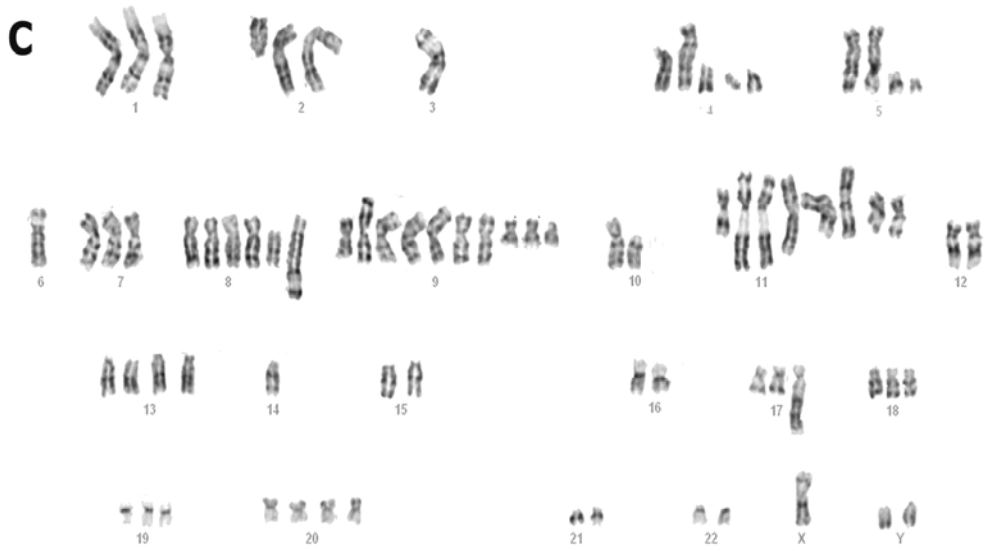

Figure 4 (continued). Representative G-banded karyotypes from UPCI:SCC131 parental cell line and Clones C and D[11].

Furthermore, most structural chromosome variations were not shared between the UPCI:SCC cell lines (Appendix H). It was challenging to describe the karyotypes of cells that displayed marker chromosome evolution, since many chromosomes contained partial broken segments from the original chromosomal rearrangements (Fig. 4). As expected, many G-banded marker chromosomes contained similar chromosomal material when painted using SKY, yet were made up of different segments from the chromosome of origin. Marker chromosomes within a clone appeared to contain segments of DNA similar to the original parental marker chromosome, but distinct from each other (Fig. 3B). In some cases, marker chromosomes from the original cell line appeared to rearrange, or evolve, by combining or breaking into segments and fusing with segments from other broken chromosomes (Fig. 3C).

[11] In: Reshmi et al. (2003). Genes Chromosomes Cancer 41:38-46. Reprinted with permission.
C) UPCI:SCC131p30 Clone D: 58,X,-X,Y,+Y,+1,-2,der(3)t(3;9)(q12;q13),-4x2,+del(4)(?),-5x2, -6x2,+der(6)t(5;6)(?;q13)x2,-8,+del(9)(p12),+der(9)t(9;14)(p21;q12)x2,-10x2,-11x2,+del(11)(p15q13), +der(11)t(4;11;17)(?;q13hsr;?),-13x2,-14,-15,-17x2,+der(17)t(2;17)(q21;q21),-18,+der(18)t(9;18) (?;p11.2),-19,+der(19)t(4;11;19)(?;q13;p13.3),-20,-21x2,+der(21)t(Y;21)(q12;q11.2),-22.

4.2. ISOLATION OF *FRA11F*

4.2.1. Mapping *FRA11F*

Previous studies by our laboratory group (HUANG *et al.* 2002) identified two breakpoint cluster regions flanking the 11q13 amplicon in our OSCC cell lines (Fig. 5). Our current goal was to identify the CFS predicted to reside within the breakpoint cluster regions. We cultured normal human blood cells in the presence of aphidicolin followed by exposure to caffeine (APCC) in order to enhance for CFS breakage.

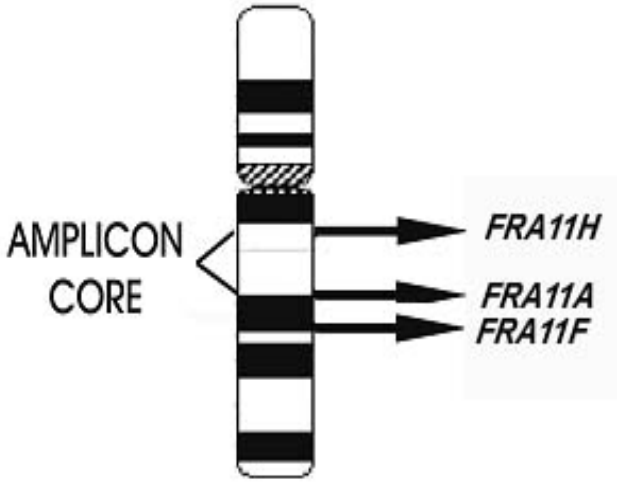

Figure 5. Proposed common fragile sites flanking the 11q13 amplicon core[12].

To be considered located within a fragile site, an individual BAC must be observed as having three distinct patterns of hybridization: 1) binding only to the proximal side of the chromosome break; 2) binding to both sides of the chromosome break, crossing the fragile site; and 3) binding only to the distal side of the chromosome break (Fig. 6). The most frequently expressed breaks meeting these criteria were localized to the region predicted for *FRA11F* on the long arm of chromosome 11 at 11q14.2. Due to the very low frequency of breakage proximal to

[12] CFS predicted from their NCBI data base locations (http://www.ncbi.nlm.nih.gov/LocusLink/). *FRA11A* is a folate-sensitive fragile site at 11q13.3.

the 11q13 amplicon core, we were unable to identify the predicted CFS, *FRA11H*. Since the purpose of this study was to characterize common fragile sites flanking 11q13, we did not use culture conditions that would induce the rare fragile site, *FRA11A*.

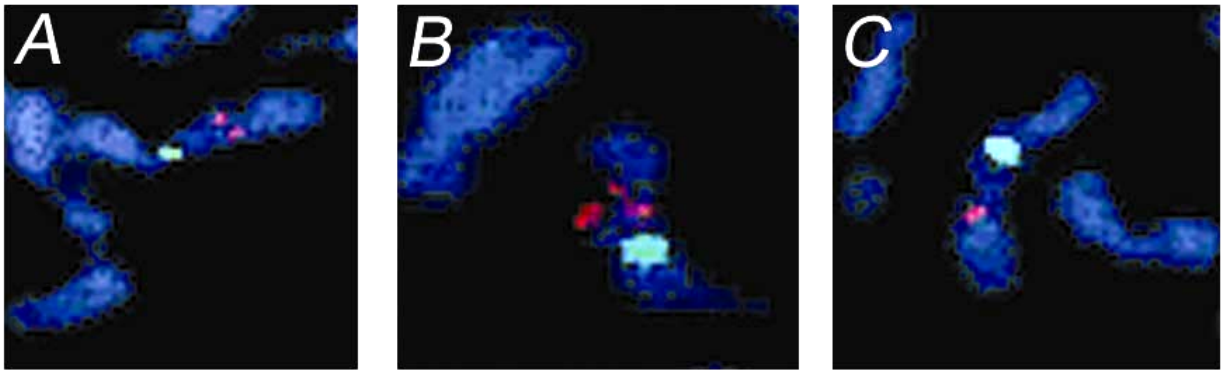

Figure 6. Localization of *FRA11F* BAC RP11-613J18 by FISH in APCC- treated normal blood cells[13]

To characterize *FRA11F*, we carried out FISH using BACs within the previously identified distal breakpoint cluster region (HUANG *et al.* 2002). The first series of probes showed hybridization patterns consistent with their positioning proximal to *FRA11F* (Fig. 7; Table 4). Of 20 BACs, seven (RP11-672A2, 483P13, 98G24, O31F2, 79B7, 118L16, and 89M14) hybridized on the centromeric side of the 11q14 break. Only RP11-372E19 was found to localize distal to the *FRA11F* fragile site. The remaining BACs within the fragile site region (RP11-281H14, 613J18, 19P3, 131C11, 89H11, 141H6, 313I2, 30C9, 18G9, 325I16, 208P3, 841F15) appeared to hybridize proximally, spanning, and distally to the fragile site with relatively equal frequencies (Fig. 7). We identified a total of 12 BACs within *FRA11F*, spanning a region of 7.5 Mb (Fig. 8; Table 4).

[13] Centromere 11 probe (CEP11, Vysis) in Spectrum-Green™; RP11-613J18 in Spectrum-Orange™ ; BAC probe proximal to the chromatid break at 11q14.2. (B) BAC probe spanning the 11q14.2 break; (C) BAC probe distal to the chromatid break.

Table 4. RP11- BAC clones in and around *FRA11F* identified by FISH.

CLONE	TOTAL # OF BREAKS	PROXIMAL (%)	SPANNING (%)	DISTAL (%)
672A2	7	7 (100)	0 (0)	0 (0)
483P13	14	14 (100)	0 (0)	0 (0)
98G24	22	22 (100)	0 (0)	0 (0)
O31F2	22	22 (100)	0 (0)	0 (0)
79B7	22	22 (100)	0 (0)	0 (0)
118L16	13	13 (100)	0 (0)	0 (0)
89M14	20	20 (100)	0 (0)	0 (0)
281H14	25	17 (68)	8 (32)	0 (0)
613J18	21	17 (81)	2 (9.5)	2 (9.5)
19P3	21	14 (66.7)	5 (23.8)	2 (9.5)
131C11	13	9 (69.2)	2 (15.4)	2 (15.4)
89H11	27	15 (55.6)	4 (14.8)	8 (29.6)
141H6	24	7 (29.2)	8 (33.3)	9 (37.5)
313I2	20	13 (65)	3 (15)	4 (20)
30C9	16	10 (62.5)	4 (25)	2 (12.5)
18G9	20	11 (55)	0 (0)	9 (45)
325I16	16	4 (25)	5 (31.3)	7 (43.7)
208P3	22	3 (13.6)	1 (4.5)	18 (81.9)
841F15	15	6 (40)	0 (0)	9 (60)
372E19	16	0 (0)	0 (0)	16 (100)

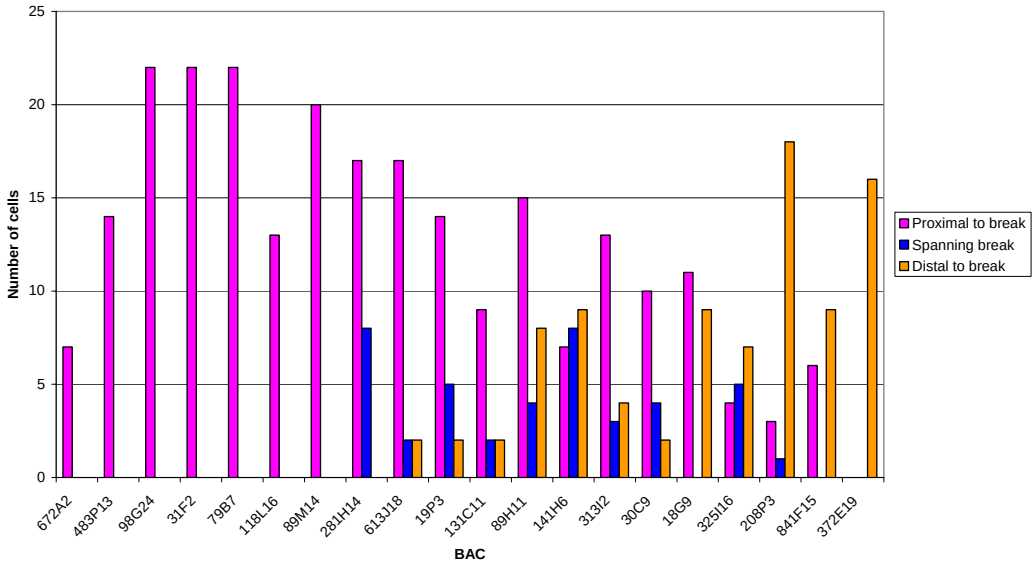

Figure 7. Distribution of BAC clones hybridized to APCC induced fragile site breakage in normal blood cells.

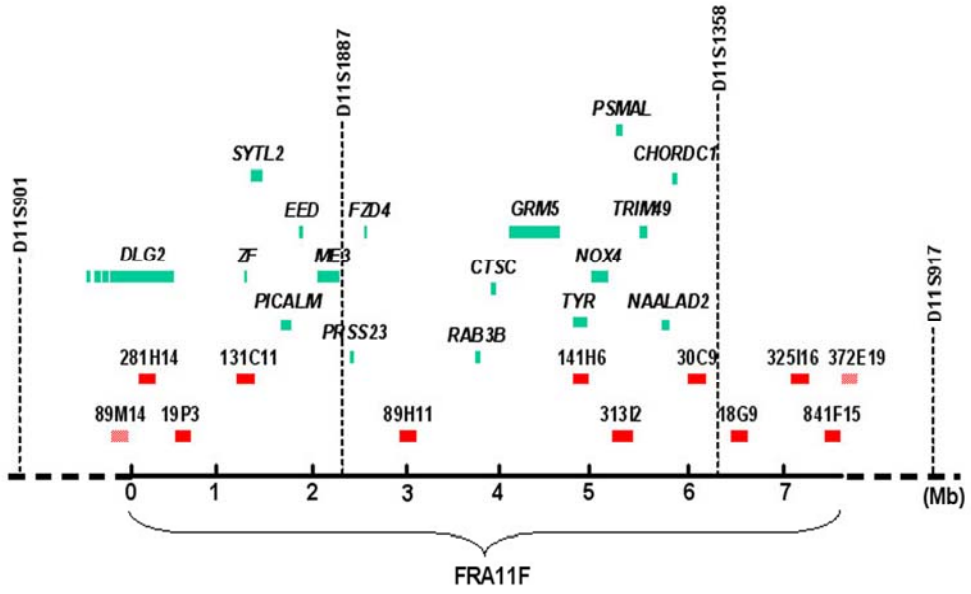

Figure 8. FISH BAC map encompassing *FRA11F* region[14].

4.2.2. Analysis of *FRA11F* in OSCC cell lines

Amplification of chromosomal band 11q13 occurs in approximately one-half of squamous cell carcinomas of the head and neck and its subset, OSCC (GOLLIN 2001). To investigate the role of *FRA11F* in 11q13 gene amplification, we carried out FISH using BACs spanning the *FRA11F* region (RP11- 208P3, 303I2, 841F15, and 281H14) along with *CCND1* (RP11-699M19) in 23 of our OSCC cell lines.

Of 12 cell lines with *CCND1* amplification, we confirmed that nine had breakage, loss, or rearrangement of *FRA11F* (Table 5). Four of five cell lines without *CCND1* amplification displayed a normal (control) hybridization pattern for *CCND1* and *RIN1* (Fig. 9A). OSCC cell lines with 11q13 amplification were divided into two categories. Six cell lines with *CCND1* amplification (UPCI:SCC029B, 056, 078, 084, 131, 136) showed complete loss of distal 11q and breakage in the proximal region of *FRA11F* on the abnormal chromosome 11 (Fig. 9B). Three

[14] FISH BAC map encompassing FRA11F region. Note that RP11-613J18 lies distal to RP11-281H14 and RP11–208P3 partially overlaps with RP11-841F15 and are not included in the map.

Table 5. Metaphase FISH results for OSCC cell lines with and without 11q13 gene amplification.

UPCI:SCC	Metaphase FISH *FRA11F / CCND1* [15]	11q13 amplification status[16]
003	complex[17]	+
016	complex	+
029B	loss / hsr	+
032	complex	+
036	not done	+
056	loss / hsr	+
070	complex	-
077	normal	-
078	loss / hsr	+
084	loss / hsr	+
099	not done	-
103	not done	+
105	complex	+
111	complex	+
114	normal	+
116	not done	-
122	complex	-
125	normal	+
131	loss / hsr	+
136	loss / hsr	+
154	complex	+
172	complex	+
182	normal	-

cell lines (UPCI:SCC056, 078, 131) revealed breaks within *FRA11F* by FISH using BACs for the proximal (RP11-281H14, 313I2) and distal regions of *FRA11F* (RP11-208P3, 841F15)(Fig.

[15] BAC clones RP11-841F15, 208P3, 303I2, and 281H14 used for *FRA11F* FISH.

[16] +, amplified; - not amplified. Amplification status based on previous quantitative microsatellite analysis (Huang et al., 2002).

[17] Complex, complex rearrangement of *FRA11F* and *CCND1*; loss, loss of *FRA11F* sequences; hsr, 11q13 amplified in the form of an hsr; normal, *FRA11F* and *CCND1* expressed hybridization pattern observed in normal metaphase cells (i.e., one *CCND1* signal centromeric to one *FRA11F* signal).

9C). The remaining cell lines expressed complex rearrangements within the 11q13 hsr and revealed variations in the hybridization pattern of the *FRA11F* sequences (Fig. 10).

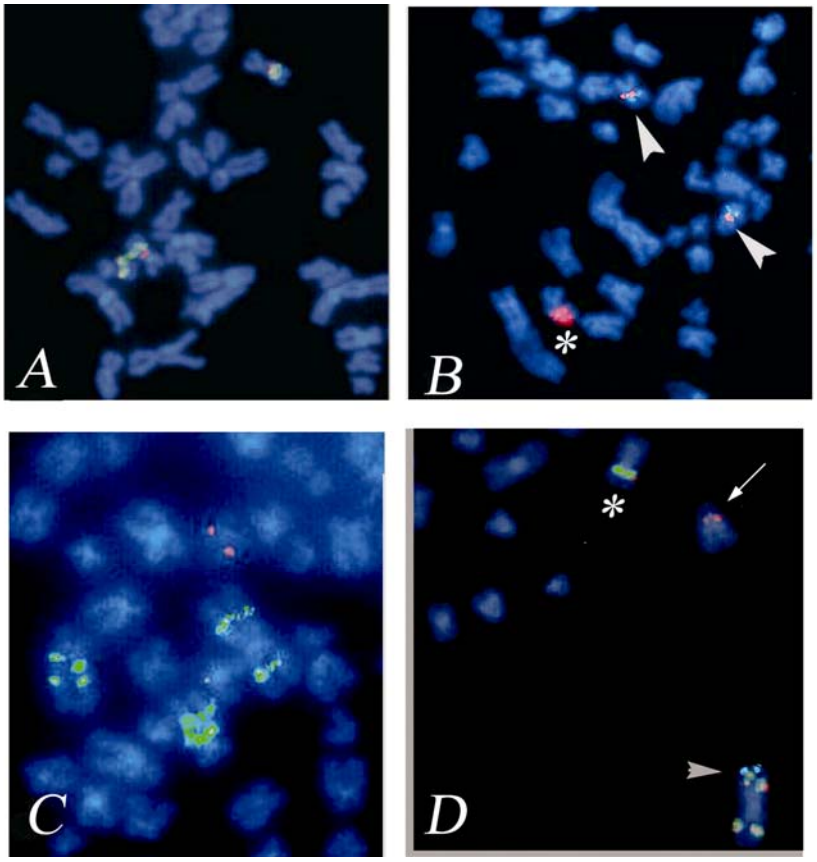

Figure 9. Hybridization patterns for *CCND1* and *FRA11F* in OSCC cell lines.
(A) UPCI:SCC116 showing three normal chromosomes 11 for *CCND1* (Spectrum-Orange™) and *FRA11F* (Spectrum-Green™); (B) UPCI:SCC136 expressing two normal copies of chromosome 11(arrowheads) and one chromosome with amplified *CCND1* in the form of an hsr, missing distal residual *FRA11F* sequences (asterisk); (C) UPCI:SCC078 showing breaks in *FRA11F* separating the proximal RP11-281H14 labeled in Spectrum-Green™ and the distal RP1189-H11 in Spectrum-Orange™. Note the loss of distal *FRA11F* and amplification of proximal *FRA11F*; (D) UPCI:SCC111 expressing a break between proximal *FRA11F* (Spectrum-Green™, asterisk) and distal *FRA11F* regions (Spectrum-Orange™, arrow). Marker chromosome with an inverted duplication of *CCND1* at the chromosome end and centromeric *FRA11F* sequences (arrowhead). Broken chromosome with an amplified end may that have resulted from breaking of an anaphase bridge.

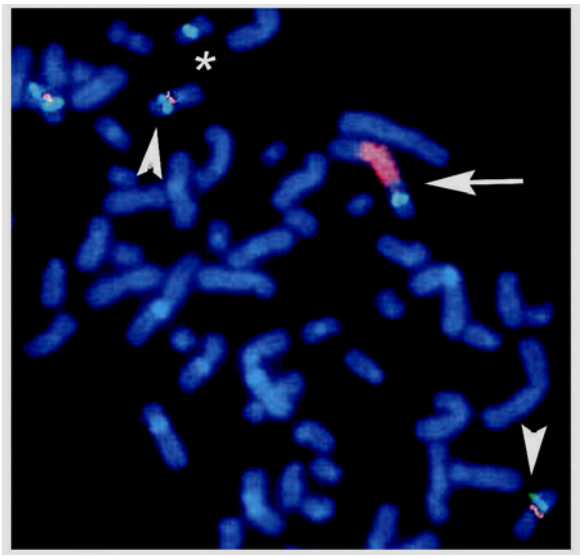

Figure 10. Atypical rearrangement pattern of *CCND1* and *FRA11F* in an OSCC cell line. UPCI:SCC016 expressing two normal chromosomes 11 (arrowheads), one derivative chromosome 11 containing *FRA11F* sequences only (asterisk), and one derivative chromosome with *FRA11F* and amplified *CCND1* in the form of an hsr (arrow).

To further understand the involvement of *FRA11F* in OSCC cell lines with complex hybridization patterns, we used three-color FISH for *CCND1*, proximal *FRA11F*, and distal *FRA11F*. BACs for proximal *FRA11F* (RP11- 281H14, 303I2) were labeled in Spectrum Green™, distal *FRA11F* (RP11- 208P38) in Spectrum Orange™, and *CCND1* (RP11-699M19) directly labeled in diethylaminocoumarin-5-dUTP 213 (DEAC 213), which is aqua. Of nine cell lines analyzed, seven (UPCI:SCC003, 032, 036, 070, 103, 111, 154) displayed chromosomes 11 with inverted duplications containing *CCND1* and *FRA11F* sequences (Figs. 11A-B). Despite lack of the inverted duplication pattern in the remaining two cell lines (UPCI:SCC105, 116), evidence of breakage or loss of *FRA11F* was observed together with *CCND1* sequences at chromosome ends expressing loss of distal 11q (Fig.11C). Taken together, our data show loss, breakage, and inverted duplication hybridization patterns involving *FRA11F* within OSCC cell lines and suggest that breakage at *FRA11F* may initiate gene amplification through the BFB cycle mechanism.

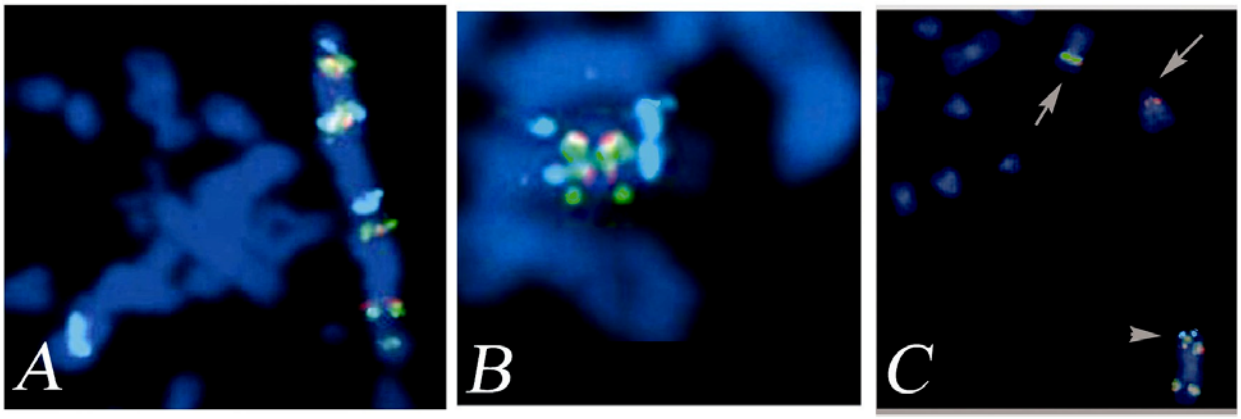

Figure 11. Inverted duplication of *FRA11F* and *CCND1* in OSCC cells.
(A) Three color FISH using *CCND1* directly labeled with DEAC213 (aqua), proximal *FRA11F* sequences in Spectrum-Green™ (RP11-281H14, RP11-313I2), and distal *FRA11F* sequences in Spectrum-Orange™ (RP11-208P3) in UPCI:SCC032 metaphase expressing an inverted duplication hybridization pattern and isolated hsrs containing *CCND1* within the complex chromosome rearrangement. (B) UPCI:SCC032 with an inverted duplication pattern showing *CCND1* flanking *FRA11F* sequences, suggesting a BFB cycle model for gene amplification. (C) UPCI:SCC111 expressing a break between the proximal *FRA11F* and distal *FRA11F* regions. Marker chromosome expressing a duplication of *CCND1* at a chromosome end with *FRA11F* sequences proximal to *CCND1*, possibly resulting from a broken anaphase bridge.

4.2.3. Helix flexibility analysis of *FRA11F*

Previous studies have demonstrated that CFS contain regions of high flexibility (MISHMAR *et al.* 1998; MORELLI *et al.* 2002), although the structural chromatin element conferring this fragility remains to be determined. To determine the flexibility status of *FRA11F*, we used the FlexStab computer program to analyze the 7.5 Mb sequence. Due to its extremely large size, the sequence of *FRA11F* was divided into two parts for flexibility analysis. Together, the 7.5 Mb region expressed 208 areas with high flexibility (Appendix G, arrows). Within *FRA11F*, significant deviation was observed compared to the >4.5 SD flexibility value (based on the value of the lowest region between 85 [3.5 Mb; x = 10.80; SD= 0.687; P<0.0001] and 123 [4.0 Mb; x = 10.95; SD= 0.717; P<0.0001]). Repeat analysis of the FlexStab program for the AT to GC ratio revealed that the CG content of *FRA11F* was approximately 37.4%, representing the lowest GC content of other CFS examined to date (MORELLI *et al.* 2002). One extremely AT- rich region (92%) was identified near a 12 kb sequence gap. Overall, repetitive elements such as LINEs (27.3%), SINEs (10%), LTRs (7.9%) and MERs (3%) accounted for greater than one-half of the

length of the *FRA11F* sequence, also demonstrating that *FRA11F* harbors the highest frequency of repeat sequences compared to other CFS described in the literature (ARLT *et al.* 2003; CALLAHAN *et al.* 2003; DENISON *et al.* 2003; HUANG *et al.* 1998; MISHMAR *et al.* 1998; MORELLI *et al.* 2002).

4.3. ASSESSING THE BFB CYCLE AS A MECHANISM FOR 11q13 GENE AMPLIFICATION

The presence of anaphase bridges in dividing tumor cells has been well documented (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; DEBATISSE *et al.* 1998; GISSELSSON *et al.* 2000; HELLMAN *et al.* 2002; HOFFELDER *et al.* 2004; SAUNDERS *et al.* 2000). Although the BFB cycle model for gene amplification is not new (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; DEBATISSE *et al.* 1998; GISSELSSON *et al.* 2000; HELLMAN *et al.* 2002; STARK 1993), no previous reports have shown direct evidence of an association between anaphase bridging and gene amplification. We previously demonstrated anaphase bridges (SAUNDERS *et al.* 2000) and 11q13 gene amplification in the form of an inverted duplication in oral cancer cells (SHUSTER *et al.* 2000)(Fig. 12). Our current investigation tests the hypothesis that 11q13 gene amplification occurs through BFB cycles. If our hypothesis is valid, the presence of anaphase bridges containing inverted duplications involving 11q13 amplicon sequences should occur more frequently in OSCC cell lines expressing the 11q13 hsr than by chance in OSCC cells without 11q13 gene amplification.

To confirm the BFB model for gene amplification, we carried out FISH using *CCND1* and *RIN1* probes on 29 OSCC cell lines with and without 11q13 amplification. Two analyses were carried out on the same chamber slide for each OSCC cell line: 1) assessment of gene copy number of *CCND1* within interphase cells to determine the frequency of copy number of CCND1 in each cell line, which enables comparison of 11q13 amplified OSCC cell lines to non-amplified cell lines; 2) determination of the total number of anaphase bridges within each cell line and the number that expressed *CCND1* and *RIN1* sequences. For this analysis, OSCC cell

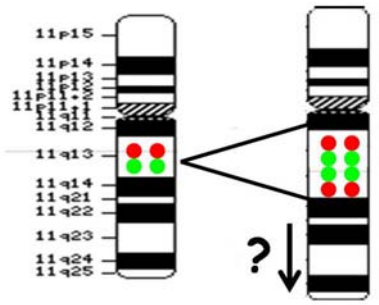

Figure 12. Inverted duplication pattern observed in BFB model of gene amplification[18].

lines were classified as having 11q13 amplification if 2% or more of interphase nuclei expressed more than 10 copies of the *CCND1* gene. Of the OSCC cell lines studied, 18 were determined to express 11q13 amplification versus 11 without 11q13 amplification (Fig. 13, Table 6). Among those cell lines defined as expressing amplification (n=18), there was a statistically significant correlation between the percentage of cells with amplification and the percentage with bridges[19]. The Spearman correlation has 0.40 ($p = 0.037$) (Appendix I). Thus, it is evident from the scatter plot that most of the correlation is due to differences between our "amplified" and "non-amplified" groups.

We observed a high frequency of 11q13 sequences within anaphase bridges in cell lines with greater than ten copies of *CCND1* (Figs.13-14, Table 6). The average frequency of anaphase bridges expressing 11q13 sequences in cell lines without 11q13 amplification was 8.2% (range 2.5% to 32.5%) compared to 26% (range 2.4% to 70%) in cell lines with 11q13 amplification. Statistical analysis of our results suggested that a significantly higher proportion of anaphase bridges contain *CCND1* and/or *RIN1* in cell lines expressing 11q13 gene amplification than in cell lines without 11q13 amplification ($p = 0.005$; t-test) (Appendix I). The finding that genes from within the 11q13 amplicon are present in the form of inverted duplications within anaphase bridges confirms that the majority of 11q13 gene amplification in OSCC occurs through the BFB cycle mechanism.

[18] Red, *RIN1* probe; green, *CCND1* probe. Left, normal chromosome 11 expressing one copy each of *RIN1* and *CCND1*; right, chromosome 11 expressing an inverted duplication, in which *RIN1* flanks *CCND1*.

[19] Statistical analyses carried out by Eleanor Feingold, Ph.D., University of Pittsburgh Graduate School of Public Health, Department of Human Genetics.

Table 6. FISH analysis of *CCND1* and *RIN1* in anaphase bridges and interphase nuclei of OSCC cells.

SCC CELL LINE[20]	Anaphase bridges containing inverted duplication pattern	Percent of interphase cells with *CCND1* gene amplification (>10 copies per cell)	*CCND1* Amplification
UMSCC001	32.5	20.4	+
UPCI:SCC003	52.5	2.9	+
UMSCC14A	7.5	0	-
UPCI:SCC016	25	52.9	+
UMSCC023	27.5	2	+
UMSCC025	5	0	-
UPCI:SCC029B	32.5	89.9	+
UPCI:SCC032	17.5	74.3	+
UPCI:SCC036	47.5	100	+
UPCI:SCC056	15.6[†]	23.4	+
UPCI:SCC066	2.5	0	-
UPCI:SCC070	2.4[§]	30.7	+
UPCI:SCC075	0	0	-
UPCI:SCC077	0[ψ]	0	-
UPCI:SCC078	2.4[§]	78.2	+
UPCI:SCC084	24.4	52	+
UPCI:SCC103	70	39	+
UPCI:SCC104	15	6	+
UPCI:SCC105	25.6	0	-
UPCI:SCC111	25	9	+
UPCI:SCC114	21.2	ND	ND
UPCI:SCC116	12.5	0	-
UPCI:SCC122	0	0.8	-
UPCI:SCC125	12.5	8.1	+
UPCI:SCC131	25	78.6	+
UPCI:SCC136	15	35.6	+
UPCI:SCC142	17.5	0	-
UPCI:SCC154	12.5	0	-
UPCI:SCC172	32.5	0	-
UPCI:SCC182	ND	26	+

[20] UM, University of Michigan; UPCI, University of Pittsburgh Cancer Institute.
[†] Only 32 anaphase bridges available for analysis.
[§] A total of 41 anaphase bridges analyzed.
[ψ] Only 24 anaphase bridges available for analysis.
[§] A total of 41 anaphase bridges analyzed.

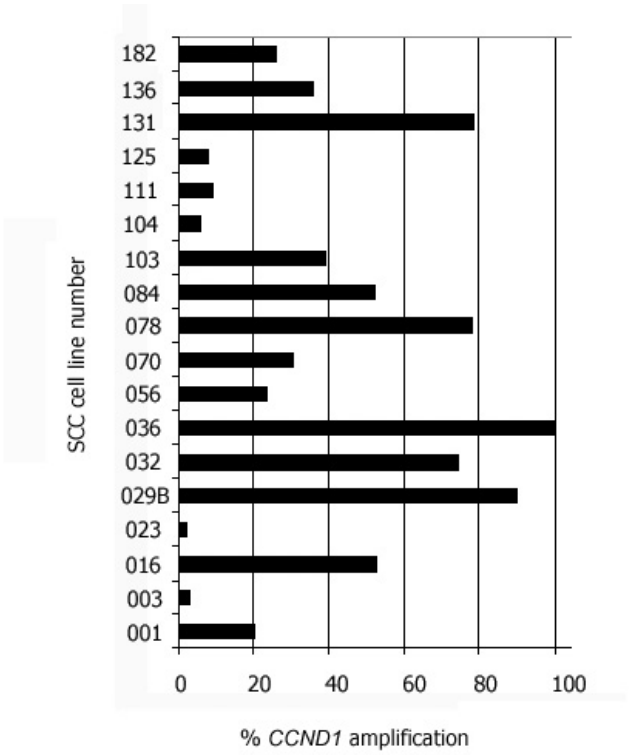

Figure 13. 11q13 gene amplification in UPCI:SCC cell lines.

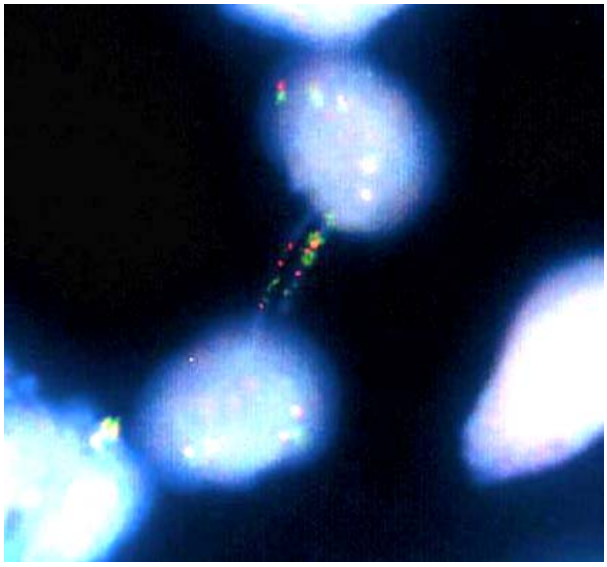

Figure 14. Inverted duplication pattern of 11q13 gene amplification in two anaphase bridges between dividing cells[21].

[21] Anaphase bridges in UPCI:SCC103 cell line. *RIN1*, Spectrum-Orange™; *CCND1*, Spectrum-Green™.

5. DISCUSSION

5.1. CIN IN OSCC CLONES

Epithelial carcinomas, such as SCCHN and their subset, OSCC, frequently exhibit complex karyotypes. The karyotypes of OSCC cells contain numerical and structural abnormalities, including balanced and unbalanced translocations, deletions, dicentric chromosomes, gene amplification in the form of hsrs, and isochromosomes. Although individual cells may express unique chromosomal rearrangements, classical and molecular cytogenetic analyses of OSCC cells have described several clonal aberrations including loss of chromosomal regions 9p21, 3p, 17p13, 11q13 amplification and/or loss of regions 4q26-q28, 6p, 8p23, 13q21, and/or 14q24 (GOLLIN 2001). As a result of these deletions and amplifications, karyotypes may express abnormalities such as whole arm chromosome loss and/or isochromosome formation of 5p, 8q, 9q, and others. Initial karyotype analysis of our OSCC cell lines by Christa Lese Martin revealed that cells were near-triploid and contained chromosome abnormalities typically observed in OSCC, along with various other structural alterations. Our findings are consistent with those of Carey et al. (1993), Shackney et al. (1995), and Jin et al. (2002), and conform to the observation that late-stage, malignant cancer cells contain between 60 and 90 chromosomes.

There are a number of ways in which cancer cells may become tetraploid, including: mitotic arrest, failed cytokinesis, and spindle defects (failed chromosome alignment followed by division of tetraploid chromosome number into several micronuclei), or endoreduplication (OKSALA and THERMAN 1974; SHACKNEY *et al.* 1995). This event, followed by subsequent chromosome loss (reviewed in Nigg, 2002) may account for the near-triploid chromosome number observed in our OSCC cell lines. In contrast to the usually quite stable karyotype of cells in hematological cancers (HEIM and MITELMAN 1995), the CIN observed as aneuploidy between individual cells has been demonstrated in a variety of carcinomas (JIN *et al.* 2002; LENGAUER *et al.* 1997b; PIHAN and DOXSEY 1999; SHACKNEY *et al.* 1995). Previous investigations from our laboratory suggest that the progressive numerical alterations in OSCC cells may result from defects in the mitotic apparatus and aberrant centrosomes (QUINTYNE *et al.*

2005; REING *et al.* 2004; SAUNDERS *et al.* 2000). Here we combined classical cytogenetics, FISH, and SKY, to compare and contrast clones derived from two of our OSCC cell lines. Consistent with previous investigations of tumor evolution (ALBERTSON *et al.* 2003), cell lines in our study demonstrated remarkable karyotype stability between the parental cell lines and daughter clones, despite expressing variations in chromosome number, unique rearrangements, and marker chromosomes that appeared to "evolve" by combining with segments of other broken chromosomes, giving rise to complex rearrangements.

Karyotype analysis of our OSCC parental cell lines and clones revealed that centromere copy number similarities between the individual clones observed by FISH did not necessarily represent the same chromosome within each cell (Fig. 3). Marker chromosome evolution in OSCC cells is consistent with previous studies demonstrating that mechanisms promoting CIN remain active in late stage tumor cells. Such factors include anaphase bridges resulting from telomere defects (GISSELSSON *et al.* 2002; GORDON *et al.* 2003) cell lines in our study demonstrated remarkable karyotype stability between the parental cell lines and daughter clones, despite expressing variations in chromosome number, unique rearrangements, and marker chromosomes that appeared to "evolve" by combining with segments of other broken chromosomes, giving rise to complex rearrangements.

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genes, susceptibility to viral integration, and the capability of acquiring drug resistance through gene amplification. In experimental mammalian cells and in human oral cancer cells, we and others have demonstrated that breakage-fusion-bridge (BFB) cycles (McCLINTOCK 1938; McCLINTOCK 1939) may result in gene amplification (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; HELLMAN *et al.* 2002; SHUSTER *et al.* 2000) as well as chromosome missegregation and aneuploidy (GISSELSSON *et al.* 2002), resulting in all forms of chromosomal instability.

Consistent with our current findings, recent studies of independently derived clones from the UPCI:SCC040 cell line demonstrated similar segregational defects between daughter clones and parental cell populations (REING *et al.* 2004). The frequency of metaphase cells with lagging chromosomes, anaphase bridges, and anaphase cells containing lagging chromosomes was remarkably conserved within the five daughter clones, although a somewhat higher incidence of multipolarity was observed. The difference in overall occurrence of segregational defects between the UPCI:SCC040 parent and cloned cells when compared to the UPCI:SCC103 cell line was striking. Unfortunately, those clones were not available for karyotype analysis. However, we show that despite common loss and gain of genetic material characteristic of OSCC tumors (CAREY *et al.* 1993; GOLLIN 2001; JIN *et al.* 2002; SHACKNEY *et al.* 1995), additional clonal alterations in chromosome structure may be present within parental and daughter cell lines, but rarely between the parental cell lines themselves. In addition, no two cells from the parent or daughter clones of either cell line expressed the identical karyotype. Although various mechanisms leading to genetic instability have been described, their overall influence with respect to carcinogenesis remains largely unknown. While defects in cell cycle checkpoints often provide a green light for abnormal tumor cells to continue through the cell cycle, other mechanisms, including BFB cycles, gene amplification, chromosomal fragile sites, and defects in the DNA repair of DSBs can be involved in promoting CIN. Thus, chromosomal instability in the form of marker chromosome evolution may result from both intrinsic chromosomal factors, such as dicentric chromosomes involved in BFB cycles (McCLINTOCK 1938; McCLINTOCK 1939), and extrinsic chromosomal factors, including multipolar spindles (SAUNDERS 2005; SAUNDERS *et al.* 2000).

5.2. BREAKAGE AT *FRA11F* AS AN INITIATING EVENT IN 11q13 GENE AMPLIFICATION

We and others have shown that amplification of band 11q13 is generally observed in the form of an hsr involving band 11q13 (BARTKOVA *et al.* 1995; GOLLIN 2001; JIN *et al.* 1998b; LESE *et al.* 1995; MICHALIDES *et al.* 1995). Other investigators have demonstrated that intracellular gene amplification may be promoted by exposing cells to certain clastogens or hypoxia, which cause DNA strand breaks (KUO *et al.* 1998; RICE *et al.* 1986; SINGER *et al.* 2000; TONNIES *et al.* 2003). In an attempt to repair these breaks, sister chromatids may fuse together and ultimately form a dicentric chromosome (Fig. 15). If both centromeres remain active, during anaphase the centromeres could either migrate to the same pole or opposite poles. In the event that the centromeres are pulled in opposing directions, an anaphase bridge is formed between the two dividing cells.

There are three possible fates for broken dicentric chromosomes resulting from anaphase bridging. The chromosome ends may 1) be repaired or stabilized through the addition of telomere sequences by telomerase; 2) fuse with other broken chromosome ends, forming derivative chromosomes that may or may not contain two centromeres; 3) fuse with each other, forming another dicentric chromosome containing an inverted duplication pattern of genes. In the event of further dicentric chromosome formation, anaphase bridges would continue through subsequent cell divisions, promoting BFB cycles. Previous studies by our laboratory demonstrated expression of the 11q13 amplicon in the form of an inverted duplication in which multiple copies of *CCND1* were flanked by *RIN1* (SHUSTER *et al.* 2000). Further investigation of our OSCC cell lines revealed dicentric anaphase chromatin bridges (SAUNDERS *et al.* 2000). Combined, these findings suggested that 11q13 gene amplification occurs via BFB cycles which may result from chromosome breakage.

There has been increasing evidence that breakage within highly flexible regions of the genome known as common fragile sites (CFS) may initiate gene amplification in human tumors (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; PIPIRAS *et al.* 1998; STARK 1993; WINDLE *et al.* 1991). We therefore set out to determine whether or not our OSCC cell lines expressed a common region prone to breakage along chromosome 11 that could trigger 11q13 gene amplification. Physical mapping studies of the 11q13 amplicon using QuMA on 30 OSCC cell

lines revealed proximal and distal breakpoint regions between D11S4178 and D11S5031 and D11S5037 and D11S1314, respectively (HUANG *et al.* 2002). Interestingly, *FRA11H* and *FRA11F* have been identified as CFS flanking the region harboring *CCND1*. Taken together, we hypothesized that one or both CFS may be highly prone to breakage in OSCC cells.

In our current investigation, we identified and characterized a CFS distal to the 11q13 amplicon. We show the most frequently broken CFS on the long arm of chromosome 11 to be *FRA11F* at 11q14.2. The very low occurrence of breakage between the centromere and 11q13

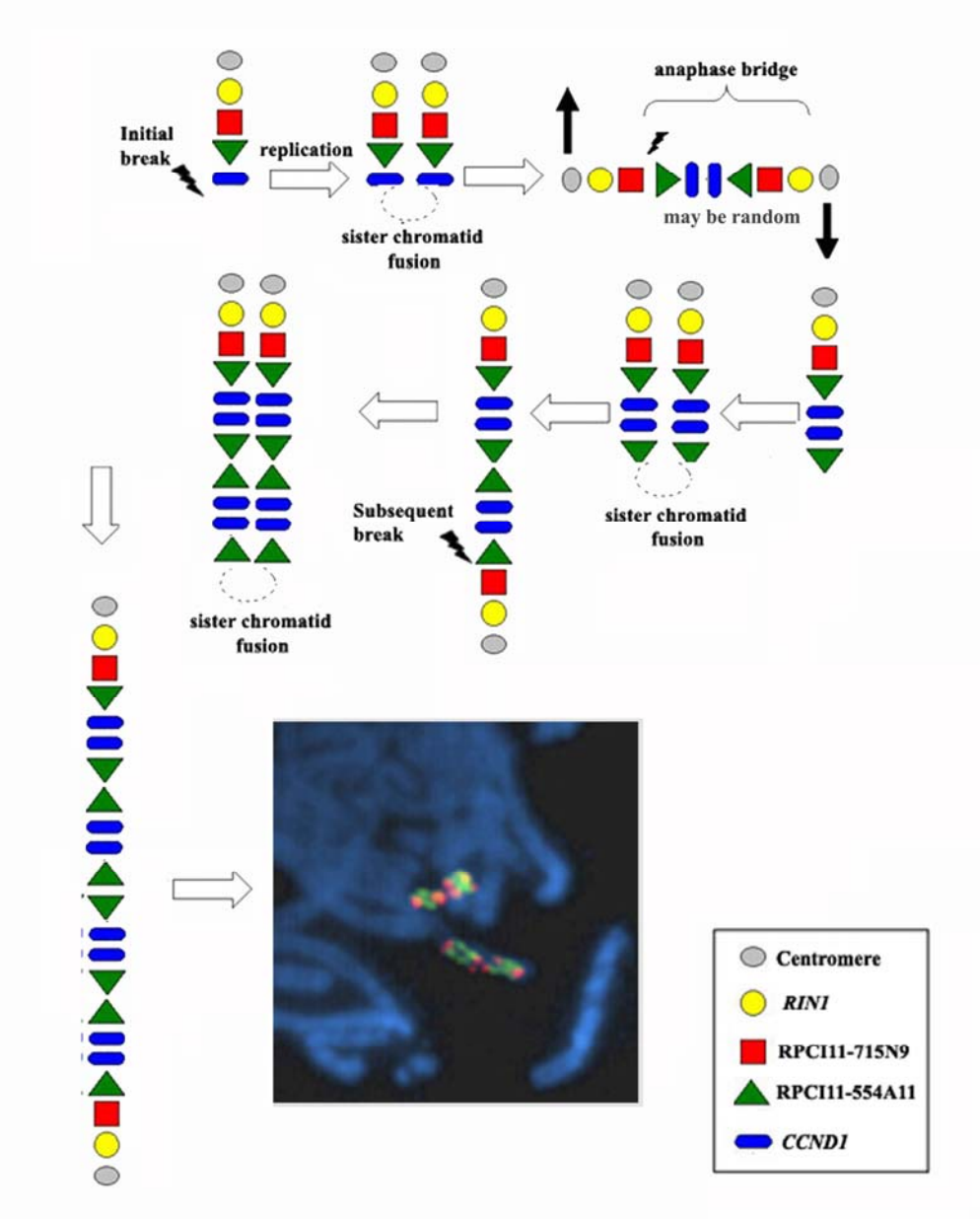


Figure 15. Proposed BFB model for OSCC cells[22].

[22] (A) Proposed BFB model for BACs proximal to 11q13 amplicon in UPCI:SCC040. Partial metaphase cell shows two chromosomes with an inverted duplication of the 11q13 amplicon. Note BFB model schematic represents only one-half of the amplicon expressed in each chromosome.

prevented physical mapping and characterization of the more proximal CFS, *FRA11H* (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene). Due to the folate-sensitive nature of the rare *FRA11A* fragile site, we were unable to physically map its exact sequence or determine its role in 11q13 gene amplification. Unlike common fragile sites, rare fragile sites cannot be induced in all individuals. A previous report suggested the presence of increased breakage of *FRA11A* in young smokers (KAO-SHAN *et al.* 1987). However, confirmation of breakage specifically within the *FRA11A* fragile site was not discussed. These investigators may actually have been observing breakage of the *FRA11F* common fragile site. If *FRA11A* is confirmed to break at a higher frequency in blood cells of smokers, physical mapping of *FRA11A* may be feasible. Following the characterization of *FRA11F*, determining its role, if any, in OSCC gene amplification may then be possible.

To compare the physical characteristics of *FRA11F* to those identified for other CFS, we characterized the sequence corresponding to the *FRA11F* region we identified by FISH (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene). A total of 12 BACs spanning 7.5 Mb corresponded to *FRA11F*, including a small 12 kb sequence gap in the genome map. We showed that breakage of *FRA11F* in APCC- induced peripheral blood cells is equally distributed among BACs spanning *FRA11F* (Figs. 7-8, Table 4). Our findings are consistent with earlier observations that the active "center" of a fragile site exhibits proximal and distal breakage with relatively equal frequencies (CALLAHAN *et al.* 2003). CFS regions have been shown to have a GC content (41%) similar to the average GC content in the human genome (MORELLI *et al.* 2002; VENTER *et al.* 2001). We found the GC content of the 7.5 Mb region defining *FRA11F* to be 37.4%, less than that described for *FRA3B, FRA6E, FRA6F, FRA7H,* and *FRA16D* (MORELLI *et al.* 2002). Since repetitive DNA sequences are also thought to play a role in contributing to the instability of fragile site regions, we analyzed *FRA11F* for these elements. We observed that repeat sequences comprised approximately one-half of the DNA content for *FRA11F*, which is a larger percentage than those identified in the five CFS characterized previously (MORELLI *et al.* 2002). Since AT base pairs are held together by one less hydrogen bond than GC base pairs, the increased percentage of AT-rich sequences within *FRA11F* suggests it may also express a higher susceptibility to breakage compared to other CFS.

Currently, there are 17 known genes within *FRA11F* (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene). The majority of genes (76%) reside in the

proximal 5.7 Mb region of *FRA11F*. Both *DLG2* and *PICALM*, a gene identified as a partner for *AF10* in the t(10;11)(p13;q14) in myeloid and lymphoid leukemias (DREYLING *et al.* 1996), contain coding regions within proximal *FRA11F*. Members of the lethal(1)discs large (*DLG*) gene family are able to convert organized epithelial tissues into muddled, undifferentiated hyperplastic overgrowths when mutated in *Drosophila* (reviewed in Watson et al., 1994). Of the tumor suppressor genes identified in *Drosophila*, only *DLG* and *l(2)gl* (lethal(2)giant larvae) have been proposed as candidates having a role in human cancers, since loss of function of either results in neoplastic transformation (DE LORENZO *et al.* 1999). One OSCC cell line, UPCI:SCC078, demonstrated amplification within the proximal region of *FRA11F* and loss of distal 11q, including distal *FRA11F*. Dual-color FISH for BACs spanning the proximal and medial *FRA11F* regions (RP11-281H14 and RP11-89H11) confirmed that a break occurred within the region of *FRA11F* containing *DLG2* (Fig. 9C). Our finding that the majority of OSCC cell lines expressing 11q13 amplification display breakage within proximal *FRA11F* suggests that disruption and/or loss of *DLG2* as well as other neighboring genes may play a role in OSCC development and/or progression.

Amplification of 11q13 in OSCC may be the consequence of losing genes distal to 11q13 following breakage at *FRA11F,* and as a secondary event resulting from the attempt of a damaged cell to repair itself through sister chromatid fusion (CIULLO *et al.* 2002; HELLMAN *et al.* 2002). The latter event would result in a dicentric chromosome containing inverted repeats of 11q13 genes within the amplicon. If both centromeres remained active on the newly formed chromosome, they may segregate to opposite poles during anaphase resulting in an anaphase bridge (MCCLINTOCK 1938; MCCLINTOCK 1939). Our current study suggests that broken chromosome ends containing *FRA11F* sequences or chromosome ends with an increase in copy number of genes proximal to lost *FRA11F* regions, eliciting subsequent BFB cycles and chromosomal instability. We previously observed dicentric chromosomes and an inverted repeat gene pattern of *RIN1* and *CCND1* within OSCC cell lines expressing *CCND1* amplification (SHUSTER *et al.* 2000). In addition, we identified variable breakpoint cluster regions in the 11q13 hsr as well as genetic heterogeneity within the amplicon of our OSCC cells (Figs. 9B-D, 10, 11). These findings provide further support for the hypothesis proposed by Hellman and colleagues (HELLMAN *et al.* 2002), that intrachromosomal gene amplification in the form of an hsr may occur by chance due to the location of the amplified genes relative to a CFS.

Our current study reveals that loss, breakage, and/or inverted duplications of *FRA11F* occurred in 15 of 23 cell lines examined. Therefore, we suggest that breakage of distal *FRA11F* at 11q14.2 may be an important event leading to amplification of the 11q13 region. We show that spontaneous breakage of *FRA11F* contributes to the complexity of the 11q13 amplicon through BFB cycles as evidenced by the presence of inverted duplications within the amplified region (Fig. 16). These data are consistent with those of Ciullo and colleagues (CIULLO *et al.* 2002), in which breakage of *FRA7I* in a T47 breast cancer cell line expressed gene amplification in the form of an hsr containing the *PIP* gene resulting from BFB cycles.

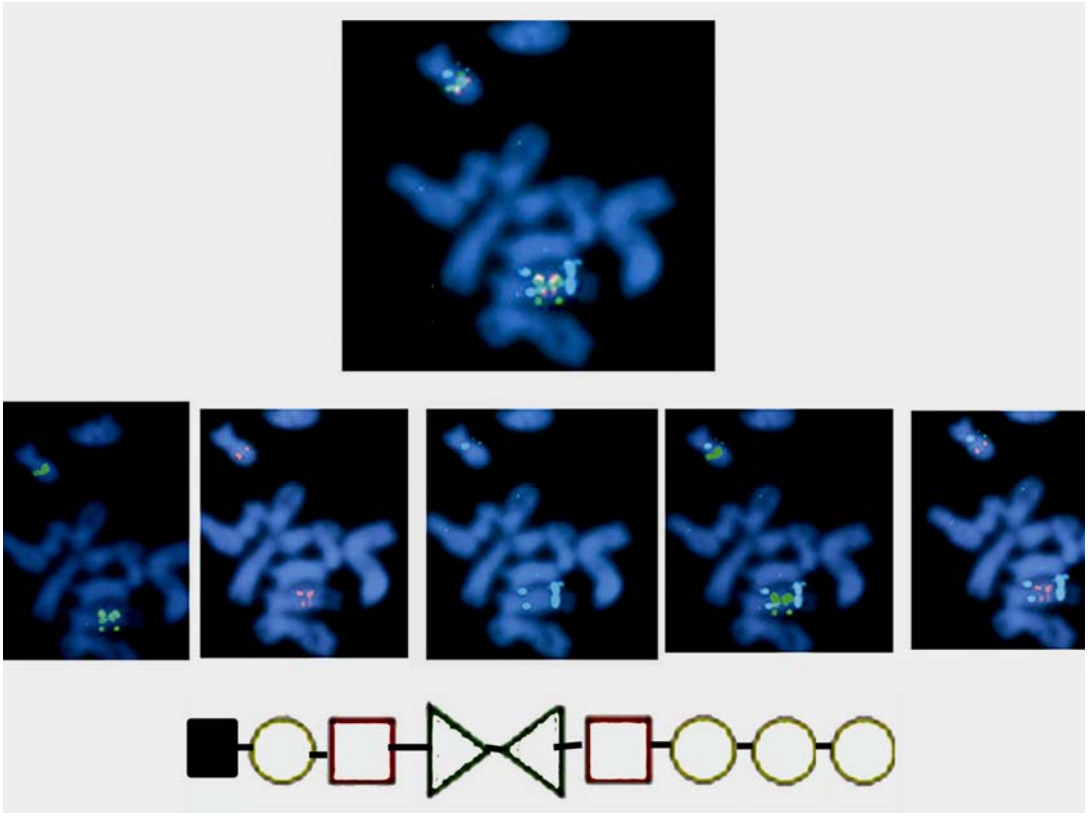


Figure 16. Metaphase FISH demonstrating inverted duplication of *CCND1* and *FRA11F* within the 11q13 amplicon[23].

[23] UPCI:SCC032 expressing an inverted duplication pattern and one normal chromosome 11. *CCND1* in aqua, proximal *FRA11F* sequences in Spectrum-Green (RP11-281H14, RP11-313I2), and distal *FRA11F* sequences in Spectrum-Orange (RP11-208P3). Panel shows 1) a merged image of *CCND1* with both proximal and distal *FRA11F* sequences; 2) distal *FRA11F* only; 3) proximal *FRA11F* only; 4) *CCND1* only; 5) proximal *FRA11F* in Spectrum-Green and *CCND1* in aqua; 6) distal *FRA11F* in Spectrum-Orange and *CCND1* in aqua. Diagram depicting an inverted duplication pattern. Filled rectangle, centromere; circle, *CCND1*; square, proximal *FRA11F* sequences; triangle, distal *FRA11F* sequences.

There are many issues that remain unresolved regarding the role of CFS in human cancer. For example, why does loss of one CFS result in gene amplification while loss of another does not? Why have individual tumor types been shown to contain LOH at multiple CFS (DENISON *et al.* 2002; PETURSDOTTIR *et al.* 2004)? Does loss of a specific region or gene(s) within a particular CFS confer a selective advantage for particular tumor types? With the exception of *FRA3B* (HUEBNER and CROCE 2003), the mechanisms by which individual CFS promote neoplastic transformation and progression are only starting to be examined. Due to resources that have become currently available through the human genome project, characterization of other CFS is more feasible.

Previous work by Casper et al. (CASPER *et al.* 2002) demonstrated the importance of *ATR* and *BRCA1* in maintaining fragile site stability. Their study concluded that fragile sites may result from defects in genes involved in DNA repair. By knocking down *BRCA1* through RNA interference, Arlt et al. (ARLT *et al.* 2004) showed that mouse and human cells with almost no functional *BRCA1* have elevated expression of CFS. Furthermore, they provide evidence that proper functioning of *BRCA1* is required for the G_2/M checkpoint to identify late replicating DNA, including CFS. These observations raise the question of whether or not breakage at CFS is causal in particular cancers, or if fragile site breakage is merely a reflection of faulty repair machinery that is unable to detect and correct these regions of stalled replication prior to cell division. Given that 1) close to half of all oral cancer cases express 11q13 amplification which is associated with decreased survival and poor response to treatment (AKERVALL *et al.* 1997; FORASTIERE *et al.* 2001; GOLLIN 2001; MICHALIDES *et al.* 1995) and 2) current cancer therapies employ agents known to cause chromosome breaks that may result in gene amplification of oncogenes within cells of relapsing individuals (CHAMPEME *et al.* 1995), a further understanding of the biological mechanism(s) involved in the amplification process is critical.

5.3. EVIDENCE IN SUPPORT OF A BFB CYCLE MODEL FOR 11q13 GENE AMPLIFICATION

The presence of anaphase bridges has been shown to be a useful biomarker for defects in chromosome segregation (HOFFELDER *et al.* 2004; LUO *et al.* 2004; MONTGOMERY *et al.* 2003). We and others have demonstrated that anaphase bridges contribute to CIN through gene amplification (GISSELSSON *et al.* 2000) and micronucleus formation with chromosome fragmentation and/or loss (GISSELSSON *et al.* 2000; HOFFELDER *et al.* 2004; KAYAL *et al.* 1993; LIVINGSTON *et al.* 1990; LUO *et al.* 2004; SAUNDERS *et al.* 2000; STICH *et al.* 1992). We further suggest that anaphase bridging promotes marker chromosome evolution (RESHMI *et al.* 2004), as this phenomenon has been reported previously in tumors exhibiting CIN (MONTGOMERY *et al.* 2003). However, the precise mechanisms by which anaphase bridges promote these events in specific tumor types remain under investigation.

We recently showed that breakage at a common fragile site, *FRA11F*, is associated with 11q13 gene amplification in oral cancer cells. In addition, we identified inverted duplications of *CCND1* flanked by *RIN1* within the 11q13 amplicon (SHUSTER *et al.* 2000), and broken chromosomes with amplified chromosome ends (Fig. 9D). Combined, our observations support the BFB cycle model for gene amplification. The current study links the findings of our previous work by providing evidence that inverted duplication sequences of *RIN1* and *CCND1* expressed in metaphase chromosomes are also expressed within a high percentage of anaphase bridges in OSCC cells. Here we carried out FISH using *CCND1* and *RIN1* probes on 29 OSCC cell lines with and without 11q13 amplification. On each chamber slide, we: 1) assessed *CCND1* gene copy number within interphase cells to confirm and compare 11q13 amplified OSCC cell lines from non-amplified cell lines in this study; 2) quantified *CCND1* and *RIN1* sequences within anaphase bridges within each cell line. OSCC cell lines negative for 11q13 amplification expressed a lower frequency of *CCND1* and *RIN1* sequences in anaphase bridges compared to cell lines with greater than ten copies of *CCND1* (Figs.13-14, Table 6). The average frequency of anaphase bridges expressing 11q13 sequences in cell lines without 11q13 amplification was 8.2% (range 2.5% to 32.5%) compared to 26% (range 2.4% to 70%) in cell lines with 11q13

amplification. Statistical analysis of our results suggested that a significantly higher proportion of 11q13 amplicon sequences occur in anaphase bridges of cell lines expressing 11q13 gene amplification than in cell lines without 11q13 amplification ($p = 0.005$; t-test) (Appendix I). The finding that genes from within the 11q13 amplicon are present in the form of inverted duplications within anaphase bridges confirms that the majority of 11q13 gene amplification in OSCC occurx through the BFB cycle mechanism.

Although we present for the first time a direct association between gene amplification and anaphase bridges in cancer cells, our current investigation cannot rule out the presence of other segregational defects contributing to anaphase bridges. Chromosome segregation requires properly functioning cell cycle checkpoints, mitotic machinery, and an efficient anaphase promoting complex (APC) to ensure that each daughter cell receives equal and identical chromosome material from their mother cell.

One of the checkpoints, the mitotic spindle checkpoint, ensures bipolar attachment of kinetochores to microtubules prior to chromosome separation at anaphase (HOYT 2001). If all kinetochores are properly attached, the checkpoint signals activation of the APC to continue with cell division. In cells with properly functioning spindle checkpoints, it has been demonstrated that a single misaligned kinetochore is able to prevent cell cycle progression (WELLS and MURRAY 1996). Genes included in this pathway are the mitotic arrest deficient (Mad) *Mad1*, *Mad2*, *Mad3/BubR1*, budding uninhibited by benzimidazole (Bub) *Bub1*, *Bub3*, *Mps1* and *Aurora-B* (reviewed in (DRAVIAM *et al.* 2004)). Proteins involved in the spindle checkpoint have shown evolutionary conservation, highlighting the importance of maintaining the delicate balance of genetic material within living cells (HOYT *et al.* 1991; LI and MURRAY 1991). Protein homologues of the Mad and Bub families have been shown to identify and associate only with lagging chromosomes that fail to align properly (CHEN *et al.* 1996; JIN *et al.* 1998a; TAYLOR *et al.* 1998; TAYLOR and MCKEON 1997). Consistent with this idea is the suggestion that lagging chromosomes may facilitate binding of Mad2 to Cdc20, inhibiting progression into anaphase (GORBSKY 2001; SHAH and CLEVELAND 2000; YU 2002). If lagging chromosomes are detected, Mad2 of the spindle checkpoint prevents separation of sister chromatids and degradation of cyclins by inhibiting APC-Cdc20 (YU 2002). Other studies have suggested that the microtubule motor, dynein, may play an important role in checkpoint inactivation by assisting in the transport of checkpoint proteins between the spindle poles and kinetochores

(reviewed in (BHARADWAJ and YU 2004). However, despite the findings that mutations in h*Bub* are present in cancer cells expressing CIN (CAHILL *et al.* 1998), loss of genes within cell cycle checkpoints by themselves has not been shown to directly cause segregational defects.

The APC is a ubiquitin ligase that targets degradation of cell cycle proteins to enable cells for mitotic exit (CASTRO *et al.* 2005). Prior to anaphase, a cohesin complex prevents premature separation of replicated sister chromatids joined in S phase until they enter anaphase (ZOU *et al.* 1999). Cohesin has been shown to contain several subunits, including Scc1p/Mcd1p, Scc3p, Smc1p, and Smc3p (MORRISON *et al.* 2003). These proteins form cohesin complexes along many sites on each chromosome (MICHAELIS *et al.* 1997). In humans, the cohesin subunits include: SMC1, SMC1β, SMC3, SCC1(RAD21), REC8, SA1, SA2, STAG3 (reviewed in Nasmyth, 2001). To enter anaphase, sister chromatids are separated by breakdown of the cohesin complex (Fig. 17). This event requires activation of securin which in turn degrades separase, then cleaves the cohesin complex at its subunits, resulting in sister chromatid separation (UHLMANN *et al.* 1999; UHLMANN *et al.* 2000; YANAGIDA 2000). Cohesin is essential for facilitating attachment of microtubules from opposite poles to sister centromeres (TANAKA *et al.* 2000). Mutant cohesion has been shown to promote monopolar attachment of sister centromeres, resulting in chromosome missegregation (GUACCI *et al.* 1997; MICHAELIS *et al.* 1997; UHLMANN and NASMYTH 1998). Errors of cohesin reformation have also been associated with CIN (HOQUE and ISHIKAWA 2002; RAO *et al.* 2001; UHLMANN 2004). One study by Jallepalli et al. (2001) observed anaphase bridges, budding nuclei, and CIN as a result of inactivating both copies of the securin/pituitary tumor-transforming 1 (*PTTG1*) gene[24], in a human colorectal cancer cell line. This suggests that cells are unable to carry out sister chromatid separation in the absence of separase cleavage by securin. In addition, failure of separase to cleave the cohesin complex may itself lead to bridge formation (HAERING *et al.* 2004).

[24] The official symbol and name for the human *SECURIN* gene.

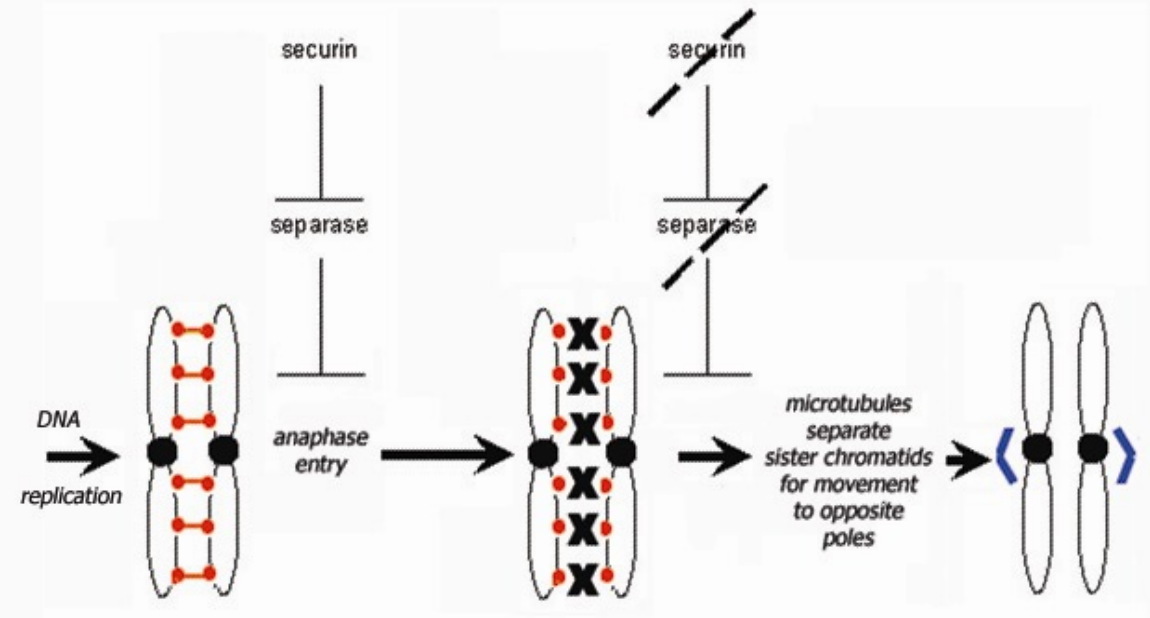


Figure 17. Degradation of the APC for chromosome separation[25].

In order for anaphase to proceed, several proteins must ubiquitinated by APC and subsequently degraded by a proteasome protein complex. In fission yeast, two mutants of centromere enhancer of position effect (cep) genes that are necessary for proteasome structure were shown to prevent sister chromatid separation, resulting in missegregation of chromosomes (JAVERZAT *et al.* 1999). Although the mechanism for this phenomenon is unclear, further studies will elucidate whether or not one or more protein targets are necessary for the resulting phenotype. However, studies in *Drosophila* revealed that mutations in a single ubiquitin-conjugating enzyme, UbcD1, resulted in dramatic increases in telomere associations (CENCI *et al.* 1997). Taken together, these studies suggest that anaphase bridges may also result from defective proteasome function.

There is also evidence to suggest an association between anaphase bridge formation and segregational errors due to abnormal numbers of mitotic spindles (GISSELSSON *et al.* 2002; GISSELSSON *et al.* 2004). Previous investigations from our laboratory identified the presence of multipolar spindles in OSCC cells (SAUNDERS *et al.* 2000). We recently demonstrated that

[25] Red connected circles, cohesion complex; blue arrows, tension from microtubules.

increases in the spindle protein, NuMA, plays a major role in promoting spindle multipolarity (QUINTYNE *et al.* 2005).

Although centrosome amplification is not essential for multipolar spindle formation (reviewed in Saunders, 2005), multipolar spindles have been shown to result from increased centrosomal amplification in mice mutant for *Brca1* (DENG 2002; XU *et al.* 1999b) and *Brca2* (TUTT *et al.* 1999; XU *et al.* 1999b). In addition, both overreplication and failed cytokinesis resulting in centrosome amplification have been associated with increases in anaphase bridging. In order for the APC complex to function properly, various substrates are required. These include, but are not limited to, Cyclin B, Cdc5/Plk1/Polo kinase, Cdc20, Aurora A kinase (CASTRO *et al.* 2005). Studies have shown that aberrant protein expression within the cell cycle, such as amplified Aurora A, Aurora B, or Plk1 may promote centrosome amplification (ZHOU *et al.* 1998). Interestingly, centrosome amplification and anaphase bridges may also be caused by overexpression of the DNA damage response genes, *ATM* and *ATR*, as well as other associated genes and proteins within these pathways (SAUNDERS *et al.* 2000; SMITH *et al.* 1998).

It was demonstrated previously that the majority of broken chromosome ends resulting from anaphase bridges are repaired by non-homologous end joining (NHEJ)(reviewed in (PASTINK *et al.* 2001). The NHEJ pathway includes DNA-dependent protein kinase (DNA-PK), Ku70, Ku80, DNA ligase-4 and XRCC4. Through this repair pathway, joining of heterologous chromosome ends may result dicentric chromosomes that are then pulled to opposite poles at anaphase, resulting in an anaphase bridge. Subsequent chromosome end fusions may occur, promoting BFB cycles and either 1) gene amplification through chromosomes that have undergone sister chromatid fusion, forming inverted duplication segments and homogeneously staining regions, or 2) CIN due to formation of other, nonhomologous dicentric chromosomes (with different chromosomal origins) (ZHIVOTOVSKY and KROEMER 2004). Current investigations are ongoing to determine the role of the specific genes in the NHEJ pathway in anaphase bridge formation resulting in CIN (C. Acilan and W.S. Saunders, unpublished work).

There are various biological mechanisms that promote anaphase bridge formation. These include: 1) formation of dicentric chromosomes through telomere dysfunction (GISSELSSON *et al.* 2000; GORDON *et al.* 2003; HACKETT *et al.* 2001), 2) centrosome amplification (DENG 2002; SAUNDERS 2005; TUTT *et al.* 1999; XU *et al.* 1999b; ZHOU *et al.* 1998), and 3) defects in genes or proteins associated with DNA damage response pathways (PARIKH and GOLLIN unpublished

work; SAUNDERS 2005; SMITH *et al.* 1998). In addition, previous investigations have suggested a BFB cycle model for gene amplification (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; HELLMAN *et al.* 2002; MCCLINTOCK 1938; MCCLINTOCK 1939; SHUSTER *et al.* 2000), proposing that gene amplification occurs through anaphase bridges resulting from dicentric chromosomes formed from sister chromatid fusion of broken chromosome ends. We and others have shown the presence of anaphase bridges in cancer cells (GISSELSSON *et al.* 2000; HOFFELDER *et al.* 2004; LUO *et al.* 2004; MONTGOMERY *et al.* 2003; REING *et al.* 2004; SAUNDERS *et al.* 2000). However, until now, a direct association between anaphase bridges and gene amplification has not been demonstrated. Our current observation of inverted duplication sequences within anaphase bridges of OSCC cells expressing 11q13 gene amplification provide conclusive evidence that at least some cases of gene amplification occur through the BFB mechanism.

6. SUMMARY

Chromosomal instability has been shown to play a significant role in the progression of human malignancies. Various factors may induce CIN through DNA double strand breaks in combination with a defective DNA damage response. In the absence of functioning cellular checkpoints, neoplastic cells with intrinsic chromosomal abnormalities are able to continue through cell division, giving rise to daughter cells that do not resemble each other or their mother cell. Similarly, extrinsic cytoskeletal aberrations such as multipolar spindles (SAUNDERS *et al.* 2000), alterations in centrosome number (GISSELSSON *et al.* 2002; SAUNDERS 2005; ZHOU *et al.* 1998) or increased expression of centrosomal proteins (BERGOGLIO *et al.* 2002; GRITSKO *et al.* 2003; LI *et al.* 2003; LINGLE *et al.* 2002; PIHAN *et al.* 1998; PIHAN *et al.* 2001; SATO *et al.* 2001; ZHOU *et al.* 1998) may play an important role in CIN. Regardless of how the observed mutator phenotype originates, propagation of these cells or chromosomal defects is maintained through BFB cycles, which may in turn promote CIN through gene amplification (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; HELLMAN *et al.* 2002; SHUSTER *et al.* 2000).

We investigated three patterns of CIN observed in OSCC: numerical chromosomal aberrations, structural chromosomal alterations, and gene amplification. The current findings demonstrate CIN in oral cancer cells, and further elucidate one mechanism by which CIN occurs. Building on previous work from our laboratory involving identification and characterization of the 11q13 amplicon (HUANG *et al.* 2002; LESE *et al.* 1995; SHUSTER *et al.* 2000) along with recent findings that cigarette smoke condensate induces double strand breaks in normal cells (LUO *et al.* 2004), we now suggest that breakage at the common fragile site, *FRA11F*, may play a key role in initiating gene amplification in OSCC cells. We mapped *FRA11F* to a 7.5 Mb region distal to the 11q13 amplicon and suggest that spontaneous breakage of *FRA11F* contributes to the complexity of the 11q13 amplicon through BFB cycles by showing loss, breakage, and rearrangements of *FRA11F* within OSCC cell lines expressing 11q13 gene amplification.

Our laboratory and others have suggested that gene amplification occurs through BFB cycles (CIULLO *et al.* 2002; COQUELLE *et al.* 1997; MCCLINTOCK 1938; MCCLINTOCK 1939;

SHUSTER *et al.* 2000), in which gene amplification occurs through anaphase bridges resulting from dicentric chromosomes formed by sister chromatid fusion of broken chromosome ends. Our current study demonstrates that sequences harbored within the 11q13 amplicon are present in the form of inverted duplication sequences. In addition, the inverted duplication sequences were found to frequently occur within anaphase bridges of OSCC cells expressing 11q13 gene amplification.

This investigation shows evidence to support our initial hypotheses that 1) breakage at a chromosomal fragile site may be an initial step for 11q13 gene amplification, and 2) gene amplification at 11q13 occurs through BFB cycles in OSCC. Although involvement of *FRA11A* in 11q13 gene amplification cannot be ruled out by the current study, our findings provide further support for the hypothesis proposed by Hellman and colleagues, that intrachromosomal gene amplification in the form of an hsr may occur by chance due to the location of the amplified genes relative to a CFS. Further studies are needed to determine a role, if any, for other fragile sites flanking the 11q region in the gene amplification process.

APPENDIX A. CELL CULTURE PROTOCOLS

M10 medium recipe

500 mL Minimum essential medium w/ Earle's salts + L-glutamine (#11095-081; GIBCO)
55 mL Fetal Bovine Serum (#16000-044; GIBCO) for 10% final concentration
5 ml Non-essential amino acids 10 mM stock (#11140-019; GIBCO)
5.55 mL L-glutamine 200 mM stock (#9317; Irvine Scientific)
694 µL Gentamicin (#9354; Irvine Scientific)

Mix together and sterilize through 0.2 micron filter ((# SLGP033RS, Millipore, Bedford, MA)
Aliquot and freeze at -20ºC

Thawing OSCC cells

1. Add 1 mL pre-warmed M10 medium to 15 mL conical tube
2. Allow cryovial to thaw quickly in 37ºC water bath (15 sec)
3. Add cells to tube containing M10 medium
4. Spin cells at 5ºC for 5 min at 1000 x g
5. Remove supernatant
6. Resuspend with 5 mL fresh M10 medium
7. Place in T25 flask with cap loosened
8. Place flask in incubator (5% CO_2 in air) at 37ºC

Passaging OSCC cells

1. Remove old medium
2. Wash cells in 1XHBSS (#9228; Irvine Scientific)
3. Add 1.5 mL 1Xtrypsin-EDTA (#9341; Irvine Scientific) to each T25
   3.0 mL T75
4. Place flask in 37ºC incubator for 1 min
5. Check flask for detached cells, agitating flask if cells still adherent
6. Once detached, add equal volume of M10 medium to flasks
7. Split cells into desired number of flasks and add M10 medium to 5 mL for T25 flask or 10 mL for each T75 flask (make sure to note passage number on flasks)
8. Return flasks to 37ºC incubator

Freezing OSCC cells

Prepare the freezing medium:
9 mL M10 medium complete
+ 1 mL DMSO for 10% final concentration (# D128-500; Fisher Scientific)

Mix together and sterilize through "DMSO safe" 0.22 micron filter (Millipore)

1. Remove the old medium
2. Wash the cells with 1XHBSS
3. Add trypsin to the flask and place the flask in a 37ºC incubator for 1 min
4. When cells become detached, add an equal volume of M10 complete medium to inactivate the trypsin
5. Place the cells in a 15 mL conical tube
6. Spin for 5 min at 1000 x g
7. Remove the supernatant by aspiration
8. Break up the pellet
9. Add 1 mL of freezing medium to the tube, resuspending the pellet
10. Place in a cryovial, making sure to label the vial with cell line information, passage number, initials, and date
11. Place the cells in the -80ºC freezer for 2 h, then store -135ºC or in liquid nitrogen vapor phase

Normal peripheral blood lymphocyte set up for chromosome analysis

1. Prewarm the peripheral blood complete medium to 37ºC in a waterbath
2. Working in the tissue culture hood, aliquot PB Max™ peripheral blood complete medium (#12557-013, GIBCO) into 5 mL per 15 mL conical tube
3. Invert the tube several times
4. Working in the tissue culture hood, aliquot the peripheral blood complete medium with 5 mL per 15 mL conical tube
5. Use a sterile gauze to remove the cap of the green top tube
6. Using a sterile glass Pasteur pipette, add 13-15 drops of blood to each culture tube
7. Cap each tube, inverting each several times
8. Place the tubes in a slant culture rack and incubate the tubes for 72 h at 37ºC

Protocol for inducing CFS in peripheral blood cells

1. Follow steps 1-7 in "Normal peripheral blood lymphocyte set up for chromosome analysis."
2. Place the tubes in a slant culture rack and incubate for 48 h at 37º
3. Add aphidicolin (APC) to blood cultures (see next page)

APC stock solution:

APC, 1 mg powder (Aphidicolin Nigrospora sphaerica, #A0781, Sigma, St. Louis, MO)
Stock 1: 1 mg APC in 14.8 mL DMSO; filter sterilize and store at -15ºC
Stock 2: 1 mL Stock 1 in 9 mL RPMI 1640 (#9140, Irvine Scientific); filter sterilize and store at -15ºC

4. Add 100 µL of Stock 2 to each culture (0.4 µM)
5. Invert the tubes and return them to the slant culture rack. Incubate the cultures for 26 h at 37ºC
6. Add caffeine

Caffeine stock solution:

Caffeine (#C0750; Sigma)
1.942 g in 100 mL RPMI (100 mM), filter sterilize

7. Remove 250 µL from each blood culture, replacing it with 250 µL caffeine solution
8. Invert the tubes and return them to the slant culture rack. Incubate the cultures for 6.5 h at 37ºC
9. Harvest the cells (NOTE: Colcemid™ is added for 2.5 h for CFS protocol)

<u>Processing chamber slides for anaphase bridge analysis</u>

1. Refer to steps 1-5 in "Passaging OSCC cells"
2. Add the appropriate amount of M10 medium to dilute the cells for 50% confluency for each chamber slide
3. Add 0.5 mL of the diluted cell pellet to each chamber slide
4. Incubate the chamber slides for 1 hr at 37ºC
5. Carefully flood the chamber slides with 1.5 mL pre-warmed M10 medium
6. Return the chamber slides to 37ºC incubator until the cells reach 70% confluency
7. Remove the M10 medium and replace it with fresh M10 medium
8. Add 5 µL (# 9311; Irvine Scientific) to each chamber slide
9. Cover each chamber slide and agitate tomix
10. Incubate chamber slides at 37ºC for 18 h (overnight)
11. Remove the medium and wash the chamber slides two times with fresh M10 to remove the Colcemid™
12. Add 2 mL of fresh M10 medium to each chamber slide
13. Incubate the chamber slides at 37ºC for 2 h (NOTE: Cell cycles will vary for each tumor type; this protocol was optimized for SCC cultures. The timing in this step may need to be increased or decreased depending on specimen)
14. Remove the medium by aspiration
15. Remove the chamber from the chamber slide
16. Place the slides in a fresh fixative solution (3:1 methanol:acetic acid) for 30 min
17. Dry the slides with forced air

18. Store the slides in the-20ºC freezer with dessicant until ready for use

APPENDIX B. HARVESTING CELLS FOR CYTOGENETIC ANALYSIS

Reagents

Colcemid™ (# 9311; Irvine Scientific)
0.075 M Hypotonic KCl (0.056g KCl in 100 mL dd H_2O)
Cold, 3:1 Methanol:acetic acid fixative (Prepare and place in -20ºC freezer)
M10 medium (for OSCC cell lines, see Appendix A)
1X trypsin/EDTA (for OSCC cell lines) (#9341; Irvine Scientific)
1X HBSS (for OSCC cell lines) (#9228; Irvine Scientific)
Peripheral blood harvest

1. Add 50 µL of Colcemid™ (# 9311; Irvine Scientific) to each 5 mL culture (10 µg/mL)
2. Invert the tubes and incubate at 37ºC for 25 min (NOTE: For APC cultures, incubate for 2.5 h)
3. Place tubes in centrifuge tubes for 7 min at 1200 x g
4. Remove the supernatant by aspiration, leaving approximately 1.5 ml in each tube
5. Resuspend the pellets by tapping the tubes
6. SLOWLY, add 5 mL pre-warmed 0.075M KCl. Begin adding hypotonic solution dropwise to each pellet, tapping LIGHLY
7. Invert the tubes one time
8. Incubate the tubes at 37ºC in a waterbath for 18 min
9. Add 5 -10 drops of cold fixative to each tube, inverting one time
10. Place tubes in centrifuge tubes for 7 min at 1200 x g
11. Remove the supernatant by aspiration, leaving approximately 1.5 ml in each tube
12. Resuspend the pellets by tapping the tubes
13. Add cold fixative to 7 mL, invert each tube one time
14. Centrifuge the tubes for 15 min at 1200 x g
15. Remove the supernatant by aspiration, leaving approximately 1.5 ml in each tube
16. Resuspend the pellets by tapping the tubes
17. Continue steps 13-16 until the pellet is white and the fixative is clear
18. Centrifuge the tubes for 5 min at 1200 x g
19. Store the pellets in the explosion proof freezer at -20ºC until use

OSCC cell harvest

1. Add 50 µL Colcemid™ to each 5 mL culture (10 µg/mL)
2. Agitate the flasks to mix, then incubate at 37ºC for 5 h
3. Remove the supernatant by aspiration
4. Wash the cells in 1XHBSS
5. Add 1.5 mL of 1X trypsin-EDTA to each T25
   3.0 mL T75
6. Place the flasks at 37ºC for 1 min
7. Check each flask for cell detachment, agitating the flask if cells are still adherent
8. Once detached, add an equal volume M10 medium to inactivate the trypsin
9. Place cells in a 15 mL conical centrifuge tube
10. Centrifuge the tubes for 7 min at 1000 x g
11. Remove the supernatant by aspiration, leaving 1.5 ml in each tube
12. Resuspend the pellet
13. SLOWLY, add 5 mL of pre-warmed 0.075M KCl dropwise to each pellet, tapping LIGHTLY
14. Incubate the tubes at 37ºC in a waterbath for 21 min
15. Add 5 -10 drops of cold fixative to each tube, inverting once
13. Add cold fixative to 7 mL, invert each tube one time
14. Centrifuge the tubes for 15 min at 1200 x g
15. Remove the supernatant by aspiration, leaving approximately 1.5 ml in each tube
16. Resuspend the pellets by tapping the tubes
17. Continue steps 13-16 until the pellet is white and the fixative is clear
18. Centrifuge the tubes for 5 min at 1200 x g
19. Store the pellets in the explosion proof freezer at -20ºC until use

APPENDIX C. G-T-G BANDING PROTOCOL

Reagents

1XPBS (# 9242; Irvine Scientific)
Giemsa stain (# G3032; Sigma, St. Louis, MO)
Gurr's buffer (# 33199-2P; Biomedical Specialties (800-269-1158))
0.25% trypsin (# 15050-65; GIBCO)
Fetal Bovine Serum (# 16000-044; GIBCO)

1. Prepare five coplin jars:

Jar 1:	5 mL 0.25 % trypsin + 60 mL 1XPBS
Jar 2:	2 mL FBS + 60 mL 1XPBS
Jar 3:	60 mL 1XPBS
Jar 4:	4 mL Giemsa stain + 60 mL Gurr's buffer
Jar 5:	60 mL Gurr's buffer

2. For OSCC cell lines, place the slides in Jar 1 for 45 – 55 sec
   Peripheral blood cells, 1 min 15 sec – 1 min 30 sec

3. Blot the slides on a paper towel, then place slides in Jar 2 for 10 sec

4. Blot the slides on a paper towel, then place slides in Jar 3 for 10 sec

5. Blot the slides on a paper towel, then place slides in Jar 4 for 4 min

6. Blot the slides on a paper towel, then place slides in Jar 5 for 1 min
7. Blow dry the slides with forced air.

APPENDIX D. PREPARATION OF BAC DNA FOR FISH PROBES

Preparation of BAC plates

Adapted from (SAMBROOK *et al.* 1989).

Reagents

975 L dd H_20
25 g Bouillon LB (Luria-Bertani), Miller (DIFCO, Becton-Dickinson)
7.5 g Bacto-Agar (DIFCO)
Autoclave

1. Place the autoclaved LB agar for plates in a 55ºC waterbath until ready to prepare the plates
2. When the medium is cool to touch, add the appropriate antibiotic

    For Rocchi probes or BACs requiring ampicillin (# A0166; Sigma):

        0.005 g ampicillin in 100 mL sterile water (50 µg/mL stock)
        For plates, 50 mL agar solution + 40 µL ampicillin stock

    For BACs requiring chloramphenicol (# C3175, 100 mg; Sigma):

        0.01 g chloramphenicol in 100 mL sterile water (100 mg/mL stock)
        For plates, 1 L LB broth + 240 µL chloramphenicol stock

3. Pour the plates and allow them to set (they may be left on the bench at room temperature overnight)
4. Streak the plates with desired BAC
5. Place the plates inverted in the warm room overnight
6. Remove the plates from the warm room approximately 16 h after culture initiation (Individual colonies should be visible)
7. Seal the plates with parafilm and store inverted at 4ºC for 1 week

Establishment of BAC cultures

1. Prepare one 15 mL conical tube per BAC by adding 5 mL autoclaved LB broth + appropriate antibiotic*
2. Select a single colony from the BAC plate and add it to the tube, stirring. Cap the tube.
3. Vortex the tube to mix
4. Loosen the cap, but tape the cap to the lip of the conical tube to ensure it is secure
5. Place the tube on a shaker in the warm room overnight

*For maxi preps, initiate 35 ml LB broth + antibiotic with 5 mL of grown BAC and place in warm room overnight

NOTE: Grown BACs may be refrigerated up to one week prior to extraction

General BAC extraction protocol

Reagents

STET: (ADD IN ORDER)
8 g sucrose + 40 mL dd H_20
5 mL of 1 M Tris base, pH 8.0
5 mL Triton X-100 (t-octylphenoxypolyethoxyethanol; T9284; Sigma)
50 mL dd H_20
1.8612 g EDTA

NOTE: There is no need to pH if the Tris base used is 8.0. Store at room temperature indefinitely

10% SDS:10 g SDS in 100 mL dd H_20
then
880 µL dd H_20
20 µL 10 N NaOH
+ 100 µL 10% SDS

7.5 M Ammonium acetate
Phenol:chloroform solution
Isopropanol
TE / RNase A:

STOCKS: 100 µL 100X TE (T9285; Sigma) in 9.9 mL dd H_20
RNase A, 100 mg/mL (#1007885; Quiagen)
1 µL RNase in 1 mL 1X TE, vortex
Store at 4ºC for 4 months

Extraction Procedure

1. Put 1 mL of grown BAC in a 1.5 mL Eppendorf tube
2. Centrifuge at high speed, 15 min
3. Remove the supernatant, drying the inside of the Eppendorf with a kimwipe
4. Resuspend the pellet in 100 µL STET
5. Add 200 µL SDS solution to each tube.
6. Gently invert each tube six times. Leave the tubes at room temperature for 5 min (test with a pipette tip to see if the solution has become"stringy")
7. Add 150 µL 7.5 M ammonium acetate to each tube, then gently invert each tube six times
8. Place the tubes on ice for 5 min
9. Centrifuge at high speed, 20 min
10. Remove the supernatant with a micropipettor and place the solution in new tubes (should have ± 400 µL)
11. Under a fume hood, add an equal volume (eg. 400 µL) of phenol:cholorform, then gently invert each tube six times
12. Centrifuge the tubes in an Eppendorf microcentrifuge at 14,000 x g for 15 min
13. CAREFULLY remove the supernatant and put it in a new tube (DO NOT disturb the bottom organic layer- if this happens, be SURE to recentrifuge before proceeding)
14. Add 0.6x the volume of isopropanol (here, 240 µL), gently invert each tube six times
15. Centrifuge the tubes in an Eppendorf microcentrifuge at 14,000 x g for 15 min
16. Decant the supernatant
17. Add an equal volume from step #10 (here, 400 µL) of COLD 70% EtOH; make sure that the pellet is floating in the ethanol
18. Centrifuge the tubes in an Eppendorf microcentrifuge at 14,000 x g for 15 min
19. Remove the EtOH and bake the tube at 60ºC until just dry (30 sec)
20. Resuspend the pellet in 15 µL TE/RNAse
21. Run a 1XTAE gel to determine the DNA concentration

BAC glycerol stock preparation

150 µL autoclaved glycerol
+ 850 µL culture grown in LB broth + antibiotic (prior to extraction)

Labeling BAC for fluorescence *in situ* hybridization (FISH)

Reagents

Vysis nick translation kit (# 32-801-300, Vysis, Downers Grove, IL)
50 nmol Spectrum Orange-dUTP™ (# 30-803-000, Vysis)
Working solution: 33 µL in sterile water (0.6 µm)

50 nmol Spectrum Green-dUTP™ (# 30-803-200, Vysis)
Diethylaminocoumarin-5-dUTP213 (DEAC213; Perkin Elmer Life and Analytical Sciences, Inc.) NOTE: Reagent must be special ordered at 10 mM concentration.

For nick translation, use 2.0 µL of 10 mM stock
+ 55.14 µL of sterile endonuclease free H_2O
Working solution is 0.35 mM DEAC213

BACs labeling with Vysis nick translation kit

17.5 µL extracted BAC DNA
2.5 µL 0.2 mM Spectrum Green-dUTP™, Spectrum Orange-dUTP™ or 0.35 mM DEAC213
5.0 µL 0.1 mM dTTP
10.0 µL dNTP mix
5.0 µL 10X nick translation buffer
+ 10.0 µL nick translation enzyme

Precipitation of labeled BAC DNA for FISH

Reagents

Human Cot-1 DNA, 500 µL (# 1581074; Roche Molecular Biochemicals, 800-262-1640)
Human placental DNA (# D3287, 10 µg/µL; Sigma)
Working: dilute 9 µL in 91µL sterile H_20
Sterile H_20
Cold, 100% EtOH
3 M sodium acetate (NaOAc), ph 5.5 (# S7899; Sigma)
Hybridization buffer: 10.0 mL molecular biology grade formamide (# F9037; Sigma)
+ 0.2 g dextran sulfate in 10 mL 4XSSC
pH to 7, store in aliquots

1. Add the reagents in order
Follow the general scheme of:

158.0 µL DNA
12.1 µL Cot-1
+ 57.5 µL pDNA
Total of the above x 0.1 equals the amount of NaOAc to add
Add the amount of NaOAc to the total volume of the solution
Total volume of the solution x 2.5 equals the amount of 100% EtOH to add

2. Mix the solution by pipetting

3. Place the tube in -135ºC freezer for 30 min
4, Centrifuge the tubes in an Eppendorf microcentrifuge at 14,000 x g for 15 min
5. Remove the supernatant and dry the inside of the tube with a kimwipe, being careful not to touch the pellet
6. Use forced air to lightly dry the pellet
7. Bake the tube at 60ºC for 5 min
8. Resuspend the pellet in 3 µL sterile water + 7 µL hybridization buffer for each slide area
9. Place the tube on a 37ºC shaker until ready to denature (see Appendix E)

APPENDIX E. FLUORESCENCE *IN SITU* HYBRIDIZATION PROTOCOL

Reagents

20XSSC: 175.3 g NaCl (BP358-212; Fisher)
88.2 g Sodium citrate (S279-3; Sigma)
800 mL dd H_20
pH to 7, add 200 mL dd H_20
Autoclave

2XSSC : 100 mL 20XSSC
+ 900 mL dd H_20
pH to 7
preheat 190 mL to 37ºC in water bath

RNase A (# R5503, 1g; Sigma)

Stock: Add 5 mL 2XSSC to the powder
Aliquot in Eppendorf microfuge tubes, 1 mL per tube
Store at -20ºC

Working: Dilute 1 mL of stock solution into 99 mL of 2XSSC in a glass bottle (or two, 50 mL conical centrifuge tubes)and lightly cap
Place the solution in a beaker of water within a waterbath at 100ºC (CRITICAL)
Aliquot into 2.5 mL tubes and store the tubes at -20ºC
For FISH, use one tube of working solution in 37.5 mL of 2XSSC, etc.

70% Formamide 140 mL Formamide (# BP227-500, Fisher Scientific)
40 mL 20XSSC
+ 20 mL dd H_20
pH to 7
preheat to 75ºC in a waterbath

Ethanol series: 70%, 80%, 100%

FISH Reagents (continued)

DAY 2, WASH 1: 0.4XSSC / 0.3% Tween 20 (polyoxyethylenesorbitan monolaurate; P5927; Sigma)

20 mL 20XSSC
950 mL dd H_20
3 mL Tween 20
dissolve, pH to 7, add 27 mL dd H_20
preheat to 72ºC in a waterbath

DAY 2, WASH 2: 2XSSC / 0.1% Tween 20

100 mL 20XSSC
850 mL dd H_20
1 mL Tween 20

dissolve, pH to 7 + 49 mL dd H_20

DAPI (25 mg) (Sigma)

Stock: 100 mL 20XSSC
850 mL dd H
1 mL Tween 20

Working: 40 µL DAPI stock
+ 50 mL 2XSSC

Antifade: PPD (# 13-169-3, 25 g; Fisher Scientific)

Working:0.15 g PPD in 15 mL 1XPBS
pH to 8, sterile filter
add 35 mL glycerol (final pH= 7.5)
Store in aliquots at -20ºC

FISH DAY 1

1. Prewarm the 2XSSC solution + RNAse aliquots to 37ºC and the 70% formamide solution to 75ºC
2. Add the RNase to the 2XSSC, and mix with a glass Pasteur pipet
3. Place the slides in RNase / 2XSSC for 30 min
4. Wash the slides 3 x 2 min in 2XSSC, at room temperature
5. Dehydrate the slides in a graded ethanol series, 70, 80, 100 for 2 min each
6. Allow the slides to air dry
7. Denature the probes at 72ºC for 5 min
8. Preanneal the probes at 37ºC in a waterbath for a maximum of 30 min
9. Denature the slides in the 70% formamide solution for 1 min 50 sec
10. Dehydrate the slides in a graded ethanol series, 70, 80, 100 for 2 min each
11. Place the slides on a 55ºC slide warmer
12. Add the probe to each slide and coverslip
13. Seal the coverslips with rubber cement
14. Place the slides in a 37ºC hybrization chamber overnight

FISH DAY 2

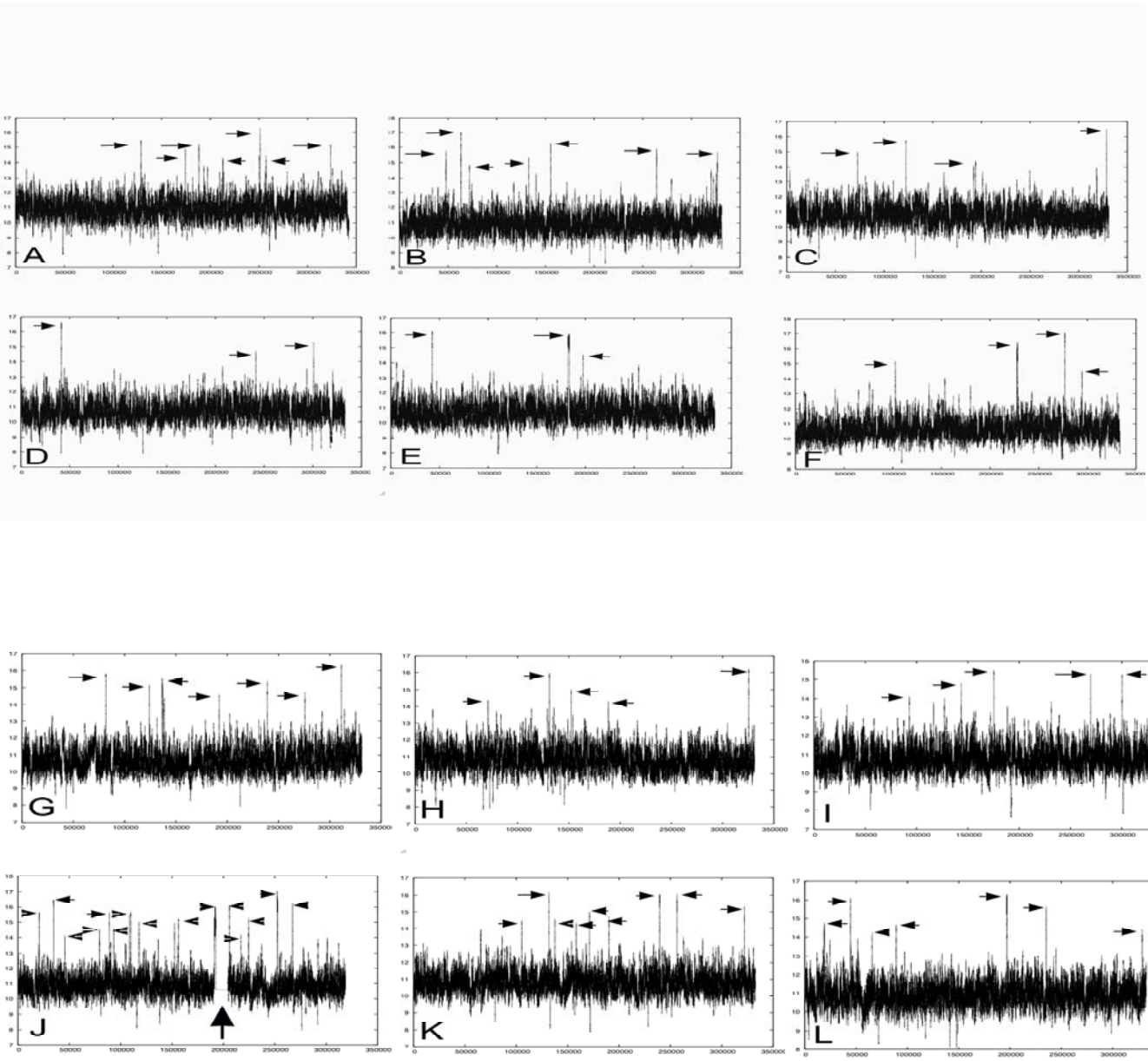
1. Remove the rubber cement and peel off the coverslips
2. Place the slides in 0.4XSSC/0.3% Tween 20 at 72ºCfor 2 min
3. Place the slides in 2XSSC/0.1% Tween 20 for 2 min at room temperature
4. Rinse the slides in 2XSSC at room temperature
5. Place the slides in the DAPI solution, 1 min
6. Rinse slides in water
7. Do not allow slides to dry. Add antifade solution to the slide
8. Coverslip the slide and seal with clear nail polish
9. Store slides at -20ºC

APPENDIX F. FLEXSTAB ANALYSIS PROTOCOL

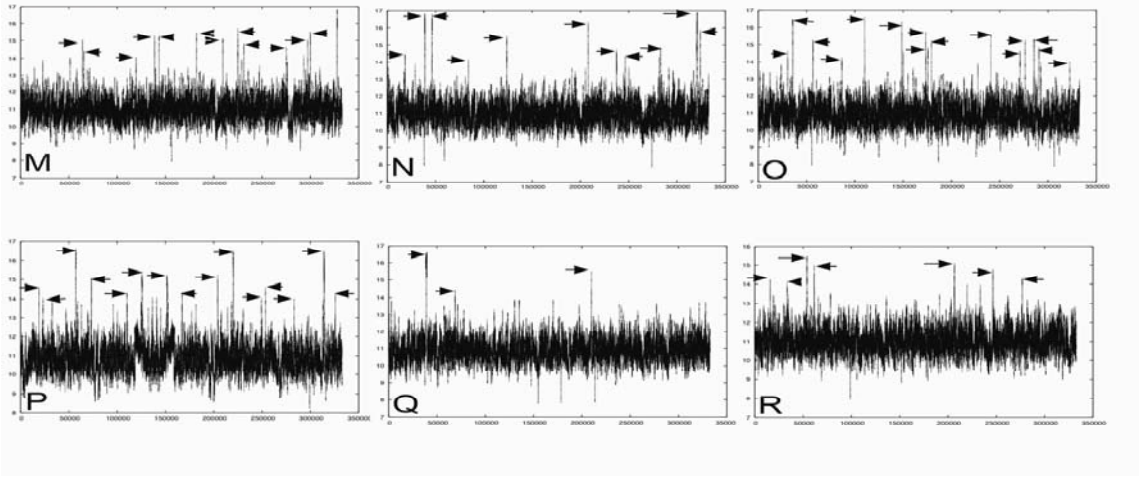
1. Install the program on UNIX as instructed at (http://leonardo.ls.huji.ac.il/departments/genesite/faculty/bkerem.htm).[§]
2. Save the input sequence file starting with ".." at the first line in the same folder
3. Run the FlexStab program with the following parameters:
   File name
   Choose GCG format (option 1)
   Window size = 100
   Shift increment (1)
   Graphic output (option 1)
   Output file name (name output file here)
   Type of analysis (option 2)
   The program will then run
4. Upon completion, there should be an output file consisting of data points. Usually, the number of data points is equal to the size of the DNA sequence (eg. 1000 bp DNA sequence will have numeric output file of 1000 data points).
5. Run a simple statistics analysis including the mean of the data value and standard deviation (SD) of the values (created by Xin Huang, Ph.D., Dept. of Human Genetics, University of Pittsburgh)
6. Choose data points that are higher than 4.5 SD as significant flexibility peaks (see Morelli et al., 2002)
7. Count the number of continuous data points in the output file for the number of total peaks
8. To get the AT/GC content, replace AT/GC in the sequence file
9. Count the number replaced
10. Calculate the total percent

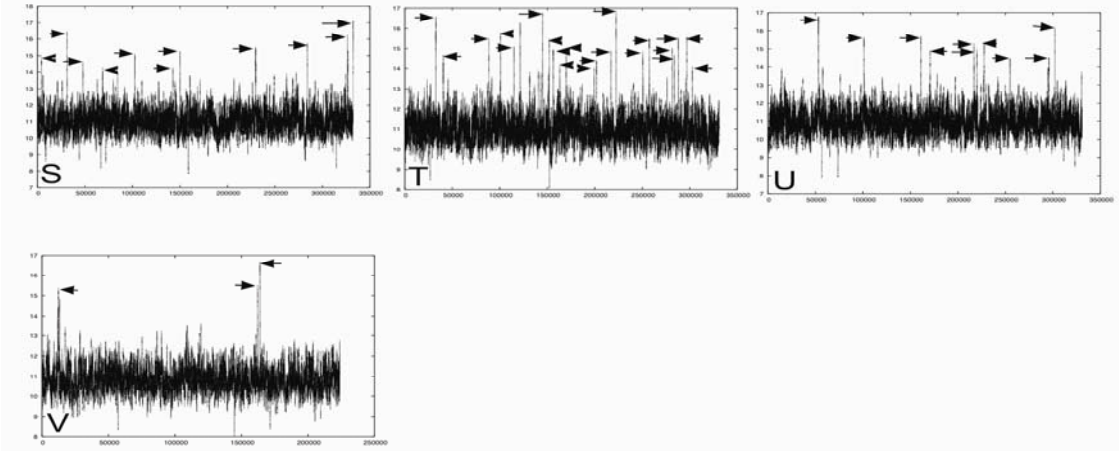
[§] FlexStab analysis carried out with the assistance of Mr. Ryan Evans and Dr. Xin Huang (Dept. of Human Genetics, University of Pittsburgh).

APPENDIX G. FLEXSTAB RESULTS[ˆ]



[ˆ] Output from FlexStab analysis program run on the *FRA11F* sequence. Arrows represent regions of high flexibility. (A) 0-342 kb; (B) 342 kb- 673 kb; (C) 673 kb- 1 Mb; (D) 1 Mb- 1.3 Mb; (E) 1.3 Mb- 1.67 Mb; (F) 1.67 Mb- 2 Mb. (G) 2.0 Mb- 2.3 Mb; (H) 2.3 Mb- 2.67 Mb; (I) 2.67 Mb- 2.98 Mb; (J) 2.98 Mb- 3.32 Mb; (K) 3.32 Mb- 3.65 Mb; (L) 3.65 Mb- 3.98 Mb. Note sequence gap (large arrow, J) of approximately 12 kb


M
N
O
P
Q
R


S
T
U
V

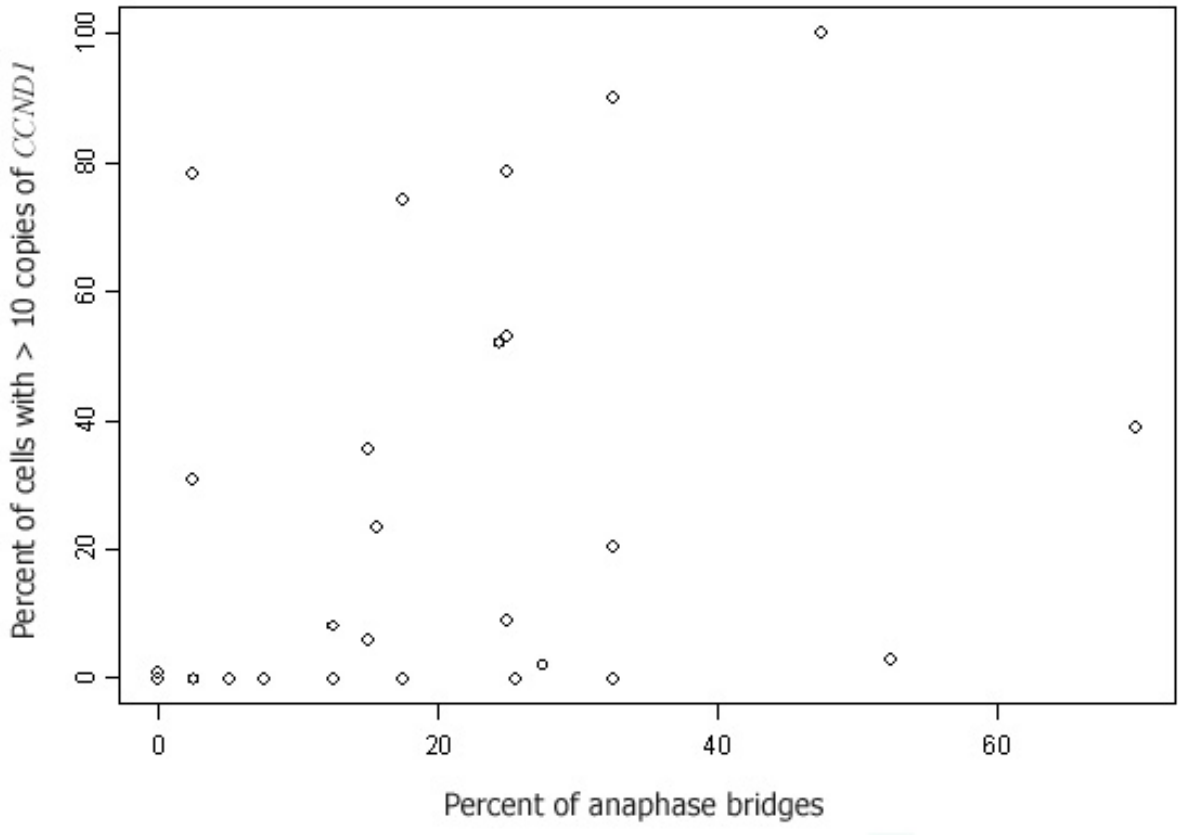
APPENDIX H. COMPOSITE KARYOTYPES OF PARENTAL AND CLONED UPCI:SCC CELL LINES

Cell line	Composite Karyotype[§]
UPCI:SCC040p16	60-75,XXY,-**X**[3],der(1)t(1;10)(p13;?)x2[2],der(1)t(1;10)(q21;?)[4],+der(1)t(1;10)(q21;?)[4],-**2,-2**[3],der(3)t(3;10)(p11;q11)x3[2],+der(3)t(3;10)(p11;q11)x2[2],**+i(3)(q10)**[2],-4[3],der(4)t(4;5)(q25;q22)[4],i(5)(p10)[4],+der(6)t(5;6)(?;q10)x2[2],+der(7)t(7;14)(p13;q11.2)[2],der(8)t(8;13)(p23;q22)[2],+der(8)t(8;13)(p23;q22)[2],der(9)t(9;19)(p10;?)x2[2],+der(9)t(9;19)(p10;?)[2],der(10)t(10;20;10)(?;?;?)[4],der(10)t(10;20)(?;?)x2[2],+der(10)t(10;20)(?;?)[2],-11[4],del(11)(q13)hsr(11)(q13)x2[4],-13,-13[4],-14,-14,-14[3],-15[4],-17[3],der(18)t(14;18)(q11.2;q11.2)[4],+der(18)t(14;18)(q11.2;q11.2)[4],-19[4],+der(20)t(16;20)(q13;q11.2)[2],-21[4],der(21)t(Y;21)(q12;q22)[3],-22[3][cp4]
UPCI:SCC040p35 Clone A	69-75,XX,-Y[3]**,**der(1)t(1;10)(p13;?)x2[2],der(1)t(1;10)(p13;?)[2],+der(1)t(1;10)(q21;?)x2[3],der(3)t(3;10)(p11;q11)[3],+der(3)t(3;10)(p11;q11)[3],der(4)t(4;5)(q25;q22)[3],-5,-5[3],+der(6)t(5;6)(?;q10)[2],+der(7)t(7;13)(q11.2;q22)[3],+der(8)t(8;13)(p23;q22)[4],**+der(8)t(8;13)(p10;q10)**[2],der(9)t(9;19)(p10;?)[3],+der(9)t(9;19)(p10;?)[3],-10[3],der(10)t(10;20;10)(?;?;?)[3],-11[4],del(11)(q13)hsr(11)(q13)x2[4],-13,-13,-13[3],-14,-14,-14[3],**der(15)ins(1;15)**[4],-17,-17[3],der(18)t(14;18)(q11.2;q11.2)[2],+der(18)t(14;18)(q11.2;q11.2)x3[2],der(20)t(13;20)(q22;q12)x2[4],-21,-21[3],der(21)t(Y;21)(q12;q11.2)[4][cp4]
UPCI:SCC040p35 Clone B	68-74,XXY,der(1)t(1;10)(p13;?)[4],der(1)t(1;10)(q21;?)[4],+der(1)t(1;10)(q21;?)[4],der(3)t(3;10)(p11;q11)[4],+der(3)t(3;10)(p11;q11)[4],del(4)(q31.1)[4],+der(4) t(4;5)(q25;q22)[3],+der(4)t(4;5)(?;q22)[2],-5[3],i(5)(p10)[3],**t(5;15;10)(?;?;?)**[3],+der(6)t(5;6)(?;q10)x3[2],der(9)t(9;19)(p10;?)x2[3],+der(9)t(9;19)(p10;?)[3],-10[3],der(10)t(10;20;10)(?;?;?)[4],-11[4],del(11)(q13)hsr(11)(q13)x2[4],-13,-13,-13[4],-14,-14,-14[4],-15,-15[3],-17[3],der(18)t(14;18)(q11.2;q11.2)x2[2],der(20)t(13;20)(q22;q12)x2[4],-21[4],der(21) t(Y;21)(q12;q21)x2[2],del(22)(q11.2)x2[2][cp4]
UPCI:SCC131p18	73-83,XY,-X[4],-2[4],der(3)t(3;16)(?;?)[2],**t(3;16)(q21;p13.1)**[2],-4,-4[5],del(4)(?)[5],**der(5)t(5;7)(q31;p13)x2**[2],-6[3],+der(7)t(X;7)(p11.2;p11.2) [2],+der(7)t(5;7)(?;?)x2[2],+der(7)t(5;7)(?;?)[2],+der(8)t(3;8;13)(?;?;?)x2[2],+der(8)t(3;8;13)(?;?;?)[2],der(9)t(9;14)(p21;q12)[2],+der(9)t(9;14)(p21;q12)x2[2], der(10)t(2;10)(p10;q10)x2[2],+der(10)t(2;10)(p10;?)[2],del(11)(q13)hsr(11)(q13)[3],der(?)t(4;11)(?;?)[2],+der(11)t(4;11;17)(?;q13;?)x2[5],+der(?)t(4;11;17)(?;?;?)x2[4],-12[3],+13[2],-14,-14[3],der(17)t(2;17)(q21;q21)[5],+der(17)t(2;17)(p13;p11.2)[2],+19[2],del(20)(q11.2)[2],-21[5],-22[3],del(22)(q11.2)x2[5][cp5]
UPCI:SCC131p33 Clone C	72-78,XY,-X[3],+Y[2],der(3)t(3;16)(q21;?)[2],der(3)t(3;16)(?;?)x2[2],-4[3].del(4)(?)[2],-5[3],+der(7)t(5;7)(?;?)[2],+der(8)t(3;8;13)(?;?;?)[2],der(9)t(9;14)(p21;q12)[2],+der(9)t(9;14)(p21;q12)[2],+der(9)t(9;14)(?;?)x2[2],+der(10)t(2;10)(p10;?)[3],+der(11)t(4;11;17)(?;q13;?)[3],+der(?)t(4;11;17)(?;?;?)x2[2],-14[3],+der(17)t(2;17)(q21;q21)[3],+20[2],+del(20)(q11.2)[2]**,**-22[3][cp3]
UPCI:SCC131p30 Clone D	56-82,XY,-X[3],+Y[3],**+1**[2],-2[4],-3,-3[3],-4,-4[4],del(4)(?)[3],-5[4],-6[4],der(9)t(9;14)(p21;q12)[2],+der(9)t(9;14)(p21;q12)x3[2],+der(9)t(9;14)(p21;?)[2],+der(9)t(9;14)(?;?)x2[3],+der(10)t(2;10)(p10;?)x2[2],der(?)t(4;11)(?;?)[2],+der(11)t(4;11;17)(?;q13;?)x2[4],+der(11)t(4;11;17)(?;q13;?)x2[3][†],+der(?)t(4;11;17)(?;?;?)x2[3],-13[3],-14,-14[3],-15[3],-16[4],-17[3],der(17)t(2;17)(q21;q21)[2],+20[2],-22,-22[3],del(22)(q11.2)[2][cp4]

[§] Rearrangements in bold face print represent abnormalities unique to an individual cell line. If not highlighted, similar aberrations were observed in other cell lines of similar lineage, but could not be included in the composite karyotype due to the number of cells analyzed.

[†] Different chromosome segments with unknown breakpoints.

APPENDIX I. CORRELATION OF *CCND1* GENE AMPLIFICATION AND ANAPHASE BRIDGES



Nonparametric Spearman correlation coefficient analyzing the percent anaphase bridges compared to percent of cells w/ > 10 copies: 0.40; p-value= 0.037[¶].

[¶] Statistical analysis carried out by Doug Potter, Ph.D. (University of Pittsburgh Cancer Institute).

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