THE DEFECTS IN THE FANCONI ANEMIA PATHWAY IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Background: The genetic mechanisms that lead to head and neck squamous cell carcinoma (HNSCC) are incompletely understood. Cancer predisposition is associated with chromosome instability and hypersensitivity to DNA damaging agents. Fanconi anemia patients are extremely sensitive to crosslinking agents and are at increased risk of developing several cancers including leukemias, gynecological, and head and neck cancers. The Fanconi Anemia proteins (FANCs) are involved in pathways necessary for crosslink damage recognition and repair. Eight FANC proteins (A/B/C/F/G/I/J/L) assemble into a nuclear complex and two other proteins, FANCD1/BRCA2 and FANCD2 act downstream of the core complex. This FA protein complex is required for the monoubiquitination of the FANCD2 protein in response to DNA damage. By testing head and neck tumors for FANC characteristics, such as hypersensitivity to DNA crosslinking agents, increased chromosome breakage FANCD2 protein ubiquitination, formation of radial figures and genomic instability, we can determine whether this specific DNA damage repair pathway is intact in the tumors.

Methods: Head and neck tumor cell lines were treated with the clastogenic crosslinking agent, diepoxybutane (DEB), and double-strand breaks and chromosomal aberrations were quantified. FANCA and FANCD2 cell lines were analyzed as positive controls and normal peripheral blood lymphocytes were used as negative controls.

Findings: HNSCC cell lines treated with DEB have an increased number of DEB-induced double-strand breaks compared to normal lymphocytes, as evidenced by increased chromosomal breaks and tri- and quadriradials, suggesting defects in the DNA damage response.

Interpretation: Patients who are hypersensitive to DNA damage are at increased risk of developing several types of cancer at an early age. Likewise, we predicted and showed that head and neck tumors have defects in the FANC pathway. This might suggest that the genes in the pathway either had germline or acquired alterations (mutations or regulatory defects), which could be tested by comparing normal cells from these patients to the tumor cells. Identifying and examining the mechanisms by which DNA damage occurs and is repaired can lead to a better understanding of genetic predisposition to cancer and to advances in early detection and treatment, therefore reducing morbidity and mortality.

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PREFACE

I would like to take this opportunity to acknowledge several people who helped me with this project. First and foremost, I would like to thank Dr. Susanne Gollin who has been a wonderful teacher and mentor. Her guidance and insight enabled me to think "outside the box" and gave me a new and different perspective in regards to cancer genetics. This is a skill that I will use in my career as a genetic counselor. I am also grateful for her thoughtful suggestions careful editing and suggestions of my thesis. She is an excellent writer, and has helped in the development of my scientific writing skills. I would also like to acknowledge my other committee members, Dr. Laura Niedernhofer and Dr. Weissfeld. I would like to thank Dr. Laura Niedernhofer for all of her help and guidance during the writing of my thesis as well as for all of the time she spent reviewing and helping me to interpret my results. Her positive attitude and support was very encouraging and reassuring. Also, I appreciate Dr. Weissfeld's interest and very thoughtful suggestions regarding my project.

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1. Introduction

Head and neck cancer

1.1.1. Overview

Cancer is a disease that is caused when regulatory mechanisms that limit cell division are defective and cells undergo unregulated division while normal cell growth and behavior is lost (46). The cell cycle is a highly regulated process that results in cell growth and division into two daughter cells. The cell cycle has four stages: G1; growth and development of the cell, S; DNA synthesis leading to chromosome replication, G2- growth and development, M; mitosis, during which chromosomes are divided between two daughter cells. Normally, the cell cycle is regulated by many signals, including external growth factors, which are proteins that are required to stimulate cell division. When cells lose their dependence on growth factors, but divide without stimulation, this can lead to cancer (20).

Head and neck cancer accounts for about 3% of all cancers. Specifically, in 2005, the American Cancer Society estimates that there will be approximately 10,000 new cases of laryngeal cancer and 29,000 new cases of oral cavity cancer with approximately 4,000 and 7,000 deaths, respectively (3). This type of cancer usually affects individuals over 50 years of age and affects twice as many males as females. Most tumors of the head and neck are squamous cell carcinomas, which are characterized by thin, flat cells. Cancers of the head and neck are further characterized by the area of the body in which they begin. These include the oral cavity, pharynx, larynx, and the salivary glands.

The most significant risk factors for head and neck cancer are tobacco (including smokeless tobacco) and alcohol use. Eighty-five percent of head and neck cancers are linked to tobacco use. People who use both tobacco and alcohol are at even greater risk for developing these cancers than people who use either tobacco or alcohol alone. Other risk factors include poor dental hygiene, environmental exposures such as inhalation of asbestos, radiation exposure, and viruses, such as HPV (human papillomavirus) and Epstein-Barr (38).

Symptoms that are common in head and neck cancer include lump or sore that does not heal, a sore throat that does not go away, difficulty swallowing, and/or a change or hoarseness in the voice. Patients are referred to a specialist, such as an Otolaryngologist after general Physicians or Dentists suspect head and neck cancer in individuals with these symptoms. The Otolaryngologist can confirm the diagnosis by a physical exam with laryngoscope to look inside the throat, MRI and/or CT scans, and a biopsy of the suspected cancerous tissue (30).

Treatment of head and neck cancer depends on the location and size of the tumor, and the patient's general health. Generally, surgery is performed to remove the cancerous tissue and radiation treatment and/or chemotherapy may be recommended, depending on the stage of the cancer (10). Prognosis also depends on the stage of the cancer. Survival rates after diagnosis of head and neck cancer are: 1 year-85%, 5 year: 58%, and 10 year: 48%. A recurrence is most likely to occur in two to three years after the original diagnosis (3).

1.1.2. Genetics of head and neck cancer

Head and neck cancer is not considered a hereditary cancer. This means that a predisposition for this type of cancer is not thought to be inherited from generation to generation. Instead, it occurs as a result of environmental exposures that can cause many genetic mutation events over time. The exact mechanisms that lead to malignancy in cells are unknown. Mutation(s) in any of the genes that maintain DNA fidelity though replication, repair, chromosome segregation and apoptosis may be responsible for tumor formation and development of cancer (22). Cells have several different mechanisms to protect against replication of abnormal cells, including cell cycle checkpoints, insurance of proper chromosome segregation, telomere maintenance, and the DNA damage response. Many different genes encode proteins that are involved in pathways that regulate cell growth, function and death (summarized in Table 1, modified from (12)). Certain individuals may have inherited genetic susceptibility to cancer development through defects in DNA damage repair pathways. Research suggests that many of these pathways may be interconnected.

Table 1: A selection of genes, defective expression of which leads to chromosome instability

Modified from Gollin, 2005 (12)

Gene name	Descriptive name	Implicated in
AURKA	Aurora kinase A	Chromosome segregation defects
AURKB	Aurora kinase B	Chromosome segregation defects
BUB1	Budding uninhibited by benzimidazoles 1 homolog	Chromosome segregation defects
BUB1B/*BUBR1*	Budding uninhibited by benzimidazoles 1 homolog beta	Chromosome segregation defects
FLJ10036	Zwilch (*Drosophila*)	Chromosome segregation defects
KNTC1	Kinetochore-associated 1; alias Rod	Chromosome segregation defects
MAD2L1	MAD2 Mitotic arrest deficient-like 1 (yeast)	Chromosome segregation defects
ZW10	ZW10 homolog centromere/kinetochore protein (*Drosophila*)	Chromosome segregation defects
ATM	Ataxia telangiectasia mutated	DNA damage response defects
ATR	Ataxia telangiectasia and Rad3 related	DNA damage response defects
BARD1	BRCA1 associated RING domain 1	DNA damage response defects
BLM	Bloom syndrome gene	DNA damage response defects
BRCA2	Breast cancer 2, early onset; aliases FANCD1, FANCB	DNA damage response defects
FANCA-L	Fanconi anemia genes A-L	DNA damage response defects
H2AFX	H2A histone family member X, H2AX	DNA damage response defects
MRE11A	MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*)	DNA damage response defects
NBS1	Nijmegen breakage syndrome 1; nibrin	DNA damage response defects
POLB	DNA polymerase beta	DNA damage response defects
TP53		DNA damage response defects
PINX1	PIN2-interacting protein 1	Telomere dysfunction
TERC	Telomerase RNA component	Telomere dysfunction
TERF1/*PIN2*	Telomeric repeat binding factor (NIMA interacting) 1; aliases PIN2, TRF, TRF1	Telomere dysfunction
CCNE1	Cyclin E	Telomere dysfunction
CDC4/*FBXW7*	F-box and WD-40 domain protein 7, archipeligo homolog (*Drosophila*)	Telomere dysfunction

There are five major DNA damage repair pathways: homologous recombination repair (HR), non-homologous end-joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) (6). In HR, sequence information that is lost due to damage in one double-stranded DNA molecule is accurately replaced by physical exchange of a segment from an intact homologous DNA molecule. (33). NHEJ also repairs double-strand breaks, by bringing broken DNA ends together and directly ligating the ends back together (7). This can result in the loss of some genetic material, i.e., the creation of deletion mutation. It is thought that cells repair the majority of double-strand breaks by NHEJ, although there is evidence that NHEJ may be coupled to HRR to generate accurate repair of double-strand breaks (31). NER repairs DNA with helix-distorting damage, including cyclobutane pyrimidine dimers and 6–4 photoproducts produced by UV light, and adducts produced by the chemotherapeutic agents, cisplatin and 4-nitroquinoline oxide (15). BER is a major DNA repair pathway protecting mammalian cells against single-base DNA damage (6). MMR proteins are primarily responsible for the post-replication correction of nucleotide mispairs and extra-helical loops. Mutational defects in *MMR* genes give rise to a mutator phenotype, microsatellite instability, and a predisposition to cancer. MMR mutations are implicated in the etiology of hereditary non-polyposis colorectal cancer (HNPCC) syndrome and a wide variety of other sporadic tumors (25).

Research shows that cancer cells commonly exhibit chromosomal instability and this instability is apparent in tumors of the oral cavity and the head and neck region (34). Chromosomal instability is the occurrence of gains and/or losses of whole chromosomes or chromosome segments which occur at increased rate than would be expected in a normal cell population (16). Chromosomal instability is thought to be the means by which normal cells

develop the features that enable them to become cancer cells (16). Cells are constantly exposed to DNA damaging agents, but have elaborate and highly conserved pathways to sense, signal, and repair this damage, therefore maintaining genomic stability (48). In addition to the DNA damage repair pathways previously described, there are signal transduction pathways triggered by DNA damage checkpoints. Through these pathways, damage is recognized and the cell cycle progression is delayed to allow DNA damage to be repaired before replication or cell division takes place. Unrepaired double-strand breaks cause chromosomal aberrations, which in turn lead to dysregulation of cell cycle control genes and further genomic instability in cells, eventually leading to tumorigenesis (19). Signaling of DNA DSBs to delay the cell cycle for repair seems to occur mainly through the checkpoint kinases, ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia Rad-3 related protein). Another kinase, DNA-PK is also a key player in the recognition of a DNA lesion (40). ATM and ATR are activated in response to a double-strand break and in turn phosphorylate other proteins involved HRR and NHEJ to prevent cell cycle progression and coordinate repair of the break.

Deleterious mutations in genes encoding proteins employed in DNA repair and/or apoptosis (e.g. *TP53*) increase genomic instability. Genomic instability increases the occurrence of new mutations, including those affecting oncogenes and tumor suppressor genes. It is thought that genomic instability drives tumor progression. The exact relationship between the DNA damage response and how it leads to chromosomal instability in unknown, however haploinsufficiency for critical DNA damage response may be important for tumorigenesis (11).

Chromosomal instability in head and neck and oral cancers has been associated with amplification of chromosome band 11q13 (13). Approximately 45% of head and neck and oral squamous cell carcinomas exhibit this DNA amplification that results in upregulation and

overexpression of genes in this region. Research has shown that Breakage-fusion-bridge (BFB) cycles may lead to gene amplification (35;39) The mechanism by which amplification occurs by BFB cycles has been described and is thought to propagate chromosomal instability (26). As a consequence of chromosome breakage, it is thought that sister chromatids fuse following replication, giving rise to dicentric chromosomes. At anaphase, the dicentric chromosome may be pulled to opposite poles resulting in breakage. Figure 1 shows how this cycle repeats and results in an amplified chromosomal region, therefore chromosomal instability.

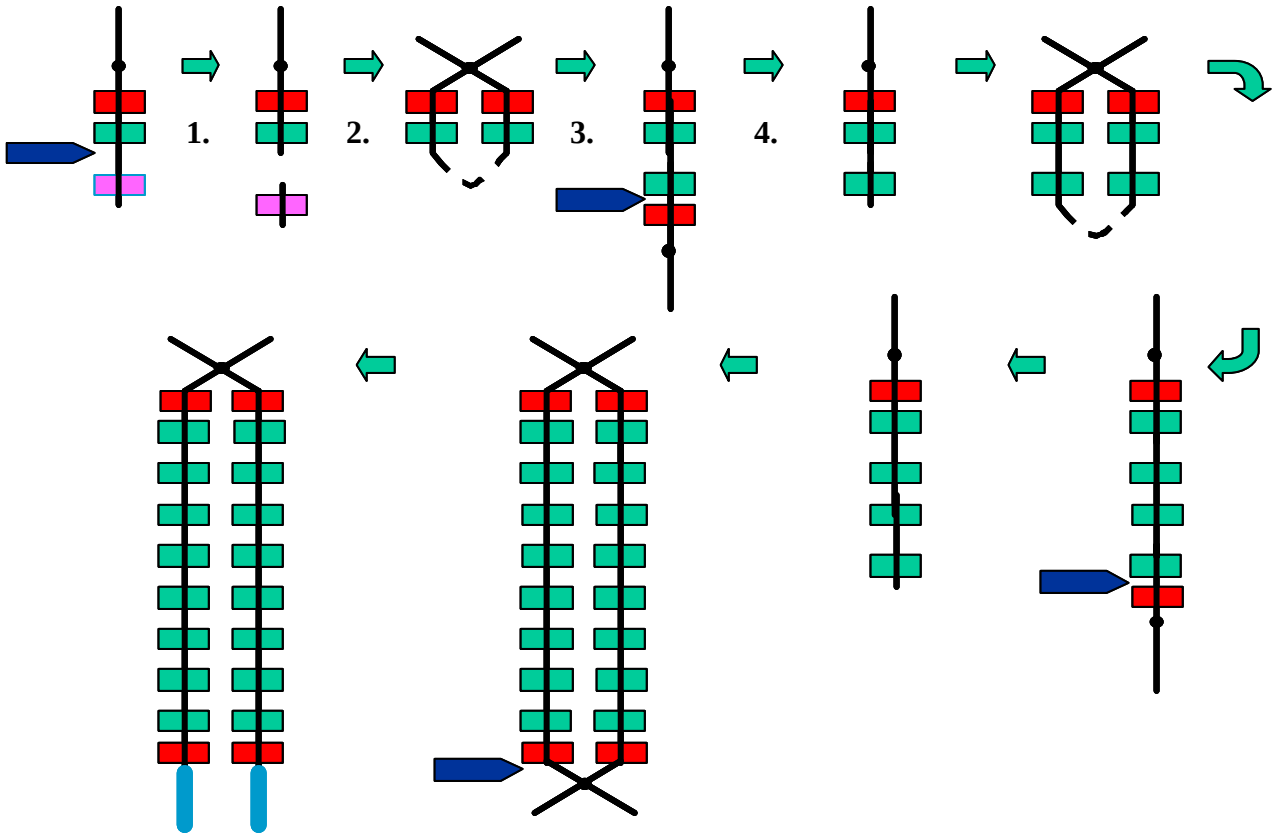

Figure 1: Schematic of breakage fusion bridge cycle

This figure shows the proposed mechanism of chromosomal amplification via breakage fusion bridge cycles. **1.** A chromosome break occurs **2.** sister chromatids fuse **3.** Formation of a dicentric chromosome **4.** Dicentric chromosome breaks and cycle repeats itself. Adapted from Gollin, SM and Reshmi, SC, unpublished.

Chromosomal breakage, via BFB, at or near chromosomal band 11q13 has been shown to result in loss of the distal segment of chromosome 11 (18). Within this region, there are genes critical for proper DNA damage repair including: *MRE11A* (11q21), *H2AFX* (11q22.3), *ATM* (11q23.2-q23.3) and *CHEK1* (11q24). As previously discussed, *ATM* is a significant part of cellular response to DNA damage, specifically DSBs. ATM protein responds to DNA damage and phosphorylates more than 15 known substrates from the DNA damage response genes including: *TP53, BRCA1, FANC, BLM, CHEK2*, and *MRE11*, which are associated with familial cancer syndromes (36). *CHEK1* also works to delay cell cycle progression when double-strand breaks are present. It is regulated by ATR and is required for the G2/M damage checkpoint (24). *MRE11* is required for DSB repair via HRR and possibly also NHEJ. It functions in the RAD50/MRE11/NBS complex which interacts with BRCA1 in repair (47). *H2AX* is also involved in DNA DSB repair. It is phosphorylated by ATM, ATR and DNA-PK and interacts with BRCA1, BRCA2, and the RAD50/MRE11/NBS complex (45). Thus loss of any of the genes in 11q23.2-q23.3, leading to haploinsufficiency, may result in dysregulation of the DNA damage response and further genomic instability.

Amplification of 11q13 itself is associated with poor prognosis (4). Haploinsuficiency of critical DNA damage response genes in the distal 11q region are thought to result in a sluggish DNA damage response (14). Other substrates from other DNA damage repair proteins downstream of ATM and previously mentioned proteins may have a similar response. Specifically, ATM phosphorylates FANC (Fanconi anemia) proteins. Individuals with the inherited disease, Fanconi Anemia have a 500-fold increased risk for head and neck cancer. Therefore, possible acquired mutations leading to haploinsufficiency of the *FANC* genes may

also play a role in development of head and neck tumors. An overview of Fanconi anemia and the relationship to head and neck cancer will be discussed in detail in the next sections.

1.1.3. Physical characteristics of patients with Fanconi anemia

Fanconi anemia(FA) is an inherited disorder characterized by physical abnormalities, bone marrow failure, and an increased risk of cancer. This genetic disorder is inherited in an autosomal recessive pattern, meaning that both parents have to be carriers of a mutation in one of the two copies of their eleven *FANC* genes and both must pass that mutated allele on to their offspring. In the general population, about 1/100,000 individuals are affected with Fanconi anemia, and 1/300 are carriers. Fanconi anemia is more common in individuals of Ashkenazi Jewish descent, who have a carrier frequency of approximately 1/100 (21).

Fanconi anemia can be difficult to diagnose due to the phenotypic variability observed in those affected and features that overlap with other genetic syndromes. Early diagnosis made at birth or in early childhood is usually based on a collection of physical characteristics by a Medical Geneticist. However, approximately 25% of individuals affected with Fanconi anemia will not have any distinguishing physical characteristics. Clinical characteristics are summarized in (43). Of the 75% of patients who are dysmorphic, the most common characteristics are short stature (51%) and abnormal skin pigmentation (55%). Another common feature is upper limb abnormalities (43%), including absent, hypoplastic, or supernumerary thumbs, clinodactyly, polydactyly, absent first metacarpal, short fingers, transverse crease, as well as hypoplastic or absent ulnae or radaii. Individuals with FA can also have eye abnormalities (23%), which

include microphthalmia, strabismus, prominent epicanthal folds, hypertelorism/hypotelorism, ptosis, slanting palpebral fissures, cataracts, epiphora, and nystagmus. Renal anomalies (21%) are also observed and include, horseshoe, hypoplastic or dysplastic kidney, hydronephosis, hydroureter, and reflux. Genitalia can also be abnormal, although this is much more common in males (32% males, 3% females). Males with FA can have hypogenitalia, undescended, absent or atrophic testes, azoospermia, phimosis, delayed puberty, although a few males with FA have fathered children. Females may have hypoplastic vulva, bicornuate uterus, absence of the uterus or vagina and ovarian atresia. Delayed menarche, irregular menses, and early menopause are seen in females, although successful pregnancies resulting in liveborn children have been reported. Gastroinstestinal and cardiopulmonary (11%) defects can include esophageal/duodenual/jejunal atresia, tracheoesophageal fistula, anteriorly placed anus, imperforate anus, persistent cloaca, Meckel's diverticulum, umbilical hernia, abnormal biliary ducts, megacolon, abdominal diastasis, Budd-Chiari syndrome, annular pancreas as well as structural defects of the heart and cardiomyopathy. Some individuals with FA may also have ear abnormalities (9%) including hearing loss which is usually conductive secondary to middle ear abnormalities and structural defects which include abnormal pinna (low-set, large, or small) and stenosis or atresia of the external auditory meatus. Lower limbs (8%) can also be abnormal and presentation may include toe syndactyly, pes planus, abnormal toes, and congenital hip dislocation. Finally, other abnormalities reported in individuals affected with FA are high-arched palate, arterial malformation, moyamoya syndrome (stenosis of blood vessels in the brain that causes paralysis of extremities, headaches, visual problems, and mental retardation), absent breast buds, absent pulmonary lobes, microcephaly, hydrocephalus, micrognathia, and vertebral abnormalities.

Many cases of Fanconi anemia are identified after a diagnosis of leukemia or bone marrow failure (44). Bone marrow failure (BMF) usually presents in the first decade of life, but age of onset is variable. BMF is a progressive decrease in peripheral blood counts, which includes thrombocytopenia (low blood platelet count that can cause spontaneous bleeding and hemorrhage), leukopenia (low count of white blood cells) and anemia (decrease in the number of red blood cells causing decrease in oxygen carrying capabilities of RBC resulting in fatigue, cardiac problems and infections). An individual with FA may also present with myelodysplastic syndrome (MDS). In childhood, MDS encompasses a diverse group of bone marrow disorders that share a common clonal defect in the stem cells. These disorders are characterized by one or more cytopenias, despite a relatively hypercellular bone marrow. MDS is rare in childhood, and most children have a rapidly progressive course with extremely poor prognosis(28). They are referred to as preleukemia because of their tendency to transform into acute myelogenous leukemia (AML). AML is common in individuals with FA. In fact, the risk for AML in FA individual is increased 785-fold (32). AML is a malignant disease of the bone marrow in which hematopoietic precursors are arrested in an early stage of development. AML is distinguished from other related blood disorders by the presence of more than 30% blasts in the blood and/or bone marrow in most AML subtypes. Prognosis is usually poor and these children require a bone marrow transplant (2).

Some individuals with FA may not be diagnosed at an early age based on absence of FA-related physical features and/or because they "escaped" leukemia development. These individuals are sometimes identified after developing certain solid tumors in their twenties or thirties. The solid tumors include head and neck, genital tract, GI tract, and skin cancer. According to the International Fanconi Anemia Registry (IFAR), 3% of FA patients developed

solid tumors at a young age (range 15-49 years old). In 2003, The Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute found that the incidence among this age range was 0.038%, which is significantly less than what has been observed in the FA population. Those patients with FA, even after BMT, are still at increased risk of developing solid tumors. Overall risks by the fourth decade in someone with FA are 90% for BMF, 10-33% for hematologic malignancies, 29% for other malignancies as well as inordinate toxicities from chemotherapy or radiation (19). Therefore, patients with Fanconi anemia are at an increased risk of several cancers, and should be followed closely by a physician and screened for these cancers accordingly.

1.1.4. Testing methods for Fanconi anemia

Genetic testing can be done to determine if an individual has Fanconi anemia. The diagnostic test which is considered "the gold standard" is chromosome breakage analysis (5). Other testing methods include full sequencing and mutation analysis and immunoblotting, which is done on a research basis at this time (37).

Individuals affected with FA have an increased amount of double-stranded chromosome breaks when exposed to DEB, due to the defects in the DNA damage repair pathway. Breaks are quantified by breaks per chromosome. Cells with more than 1.0 average break per chromosome are considered to have increased breakage. The presence of greater than 30% cells with radial formation is diagnostic for FA (5). Chromosome breakage is analyzed by obtaining peripheral blood from the suspected affected individual and culturing cells in the presence of the cross-linking agent, DEB. Untreated cells from the patient are also cultured, but without DEB and used as a positive control. Then, a total of fifty metaphase cells from lymphocytes are scored and analyzed for chromosomal breakage including the formation of radials, the characteristic finding

in this disease. Results are compared to the positive control and a normal control that have been tested in parallel. The normal control is a sex and age matched individual with no history of FA. These cells are cultured and one flask is treated with DEB and the other is left untreated. The cultures without the DNA clastogenic agent are used to measure the spontaneous breakage rate. Results are reported as either average number of breaks/cell or as *x* number of cells with 1,2,3...>8 breaks. The number of cells with radial formation is recorded. In addition to using this test to confirm a diagnosis of FA, prenatal testing can be performed. This is offered for fetuses at 25% risk and is performed via chorionic villus sampling (CVS) or amniocentesis. Affected fetuses will have increased breakage in chorionic villi or amniocytes (1).

Even though the chromosome breakage test is considered the "gold standard", there are limitations. For instance, the result may be complicated by mosaicism. Mosaicism refers to the reversion of the inherited FA mutation in a subpopulation of patient's cells. As a result of the reversion, these cells are no longer sensitive to crosslinking agents and therefore appear normal in a chromosome breakage test, giving a false negative DEB/MMC result. In individuals with a negative DEB/MMC test in whom a high degree of clinical suspicion remains, DEB/MMC testing could be performed on an alternative cell type, such as skin fibroblasts, to establish the diagnosis. Also, this test cannot detect FA heterozygotes (carriers).

Another method that has been used in the diagnosis and study of FA is immunodetection of ubiquitinated FANCD2. FANCD2 monoubiquitination is essential for the functional integrity of the FA pathway as measured by resistance to MMC or DEB. Because FANCD2 monoubiquitination is intact in other bone marrow failure syndromes and chromosomal breakage syndromes tested to date, evaluation of FANCD2 monoubiquitination by immunoblotting provides a rapid diagnostic test for Fanconi anemia. Limitations of this test include the likelihood

that rare FA subtype FANCD1 would be missed by this approach, as could individuals with somatic mosaicism. Also, this test is only available on a research basis at this time.

Molecular genetic testing is also available. Nine of the eleven genes in the Fanconi complementation group have been identified and mutation analysis is available (Table 2).

Table 2: Summary of molecular testing in Fanconi anemia

Complementation Group	Chromosome Locus	Test Methods	Mutations Detected	Detection Rate	Test Availability
FANCA	16q24.3	Sequence analysis	Sequence Alterations	66%	Clinical
FANCB	X	Mutation analysis	Sequence Alterations	0.8%	Research
FANCC	9q22.3	Sequence analysis	Sequence Alterations IVS4 +4 A to T	9.6%	Clinical
FANCD1/BRCA2	13q12.3	Direct DNA[1]	Sequence Alterations	3.3%	Research
FANCD2	3p25.3	Direct DNA	Sequence Alterations	3.3%	Research
FANCE	6p22-p21	Direct DNA	Sequence Alterations	2.5%	Research
FANCF	11p15	Sequence analysis	Sequence Alterations	2.1%	Clinical
FANCG	9p13	Sequence analysis	Sequence Alterations	8.8%	Clinical
FANCI	Unknown	N/A	Sequence Alterations	Unknown	Research
FANCJ	Unknown	N/A	Sequence Alterations	Unknown	Research
FANCL	2p16.1	Direct DNA	Sequence Alterations	0.4%	Research

1-Direct DNA testing may include mutation analysis, mutation scanning, sequence analysis

In addition to confirming a suspected diagnosis of FA in an individual, molecular testing can also be used for carrier testing and prenatal diagnosis. When using molecular testing, there are issues to consider regarding interpretation of results (Adapted from the ACMG Recommendations for Standards for Interpretation of Sequence Variations 2000). The types of sequence alterations that may be detected can be reported in five categories:

1. Pathogenic sequence alteration reported in the literature
2. Sequence alteration predicted to be pathogenic, but not reported in the literature
3. Unknown sequence alteration of unpredictable clinical significance
4. Sequence alteration predicted to be benign, but not reported in the literature
5. Benign sequence alteration reported in the literature

Therefore, there can be some uncertainty when reporting results to patients due to the limited knowledge about FA mutations. There are a number of possibilities to consider if a sequence alteration is not detected. These include the following: the patient does not have a mutation in the tested gene (e.g., a sequence alteration exists in another gene at another locus), the patient has a sequence alteration that cannot be detected by sequence analysis (e.g., a large deletion, a splice site deletion), the patient has a sequence alteration in a region of the gene (e.g., an intron or regulatory region) not covered by the laboratory test. There are also limitations with molecular testing. Molecular testing is complicated by the number of possible associated genes and by the large number of possible mutations in each gene, and the large size of many of the FA genes. Also, *BRCA2* mutation analysis and sequencing is available clinically for hereditary breast/ovarian cancer; however, information is limited regarding its possible application in FA.

1.1.5. Management of Fanconi Anemia

A multidisciplinary approach is used to manage an individual diagnosed with FA as summarized in Fanconi Anemia: Standards for Clinical Care (2). The physical abnormalities can be assessed and monitored. These assessments include kidney/renal ultrasound to rule out kidney

abnormalities, formal hearing test, developmental assessment in young children, ophthalmology referral, genetics evaluation, referral to an Endocrinologist to monitor growth and development.

There are also recommendations to manage bone marrow failure. Individuals with FA should be followed by a Hematologist. HLA-typing of siblings and parents should be performed in preparation for BMT. Also, assessment of liver and kidney function, iron levels and blood counts should be measured regularly, as well as bone marrow aspiration annually to evaluate cell morphology and to identify cytogenetic abnormalities. When an individual has bone marrow failure, androgen administration can help the blood counts, but responses may be transient and side effects can be problematic. Ultimately, bone marrow transplantation is warranted. Hematopoietic stem cell transplantation is the only curative therapy for the hematological manifestations of FA. Since individuals with FA are exquisitely sensitive to the toxicity of the usual chemotherapy and radiation regimens used in preparation for BMT, reduced doses are typically used. There are limitations to BMT. It is often difficult to find a donor and the procedure has about a 25% mortality rate due to graft versus host disease and infections as a result of immunosuppression. Also, patients remain at risk for solid tumor development.

There are also management issues to consider regarding cancer prevention and treatment. FA individuals should avoid toxic agents associated with tumorigenesis, and radiographic studies should be minimized, as these exposures are known to cause DNA damage. Additional cancer surveillance is also warranted. Recommendations include annual rectal examinations, frequent dental and oropharyngeal examinations, annual esophageal endoscopy may be considered, females should have an annual gynecological examination with pap smears, and prompt and aggressive workup for any symptoms suggestive of a malignancy should be pursued. Treatment of malignancies is challenging as a result of the increased toxicity associated with chemotherapy

and radiation therapy in individuals with FA. Treatment with decreased doses or modified regimens at experienced centers may be possible.

Molecular aspects of Fanconi anemia

1.1.6. Molecular Pathogenesis

Eight FANC proteins (A/B/C/F/G/I/J/L) assemble in a nuclear complex and two other proteins, FANCD1/BRCA2 and FANCD2 act downstream of the core complex. The products of six cloned FA genes (A, C, D2, E, F, G) are orphan proteins with little or no homology to each other or to other known proteins. *FANCG* is identical to the human *XRCC9* gene, which was identified in a screen for genes that could correct chromosomal instability following DNA damage in Chinese hamster UV40 cells (23). The FANCD2 protein has lower eukaryote homologues — in *Drosophila*, *Arabidopsis*, and *C. elegans* (42). Recent evidence supports a model whereby the proteins of seven cloned FA genes interact in a common cellular pathway. The gene for *FANCD1* was recently identified as the tumor suppressor/breast cancer susceptibility gene, *BRCA2* (9). FANCD1/BRCA2 is not required for FANCD2 monoubiquitination, but is still required for resistance to crosslinking agents like mitomycin C (MMC) or diepoxybutane (DEB). Recently, *FANCL*, was identified as the E3 ligase required for FANCD2 monoubiquitination (17). FANCD2 is also phosphorylated by the ataxia-telangiectasia kinase, ATM, in a process that regulates a radiation-induced cell cycle checkpoint (41). The FA-I and FA-J complementation groups have recently been described, though the genes have yet to be identified [49].

At least six of the FA proteins (A, C, E, F, G, and L) are assembled in a nuclear complex. This FA protein complex is required for the monoubiquitination of the FANCD2 protein in response to DNA damage (Figure 2). The FANCD2 protein has two functions: 1) it is

monoubiquitinated in response to DNA crosslink damage leading to its interaction with BRCA2 and function in DNA damage repair and 2) it is also phosphorylated by ATM, leading to the activation of an S phase checkpoint response. FANCD2 has two forms: FANCD2-S (short form) and FANCD2-L (long form). In response to DNA damage, this complex monoubiquitinates the FANCD2 protein promoting its translocation to *BRCA1* repair foci. Ubiquitination of FANCD2 converts it from the –S to the –L form and activates the protein (8).

Disruption of this pathway leads to the common cellular and clinical abnormalities observed in FA [9]. The FA genes and their products associate to form a pathway that interacts with several DNA damage response proteins involved in cell cycle checkpoints or DNA damage repair. These are thought to include BLM, ATM, BRCA1/2, XPF, and the MRE11/RAD50/NBS1 complex. The precise function of this interaction is still unknown.

1.1.7. Fanconi repair pathway

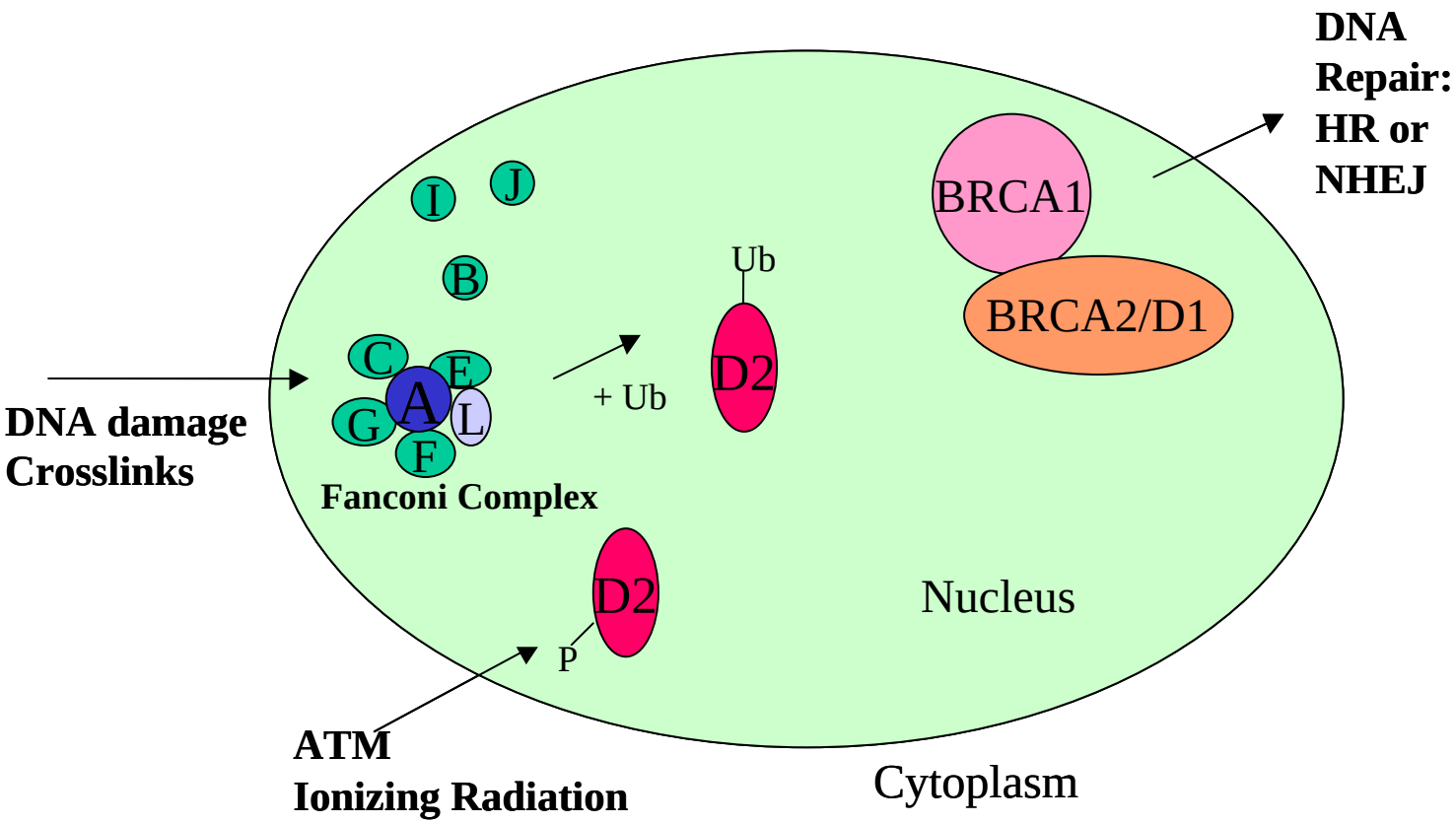

Figure 2: FA genes in response to DNA damage

In response to DNA damage, the Fanconi D2 (FANCD2) protein is monoubiquitinated the Fanconi L protein in a process requiring the core complex containing Fanconi proteins A, C, E, F, G, and L. Following monoubiquitination, FANCD2 associates with the tumor suppressor proteins BRCA1 and BRCA2/FANCD1 where it promotes DNA repair. ATM-dependent FANCD2 phosphorylation plays a role in radiation-induced cell cycle arrest. (Modified from 8)

FA is diagnosed by sensitivity to interstrand crosslink (ICL) damage. Crosslinking agents are DNA damaging drugs that join opposite strands of the DNA double helix and form covalent bridges known as ICLs. ICLs prevent strand separation and effect transcription and replication. Both strands of DNA are affected. Therefore, there is no undamaged strand present to act as a template during repair of the DNA damage. ICL repair is thought to be very complex and proteins from different repair pathways are utilized to cope with the ICLs including NER (nucleotide excision repair), TCR (transcription-coupled repair), and mismatch repair pathways. The exact mechanism by which ICL repair occurs is unknown, although it has been suggested that ICL repair takes place during S phase of the cell cycle (27). If there is an ICL, the replication fork stalls resulting in the formation of a DSB. The ICL must be excised, likely by the NER-endonuclease ERCC1-XPF before the replication-induced DSB can be repaired and replication continues (29). The Fanconi pathway is activated in response to certain kinds of DNA damaging agents that cause double-strand breaks in DNA, such as crosslinking agents. In FA cells that are unable to repair ICL-induced, replication-dependent DSBs, chromosome breaks may be rejoined by nonhomologous end-joining (NHEJ). This can result in correct rejoining of a chromosome, or incorrect joining of different broken chromosomes, leading to chromosome translocations that can lead to quadriradial formation, triradials and other chromosomal aberrations indicative of error-prone DSB repair (dicentrics, giants, chromosome gaps). In the absence of NHEJ, chromatid breaks accumulate and persist, without rejoining in aberrant structures.

Summary

Squamous cell carcinoma of the head and neck has not been identified as part of an inherited cancer syndrome. This specific cancer has been mostly attributed to environmental factors, such as tobacco and alcohol use. However, it is possible that HNSCC cases may be explained by genetic factors. Knowledge of the high incidence of HNSCC in individuals diagnosed with Fanconi anemia, leads to the possibility that individuals diagnosed with HNSCC may have defects in the FA genes. Also, because of the complexity of diagnosing FA, it is possible that atypical (young, non-smokers, non-drinkers) patients who develop HNSCC may have Fanconi anemia. Finally, the increasing knowledge of the molecular aspects of DNA damage repair pathways could lead to a better understanding of the development of cancer and in patients with HNSCC as well as improve FA diagnosis.

2. Hypothesis

Head and neck squamous cell carcinoma cell lines exhibit defects in the Fanconi anemia pathway for DNA damage repair.

3. Specific Aims

1. To determine if head and neck cancer cell lines exhibit an increased susceptibility to chromosome breakage and radial formation upon exposure to DNA crosslinking agents, as occurs in Fanconi anemia cells.

2. To determine if head and neck cancer cells are deficient in the damage response to DNA interstrand crosslinks by testing for the presence of FANCD2 ubiquitination in the presence of DEB.

3. To compare the molecular results with clinical information about the patients corresponding to cell lines to determine if there is a possible susceptibility to head and neck and related cancers because of a deficient DNA damage response pathway.

4. Materials and Methods

Patient Cell Lines

Cell lines derived from tumors of consenting patients who had surgical removal of a head and neck squamous cell carcinoma between April 1994 and October 1995 were utilized and given an identification number. These include: UPCI:SCC078, UPCI:SCC084, UPCI:SCC116, UPCI:SCC122, UPCI:SCC125, UPCI:SCC131, UPCI:SCC136, and UPCI:SCC142. Table 3 summarizes the known clinical information about these patients. Two Fanconi anemia cell lines (SV40-transformed dermal fibroblasts) from the NIGMS Coriell Cell Repository were used. This included a cell line form a patient with genotype FANCA-/- (GM06914) and a cell line from a patient with genotype FANCD2-/- (GM16633). Control peripheral blood from a anonymous normal male <age 40 with no family history of FA was obtained in June 2004.

Table 3 Summary of clinical characteristics, personal and family information from patients whose tumors were examined

Cell line UPCI:SCC	Sex	Age at diagnosis	Risk Factors	Family history of cancer	Primary cancer
078	M	60	Alcohol Worked in Mill	Brother-Colon Ca, Prostate Father-Throat	Retromolar trigone
084	M	52	Tobacco Alcohol	None	Retromolar trigone
116	M	57	Tobacco Alcohol Worked in coal mine	Father- Prostate	Alveolar Ridge
122	M	63	Tobacco Alcohol	None	Tongue
125	F	78	Tobacco	None	Alveolar Ridge
131	M	73	Tobacco Alcohol Worked in coal mine	None	FOM
136	F	64	Tobacco	None	Floor of Mouth
142	M	58	Tobacco Alcohol	None	Tonsil

Cell Culture

Cells were cultured in MEM medium (Invitrogen, Gaithersburg, MD) with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA) and antibiotics as described previously by Shuster et al., [2000]. Once established, two T-25 flasks per cell line were used for experimentation. The protocol for testing peripheral blood samples for chromosome aberrations was adapted from the described method in the AGT Cytogenetics Manual (1). Fifty nanograms

of DEB was added to one of the flasks of each cell line and the corresponding flask of each of the cell lines was used as a control. The flasks were incubated at 37ºC for 48 hours and harvested for cytogenetic studies following exposure to 50 µg Colcemid™ (Irvine Scientific, Santa Ana, CA) for 4.5 hours. After removal from flasks, cells were treated with a hypotonic 0.075M KCl solution for 18 min at 37°C, then fixed with a 3:1 methanol:acetic acid fixative for 20 min at room temperature. Cells were washed two more times in this fixative, and fixed cell pellets were stored at -20°C prior to use. Slides were made and solid stained according to the conventional Geimsa staining method (AGT Cytogenetics manual pg. 264). Metaphase spreads on the slides were analyzed and the aberrations were scored.

Scoring of chromosome aberrations

Slides made from each cell line were coded and scored by two separate individuals. These individuals were unaware of which cell line they were looking at and whether it was treated with DEB. Each individual analyzed 50 metaphase spreads from 50 different cells in each cell line, treated and untreated. A standard cytogenetic scoring sheet was used to tally chromosome

aberrations. Aberrations that were scored as a single break include: chromatid breaks/gaps, minutes, dots, and fragments and aberrations that were scored as two breaks included: triradials, quadriradials, dicentrics, giants, and chromosome breaks. Results were presented as average number of breaks per chromosome and adjusted for anueploidy of the tumor cell lines. Cell lines with >1 break/chromosome were categorized as having increased breakage and those with <0.5 breaks/chromosome were considered normal as per the standard scoring method used in the AGT manual.

Statistical Analysis

After the results from the blinded breakage analysis was completed, the Wilcoxon rank sum test was done to compare the overall mean of chromosome aberrations between cases and controls. This test is used to compare two groups that are not normally distributed. This test showed the effect the DEB treatment had on the cell lines and their ability to repair breaks and were graphed to reflect this comparison.

A calculation of baseline genomic instability was done to compare the average breaks per chromosome in the untreated cell lines to the average breaks per chromosome in the untreated control peripheral blood.

Immunoblots

Cell Lysates

Each cell line was grown to 70-80% confluence in two T-75 flasks as described above. Of the two flasks for each of the cell lines, one flask from each cell line was treated with 50ng of DEB and the other was not treated and used as a control. The cells were scraped from the flask and centrifuged to make cell pellets and centrifuged. The cell pellets were washed three times with PBS and centrifuged to remove supernatant and transferred to eppendorf tubes and washed with PBS. Cells were microcentrifuged to remove any debris in the supernatant. The supernatant was then removed and then suspended in lysis buffer (see reagents below) for 20 min on ice followed by centrifugation at 4^{o}C for 15 min. Protein concentrations were measured on a spectrophotometer using Bradfords reagent using 10ul of cell lysate + 200ul of Bradford reagent + 800 ul of deionized water. Protein concentrations were equalized to 1ug/ul and 12-20ul of loading dye was added. Samples were set in boiling water for 5 min and stored in the freezer at -20^{o}C for further use.

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Gel Preparation

A 7% polyacrylamide running gel was made as outlined below and polymerized for approximately 20 minutes. The stacking gel (preparation outlined below) was then poured on top of the running gel, to accomodate a comb. The comb was then removed, 1X SDS electrophoresis buffer was poured into the apparatus. The wells of the apparatus were filled with

the sample and dye and a protein marker loaded in one of them. Samples were run for approximately 90 minutes at 120V to observe migration of bands. The blots were then transferred to polyvinylidene fluoride (PVDF) microporous membrane (Millipore), which was pretreated with methanol and transfer buffer. Blots were then stained with amido black to detect total protein and then destained with methanol.

RUNNING GEL		**STACKING GEL**	
Distilled water	7.6 ml	Distilled water	3ml
Tris pH 8.8 (1.5M)	3.75ml	Tris pH 6.8 (0.5M)	1.25ml
30% acryl, 8% BA	3.5ml	30% acryl, 8% BA	650ul
10% SDS	150ul	10% SDS	50ul
10% APS	80ul	10% APS	35ul
Temed	14ul	Temed	7ul

Probing with antibodies

Blots were washed with three changes of TBST (see reagents below) three times over 5-15 min. Membranes were blocked with 15 ml of 5% milk solution (powdered nonfat dry milk) in TBST and allowed to rock at room temperature for 1 -1.5 hours. Blots were again washed with three changes of TBST three times for 5 min. Primary antibody-mouse monoclonal FANCD2 (FI-17; Santa Cruz Biotech) was added to a staining dish at room temperature and allowed to rock overnight. The primary antibody was removed and the blots were washed with three changes of TBST three times at 5-10 minutes. The secondary antibody, rabbit anti-mouse IgG-HRP at a concentration of 1/3000 was added with 5% milk solution to the staining dish and allowed to rock for 1.5 hours. The secondary antibody was removed and then washed again with three changes of TBST, three times for 5 min each. Western blot chemiluminescence reagents (500ul

each) were added and developed in the dark at room temperature on film (Blue Sensitive Medical Research Grade X-Ray Film, LPS, Inc.).

Reagents:

Resolving buffer (1.5M)
72.6gms of Tris base in 400 ml of distilled water. Adjust pH to 8.8
Make sure that initially you dissolve the tris base in 350 ml of d/w as at least 35-50 ml of HCl will be used to bring the pH to 8.8.

Stacking Buffer (0.5M)
Dissolve 6 grams of Tris base in 100 ml of distilled water. Adjust pH to 6.8.

10% SDS
Dissolve 10 grams of SDS in 100 ml of distilled water.

10% APS
Dissolve 1 gm of APS in 10 ml of distilled water.

1X SDS Electrophoresis buffer.

Sr No	Reagent	Amount
1	Tris base	3.05 grams
2	Glycine	14.4 grams
3	SDS	1 gm
4	Distilled water	1.0 L

1X Transfer buffer

Sr No	Reagent	Amount	
1	Tris base	18.2 grams	4.55g
2	Glycine	86.5 grams	21.63g
3	Methanol	400 ml	100ml
4	Distilled water	4.0 L	1.0L

!!! Always add methanol at the end.

1X TBST

Sr No	Reagent	Amount
1	Tris base	6.05 grams
2	NaCl	14.6 grams
3	HCl	2.8 ml
4	Tween 20	1.0 ml
5	Distilled water	2.0 L

1X Sample Buffer

Sr No	Reagent	Amount
1	Stacking buffer	2.5 ml
2	10% SDS	4.0 ml
3	Glycerol	2.0 ml
4	Beta mercaptoethanol	1.0 ml
5	Distilled water	0.5 ml
	Total	10.0 ml

Lysis Buffer

Sr No	Reagent	Amount
1	1 M Tris base pH .4	5.0 ml
2	5 M NaCl	15.0 ml
3	0.5M EDTA	5.0 ml
4	Triton x100	1%

Store at 4^{o}C.

Protease inhibitor

Sr No	Reagent	Amount
1	Ethanol	1 ml
2	aprotinine	5.0 mg
3	leupeptine	10.0 mg

Protease inhibitor (2)

Sr No	Reagent	Amount
1	Lysis Buffer	1 ml
2	Pepstatin	4ml
3	Leupeptine	40ml
4	DTT	5ml
5	PMSF	5ml

Good for one day. Thaw DTT and PMSF completely in water bath before using.

Leupeptin
Dissolve to 10mg/ml in dH2O.
Pepstatin
Dissolve to 10mg/ml in dH2O.
DTT (1M)
Boil in dH2O.
PMSF (100mM)
Dissolve in isopropanol.

5. Results

Chromosome Breakage

Eleven total cell lines were examined to compare the effect of the cross-linking agent, DEB, on DNA damage repair. Cells with a competent DNA damage response, specifically in the FANC pathway, should not express increased chromosome breakage when exposed to this agent. Figure 3 shows the average number of breaks per chromosome in each cell line, comparing those treated with DEB to matched untreated controls. Both FANCA-/- and FANCD2-/- cell lines show the largest induction of breakage after exposure to DEB. UPCI:SCC125, UPCI:SCC131, and UPCI:SCC142 all show significant increased breakage (>1.0 breaks per chromosome). UPCI:SCC084, UPCI:SCC122, and UPCI:SCC136 show moderate breakage (0.5> breaks per chromosome <1.0). Human peripheral blood lymphocytes (HPBL) cells show an increase in breaks in the treated cell lines compared to controls, but still fall within the normal breakage range (<0.5 breaks per chromosome). UPCI:SCC078 has slightly higher breakage in the untreated cells than in the treated, indicating spontaneous genomic instability. SCC116 also has slightly higher breakage in the untreated cells than in the treated, but also has a much higher level of breakage in the untreated cells when compared to the other cell lines. The percent of cells with radial formation was also calculated. The FANCD2-/- cell line had 10% of cells with radial formation and the FANCA-/- cell line had 36% of cells with radial formation when exposed to DEB. Only UPCI:SCC142 showed radial formation in response to DEB and this was observed in 3% of cells.

The baseline, or spontaneous rate of genomic instability in these cells lines was determined by comparing the mean number of breaks per chromosome in the untreated cell lines to the untreated peripheral blood lymphocytes. UPCI:SCC084 had a lower number of breaks in the untreated cell line when compared to untreated HPBL. All of the other cell lines showed increased breakage and therefore, more genomic instability in the untreated cell lines compared to HPBL, with UPCI:SCC116 having the highest number of chromosome breaks when untreated.

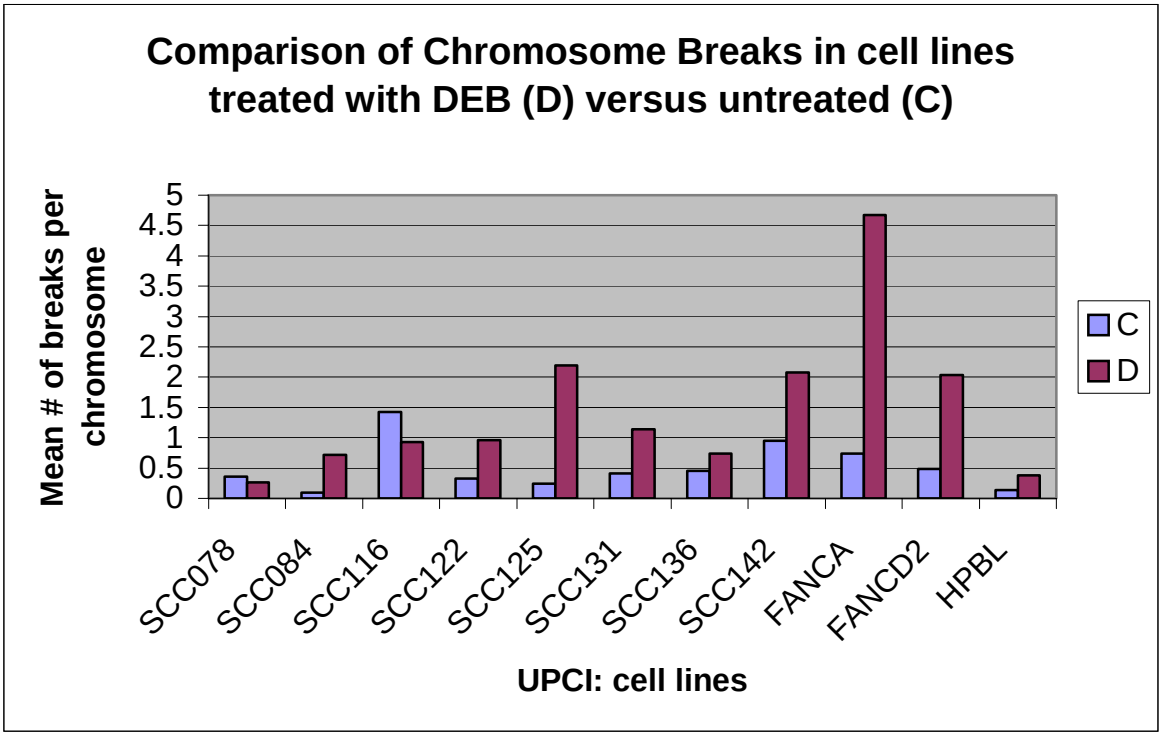


Figure 3: Average chromosome breaks in treated versus untreated HNSCC cell lines

Note that FANCA and FANCD2 have the highest induction of breakage when treated with DEB (D). All other cell lines show an increase in breakage except UPCI:SCC078 and UPCI:SCC116.

Types of aberrations were tallied in each cell line for both treated and untreated and results are shown in Table 4. Note that there are several aberrations that involve two chromosome breaks indicated by formation of rings, dicentrics, giants, chromosome breaks and

radials. In these cell lines the most frequent aberrations observed are minutes and dots. Only UPCI:SCC142 and FANCA and FANCD2 showed radial formation.

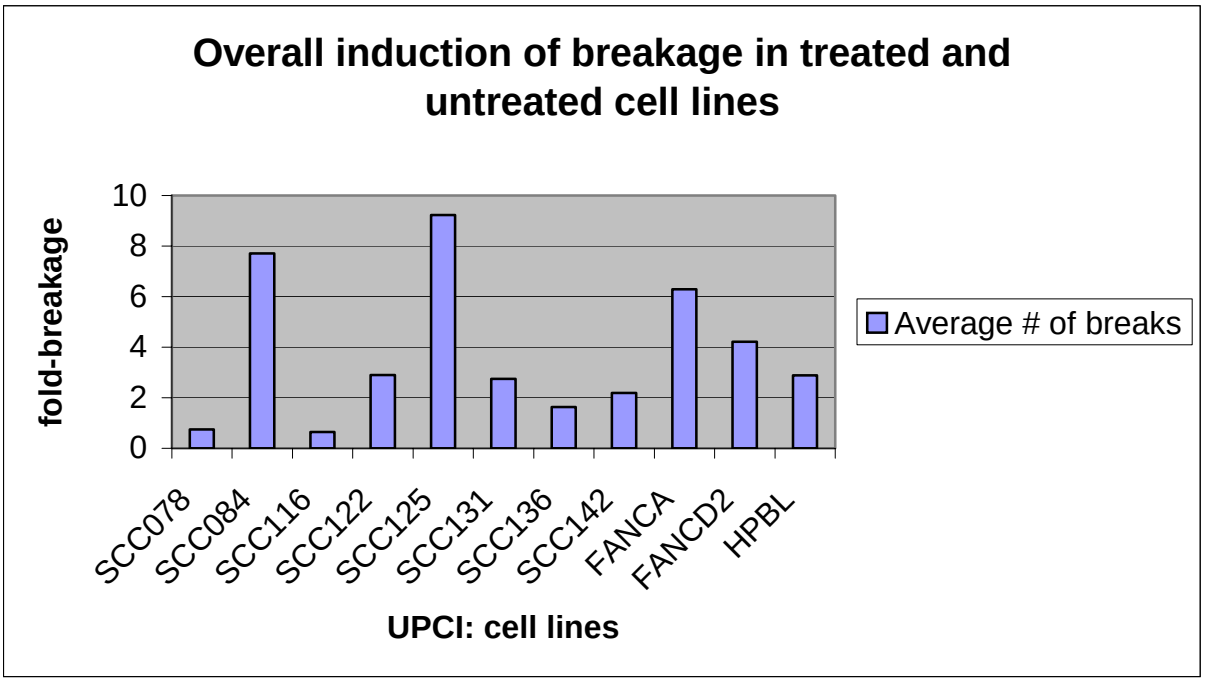


Figure 4: Overall increase in breakage in cell lines after DEB treatment

The FANC cell lines and UPCI:SCC084 and UPCI:SCC125 had the largest induction of breakage after treatment with DEB. This suggests that these cell lines have the greatest sensitivity to crosslinking agents.

Table 4: Summary of frequency of the types of chromosome aberrations

Cell line	Chromosome Breaks	Chromatid Breaks	Radials	Giant	Ring	Minutes	Dot	Dicentric	Fragment
HPBL control	**0**	**11**	**0**	**4**	**0**	**5**	**2**	**0**	**1**
HPBL treated	**7**	**4**	**0**	**3**	**0**	**23**	**5**	**0**	**1**
078 control	**0**	**4**	**0**	**1**	**1**	**4**	**1**	**0**	**0**
078 DEB	**0**	**11**	**1**	**4**	**0**	**5**	**2**	**0**	**1**
084 control	**0**	**3**	**0**	**0**	**0**	**1**	**4**	**1**	**0**
084 DEB	**5**	**4**	**0**	**0**	**1**	**38**	**8**	**0**	**17**
116 control	**4**	**23**	**0**	**6**	**2**	**34**	**7**	**8**	**4**
116 DEB	**63**	**48**	**0**	**5**	**2**	**20**	**14**	**1**	**22**
122 control	**3**	**11**	**0**	**0**	**0**	**18**	**1**	**1**	**4**
122 DEB	**11**	**15**	**0**	**8**	**0**	**45**	**4**	**2**	**2**
125 control	**2**	**6**	**0**	**2**	**1**	**2**	**7**	**1**	**0**
125 DEB	**19**	**23**	**0**	**8**	**9**	**22**	**30**	**27**	**0**
131 control	**5**	**11**	**0**	**1**	**0**	**16**	**3**	**4**	**4**
131 DEB	**5**	**34**	**0**	**0**	**2**	**36**	**33**	**1**	**11**
136 control	**4**	**4**	**0**	**7**	**0**	**16**	**6**	**1**	**8**
136 DEB	**2**	**13**	**0**	**4**	**10**	**12**	**12**	**8**	**4**
142 control	**8**	**25**	**0**	**0**	**1**	**33**	**19**	**8**	**5**
142 DEB	**39**	**85**	**4**	**7**	**2**	**27**	**8**	**5**	**3**
FANCD2 control	**1**	**6**	**0**	**3**	**1**	**15**	**8**	**7**	**4**
FANCD2 DEB	**35**	**62**	**16**	**9**	**2**	**15**	**7**	**5**	**13**
FANCA control	**9**	**15**	**0**	**5**	**0**	**9**	**18**	**10**	**2**
FANCA DEB	**74**	**80**	**52**	**25**	**7**	**14**	**17**	**14**	**12**

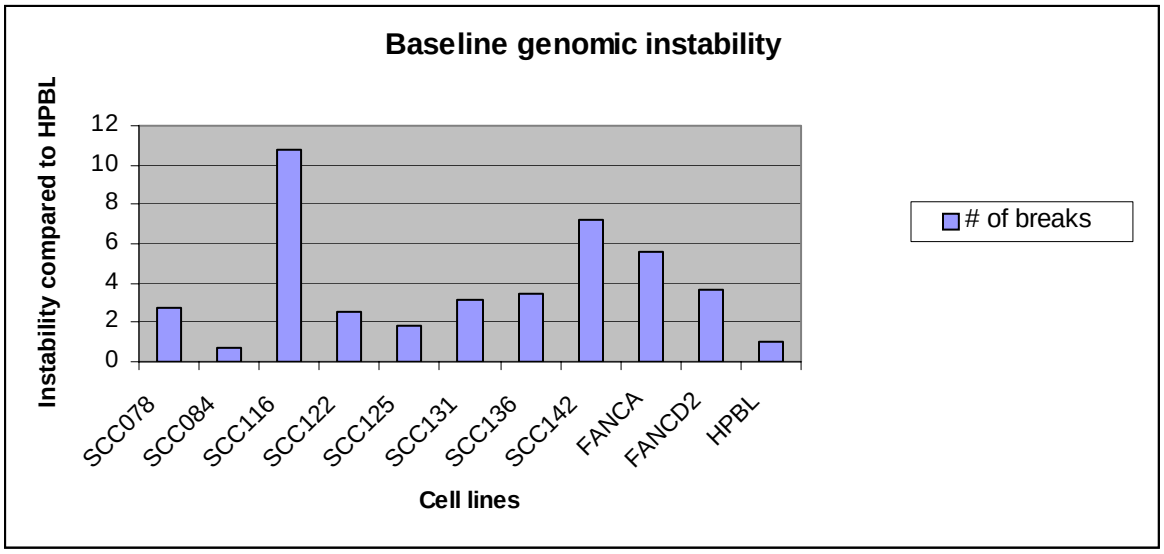
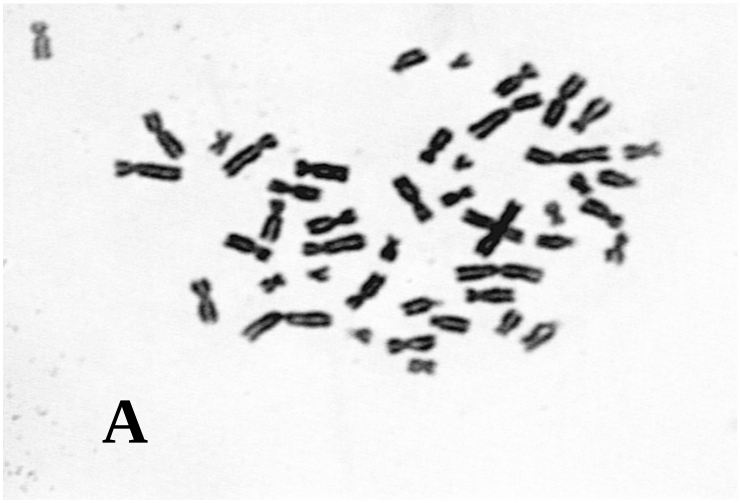
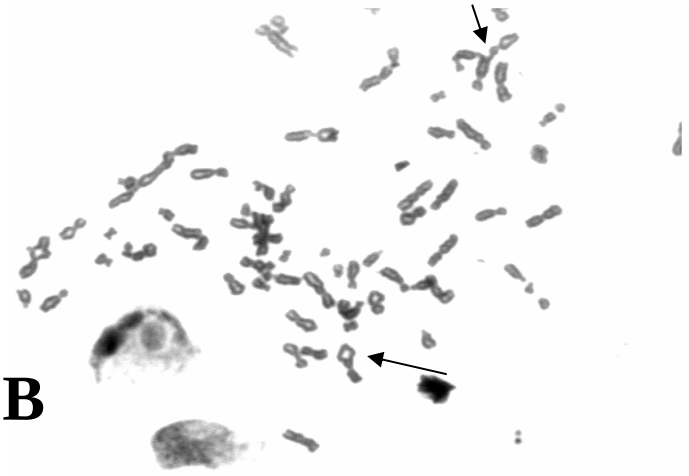


Figure 5: Baseline genomic instability in cell lines compared to HPBL

Not that UPCI:SCC116 had the highest rate of spontaneous instability, FANCA, FANCD2, andUPCI:SCC142 also have a high rate of genomic instability, and all other UPCI:SCC's have spontaneous instability except for UPCI:SCC084 when compared to the spontaneous instability in the human peripheral blood lymphocyte cells (HPBL).





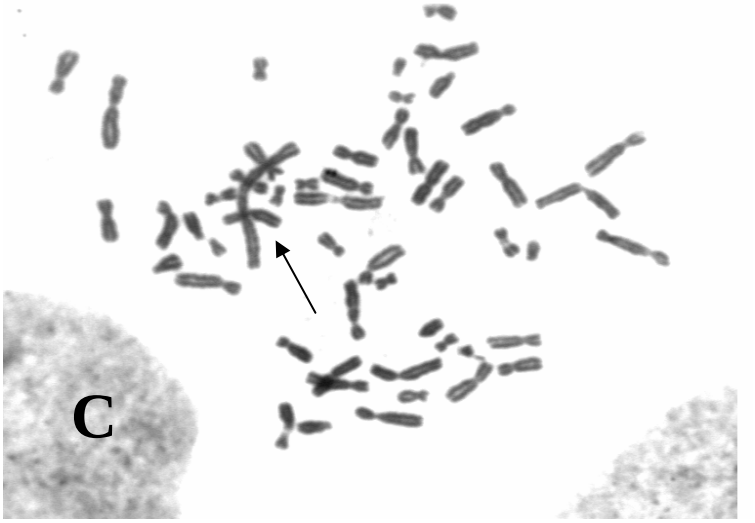


Figure 5: Chromosome Breakage

Select metaphase spreads comparing chromosome breakage in the presence of DEB. (5A) Control peripheral blood lymphocytes do 5B) FANCD2-/- cell line has increased chromosome breakage and a defective DNA damage response in the presence of a cross-linking agent. Arrows not have any chromosome breakage and therefore have a normal DNA damage response in the presence of a cross-linking agent. (show radial formation and single and double-strand breaks. (5C) Cell line SCC142 in response to DEB. There is increased breakage, but it is not as severe as in the FA cells. Arrow shows fused chromosomes as a result of breakage.

Western Blots

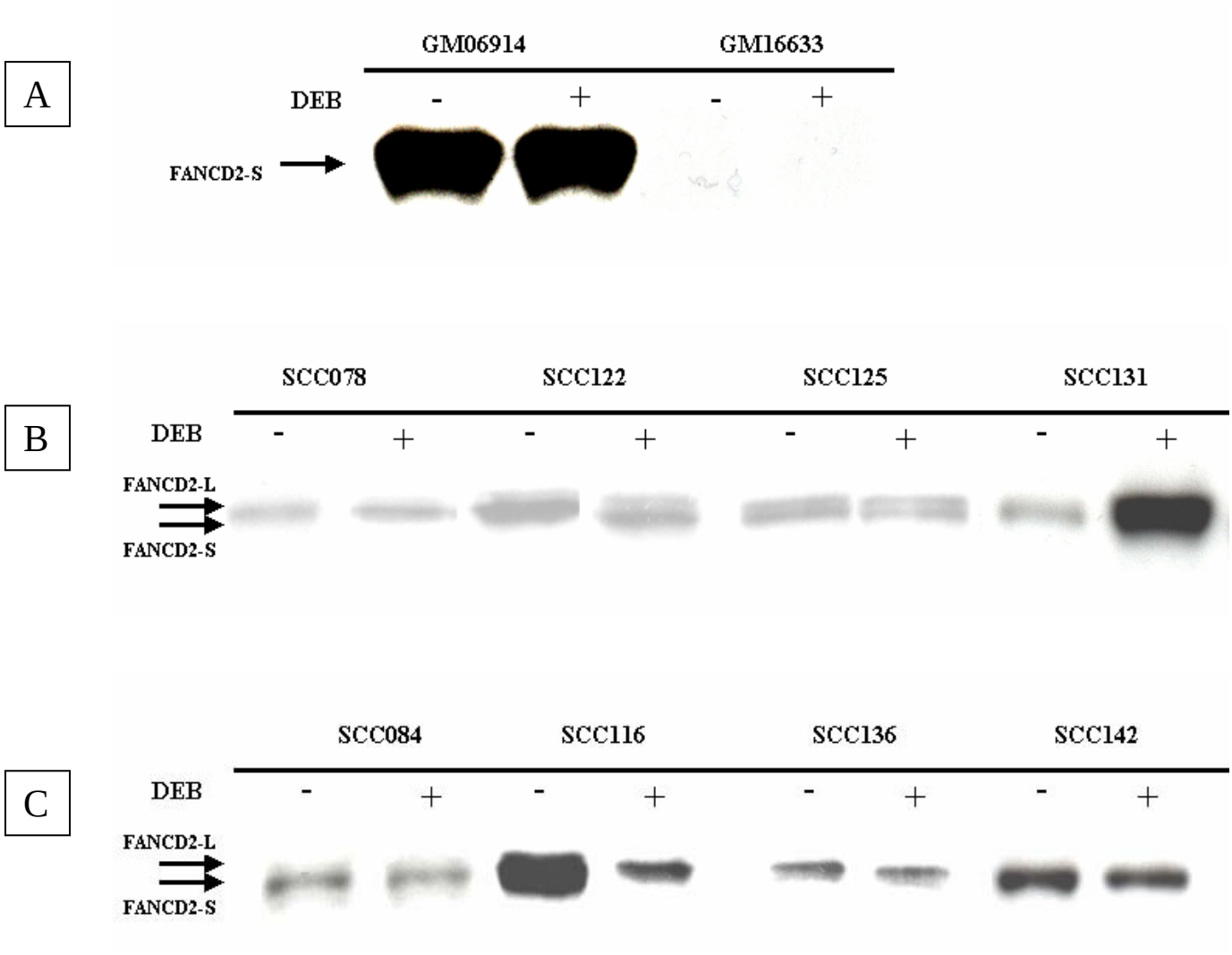


Figure 6: Western Blots for FANCD2

Immunoblotting was performed to assess the presence and ubiquitination of FANCD-2 protein in the UPCI:SCC cell lines. Two FANC cell lines (FANCA-/- and FANCD2-/-) were used as negative controls. Cellular proteins were separated and probed with anti-FANCD2 antibody. These results are not conclusive, but interpretation is as follows: (A) *FANCA*-/- (GM06914) shows *FANCD2* protein expression, but not ubiquitination of *FANCD2*. *FANCD2* -/- shows no expression of the *FANCD2* protein. (B) shows a selection of SCC cell lines. All show *FANCD2* protein expression, and SCC078, SCC122, and SCC125 show ubiquitination of *FANCD2*. (C) shows another selection of cell lines, all of which show *FANCD2* protein expression. None of these cell lines show ubiquitination of *FANCD2*. Arrows show the FANCD2 protein (FANCD2-S) and the ubiquitination (FANCD2-L).

6. Summary of results in relation to 11q13

Table 5 summarizes the results and the relationship to 11q13 status. The UPCI:SCC cell lines used for experimentation were chosen based on whether they have 11q13 amplification and/or distal loss of 11q. Because several of the genes on distal 11q are involved in DNA damage repair and also may interact with the FANC pathway, we wanted to see if there was any correlation between 11q loss and defects in the FANC pathway.

Table 5: Summary of results

Cell Line	11q13 status	Baseline Genomic Instability (compared to HPBL control)	DEB induction (increased breaks per chromosome)	Presence of FANCD2 protein	Ubiquitination of FANCD2
FANCD2	Unknown	3.7 X increased	High (>1.0)	No	No
FANCA	Unknown	5.6 X increased	High (>1.0)	Yes	No
SCC116	No gene amplification, No gene loss	10.7 X increased	None (treated>untreated)	Yes	No
SCC122	No gene amplification, gene loss	2.5 X increased	Moderate (>0.5, <1.0)	Yes	Yes
SCC125	No gene amplification, gene loss	1.8 X increased	High (>1.0)	Yes	Yes
SCC142	No gene amplification, gene loss	7.2 X increased	High (>1.0)	Yes	No
SCC078	Gene amplification, gene loss	2.7 X increased	None (<0.5)	Yes	Yes
SCC084	Gene amplification, gene loss	0.7 X increased	High (>1.0)	Yes	No
SCC131	Gene amplification, gene loss	3.1 X increased	High (>1.0)	Yes	No
SCC 136	Gene amplification, gene loss	3.7 X increased	Moderate (>0.5, <1.0)	Yes	No

7. Discussion

Eleven cell lines (summarized in Table 3) were examined to measure the DNA damage response to the crosslinking agent, DEB. Cells with a competent DNA damage response, specifically in the FANC pathway , should not have increased chromosome breakage compared to normal human lymphocytes when exposed to this agent and are expected to show FANCD2 ubiquitination by western blot. Cell lines that show increased breakage in the presence of DEB may have a defect in the FANC pathway, which may correlate with lack of *FANCD2* ubiquitination. Alternatively, FANCD2 may be ubiquitinated but not be fully functional, or there may be a mutant protein downstream of *FANC* (i.e. *BRCA1*), which results in an inability to repair the DNA damage, leading to increased chromosomal aberrations. Figure 3 shows the average number of breaks per chromosome in each cell line comparing cells treated with DEB to those left untreated and figure 6 shows the western blots. The UPCI:SCC cell lines were grouped according to their breakage status after treatment with DEB for analysis. This method of grouping was chosen as breakage analysis is the "gold standard" in diagnosing FA. UPCI:SCC125, UPCI:SCC131, and UPCI:SCC142 all show DEB-induced breaks compared to normal cells, characterized by >1.0 breaks per chromosome. UPCI:SCC084, UPCI:SCC122, and UPCI:SCC136 show moderate breakage, characterized as >0.5 breaks per chromosome <1.0. UPCI:SCC116 also shows moderate breakage, but has a very high rate of genomic instability. UPCI:SCC078 has a low level of breakage after treatment with DEB. Western blots were done to confirm the DNA damage response. These results are inconclusive, but interpretation of the results are discussed. The specific results of each UPCI:SCC cell line and the controls are discussed below.

As expected, both FANCA-/- and FANCD2-/- cell lines show the largest induction of breakage when treated with DEB. FANCA-/- shows approximately six-fold increase in breakage with 36% of cells with radial formation FANCD2-/- shows approximately a four-fold increase in breakage with 10% of cells with radial formation, both in keeping with the formal diagnosis of Fanconi anemia. Western blot supports these results, as FANCD2-/- does not show any FANCD2 protein expression and FANCA-/- shows the presence of FANCD2, but no ubiquitination. The complete absence of FANCD2 protein results in no interaction with BRCA proteins to repair DNA damage, hence the increased DEB-induced breaks and radials in the null cell line. In the absence of FANCA protein function, the FANC complex cannot activate FANCD2 and therefore results in undetectable levels of the ubiquitinated form of FANCD2 and deficient damage repair.

The group of UPCI:SCC cell lines that show the largest induction of breakage, are not similar in FANCD2 protein expression. UPCI:SCC125 shows approximately a ten-fold increase in breakage ($p<0.01$), but no radial formation. By western blot analysis UPCI:SCC125 shows ubiquitination of FANCD2 in the presence of DEB. This indicates that the FANC pathway is likely intact, and the breaks are a result of a defect downstream from the FA pathway. This cell line is known to have distal chromosome 11q13 loss, so the loss of *ATM*, *CHEK1*, *H2AX*, and *MRE11*, which all play a role in DSB repair may contribute to the increased number breaks observed. It should also be noted that UPCI:SCC125 is a very heterogeneous tumor cell line, as the percent of cells with loss are almost equal to the percent of cells without loss. Therefore, these results may reflect the specific cell population tested, and not that of the entire tumor. It is possible that the cell population represented originated from a cell with distal 11q loss. UPCI:SCC131 shows approximately a three-fold increase in breakage ($p< 0.01$) and no radial

formation. UPCI:SCC131 does not show FANCD2 monoubiquitination in the presence of DEB. The FANCD2 protein is present, but its activity is unknown due to the lack of ubiquitination. It is possible that the protein is non-functional. This could mean that there could be a defect in any of the proteins in the FANC complex. This cell line is known to have 11q13 amplification and loss. Therefore, loss of distal 11q and possible haploinsufficiency of the genes could also contribute to the increased level of breakage via a defect in DSB repair. UPCI:SCC142 shows approximately a two-fold increase in breakage ($p<0.01$) and 3% of cells with radial formation. UPCI:SCC142 also has a high level of breaks in the untreated cell line, indicating spontaneous genomic instability. Western blot analysis of UPCI:SCC142 shows presence of the FANCD2 protein, but no ubiquitination. This suggests that like in UPCI:SCC131, the FANCD2 protein may be non-functional as a result of a defect in FANCD2 itself or a defect in the FANC complex that ubiquitinates FANCD2. UPCI:SCC142 is known to have 11q loss, which also might contribute to defects in DNA damage repair. Even though all of these cells lines show increased breakage similar to the amount of breakage seen in cells of an individual diagnosed with FA, the absence of significant radial formation suggests that the breakage is not a result of "classical" defects in the FANC pathway. All of these cell lines also have distal 11q loss, but the ubiquitination results are not consistent. Since UPCI:SCC125 shows FANCD2 ubiquitination, it is possible the tumor may have already had a defect or acquired a mutation in one of the genes on distal 11q (i.e. *MRE11*) that acts downstream of the FANC pathway. This results in breakage, but an intact FANC pathway. In UPCI:SCC131 and UPCI:SCC142, it is appears that the breakage results from a defect upstream of the FANC pathway, resulting in no ubiquitination of FANCD2.

The UPCI:SCC cell lines that show moderate breakage, are not consistent with defects in the FANC pathway. UPCI:SCC084 shows approximately a seven-fold increase in the number of cells with breaks ($p<0.01$) but no radial formation. This cell line shows low spontaneous genomic instability, and a definite increase in breakage when exposed to DEB. By western blot, there is not FANCD2 ubiquitination. This suggests that breakage could have occurred as a result of a defect in the FANC complex or as a result of a defect upstream in the pathway. UPCI:SCC084 is known to have both 11q13 amplification and loss. Loss of DNA damage response genes at this location could also contribute to the observed increased number of breaks UPCI:SCC122 shows approximately a two-fold increase in breakage ($p< 0.01$) and no radial formation. Western blot analysis shows ubiquitination of FANCD2, which shows the FANC pathway is intact in this cell line. The breaks may have result from a defect in DNA damage repair downstream of the FANC pathway. Because this cell line has 11q loss, the loss of DNA damage repair genes, specifically *MRE11*, which participates in the repair DSBs, may be responsible for in the increased number of breaks without radial formation. UPCI:SCC136 shows less than a two-fold increase in breakage ($p=0.08$) and no radial formation. Western blot analysis shows no ubiquitination of FANCD2. This indicates that there may be defect elsewhere in the pathway that caused defective DNA damage repair, especially since the increase in breakage was not statistically significant in the presence of DEB. The FANCD2 protein may not be activated because of defects elsewhere in the pathway. UPCI:SCC136 is known to have 11q13 amplification and distal loss, which may account for other defects in the pathway.

UPCI:SCC078 has slightly higher breakage in the untreated cells than in the treated. This increase in breakage was not statistically significant (p-value=0.59). Since UPCI:SCC078 untreated cell line showed a higher incidence of spontaneous breaks, this indicates that this

particular cell line has baseline genomic instability that is increased when compared to other cell lines used, and breakage levels are not increased in the presence of a crosslinking agent. Western blot analysis of UPCI:SCC078 shows that in the presence of DEB, FANCD2 is ubiquitinated. This suggests that in UPCI:SCC078, the FANC pathway is intact and is able to repair damage caused by a crosslinking agent. Therefore, there may be a defect elsewhere in the DNA damage response pathway that lead to tumor development. UPCI:SCC078 is known to have 11q13 amplification and distal loss. Loss of DNA damage repair genes at this location may explain the higher baseline genomic instability as well as well as the intact Fanconi pathway.

UPCI:SCC116 also has slightly higher breakage in the untreated cells than in the treated cells, which is statistically significant ($p< 0.01$). This cell line also has a much higher level of breakage in the untreated cells when compared to the other cell lines. Therefore, UPCI:SCC116 cell line has a high rate of genomic instability and it is not increased in the presence of crosslinking agent. Western blot confirms the presence of FANCD2, but does not show ubiquitination in the presence of DEB. Even though FANCD2 can be detected by western blot, the protein function is unknown due to lack of ubiquitination. It is possible that it is a non-functional protein, or that there is a defect in the FANC complex that results in absence of ubiquitination of FANCD2. SCC116 does not have 11q13 amplification or distal loss. These results combined with the increased chromosome breakage/genomic instability in the untreated cell line suggest that there is a defect elsewhere in the DNA damage response and does not include the DNA damage repair genes on chromosome 11.

HPBL cells show an increase in breaks in the treated cell lines compared to controls, but still fall within the normal breakage range (<0.5 breaks per chromosome).

The results of this study show that head and neck tumor cell lines clearly exhibit defects in DNA damage response as illustrated by increased chromosome breakage in the presence of the DNA damaging agent, DEB. However, the clinical diagnosis of Fanconi anemia is made only when there are >10% cells with radial formation as other syndromes (i.e. Nijmegen breakage syndrome (NBS)) will show increased breakage in the presence of a crosslinking agent. Western blot analysis confirmed that the FANCD2 protein is present, but is not consistently ubiquitinated/activated in the cell lines. It is unlikely that any of the patients whose tumors were used to derive the UPCI:SCC cell lines have/had FA. This is expected, as these patients had environmental exposures associated with HNSCC (tobacco use, alcohol use, and job-related exposures) and their cancers were diagnosed at a later age. These characteristics do not correlate with a hereditary predisposition to HNSCC or FA, in which early onset of HNSCC (<age 40) usually in the absence of environmental exposures is observed. However, in the UPCI:SCC cell lines tested, the presence of increased spontaneous and DEB-induced breaks, as well as occasional defects in FANCD2 ubiquitination is consistent with a defect in the FANC pathway specifically in the HNSCC tumor. This could be a result of 1) two acquired mutations in the cell that eventually led to the tumor, 2) a single mutation in patients who are/were carriers (heterozygous) for FA mutations that also acquired a mutation or had somatic loss, 3) patients who have germline FA mutations, or 4) patients whose tumors are haploinsufficient for the FANC genes. It is also possible that the increased breakage could have resulted from DNA damage response defects upstream or downstream in the repair pathway and that they play a role in the development of HNSCC. However, the defects in the Fanconi pathway do not correlate with 11q13 amplification and/or loss patterns. These results could possibly be confounded by the fact that cell lines can represent asynchronous cell populations due to clonal expansion and

may only adequately represent part of the tumor. Also, cells may not be dividing at the same rate and may not have been exposed to DEB at the same point in the cell cycle. It is also possible that in the presence of DEB, cells may have undergone apoptosis and the cell population we tested was a fraction of the original cell population. Finally, it is not completely understood as to how all of the DNA damage repair genes interact with each other, which makes it difficult to determine where the damage was not repaired.

Therefore, further studies are warranted. The other FANC proteins can be tested by western blot to determine defects in the core complex. This could explain why many of the UPCI:SCC cell lines are not ubiquitinated in response to cross linking agents.

Fluorescence *in situ* hybridization (FISH) of *FANCD2* on chromosome 3p25.3 should be performed to determine copy number of the gene. Many head and neck tumors also exhibit loss of 3p and therefore *FANCD2* may be lost on one or more chromosomes leading to defects in DNA damage repair. This test also can detect haploinsufficiency of *FANCD2* in tumor cells. Cell lines with overall loss of *FANCD2* might correlate with the cell lines already known to show defects in the FANC repair pathway such as those with increased breakage or those which are not ubiquitinated in response to DNA damage.

A functional assay for detection of BRCA foci should also be performed. When FANCD2 is ubiquitinated, it interacts with BRCA1. BRCA1 forms nuclear foci in response to DNA damage. Therefore, if the FA pathway is completely intact, nuclear focus formation would be expected in response to damage and functional FANCD2 will colocalize with these BRCA1 foci. This would confirm the possibility that the FANC pathway is intact in some cell lines and that the damage is not repaired as a result of defects somewhere downstream in the pathway.

Study and correlation of other genes involved in DNA damage repair is also warranted. *ATM* and *ATR* are especially important to study as they are known to activate the FANC pathway. Knowing if there are defects in those specific DNA damage repair genes could give more information about the apparent defects in the FA genes in the HNSCC cell lines.

It also important to study the relationship between HNSCC and FA clinically. Because of the rarity and variability of FA, it is often not diagnosed until the patient is diagnosed with cancer. Therefore, FA should be considered in the case of an atypical clinical presentation of head and neck cancer and/or if other associated cancers are present (i.e. esophageal, cervical). Physicians should be suspicious of patients who present with HNSCC who are at a young age (<40), non-smokers, and non-drinkers. Because individuals with FA are hypersensitive to radiation and chemotherapy when crosslinking agents are used (such as Cisplatin), general adjuvant treatment for HNSCC would be extremely toxic and lethal to the patient. A detailed family history should be obtained and genetic counseling for risks provided to family members.

Currently, only mutation analysis can detect carriers/heterozygotes of FA. *BRCA2* heterozygotes are at an increased risk to develop breast, ovarian, pancreatic, prostate, and skin cancers. Because *BRCA2* is an FA gene as well, it is possible that heterozygous carriers of mutations in the other FA genes (A, B, C, D2, *etc*.) could also have an increased risk for cancer. Further investigation might reveal the underlying defects in HNSCC as well as other cancers that are currently considered sporadic.

Public Health Significance

Head and neck cancer (including oral cancer) is expected to account for 11,000 deaths in the United States in 2005. While this specific type of cancer is considered sporadic and strongly associated with tobacco and alcohol use, it is suspected that there are underlying genetic mechanisms that predispose individuals to head and neck cancer. Otherwise, all individuals with these exposures would have the disease. Examining the association between FA and HNSCC increases our understanding the genetic mechanisms that lead to head and neck cancer in the general population. This has both clinical and scientific value.

First, knowing that a patient has an underlying susceptibility to develop HNSCC can allow for more frequent HNSCC screening. Currently, there are no recommended regular early detection programs that screen for HNSCC. This results in many HNSCC being diagnosed at a later pathological stage where surgery may not be an option, or where chemotherapy and/or radiation may not be successful. This in turn leads to the high mortality rate and low five-year survival rate for individuals diagnosed with HNSCC. As discussed previously, survival rate is significantly increased when HNSCC is detected and treated at an early stage. Development of screening programs, like the increased screening recommended in FA patients, would allow for earlier detection and increased survival as well as decreased morbidity.

Treatment and management may also be different in a patient with an underlying genetic susceptibility for HNSCC. Understanding the biological development of cancer could lead to better use of current treatments/therapies or could lead to the development of better treatments. In most patients diagnosed with HNSCC, adjuvant chemotherapy and radiation is given after surgery. Individuals with FA are sensitive to radiation and hypersensitive to certain chemotherapeutic agents. Therefore, patients with defects in DNA damage repair due to a

mutation in the FA pathway might also have these sensitivities to treatment. In contrast, if the FA pathway is specifically affected in HNSCC tumors, this offers the opportunity to specifically treat this class of tumors with chemotherapeutic agents that cause DNA interstrand crosslinks, as FA deficient cells will be hypersensitive to these agents.

Individuals who have a mutation in the FA pathway and therefore DNA damage defects, may also have increased risk to develop other cancers. Therefore, understanding the exact mechanisms that cause HNSCC as well as understanding the function of the FA genes could lead to a better understanding of the development of other types of cancers and their relationship to HNSCC. Again, this understanding would lead to better risk assessment of cancer and therefore increased screening and earlier detection of specific cancers as well as use of safer therapies and reduced morbidity and mortality.

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