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Biological and Functional Consequences of Single Nucleotide Polymorphisms of the *MGMT* Gene

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Biological and Functional Consequences of Single Nucleotide Polymorphisms of the *O^6-Methylguanine-DNA-Methyltransferase* Gene

by

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Dedication

Dedicated to my husband Michael, for supporting me during the hardest time of my life, and to my parents for always pushing me to reach my highest potential.

Thank you from the bottom of my heart.

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Biological and Functional Consequences of Single Nucleotide Polymorphisms of the *O^6-Methylguanine-DNA-Methyltransferase* Gene

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Single nucleotide polymorphisms (SNPs) in DNA repair genes could alter the transcriptional levels, structure and function of DNA repair proteins and alter DNA repair proficiency. Consequently, these SNPs could significantly influence the level of genetic damage, which is an early critical factor in the cascade of events leading to cancer. Molecular epidemiological studies indicate that SNPs in the *0^6-Methylguanine-DNA-Methyltransferase* (*MGMT*) gene, which repairs alkyl adducts at the 0^6-position of guanine, may be associated with an increased risk of lung cancer. However, the functional and biological significance of these SNPs has yet to be systematically characterized. We used two biologically relevant endpoints in an exposed population of 350 individuals to determine the association between genetic damage and SNPs in *MGMT*; chromosome aberrations to examine macrolesions, and mutation frequency to examine microlesions. In addition, we used the luciferase reporter assay to determine effects of SNPs in the promoter/enhancer region of *MGMT* on promoter activity. Coding SNPs had a

marginal effect on macrolesion damage after exposure to alkylating agents. However a strong effect of coding SNPs on microlesion damage was observed. The luciferase expression data demonstrated a significant increase in promoter activity in the presence of the enhancer SNP compared to the wild-type form. This indicates that, in a biological system, inheritance of one copy of these SNPs could affect the level of genetic damage, especially after accumulated exposure to alkylating agents. While the coding SNPs evaluated are predicted to be detrimental, as indicated by the accumulation of genetic damage, the promoter/enhancer SNP evaluated may be protective as levels of cellular protein would be increased.

Table of Contents

List of Tables

List of Figures

List of Illustrations

Chapter 1: INTRODUCTION

THE HUMAN GENOME PROJECT/SNP PROJECT

Since 1977, when Frederick Sanger at the U.K Research Council and Allan Maxam and Walter Gilbert at Harvard University first developed efficient methods for DNA sequencing, scientist and scholars have dreamed of mapping the human genome (Maxam and Gilbert 1977, Sanger et al., 1977). In 1982, Akiyoshi Wada first proposed automated sequencing and by 1986, the U.S. Department of Energy (DoE) was discussing the feasibility of sequencing the full human genome. The same year, Renato Dulbecco of the Salk Institute proposed sequencing the human genome for cancer research (Dulbecco 1986) and Leroy Hood of Caltech announced the first automated DNA sequencing machine (Smith et al., 1986). In 1988, the NIH took over the Human Genome Project from the DoE and named DNA research pioneer James Watson as the head. As part of its final 5-year review before the release of the human genetic sequence, NIH initiated single nucleotide polymorphism (SNP, single nucleotide change that occurs in greater than 1% of the population) mapping goals in order to delineate genetic variation between individuals. This map, combined with the human genome sequence, would give researchers a better tool to examine the interplay between genetics, environment, and disease risk as first proposed by Dulbecco (Dulbecco 1986).

The final mapped human genome was published separately by J. Craig Venter and researchers at Celera (Venter et al., 2001), and Francis Collins and researchers backed by NIH (Lander et al., 2001). The same year, a group from Cold Springs Harbor published a high-density single nucleotide polymorphism (SNP) map of the human genome (Sachidanandam et al., 2001). The goal of this project was to identify variations within

the human genome that can affect disease risk or response to therapies, toxicants or stress, but did not include cases in which a minor allele is necessary and/or sufficient for specific disease development. This lead to a flurry of research into diseases known to be affected by environmental factors but also appeared to have a genetic component, such as heart disease, cancer, birth defects, addiction, Alzheimer's, and Parkinson's. For the first time, scientists had a powerful tool to determine the effect that genetics have on an individual's response to toxicants and subsequent disease development.

DNA ALKYLATION

Alkylators are a common and detrimental source of cellular damage and can react at all oxygen (O) and nitrogen (N) atoms within nucelobases and O atoms within phosphodiesters (Drabløs et al., 2004). Approximately 45%-75% of total exposure to alkylators is due to endogenous production of these compounds (Tricker 1997).

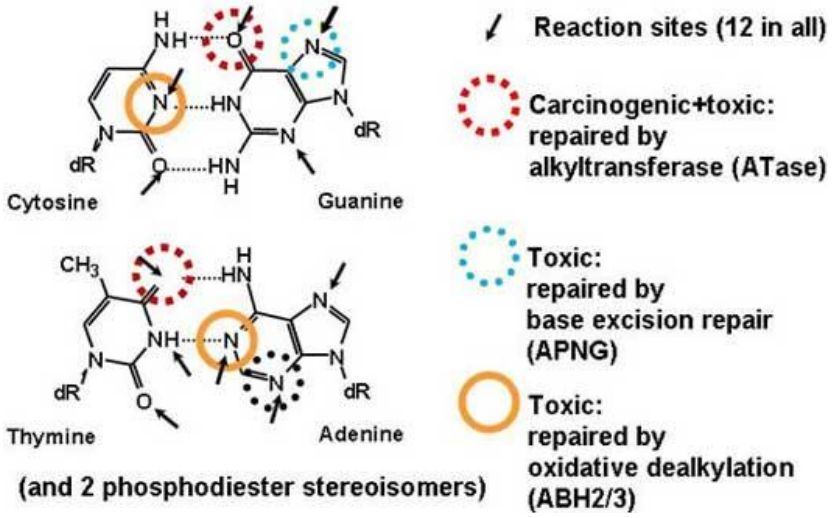

Illustration 1: DNA-Alkylation reactive sites (Source: Paterson Institute for Cancer Research)

Endogenous sources of alkylating agents include S-adenosylmethionine (SAM) and nitrosation of glycine derivatives or bile acids (reviewed by Drabløs et al., 2004). SAM is a common methyl donor in various biochemical reactions, and can react with nuclear DNA to give rise to predominantly 7-methylguanine and 3-methyladenine, as well as O^6-methylguanine (Barrows and Magee 1982; Rydberg and Lindahl 1982). Nitrosation of amino acid derivates, specifically α-lactones such as glycine, occurs in the stomach and large intestine, leading to O^6-methylguanine or O^6-carboxymethylguanine DNA adducts in these tissues (Shuker and Margison 1997). Nitrosation of the cholic acid amides in bile also occurs within the stomach, forming predominantly O^6-methylguanine DNA adducts (Busby et al., 1985). These compounds have been linked with gastric ulcers and gastrointestinal cancers. Interestingly, *E. coli* bacteria within the large intestine are also capable of producing *N*-alkyl-*N*-nitroso compounds and, while not strictly an endogenous source, it further increases the body burden of compounds capable of producing O^6-methylguanine DNA adducts created regardless of exposure (Taverna and Sedgwick 1996). Adduct levels between individuals vary at least 100-fold but does not correlate with cancer risk. However, levels of the direct reversal repair protein O^6-Methylguanine-DNA-Methyltransferase (MGMT) protein within the intestinal tissue, which is responsible for repairing much of the DNA alkylation damage, correlates strongly with cancer risk (reviewed by Povey et al., 2002).

Chemical alkylators are formed naturally in the environment and from man-made sources. Exposure to various types of alkylators, such as halocarbons, N-nitroso compounds, and alkylating drugs, have been shown to be carcinogenic and/or mutagenic depending on the base site (reviewed by Drabløs et al., 2004). Halocarbons include compounds such as chloromethane and iodomethane; both are formed in terrestrial

environments from plant and fungi sources and produced industrially (Vaughan et al., 1993; Mohamed et al., 2002; Ballschmiter 2003; Hamilton et al., 2003). Chloromethane alone contributes up to five million tons of alkylating agents to the ambient environment each year. These compounds have been shown to be both mutagenic and carcinogenic, although carcinogenicity data in humans is incomplete (Bolt and Gansewendt 1993).

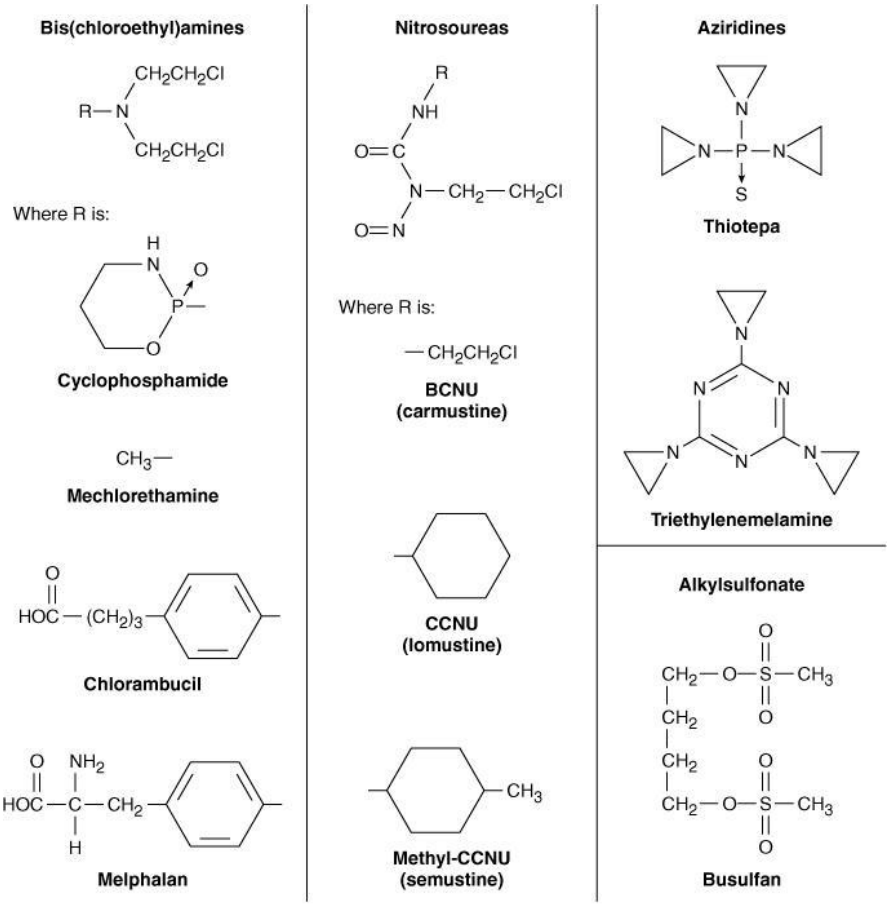

Illustration 2: Examples of various exogenous alkylating agents (Chu and Sartorelli 2011)

N-nitroso compounds also contribute significantly to an individual's alkylator adduct burden. These compounds are produced mainly during curing and drying of tobacco, and can be found in main stream and side stream smoke after combustion (Drabløs et al., 2004). 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are tobacco-specific nitrosamines and are considered the most mutagenic of all tobacco N-nitroso compounds. These compounds are metabolized by various cytochrome P450 (CYP450) enzymes. For example, the metabolic activation of NNK to DNA adducts proceeds via α-hydroxylation (hydroxylation of the carbon adjacent to the N-nitroso group). This reaction occurs either at the methyl or methylene carbon of NNK (reviewed by Hecht 1998). α-Hydroxylation at the methyl carbon produces α-hydroxymethyl NNK, which spontaneously loses formaldehyde producing α-pyridyloxobutyldiazohydroxide. This compound reacts with DNA forming stable pyrdyloxobutylation adducts (Hecht 1998). α-Hydroxylation at the methylene carbon results in formation of α-methylene hydroxy NNK, which spontaneously produces methane diazohydroxide. This compound is converted to methane diazonium ion (Hecht 1998). Metabolites give rise to various DNA adducts, such as O^6-methylguanine (O^6-meG), N7-methylguanine (N^7-meG) and N^3-methyladenine (N^3-meA) (Hecht 1999). Other sources of N-nitroso compounds include

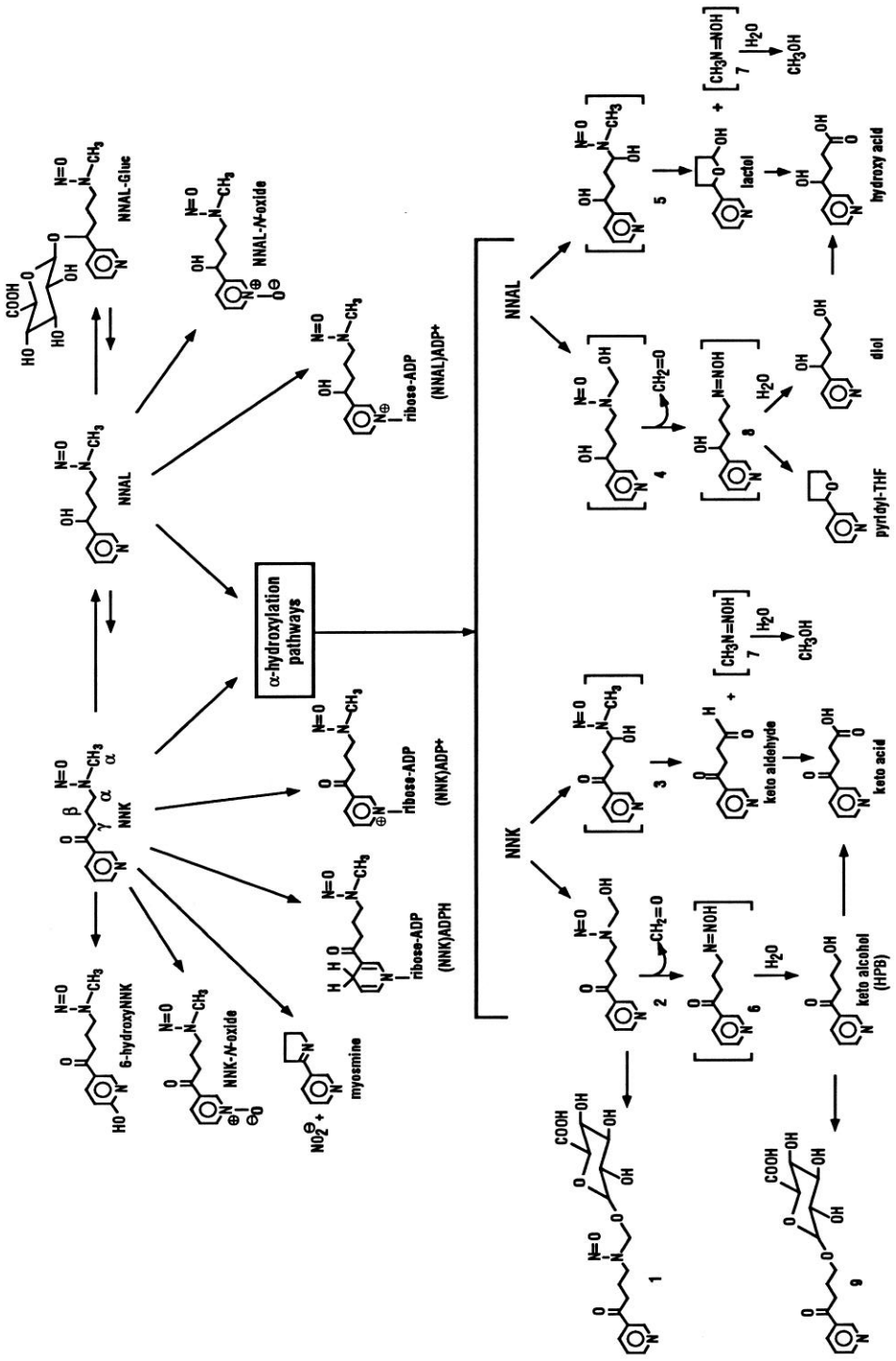

Illustration 3: Metabolism of NNK and NNAL (Hecht 1998)

myosmine in fruits, vegetables and milk as well as a large variety of N-nitroso compounds in cured foods and fish (Tyroller et al., 2002; Goldman and Shields 2003).

In addition to these incidental exposures, many prescription drugs also are functional alkylators. Antibiotics (streptozotocin) and chemotherapeutics (carmustine, lomustine, and temozolomide) are strong alkylators as they do not need to be activated by CYP450s (Drabløs et al., 2004). Bifunctional alkylators, such as carmustine, exert a complex effect on DNA as both active sites can react with DNA or other proteins, forming inter- and intra-strand crosslinks as well as DNA-protein crosslinks.

CARCINOGENICITY OF O^6-ALKYLGUANINE ADDUCTS

The O^6-meG adduct is considered one of the most mutagenic DNA lesions, and its carcinogenic effects are well documented (Margison et al., 2002). This adduct presumably alters the hydrogen bonding of the adducted guanine so that, during replication, miscoding occurs, and a thymidine instead of a cytosine is incorporated opposite the O^6-meG base. Mismatch DNA repair then recognizes the O^6-meG:T mispair, initiates long-patch excision and the gap is filled by polymerase δ or ε, reincorporating a thymidine (Margison et al., 2002). This leads to "futile cycling", which often induces recombinational repair that can lead to the formation of chromosome aberrations (CA), sister chromatid exchanges, translocations and deletions If, on the other hand, DNA replication occurs, an adenine is incorporated across from the thymidine during the second cell cycle, giving rise to a G→A point transition mutation (Margison et al., 2002). It was paradigmatically believed that methylated bases, notably

O^6-meG, are mainly a source of point mutations (Loveless 1969; Natarajan et al., 1984; Margison et al., 2002).

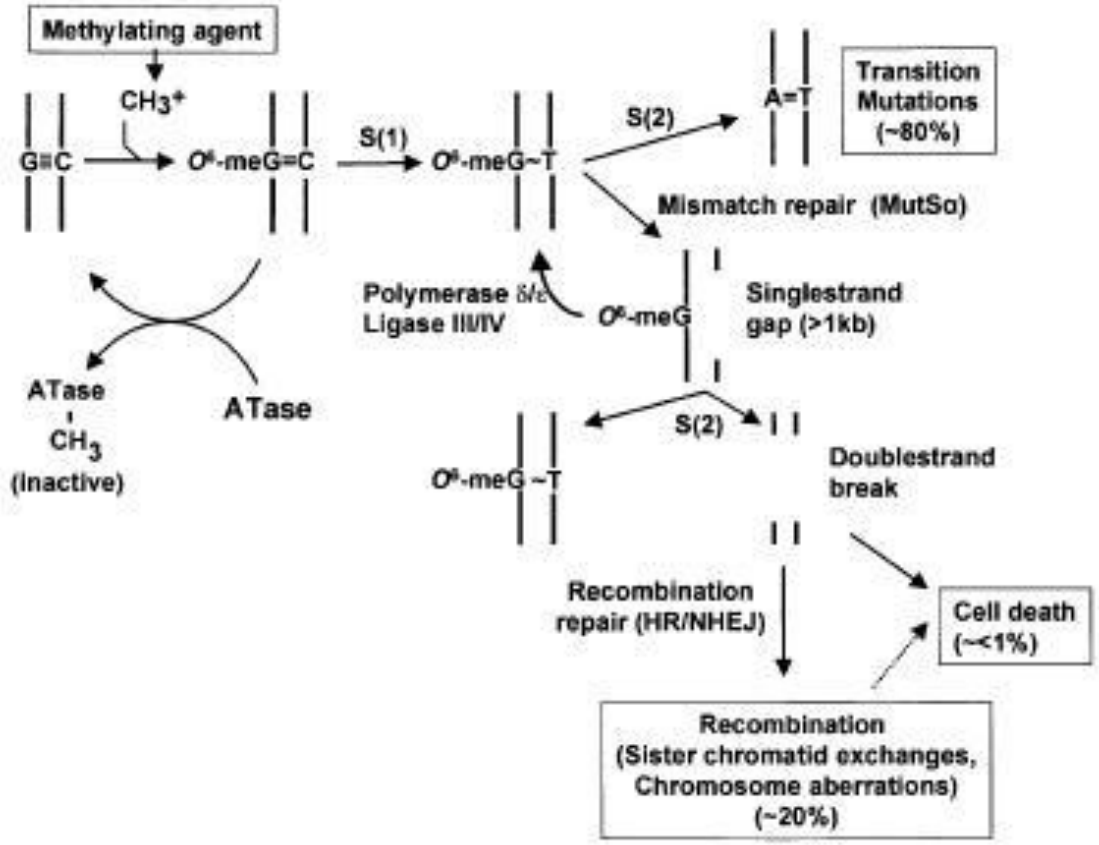

Illustration 4: DNA damage endpoints resulting from the DNA adduct O^6-methylguanine (Margison et al., 2002)

With the availability of repair-deficient mutants and advent of gene manipulation technology, this concept has been reassessed and modified. It is now clear that persistence of O^6-meG, due to poor repair by MGMT, not only leads to point mutations but also to CA (Kaina 2004). Using isogenic Chinese hamster cell lines either not

expressing MGMT (Mex^-), expressing MGMT (Mex^+) or exhibiting the tolerance phenotype (Mex^- methylation resistant), Kaina et al., (1997) reported that if O^6-meG is not repaired by MGMT, the lesion not only generates gene mutations, but also CA. When all three cell lines (Mex^-, Mex^+, and Mex^- methylation resistant) were compared as to their clastogenic response to the O^6-MeG producing agent MNNG, Mex^- cells were more sensitive than Mex^+ cells to MNNG-induced CA, clearly indicating that O^6-MeG is an important clastogenic lesion (Kaina et al., 1997; Kaina 2004).

DIRECT REVERSAL REPAIR

Direct reversal repair (DRR) is the primary pathway responsible for repairing alkylation damage and is considered the simplest of all repair mechanisms because the pathway does not require DNA nicking, polymerization or ligation. In humans, the *O^6-Methylguanine-DNA-Methyltransferase* (*MGMT*) gene is the only member of this family. The ~350kb gene on chromosome 10q26 codes for a 207 amino acid protein with a molecular weight of 22kDa (reviewed by Mishina et al., 2006). The active site is simple compared to other DNA repair proteins, consisting only of a reactive cysteine residue at position 145. The protein is responsible for repairing alkylation damage at the 0^6 position of guanine in B-DNA, but can repair O^4-methylthymine as well, albeit at a 35-fold lower rate (Paalman et al., 1997). The protein is capable of recognition and repair of not only O^6-methylguanine, but also bulkier adducts such as O^6-[4-oxo-4-(3-pyridyl)butyl]guanine (O^6-pobG) resulting from NNK metabolism, ethyl-, n-propyl-, n-butyl-, 2-chloroethyl-, 2-hydroxyethyl-, iso-propyl- and iso-butyl adducts resulting from various endogenous and exogenous n-nitroso compounds (Pegg 2000).

The NMR protein structure, both alone and bound to an O^6-methylguanine base, have shed light on the repair mechanisms of this protein. The N-terminal portion of the protein (AA 1-85) contains a bound Zinc(II) ion coordinated by Cys5, Cys24, His29 and His85 in a tetrahedral formation (Daniels et al., 2000). The ion preserves structural integrity and serves to lower the pKa of the Cys145, increasing the reactivity of the protein (Guengerich et al., 2003, Rasimas et al., 2003a). The C-terminal domain (AA 86-207) contains the conserved active site IPCHRV and the helix-turn-helix DNA binding motif with the DNA damage recognition Tyr114 residue (Wintjens and Rooman, 1996; (Daniels et al., 2000). Hydrogen bonding among Asn137, Val139, Ile143 and Cys 145 confers stability to the protein and serve to further lower the pKa of the reactive cysteine (Daniels et al., 2000). The current repair hypothesis involves flipping the adducted base out of the DNA helix and into the active site pocket in the protein. Protein-DNA interactions are primarily through the DNA phosphate backbone within the minor groove (Daniels et al., 2004; Duguid et al., 2005). Tyr114 disrupts the normal B-DNA helix by interacting with the 3' phosphate at the flipped base site (Daniels et al., 2004). The adducted base is flipped into the active site while Arg128 invades the helical stack to replace the missing base pair. In addition, Tyr114 hydrogen bonds to the N3 position of the adducted guanine, serving to select this base over others for repair (Daniels et al., 2004). Additional hydrogen bonding by Val148 and Cys145 to the N^2 position and Ser159 to the O^6 position stabilizes the repair transitional state. The mechanism of adduct transfer is not fully understood but it is thought to involve formation of a thiolate derivative from Cys145 acting as a nucleophile to displace the alkyl group and transfer it to the active cysteine (Pegg 2000).

Because the alkyl group becomes covalently bound to Cys145 after repair, a single MGMT protein molecule is only capable of repairing one adduct. Disruption of a salt bridge between Asn137 and Met134 destabilizes the protein and signals for ubiquitination and subsequent degradation by protease V8 (Oh et al., 1996, Daniels et al., 2000, Rasimas et al., 2003b).

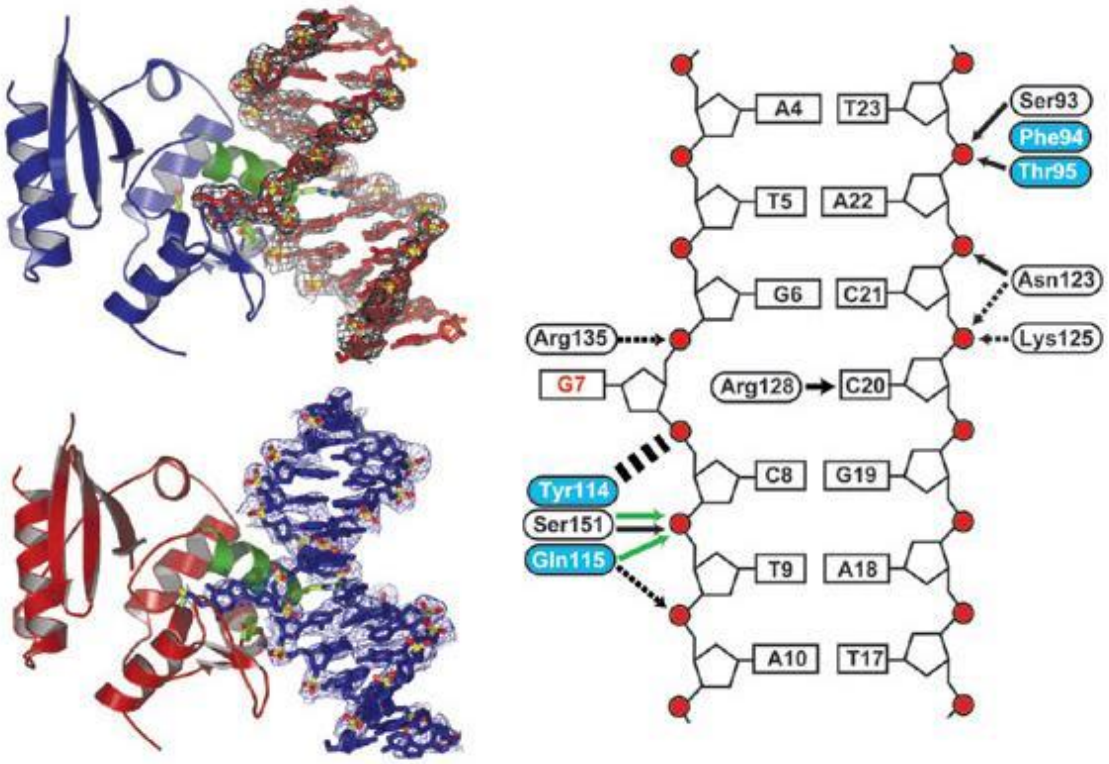

Illustration 5: Nucleotide flipping of the MGMT protein. Relevant amino acids are noted; dotted lines are hydrogen bonds (Daniels et al., 2004)

There is also evidence that the adducted MGMT protein acts as a negative regulator for the estrogen receptor (Teo et al., 2001). Treatment of ER+/MGMT+ cells with O^6-benzylguanine (O^6-beG) inhibits estrogen-mediated cell growth. Protein

alkylation results in a protein conformational change, exposing residues 99-107 containing the LXXLL motif, which is found in a majority of steroid coactivators to facilitate binding to nuclear hormone receptors (Oh et al., 1996, Teo et al., 2001).

MGMT Activity and Cancer Development

The contribution of low MGMT activity to the development of lung cancer, especially adenocarcinoma, has been investigated by several laboratories. Promoter hypermethylation of the *MGMT* gene, which decreases MGMT expression, was associated with poor prognosis in smoking lung cancer patients (Hayashi et al., 2002; Brabender et al., 2003). Promoter hypermethylation was also correlated with tumor grade (Leng et al., 2011). In addition, promoter hypermethylation was associated with a shift in the mutational spectrum of the *TP53* gene, leading to more G → A transitions in non small-cell lung cancer (NSCLC) tumor tissue (Wolf et al., 2001). Loss of the MGMT protein within tumor cells was also correlated with nodal metastasis as well as p53 overexpression in adenocarcinomas (Myong 2010).

SNPs and cancer risk

Single nucleotide polymorphisms (SNPs) in DNA repair genes can plausibly account for interindividual variability in DNA repair capacity. SNPs occurring in the coding region of a gene (cSNPs) have the potential to alter the amino acid composition and could alter the structure and/or function of the resulting protein. As such, cSNPs in DNA repair genes could potentially affect the level of genetic damage resulting from exposure to mutagens and carcinogens. Coding SNPs in the nucleotide excision repair

genes *XPA* and *XPD* have been shown to increase lung cancer risk in smokers (Benhamou and Sarasin 2002; Zhou et al., 2002; Wu et al., 2003).

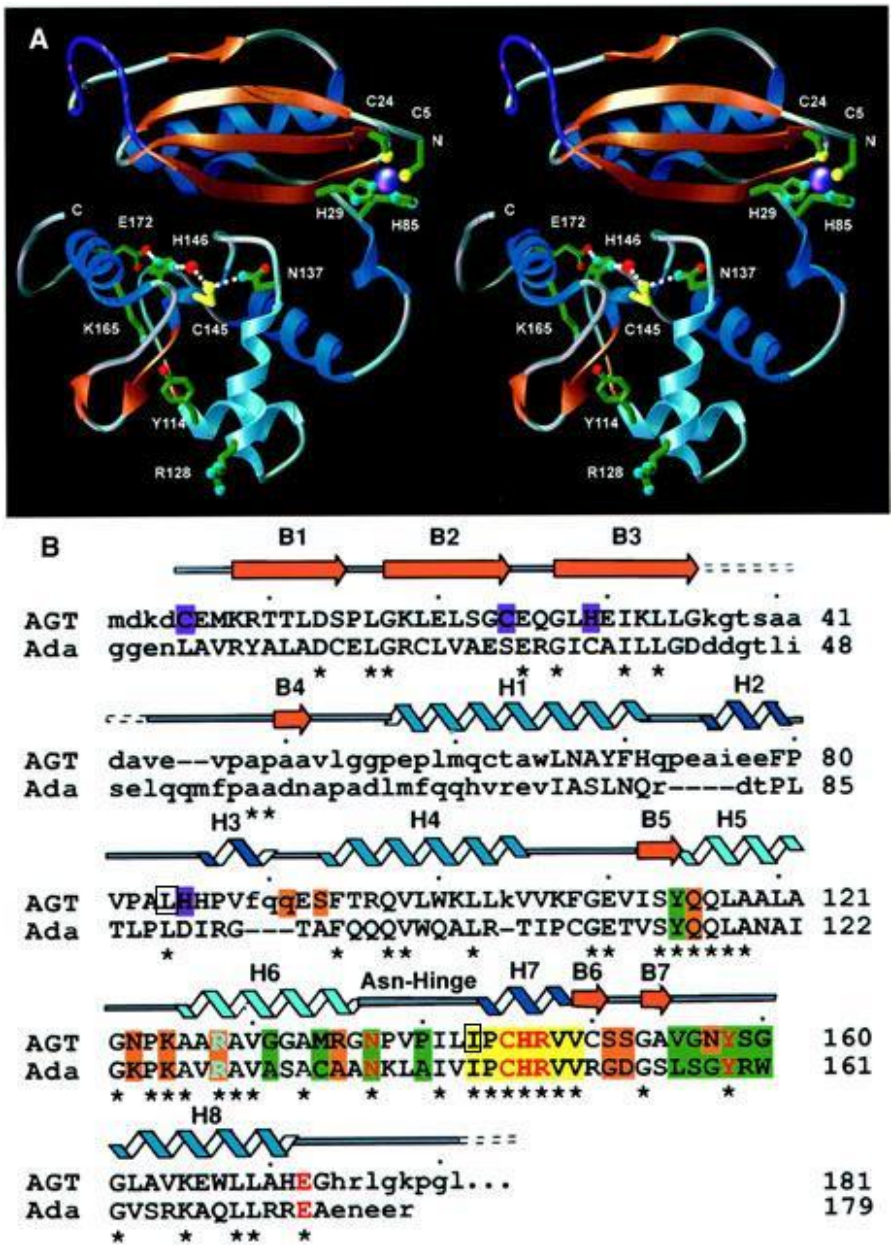

Illustration 6: Crystal structure and protein sequence of MGMT, empty boxes indicate position of cSNPs of interest (Daniels et al., 2000)

Our laboratory has recently reported that the 312N polymorphism in the *XPD* gene significantly increases the frequency of chromosome aberrations (CA) in circulating PBLs of smokers (Affatato et al., 2004). Studies investigating the effect of cSNPs in the base excision repair genes *OGG1* and *XRCC1* have shown that cSNPs in these genes increase the risk for lung and for head and neck cancers in certain subgroups of individuals (Sugimura et al., 1999; Sturgis et al., 1999; David-Beabes and London 2001; Olshan et al., 2001). In addition to the well studied cSNPs, other SNPs occur in promoter regions, enhancer regions, or other regulatory elements of many genes. SNPs in these non-coding regions can result in a change in basal expression levels or could alter transcriptional or translational response to genotoxic agents. For example Gazzoli (2003) demonstrated that two SNPs in the promoter of the *MSH6* gene altered two SP-1 binding sites and resulted in a 50% reduction in promoter activity as well as sensitization to transcriptional silencing via DNA methylation (Gazzoli and Kolodner 2003). Several cSNPs in the *MGMT* gene have been reported, but only the linked variants I143V and K178R polymorphisms have been investigated as risk modifiers for lung cancer. The I143V cSNP was reported to be associated with a two-fold increase in risk for the development of this disease (Kaur et al., 2000; Cohet et al., 2004). Although these studies have shown associations between this cSNP in *MGMT* gene and lung cancer risk, their functional significance remains to be systematically elucidated.

MGMT EXPRESSION VARIABILITY AND CHEMOTHERAPEUTIC RESPONSE

In addition to their potential role as cancer risk modifiers, SNPs in the *MGMT* gene could also play a significant role in the response of patients treated with therapeutic alkylating agents. As such, these SNPs may have the potential to influence sensitivity to alkylation chemotherapy. Support for this hypothesis stems from studies indicating that reduced MGMT activity is associated with improved response to treatment with alkylating agents, and that tumor cells expressing little or no MGMT are very sensitive to such treatments (Mineura et al., 1995; Anda et al., 2003; Balaña et al., 2003; Ma et al., 2003; Madhusudan and Middleton 2005; McCormack et al., 2011). Additional support stems from studies showing that a low tumor MGMT level is a predictive marker of survival in patients with malignancies treated with the chloroethylating agent BCNU, which results in the formation of the O^6-cholorethylguanine adduct that is also repaired by MGMT (Jaeckle et al., 1998). Low MGMT levels were associated with improved response to alkylation chemotherapy in glioma patients, and with prolonged survival of treated patients (Anda et al., 2003; Balaña et al., 2003). Only a few studies have addressed the relationship between *MGMT* polymorphisms and response to alkylation chemotherapy. In one study, the variant MGMT protein corresponding to the G160R polymorphism was less sensitive to inactivation by O^6-benzylguanine (BG). BG is a potent specific inhibitor of MGMT that has been used to enhance sensitivity to chemotherapeutic alkylating agents (Wu et al., 1999). In another small study of 52 melanoma patients treated with dacarbazine, Ma et al., (2003) reported no significant association between *MGMT* polymorphisms and clinical response to treatment. Recently, however, in a large study of colorectal cancer patients, a significant association between the MGMT 84F variant and better prognosis in patients receiving chemotherapy was

observed (Moreno et al., 2006). Based on the above observations, we hypothesize that SNPs in the promoter and coding regions of *MGMT* could alter the expression levels and/or functions of the resulting protein.

TRANSCRIPTIONAL REGULATION OF *MGMT*

The *MGMT* promoter was first characterized by Harris et al., (1991). The region consists of 1197 nucleotides, is G-C rich and contains no TATA or CAAT boxes. Two AP1, two AP2, two glucocorticoid response elements (GRE), one heat shock promoter element (HSP), and multiple SP1 sites were identified, along with the putative transcriptional start site (Harris et al., 1991). In addition, an enhancer element is also present, as denoted by CCGCCC tandem repeats, located within the untranslated exon 1.

Transcriptional regulation occurs through multiple pathways. Binding of NF-κB, at -766 and -90, increases mRNA and protein levels independent of methylation status, leading to resistance to alkylating agents (Lavon et al., 2007). Although the biological significance is still unclear, glucocorticoid receptors (GRs) can bind to the GREs present in the promoter region and activate transcription (Biswas et al., 1999). Induction of the Protein Kinase C (PKC) pathway increased mRNA levels up to 5-fold, most likely through activation of AP-1 (Boldogh et al., 1998). Overexpression of p53 can downregulate promoter activity, however this has not been shown in a biologically relevant system in humans (Harris et al., 1996). In addition, *MGMT* transcription can be regulated by CPB/p300 histone acetylation (Bhakat and Mitra 2000). Acetylation of histones H3 and H4 is associated with loose chromatin structure and transcriptional activation of *MGMT*, specifically within a region of -310 to +10 relative to the transcription start site (Danam et al., 2005). Hypermethylation of CpG islands from -245

to +225 is associated with protein loss in cancer cell lines, specifically -249 to -103 and +107 to +196 (Qian et al., 1995, Qian and Brent 1997).

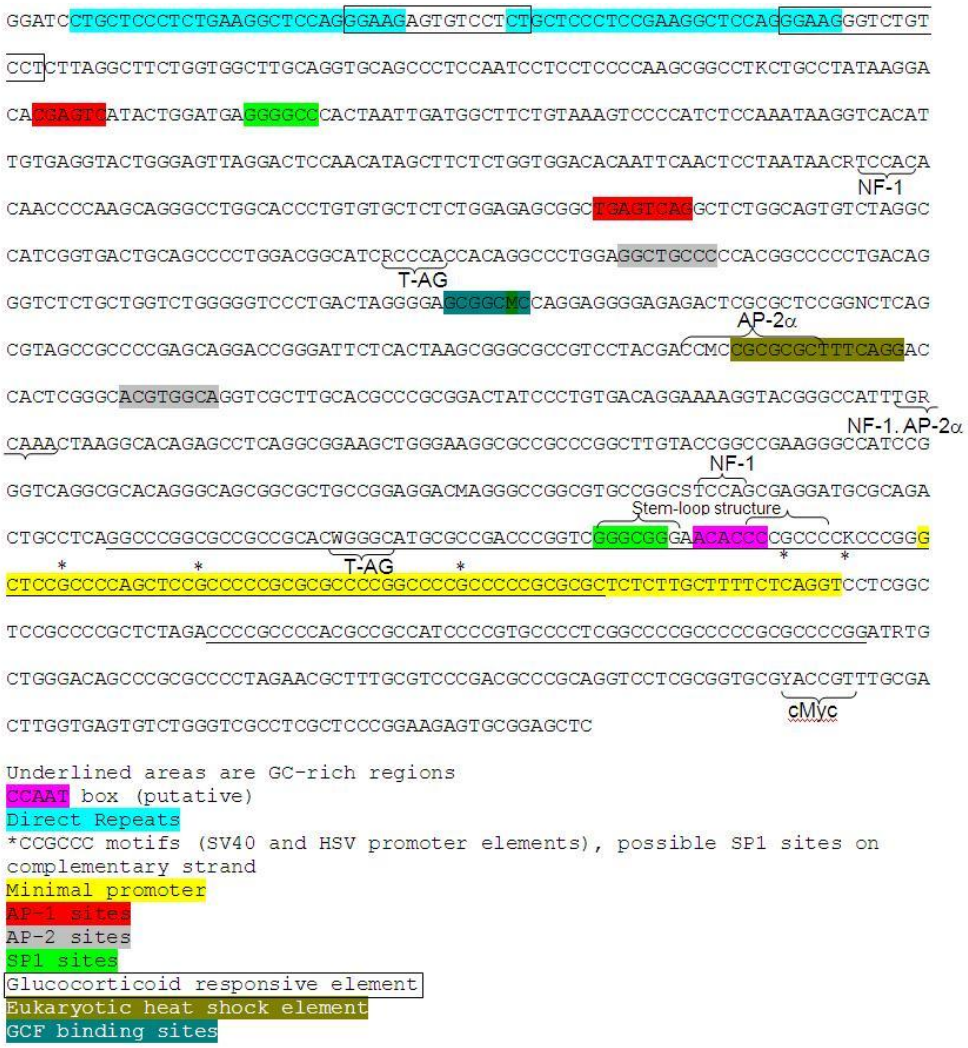

Illustration 7: Characterization of the *MGMT* promoter (Harris et al., 1991)

The Methyl-CpG Binding Proteins MeCP2 and MBD1 bind to the hypermethylated areas and repress transcription. This leads to increased sensitivity to alkylating agents due to the inability to repair the O^6-meG adduct (Danam et al., 2001; Hegi et al., 2005). The

combination of histone acetylation and methylation status results in tight epigenetic control of *MGMT* mRNA levels; demethylation of *mer*- cells results in an increase in histone acetylation and an increase in mRNA (Danam et al., 2005). This is due to the recruitment of histone deacetylase complexes to hypermethylated DNA (Nan et al., 1998, Ng et al., 2000)

CELLULAR LOCALIZATION OF MGMT

There is limited information on the localization and nuclear retention of the MGMT protein. Studies have indicated that MGMT is a nuclear protein (Ayi et al., 1992; Lee et al., 1992; Brent et al., 1993) and that nuclear localization of MGMT is a two-step process (Lim and Li, 1996). The first step involves the translocation of the protein from the cytosol to the nucleus, and the second involves the nuclear retention of MGMT, through binding with DNA, to prevent its export from the nucleus back to the cytoplasm (Lim and Li., 1996). After introducing mutations at the lysine and arginine residues within the putative nuclear localization sequences (KLLKVVK 101-107 and PKAAR 124-128), Lim and Li (1996) reported that the PKAAR sequence was primarily responsible for nuclear targeting, although the sequence alone had no nuclear targeting properties. The K125L mutation within the PKAAR sequence is 100% repair-capable in cell extract assays, however the MGMT protein remained predominantly in the cytosol, presumably because of impaired binding of the mutant protein to nuclear DNA. This lead to the inability to repair O^6-meG adducts (Lim and Li, 1996). Altered DNA binding was also observed with other MGMT mutations, including R128L, K101L and K104L, and was attributed to possible gross structural alteration of the mutant proteins (Lim and

Li, 1996). As the O^6-meG lesion is preferentially repaired at trascriptionally active sites (Thomale et al., 1994), the MGMT protein is bound to DNA at the highest concentration in trascriptionally active DNA (Ali et al., 1998). MGMT is present at the highest concentration during S and G2/M phases, and in lower concentrations during G0 or G1 (Ali et al., 1998). Cell staining was characterized by low level speckling within the nucleus at G0 and G1, followed by intense nuclear staining during the S phase and diffuse staining at G2/M (Ali et al., 1998).

SMOKERS AS A MODEL POPULATION FOR EXPOSURE TO ALKYLATING AGENTS

Tobacco smoking has long been known to be associated with tumors in several organs including the oral cavity, larynx, esophagus, lung, pancreas, kidney and bladder (IARC 1986). Despite this, respiratory cancer is expected to be the leading cause of death among both males and females in 2011, and will be the 2nd most common tumor diagnosed among both males (after prostate cancer) and females (after breast cancer) (ACS 2011). Smokers are exposed to a variety of known carcinogens including the alkylating tobacco-specific nitrosamines such as NNK (Spiegelhalder and Bartsch 1996). Exposure to tobacco smoke, therefore, provides an excellent human model for the proposed studies because: (a) the repair of the DNA damage produced by alkylating nitrosamines in tobacco smoke involves the direct reversal DNA repair pathway in which MGMT plays a major role; (b) millions are exposed to tobacco smoke; (c) smokers are easy to identify; and (d) we have found that most smokers are willing to participate in studies such as those we propose.

CHROMOSOME ABERRATIONS (CA) AS A BIOMARKER OF EFFECT

Cancer is the result of the accumulation of multiple genetic changes. Each alteration, whether an initiating or a progression-associated event, may be mediated through a gross chromosomal change and therefore has the potential to be cytogenetically detected (Solomon et al., 1991). Chromosome aberrations and sister chromatid exchanges in PBLs have been shown to detect susceptibility to DNA damage, and are used as internal indicators to document genotoxic effects in exposed populations. Chromosome aberrations are produced by errors in DNA repair in unreplicated DNA, while chromatid type aberrations are produced by errors in DNA repair in cells that have already replicated at the time of exposure to the clastogenic agent.

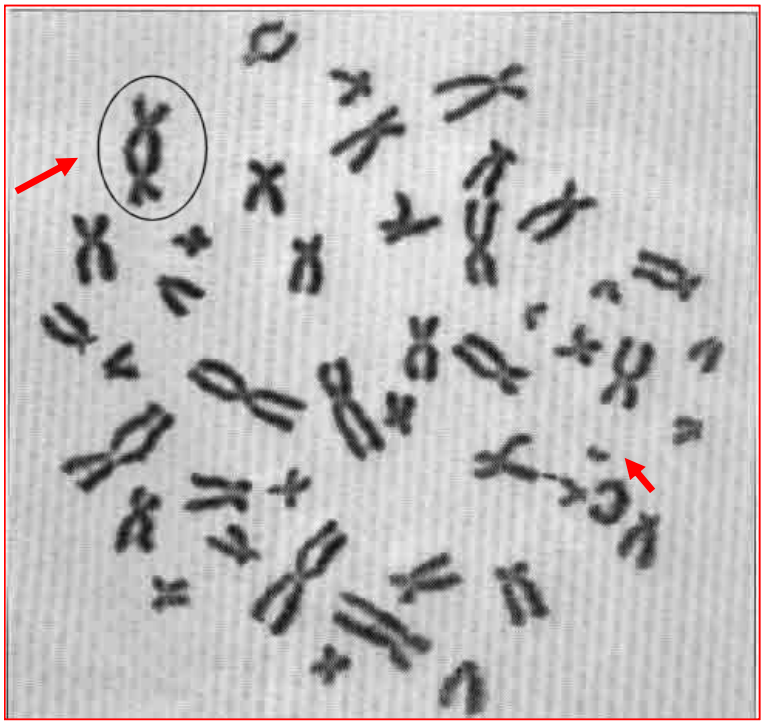

Illustration 8: Example of chromatid and chromosome type aberrations (courtesy of Randa El-Zein, MD Anderson Cancer Center, Houston Texas)

An increased frequency of aberrations has generally been considered indicative of increased cancer risk for those exposed to damage-inducing agents (Hsu 1989). Bonassi et al., and Hagmar et al., in separate independent prospective studies reported a significant increase in the mortality ratio for all cancers in subjects who had previously shown increased levels of chromosomal aberrations in their lymphocytes.

Importantly, the data from both these studies, indicated that the frequency of chromosome instability in peripheral blood lymphocytes is a relevant biomarker for cancer risk in humans, reflecting early biological effects of genotoxic carcinogens and individual cancer susceptibility (Hagmar et al., 1998, Bonassi et al., 1995, 2000). There is considerable interindividual variation in sensitivity to environmental mutagens. Within the general population, this variation may be attributed to varying degrees of DNA maintenance capability (Oesch et al., 1987). One way to detect this variation is through the mutagen sensitivity assay developed by Hsu et al. Mutagen sensitivity is an intrinsic biomarker, which has been shown to determine a cancer susceptible phenotype (Spitz and Bondy 1993).

The mutagen-sensitivity assay is a well-validated method that is used to detect potential variations in susceptibility to the effects of genotoxic agents. This assay is based on the quantitation of mutagen-induced CA in cultured peripheral blood lymphocytes obtained from study subjects. The mutagen-sensitivity assay reflects the individual's sensitivity to the mutagen used in the assay, as well as the DNA repair capacity in that individual (Hsu et al., 1989). Population studies, using phenotypic DNA repair assays, have demonstrated interindividual variability in the ability to repair DNA (Oesch et al., 1987; Takano et al., 1991) and have documented associations between reduced DNA

repair and susceptibility to cancers at several sites (Spitz and Bondy 1993; Wei et al., 1994, 1996). Several investigators have reported that individuals with lung cancers show higher mutagen-sensitivity within surrogate cells such as PBLs compared to control subjects that do not have cancer (Spitz et al., 1995; Wu et al., 1998, 2000; Li et al., 2001; Schmezer et al., 2001; Shen et al., 2003; Zheng et al., 2003). Other investigators have utilized this assay for other tobacco-related cancers and have found associations between CA levels and head/neck cancer (Schantz et al., 1989, 1997, 2000; Cloos et al., 1996; Wu et al., 1998; Xiong et al., 2007) and bladder cancer (Aben et al., 2000). In addition, many laboratories, including ours, are using the mutagen-sensitivity assay to examine the role that polymorphisms in susceptibility genes might play in the cellular response to environmental toxicants (Abdel-Rahman et al., 2001, 2003, 2005; Wang et al., 2003; Affatato et al., 2004; Hill et al., 2005; Iarmarcovai et al., 2006; Laczmanska et al., 2006, 2007).

THE X-LINKED HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE (*HPRT*) ASSAY AS A BIOMARKER OF EFFECT

Evaluation of the rates of somatic cell mutations have been used to monitor genetic damage caused by either endogenous or environmental factors (Cole and Skopek 1994). Furthermore, mutations at reporter genes provide realistic markers for genetic toxicology in that mutagen metabolism and delivery to genetic targets are assessed and individual differences in mutagen susceptibility may be determined (Albertini et al., 1990). The X-linked hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) gene is located on chromosome Xq (Henderson 1969) and is one of the most well developed reporter systems. The *HPRT* gene is constitutively expressed and functions in the purine

salvage pathway where it phosphorylates its normal substrate, as well as cytotoxic purine analogues. Cells lacking this enzyme are resistant to these analogues (Albertini 1985, Stout and Caskey 1985). Studies of mutations at the *HPRT* gene have provided insights into several aspects of somatic mutations *in vivo*, including molecular mechanisms of mutagenesis, the relationship between DNA damage and mutation, and individual susceptibility factors such as DNA repair capacity (Fuscoe et al., 1992, Rainville 1995). In addition to serving as an exposure indicator, the *HPRT* assay has also been hypothesized to be an effect indicator where mutations may arise as a result of chronic exposures and these elevated mutant frequencies could be stable over many years (Perera et al., 1994). Elevated levels of mutant frequencies at the *HPRT* locus has been described in cancer prone patients with ataxia telangiectasia or xeroderma pigmentosum (Cole et al., 1992) in whom defects in DNA repair have been implicated.

LUCIFERASE ASSAY SYSTEM

In 1979, McElroy and Deluca purified and characterized the bioluminescent firefly luciferase gene for use in basic science research (McElroy and Deluca 1983). Cloning and inserting the gene into a plasmid to be used in reporter expression studies gave multiple advantages over other expression vectors such as β-galactosidase (β-gal) and chloramphenicol acetyl transferase (CAT). The luciferase assay is quicker and simpler, requiring a fifth of the amount of time and only a tenth of the number of cells as compared to the CAT assay (Williams et al., 1989). Quantitating luciferase expression is a simple single step process using a luminometer, as opposed to the CAT assay which is a multi-step analysis process, requiring incubating extracts with radioactive chloramphenicol, purifying the product by thin-layer chromatography and quantitation

using a scintillation counter. In addition, due to the increased sensitivity of the luciferase assay, data can still be collected for low activity promoters such as interleukin-2 (Williams et al., 1989).

For gene expression studies, the promoter sequence of interest is cloned into a plasmid 5' of the luciferase gene. The resulting expression vector is transiently transfected into actively growing cells and the cell extract is assayed for protein activity after 24-48 hours. This is an ideal system to examine promoter activity under a variety of experimental conditions because the protein is not present in mammalian cells, is non-toxic, and simple to assay.

RESEARCH OBJECTIVES

The objective of this research was to elucidate the biological and functional consequences of single nucleotide polymorphisms within the direct reversal repair gene *MGMT*. SNPs in this DNA repair gene could alter the transcriptional activity, structure and function thus altering DNA repair proficiency. Consequently, these SNPs could significantly influence the level of genetic damage, which is an early critical factor in the cascade of events leading to cancer. Previous studies indicate an increased risk for various types of cancers associated with carcinogen exposure and inheritance of one or more SNPs. However, little was known regarding the functional consequences of these SNPs or their effect on markers upstream from cancer development such as gene mutations or DNA damage. We used two biologically relevant endpoints in an exposed population to determine the correlation between SNPs in *MGMT* and genetic damage. In addition, we used the luciferase reporter assay to determine effects of SNPs in the promoter/enhancer region of *MGMT* on promoter activity.

The well validated mutagen-sensitivity assay was used to test the hypothesis that SNPs in the MGMT protein alter protein function, leading to an increase in unrepaired O^6-meG and thus chromosome aberrations. Primary lymphocytes from a control population of non-smokers and an exposed population of smokers were treated with the tobacco-specific nitrosamine NNK, which causes O^6-meG adducts. Cells were harvested pre-exposure, 1 hour after exposure and 24 hours after exposure and chromosome aberrations were analyzed according to standard procedure (ISCN, 1985). Data were correlated with demographic factors such as age, gender, ethnicity and smoking status, as well as *MGMT* genotype. These experiments will provide some insight into the potential effects of *MGMT* variants on macrolesion DNA damage in an exposed and non-exposed population.

In addition to chromosome breaks, unrepaired O^6-meG adducts also result in mutations. We used the *Hypoxanthine Phosphoribosyl Transferase (HPRT)* T-cell mutation assay to determine alterations in mutation frequency or mutation spectrum in a population of smokers and non-smokers re-recruited from aim 1 based on *MGMT* genotype status. Primary lymphocytes were harvested and plated in either cloning efficiency (CE) medium or CE medium containing 6-thioguanine (6-TG) for *HPRT* mutant selection. Selected *HPRT* mutation positive clones were further propagated and the RNA was reverse transcribed and sent for sequencing to determine the spectra of inactivating mutations. Data were correlated with demographic factors and *MGMT* genotype. These studies will delineate effects of *MGMT* SNPs on microlesion DNA damage as well as any alterations in specific mutagenic events.

We used the Dual-Luciferase Assay system to investigate the role of SNPs in the promoter/enhancer region of the *MGMT* gene on transcriptional activity pre- and post-exposure. Normal Human Bronchial Epithelial cells (NHBE) were co-transfected with the

control *Renilla* plasmid under the control of the strong ubiquitous SV40 promoter and the experimental *Luciferase* plasmid under the control of the SV40 promoter, the wild-type referent *MGMT* minimal promoter/enhancer or a variant containing the C → T SNP within the enhancer region at position 1099. Half the cells were exposed to mainstream tobacco smoke to determine any inducibility of the *MGMT* gene under exposure conditions. These data will examine functional consequences within the non-coding region of the *MGMT* gene.

Results from these experiments further delineated potential effects of SNPs within the direct reversal repair gene *MGMT*. Scientifically, this work adds to the continually growing knowledge database regarding consequences of genetic variants and genetic damage upstream of disease. From a population health standpoint, this work serves to identify potential risk factors for disease.

Chapter 2: MATERIALS AND METHODS

MATERIALS

Table 1: Materials

Item	Company	Catalog #
10x PCR Buffer	Promega	N/A
10X TBE buffer	Invitrogen	15581-044
6-Thioguanine	Sigma	A4882
Acetic Acid, Glacial	Fisher Scientific	A38-212
alamarBlue®	Trek Diagnostics	00-100
Ampicillin	Sigma	A9393-5G
Antarctic Phosphatase	New England Biolabs	M0289S
BEGM Bullet Kit (Medium and Supplements)	Lonza	CC-3170
Cell Lysis Buffer	Puregene	158906
Colcimid KaryoMAX	Invitrogen	15212-012
Defined Fetal Bovine Serum	Hyclone	SH30070.03
DMSO	Sigma	472301
DNA Hydration Solution	Puregene	158914
dNTPs	GE Healthcare	28406551
Dual-Luciferase® Reporter Assay System	Promega	E1910

EndoFree Plasmid Maxi Kit	Qiagen	12362
Ethidium Bromide	CLP	5450
FailSafe PCR System	Epicentre	FS99100
*Hind*III Restriction Enzyme	New England Biolabs	R0104S
Histopaque®-1077	Sigma	10771
HL-1 Serum-Free Medium	Cambrex	77201
Human Recombinant IL-2	Fisher Scientific	CB40043
	R&D Systems	202-IL
Human T-Stim with PHA	Fisher Scientific	CB40045
KaryoMAX Giemsa Stain	Invitrogen	10092-013
Kentucky Reference 3R4F Cigarettes	University of Kentucky Tobacco Research Institute	
*Kpn*I Restriction Enzyme	New England Biolabs	R0142S
L-Glutamine	Invitrogen	25030-081
Lipofectamine™ 2000	Invitrogen	11668-019
MEM Non-Essential Amino Acids Solution	Invitrogen	11140-050
MEM Sodium Pyruvate Solution	Invitrogen	11360-070
Methanol	Fisher Scientific	A412-4
$MgCl_2$	Promega	N/A
NNK	NCI Chemical Carcinogen Repository	E0698

Nusieve 3:1 Agarose	Cambrex	50090
Penicillin-Streptomycin	Invitrogen	15140-122
pGL4.10[luc2] Vector	Promega	E6651
Phosphate Buffered Saline	Sigma	P3813
Phytohemagglutinin (PHA) 15 – Reagent grade	Remel	30852701
Phytohemagglutinin (PHA) 16 – Purified	Remel	30852801
Phytohemagglutinin (PHA), M form, liquid	Invitrogen	10576-015
Potassium Chloride	Sigma	P9333
Potassium Phosphate Monobasic	Sigma	P5655
pRL-SV40 Vector	Promega	E2231
Proteinase K	Sigma	P2308
QIAprep Spin Miniprep Kit	Qiagen	27104
QIAquick Gel Extraction kit	Qiagen	28704
QIAquick PCR Purification Kit	Qiagen	28106
Qualified Fetal Bovine Serum	Invitrogen	26140-079
RBC Lysis Solution	Puregene	158902
RETROscript Reverse Transcription kit	Applied Biosystems	1710
Ribonuclease A	Sigma	R5125
RNAqueous RNA Isolation Kit	Applied Biosystems	1912

RPMI Medium 1640	Invitrogen	22400-105
*Sap*I Restriction enzyme	New England Biolabs	R0569S
Sodium Chloride	Sigma	S7653
Sodium dodecyl sulfate, 20% W/V	VWR	IB07064
T4 Ligase	New England Biolabs	M0202S
Taq DNA Polymerase	Promega	N/A
TaqMan® Universal PCR Master Mix	Applied Biosystems	4304437

STUDY SUBJECTS AND COLLECTION OF BLOOD SAMPLES

The subjects for this research were recruited, without regard to age, sex or ethnicity, from the smoking and non-smoking staff and student population attending the University of Texas Medical Branch (UTMB) in Galveston, Texas. These individuals had responded to posted notices throughout campus. Individuals were defined as non-smokers if they had smoked less than 100 cigarettes during their lifetime. Individuals were defined as current smokers if they had smoked at least three cigarettes per day for at least one year prior to enrollment in the study. Ex-smokers reported no smoking within the past 6 months or more. The study protocol was approved by the UTMB Institutional Review Board. All study subjects signed a consent form that described the purpose of the study. They were asked to fill out a questionnaire that provided demographic, occupational, and medical information. Information was collected regarding smoking habits, including number of cigarettes per day, preferred brand, duration of smoking,

former tobacco use, and use of other tobacco products. Exclusion criteria for all volunteers included a recent acute viral or bacterial infection, a major chronic illness such as cancer or an autoimmune disorder, recent blood transfusion, treatment with mutagenic agents such as chemotherapy or radiotherapy, excessive alcohol consumption, and employment involving exposure to potentially mutagenic agents. Because of these criteria, only apparently healthy volunteers were included in the study to control for potential confounders. A blood sample (50-70 ml) was obtained from each volunteer.

DNA ISOLATION

Nine ml of RBC lysis solution was mixed with 1.5 ml of whole blood and incubated at room temperature for 10 min. Tubes were inverted 2-3 times during the incubation. Tubes were centrifuged at 350 x g for 10 minutes and the supernatant was aspirated. After vortexing, cells were incubated overnight at 37°C with 3 ml cell lysis buffer, 100 µl 20% SDS, and 50 µl proteinase K. Forty ml RNAse A was added and tubes were incubated for a further 45 min at 37°C. The solution was put on ice for 5 min, then vortexed with 1.5 ml/tube of saturated NaCl solution. Tubes were centrifuged at 1400xg for 15 minutes. The supernatant was added to new tubes with 100% cold ethanol and inverted to precipitate the DNA. Tubes were centrifuged at 1000xg for 5 minutes and DNA was washed with 3 mL 70% ethanol. DNA was precipitated by centrifugation at 1000xg for 3 minutes. The ethanol was poured off and the DNA allowed to air dry for 10-15 minutes to evaporate any remaining ethanol. The DNA was resuspended in 250µl of DNA Hydration solution and incubated at 37°C overnight. DNA was stored at -80°C. Two DNA isolation procedures were performed for each subject.

ANALYSIS OF POLYMORPHISMS IN *MGMT*

We used our recently developed PCR-RFLP and TaqMan® based assays for determining the *MGMT* L84F polymorphism and the I143V polymorphism, respectively (Hill et al., 2005, 2007). The C1099T SNP was genotyped by direct sequencing.

CTT → TTT Leu→ Phe at codon 84 (L84F, rs12917)

For the analysis of the L84F polymorphism, two primers, 5'-TTCTGCTGCACAGCTAGTTGAG -3' (sense) and 5'-GCCAAAACTAACACAAGTGTTGG -3' (antisense), were used to generate a 499 base pair amplicon. The 50 µl PCR reaction mixture for the reaction consisted of ~50 ng of genomic DNA, 200 µM dNTPs, 1x-PCR buffer solution, 1.0 mM $MgCl_2$, 5 pmol of each primer, and 1 U of Taq DNA polymerase (Promega, Madison, WI). The PCR conditions consisted of an initial melting step of 94°C for 5 min, followed by 30 cycles of melting at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec. A final extension step at 72°C for 5 min terminated the process.

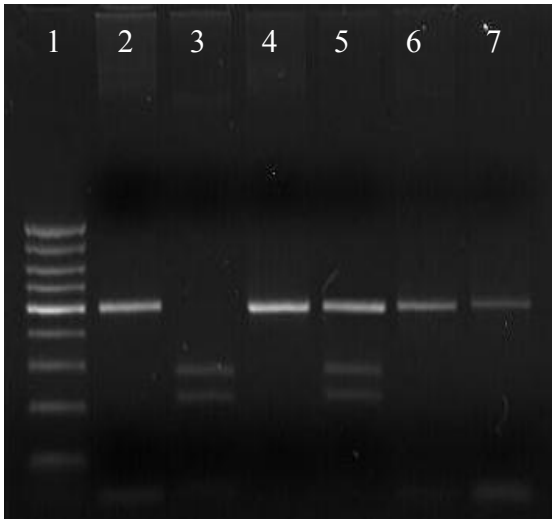

Figure 1: Representative gel demonstrating a homozygous wild type (lane 2), heterozygous (lane 5) and homozygous variant (lane 3) individual for the L84F SNP. This SNP introduced a SapI cut site, which was used to distinguish between the two alleles.

Five µl of the PCR product was digested at 37°C overnight with 2 U of *Sap*I (NEB, Ipswich, MA) in a total volume of 25 µl. The digested product was run on a 1% agarose gel at 85 V for 1 hr. The genotypes were identified according to the banding pattern observed. The reference (wild-type) allele is recognized by two bands at 283 bp and 216 bp. The variant 84F allele is identified by the absence of the *Sap*I cutting site. For quality control, representative samples of both the reference and the variant alleles were confirmed by direct sequencing.

ATC → GTC Ile→ Val at codon 143 (I143V, rs17406533)

For the analysis of the I143V polymorphism, using the Taqman®-based assay (Hill et al., 2005), fluorescent probes were designed to anneal to the polymorphic site, depending on the sequence, and labeled with either the FAM or VIC fluorophore and an appropriate quencher. The probe for the reference sequence was 5'-CATCCTCATCCCGTGC-3', where the underlined base anneals with the reference nucleotide. The probe for the variant sequence was 5'- CATCCTCGTCCCGTG-3', where the underlined base anneals with the variant nucleotide. The forward PCR primer used was 5'-CCAAAGACCTCGTTGTCCAGAT-3' and the reverse primer used was 5'-CGCTGCTGCAGACCACTCT-3'. The PCR reaction consisted of TaqMan® universal master mix, template DNA, and target assay mix in a total reaction volume of 50µl. Thermal cycling was carried out on an ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) in our Molecular Genomics Core facility under factory defaults (50°C, 2 min; 95°C, 10 min; and 40 cycles at 95°C for 15 sec and 60°C for 1 min). Designation of referent and polymorphic forms was determined by the FAM to VIC ratio. For quality control, representative samples of both the referent and the variant alleles were confirmed by direct sequencing.

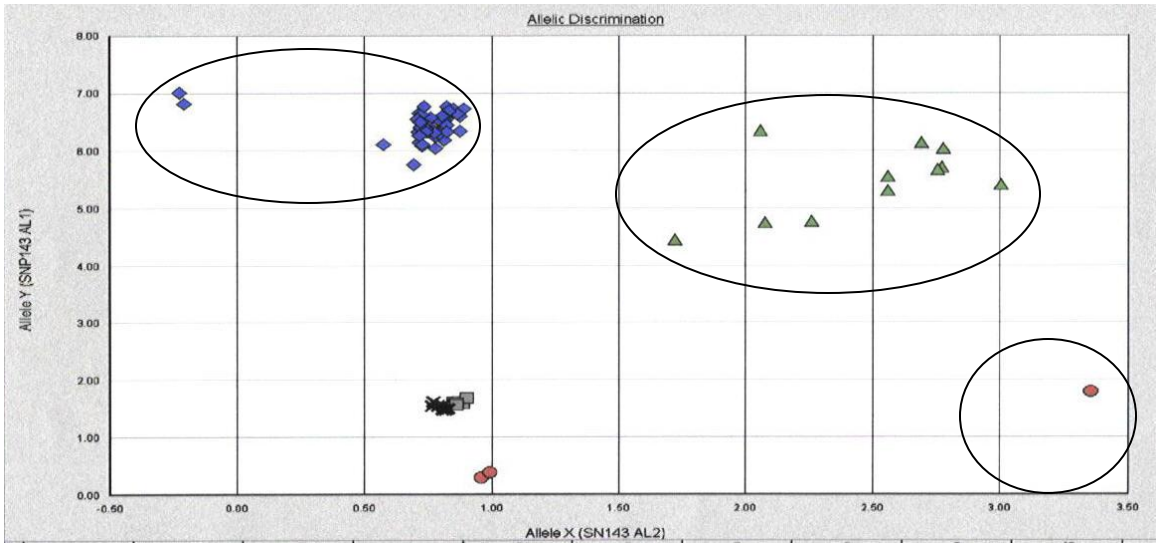

Figure 2: Representative output for the TaqMan assay for genotyping of the I143V polymorphism. Homozygous wild type is denoted by the blue diamonds, heterozygous is denoted by the green triangles and homozygous variant is denoted by the red circles.

C → T at position 1099 (rs 16906252, reference sequence X61657)

PCR and direct sequencing was used to determine the genotype at the 1099 position within the promoter/enhancer region of *MGMT*. The 5' primer sequence was located within exon 1, with the sequence 5'ACAGCCCGCGCCCCTAGAACG-3'. The 3' primer was located within intron 1, with the sequence 5'-CGGCGAAGTGAGGGCGCCTGC-3'. The exonic primer resulted in more reliable amplification compared to similar primers farther upstream of the SNP. The FailSafe™ PCR System from Epicentre Biotechnologies (Madison, WI) was used to amplify the fragment according to manufacturer's instructions. Briefly, 2 µl purified DNA was mixed on ice with 0.3 µl of a 20 µM stock primer solution, 0.5 µl FailSafe PCR Enzyme Mix and water, to give a volume of 25 µl. To this, 25 µl FailSafe PCR 2x Premix J was added, vortexed and returned to ice. The tubes were placed in a pre-warmed 96°C PCR

thermal cycler. The PCR program was as follows: 96°C for 60 sec, 95°C for 2 min followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, and 70°C for 60 sec. Finally, an extension step was performed at 72°C for 10 min. A volume of 5 μL was run on a 3% agarose gel with 0.5 μg/mL ethidium bromide at 100V for 60 min in 1x TBE buffer. Bands were visualized using the AlphaImager 2200 (Alpha Innotech, San Leandro, CA).

After verification to ensure the presence of only one band per PCR reaction, the remainder was purified using the QIAquick PCR purification procedure (QIAGEN Inc.; Valencia, CA). Purified amplicons were cycle-sequenced in both directions using the BigDye® Terminator ver. 3.1 chemistry (Applied Biosystems Inc.; Foster City, CA) and analyzed on an ABI PRISM 3100-Avant® Genetic Analyzer at our Recombinant DNA Laboratory core facility in the Sealy Center for Molecular Science. The PCR primers were used for the cycle sequencing reactions.

CYTOGENETIC CULTURES FOR THE ANALYSIS OF CHROMOSOME ABERRATIONS

Cultures for cytogenetic assays were established according to standard procedures (Evans and O'Riordan, 1975). Aliquots of 1 ml of blood were cultured with 9 ml of RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). Stimulation of PBLs was accomplished by the addition of 0.18 mg/ml PHA. Three cultures were started for each subject; one culture was not treated to give a baseline in vivo CA frequency and the other cultures were used for the mutagen-sensitivity assay.

Treatment of Cells with NNK

After forty-six hrs, the cells in the treated cultures were centrifuged and the growth medium reserved. The PBLs were then resuspended in 5 ml serum-free RPMI 1640 supplemented with 0.24 mM NNK (CAS No 64091-91-4, National Cancer Institute, Midwest Carcinogen Repository, Kansas City, MO) and incubated at 37^{o}C in the presence of 5% CO_2 for 1 hr. Our laboratory has previously demonstrated the ability of cultured lymphocytes to metabolize NNK and induce genetic damage at this concentration without significant toxicity and without affecting cell viability (Abdel-Rahman and El-Zein 2000). Following NNK treatment, the cells were washed twice with serum-free RPMI 1640, transferred to clean tubes, and resuspended in the original growth medium until harvested. Harvesting was performed at 1 hr and 24 hrs after NNK treatment. The untreated culture was harvested with the 24 hr post-treatment culture.

Cell Harvesting and Slide Preparation

Prior to harvest, cells from all cultures were treated with 0.1 μg/ml colcemid (Gibco-Invitrogen) for 1 hr at 37°C and 5% CO_2 to arrest the cells in metaphase. The cultures were centrifuged at 300 x g for 10 min. The medium was aspirated and cells were resuspended in hypotonic solution (0.075 M potassium chloride) and incubated at 37°C water bath for 30 min. One mL of freezing cold Carnoy's fixative (3:1 methanol:acetic acid), was added and cultures were centrifuged at 300 x g for 10 minutes. Cultures were washed twice with 10 mL freezing cold fixative. Finally, cells were resuspended in 1 mL cold fixative and stored at -80°C. Slides for cytogenetic analysis were then prepared in duplicate by spreading the fixed cells on the slides and staining with 2.5% Giemsa in 0.025% potassium phosphate monobasic buffer (pH 6.8) for 5 minutes followed by two washes with deionized water. Slides were coded before scoring

to protect against scorer bias. Fifty metaphase cells on each slide were scored for CAs using a Nikon 400 light microscope according to standard procedures (ISCN, 1985). Aberrations were recorded as chromosome breaks or frank chromatid breaks. Chromatid breaks were counted as one break and chromosome breaks as two breaks.

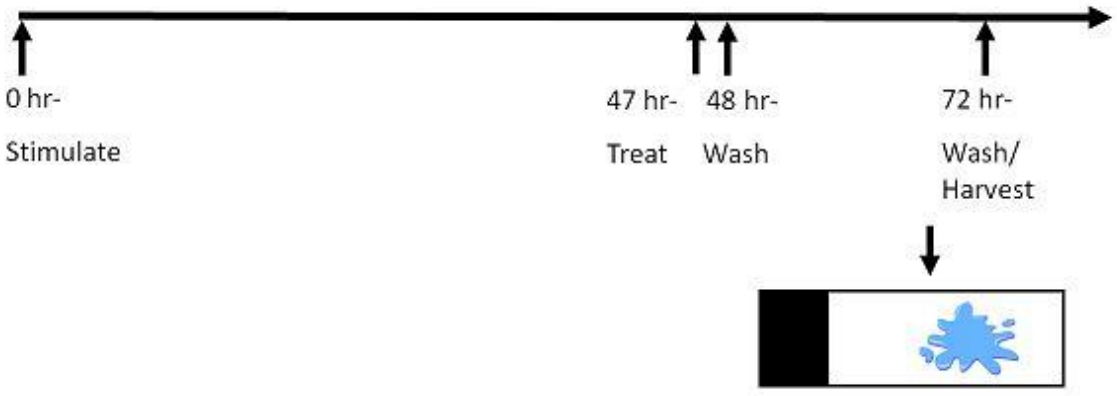

Figure 3: Timeline for whole blood cell cultures, NNK treatment and harvest

HYPOXANTHINE PHOSPHORIBOSYL TRANSFERASE (HPRT) T-CELL MUTATION ASSAY

The *HPRT* assay was performed according to the protocol originally developed by Albertini (2000) with modifications as described below. To rapidly and objectively identify *HPRT* gene mutants in human lymphocytes, we modified a fluorescent detection method originally developed by Dobrovolsky et al., (2000) for identifying murine *Hprt* mutant clones. Lymphocytes were isolated from each blood sample using Histopaque®-1077 according to the manufacturer's recommendations. Lymphocytes were washed with PBS, resuspended in RPMI 1640, counted, and 15 x 10^6 lymphocytes were then cultured for 40 h in a 50 mL polypropylene conical tube in stimulation medium consisting of

68.5% RPMI 1640, 20% HL-1, 10% FBS, 1% 200mM L-glutamine, and 0.5% PHA. For the cloning efficiency and the mutant selection cultures, lethally irradiated 36x4 cells (IR cells) were used as feeder cells at a concentration of 20,000 cells/well in all culture plates. To determine cloning efficiency, which measures the ability of lymphocytes to grow in culture, six experimental isolated lymphocytes per well were plated with 20,000 IR cells in cloning efficiency (CE) medium for a total of two plates per sample using Costar 96-well plates (Fisher; Hampton, NH). CE medium consisted of 59% RPMI 1640, 20% HL-1, 10% FBS, 10% human T-STIM, 1% 200mM L-glutamine, and 0.0625% PHA. Alternatively, CE medium was composed of 67% RPMI, 20% HL-1, 10% FBS, 1% each of MEM, sodium pyruvate, and L-glutamine, 0.0625% PHA and 10U/mL IL-2. It was necessary to use this variation when T-Stim became unavailable halfway through the study. Four samples were repeated using the new CE medium to ensure comparable cloning efficiency. The remaining cells from each sample were used for *HPRT* mutant selection using TG. They were plated in four to seven 96-well plates with 20,000 experimental cells and 20,000 IR cells per well in CE medium containing 2×10^{-5} M TG. In addition, 20,000 IR cells per well were plated in CE medium without either experimental cells or TG for a total of four IR plates per experiment. These IR plates were used as controls for background fluorescence values, as described below, and to ensure that the feeder cells were lethally irradiated.

After 12 days of growth, 50 µL of CE medium supplemented with 2.5% AlamarBlue® (aB) was added to each well, giving a final concentration of 0.5% aB. AlamarBlue is an indicator dye formulated to quantitatively measure proliferation of various cell types. The dye diffuses into proliferating cells and is metabolized via a redox reaction to a fluorescent molecule. A properly configured plate reader can sensitively

detect increases in fluorescence as an indicator of cell proliferation. This method has advantages over the traditional visual method including rapid screening (on average 50% faster) and objective scoring of potentially positive wells. On day 13, plates were read on a Tecan GENios Pro microplate reader with an excitation of 535 nm, emission of 590 nm, integration time of 40 μs and 10 reads per well. The gain was optimized for each experimental day against a randomly selected CE plate. The mean fluorescence value (FV) and standard deviation was calculated for the four IR plates. The standard deviation was multiplied by three and added to the mean FV to give a positive cutoff value for CE plates. CE wells were considered positive for growth if the FV was higher than this calculated value. For the mutant selection plates containing TG, the median value of each plate was calculated. Wells that were 25% or more above that value were scored visually to confirm a positive clone.

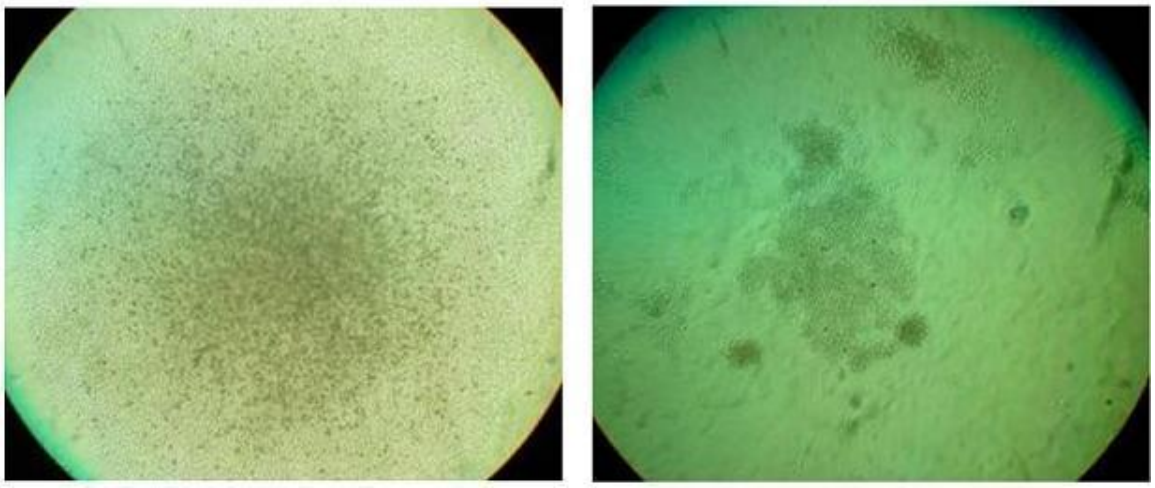

Figure 4: Visual confirmation of an alive (left) and dead (right) *HPRT* mutation assay well.

For the CE plates, there was a significant correlation (Pearson's $r^2 = 0.80$, $p < 0.001$) between visually identified positive wells (the traditional approach) and those clones exhibiting ≥3 standard deviations away from the mean FV of our baseline negative wells in the IR plates (automated approach). The mutant frequency (MF) was calculated according to the method of Cochrane and Skopek using the ratio of the negative natural log of the proportion of negative wells in mutant selection plates to the negative natural log of the proportion of negative wells in CE plates (MF = -ln(Po)TG / -ln(Po)CE) (Cochrane and Skopek 1994).

Mutation Spectra Analysis

To generate data to analyze the spectra of *HPRT* mutations, we developed a rapid and simple technique. Up to ten positive TG clones per subject were randomly selected and grown for a further eight days in 1 mL of CE medium supplemented with 2×10^{-5} M TG in 24-well plates to allow for additional clonal expansion. Expanded clones were then removed from the plates and pelleted at 300 x g for 10 minutes using a tabletop centrifuge. Total RNA was isolated using the RNAqueous kit according to the manufacturer's recommendation (Ambion, Inc, Austin, TX). Total RNA was reverse-transcribed to cDNA using the RETROScript® protocol with initial heat denaturation at 75°C for 3 minutes and random hexamer primers, following the manufacturer's protocol.

To enrich for the mutant *HPRT* gene transcripts, two rounds of PCR were performed using primers that are reported 5'-3' with numbers corresponding to the position of the 3' base in each primer in the published mRNA sequence (NM_000194; National Center for Biotechnology Information [NCBI], Bethesda, MD), relative to the first base in the coding sequence: HPRT1S-GCGCCTCCGCCTCCTCCTCTG CTC (-55), HPRT1AS-AGGCTCATAGTGCAAATAAAC AGTT (825). The PCR reaction

contained 2 μL total RNA, 0.2 mM dNTP mix, 2 mM MgCl2, 0.5 μM of each primer, 1x PCR buffer, and 0.0015U Taq, and water up to a 50 μL volume. The thermal cycling profile was as follows: 95°C for 45 sec, 62°C for 1 min, 69°C for 2 min for 35 cycles. A second round of the PCR, using nested primers and unpurified, amplified material (1-2 μl) from the first round of the PCR, along with the same concentration of PCR reagents, generated an adequate amount of template for DNA sequence analysis: HPRT1SNEST-CCACCGGCTTCCTCCTCCTG (-33), HPRT1ASNEST-GATAATTTTACTGGCGATGTCAA (698). The thermal cycling profile was as follows: 95°C for 30 sec, 63°C for 30 sec, 69°C for 1 min for 35 cycles. PCR products (731 bp in length), encompassing the entire *HPRT* coding sequence, were verified using gel electrophoresis. A volume of 5 μL was run on a 1% agarose gel with 0.5 μg/mL ethidium bromide at 100V for 45 minutes in 1x TBE buffer. Bands were visualized using the AlphaImager 2200 (Alpha Innotech, San Leandro, CA).

After verification to ensure the presence of only one band per PCR reaction, the remainder was purified using the QIAquick PCR purification procedure (QIAGEN Inc.; Valencia, CA). Purified amplicons were cycle-sequenced in both directions using the BigDye® Terminator ver. 3.1 chemistry (Applied Biosystems Inc.; Foster City, CA) and analyzed on an ABI PRISM 3100-Avant® Genetic Analyzer at our Recombinant DNA Laboratory core facility in the NIEHS Environmental Toxicology Center. Primers that were used for cycle-sequencing were either the second round PCR primers or this additional set of internal primers designed specifically for sequencing reactions: HPRT1Sseq-CTCCTCCTGAGCAGTCAG (-24), HPRT1ASseq-TTCCAAACTCAACTTGAAC (664). DNA sequence chromatograms were proofed for quality using the Lasergene version 6 SeqMan II program (DNASTAR Inc., Madison,

WI). The resulting DNA sequences were aligned using the Clustal V algorithm (default parameter settings) in the Lasergene version 6 MegAlign program (DNASTAR Inc.) with the published, functional HPRT1 coding sequence. Inactivating mutations were then mapped to the coding sequence using these alignments.

The cDNA template was simultaneously verified by amplifying the *β-ACTIN* coding sequence (NCBI, NM_001101) using the first round PCR primers β-ACTINS-GTCCGCCCCGCGAGCACAGAG (-50) and β-ACTINAS-AGGGGCCGGACTCGTCATACTCCTG (1077) followed by the second round PCR primers β-ACTINSNEST-ATGATATCGCCGCGCTCGTCGTC (7) and β-ACTINASNEST-ATCTCCTTCTGCATCCTGTCG (929). PCR parameters (concentrations and cycling conditions) were the same as for the *HPRT* sequence.

Mutations were classified as single base pair substitutions, tandem base pair substitutions, insertions (single to multi-base), deletions (single to multi-base), apparent splicing errors (e.g. intronic insertion, exon skipping, exon duplication) or none apparent (i.e. identified mutant with no apparent amino acid sequence change).

GENERATION OF *MGMT*-DRIVEN LUCIFERASE EXPRESSION VECTORS

A wild-type and variant clone of the minimal promoter/enhancer region was a kind gift from Dr. Marek Rusin, Maria Skłodowska-Curie Memorial Institute, Gliwice Poland (Krześniak et al., 2004). 1 µl of the plasmid preparation was transformed into 50 µl of Subcloning Efficiency DH5α bacterial cells (Invitrogen Inc.). Bacterial cells were selected using 100 µg/ml ampicillin on LB agar plates. At the same time, 1 µl of pGL4-Basic plasmid (Promega Inc.) or 1 µl of pGL4-SV40 *Renilla* was transformed into 50 µl of Subcloning Efficiency DH5α bacterial cells. The pGL4-SV40 *Renilla* plasmid contains

the fluorescent *Renilla* gene under the control of the strong ubiquitous SV40 promoter to serve as a measure of transfection efficiency. Colonies were selected and grown in 2 ml LB broth at 37°C for 12-16 hours. Plasmids were isolated using the Qiagen MiniPrep Kit and quantified at 260nm wavelength. 15 µl of each construct was digested with 4 µl *Hind*III and 2 µl *Kpn*I in a total reaction volume of 40 µl at 37°C for one hour. 1.5 µl of the vector was digested with 1.5 µl each of *Hind*III and *Kpn*I at 37°C for one hour in a total reaction volume of 20 µl. The vector was subsequently dephosphorylated at 37°C for 15 min using 2.5 µl AP buffer and 1 µl Antarctic Phosphatase for a total reaction volume of 25 µl. Antarctic Phosphatase was then heat killed by incubation of the reaction at 65°C for 30 min. A 1% gel run at 100V for one hour was used to verify the cutting and purify the inserts and vector from uncut plasmid. The Qiagen Gel Extraction Kit purified the DNA from the agar. The purified insert and vector were ligated in a 5:1 ratio overnight using 1 µl T4 Ligase and 2 µl reaction buffer, for a total reaction volume of 20 µl. The resulting plasmids was transformed into DH5α cells and grown overnight using 100 µg/ml ampicillin on LB agar plates. Individual colonies were selected and grown in 2 mL LB broth at 37°C for 8 hours. 100 µl of the starter culture was added to 100 ml LB broth and grown for 12 hours. Plasmids were purified from the culture with the Qiagen Endo-Free MaxiPrep Kit. The plasmids were subsequently sequenced using primers that annealed to the vector to verify that the plasmid carries either the wild-type or variant sequence and to ensure no random mutations were introduced. Plasmids were stored at -20ºC until transfection.

TRANSIENT TRANSFECTION OF NHBE CELLS AND LUCIFERASE ASSAY

Normal Human Bronchial Epithelial (NHBE) cells were ordered from Lonza (Walkersville, MD) as a frozen cryovial. Cells were stored in liquid nitrogen until ready for use. 1 ml/5cm^2 BEBM medium with all supplements was added to T-75 flasks and allowed to equilibrate in a humidified 37°C/5% CO_2 incubator for 60 min. Cells were quickly thawed at 37°C and seeded at a density of 3,500 cells/cm^2. The medium was changed one day after plating, and every other day thereafter. Medium was always pre-warmed to 37°C before adding to the flask.

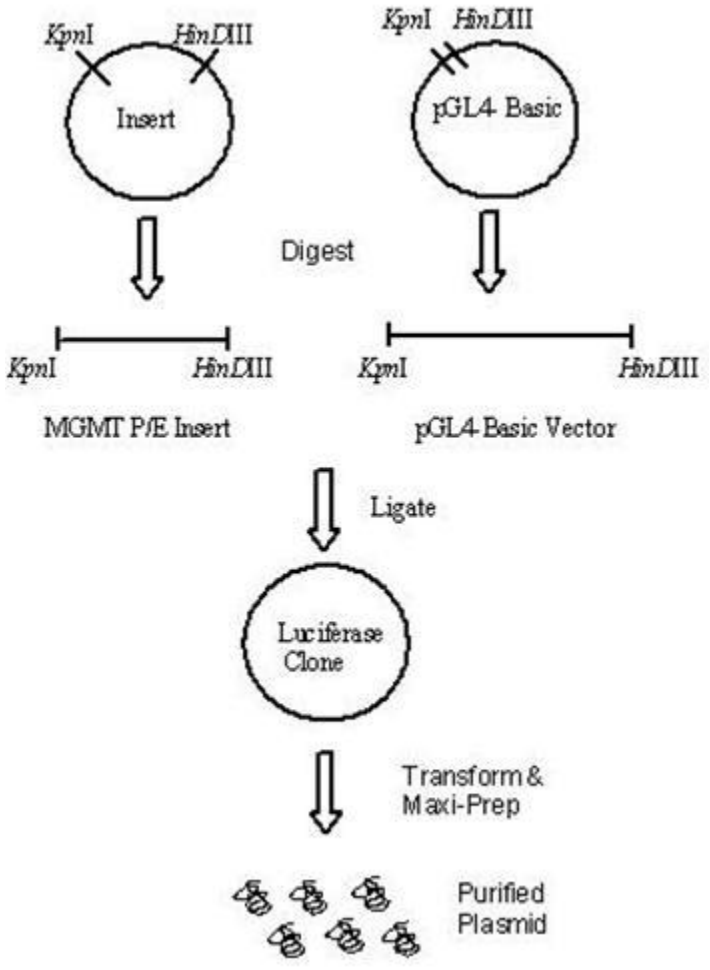

Figure 5: Generation of the luciferase vector under the control of the *MGMT* wild-type or variant promoter

Six days after plating, cells were trypsinized, counted, and plated in fresh medium in 6-well plates at a density of 3,500 cells/cm^2 and a volume of 1 ml/5cm^2. Medium was changed the day after plating and every other day thereafter. Plates were prepared for each vector for 24 hours post transfection, 48 hours post transfection, and smoke-exposed 48 hours post transfection. At 95% confluency, NHBE cells were transfected with the above mentioned constructs for 4.5 hours in a mixture of 1 μg luciferase vector and 0.5 μg *Renilla* vector per well with 6 μg Lipofectamine / μg DNA. Medium was changed and cells were allowed to recover for 24 hours. At this time, the baseline expression plates were harvested. Medium was aspirated and cells rinsed with phosphate buffered saline and incubated with 200 μl 1x lysis buffer. Debris was spun down at 12,000 x g for 1 minute and the lysate was stored at -80°C. In addition, smoke-exposed plates were transferred to the Inhalation Facility Core Laboratory incubators and placed in a 14x10x4 inch plastic container pre-warmed to 37°C on a rocker. Plates were exposed to Kentucky Reference 3R4F cigarettes (University of Kentucky Tobacco Research Institute, Lexington, KY) at a rate of 2 cigarettes/hour and airflow equilibrated to 5 minutes/cigarette for 5 hours. Plates were then returned to the incubator and allowed to recover for 19 hours. Unexposed plates remained at 37°C for the duration. Cell harvesting was performed as previously stated. Luciferase activity was measured using the Dual-Luciferase Assay Kit (Promega, Inc.) using a Tecan GENios Pro microplate reader. Luminescence was measured in relative light units (RLUs). The relative luciferase activity in each sample was normalized to the relative *Renilla* activity in order to control for transfection efficiency. All experiments were performed in triplicate.

Chapter 3: RESULTS

ANALYSIS OF CHROMOSOME ABERRATIONS

The goal of this study was to determine the relationship between SNPS in the coding region of the *MGMT* gene and genetic damage in the form of chromosome aberrations in smoking and non-smoking populations. As the coding SNPs are predicted to alter protein function, we hypothesize that they will alter levels of CA in smokers, independent of other demographic factors (i.e. age, gender).

Demographics of the study population

Demographic information for the study population is presented in Table 2. The study population included 271 females (73%) and 102 males (27%). The majority of individuals (62%) were Non-Hispanic White (n=230) while African-Americans (n=60), Hispanics (n=57) and people of Asian descent (n=26) made up 38% of the population. There were 193 non-smokers, 81 ex-smokers and 99 smokers. Current and former smokers had smoked between 3 and 50 cigarettes per day (mean±SE 17.88±0.79) for a minimum of one year (mean±SE 18.76±0.99 years) before participating in the study. The participants' age ranged from 18 to 88 years, with a median of 38 years and a mean (±SE) of 40.14±0.79 years. Smoking rates were proportional between genders (χ^2 $p>0.53$). Ex-smokers were significantly older (47.11±1.64) than non-smokers (37.31±1.06) or smokers (39.97±1.49) ($p<0.0001$). Older individuals tended to smoke significantly more cigarettes per day than younger individuals (Spearman $\sigma \pm 95\%$ CI = 0.37±0.12, $p<0.0001$). Overall, males were older than females (38.65±0.83years versus 44.11±1.81 years, $p<0.002$). Males had smoked significantly more cigarettes per day than females in the ex-smoker group (17.69±1.30 vs. 25.08±2.71, $p<0.007$) but not in the

current smokers (15.43±1.05 vs. 18.10±2.04, p>0.20). Male smokers were significantly older than female smokers regardless of current smoking status (48.31±2.57 vs. 40.99±1.13, p<0.003).

Table 2: Selected demographics of the study population evaluated for chromosome aberrations

	Non-Smokers (%)	**Ex-Smokers (%)**	**Smokers (%)**	**P-Value**
Age	37.31±1.06	47.11±1.64	39.97±1.49	<0.0001
Gender				
Female	145 (53.5%)	57 (21%)	69 (25.5%)	
Male	48 (47%)	24 (23.5%)	30 (29.5%)	0.52
Ethnicity				
White	106 (46%)	57 (24.8%)	67 (29.2%)	
Af-Am	39 (65%)	12 (20%)	9 (15%)	
Hispanic	27 (47.4%)	11 (19.3%)	19 (33.3%)	
Asian	21 (80.8%)	1 (3.8%)	4 (15.4%)	0.06

After correcting for these variables, males who smoked at the time of enrollment had significantly more pack-years (packs per day times number of years smoked) (29.53±4.95) than females (15.39±1.67, p<0.001). This was not the case for ex-smokers (25.61±7.07 vs. 16.88±2.22, p>0.13). Male non-smokers, however, were not significantly older than female non-smokers (39.39±2.39 years vs. 36.62±1.18 years, p>0.26). For the analysis of smoking rates among ethnicities, smokers of Asian descent were excluded due to the small sample number (n=5). Years of smoking did not differ

between the ethnicities independent of current smoking status ($p>0.08$). Non-Hispanic Whites and African-Americans were significantly older than Hispanics (45.06±1.34 and 44.71±3.25 vs. 36.97±2.72, $p<0.03$). Non-Hispanic whites had the highest number of cigarettes per day (19.40±0.94) versus Hispanics (14.62±1.94) or African-Americans (14.52±2.28) ($p<0.025$). Therefore, non-Hispanic Whites had a significant increase in pack-years (22.99±1.89) compared to other ethnicities (14.09±4.59 for African-Americans, 10.61±3.97 for Hispanics, $p<0.01$).

MGMT Genotypes and Allele Frequency

The frequency of the variant 84F allele (rs12917) in this study population was 0.144, which was similar to the allele frequency reported in other studies (Egyhazi et al., 2002; Krześniak et al., 2004). The frequency of the variant 143V allele (rs17406533) was 0.103. This frequency was also similar to that reported in other published studies (Kaur et al., 2000, Ford et al., 2000). There was no significant difference in age between the wild-type and polymorphic forms at codon 84 (wild-type 40.01±0.92; heterozygous 40.63±1.65; homozygous variant 40.40±4.85, $p>0.95$) or codon 143 (wild-type 41.09±0.90; heterozygous 38.58±1.98; homozygous variant 44.33±6.22, $p>0.44$). In addition, there was no significant difference in allele frequencies for either of these cSNPs between smokers and non-smokers ($p>0.36$ for the L84F and $p>0.69$ for the I143V) or between males and females ($p>0.38$ for the L84F $p>0.68$ for the I143V). No linkage disequilibrium between the cSNPs was apparent ($D'=0.07$, $p>0.30$).

Effect of MGMT L84F and I143V cSNPs on background CA frequencies

In the study population, the baseline (background) in vivo CA frequency was not normally distributed and ranged from 0 to 7 breaks, with an average of 0.91±0.05 breaks

and median of 1 break per 50 cells. There was no association between ethnicity and CA levels ($p>0.22$). Age was not associated with baseline CA values. Linear regression demonstrated an estimated slope (95% CI) of -0.0024±0.007 and Spearman $\sigma = 0.001$ ($p>0.36$). There was no significant effect of gender alone on baseline CA values (0.93±0.06 vs. 0.89±0.11, $p>0.76$). Lastly, no significant effect of smoking status on CA frequency was apparent (non-smokers 0.89±0.07, ex-smokers 1.06±0.12, smokers 0.85±0.11, $p>0.37$). Although the observed in vivo CA frequencies were slightly higher for individuals with the 84F polymorphism (1.01±0.11) compared to individuals homozygous for the reference allele (0.88±0.06), the difference was not statistically significant ($p>0.32$). No significant differences in baseline CA frequencies were apparent between individuals with these polymorphisms compared to individuals homozygous for the referent allele when stratified based on smoking (non-smokers 0.86±0.08 vs. 1.0±0.14 $p>0.42$; smokers 0.91±0.10 vs. 1.02±0.17 $p>0.57$). There was no significant difference between wild-type and variant at codon 84 within females ($p>0.11$) nor males ($p>0.48$). In addition, there was no significant effect of the 143V variant on background CA (0.92±0.06 vs. 0.85±0.14, $p>0.63$). Furthermore, no significant differences between homozygous wild-type or variant cells were seen after gender stratification (females 0.91±0.07 vs. 0.91±0.18 $p>0.99$; males 0.93±0.13 vs. 0.68±0.24, $p>0.40$). In the non-smokers, cells from individuals with the 143V variant had a significant decrease in the median CA level compared to cells from wild-type individuals ($p<0.03$) however this was not seen in smokers ($p>0.41$).

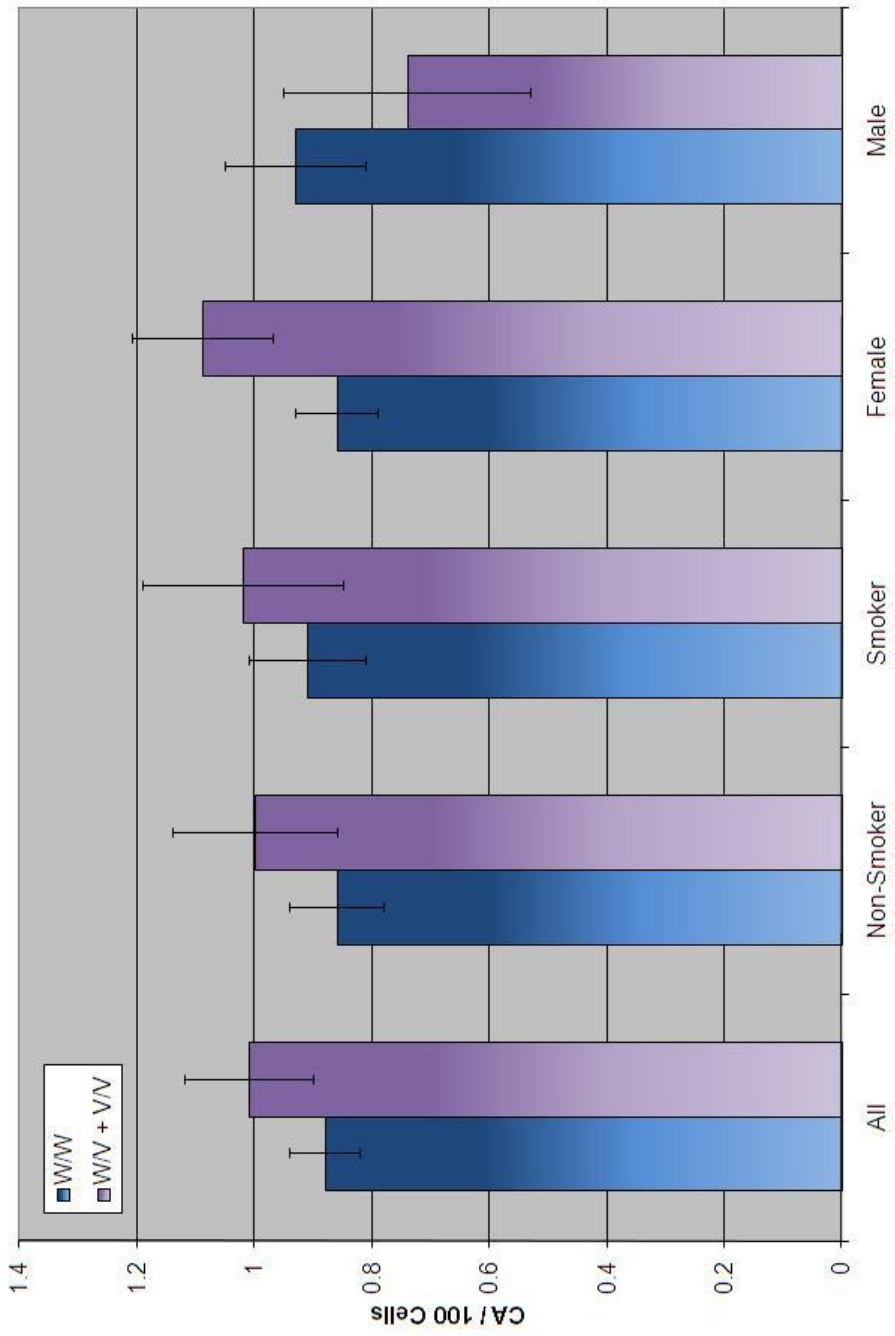

Figure 6: Background CA levels stratified by L84F genotype, smoking and gender

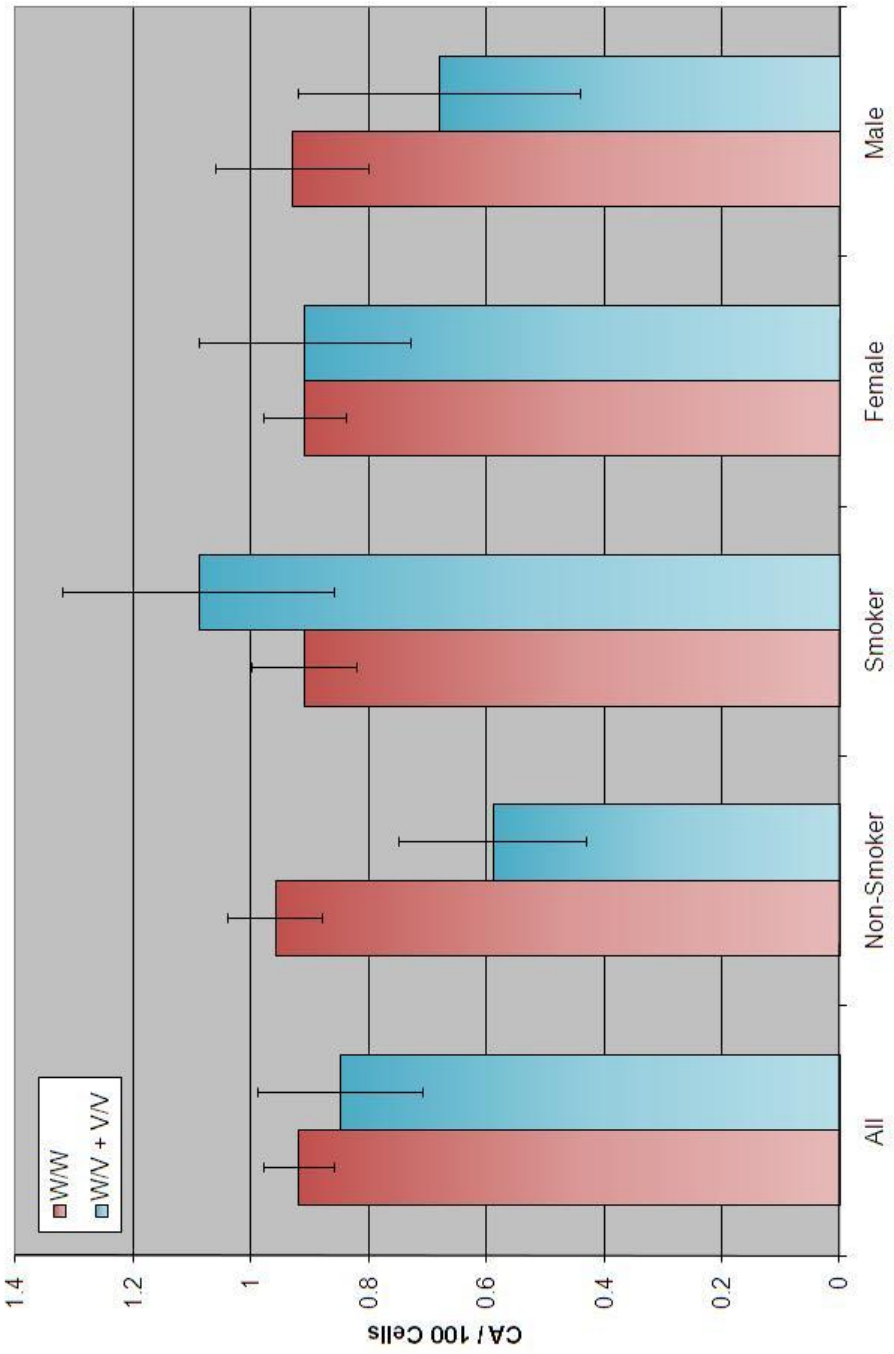

Figure 7: Background CA levels stratified by I143V genotype, smoking and gender

Effect of MGMT L84F and I143V cSNPs on NNK-induced CA frequencies

Using the mutagen-sensitivity assay, with NNK as the test mutagen, the frequencies of NNK-induced CAs in the total population after 1 hour of exposure, controlling for the background, ranged between 0 to 9 breaks with an average of 2.28 breaks and a median of 2 breaks per 50 cells. Age was marginally associated with NNK-induced CA values at this time point. Linear regression demonstrated an estimated slope (95% CI) of 0.0103±0.0118 ($p>0.08$) and Spearman $\sigma = 0.1093$ ($p<0.04$). There was no significant difference in mutagen-induced CA levels between females (2.35±0.11) and males (2.13±0.17 $p>0.28$) or between the different ethnicities ($p>0.48$). No association was seen with smoking at this time point (non-smokers 2.19±0.13, ex-smokers 2.53±0.19, smokers 2.28±0.18, $p>0.35$). In addition, there was no association between wild-type and variant cells for the 84F SNP (2.24±0.11 vs. 2.29±0.21, $p>0.86$). After stratification based on smoking status, the 84F variant was not associated with mutagen-induced CA (non-smokers 2.16±0.14 vs. 2.29±0.31, $p>0.68$; smokers 2.42±0.15 vs. 2.33±0.25, $p>0.74$). There was no significant difference between the wild-type and variant individuals in cells from females (2.31±0.12 vs. 2.45±0.23, $p>0.59$) or from males (2.20±0.19 vs. 1.87±0.37, $p>0.41$). There was also no significant difference between cells wild-type and variant for the 143V SNP (2.30±0.10 vs. 2.23±0.20, $p<0.76$). This was true in both the non-smokers (2.17±0.14 vs. 2.28±0.32, $p>0.76$) and smokers (2.44±0.14 vs. 2.18±0.26, $p>0.43$). There was no significant difference in mutagen-induced CA between wild-type and variant cells in either females (2.35±0.12 vs. 2.37±0.24, $p>0.93$) or males (2.18±0.19 vs. 1.89±0.38, $p>0.51$).

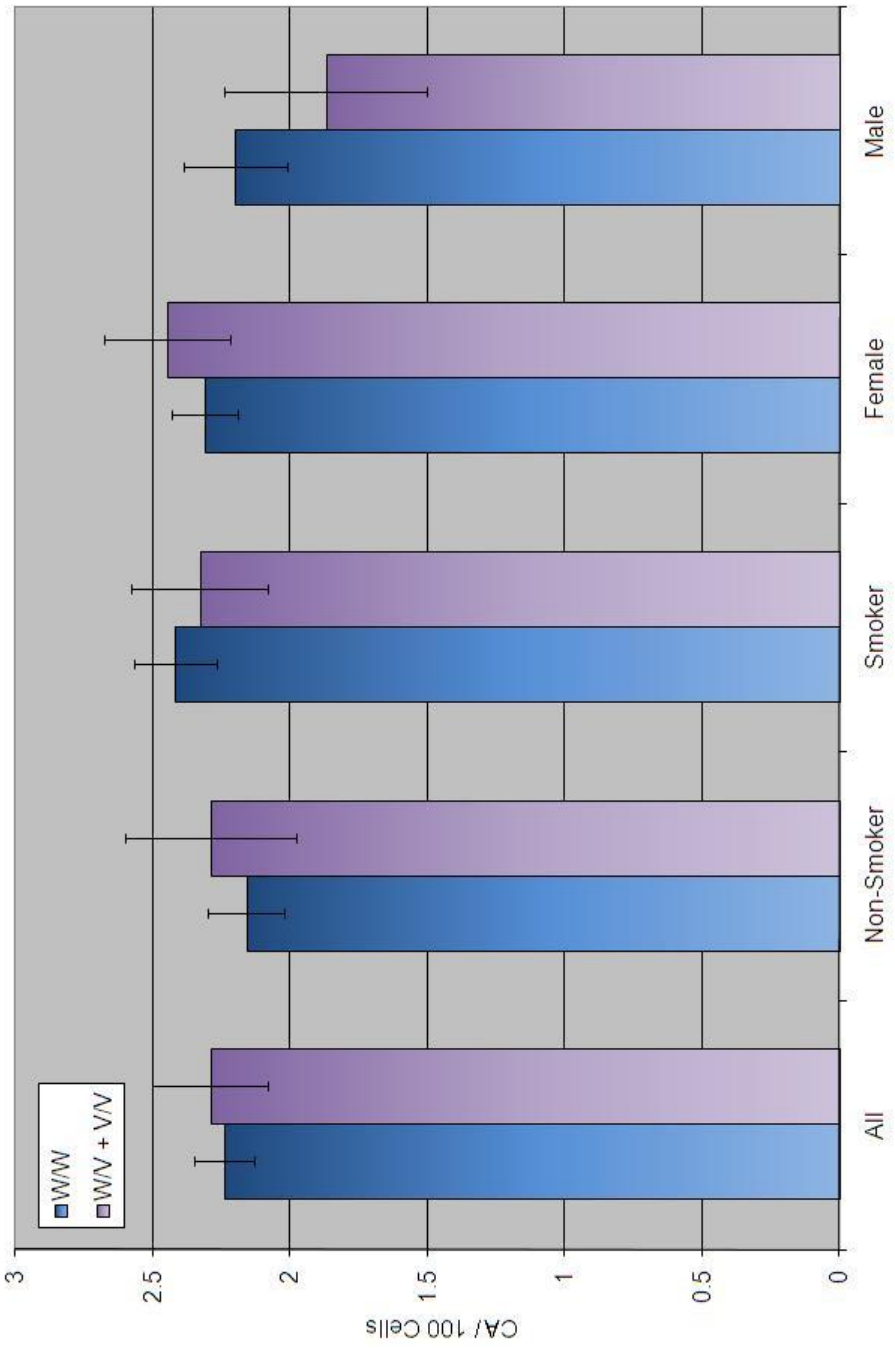

Figure 8: CA levels one hour after NNK exposure stratified by L84F genotype, smoking and gender

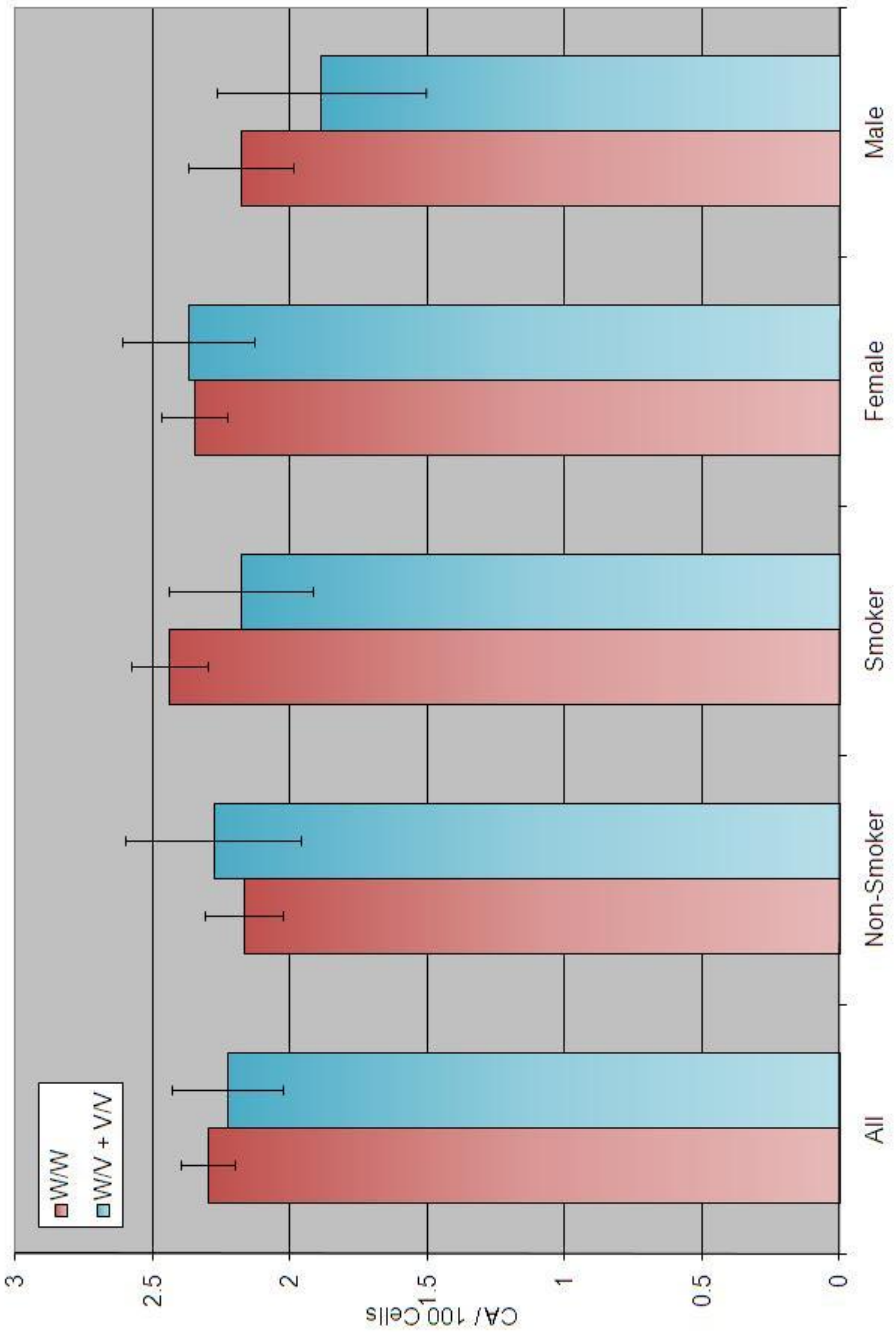

Figure 9: CA levels one hour after NNK exposure stratified by I143V genotype, smoking and gender

The frequency of NNK-induced CA 24 hours after exposure, controlling for the background CA, ranged from 0 to 14 breaks, with an average of 3.42 and a median of 3 per 50 cells. Age was not associated with CA values. Linear regression demonstrated an estimated slope (95% CI) of 0.0048±0.0168 ($p>0.57$) and Spearman $\sigma = 0.0337$ ($p>0.51$) There was no significant difference in CA levels between females (3.42±0.15) and males (3.43±0.26 $p>0.99$). CA levels were comparable between non-smokers (3.41±0.18), ex-smokers (3.79±0.27) and current smokers (3.16±0.25) ($p>0.18$). Also at this time point, non-Hispanic whites had a significantly lower CA frequency than Hispanics (3.08±0.16 vs. 4.36±0.31, $p<0.003$). At this time point, cells from individuals with at least one copy of the 84F variant had a significant increase in CA frequency compared to cells from wild-type individuals (3.09±0.14 vs. 3.81±0.26, $p<0.02$). After stratification based on smoking status, the 84F variant was not associated with CA (non-smokers 3.30±0.19 vs. 3.80±0.41, $p>0.23$; smokers 3.32±0.22 vs. 3.79±0.36, $p>0.27$). No gender effect was seen for this time point, with no significant difference between the wild-type and variant individuals in either females (3.29±0.16 vs. 3.84±0.31, $p>0.10$) or males (3.35±0.29 vs. 3.65±0.59, $p>0.64$). There was no significant difference between cells that were wild-type or variant for the 143V SNP at this time point (3.29±0.14 vs. 3.26±0.32, $p<0.92$). This was true in both the non-smokers (3.29±0.18 vs. 3.0±0.53, $p>0.51$) and smokers (3.29±0.22 vs. 3.51±0.38, $p>0.65$). In females, cells from individuals carrying the variant 143V had a significant decrease in median CA compared to cells from individuals wild-type at this locus ($p<0.03$). In males, cells from individuals with the variant 143V allele had a significant increase in median CA compared to wild-type cells ($p<0.02$).

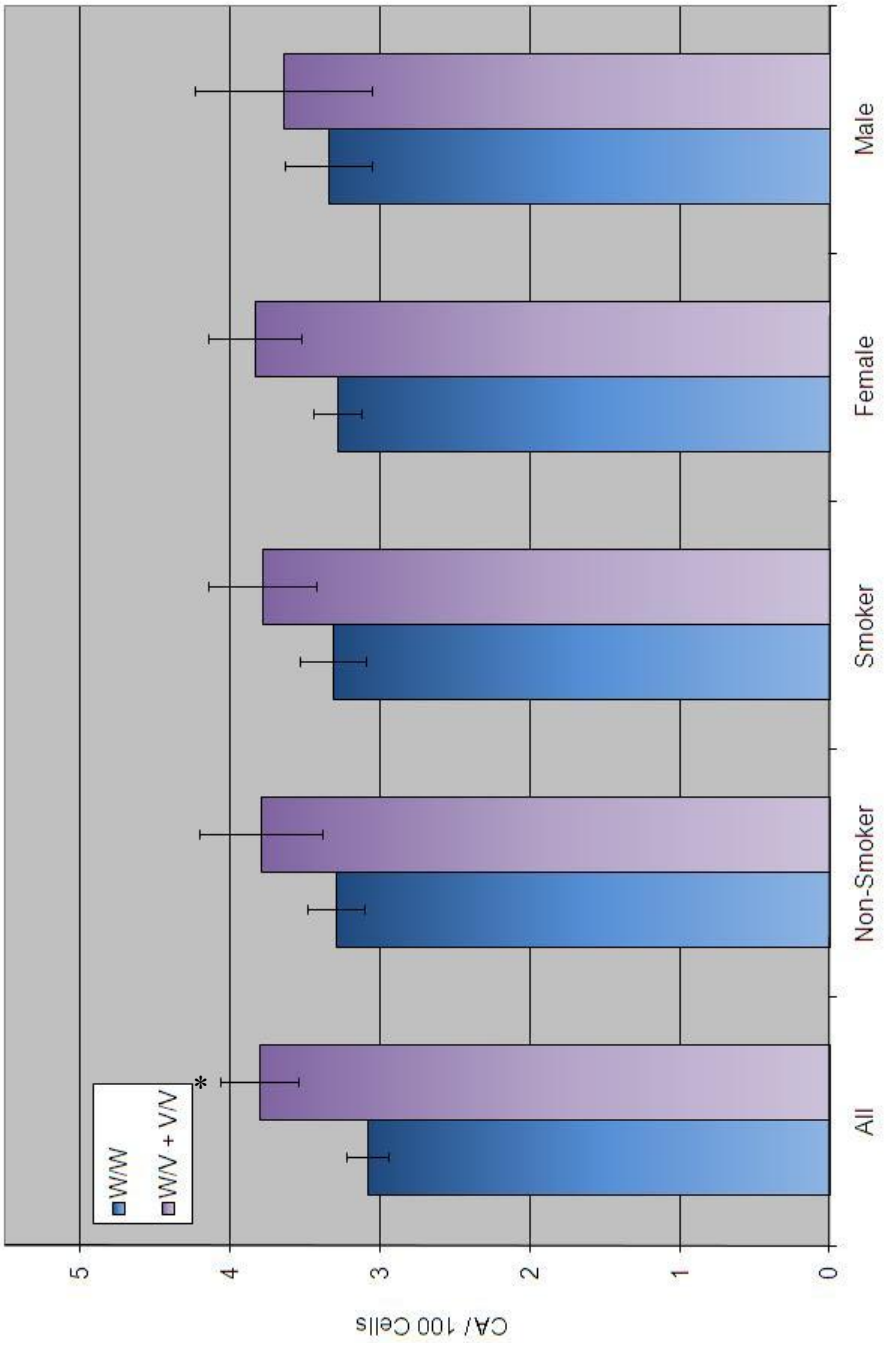

Figure 10: CA levels 24 hours after NNK exposure stratified by L84F genotype, smoking and gender.

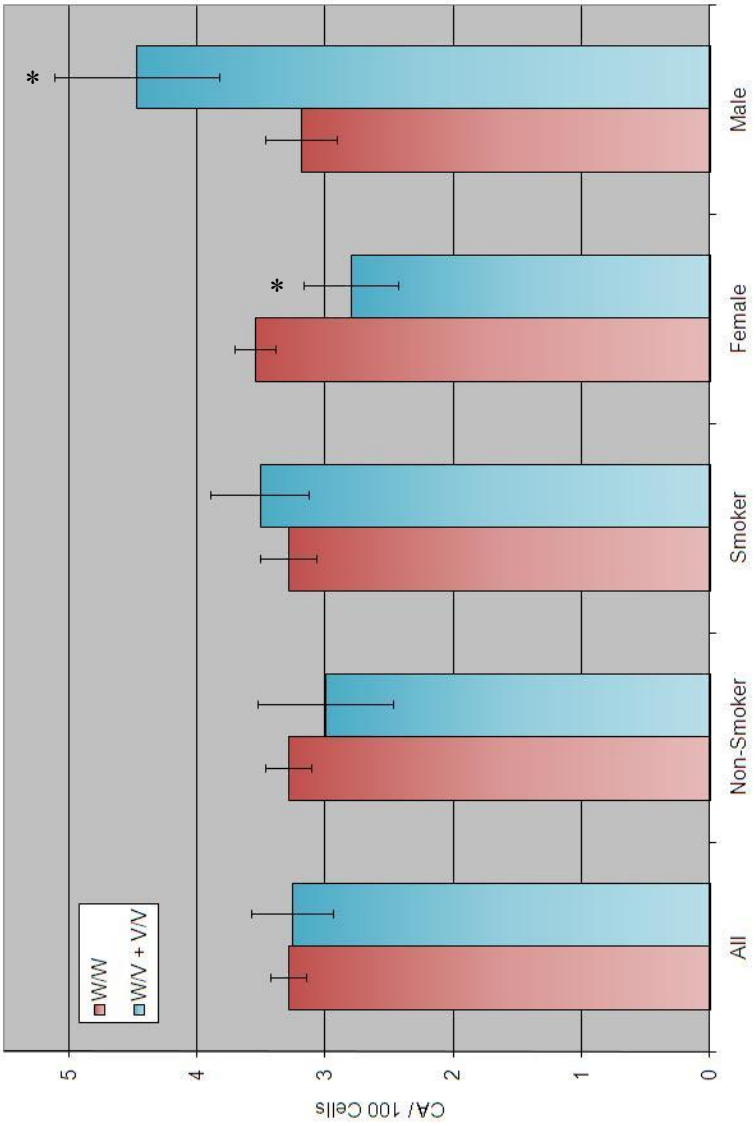

Figure 11: CA levels 24 hours after NNK exposure stratified by I143V genotype, smoking and gender

HYPOXANTHINE PHOSPHORIBOSYL TRANSFERASE (HPRT) T-CELL MUTATION ASSAY

The goal of this study was to determine the relationship between SNPs in the coding region and P/E region and genetic damage in the form of *HPRT* mutations as a model for global mutation rates in exposed and unexposed populations. As we saw a small but significant increase in NNK-induced CA in individuals with *MGMT* polymorphisms, we hypothesize that these SNPs could alter mutation frequency in smokers, independent of other demographic factors (i.e. age, gender). Since high MGMT level and activity may protect against G → A transition mutations, we also hypothesize that this mutation type will be more prevalent in individuals with one of the coding SNPs. The C1099T SNP was previously reported to increase promoter activity, and thus is predicted to protect against mutations due to higher MGMT protein levels.

Demographics of the Study Population Evaluated for Genotoxic Effects Using the *HPRT* Assay

The study population was a subset of the total population and included 55 females (77%) and 16 males (23%) for a total of 71 subjects. The majority of individuals (68%) were White non-Hispanics (n=48) while African-Americans (n=7), Hispanics (n=4) and Asians (n=12) made up 32% of the population. There were 41 non-smokers and 30 smokers. To reduce confounders, ex-smokers were not included in this portion of the study. Smokers had smoked between 3 and 40 cigarettes per day (mean±SD 19±10) for a minimum of three years (mean±SD 22±10 years). The age of the participants ranged from 21 to 69 years, with a mean±SD of 37±11 years. There was a significant difference between the mean ages of smokers and non-smokers, with non-smokers tending to be slightly younger (34±1.42 versus 40±1.18, $p<0.04$). In addition, males were slightly

older than females (35±11 versus 41±12, $p<0.04$). There was no significant age difference between the genotype groups ($p>0.10$). There was no significant difference in the smoking habits (total number of years of smoking, number of cigarettes smoked per day, and pack years, defined as packs smoked per day times the number of smoking years) between males and females, between genotype groups or between the different ethnicities ($p>0.05$). However, the number of cigarettes smoked per day tended to increase with increasing age ($p<0.02$,). There was no statistical difference in mean age between the genotype groups.

Table 3: Selected demographics of the study population evaluated for *HPRT* mutation frequency

	Non-Smokers (%)	**Smokers (%)**	**P-Value**
Age	34±1.42	40±1.18	<0.04
Gender			
Female	33 (60%)	22 (40%)	
Male	8 (50%)	8 (50%)	0.47
Ethnicity			
White	25 (52%)	23 (48%)	
Af-Am	2 (28.5%)	5 (71.5%)	
Hispanic	4 (100%)	0 (0%)	
Asian	10 (83%)	2 (17%)	<0.03

Because some re-recruitment for this study was performed after genotype analysis, the genotypes for the 84F and 143V variants are in apparent Hardy-Weinberg disequilibrium. However, in the entire study population (n=373), the allele frequency for these polymorphisms was 0.14 and 0.10, respectively, which is concordant with previously published studies (Harris et al., 1991; Ford et al., 2000; Egyhazi et al., 2002; Krześniak et al., 2004). Analysis for linkage disequilibrium was performed for these subjects, and no linkage between the two polymorphisms was observed (D' = 0.07, $p>0.61$). The allele frequency for the C1099T SNP was below expected at 0.03. Due to the relatively low numbers of individuals with the 1099T, there was apparent disequilibrium between this SNP and the 84F ($p<0.0001$).

Effect of Age, Smoking and Gender on Cloning Efficiency (CE) and *HPRT* Mutation Frequency (MF)

Since the main effect variables of interest in this study (age, smoking status, gender and genotype) were not correlated, statistical analyses were conducted to evaluate the effect of each of these parameters individually. As documented in our laboratory and in prior studies, we found a significant inverse correlation between cloning efficiency (CE) values and mutation frequency (MF) (Spearman $\sigma = -0.385$, $p<0.001$) (Jones et al., 1993, 1995; Hill et al., 2007; Havla et al., 2009). We also determined that age alone was not an independent predictor of the CE (Spearman $\sigma = 0.13$, $p>0.28$) nor were any of the other variables of interest (smoking $p>0.60$, the L84F SNP $p>0.14$, the I143V SNP $p>0.31$, the C1099T SNP $p>0.47$ and gender $p>0.68$.). Since the final MF was adjusted to account for the variability in CE, and there were no associations between the variables of interest and the CE values, these were not included in the rest of the analyses.

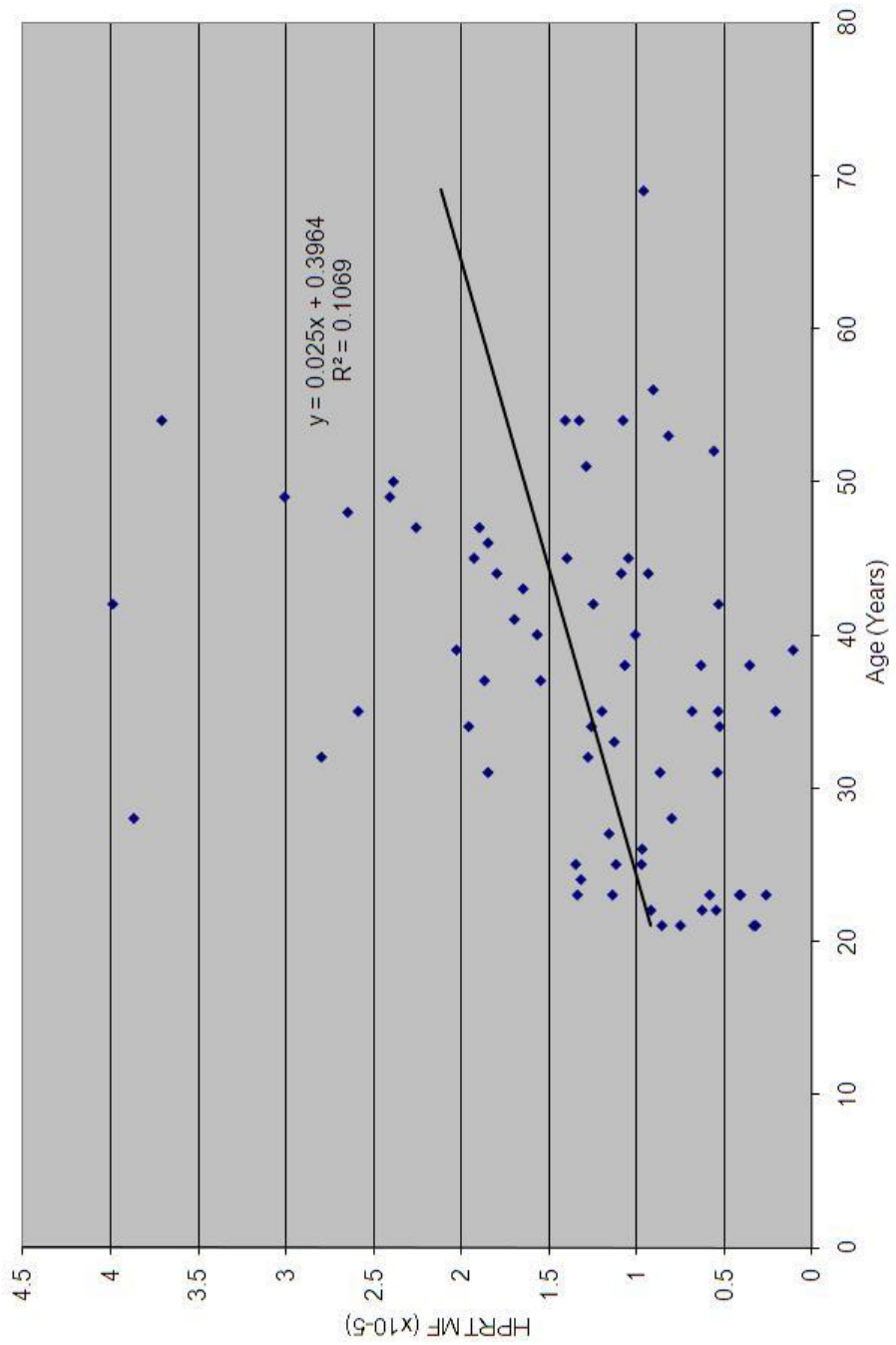

Figure 12: Linear correlation between age and *HPRT* mutation frequency

Similar to prior studies (Jones et al., 1995, Hill et al., 2007, Havla et al., 2009), age was an independent predictor of MF (Spearman σ =0.41, $p<0.0005$) with the linear estimate of MF ($x10^{-5}$) = 0.025(Age in years) + 0.40, giving a small, approximately 2.5%, increase in background MF per year. In this study, smokers had a significantly higher ($p<0.005$) MF compared to non-smokers. This increase was only significant for the female smokers compared to the female non-smokers ($p<0.01$). There was no significant increase in MF between male smokers versus male non-smokers ($p>0.15$). Although we had a total of only 16 male subjects in this study, our findings are compatible with some previously published studies (Hüttner et al., 1995; Meng et al., 2007).

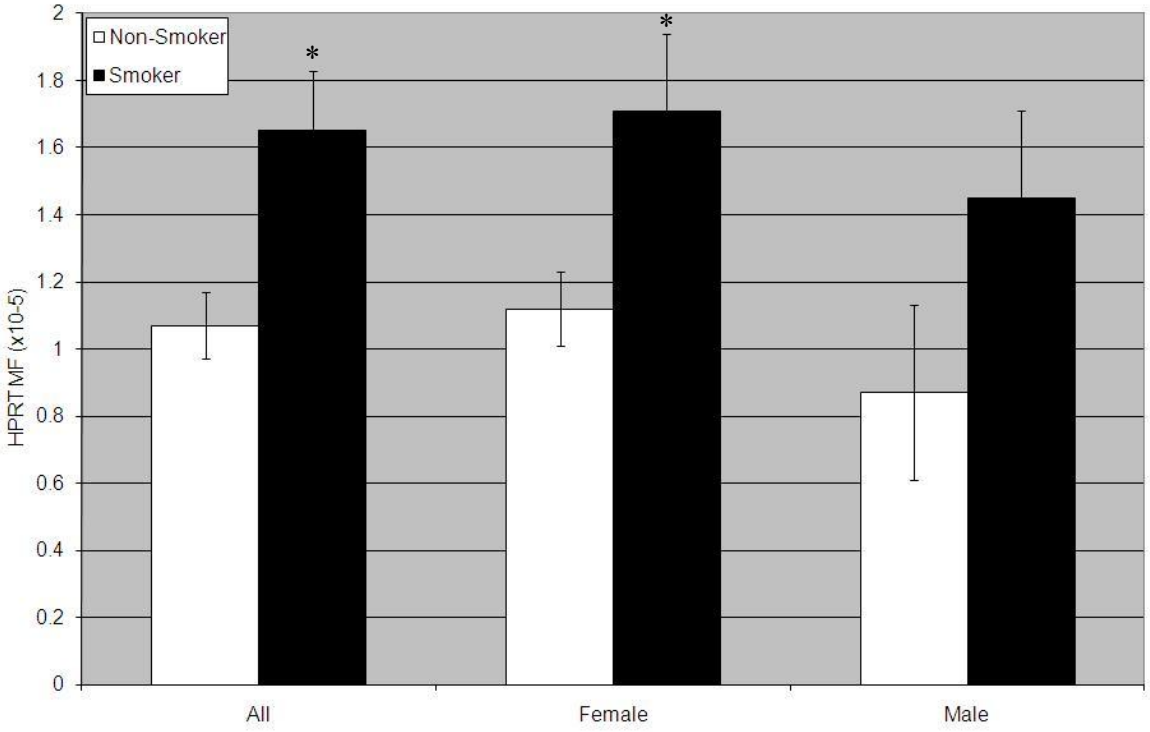

Figure 13: Increased *HPRT* MF in smokers, stratified by gender

Effect of *MGMT* Genotype on *HPRT* Mutation Frequency (MF)

Of the two coding polymorphisms in the *MGMT* gene that we investigated, only the 84F polymorphism was significantly associated with increased MF (about a 67% increase; $p < 0.0005$). This effect was enhanced by smoking status. Cells from smokers who carried at least one variant 84F allele had a significantly higher MF (about a 90% increase; $p<0.002$) compared to cells from smokers who were homozygous for the wild-type L84 allele. There was no significant influence of the 84F polymorphism on MF in individuals who were non-smokers ($p=0.06$). The subjects who were homozygous variant for the 84F were all smokers, therefore, we could not determine whether there was a significant gene-dose effect independent of smoking. We re-evaluated the influence of smoking on *HPRT* MF stratified by genotype. Smoking continued to be an independent factor in predicting MF, but this effect was more pronounced in individuals with the 84F variant. Homozygous wild-type individuals who smoked had a 36% increase in MF compared to non-smokers ($p<0.05$) while smokers who carried at least one copy of the 84F variant had a 51% increase in MF compared to non-smokers ($p<0.02$).

In contrast to our results with the 84F polymorphism, we observed no significant effect for the I143V polymorphism on MF ($p>0.30$). Further stratification by smoking status indicated that there was no significant influence of this polymorphism on MF, either in smokers or in non-smokers, or females versus males.

Our data regarding the relationship between the C1099T SNP and *HPRT* MF was inconclusive. This was not surprising given the low frequency of this SNP that we observed in our study sample (allele frequency = 0.03). We were unable to correlate the

C1099T promoter SNP with *HPRT* MF levels due to the lower than expected frequency and apparent disequilibrium with the cSNP at codon 84.

We further conducted a multiple regression analysis using the main effect variables that were significantly associated with MF (smoking, age and the 84F polymorphism), in an effort to predict the combined effect of these variables on *HPRT* MF. Smoking and genotype were run as dichotomous categorical independent variables and age as a continuous variable. Using this model, genotype and smoking status remained statistically significant in predicting MF ($p<0.01$). Therefore, age was not included in the final model due to the very small increase in R^2. The final estimated linear model was *HPRT* MF ($\times 10^{-5}$) = 0.87 + 0.737 (84F polymorphism) + 0.563 (smoking), $R^2 = 0.27$. The estimated background MF of 0.87×10^{-5} was slightly lower but consistent with our actual observed background HPRT MF of 0.95×10^{-5} (observed in non-smokers who are homozygous for the wild-type L84 allele, $p>0.36$).

Relationship between Age, Smoking, or Genotype and *HPRT* Mutation Spectrum

We analyzed 144 clones from 26 individuals to determine the type of *HPRT* gene mutations. No previously unknown mutations were found in this study compared to what has been reported in the currently available database (Cariello 1994). The distributions of presumed inactivating *HPRT* gene mutations between smokers and non-smokers were not statistically different (likelihood ratio $p>0.67$). Due to the low frequency of the C1099T SNP, the mutation spectrum was not analyzed for this SNP.

In non-smokers, the characteristics of the total mutations were: 48% base substitutions, 14% presumed splicing errors, 23% deletions, 4% insertions, and 11% with no observed change. A clone from two individuals had tandem base substitutions. The majority of base pair substitutions occurred at A (39.3%) or G (33%) bases on the

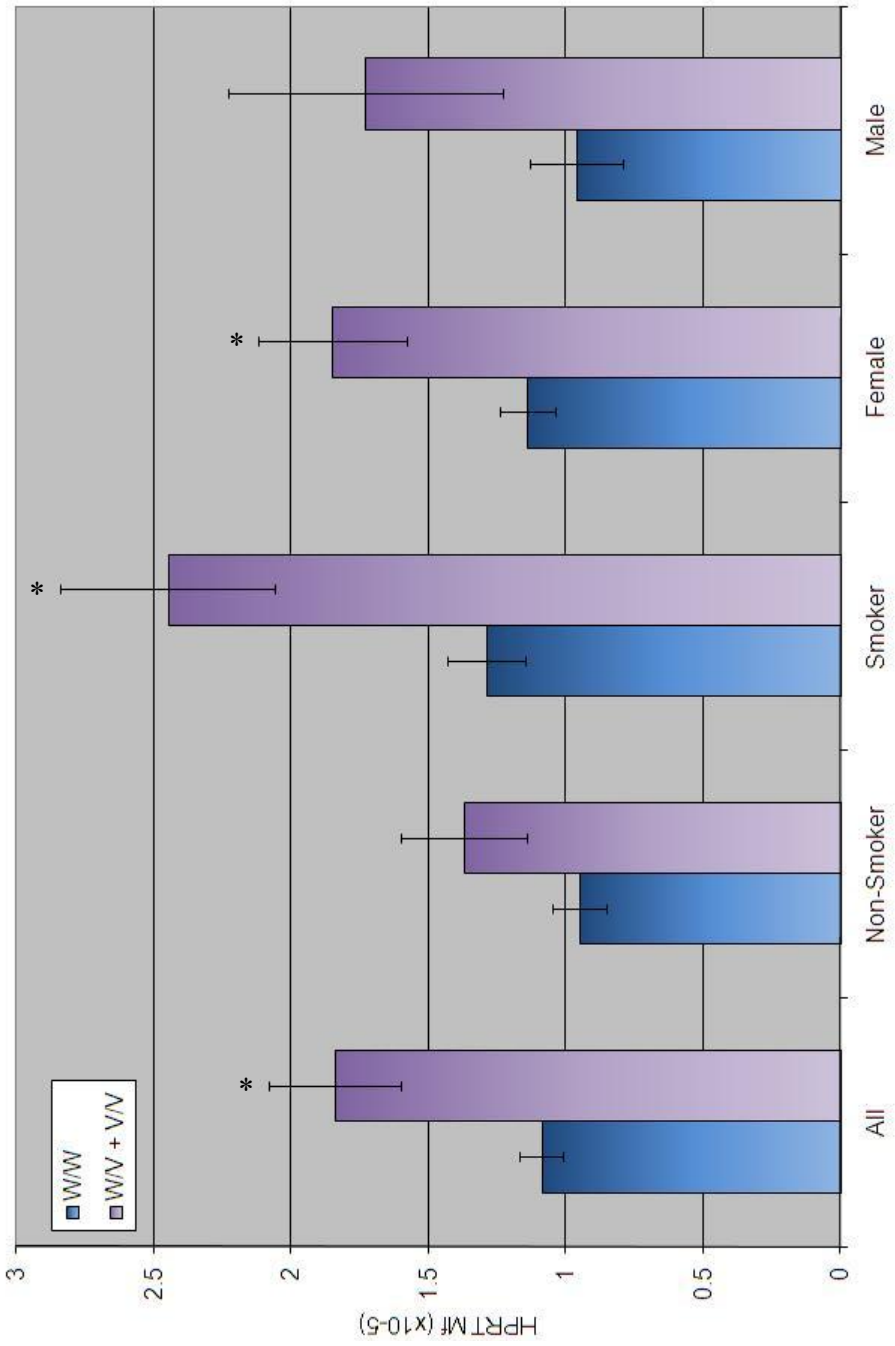

Figure 14: Comparison of *HPRT* MF stratified by L84F genotype, smoking and gender

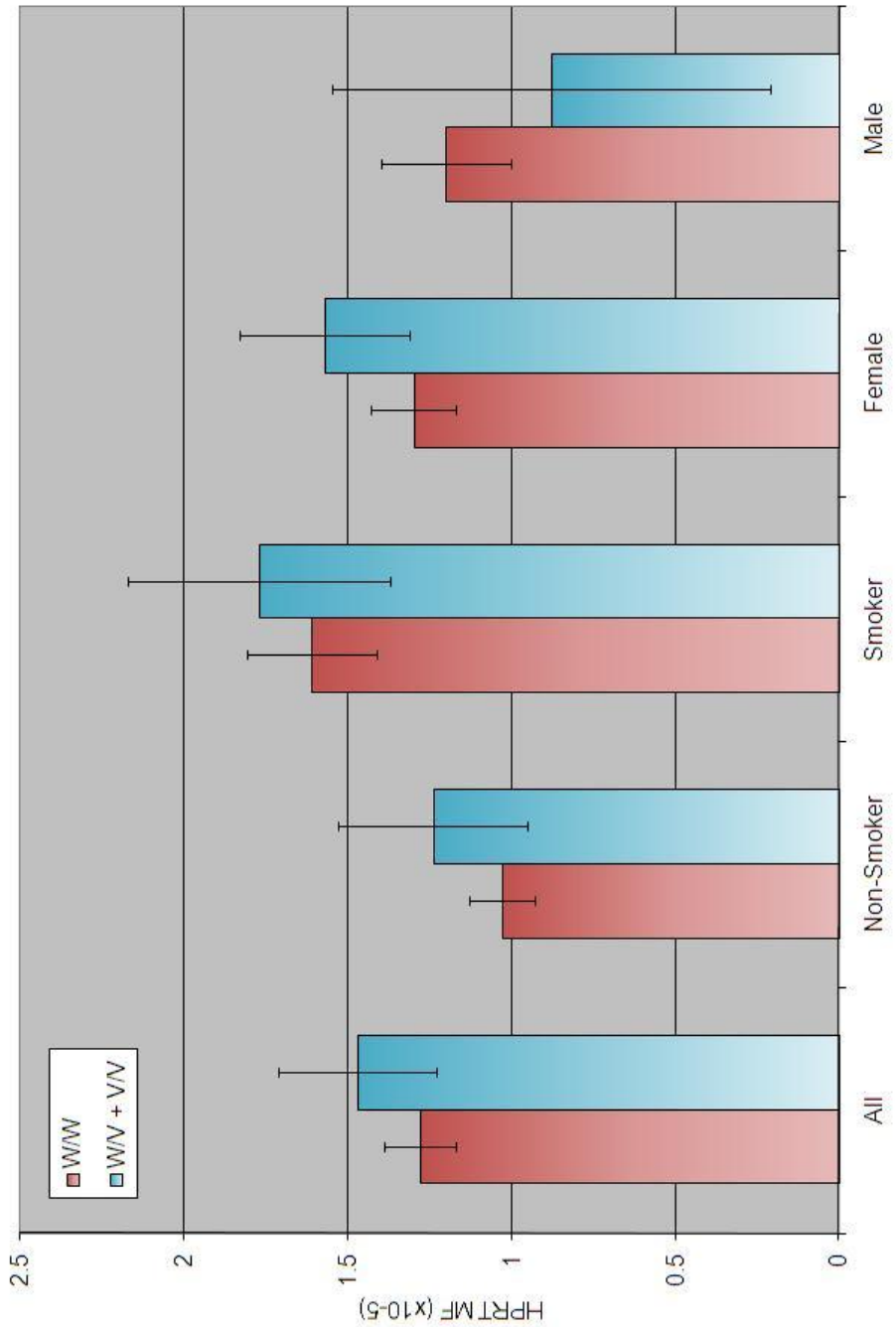

Figure 15: Comparison of *HPRT* MF stratified by I43V genotype, gender and smoking.

transcribed strand. Half of the base substitutions were transition mutation events. Of the splice variants, half were insertions. Three splice variants (60%) were small insertions resulting in a frame shift or insertion of one amino acid. The remainder resulted in either a duplication of exon 3 or a partial insertion of intron 1, 4 or 5. All deletion splice variants were complete loss of exons 2-3, 6 or 8. Out of 17 intra-exonic deletions, 9 (53%) were large deletions (encompassing at least one codon). All insertions were 1-3 base pairs in exon 2.

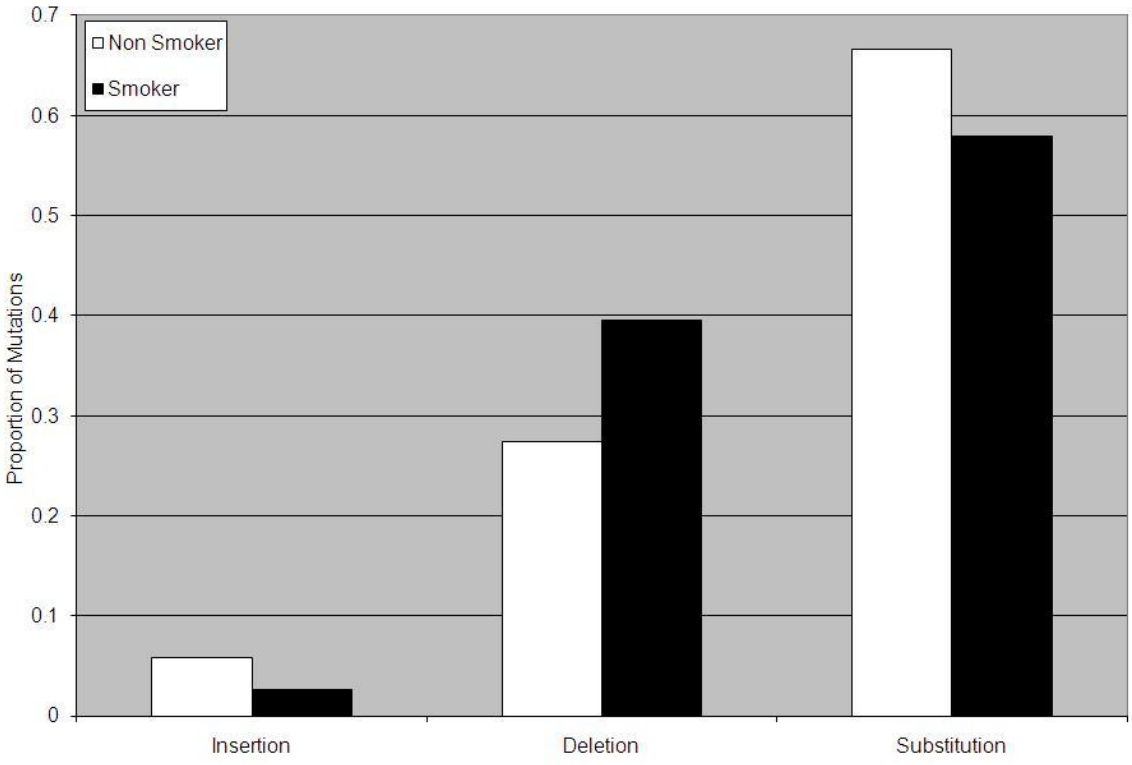

Figure 16: Effect of smoking on the *HPRT* mutation spectrum

In smokers, the characteristics of the total mutations were: 41% base substitutions, 21% presumed splice variants, 20% deletions, 7% insertions, and 11% with

no detectable change. A majority of base pair substitutions occurred at G (48%) base on the transcribed strand. 53% of base substitutions were transition mutation events. Of the 15 splice variants, 12 (80%) were deletions, resulting in loss of exon 2-3, 4, 5, 7 or 8. Six of 13 (46%) of the deletions were small (≤3 nucleotides). Smokers had three small insertions. There was no significant difference in the proportion of transitions or transversions between smokers and non-smokers.

The influence of the L84F polymorphism on the *HPRT* mutation spectrum was also evaluated, regardless of smoking status, but it was not statistically significant ($p>0.23$). In cells from individuals homozygous for the wild-type L84 allele, the characteristics of the total mutations were: 39% substitutions, 24% presumed splice variants, 23% deletions, 2% insertions and 12% with no detectable mutation. A majority of the base substitutions were at A (28%) or G (38%) bases in the transcribed strand. 45% of base substitution mutations were transition events. Thirteen of 19 (68%) of the splice variants resulted in deletions and loss of exons 2-3, 4, 6, 7 or 8. Ten of 18 (55%) of deletions were small deletions, resulting in a frameshift or a loss of one amino acid. Two insertions were 1 bp frameshifts.

In cells from individuals carrying the 84F variant allele, regardless of the smoking status, the distribution of total mutations was 52% base substitutions, 9% presumed splice variants, 21% deletions, 9% insertions, and 9% with no detectable change. A majority of base substitutions occurred at A (26%) or G (45%) on the transcribed strand. 56% of base substitutions were transition events. Six of 8 (75%) of the splice variants resulted in a deletion, and loss of exons 2-3, 4, 5 or 8. Nine of 13 (69%) of the deletions were large (greater than one codon triplet). All insertions were 3 bp or less.

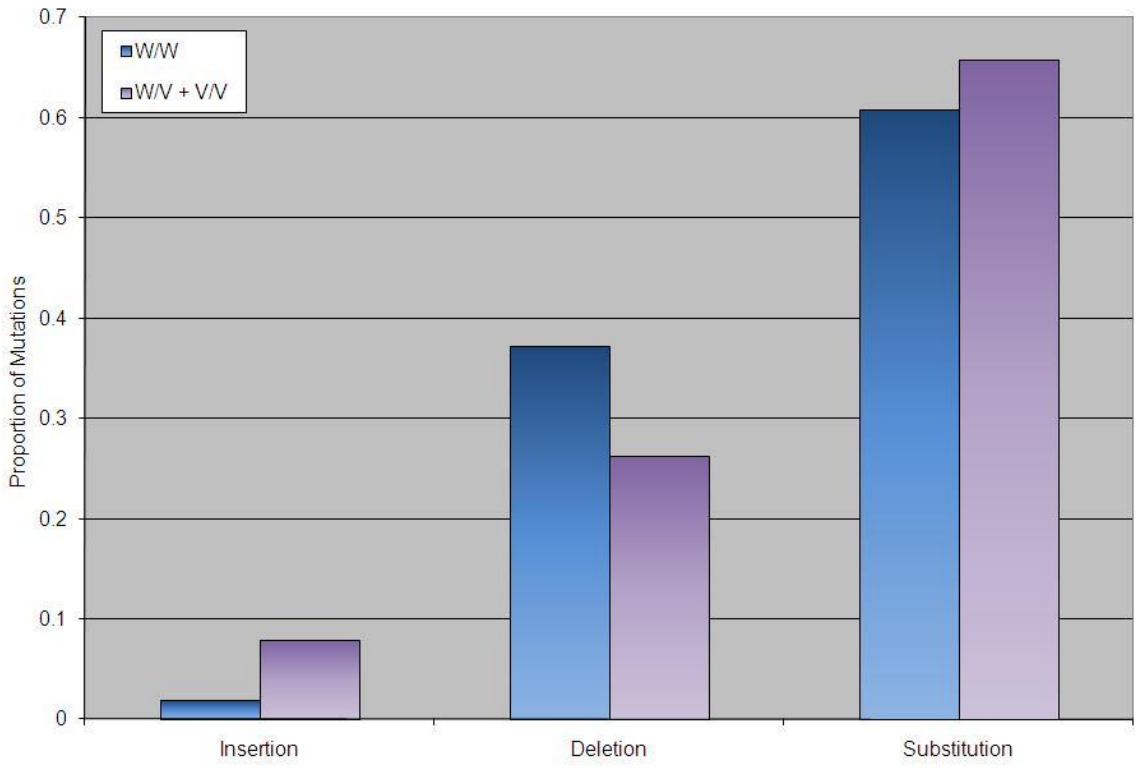

Figure 17: Effect of the L84F SNP on the *HPRT* mutation spectrum

Since MGMT protects mainly against G → A transition mutations, we specifically looked for these mutations to determine if, among all point mutations, the G → A was more common among individuals with the 84F genotype. There was no significant difference between wild-type or variant individuals in the proportion of G → A mutation rates ($p>0.10$). Sequencing is limited to cDNA, therefore it is unknown if any splice variant mutations could have been the result of G → A transition mutations.

Since smokers are exposed to a larger number and amount of mutagenic agents compared to non-smokers, we hypothesized that the influence of polymorphisms in the MGMT protein on the spectrum of inactivating mutations would only be seen under this increased carcinogenic burden. We therefore re-ran the statistical analysis for the 84F

polymorphism only in smokers, considering the hypothesis that, due to the increase in exposure to mutagenic agents, we would be more likely to see an effect. However, there was no effect of the 84F polymorphism on the mutation spectrum in smokers ($p>0.50$).

When the effect of the I143V polymorphism on the mutation spectrum was evaluated, there was no significant difference between cells from individuals who were homozygous for the wild-type I143 allele and cells from individuals with the 143V variant allele ($p>0.34$). The distribution of total mutations in cells from homozygous wild-type individuals was 49% base substitutions, 17% presumed splice variants, 23% deletions, 4% insertions, and 7% with no detectable change. A majority of base substitutions occurred at A (31%) or G (32%) on the transcribed strand. 44% of base substitutions were transition events. 57% of the splice variants resulted in a deletion, and loss of exons 2-3, 5, 7 or 8. 47% of the deletions were large (greater than one codon triplet). All insertions were 3 bp or less.

The distribution of total mutations in cells from heterozygous variant individuals was 39% base substitutions, 21% presumed splice variants, 18% deletions, 11% insertions, and 11% with no detectable change. A majority of base substitutions occurred at A (38%) or G (38%) on the transcribed strand. 62.5% of base substitutions were transition events. 80% of the splice variants resulted in a deletion, and loss of exon 2-3, 4, 7 or 8. 67% of the deletions were large (greater than one codon triplet). All insertions were 3 bp or less. There were no homozygous variant individuals in this portion of the study.

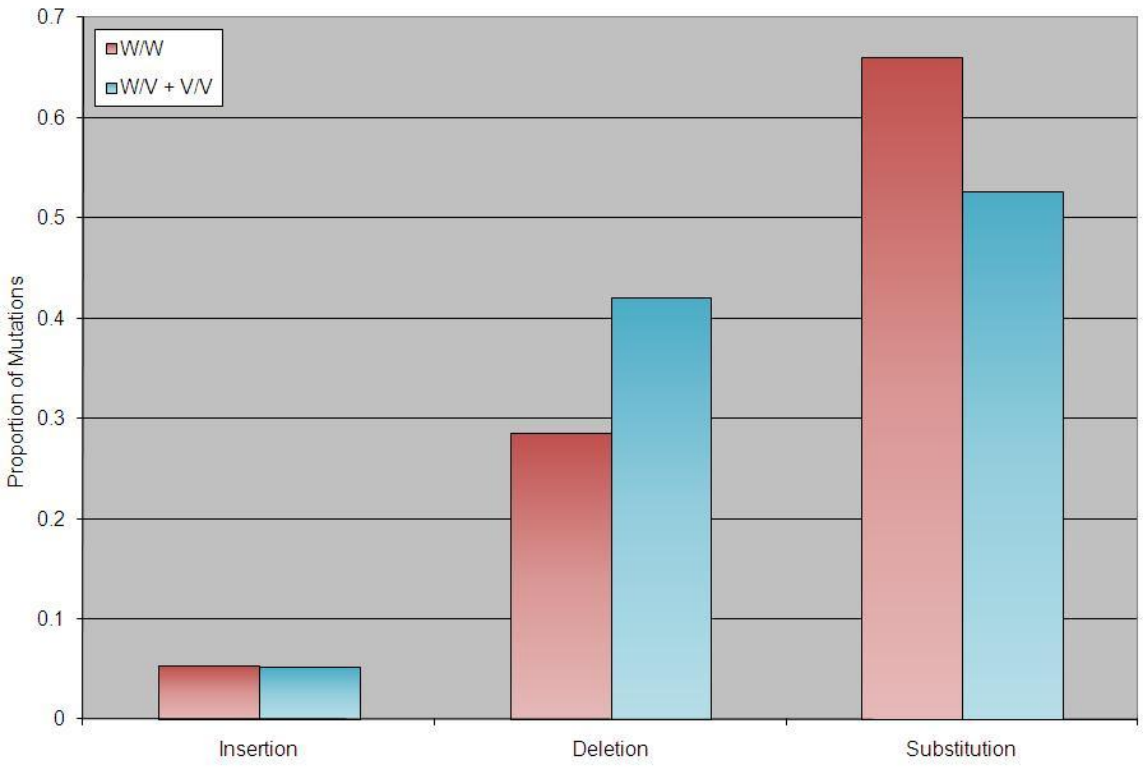

Figure 18: Effect of the I143V SNP on the *HPRT* mutation spectrum

RELATIONSHIP BETWEEN ENHANCER SNPS AND PROMOTER ACTIVITY

The goal of this study was to determine the effect of the C1099T SNP on promoter activity in a biologically relevant cell system. As this SNP was previously reported to increase promoter activity (Krześniak et al., 2004) in various cancer cell lines, we hypothesize that these data will be replicated in normal human bronchial epithelial cells. Data regarding the inducibility of *MGMT* is conflicting via exposure to methylating agents or DNA damage alone; however we propose that we will see an increase in promoter activity after exposure to whole mainstream cigarette smoke.

In order to determine any potential effect that the C1099T SNP has on promoter activity in the presence and absence of O^6-meG producing compounds, the wild-type and variant forms of the minimal *MGMT* promoter/enhancer region was cloned into the pGL-4 Basic vector, 5' to the luciferase gene and transfected into normal human bronchial epithelial cells. The luciferase gene under the control of the strong ubiquitous SV40 promoter was used as the positive control. Luminescence values depicting levels of expression are reported in figure 19.

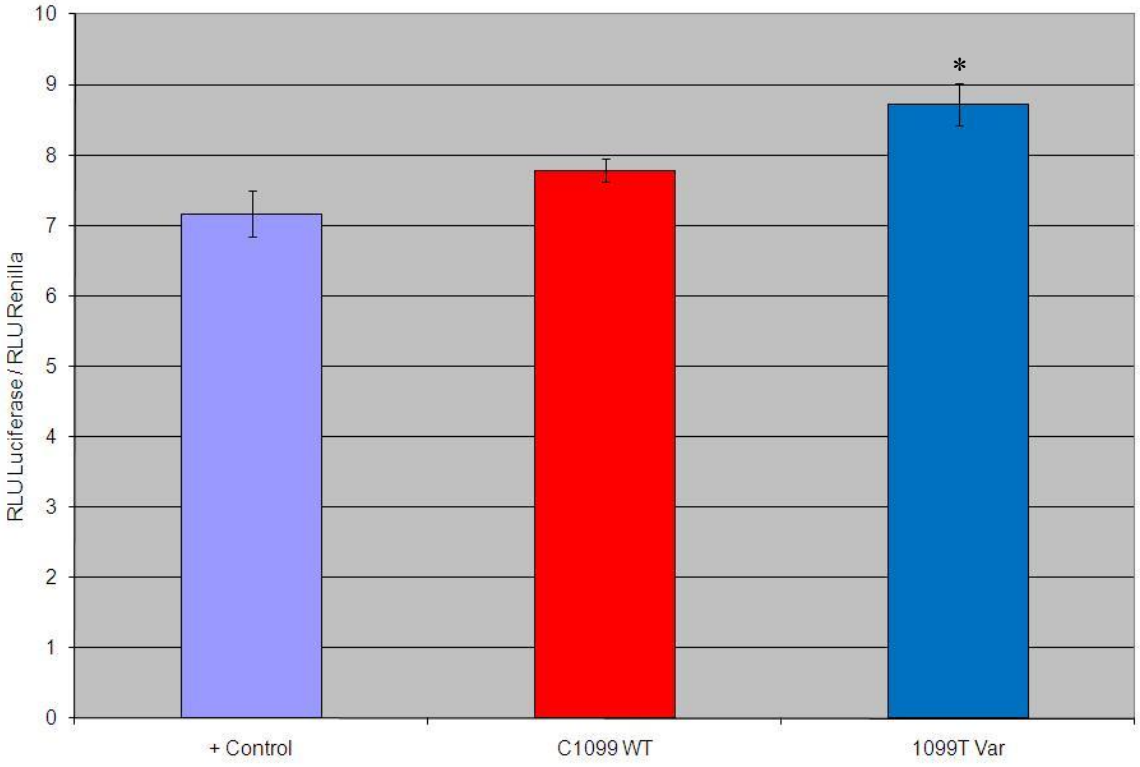

Figure 19: Effect of the 1099T enhancer SNP on transcriptional activity.

Although surprising, luciferase expression under control of the *MGMT* wild-type P/E promoter was not significantly different than the positive control SV40 promoter in NHBE cells, indicating strong *MGMT* promoter activity ($p>0.07$). As reported

previously (Krześniak et al., 2004), the 1099T SNP in the P/E region was associated with a 13% increase in promoter activity ($p<0.009$). Surprisingly, exposure to mainstream tobacco smoke completely shut down promoter activity for both the experimental plasmids and the control *Renilla* plasmid (data not shown). This may be due to several factors, including cell toxicity or deactivation of all but necessary transcription while the cells recover.

CHAPTER 4: DISCUSSION AND FUTURE STUDIES

The goal of this research was to elucidate the biological and functional consequences of single nucleotide polymorphisms within the direct reversal repair gene *MGMT*. Previous studies indicate an increased risk for various types of cancers associated with carcinogen exposure and inheritance of one or more SNPs. However, little was known regarding the functional consequences of these SNPs or their effect on markers upstream from cancer development such as gene mutations or DNA damage. We used the well-validated mutagen-sensitivity assay and the *HPRT* gene mutation assay as biomarkers of effect to determine influence of age, gender, exposure status and *MGMT* cSNPs on these endpoints. In addition, referent and variant constructs of the promoter/enhancer region were created in order to determine effects of a SNP in this area on promoter activity.

RELATIONSHIP BETWEEN *MGMT* POLYMORPHISMS AND CHROMOSOME ABERRATIONS AS A BIOMARKER OF EFFECT

The goal of this study was to investigate the relationship between two cSNPs in the *MGMT* gene and levels of genetic damage associated with exposure to tobacco carcinogens. Several cSNPs in MGMT have been described, including L84F, I143V, K178R, (which was in linkage disequilibrium with I143V) and the rare G160R. In this study we focused on the L84F cSNP, due to its relatively high reported frequency in humans and could thus affect a large segment of the general population. We also focused on the I143V cSNP, because of its close proximity to the active cysteine residue at position 145 of the protein. After nucleotide flipping, the DNA adduct is transferred from the base to the active cysteine at position 145. Our data indicate that the presence of

the 84F allele results in an overall increase in NNK-induced CAs but not in particular subgroups. Although there was no overall difference in NNK-induced CAs in cells from individuals who carry the 143V allele, there were significant differences in the response to the mutagenic effects of NNK in specific subgroups of individuals.

The mutagen-sensitivity assay is a well-validated method that is used to detect potential variations in susceptibility to the effects of genotoxic agents. This assay is based on the quantitation of mutagen-induced CA in cultured peripheral blood lymphocytes obtained from study subjects. The mutagen-sensitivity assay reflects the individual's sensitivity to the mutagen used in the assay, as well as the DNA repair capacity in that individual (Hsu et al., 1989). Population studies, using phenotypic DNA repair assays, have demonstrated interindividual variability in the ability to repair DNA (Oesch et al., 1987; Takano et al., 1991) and have documented associations between reduced DNA repair and susceptibility to cancers at several sites (Spitz and Bondy, 1993; Wei et al., 1994, 1996). Several investigators have reported that individuals with lung cancers show higher mutagen-sensitivity within surrogate cells such as PBLs compared to control subjects that do not have cancer (Spitz et al., 1995; Wu et al., 1998, 2000, 2007; Li et al., 2001; Schmezer et al., 2001; Zheng et al., 2003; Shen et al., 2003; Kosti et al., 2010; Liu et al., 2010; Patel et al., 2010). Other investigators have utilized this assay for other tobacco-related cancers and have found associations between mutagen-induced CA levels and head/neck cancer (Xiong et al., 2007; Cloos et al., 1996; Schantz et al., 2000; Wu et al., 1998) and bladder cancer (Aben et al., 2000). In addition, many laboratories, including ours, are using the mutagen-sensitivity assay to examine the role that polymorphisms in susceptibility genes might play in the cellular response to environmental toxicants (Abdel-Rahman et al., 2001, 2003, 2005; Wang et al., 2003;

Affatato et al., 2004; Hill et al., 2005; Iarmarcovai et al., 2006; Laczmanska et al., 2006, 2007).

To our knowledge, this is the first investigation that addressed the effect of these SNPs on cytogenetic damage induced by exposure to specific tobacco carcinogens. Our data are supportive of epidemiological studies that examined the effect of SNPs in *MGMT* on lung cancer risk. Due to the variability of sample size, tumor grade, and exposure to potentially carcinogenic compounds, data from these studies are at best inconsistent. Two studies reported no significant difference in lung cancer risk for individuals with the 84F or the 143V (Krześniak et al., 2004; Chae et al., 2006), or the K178R polymorphism (which is in linkage disequilibrium with the 143V polymorphism) (Yang et al., 2004). Wang et al. reported an increased 84F and 143V variant allele frequency in non-Hispanic white lung cancer cases compared to controls (Wang et al., 2006). In addition, there was a synergistic effect within the 4 SNPs examined; having a combination of SNPs was associated with an increase in lung cancer risk compared to having none of these SNPs. This effect was most pronounced in women and current smokers (Wang et al., 2006). Two other studies examined the effect of the I143V polymorphism and are in general agreement that this cSNP was associated with about a two-fold increase in lung cancer risk (Kaur et al., 2000; Cohet et al., 2004). In contrast, Zienolddiny reported that the K178R variant was associated with lower PAH-DNA adduct levels within normal lung tissue (Zienolddiny et al., 2006) and Crosbie et al., reported a lower risk of lung cancer with the 178R variant (Crosbie et al., 2008). Only one other study has examined the association between SNPs in MGMT and DNA damage response after exposure. Background, but not gamma-radiation induced single strand

breaks (SSBs), were more prevalent in PBLs of cells from individuals with the variant 84F allele (Rzeszowska-Wolny et al., 2005).

Our data are also in agreement with animal studies with transgenic mice deficient in Mgmt. Studies have indicated that the Mgmt deficient mouse is more sensitive to nitrosamine-induced lung tumors (Iwakuma et al., 1997). Furthermore, a transgenic mouse line lacking mouse Mgmt but expressing human MGMT showed resistance to NNK-induced lung tumors compared to non-transgenic mice (Liu et al., 1999). In this study, MGMT positive mice also demonstrated lower levels of O^6-meG adducts in lung tissue and fewer K-*ras* G → A transition mutations (Liu et al., 1999). In humans, reduced MGMT activity, as manifested by decreased expression due to promoter hypermethylation, is associated with increasing non-small cell lung cancer (NSCLC) tumor grade and an increase in G→ A transitions in the *TP53* gene and at CpG sites (Wolf et al., 2001; Pulling et al., 2003; Topaloglu et al., 2004; Ekim et al., 2011). These data support our findings and demonstrates the importance of proper *MGMT* regulation, expression, and activity as well as the potential carcinogenicity of unrepaired O^6-meG adducts.

As MGMT protein levels vary greatly among tissues within the same individual, as well as between individuals, data may be confounded and obscure associations with these SNPs. It is possible that the SNPs themselves can cause allelic imbalance, affecting protein levels. Margison et al., (2005) demonstrated an allelic imbalance in four of seven PBL samples from patients participating in a bronchoscopy study. The wild-type referent lysine at codon 178 was over-expressed compared to the variant arginine in heterozygous individuals. The lower mRNA levels could result in decreased protein levels, conferring sensitivity to alkylation damage within cells variant at codon 143/178.

Due to a smaller percentage of unrepaired O^6-alkylguanine adducts being converted to chromosome aberrations versus mutations, any potential effects of the SNPs of interest was expected to be small. We demonstrated a significant increase in NNK-induced CA in individuals with the 84F variant 24 hours following NNK-exposure. No difference was seen 1 hour after exposure due to the mechanism of CA formation; cells require one round of DNA replication to convert the nick left from mismatch repair into a chromosome or chromatid type break (Margison et al., 2002). Therefore it was crucial to investigate mutation rates as well as CA levels, by using the *HPRT* mutation assay.

RELATIONSHIP BETWEEN *MGMT* POLYMORPHISMS AND THE *HPRT* MUTATION ASSAY AS A BIOMARKER OF EFFECT

The primary goal of this research was to investigate the potential influence of two cSNPs in the *MGMT* gene and one SNP in the enhancer region on frequencies of *in vivo* somatic cell mutations in lymphocytes of individuals exposed to alkylating agents such as those present in tobacco smoke. The *HPRT* cloning assay was used in this study because it is one of the few sensitive assays available for the *in vivo* measurement of somatic cell mutations in humans (Compton et al., 1991; Albertini 2001). Relying on the X-linked *HPRT* gene as an endogenous reporter of possible genome-wide somatic cell mutations, researchers have used the assay to examine the association between somatic cell mutations and exposure to environmental, occupational, or therapeutic agents, or other phenomena, such as age. Unfortunately, because the assay is labor- and time-intensive, and the scoring of assay plates using the conventional visual approach is prone to subjective analysis, the assay is not widely used except in highly specialized laboratories. In an effort to make the assay more convenient for use in less specialized settings and to

make it less time-consuming and more objective, we refined a semi-automated method (Dobrovolsky et al., 2000) in order to address these issues. Our approach involved discriminating proliferating lymphocyte clones from non-proliferating lymphocytes using an indicator dye, alamarBlue®, that is metabolically reduced by proliferating cells to a fluorescent form. A properly configured microplate reader was then used to identify those wells that contain proliferating lymphocyte clones. In addition, we have also streamlined the approach used to generate *HPRT* mutation spectra. These improvements resulted in a time-saving of approximately 50% per assay. We utilized these new techniques to investigate the effect of age, smoking, and inherited polymorphisms in the *MGMT* gene on the frequency and spectra of mutations at the *HPRT* gene locus in human lymphocytes.

Consistent with reports by other investigators, we found a significant correlation between age and increased *HPRT* MF (King et al., 1994; Jones et al., 1995; Morley 1998; Liu et al., 1997). These earlier studies consistently indicated that *HPRT* MFs increase linearly with age at a rate of approximately 1-2% per year (Vrieling et al., 1992; King et al., 1994; Cole and Skopek 1994; Jones et al., 1995). We found a significant age-related increase in MFs with an annual increase of about 1.8% or 0.18 mutagenic events per million cells. Our data also indicate that the MFs are negatively correlated with cloning efficiency (CE) values. This correlation was controlled for using the method of Cochrane and Skopek (1992) to adjust final MF values by the CE. In accordance with earlier reports, there was no impact of age on CE (King et al., 1994; Kumar et al., 2004). This indicates that the observed increase in MFs with age is not due to a CE-age confounder.

Many groups have investigated the effect of smoking on *HPRT* mutant frequency and on the spectra of mutations in smokers using the *HPRT* mutant lymphocyte assay. A

few studies found no association between smoking and increased MF (Davies et al., 1992; Branda et al., 1993). However, many other studies indicated an increase in MF in smokers compared to non-smokers (Vrieling 1992; Jones et al., 1993; Duthie et al., 1995; Jones et al., 1995; Podlutsky et al., 1999; Curry et al., 1999; Hou et al., 1999; Kumar et al., 2004). Our results are concordant with the published studies that indicate an increase in *HPRT* MF in smokers compared to non-smokers. This increase in MF is, presumably, a result of increased exposure to methylating agents, polycyclic aromatic hydrocarbons (PAHs) and other carcinogens found in tobacco smoke.

We observed an increase in *HPRT* MF in female smokers compared to non-smokers but no such observation was present in males (Hill et al., 2007). While the increase in *HPRT* MF could be indicative of a gender difference in the metabolism of NNK, it could be indicative of a gender difference in DNA repair efficiency. Hüttner et al. (1995) reported that the increased MF in smokers was only present in females, although this was not confirmed in other studies (Hüttner et al., 1995). Other investigators have attempted to elucidate secondary mechanisms which could contribute to increased sensitivity in females. For example, comparisons of cytochrome P450 (CYP) expression in normal lung tissue and in lung tumors reveal some startling differences between males and females. Specifically, studies have shown that *CYP1A1* expression in lung tissue was twice as high in females as in males (Mollerup et al., 1999). In the same study, it was also reported that females had higher levels of bulky DNA adducts in both tumor and non-tumor tissues than males. The number of adducts correlated with *CYP1A1* expression (Mollerup et al., 1999). It should be pointed out that *CYP1A1*, which plays a role in NNK bioactivation, is known to be induced by smoking (Smith et al., 2001). Such induction could lead to an increased adduct burden in females

compared to males. This is consistent with results from another study, in a Taiwanese population, that documented higher levels of bulky adducts in non-smoking female lung cancer patients compared to non-smoking male lung cancer patients (Cheng et al., 2001). Although these studies examined bulky adduct formation rather than mutagenic events, the observations are consistent with increased activation of NNK in women, either due to higher *CYP1A1* expression and presumably higher activity, or a reduced capacity for the repair of NNK-induced genetic damage. The high bulky adduct load could lead to elevated frequencies of mutagenic events.

Another possible explanation for the observed gender difference with NNK is the role that estrogens and estrogen receptors (ERs) might play in the carcinogenic process. In a human population study, an association was found between estrogens and the development of adenocarcinoma, the most prevalent type of lung cancer in women (Taioli and Wynder 1994). Specifically, women who had an early menopause were found to be at decreased risk for adenocarcinoma, while women on estrogen therapy were at increased risk. Normally, women have both ERα and ERβ in tumor tissues while men predominantly have ERβ, which results in gender differences in response to estrogens (Fasco et al., 2002). In addition, there is a multiplicative effect in women of hormone replacement therapy and smoking on the risk of developing adenocarcinoma of the lung (Taioli and Wynder 1994). In concordance with this, treatment of two *MGMT* hypermethylated lung cancer cell lines with 17β-estradiol restored normal *MGMT* methylation patterns and mRNA expression (Lai et al., 2009). This pattern of lower hypermethylation rates in women, either younger or taking hormone replacement therapy, coincides with development of adenocarcinoma. Because of the close correlation between exposure to NNK and the development of adenocarcinoma of the lung (Hecht et

al., 1998), our data could provide a possible explanation for the increased incidence of adenocarcinoma that has been observed in female smokers (Payne 2001). The combination of a slight decrease in DNA repair efficiency, increased carcinogen activation, and hormone interaction could lead to an increased risk in females of adduct development, DNA damage and, ultimately, cancer development.

There is still some controversy surrounding the effect of smoking on the *HPRT* mutation spectrum. The majority of the research indicates that there is no significant change in the overall *HPRT* mutation spectrum with smoking exposure (Vrieling et al., 1992; Burkhart-Shultz et al., 1996; Podlutsky et al., 1999, Hackman et al., 2000). Our findings are consistent with these studies, supporting no significant difference in the mutation spectrum between smokers and non-smokers. Podlutsky et al., demonstrated no shift in the complete *HPRT* mutation spectrum in smokers compared to non-smokers, but reported a higher percentage of base substitutions (72% for non-smokers; 78% for smokers) than the levels we observed in our study (52% for non-smokers and 37% for smokers). This difference, which was observed in the magnitude of the response, could be attributed to the fact that, in the studies of Podlutsky et al., only full length transcripts were sequenced. As such, large insertions and deletions could not be detected. However, they also concluded that there was a higher frequency of transversions in smokers, particularly G → T transversions (Podlutsky et al., 1991). Our study demonstrated no difference in the transition/transversion ratio between smokers and non-smokers. Børresen et al., (1990) and Burkhart-Schultz et al., (1996) also found no difference in the mutation spectrum for base substitutions between smokers and non-smokers. On the other hand, Hackman et al., (2000) reported a difference in the *HPRT* mutation spectrum between smoking and non-smoking subjects who had been diagnosed with lung cancer.

They reported a significant difference in the proportion of transition and transversion mutations between these smokers and non-smokers. Our research, however, was not comparable to studies of cancer patients since we used only apparently healthy individuals.

To our knowledge, our project was the first to address the relationship between germline polymorphisms in *MGMT* and somatic cell gene mutations in humans. We focused on the L84F cSNP, because this polymorphism was found at a relatively high frequency in the general population (Krześniak et al., 2004; Egyhazi et al., 2002), and also because of our findings indicating that this polymorphism confers increased sensitivity to genetic damage induced by the alkylating tobacco-specific carcinogen, NNK (Hill et al., 2005). We also studied the I143V cSNP because of its close proximity to the active cysteine residue at position 145 of the MGMT protein, which is essential for protein function (Kaur et al., 2000). Our data indicate that, while there was no significant association between the I143V polymorphism and *HPRT* MF, cells from individuals with at least one copy of the 84F variant allele did show an increase in MF compared to cells from individuals homozygous for the referent wild-type L84 allele. This effect, however, was only statistically significant in smokers.

These results suggest a possible gene-environment interaction. The 84F polymorphism could affect the phenotype of the MGMT protein, resulting in suboptimal repair of genetic damage in the presence of exposure to alkylating agents, such as those found in tobacco smoke. These results also indicate that this cSNP does not completely abolish MGMT activity, but rather causes only a slight decrease in such activity. Such a slight decrease in activity due to this polymorphism could manifest as an increase in MF after exposure to alkylating agents. Our observation was consistent with other investigators

who demonstrated that Chinese hamster ovary cells with low MGMT activity, when treated with the alkylating agent dacarbazine (Psaroudi and Kyrtopoulos 2000) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Yang et al., 1994), had a significant increase in *HPRT* MF and an increase in G → A transitions compared to cells with high MGMT activity. Furthermore, our findings are consistent with those of other investigators, who reported that depletion of MGMT by pretreatment of cells with O^6-benzylguanine, a selective MGMT inhibitor, increased the toxicity and mutagenicity of the alkylating agents N-ethyl-N-nitrosourea (ENU) (Tong et al., 1997) and MNNG (Lukash et al., 1991). While our results indicating no significant effect for the I143V polymorphism on *HPRT* MF was unexpected, given that this polymorphism is in close proximity to the active cysteine moiety at position 145 of the MGMT protein, the results are in agreement with our previous observations indicating no significant overall effect of this polymorphism on chromosome aberration (CA) levels (Hill et al., 2005). Specifically, this polymorphism was not significantly associated with increases in baseline CA levels based on smoking status (Hill et al., 2005). It should be pointed out, however, that CA and mutation frequency represent two different types of lesions. The *HPRT* gene mutation assay detects "microlesion" changes in DNA, such as point mutations or small deletions, whereas cytogenetic endpoints chiefly reflect "macrolesions" that can result from recombinational events or errors in chromosomal segregation. Therefore, a direct comparison between the results of the two endpoints was not totally appropriate.

Because a decrease in MGMT activity could lead to an increase in G→A transition mutations (Margison et al., 2002), we hypothesized that cells from individuals with a polymorphism that alters MGMT activity would have increased levels of base

substitution mutations and G → A transitions. In addition, we hypothesized that this effect might be exacerbated by smoking. Consistent with these hypotheses, our data suggest a possible increase, although it was not statistically significant, towards an increase in base substitution mutation frequency in cells with the 84F polymorphism compared to cells homozygous for the wild-type L84 allele, regardless of the smoking status ($p=0.23$). In addition, because we measured mutations rather than actual adduct levels, we do not know if the observed mutation was a result of an unrepaired adduct on the transcribed or non-transcribed strand. Because of the relatively small sample size of the current study, we could not evaluate the interaction between this polymorphism and smoking on the mutation spectra. Our findings are consistent with the hypothesis that the L84F polymorphism causes only a subtle reduction in MGMT activity. Nevertheless, regardless of the type of mutations, it is clear from the observed increase in *HPRT* MF that the 84F polymorphism could negatively impact the repair of DNA damage. While the exact mechanism by which this polymorphism might exert its effect is still not clear, one possibility is that the 84F polymorphism could affect zinc binding, which is needed for full MGMT activity (Rasimas et al., 2003a). Native protein contains one zinc molecule and is coordinated through four residues, including H85 (Daniels et al., 2000). The bulky phenylalanine amino acid at residue 84 could inhibit coordination of the zinc atom by H85.

Our data indicate that individuals with the 1099C SNP had a two-fold increase in MF compared to individuals homozygous for the wild-type C1099 allele ($p<0.04$), however, these data may be biased due to environmental and genetic factors. We found that each person with the variant 1099C allele was also variant for the 84F SNP. Within our data set, the 84F SNP alone was associated with higher *HPRT* MF compared to

homozygous wild-type individuals. In addition, the individuals with the 1099T SNP were all smokers and over the median age of this population. Therefore, while these preliminary studies may indicate a potential effect of the 1099T SNP on genetic damage, a firm conclusion in this regards cannot currently be made. A larger sample size is required to further understand the mechanism by which this SNP exerts its effect, both independently, and in conjunction with the 84F codon SNP and their interaction with age and smoke exposure on *HPRT* MF. Due to this linkage disequilibrium and low SNP frequency, we chose to focus on the promoter SNP for functional studies to determine any effect on promoter activity.

USE OF THE LUCIFERASE ASSAY TO DETERMINE EFFECT OF PROMOTER *HPRT* POLYMORPHISMS ON BACKGROUND AND MUTAGEN-INDUCED EXPRESSION

The goal of this study was to determine the effect of the C1099T SNP on promoter activity in a biologically relevant cell system. Data regarding the inducibility of *MGMT* is conflicting; there is limited research on the association between human *MGMT* expression before or after exposure to various alkylating agents in a biologically relevant system. NIH 3T3 cells and HeLa S3 cells both demonstrated no inducibility of the MGMT protein 4-12 hours after exposure to 5 μM MNNG (Fritz et al., 1991). Using the CAT assay, Grombacher et al., (1996) showed no inducibility of the human *MGMT* promoter after exposure to 60 μM of the alkylating MNNG or ionizing radiation in HeLa S3 cells (Grombacher et al., 1996). In human population studies, two research groups have demonstrated an increase in MGMT protein levels within tumor tissue of the lung or esophagus in smokers (Mattern et al., 1998; Nozoe et al., 2002). In contrast, Povey et al., (2006) showed a statistically significant lower MGMT activity in smokers compared to

non-smokers within normal bronchial epithelium. In addition, epithelial cells from a patient with oral leukoplakia and histological evidence of hyperplasia exposed to smokeless tobacco extract resulted in a decrease in MGMT protein expression (Rohatgi et al., 2005). Whether these effects are due to a cell selection process during tumor development or a normal cellular response to environmental tobacco smoke is unclear. Although the data seem conflicting, high MGMT levels have been seen only within tumor tissue, while a decrease in expression is evident in non-cancerous tissue after exposure

Polymorphisms in the promoter and enhancer regions are capable of altering basal or induced promoter activity due to the creation or destruction of transcriptional or repressive binding sites. Multiple SNPs in the P/E region of *MGMT* have been identified but researchers have only begun to study them in regards to methylation patterns, promoter activity and protein levels. A variant at the 1099 position (ref X61657, Harris et al., 1991) results in a C → T change in the palindrome GGTGCGCACC (Rusin et al., 1999). This particular SNP was associated with a statistically significant 16% increase in promoter activity in A549 cells, but not with lung cancer incidence within a small Polish population (Krześniak et al., 2004). These data were confirmed in our study using the biologically relevant human bronchial epithelial cells rather than a cancer cell line. Our data indicate a 13% increase in promoter activity with the variant T allele as compared to the referent C allele. This particular SNP was of interest because it potentially resides in a c-Myc binding site (TESS analysis, unpublished data). While TESS indicates the presence of the c-Myc binding site with the referent C allele, mutating the sequence to include the T variant allele could theoretically destroy the binding site. Unfortunately no

data is available regarding the activity of c-Myc on the *MGMT* promoter and will be a fruitful avenue for future studies.

Although unlikely during this study, promoter SNPs can also affect the epigenetic status of *MGMT* and influence the gene's transcriptional regulation. For example, it is known that, like most genes, *MGMT* transcription is, in part, regulated by CpG methylation, and this affects MGMT activity (Qian et al., 1995; Qian and Brent 1997; Watts et al., 1997; Bhakat and Mitra 2003). Recent studies with other genes demonstrate relationships between SNPs in the promoter regions and CpG methylation. For example, Taylor et al. (2007) recently reported a significant association between the methylation profile of the promoter of the *LRP1B* gene and the presence of a G to C SNP (rs1375610) in the promoter region of the gene. The odds of methylation at this site were twice as high for the C allele versus the G allele (Taylor et al., 2007). Savage et al., also recently reported that the EX16+88G>A (rs998075) SNP in the *IGF2R* gene alters CpG methylation, providing further evidence of functional effects of promoter SNPs on methylation status (Savage et al., 2007). As with the examples cited above, it is plausible to hypothesize that SNPs in the *MGMT* P/E region could significantly impact CpG methylation. Consistent with this hypothesis, a recent study by Ogino et al., found a significant association between the C>T SNP (rs16906252), promoter methylation, and reduced expression of MGMT in tumors of colorectal cancer patients (Ogino et al., 2007). In addition, the variant allele was significantly associated with increased methylation in adenocarcinoma tissue and in sputum samples from current smokers (Leng et al., 2011). The exact mechanism, however, is still unclear and warrants further investigation. The epigenetic effects of SNPs on P/E regions will be a fruitful avenue for future studies.

We were interested in determining effects of whole mainstream smoke exposure on *MGMT* promoter activity. Exposure resulted in a 100% decrease in promoter activity not only for the *MGMT* promoter, but also the positive internal control SV40 promoter, indicating a complete downregulation of transcriptional activity. Although cells did not exhibit typical changes associated with toxicity (rounding, vacuolorization, lack of filopodia), it is possible that the cells were undergoing a toxic response that we did not evaluate in our study. Although the exposure time we selected was comparable to other studies (Albino et al., 2006) the cells were allowed to recover for a longer period of time (19 hours versus 1 hour). In addition, cells were exposed in complete medium rather than PBS and the medium was not changed after the exposure time was completed. Oxidative stress from cigarette smoking is responsible for protein oxidation, leading to modification of amino acid residues and accumulation of protein carbonyl adducts within the plasma of exposed individuals (Reznick et al., 1992; Berlett and Stadtman 1997; Lee et al., 1998; Marangon et al., 1999; Pignatelli et al., 2001). In vitro exposure of plasma to cigarette smoke results in formation of protein carbonyl adducts, altering protein structure and function (Reznick et al., 1992; Cross et al., 1993; Panda et al., 1999). It is possible that protein oxidation during cigarette smoke exposure depleted functional serum proteins or other required supplements, preventing the cells from adapting and recovering after exposure.

We did, however, demonstrate a significant 13% increase in promoter activity with the variant T allele compared to the referent C allele. This is in concordance with previous findings (Krześniak et al., 2004). Other SNPs within the promoter/enhancer region of the gene, which reside in presumed protein binding sites and CpG islands, will be of interest in future studies.

CONCLUSIONS AND FUTURE DIRECTIONS

The goal of this research was to elucidate the biological and functional effects of polymorphisms both within the MGMT protein and 5' upstream of the gene within the promoter region. The data demonstrated a detrimental effect of the coding SNP at position 84 within the protein, resulting in an increase in genetic damage. In addition, the variant at position 1099 in the promoter/enhancer region increased transcriptional activity. While these studies threw more light on the effect of polymorphisms on the function of a critical protein, they also open the door for additional investigations. For example, with the advancement in the human genome project, it is clear that SNPs do not exist independently but exist in linkage disequilibrium with other SNPs. These combinations form haplotypes, which not only are inherited as a group but also exert their effects as a group. Laboratories are beginning to investigate haplotypes as combined risk factors for disease, as well as in relation to drug treatment. Our laboratory is currently performing haplotype analysis on the *MGMT* promoter as an area for future study. In addition, to SNPs in the promoter affecting transcriptional regulation directly (as shown via luciferase constructs), these SNPs may also alter methylation patterns within healthy cells and tumor cells. The C>T SNP (rs16906252) was associated with promoter methylation and reduced expression of MGMT in tumors of colorectal cancer patients, within adenocarcinomas tissue, and in sputum samples from current smokers (Ogino et al., 2007; Leng et al., 2011). The work presented in this dissertation opens the door for future studies in that direction.

Appendix

Table 4: Chromosome Aberration Data

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-123	M	White	68	Ex	15	30	22	W/W	W/W	1	1	2
S-124	F	White	60	Non	0	0	0	W/W	W/W	0	3	3
S-125	M	Af-Am	68	Non	0	0	0	W/W	W/W	0	6	1
S-126	M	White	55	Curr	40	20	40	W/W	W/W	0	3	2
S-127	F	White	64	Non	0	0	0	W/v	W/W	1	0	3
S-128	M	Hispanic	75	Ex	20	20	20	W/W	W/W	0	4	0
S-129	M	Af-Am	50	Ex	3	10	2	W/v	W/W	1	2	4
S-130	M	White	69	Ex	10	20	10	W/W	W/W	1	2	4
S-131	F	White	71	Ex	30	10	15	W/v	W/W	2	2	8
S-132	M	White	73	Curr	50	30	75	W/W	W/W	0	2	2
S-133	F	White	72	Ex	41	10	20	W/v	W/W	0	1	5
S-134	M	Af-Am	49	Non	0	0	0	W/W	W/W	0	2	2
S-135	M	White	77	Ex	11	40	22	W/W	W/W	1	0	7
S-136	F	White	67	Curr	55	5	14	W/W	W/W	0	4	1
S-137	F	White	56	Ex	34	10	17	W/W	W/v	0	1	3
S-138	M	White	68	Ex	50	40	100	W/W	W/W	1	1	7
S-139	F	Af-Am	27	Ex	4	10	2	W/W	W/W	1	1	5
S-140	F	White	42	Non	0	0	0	W/v	W/W	2	5	2
S-141	F	White	43	Non	0	0	0	W/W	W/v	0	2	5
S-142	F	White	49	Curr	30	15	22	W/W	W/W	1	0	3
S-144	M	White	40	Curr	27	5	7	W/v	W/v	0	0	11
S-145	F	White	74	Ex	30	5	8	W/v	W/W	1	2	6
S-146	M	White	75	Curr	60	20	60	W/W	W/W	0	1	2
S-147	M	White	73	Non	0	0	0	W/v	W/W	1	0	0

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-148	F	White	61	Curr	30	20	30	W/W	V/v	1	2	1
S-149	F	Af-Am	35	Non	0	0	0	W/W	W/v	0	2	0
S-150	F	White	52	Non	0	0	0	W/W	W/v	1	3	1
S-156	F	White	35	Non	0	0	0	W/W	W/v	3	5	0
S-157	M	White	35	Non	0	0	0	W/W	W/v	0	1	6
S-158	M	White	74	Ex	50	50	125	W/W	W/W	0	4	0
S-159	M	White	84	Ex	20	30	30	W/W	W/W	1	6	0
S-160	M	White	66	Ex	45	40	90	W/W	W/W	4	0	0
S-161	F	White	68	Curr	10	50	25	W/W	W/W	0	2	0
S-162	F	White	80	Non	0	0	0	W/W	V/v	0	0	3
S-163	F	White	73	Non	0	0	0	W/W	W/W	1	2	2
S-165	F	Asian	31	Non	0	0	0	W/W	W/W	0	0	2
S-166	M	White	42	Ex	12	30	18	W/W	W/v	0	3	2
S-168	M	Hispanic	26	Curr	9	20	9	W/W	W/W	0	3	2
S-169	M	Af-Am	67	Curr	50	20	50	W/v	W/W	0	4	7
S-170	M	White	45	Curr	30	10	75	W/v	W/W	0	1	2
S-171	M	White	62	Ex	10	40	20	W/v	W/W	0	1	2
S-180	F	White	51	Non	0	0	0	W/W	W/W	2	3	2
S-181	F	Af-Am	88	Non	0	0	0	W/W	W/W	1	0	6
S-185	F	White	62	Ex	50	20	50	W/v	W/W	1	2	3
S-186	F	Af-Am	63	Curr	25	30	38	W/W	W/v	2	4	3
S-187	F	White	65	Non	0	0	0	W/v	W/v	1	3	4
S-188	M	White	80	Curr	50	20	50	W/W	W/W	1	6	6
S-189	F	White	79	Non	0	0	0	W/W	W/W	1	2	5
S-190	M	White	51	Non	0	0	0	V/v	W/W	1	3	6
S-192	M	White	55	Ex	32	30	48	W/W	W/W	3	0	7
S-193	F	White	55	Ex	39	20	39	W/W	W/W	2	6	0
S-194	M	Hispanic	46	Non	0	0	0	W/W	W/W	0	1	6
S-195	F	Af-Am	23	Non	0	0	0	W/v	W/W	2	0	4

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-196	F	White	26	Curr	11	15	8	W/W	W/W	0	0	2
S-197	F	White	46	Curr	32	25	40	W/v	W/W	0	9	1
S-198	F	Af-Am	45	Curr	15	12	9	W/W	W/W	0	1	3
S-199	M	White	47	Curr	30	20	30	W/W	W/W	0	3	4
S-200	M	White	72	Curr	50	10	25	W/W	W/W	0	1	2
S-201	M	Af-Am	79	Ex	34	10	17	W/W	W/W	0	2	8
S-202	M	Asian	30	Curr	13	20	13	W/W	W/W	3	0	10
S-203	M	Hispanic	41	Ex	25	15	19	W/v	W/W	0	5	0
S-204	F	Hispanic	24	Non	0	0	0	W/W	W/W	0	0	6
S-205	F	Hispanic	37	Curr	25	20	25	W/v	W/W	1	0	2
S-206	M	Hispanic	29	Non	0	0	0	W/v	W/v	0	0	7
S-207	F	White	22	Non	0	0	0	V/v		0	1	1
S-208	M	Hispanic	28	Curr	10	20	10	W/W	W/W	1	0	3
S-211	M	White	24	Non	0	0	0	W/W	W/W	3	0	2
S-212	F	White	24	Non	0	0	0	W/W	W/W	0	1	4
S-213	F	White	28	Curr	5	5	1	W/W	W/v	1	1	2
S-214	F	Hispanic	37	Ex	9			W/v	W/W	0	2	0
S-215	F	White	25	Non	0	0	0	W/W	W/W	1	2	3
S-216	M	White	53	Curr	30	40	60	W/W	W/W	0	3	1
S-217	M	White	30	Non	0	0	0	W/W	W/W	1	2	1
S-218	M	White	42	Curr	20	20	20	W/W	W/W	0	1	0
S-219	M	White	24	Ex	1	10	1	W/W	W/W	1	3	0
S-220	F	White	40	Curr	21	15	16	W/W	W/W	0	7	0
S-221	M	White	53	Non	0	0	0	W/v	W/v	0	2	7
S-223	F	Af-Am	26	Non	0	0	0	W/W	W/W	0	1	0
S-224	F	White	48	Curr	30	20	30	W/v	W/v	2	2	1
S-225	F	White	23	Non	0	0	0	W/W	W/W	0	1	2
S-226	M	Asian	25	Non	0	0	0	W/W	W/v	0	2	2
S-227	F	White	40	Curr	22	20	22	W/v	W/W	0	3	2

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-228	F	White	28	Curr	15	10	8	W/W	W/v	2	1	4
S-229	F	Hispanic	47	Ex	12	20	12	W/W	W/W	3	1	5
S-230	F	White	23	Non	0	0	0	W/W	W/W	0	5	1
S-233	F	Asian	32	Non	0	0	0	W/W	W/W	0	1	5
S-234	M	White	47	Curr	25	20	25	W/W	W/W	0	4	6
S-235	F	White	36	Non	0	0	0	W/W	W/W	2	2	3
S-236	F	White	27	Curr	10	10	5	W/v	W/W	0	2	4
S-237	F	White	22	Non	0	0	0	W/W	W/W	0	1	2
S-241	F	Af-Am	37	Non	0	0	0	W/v	W/W	0	1	6
S-242	F	White	42	Ex	17	20	17		W/W	0	6	0
S-243	F	Hispanic	28	Non	0	0	0	W/W	W/W	1	0	2
S-244	F	White	30	Non	0	0	0	W/W	W/W	2	1	5
S-245	F	White	22	Non	0	0	0	W/W	W/v	0	2	1
S-246	M	White	33	Curr	9	15	7	W/v	W/v	2	0	3
S-247	F	White	28	Curr	16	18	14	W/v	W/W	1	0	5
S-260	M	White	77	Curr	69	20	69	W/W	W/W	1	2	1
S-262	F	White	22	Non	0	0	0	W/W		0	1	1
S-263	F	White	23	Non	0	0	0	W/W	W/W	1	2	2
S-264	F	White	24	Non	0	0	0		W/W	1	0	2
S-265	F	White	62	Ex	8	3	1	W/v	W/W	2	1	8
S-266	M	White	44	Curr	40	50	100	W/W	W/W	0	2	1
S-267	M	Hispanic	45	Ex	15	50		W/W	W/W	1	1	2
S-268	M	White	54	Non	0	0	0	W/W	W/v	0	2	0
S-269	F	White	70	Non	0	0	0	W/W	W/W	0	1	6
S-270	M	Hispanic	29	Curr	5	2	1		W/W	1	1	2
S-271	F	White	45	Ex	20	30	30	W/W	W/W	1	1	3
S-272	F	White	36	Ex	9	20	20	W/W	W/W	1	2	2
S-273	F	Af-Am	40	Non	0	0	0	W/W	W/W	1	1	2
S-274	F	Asian	22	Curr	9	10	4	W/W		0	7	2

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-317	M	White	67	Non	0	0	0	W/W	W/v	0	1	2
S-318	F	Af-Am	58	Curr	26	3	4	W/v	W/W	0	0	6
S-319	M	Asian	72	Non	0	0	0	W/W	W/W	2	1	7
S-320	F	White	39	Non	0	0	0	W/W	W/W	0	4	3
S-321	F	Hispanic	51	Non	0	0	0	W/v	W/W	2	1	8
S-323	F	White	37	Curr	19	20	19	W/W		1	0	2
S-328	F	White	27	Curr	2	9	1	W/W	W/W	0	5	3
S-329	F	White	44	Curr	30	30	45	W/W	V/v	0	5	3
S-331	F	White	71	Ex	40	40	80	W/v	W/W	0	4	1
S-332	M	White	67	Non	0	0	0	W/W	W/W	2	2	6
S-334	M	Af-Am	65	Non	0	0	0	W/W	W/W	0	2	1
S-343	F	Asian	43	Non	0	0	0	W/W	W/W	1	1	9
S-344	F	White	53	Ex	12	30	18	W/W	W/W	2	3	5
S-345	F	White	41	Non	0	0	0	W/W	W/W	1	2	4
S-346	F	White	22	Non	0	0	0	W/W	W/W	1	5	3
S-347	F	Hispanic	46	Non	0	0	0	W/v	W/v	1	1	2
S-348	F	White	22	Non	0	0	0	W/W	W/W	1	2	7
S-367	M	White	25	Non	0	0	0	W/W	W/W	7	0	0
S-368	F	White	22	Non	0	0	0	W/W	W/W	1	0	5
S-370	F	Af-Am	39	Curr	22	15	22	W/W	W/W	2	2	9
S-371	F	White	44	Non	0	0	0	W/W	W/W	0	2	3
S-372	F	White	48	Curr	27	20	40	W/v	W/W	1	0	3
S-373	F	White	46	Ex	23	20	23	W/W	W/W	1	5	0
S-374	F	Hispanic	30	Non	0	0	0	W/W	W/v	0	2	1
S-375	F	White	41	Non	0	0	0	W/W	W/W	1	0	5
S-376	F	White	42	Non	0	0	0	W/W	W/v	0	1	1
S-377	F	Af-Am	42	Non	0	0	0	W/W	W/W	1	1	2
S-378	F	White	43	Non	0	0	0	W/W	W/v	0	3	2
S-379	M	White	31	Non	0	0	0	W/v	W/W	1	0	5

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-380	F	Hispanic	27	Non	0	0	0	W/W	W/W	1	0	1
S-381	F	White	46	Ex	28	20	28	W/v	W/v	1	2	4
S-383	F	Af-Am	44	Curr	26	15	20	W/v	W/W	0	5	3
S-384	F	Af-Am	39	Curr	15	15	12	W/W	W/W	0	1	1
S-385	F	Af-Am	47	Curr	15	15	12	W/W	W/W	1	1	2
S-386	F	White	39	Ex	7	15	5	W/W	W/W	0	6	2
S-387	F	Asian	34	Curr	18	20	18	W/W	W/W	1	0	2
S-388	F	White	24	Curr	7	5	2	W/W	W/W	1	1	3
S-389	F	White	22	Non	0	0	0	W/v	W/W	1	0	4
S-390	M	White	22	Ex	5	2	1	W/v	W/v	0	6	2
S-391	F	White	42	Curr	25	12	13	W/W	W/v	2	2	5
S-393	F	White	34	Non	0	0	0	W/W	W/W	1	4	3
S-394	F	White	26	Non	0	0	0	W/W	W/v	1	4	4
S-395	F	White	27	Ex	6	5	1	W/W	W/W	7	3	0
S-396	F	White	29	Non	0	0	0	W/W	W/W	1	1	3
S-397	F	White	28	Ex	12	15	8	W/v	W/v	7	1	9
S-399	F	Hispanic	53	Non	0	0	0	W/W	W/W	3	4	4
S-400	F	White	49	Non	0	0	0	W/W	W/v	1	0	6
S-401	F	White	52	Non	0	0	0	W/W	W/W	0	2	3
S-402	F	Af-Am	45	Ex	25	3	6	V/v	W/W	1	0	4
S-403	F	White	37	Ex	3	10	2	W/W	W/W	0	1	2
S-404	F	White	25	Curr	10	20	10	W/W	W/W	1	0	4
S-405	F	Hispanic	51	Curr	26	20	26	W/W	W/W	1	5	2
S-406	F	Hispanic	40	Curr	22	7	6	W/W	W/v	2	2	5
S-407	F	Af-Am	35	Non	0	0	0	W/W	W/W	1	3	3
S-408	F	White	24	Curr	6	4	2	W/W	W/W	0	3	2
S-409	M	White	42	Non	0	0	0	W/W	W/W	1	4	0
S-410	F	Hispanic	42	Curr	20	3	4	W/W	W/W	1	0	2
S-411	F	White	51	Non	0	0	0	W/v	W/W	2	2	9

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-412	F	White	48	Non	0	0	0	V/v	W/W	0	5	2
S-413	M	White	31	Non	0	0	0	W/v	W/W	1	4	2
S-414	F	Af-Am	57	Non	0	0	0	W/W	W/W	2	3	5
S-415	F	Af-Am	51	Ex	27	10	13	W/v	W/W	0	2	2
S-416	F	Hispanic	41	Curr	20	15	15	W/W	W/W	1	3	4
S-417	F	White	44	Ex	30	30	45	W/W	W/v	0	4	3
S-418	F	White	64	Ex	35	40	70	W/W	W/v	0	3	3
S-419	F	White	24	Non	0	0	0	W/W	W/W	1	5	1
S-420	F	White	58	Non	0	0	0	W/W	W/v	2	3	13
S-421	F	White	49	Ex	10	40	20	W/W	W/W	0	4	6
S-422	F	White	48	Ex	22	20	22	W/W	W/v	1	2	4
S-423	F	White	59	Ex	10	15	8	W/v	W/W	0	5	3
S-424	F	White	36	Curr	19	20	19	W/W	W/W	2	3	6
S-425	M	Asian	25	Non	0	0	0	W/W	W/W	2	3	5
S-426	F	White	23	Non	0	0	0	W/W	W/W	1	2	8
S-427	F	Asian	22	Non	0	0	0	W/W	W/W	1	1	5
S-428	F	White	44	Curr	26	10	13	W/W	W/W	2	3	6
S-429	M	White	44	Curr	28	20	28	W/W	W/W	0	2	5
S-430	F	White	27	Ex	6	3	2	W/v	W/W	2	2	9
S-431	F	White	36	Curr	15	20	15	W/W	W/W	3	2	14
S-432	F	Hispanic	34	Non	0	0	0	W/v	W/W	3	7	4
S-433	F	Hispanic	46	Non	0	0	0	W/W	W/W	0	3	3
S-434	F	Asian	53	Non	0	0	0	W/W	W/W	0	5	3
S-435	F	Hispanic	36	Non	0	0	0	W/W	W/W	3	4	5
S-436	F	White	44	Non	0	0	0	W/W	W/v	1	1	3
S-437	F	Af-Am	21	Non	0	0	0	W/W	W/W	2	1	8
S-438	M	White	59	Non	0	0	0	W/W	W/W	1	0	1
S-439	F	White	44	Curr	30	20	30	W/W	W/W	2	1	5
S-440	M	White	25	Non	0	0	0	W/W	W/W	1	0	2

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-441	F	White	50	Ex	12	20	12	W/W	W/W	1	2	8
S-442	M	White	34	Curr	12	20	12	W/W	W/v	1	2	2
S-443	F	Hispanic	26	Non	0	0	0	W/W	W/W	1	2	5
S-444	F	Hispanic	39	Ex	20	15	10	W/v	W/W	1	0	3
S-445	M	White	30	Non	0	0	0	W/W	W/W	1	1	4
S-446	F	White	40	Non	0	0	0	W/W	W/W	2	1	3
S-447	F	Asian	22	Non	0	0	0	W/W	W/W	0	3	7
S-448	F	Hispanic	22	Curr	1	3	1	W/v	W/W	1	4	3
S-449	F	White	31	Ex	10	5	5	W/W	W/W	0	3	2
S-450	F	White	47	Ex	15	15	15	W/W	W/W	0	4	4
S-451	M	Hispanic	43	Curr	25	20	30	W/W	W/W	3	7	4
S-452	F	White	47	Ex	30	22	45	W/v	W/W	1	3	9
S-453	F	White	36	Ex	15	22	22	W/W	W/W	1	4	4
S-454	F	White	50	Ex	2	20	2	W/W	W/W	1	2	4
S-455	F	White	55	Ex	18	30	36	W/W	W/W	0	3	4
S-467	M	White	42	Curr	25	40	50	W/W	W/W	2	3	5
S-469	F	White	41	Non	0	0	0	W/W	W/W	1	4	0
S-470	F	White	24	Ex	5	30	2	W/W	W/v	1	4	6
S-472	F	White	27	Non	0	0	0	W/W	W/W	0	0	0
S-475	F	Af-Am	25	Non	0	0	0	V/v	W/W	1	4	3
S-480	M	Asian	40	Non	0	0	0	W/W	W/W	1	3	6
S-484	F	White	21	Non	0	0	0	W/W	W/W	0	1	2
S-490	F	White	60	Non	0	0	0	W/v	W/W	0	7	0
S-494	F	White	37	Non	0	0	0	W/W	W/W	0	4	3
S-496	F	White	34	Non	0	0	0	W/W	W/W	0	3	0
S-497	F	White	28	Non	0	0	0	W/W	W/W	0	0	2
S-505	F	White	22	Non	0	0	0	W/W	W/W	1	0	4
S-506	F	White	32	Non	0	0	0	W/W	W/W	1	1	3
S-507	F	White	31	Non	0	0	0	W/W	W/W	1	4	0

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-509	F	Hispanic	40	Curr	23	3	11	W/W	W/W	2	3	5
S-521	F	Af-Am	46	Non	0	0	0	W/W	W/W	1	3	5
S-524	F	Af-Am	37	Non	0	0	0	W/W	W/W	1	0	7
S-525	F	White	49	Curr	6	12	3	W/v	W/W	3	2	9
S-526	F	White	26	Curr	12	10	6	W/v	W/v	2	1	2
S-527	M	White	32	Non	0	0	0	W/W	W/v	0	5	5
S-536	M	Hispanic	28	Curr	7	1	1	W/W	W/W	2	2	2
S-537	M	Asian	31	Curr	3	10	2	W/v	W/W	1	2	0
S-538	F	White	34	Curr	18	20	18	W/v	W/W	1	2	2
S-540	F	Af-Am	45	Non	0	0	0	W/W	W/W	1	5	5
S-541	F	White	49	Non	0	0	0	W/W	W/W	4	0	4
S-542	F	White	29	Curr	3	1	1	W/W	W/W	3	0	6
S-544	F	White	51	Curr	30	20	30	V/v	W/W	2	5	5
S-547	M	Hispanic	27	Non	0	0	0	W/v	W/v	4	0	8
S-548	M	Hispanic	23	Non	0	0	0	W/W	W/W	2	2	3
S-550	F	White	39	Curr	24	20	24	V/v	W/W	0	2	0
S-551	F	Hispanic	31	Non	0	0	0	W/W	W/W	1	0	5
S-553	M	Hispanic	24	Curr	7	5	3	W/W	W/v	2	2	5
S-554	F	Asian	28	Non	0	0	0	W/W	W/W	0	6	2
S-555	F	Asian	27	Non	0	0	0	W/W	W/W	2	1	6
S-556	F	White	41	Non	0	0	0	W/W	W/W	0	2	0
S-557	F	Hispanic	26	Non	0	0	0	W/v	W/W	1	2	3
S-558	F	White	42	Ex	6	10	3	W/W	W/W	2	1	4
S-559	M	White	27	Non	0	0	0	W/W	W/W	0	6	0
S-560	M	White	23	Non	0	0	0	W/W	W/W	2	1	5
S-561	F	Hispanic	21	Curr	2	7	1	W/W	W/W	0	2	1
S-562	F	Hispanic	20	Non	0	0	0	W/v	W/W	2	0	3
S-563	F	White	30	Curr	10	15	7	W/W	W/W	5	4	2
S-564	M	Hispanic	40	Curr	23	5	11	W/W	W/v	1	0	5

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-565	F	White	47	Curr	34	30	51	V/v	W/W	1	2	1
S-566	M	White	61	Non	0	0	0	W/W	W/W	1	5	0
S-567	F	White	61	Ex	24	20	24	W/W	W/W	1	3	3
S-568	M	White	64	Ex	10	20	10	W/W	W/W	0	1	1
S-569	F	Hispanic	32	Curr	20	20	20	W/W	W/W	0	1	5
S-573	F	Af-Am	50	Non	0	0	0	W/v	W/W	3	8	0
S-574	F	Af-Am	38	Non	0	0	0	W/v	W/W	0	1	5
S-578	M	Hispanic	46	Curr	20	5	6	W/v	W/W	2	2	3
S-579	M	White	24	Ex	4	20	4	W/W	W/W	1	3	9
S-580	F	White	58	Non	0	0	0	W/W	W/W	0	3	4
S-581	F	White	25	Non	0	0	0	W/v	W/W	1	4	3
S-582	F	White	50	Non	0	0	0	W/v	W/W	1	4	4
S-583	F	White	45	Curr	30	15	22	W/W	W/W	0	2	2
S-584	F	Hispanic	25	Non	0	0	0	W/v	W/W	1	6	4
S-585	F	White	35	Non	0	0	0	W/W	W/W	0	5	3
S-586	M	White	34	Curr	18	15	13	W/W	W/W	1	5	2
S-587	F	White	50	Non	0	0	0	W/W	W/W	1	3	5
S-588	F	Hispanic	45	Ex	20	8	10	W/v	W/W	0	3	7
S-589	M	White	38	Non	0	0	0	W/W	W/v	1	2	4
S-590	F	White	47	Curr	31	20	31	W/W	W/v	1	2	6
S-591	F	White	23	Non	0	0	0	W/W	W/W	2	3	5
S-592	F	Hispanic	54	Non	0	0	0	W/W	W/W	1	4	6
S-593	F	White	38	Curr	12	10	6	W/W	W/W	0	3	3
S-594	F	Af-Am	54	Non	0	0	0	W/W	W/W	1	5	2
S-601	F	Hispanic	53	Non	0	0	0	W/W	W/W	1	2	1
S-603	M	Asian	24	Non	0	0	0	W/v	W/W	0	1	3
S-604	F	White	54	Non	0	0	0	W/v	W/W	0	2	5
S-605	M	Asian	39	Non	0	0	0	W/W	W/W	1	0	3
S-606	F	Asian	35	Non	0	0	0	W/W	W/W	0	1	2

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-607	F	White	24	Non	0	0	0	W/v	W/v	0	0	2
S-608	F	White	49	Ex	19	20	19	W/W	W/v	0	1	1
S-609	F	Hispanic	37	Non	0	0	0	W/v	W/W	1	1	2
S-610	F	White	30	Curr	8	20	8	W/W	W/W	0	3	2
S-611	F	Af-Am	29	Non	0	0	0	W/W	W/W	0	4	0
S-612	F	White	46	Non	0	0	0	V/v	W/W	1	2	4
S-613	F	Asian	22	Non	0	0	0	W/W	W/W	0	1	5
S-614	M	White	23	Curr	4	20	4	W/v	W/v	0	2	2
S-617	F	Asian	21	Non	0	0	0	W/W	W/W	0	2	1
S-618	F	Af-Am	44	Non	0	0	0	W/W	W/W	3	4	5
S-619	F	Hispanic	38	Non	0	0	0	W/W	W/W	2	3	6
S-621	M	Af-Am	42	Ex	5	15	2	W/W	W/v	1	3	8
S-623	F	Af-Am	35	Ex	19	15	17	W/W	W/W	3	7	4
S-624	F	Af-Am	21	Non	0	0	0	W/v		1	3	9
S-625	F	Af-Am	21	Non	0	0	0	W/W		0	3	4
S-626	F	Af-Am	37	Ex	15	40	30	W/W	W/W	1	2	4
S-627	F	Af-Am	49	Ex	30	20	30	W/v		2	1	7
S-628	M	Af-Am	44	Non	0	0	0	W/W		1	0	3
S-629	M	White	33	Non	0	0	0	W/W	V/v	1	3	4
S-630	F	Hispanic	21	Ex	8	30	12	W/W		1	2	8
S-631	M	Af-Am	19	Non	0	0	0	W/W		1	2	2
S-632	F	Af-Am	29	Ex	5	7	1	W/W		1	2	5
S-633	F	Hispanic	48	Ex	7	20	7	W/W		1	4	2
S-634	F	Asian	21	Ex	2	6	1	W/W		1	2	4
S-635	F	White	25	Non	0	0	0	W/W	W/W	1	0	4
S-636	F	White	20	Curr	4	20	4	W/W	W/W	0	2	4
S-637	M	Af-Am	20	Ex	3	20	3	W/W		1	2	7
S-638	F	White	28	Ex	8	22	9	W/v	W/v	1	3	2
S-639	F	Af-Am	31	Non	0	0	0	W/v		1	4	3

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-648	F	Af-Am	36	Curr	10	10	5	W/W	W/W	0	3	3
S-653	F	White	43	Non	0	0	0	W/W	W/W	0	0	2
S-654	F	White	24	Non	0	0	0	W/W	V/v	0	2	0
S-657	F	White	40	Ex	5	10	2	W/W	W/W	0	4	0
S-660	M	Asian	31	Non	0	0	0	W/v	W/W	0	2	0
S-661	F	White	26	Non	0	0	0	W/W	W/W	0	4	0
S-663	F	White	36	Ex	10	20	10	W/W	W/W	2	0	4
S-664	F	White	24	Non	0	0	0	W/v	V/v	0	2	0
S-665	M	White	24	Non	0	0	0	W/W	W/W	0	0	2
S-666	F	White	39	Curr	20	25	9	W/W	W/v	0	0	4
S-667	F	White	28	Curr	12	20	12	W/W	W/W	1	1	2
S-671	F	Af-Am	25	Non	0	0	0	W/v		2	3	9
S-672	F	White	47	Non	0	0	0	W/W	W/v	0	9	0
S-674	F	Af-Am	48	Non	0	0	0	W/W		3	4	6
S-675	M	Af-Am	64	Non	0	0	0	W/W		1	4	5
S-676	F	Af-Am	62	Non	0	0	0	W/W		3	2	5
S-677	M	White	69	Non	0	0	0	W/W	W/W	1	3	2
S-678	M	Hispanic	19	Ex	2	20	2	W/W		1	3	4
S-679	F	Af-Am	23	Non	0	0	0	W/W	W/W	0	3	3
S-680	F	Af-Am	23	Non	0	0	0	W/W	W/W	1	3	8
S-681	F	Hispanic	38	Non	0	0	0	W/W	W/W	0	2	2
S-682	F	White	21	Curr	3	3	1	W/W	W/W	0	2	3
S-684	F	White	19	Non	0	0	0	W/W	W/W	2	2	2
S-686	F	Af-Am	26	Non	0	0	0	W/W	W/W	0	1	0
S-687	F	White	35	Curr	18	15	9	W/W	W/W	0	4	0
S-688	F	White	21	Non	0	0	0	W/v	W/v	0	2	0
S-689	M	Hispanic	21	Non	0	0	0	W/v	W/W	2	2	6
S-690	F	White	44	Ex	13	20	13	W/v	W/W	2	4	0
S-692	F	White	23	Curr	2	10	1	W/W	W/W	0	4	0

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-693	F	Af-Am	30	Non	0	0	0	V/v	W/W	1	5	1
S-694	F	Af-Am	29	Non	0	0	0	W/v		1	1	9
S-695	M	Asian	43	Non	0	0	0	W/v	W/W	0	0	0
S-696	F	Hispanic	25	Curr	1	10	1	W/W	W/W	0	3	0
S-700	F	White	29	Curr	11	20	11	W/v	W/v	0	2	2
S-701	F	White	29	Curr	11	15	8	W/W	W/v	0	1	0
S-702	F	Hispanic	19	Non	0	0	0	W/W	W/W	0	1	3
S-704	F	White	28	Non	0	0	0	W/v	W/W	0	1	2
S-705	F	White	24	Non	0	0	0	W/W	W/v	1	4	0
S-708	M	Asian	27	Non	0	0	0	W/W	W/W	0	1	2
S-709	F	White	59	Ex	20	6	6	W/v	W/W	2	1	6
S-710	M	White	33	Non	0	0	0	W/W	W/W	0	6	2
S-715	F	White	39	Curr	24	20	24	W/v	W/v	1	5	2
S-717	F	White	27	Ex	8	14	6	W/v	W/W	2	2	5
S-718	M	White	31	Non	0	0	0	W/W	W/W	0	1	4
S-725	F	White	42	Ex	8	10	4	W/W	W/W	0	2	0
S-726	F	White	20	Non	0	0	0	W/W	W/W	1	0	3
S-727	M	Asian	24	Non	0	0	0	W/W		1	2	8
S-728	F	White	37	Non	0	0	0	W/W	W/v	1	4	3
S-729	M	Af-Am	18	Non	0	0	0	W/W		1	2	8
S-730	F	Hispanic	33	Non	0	0	0	W/W		1	2	5
S-731	M	Hispanic	50	Ex	23	30	22	W/v		1	4	6
S-732	M	Af-Am	37	Ex	2	10	1	W/W		1	2	6
S-733	F	White	46	Non	0	0	0	W/W	W/W	1	2	5
S-734	F	Af-Am	52	Non	0	0	0	W/W		2	1	7
S-736	F	White	47	Ex	10	20	10	W/W	W/W	1	2	4
S-744	F	Af-Am	38	Non	0	0	0	W/v	W/W	2	1	7
S-754	F	White	28	Curr	10	3	2	W/v	W/v	2	3	3
S-764	F	Hispanic	27	Curr	4	10	2	W/W	W/W	0	2	4

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	Pack-Year	MGMT84	MGMT143	Break0	Break48	B72RR
S-785	F	White	58	Curr	37	40	74	W/W	W/W	0	1	2

Table 5: *HPRT* Data

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/Day	PY	C1099T	MGMT84	MGMT143	CE	Clonal
S-157	M	White	35	Non	0	0	0	W/W	W/W	W/v	0.1730	0.21
S-227	F	White	45	Curr	27	20	27		W/v	W/v	0.1805	1.05
S-505	F	White	22	Non	0	0	0	W/W	W/W	W/W	0.1612	0.549
S-544	F	White	54	Curr	33	20	33	W/W	v/v	W/v	0.1358	3.71
S-550	F	White	42	Curr	27	20	27	W/v	v/v		0.1523	3.99
S-565	F	White	47	Curr	34	30	51	W/v	v/v	W/W	0.2209	1.9
S-571	F	White	34	Non	0	0	0	W/W	W/W	W/v	0.1208	1.96
S-574	F	White	38	Non	0	0	0	W/W	W/v	W/W	0.2418	0.636
S-576	F	White	34	Non	0	0	0		W/W	W/W	0.2381	1.26
S-586	M	White	34	Curr	18	15	13	W/W	W/W	W/W	0.2455	0.529
S-591	F	White	23	Non	0	0	0	W/W	W/W	W/W	0.3398	0.586
S-593	F	White	38	Curr	12	10	6		W/W	W/W	0.4621	1.07
S-595	F	White	54	Non	0	0	0	W/W	W/v	W/W	0.3945	1.41
S-596	M	White	54	Curr	23	20	23		W/W	W/W	0.3332	1.08
S-597	F	White	52	Non	0	0	0	W/W	W/W	W/W	0.4141	0.562
S-598	M	White	39	Non	0	0	0	W/W	W/W	W/W	0.6931	0.11
S-605	M	Asian	39	Non	0	0	0	W/W	W/W	W/W	0.1480	2.03
S-606	F	Asian	35	Non	0	0	0		W/W	W/W	0.2381	1.2
S-617	F	Asian	21	Non	0	0	0	W/W	W/W	W/W	0.3269	0.323
S-618	F	White	44	Non	0	0	0		W/W	W/W	0.3332	1.09
S-619	F	Hispanic	38	Non	0	0	0	W/W	W/W	W/W	0.7607	0.358
S-623	F	White	35	Curr	19	15	17	W/W	W/W	W/W	0.5519	0.686
S-650	M	White	40	Curr	20	30	30	W/v	W/v	W/W	0.2310	1.57
S-660	M	Asian	31	Non	0	0	0	W/W	W/v	W/W	0.1780	0.542
S-677	M	White	69	Non	0	0	0		W/W	W/W	0.6931	0.963
S-679	F	White	23	Non	0	0	0		W/W	W/W	0.1706	1.14

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/ Day	PY	C1099T	MGMT84	MGMT143	CE	Clonal
S-680	F	White	23	Non	0	0	0		W/W	W/W	0.1319	1.34
S-682	F	White	21	Curr	3	3	1	W/W	W/W	W/W	0.1300	0.335
S-683	F	Hispanic	22	Non	0	0	0	W/W	W/W	W/W	0.2381	0.629
S-686	F	Af-Am	26	Non	0	0	0		W/W	W/W	0.1054	0.971
S-687	F	White	35	Curr	18	15	9	W/W	W/W	W/W	0.0811	0.537
S-694	F	White	31	Non	0	0	0	W/W	W/v		0.2533	0.869
S-695	M	Asian	45	Non	0	0	0	W/W	W/v		0.2145	1.93
S-697	F	Asian	48	Non	0	0	0	W/W	W/v	W/W	0.2670	2.65
S-698	F	White	44	Non	0	0	0		W/W	W/W	0.4040	0.936
S-699	M	White	49	Curr	30	20	30		W/v	W/W	0.1780	3.01
S-703	F	White	50	Non	0	0	0	W/W	W/v	W/W	0.1038	2.39
S-707	F	White	35	Non	0	0	0	W/W	W/v	W/v	0.1319	2.59
S-713	F	White	25	Curr	8	15	6	W/W	W/W	W/v	0.4488	1.35
S-738	F	White	21	Non	0	0	0	W/W	W/W	W/v	0.4040	0.753
S-740	F	White	23	Non	0	0	0	W/W	W/W	W/W	0.4925	0.264
S-752	F	White	25	Non	0	0	0		W/v	W/W	0.1480	1.12
S-753	F	White	33	Curr	15	20	15		W/W	W/v	0.4141	1.13
S-758	F	Hispanic	23	Non	0	0	0	W/W	W/v	W/v	0.2573	0.409
S-759	F	Asian	31	Non	0	0	0	W/W	W/W	W/v	0.1805	1.85
S-769	M	White	23	Non	0	0	0	W/W	W/W	W/W	0.5100	0.417
S-770	F	White	42	Curr	29	20	29		W/W	W/W	0.7607	1.25
S-776	F	White	54	Curr	40	30	60		W/v	W/W	0.4488	1.33
S-778	F	Asian	42	Curr	30	40	60	W/W	W/W	W/W	0.3398	0.535
S-779	F	White	45	Curr	32	20	32	W/W	W/W	W/W	0.3269	1.4
S-782	F	White	49	Curr	15	12	9	W/W	W/W	W/W	0.1339	2.41
S-783	M	Af-Am	56	Curr	41	15	30	W/W	W/W	W/W	0.1029	0.908
S-790	F	White	44	Curr	25	20	25	W/W	W/W	W/W	0.2986	1.8
S-794	F	Hispanic	22	Non	0	0	0	W/W	W/W	W/v	0.2310	0.921
S-799	F	Af-Am	51	Non	0	0	0		W/W	W/W	0.3537	1.29

ID	Sex	Ethnicity	Age	Smoking	Yrs Smoked	Cig/Day	PY	C1099T	MGMT84	MGMT143	CE	Clonal
S-800	F	White	32	Non	0	0	0		W/W	W/v	0.1857	1.28
S-807	F	White	40	Non	0	0	0		W/v	W/W	0.2656	1.01
S-808	M	Asian	53	Non	0	0	0	W/W	W/W	W/W	0.3094	0.821
S-809	F	Asian	25	Non	0	0	0		W/v	W/W	0.2836912	0.975
S-816	F	Af-Am	28	Curr	10	5	3	W/W	W/W	W/W	0.2053	0.802
S-820	F	White	46	Non	0	0	0	W/W	W/W	W/W	0.1682	1.85
S-829	F	White	32	Curr	17	15	12	W/W	W/W		0.1208	2.8
S-831	M	Asian	43	Curr	20	12	12	W/W	W/W		0.1805	1.65
S-835	F	White	21	Non	0	0	0	W/W	W/W	W/W	0.2319	0.858
S-836	M	White	24	Curr	7	20	7		W/W	W/W	0.1545	1.32
S-847	F	Af-Am	41	Curr	20	40	40	W/W	W/v	W/W	0.2986	1.7
S-849	F	Asian	27	Non	0	0	0		W/W	W/W	0.1480	1.16
S-858	F	Af-Am	28	Curr	12	10	6	W/W	W/v	W/W	0.0550	3.87
S-861	F	Af-Am	47	Curr	32	40	64	W/W	W/W	W/W	0.1857	2.26
S-862	M	White	37	Curr	20	10	10		W/W	W/v	0.2935	1.55
S-863	F	White	37	Curr	12	9	5		W/W	W/v	0.2573	1.87

Table 6: Mutation Spectrum Data

ID	Type of Mutation	Change	Type	Location	Protein Consequences	Comments
S224	deletion	--		28-32	Partial Exon 2	
S224	deletion	22 bp		540-562	Partial Exon 8	
S224	deletion	-AA		375-376	Partial Exon 4	
S224	deletion SV	--		385-402	Exon 5 Loss	
S224	deletion SV	--		28-318	Exons 2 and 3 Loss	
S224	Substitution	G -> C	Transversion	118	Gly -> Arg	
S224	Substitution	TG -> AT		170-171	Met -> Asn	
S227	deletion SV	--		28-318	Exons 2 and 3 Loss	
S227	deletion SV	--		319-384	Exon 4 Loss	
S227	Insertion	--- -> T		375	--	
S227	insertion SV	--		28708-28745	Partial Intron 4 (751-788)	
S227	Substitution	G ->A	Transition	197	Cys -> Tyr	

ID	Type of Mutation	Change	Type	Location	Protein Consequences	Comments
S227	Substitution	T -> A	Transversion	295	Phe -> Ile	
S544	deletion	-CTGAG		86-90	Partial Exon 2	
S544	N/A	--		--	--	
S544	Substitution	C -> T	Transition	508	Arg -> Ter	
S544	Substitution	G -> A	Transition	617	Cys -> Tyr	
S544	Substitution	G -> T	Transversion	617	Cys -> Phe	
S544	Substitution	G -> T	Transversion	626	Ser -> Ile	
S545	Substitution	C -> A	Transversion	17	Pro -> His	
S545	Substitution	G -> A	Transition	46	Gly -> Ser	
S545	Substitution	GC -> AT	Transition	429-430	Met/Glu -> Ile/Ter	
S550	deletion	- TTTGGA		93-98	Partial Exon 2	
S550	deletion SV	--		319-384	Exon 4 Loss	
S550	Insertion	-- -> TAT		574	--	
S550	Insertion	-- -> TC		444	--	
S550	insertion SV			41444-41454	Partial Intron 8	
S550	N/A	--		--	--	
S550	Substitution	C -> T	Transition	151	Arg -> Ter	
S550	Substitution	G -> A	Transition	580	Asp -> Asn	
S550	Substitution	T -> C	Transition	533	Phe -> Ser	
S694	deletion	16 bp		610-626	Partial Exon 9	
S694	deletion	2 bp		436-437	Partial Exon 6	
S694	deletion	7 bp		564-570	Partial Exon 8	
S694	N/A	--		--	--	
S694	N/A	--		--	--	
S694	Substitution	A -> G	Transition	530	Thr -> Ala	
S695	deletion SV	--		319-384	Exon 4 Loss	
S695	N/A	--		--	--	
S695	N/A	--		--	--	
S695	Substitution	A -> G	Transition	611	His -> Arg	
S695	Substitution	G -> A	Transition	143	Arg -> His	
S703	deletion	1 bp		7	?	
S703	deletion SV	77 bp		533-609	missing exon 8	
S703	insertion	3 bp		at 115	?	
S703	insertion SV	36 bp		at 28	?	segment of intron 1 from 1719-1752
S703	substitution	A --> G	Transition	404	D --> G at 135	2 mutations in this sequence
S703	substitution	A --> G	Transition	496	K --> E at 166	2 mutations in this sequence
S703	substitution	A --> G	Transition	611	H --> R at 610	
S703	substitution	A --> T	Transversion	602	D --> V at 201	2 mutations in this sequence
S703	substitution	C --> T	Transition	605	L --> S at 202	
S703	substitution	G --> A	Transition	119	G --> E at 40	
S703	substitution	G --> A	Transition	539	G --> E at 180	2 mutations in this sequence

ID	Type of Mutation	Change	Type	Location	Protein Consequences	Comments
S703	substitution	G --> A	Transition	617	C --> Y at 206	
S703	substitution	G --> C	Transversion	135	R --> S at 45	
S753	deletion	1 bp		596	?	
S753	deletion SV	77 bp		533-609	missing exon 8	
S753	substitution	C --> T	Transition	113	P --> L at 38	
S770	deletion	8 bp		589-596	?	
S770	N/A	N/A		N/A	N/A	no apparent change
S770	substitution	A --> T	Transversion	44	D --> V at 135	
S770	substitution	T --> G	Transversion	51	Y --> stop at 17	
S793	deletion	1 bp		527	?	
S793	deletion	11 bp		490-500	?	
S793	deletion	17 bp		610-626	?	
S793	N/A	N/A		N/A	N/A	no apparent change
S793	substitution	G --> C	Transversion	46	G --> R at 16	2 mutations in this sequence
S793	substitution	G --> C	Transversion	190	A --> P at 64	2 mutations in this sequence
S794	deletion SV	291 bp		28-318	missing exons 2-3	
S794	substitution	A --> G	Transition	133	R --> G at 45	
S794	substitution	A --> T	Transversion	581	D --> V at 194	
S794	substitution	AG --> TC		45-46	G --> R at 16	
S799	deletion	1 bp		187	?	
S799	deletion SV	77 bp		533-609	missing exon 8	
S799	insertion	1 bp		at 125	?	
S799	insertion SV	125 bp		at 404	?	segment of intron 5 from 32812 to 32936
S799	N/A	N/A		N/A	N/A	no apparent change
S799	substitution	G --> C	Transversion	130	D --> H at 44	
S799	substitution	G --> T	Transversion	14	S --> I at 5	
S799	substitution	GC --> TT		148-149	A --> F at 50	
S807	deletion	1 bp		537	?	
S807	deletion	14 bp		158-171	?	
S807	insertion SV	184 bp		at 318	?	repeat of exon 3
S807	N/A	N/A		N/A	N/A	no apparent change
S807	substitution	A --> G	Transition	167	E --> G at 56	4 mutations in this sequence
S807	substitution	A --> G	Transition	331	T --> A at 111	4 mutations in this sequence
S807	substitution	A --> T	Transversion	349	I --> F at 117	4 mutations in this sequence
S807	substitution	C --> G	Transversion	551	P --> R at 184	
S807	substitution	C --> T	Transition	202	L --> F at 68	
S807	substitution	G --> A	Transition	197	C --> Y at 66	4 mutations in this sequence
S807	substitution	G --> C	Transversion	208	G --> R at 70	
S808	deletion	1 bp		610	?	
S808	insertion	2 bp		at 82	?	
S808	substitution	A --> T	Transversion	611	H --> L at 204	

ID	Type of Mutation	Change	Type	Location	Protein Consequences	Comments
S808	substitution	C --> A	Transversion	366	no change	no apparent change
S808	substitution	G --> T	Transversion	569	G --> V at 190	
S808	substitution	T --> A	Transversion	398	V --> E at 133	
S808	substitution	T --> G	Transversion	95	L --> W at 32	
S809	deletion	1 bp		631	?	
S809	deletion	4 bp		83-86	?	
S809	deletion SV	82 bp		404-485	missing exon 6	
S809	insertion SV	67 bp		at 404	?	segment of intron 5 - splicing error
S809	N/A	N/A		N/A	N/A	no apparent change
S809	substitution	C --> T	Transition	151	R --> stop at 51	
S809	substitution	T --> A	Transversion	29	I --> N at 10	
S829	deletion	1 bp		17	?	
S829	deletion SV	291 bp		28-318	missing exons 2-3	
S829	deletion SV	77 bp		533-609	missing exon 8	
S829	N/A	N/A		N/A	N/A	no apparent change
S829	substitution	A --> G	Transition	611	H --> R at 204	
S829	substitution	T --> A	Transversion	542	F --> Y at 181	
S831	deletion	1 bp		346	?	
S831	deletion SV	291 bp		28-318	missing exons 2-3	
S831	deletion SV	47 bp		486-532	missing exon 7	
S831	substitution	G --> C	Transversion	208	G --> R at 70	
S831	substitution	G --> T	Transversion	97	E --> stop at 33	
S835	deletion	38 bp		603-640	?	
S835	deletion	44 bp		29-72	?	
S836	deletion	1 bp		555	?	
S836	deletion SV	47 bp		486-532	missing exon 7	
S836	insertion SV	49 bp		28	?	segment of exon 1 & intron 1 from 1692-1751
S836	substitution	C --> T	Transition	508	R --> stop at 170	
S847	deletion	12 bp		285-296	?	
S847	deletion	24 bp		149-172	?	
S847	deletion SV	18 bp		385-403	missing exon 5	
S847	substitution	A --> T	Transversion	602	D --> V at 201	
S847	substitution	G --> T	Transversion	517	G --> stop at 173	
S849	deletion	3 bp		59-61	?	
S849	deletion	66 bp		323-388	?	
S849	insertion SV	38 bp		at 385	?	segment of intron 4 from 28706-28745
S849	substitution	T --> A	Transversion	473	V --> D at 158	
S858	substitution	G --> A	Transition	617	C --> Y at 206	
S858	substitution	T --> A	Transversion	459	Y --> stop at 153	
S861	deletion	1 bp		478	?	
S861	deletion SV	77 bp		533-609	missing exon 8	
S861	N/A	N/A		N/A	N/A	no apparent change

ID	Type of Mutation	Change	Type	Location	Protein Consequences	Comments
S861	substitution	G --> A	Transition	119	G --> E at 40	
S862	deletion	21 bp		533-553	?	
S862	N/A	N/A		N/A	N/A	no apparent change
S862	substitution	A --> G	Transition	401	E --> G at 134	
S862	substitution	G --> A	Transition	148	A --> T at 50	
S863	deletion SV	66 bp		319-384	missing exon 4	
S863	N/A	N/A		N/A	N/A	no apparent change
S863	N/A	N/A		N/A	N/A	no apparent change

Table 7: Luciferase Data (3 experiments)

	Luciferase	Renilla	Normalized
SV40 + Control	105039	14627	7.1811718
	126644	18559	6.823859
	97682	13061	7.4789067
WT Unexposed	11359	1470	7.7272109
	23200	2913	7.964298
	13939	1821	7.6545854
Var Unexposed	15542	1773	8.7659334
	18713	2082	8.9879923
	17671	2102	8.4067555
Wt Exposed	54	28	1.9285714
	88	1	88
	18	31	0.5806452
Var Exposed	77	5	15.4
	25	8	3.125
	91	1	91

LIST OF ABBREVIATIONS

aB : AlamarBlue®
ABI : Applied Biosystems
AP : Antarctic phosphatase
AP1 : Activator Protein 1
AP2 : Activator Protein 2
BCNU : Bis-chloroethylnitrosourea
BG : O^6-benzylguanine
BP : Base Pair
CA : Chromosome Aberration
CAT : chloramphenicol acetyl transferase
cDNA : coding deoxyribonucleic acid
CE : Cloning Efficiency
C-myc : myelocytomatosis
CPB : CREB Binding Protein
DNA : Deoxyribonucleic Acid
DoE : Department of Energy
ENU : N-ethyl-N-nitrosourea
ER : estrogen receptor
FBS : Fetal Bovine Serum
FV : fluorescence value
GR : glucocorticoid receptors
GRE : glucocorticoid response element
HPRT : Hypoxanthine-guanine Phosphoribosyl Transferase
HSP : heat shock promoter element
IGF2R : insulin-like growth factor 2 receptor
IL-2 : Interleukin 2
IR : Irradiated
LB : Lysogeny Broth
LRP1B : Low-density lipoprotein receptor-related protein 1B
MBD1 : Methyl-CpG-binding domain protein 1
MeCP2 : Methyl CpG Binding Protein 2
MEM : Minimal Essential Medium
MF : Mutant Frequency
MGMT : O^6-Methylguanine-DNA-Methyltransferase
MNNG : N-methyl-N'-nitro-N-nitrosoguanidine

mRNA : messenger ribonucleic acid
MSH6 : MutS α Homologue
N^3-meA : N^3-methyladenine
N^7-meG : N^7-methylguanine
NHBE : Normal Human Bronchial Epithelial
NIH : National Institute of Health
NNAL : 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK : 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NSCLC : non small-cell lung cancer
O^6-meG : O^6-methylguanine
O^6-pobG : O^6-[4-oxo-4-(3-pyridyl)butyl]guanine
OGG1 : 8-Oxoguanine Glycosylase
P/E : Promoter/ Enhancer
PBL : Peripheral blood lymphocytes
PBS : Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PHA : Phytohemagglutinin
PKC : Protein Kinase C
RBC : red blood cell
RFLP : Restriction Fragment Length Polymorphism
RLU : Relative Light Units
SAM : S-adenosylmethionine
SNP : Single Nucleotide Polymorphism
SP-1 : Specificity Protein 1
SV40 : Simian vacuolating virus 40
TG : 6-Thioguanine
XPA : Xeroderma Pigmentosum complementation group A
XPD : Xeroderma Pigmentosum complementation group D
XRCC1 : X-ray repair cross-complementing protein 1

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