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Determining epigenetic instability in normal and diseased human vulva

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DETERMINING EPIGENETIC INSTABILITY IN CELLS OF NORMAL AND DISEASED HUMAN VULVA

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ABSTRACT

Epigenetics is defined as the study of heritable changes of DNA. One such component of epigenetic regulation is DNA methylation in humans. In neoplastic cells epigenetic controls are often dysregulated, especially in the promoter region of CpG islands. Global hypomethylation along with region specific hypermethylation of CpG islands in the promoter region of tumor suppressor is often indicative of neoplastic cells. In cancer, CpG island cytosine hypermethylation has been observed in more than fifty genes, including known tumor suppressor and DNA repair genes.

Squamous cell carcinoma (SCC), lichen sclerosis (LS), and adjacent normal tissues were obtained by radical vulvectomies of over one hundred patients. Normal unassociated tissues were also collected in the same manner. The disease process of LS provides an environment conducive to oxidative damage and increases in free radicals. Increased methylation in the promoter regions of specific tumor suppressor and DNA repair genes were anticipated to display a progression to malignancy from normal tissue to LS to SCC.

Hypermethylation patterns of p16, p15, O^6 methyl guanine methyl transferase (MGMT), glutathione S-transferase pi (GSTP1) were examined by methylation specific polymerase chain reaction (MSP) to obtain an etiological model of vulvar cancer. SCC samples exhibited 26% and 34% methylation in p16 and p15 genes. LS samples displayed 22% and 31% methylation in p16 and p15. The level of hypermethylation in SCC and LS associated samples was significantly different from normal samples in both p16 and p15 genes, suggesting that silencing of these two genes is an early and important

event in vulvar squamous cell carcinoma. GSTP1 and MGMT were not found to have a statistically significant difference in any of the tissues tested.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The modern world has brought about much advancement in the sciences. These advances have brought about many chemicals that are used in processing and development of many of the products that we consume regularly. Due to the advent of modern technology, new chemicals enter the environment daily. Governmental regulatory agencies have done much to combat the release of dangerous chemicals, but still many of these are often released into the environment at extremely low levels that are difficult to monitor and detect. It has been estimated that the number of organic chemicals that are continually being brought into the environment may include more that 100,000 chemicals (Pitot and Dragan 2001). One can be exposed to chemicals by many routes. Contamination of waste, water, food supplies, and air are common routes by which one might be exposed. In fact, chemical exposure occurs in every nation to every person on a daily basis though multiple pathways. Developing countries that do not have stringent environmental regulations are most concerning. These countries, along with large consumers such as the United States, release contaminants that can often travel globally affecting those in other regions of the world.

Many of the properties, routes of exposure, and toxicological effects of toxic chemicals are known. Modern science has given us the ability to classify thousands of chemicals and determine the detrimental effects of exposure. Xenobiotics are substances, natural and manmade, that are foreign to a biological system (Eaton and Klaassen 2001).

The science of toxicology focuses on the adverse effects of xenobiotics on living organisms. Although science, in particular toxicology, has given us a greater understanding of exposures and chemicals, much is still not understood. Modern toxicology could not possibly determine all deleterious effects of chemicals on animals, humans, or the environment. Furthermore, regulation regarding the release of known hazardous chemicals cannot control all possible exposures.

Exogenous agents, including cigarette smoke, dietary factors, occupational and environmental chemical exposures, and biologic agents, are causative factors in many cancers (Moore, Huang et al. 2003). Direct associations between exogenous agents and cancer were first made over 200 years ago The first report of carcinogenesis associated with chemicals is attributed to the English physician Percivall Pott (Pitot and Dragan 2001). Pott described a correlation between scrotum cancer and chimney sweeps, which was caused by soot exposure over the course of their lifetime. Pott was in essence the forefather of the branch of toxicology which we refer to as chemical carcinogenesis. Chemical carcinogenesis is the study of exogenous chemicals and their ability to induce cancer.

1.2 Genetics vs. Epigentics

The genome is controlled by many processes which regulate gene expression. It is now evident that two forms of information are encoded in the genome; genetic and epigenetic. Genetic information, which is organized using nucleotide bases, provides the essential instructions for manufacturing proteins. Epigenetic information provides additional instructions that facilitate how genetic information will be utilized. Epigenetic changes are those that are defined as heritable changes of DNA, not involving changes in

DNA sequence, that regulate gene expression (Dunn, Verma et al. 2003). The far more recent study of epigenetics is beginning to answer many questions about gene regulation that genetics could not explain. DNA methylation and histone modification are the major epigenetic mechanisms that can affect gene expression in mammals(Wolffe and Matzke 1999).

1.3 DNA Damage

DNA damage can be induced by one of two phenomenons: damage from endogenous agents and mistakes occurring in cell replication and repair. Agents that damage DNA include ionizing radiation, ultraviolet light, chemicals, and others such as hydrocarbons. These chemicals react with DNA and may cause damage such as adduct formation, oxidative alteration, and strand breakage (Gregus and Klaasen 2001).

Damage to DNA usually involves the alteration of genes in somatic cells. Somatic cell mutations affect a given cell and every cell in its line after division, as opposed to germ cells which affect every cell in an individual's offspring. Somatic cell mutations occur during chromosomal replication when cells are dividing. During normal chromosomal replication, nucleotides are copied within cells with great precision. In the course of copying a few billion nucleotides, fewer than one hundred errors are likely to be made, most of them inconsequential (Varmus and Weinberg 1993). Table 1.1 lists some of the genetic variations that might occur in the genetic sequence if replication is not faultless.

Table 1.1 DNA mutations

Mutation Classification	Mutation Type	Effects
Genetic	Single Base Substitution (Missense)	Amino acid substitution, new gene product with altered activity
	Single Base Substitution (Nonsense)	Premature translational termination, truncated protein, altered regulation of normal gene product
	Frame Shift	Shift in reading frame, Multiple amino acid substitutions, Premature translational termination
	Insertion	Addition of multiple amino acids, Transcriptional and/or translational termination
	Inversion	Amino acid substitution
	Deletion	Loss of amino acids, Shift of reading frame
	Double Strand Breaks	Premature translational termination
	Single Strand Breaks	Premature translational termination
	Translocation	Addition of multiple amino acids, Loss of amino acids, Transcriptional and/or translational termination or up regulation, chimeric gene products
	Amplification	Addition of multiple amino acids, Transcriptional and/or translational up regulation
	Aneuploidy	Loss or Gain of Chromosomes
	Adduct	Secondary Alterations
Epigenetic	Methylation	Up-regulation or down-regulation of gene expression
	Acetylation	Up-regulation or down-regulation of gene expression

If a mistake has occurred during replication, the cell copes with the DNA damage in one of three ways. In the case of extensive damage the cell will undergo a death pathway. If the damage is extensive necrosis will occur. Necrosis is a pathological process in which cells lose membrane integrity and die. The loss of integrity often induces an inflammatory response. Apoptosis is a programmed cell death that results from an energy dependent, endogenous cellular process. Membrane integrity is preserved and inflammatory reactions are minimized. In the event that the damage is less severe, the cell enters one of its many repair processes that are part of a generalized cellular DNA damage response network (Preston and Hoffmann 2001).

1.4 DNA Repair

Three general categories of recovery phenomena are direct reversal, excision repair, and tolerence (Cleaver 1984). All three deal with damaged DNA bases and DNA strand breaks.

Direct chemical reversal is a mechanism that is very common in the repair of damaged bases. It often involves enzymes such as DNA photolyase which cleave adjacent pyrimidines dimerized by UV light (Gregus and Klaasen 2001). Minor adducts, including methyl groups, are often removed by DNA repair proteins encoded by the methyl guanine methyl transferase (MGMT) gene (Pitot and Dragan 2001).

Excision Repair includes two forms of repair: base excision repair and nucleotide excision repair. Base excision repair, like direct chemical reversal, removes the damaged base by a DNA glycosylase. The glycosylase removes the damaged base, creating a gap in the sequence. DNA polymerase then fills in the gap with the correct base, and then the break is ligated to the parental strand. Nucleotide excision repair differs from Base

excision repair in that a larger segment of DNA is removed even if only one base is to be corrected. Nucleotide excision is responsible for removal of most bulky adducts from DNA. In this case, the DNA is unwound at the site of repair producing a bubble. Enzymes then remove the affected base and several bases surrounding it. Using the opposite strand as a template, DNA polymerase fills in the excised nucleotides with correct matches. DNA binds the new strand into the backbone.

Mismatch repair occurs after DNA replication and is a final check of the sequence. It deals with correcting mismatches of normal bases formed during DNA replication, genetic recombination, and as a result of DNA damage induced by chemical and physical agents (Preston and Hoffmann 2001). The strand is checked for base pair matches, using enzymes involved in excision repair and other specialized enzymes. DNA strand breaks are corrected according to the type of break. Single-strand breaks are corrected using the same systems as used in Base-Excision Repair. Double-strand breaks are repaired via two methods: direct joining and homologous recombination. Direct joining of the broken ends requires a protein that recognizes and binds the exposed ends, finally bringing them together for ligating. Errors in direct joining are associated with translocations that are associated with many cancers. Homologous recombination uses information found in the sister chromatid, or a homologous chromosome, to repair the break. Two proteins used in homologous recombination are encoded by the tumor suppressor genes BRCA-1 and BRCA-2. Research has shown that inherited mutations in either of these genes predispose women to breast cancer.

Tolerance mechanisms evade the damage without making repairs (Pitot and Dragan 2001). If the damage in the DNA strand cannot be corrected immediately, it may

be left. In the case of DNA damage, such as a pyrimidine dimer for example, DNA synthesis may be blocked. DNA replication may be able to resume downstream of a dimer, leaving a gap of single-stranded unreplicated DNA (Montelone 1998). If the cell divides, by filling in the gap, the dimer may be carried on through cell division.

1.5 Genomic Instability

Genomic instability is characterized by an increased frequency of mutations such that an instable genome results. Many pathways to genomic instability have been established. Germ line mutations and somatic mutations have been implicated in dysregulation of cell cycle controls. Cell cycle checkpoint systems are employed by the cell to maintain genomic integrity and proper cellular function. In eukaryotes, two checkpoints prevent chromosome segregation when DNA replication is inhibited or DNA is damaged (Murakami and Nurse 2000). One of the checkpoints works before entry into mitosis and the other before chromosome duplication. If the checkpoint systems are not working properly, then full complements are not transferred to daughter cells during replication.

In the event that a gene is mutated in the cell, or if a mutated gene is inherited our diploid nature initially will minimize the impact, except for dominant cases (Anderson 2001). The cell will use the genetic information from the remaining normal allele. If the normal allele is also lost, genomic damage is initiated. If severe damage is acquired in an essential gene, the cell will go through a death pathway. If the damage is not severe enough to initiate a death pathway then errors will continue to be made in the genome resulting in increase instability.

1.6 Carcinogenesis

Cancers acquire multiple genetic changes in DNA nucleotide sequence often resulting in chromosomal abnormalities. Many cancers also undergo epigenetic changes that can have profound effects on gene expression (Jones and Baylin 2002). It has been suggested that the acquisition of some form of inherent genomic instability (defined as a mutator phenotype of hypermutation) is a hallmark of tumorigenesis (Hanahan and Weinberg 2000). It is assumed therefore, that genomic instability is the driving force behind tumor formation. Arguments have been raised by some that genomic instability is not necessary for tumorigenesis to occur. Sieber et. al. argue that early tumors grow with a normal mutation rate (Sieber, Heinimann et al. 2003). Genomic instability is then acquired and spreads throughout the tumor, therefore giving it its characteristic growth advantage. Vogelstein presented a colorectal cancer progression pathway, which defined cancer as a clear, well-defined process from normal to cancerous cells due to the acquisition of genomic instability. Some believe cancer formation to be a much more chaotic process, with many routes to genomic instability, undergoing many less significant events. Factors such as genomic destabilization, Darwinian evolution, and natural selection for invasive, proliferating populations of cells are the essence of cancer (Anderson 2001).

1.7 Epigenetic Information

The three main types of epigenetic information are cytosine DNA methylation, genomic imprinting, and histone modifications (Feinberg and Tycko 2004).

1.8 Cytosine DNA Methylation

Methylation of cytosine is the only naturally occurring modification of DNA in mammals (Moore, Huang et al. 2003) and is perhaps the most critical component of epigenetic regulation in mammalian cells. In higher order mammals DNA methylation usually occurs at CpG dinucleotides which are frequently clustered in regions of about 1-2kb in length. These C*pG islands* are located in or near the promoter and exon regions of genes (Laird 1997; Jones and Laird 1999; Esteller and Herman 2002; Li, Hursting et al. 2003). Abnormal DNA methylation patterns are a hallmark of most cancers, including those of high proportion in the United States such as colon, lung, prostate, and breast cancer (Baylin, Herman et al. 1998; Esteller, Corn et al. 2001; Ross 2003). Common characteristics of mammalian tumor cells include widespread global hypomethylation and region specific hypomethylation and hypermethylation.

Cytosine methylation is catalyzed by one of three DNA transferases (*DNMT1*, *DNMT3A*, *DNMT3B*), which transfer methyl groups from S-adenosylmethionines (SAM) to the 5' carbon of cytosine residues (Li, Hursting et al. 2003). *DNMT1* is the most abundant methyltransferase and is responsible for global DNA methylation after replication. Figure 1.1 illustrates the mechanism of DNMT methylation via the SAM pathway (Strathdee and Brown 2002).

Mechanisms for promoter hypemethylation and the resulting loss of gene expression are being investigated. The thought is that de novo methylation initiates the silencing of genes. The methylated gene then recruits methyl binding proteins (MBP) and histone de-acetylases. A condensed chromatin structure results that silences the associated gene.

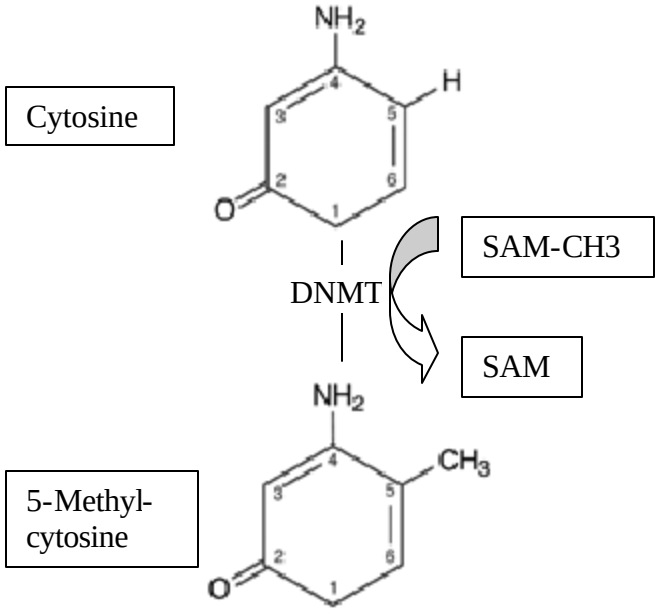

Figure 1.1 The mechanism of DNMT methylation (Strathdee and Brown 2002).

1.8.1 Regional Hypomethylation

Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells (Feinberg and Tycko 2004). Hypomethylation of DNA has been recognized to have mechanistic implications in the genome.

Hypomethylation has been associated with cancer, by activating proto-oncogene. Proto-oncogene are normally methylated and silenced within the genome. Cancer has been associated with the activation of proto-oncogenes. The oncogene H-ras is a classic example of a gene affected by hypomethylation (Feinberg and Tycko 2004). H-ras encodes a protein that regulates signal transduction to the cell nucleus, regulating cell division. A possible consequence of hypmethylation in the genome is genomic instability, resulting in a loss of heterozygosity (LOH) (Chen, Pettersson et al. 1998).

LOH is characterized by the loss of one allele at a specific locus (site on the chromosome) which can activate proto-oncogenes.

1.8.2 Regional Hypermethylation.

Increased methylation is observed in the promoters of many genes in cancer cells (Dunn 2003). This promoter methylation has been shown to correlate with inhibition of transcription (Verma, Dunn et al. 2003) and the inactivation of tumor suppressor genes, which correlates with cancer progression.

A small set of cellular genes are the targets for genetic alterations that initiate neoplastic transformations (Gregus and Klaasen 2001). Tumor-suppressor genes are crucial in the regulation of cell division. Tumor-suppressors encode proteins that inhibit the progression of cells in the division cycle (Gregus and Klaasen 2001). Tumor suppressors, unlike proto-oncogenes, are activated in the normal genome. When DNA damage is detected by a tumor suppressor gene it can either halt cell division until the damage is corrected, or stimulate the cells to go through an apoptotic pathway. When tumor suppressors are not functioning correctly, cells proliferate in an uncontrolled fashion accumulating further DNA damage. Inactivation of tumor suppression may be accomplished by faulty nucleotide-excision repair, base-excision repair, mismatch repair, chromosomal repair, or changes in the status of CpG-island methylation. A normal cell has two alleles of a tumor suppressor gene. Most tumor suppressor genes are recessive and must be inactivated by a two hit method, which disables both alleles. Knudsons's two-hit hypothesis is detailed in Fig. 1.2 (Jones and Laird 1999; Jain 2003). Inactivation of tumor suppressor genes is associated with considerable tissue specificity for cancer development (Pitot and Dragan 2001).

The best recognized tumor suppressor involved in carcinogenesis is p53. The p53 gene is a tetramer that is an exception to the two-hit hypothesis. Because of its structure it can be inactivated by one hit. p53 is activated in response to several malignancy-associated stress signals, resulting in the inhibition of tumor-cell growth (Balint and Vousden 2001; Vousden and Lu 2002). p53 may respond to stress using a variety of responses such as halt of cell proliferation, apoptosis, and repair of genetic damage. Tumor mutations associated with p53 are almost completely single base substitutions. Mutations in the p53 gene are found in fifty percent of human tumors and in a variety of induced cancers (Gregus and Klaasen 2001).

1.9 Genomic Imprinting

Genomic imprinting is a parent-of-origin-specific allele silencing, or relative silencing of one parental allele compared with the other parental allele (Feinberg and Tycko 2004). DNA methylation is a vital molecular mechanism of imprinting. It is DNA methylation that marks the imprinted genes differently on egg and sperm. Inheritance of these epigenetic marks leads to differential gene expression (Reik, Collick et al. 1987). The germ line has the role of resetting imprints such that in mature gametes they reflect the sex of that germ line (Reik and Walter 2001).

A number of pediatric solid tumors are associated with LOH of genes that are known to be imprinted in normal tissue (Dunn 2003). One mechanism for loss of imprinting (LOI) is hypermethylation of the tumor suppressor and transcription regulator H19 (Verona and Bartolomei 2003), which allows activation of the normally silent maternal allele of IGF2 (Cui, Onyango et al. 2002).

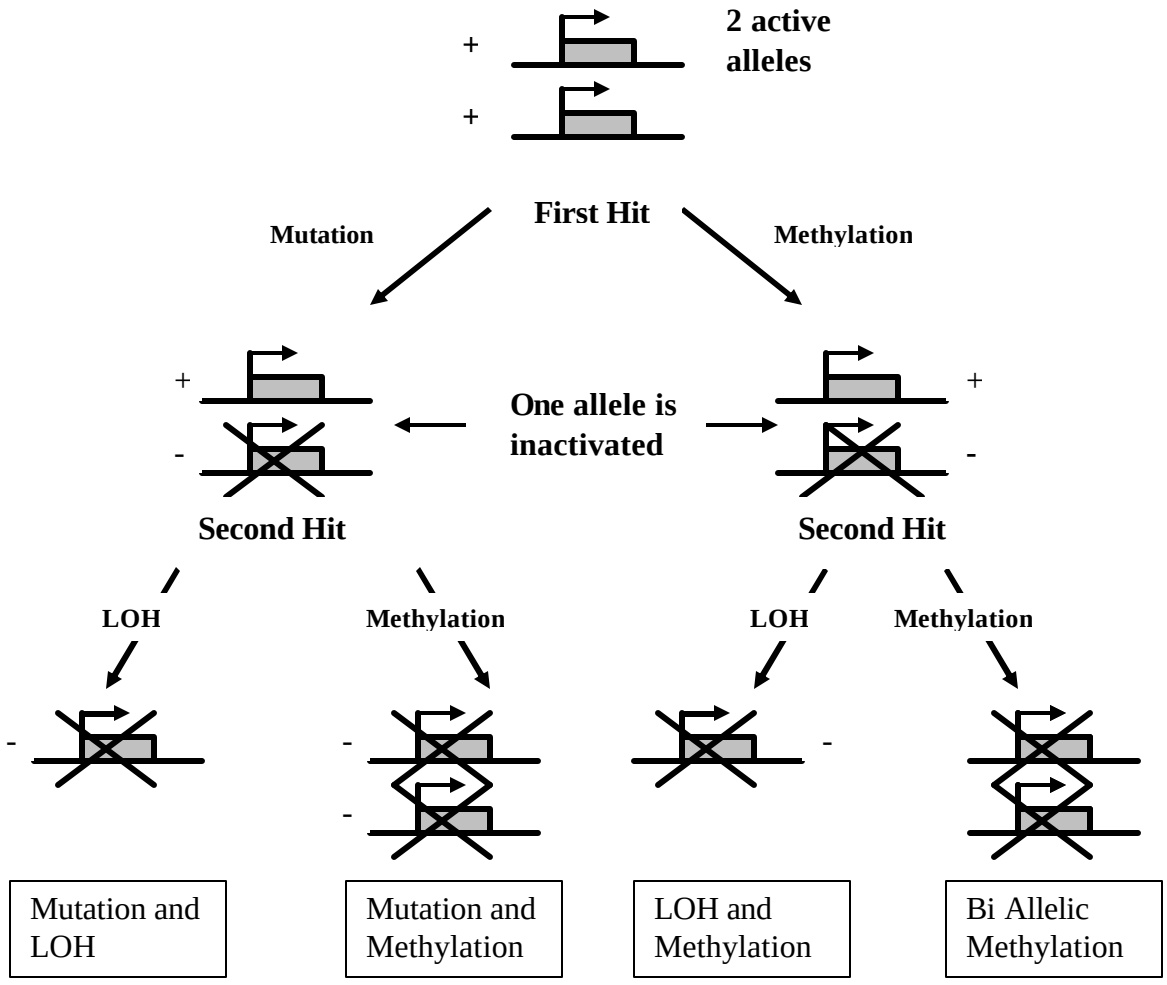

Figure 1.2 Knudson's hypothesis. (Adapted Jones and Laird 1999)

1.10 Histone Modifications

The typical eukaryotic chromosome contains 1 to 20cm of DNA packed into a dense structure called chromatin. Histones are proteins that aggregate with DNA to make the basic structural subunit of chromatin which are called nucleosomes. The amino-terminal tails of histones protrude from the nucleosome and are subject to chemical modifications including phosphorylation, acetylation, and methylation (Jenuwein and Allis 2001; Kondo, Shen et al. 2003). The histone code uses chromatic location to

determine the expression status of individual genes. Modifications may alter gene expression by altering the regulatory factors associated with chromatin. DNA methylation enables the conversion of histones to a nonacetylated state through histone deacetylases (HDAC), which is thought to result in the production of compacted chromatin that is resistant to transcription (Nan, Campoy et al. 1997; Nan, Ng et al. 1998).

Histone acetylation is maintained by a balance between the activities of two enzyme families, the histone acetyltransferases and histone deacetylases (HDACs) (Turner 1998). A lack of balance in the two enzymes can lead to changes in the genome. The silencing of gene expression is associated with deacetylated histones, which are often found to be associated with regions of DNA methylation as well as methylation at the lysine 4 residue of histone 3 (Thiagalingam, Cheng et al. 2003). Histone H3-lysine 9 methylation has been associated with gene silencing in cancer cells of the tumor suppressor genes p14ARF and p16INK4 (Nguyen, Weisenberger et al. 2002). Death-associated protein kinase (DAPK) is a tumor suppressor associated with positive regulation of apoptosis and tumor necrosis factors. DNA methylation and histone deacetylation were found to be associated with silenced DAPK expression in colorectal and gastric cancers (Satoh, Toyota et al. 2002).

1.11 Causes of Aberrant Methylation

Possible causes of atypical methylation within the genome are numerous. Links to diet and the polymorphism of methylenetratrahydrofolate reductase (MTHFR) have been established in increased colorectal cancer (Dunn, Verma et al. 2003; Feinberg and Tycko 2004). Folate is an important mediator in methyl group metabolism, which is an

important factor in the formation of methyl donor S-adenosylmethionine (SAM). A lack of folate could result in hypomethylation. The resulting hypomethylation could contribute to cancer risk by increasing the rate of chromosomal breakage caused by uracil misincorporation during DNA synthesis or by decreasing DNA methylation (Herbert 1986; Moore, Huang et al. 2003).

De Novo methylation of genes is considered to be primarily an epigenetic event, but methylation can also be influenced by exogenous factors. Many common environmental carcinogens have been shown to alter DNA methylation patterns. An association between nickel exposure, epigenetic silencing of the tumor suppressor gene (p16), and tumorigenesis was reported (Sutherland and Costa 2003). Arsenic and other elements, found in environmental settings induces hyper- and hypomethylation *in vivo* (Moore, Huang et al. 2003). Chemotherapeutic agents (Moore, Huang et al. 2003), tobacco smoke, and numerous viruses such as human papilloma virus (Muegge, Young et al. 2003; Verma 2003) have all been associated with aberrant methylation and cancer.

1.12 Properties and Processes of the Epidermis

The epidermis is a stratified squamous epithelium consisting of four biochemically and morphologically distinct layers (Serewko, Popa et al. 2002). The basal layer consists of a single layer of proliferating keatinocytes attached to the basement membrane. When the basal cells commit to terminal differentiation they withdraw from the cell cycle and down regulate specific genes such as the cyclin dependent kinase-1 (cdk-1.) These differentiating cells then pass into the suprabasal layers to form the spinous and granular layers that express genes specific to differentiating cells. The granular cells commit to apoptosis, thus forming the cross-

linked envelope characteristic of the stratum corneum (Serewko, Popa et al. 2002). This complex process requires the coordinated activation and repression of specific genes. Disruption of cell differentiation is known to accompany neoplasia (Hartwell and Kastan 1994). Several pathways are present that can alter genes that regulate cellular processes, such as growth regulation, apoptosis, and terminal differentiation (Serewko, Popa et al. 2002). Tumor suppressors and DNA repair genes are know to control such processes.

1.13 Lichen Sclerosus (LS)

Lichen sclerosus is a dermatologic inflammation affecting genital and perianal areas. It is a chronic, destructive dermatosis with a predilection for the vulva (Carlson, Lamb et al. 1998). LS is a non-contagious condition, which is classified into the general category of vulvodynia (chronic vulvar pain). This non-neoplastic disorder is characterized by a white thickening of the skin of the vulva, which eventually leads to scaring and lesions. It is usually localized to the perineum, labia, fourchette, and clitoris, and does not affect the vagina. Symptoms include pruritis (severe itching), burning pain, dyspareunia (pain with intercourse), vaginal discharge, and anal or genital bleeding (Loening-Baucke 1991). Most frequently, treatment involves the use of topical corticosteroids. Persistence despite most medical treatments is problematic (Carlson, Ambros et al. 1998).

Symptomatic vulvar lichen sclerosus typically affects peri-menopausal women with a mean age of 54 years (Wallace 1971; Carlson, Ambros et al. 1998), but may also affect prepubertal girls (Wallace 1971; Carlson, Ambros et al. 1998; Neill, Tatnall et al. 2002). The etiology of LS remains unknown, although infectious, genetic, and autoimmune causes have been suggested (Nyirjesy 2002). Of particular concern in

diseases such as LS is the importance of chronic inflammation and scarring in relation oncogenesis (Carlson, Ambros et al. 1998). LS is know to have numerous macrophages (Carlson and Edenhamn 2000) which could produce free radicals that are known mutagens possibly leading to genetic or epigenetic alterations. Advancement to squamous cell carcinoma (SCC) could occur via genetic instabilities in tumor suppressors and other genes.

SCC is the most common malignancy described in involvement with LS. Reports of squamous cell carcinoma arising from patients with LS in a clinical setting are prevalent. Agreements between the two differ greatly. One such study found that the magnitude of this risk is about 5% or less in patients with lifelong LS (Wallace 1971; Derrick, Ridley et al. 2000; Neill, Tatnall et al. 2002). Another study conducted by (Carlson, Ambros et al. 1998) found a higher incidence. It was found that of patients with symptomatic LS, 21% developed invasive SCC. Of those developing SCC 9% were preceded by vulvar intraepithelial neoplasia (VIN) which is a precursory lesion of SCC. However, histological evaluations of SCC have found that about 60% occur on a background of LS (Vilmer, Cavelier-Balloy et al. 1998), leading one to contemplate the association between LS and oncogenesis.

1.14 Squamous Cell Carcinoma

Squamous cell carcinoma of the vulva is a cancerous tumor that can affect women of all ages. Approximately 90% of vulvar tumors are squamous cell carcinoma (Canavan and Cohen 2002). Its incidence is about one-eighth that of cervical carcinoma, but increases as a function of age to a peak of 20 per 100,000 after age 75 (Mabuchi, Bross et al. 1985; Brinton, Nasca et al. 1990). It is characterized by red, pink, or white nodules or

plaques, appearing on the labia, clitoris, or the perineum. A wart-like or rough ulcerated appearance is common. Symptoms are similar in nature to LS, but often more severe. SCC is usually slow growing and may begin with a precancerous condition known as vulvar intraepithelial neoplasia (VIN) or dysplasia (Carlson, Ambros et al. 1998).

There are two clinicopathological types of vulvar squamous cell carcinoma. Human papillomavirus (HPV)-positive and HPV-negative, which can be distinguished to some degree on routine histology (Scurry and Vanin 1997). Table 1.2 depicts the characteristics of the two types (Crum 1992).

Table 1.2 Models of vulvar cancer (Adapted Crum, et al, 1992.)

Characteristic	Type 1	Type 2
Age	Younger (35 to 65 years old)	Older (55 to 85 years old)
Pre-existing lesion	Vulvar intraepithelial neoplasia	Vulvar inflammation, lichen sclerosis, differentiated VIN
Cervical neoplasia	High association	Low association
Cofactors	Age, immune status, viral integration, possibly mutated genes	Vulvar atypia, possibly mutated genes
HPV DNA	Frequent (>60 percent)	Seldom (<15 percent)
History of condyloma	Strong association	Rare association
History of STD	Strong association	Rare association
Cigarette smoking	High incidence	Low incidence

1.15 Polymerase Chain Reaction

The polymerase chain reaction is a commonly used method in molecular genetics. It is a powerful method by which a target sequence of nucleic acids may be amplified. DNA sequences usually 50 to 2,000 nucleotides long are “selected” from an original template such as genomic DNA using oligonucleotide primers. The DNA is amplified in

a solution of reagents that enhance the effectiveness of amplification. PCR reactions include taq DNA polymerase, MgCl2, PCR buffer, deoxynucleotide triphosphates (dNTP's), and template DNA. A DNA thermal cycler is used to provide varying temperatures for the denaturation, annealing, and extension of the DNA sequences. Amplification is carried out over numerous cycles to produce the desired number of copies. Each DNA template produces one copy for each cycle. In the following cycle the new strand along with its template act as a template, so in theory the DNA of interest will double with each cycle performed. PCR will amplify DNA exponentially providing the essential reagents and DNA are present.

Oligonucleotide (oligo) primers are necessary for polymerase chain reaction. They are usually short sequences 20-30 nucleotides in total length that are created in a laboratory. In standard PCR two primers are used. These primers, referred to as forward and reverse are used to target the nucleotides at either end of the targeted DNA sequence.

Nested PCR adds a step to increase annealing efficiency which is especially useful when final sequences of less than 200 base pair are expected. The two-stage nested PCR approach improves the sensitivity to detect methylated alleles by greater than 50 fold over the original method (Palmisano, Divine et al. 2000). This first PCR uses oligos that amplify a region of DNA that is slightly larger than the final product. This region overlaps the final sequence by 50 to 100 base pair on both ends of the forward and reverse primer. A second nested PCR is then run using the product from the first PCR as template. This run actually produces the final section (gene) of interest. Figure 1.3 illustrates PCR.

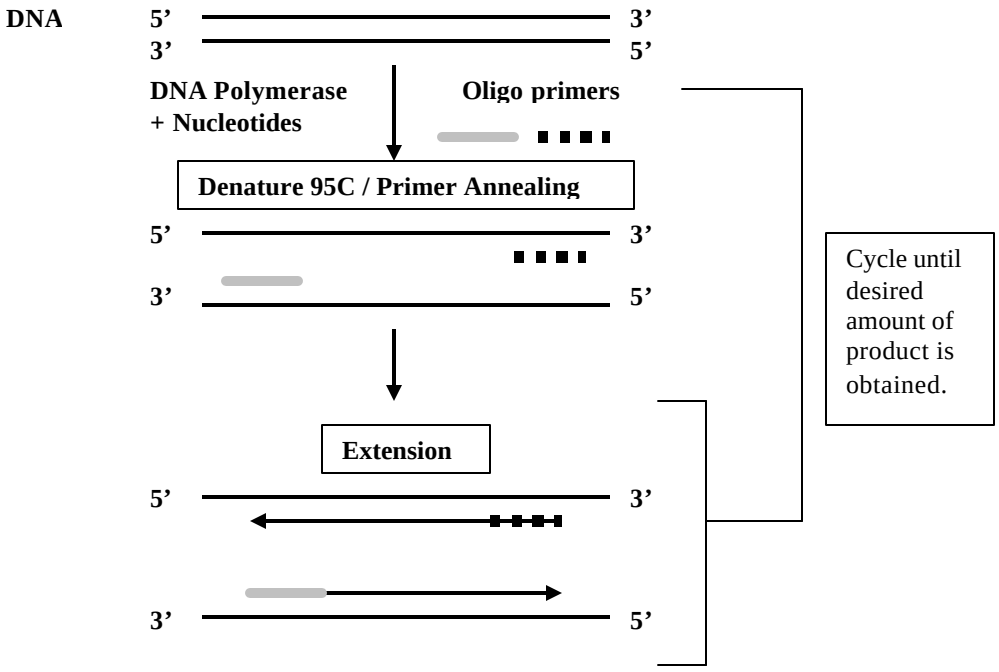

Figure 1.3 Polymerase Chain Reaction

1.16 Methylation Specific Polymerase Chain Reaction (MSP)

The most widely used assay for detecting methylation is methylation-specific polymerase chain reaction (MSP), developed at The Johns Hopkins University (Herman, Graff et al. 1996). This method involves the investigation of the CpG islands by utilizing slight variations in traditional PCR methods. After isolation the DNA is treated with sodium bisulfite to convert all unmethylated cytosines to uracils. Methylated cytosines remain intact. PCR is then carried out using primers that are specific for either the methylated or unmethylated DNA. If the primer has matched successfully then amplification will occur. Electrophoresis is then used to resolve the amplification

products on a 2-3% agarose gel. If methylation is present bands will be seen when amplified with a methylated primer.

1.17 Gene Selection

The purpose of this study was to determine the correlation of vulvar cancer with promoter methylation of selected genes. Based on published data MSP was chosen as an effective means of evaluating the promoter region of tumor suppressors. Two tumor suppressors and two DNA repair genes that are implicated with many types of cancer were chosen for this study based on published data. The two tumor suppressors chosen were p16 (INK4a) and p15 (INK4b). The two DNA repair genes selected were O^6 methyl guanine methyl transferase (MGMT) and glutathione S-transferase pi (GSTP1). Table 1.3 lists genes that are commonly affected by aberrant methylation.

1.17.1 p16 (INK4a) and p15 (INK4b)

Loss of cell cycle regulation through changes in the cyclin D/retionoblastoma (pRb) pathway is common in human neoplasia (Carlson, Ambros et al. 1998; Wong 2001). p16 encodes cyclin-dependent kinase (CDK) inhibitors 4 and 6, which negatively regulate G1-S transition of the proliferating cells by contributing to the maintenance of pRb in an active state (Hartwell and Kastan 1994; Morgan 1995; Xing, Nie et al. 1999).

A lack of the p16 inhibitory effect on CDK enzymes predisposes cells to uncontrolled growth. Previous work on epigenetic silencing of p16(INK4a) in vulva disorders detected aberrant methylation in 68% of VC, 69.2% of VIN, and 42.8% of LS cases (Lerma, Esteller et al. 2002) using MSPCR. Another study found p16 silenced in 36% of vulvar SCC cases (Gasco, Sullivan et al. 2002). Epigenetic inactivation most

likely represents an early event that may occur in clinically benign lesions such as LS (Lerma, Esteller et al. 2002).

p15 has much in common with it's sibling p16. It is not only adjacent to p16 on chromosome 9p21, but it also encodes a cyclin-dependent kinase inhibor. p15 is unlike p16 in that it is pRb independent. Its mode of action is to bind to the CDK enzyme preventing p27 association (Xing, Nie et al. 1999). p27 then binds to and inactivates the cyclin E-CDK2 complex, thereby blockin the cell cycle at the G1-S boundary (Reynisdottir and Massague 1997).

1.17.2 O^6 Methyl Guanine Methyl Transferase (MGMT)

MGMT plays a critical role in DNA repair pathways by removing alkyl adducts from methyl groups. The MGMT DNA repair protein is responsible for the removal of mutagenic and cytotoxic adducts from the O^6 position of guanine (Esteller, Toyota et al. 2000). O^6-methylguanine creates a mispair with thymine during DNA replication and if the adduct is not removed a conversion from a G→C pair to an A→T pair results (Esteller, Toyota et al. 2000). The loss of expression however, is almost always due to epigenetic silencing of the gene via methylation pathways.

1.17.3 Glutathione S-transferase pi (GSTP1)

GST genes comprise many isoenzymes that are essential in cellular protection against mutagenic and carcinogenic agents. Repression of transcription accompanying CpG island hypermethylation has been predisposed to be mediated by methyl-CpG binding domain (MBG) proteins (Bakker, Lin et al. 2002). In essence the MBG protein competes for access to the promoter region of the gene so that the GSTP1 encoded protein cannot bind.

Table 1.3 Genes affected by CpG methylation.

Gene	Function	Associated Tumor Types	Notes
$p16^{INK4a}$	Encode cyclin-dependent kinase inhibitors, which regulate G_1-S transition, associated with aberrant p53 expression and associated apoptosis	Lung (non-small cell lung cancer), vulvar, colon, lymphomas, bladder, esophagus, stomach, renal, others	Epigenetic factors more significant than genetic mutations
p15	Encode cyclin-dependent kinase inhibitors, which regulate G_1-S transition. Independent of pRb	Acute myeloid leukemia, Acute lymphoblastic leukemia, glioblastoma brain cancer, colon, lung, breast, others	p15 is located on chromosome 9p21 with p16, but methylation of both genes is rare
GSTP1	Enzymes involved in the detoxification of xenobiotics and oxygen radicals, may help defend hepatocytes against reactive oxygen species	Prostate, liver, colon, breast, kidney, others	Located on chromosome 11q13, not traditionally considered to be a tumor suppressor, but high levels of hypermethylation are found in solid tumors
MGMT	DNA repair protein, which removes alkyl adducts from a methyl group to an active cytosine in its own sequence. The reaction inactivates the MGMT molecule for each lesion repaired	Brain, head and neck, breast, prostate, renal, colon, non-Hodgkin's lymphoma, others	Associated with the DNA methyltransferase deficient phenotype, Increased K-ras mutation

CHAPTER 2

MATERIALS AND METHODS

2.1 Genomic DNA

Normal and tumor vulvar tissues were obtained via radical vulvectomies from over 100 patients. Due to the nature of a radical vulvectomy surrounding normal tissues, lichen sclerosis tissues, and other tissue types were obtained when in close proximity to the squamous cell tissue. In addition to the collection of tumor and the associated surrounding tissue normal tissue was collected from five patients with no apparent vulvar ailment. This gave the opportunity to evaluate true normal tissue as an unmethylated same tissue control. The patient tissues were histologically evaluated by Dr. Andrew Carlson of Albany Medical College and the corresponding histological condition was noted. The tissues for each patient were then separated according to condition and packaged for various analyses. Tissues that were to be analyzed in by us were stored at -80C and shipped on dry ice. Upon arrival the samples were again placed in a freezer at -80C. Dr. Carlson has analyzed the tissues for protein expression and will be comparing this data with the corresponding methylation data.

2.2 DNA Isolation

DNA was isolated from the specimens by standard phenol and chloroform extraction procedures (Xing, Nie et al. 1999). This protocol is the most commonly used method of purifying and concentrating DNA preparations. Approximately 500 mg of tissue was minced and then frozen in liquid nitrogen. The tissue was then crushed under liquid nitrogen using a mortar and pestle until a fine powder was obtained. The tissue

was then suspended in digestion buffer to approximately 1mL and left to digest at 50°C for 12 hours in capped tubes. The digest was extracted with phenol chloroform and cold ethanol. The DNA precipitates were then centrifuged and the supernatant containing the DNA was collected.

The second method used for genomic DNA extraction was the QIAamp DNA Mini Kit (Qiagen). The QIAamp kit was used as an efficient and quicker alternative which was useful due to the large number of samples that were isolated. The samples were then cleaned using standard ethanol precipitation and re-suspended in TE buffer.

2.2.1 DNA Quantification

Spectrophotometric detection is the most widely used method to determine DNA concentration (Adams 2003). The isolated DNA was analyzed for concentration and purity using a Cary 100 Bio spectrophotometer. Of the various methods is the calculation of the ratio of absorbance at 260 nm to the absorbance at 280 nm with a 1cm path length. The ratio is used to approximate the percentage of absorbing molecules in the sample that are protein. A sample of pure nucleic acid will have an absorbance ratio of 1.95. Pure protein on the other hand will absorb at 0.57. For this study the sample absorbances all fell very close to the 1.9 value, which confirms a relatively pure nucleic acid sample.

2.3 Bisulfite Modification

Approximately 2µg of DNA was denatured in a final concentration of 0.2M NaOH for 10 min. at 37C to create single stranded DNA. Thirty microliters of 10mM hydroquinone and 520 µl of freshly prepared 3M sodium bisulfite (pH 5.0) was added, then samples were covered with mineral oil and and incubated at 50C for 16 hours. The DNA was then purified using the Wizard DNA purification kit (Promega) and

resuspended in 50ul of heated water (60-70C). NaOH was added to a final concentration of 0.3M and glycogen (Boehringer) was used as a carrier. DNA was then ethanol precipitated as normal and resuspended in 20µl of water. Samples were stored at -80C until use.

2.4 Reagents

Reagents were chosen based on peer reviews of publications from labs that employ the methylation specific polymerase chain reaction assay (MSP). Amplitaq Gold (Applied Biosystems) is the thermostable taq polymerase that was chosen for the assay. This polymerase is often used in sensitive applications to reduce all possibility of non-specific annealing of primers which might cause false positive results. The primers used in the assay were purchased from BioServe Biotechnologies (Laurel, MD). Primers were diluted upon arrival with TE buffer to 0.5 nano moles per microliter. The four dNTP's used in were diluted in water to a working concentration of 25mM each. Unmethylated and methylated controls were purchased from Serologicals Corporation (United Kingdom). All reagents were stored in a standard freezer at -20C and thawed on ice before use.

2.5 Methylation Specific PCR Primer Selection

Gene sequences were obtained by searching the PubMed nucleotide database, which is a service of the National Library of Medicine (http://www.ncbi.nlm.nih.gov/PubMed/.)

Nested PCR was chosen as the method of choice for three of the four sites. This "two stage" PCR approach improves the sensitivity to detect methylated alleles by greater than 50 fold over the original method (one methylated allele in greater than 50,000

methylated alleles) (Palmisano, Divine et al. 2000). The two nested primers recognize the bisulfite-modified template but do not discriminate between the methylated and unmethylated alleles. It in essence "cradles" the final sequence allowing for improved final amplification. The nested primers for p16 and MGMT were reported previously by (Palmisano, Divine et al. 2000). The GSTP1 nested primer was chosen by analyzing the area around the bisulfite-modified sequence and choosing a length twenty to twenty five nucleotides still within the promoter region of the gene.

For MSP, two pairs of primers are needed, one of them specific for the bisulfite-modified / methylated DNA (M pair) and one for the modified / unmethylated DNA (U pair.) For each sample two PCR's must be performed with each pair of primers. Amplification with the U pair indicates no methylation of the CpG island. Amplification with the M pair and amplification with both M and U pairs indicates methylation. Primer sequences for the amplification of methylated and unmethylated alleles of p16 (Herman, Graff et al. 1996), MGMT (Esteller, Toyota et al. 2000), and GSTP1 (Esteller, Corn et al. 1998) were chosen based on published data. Primer sequences for the methylated and unmethylated alleles of p15 were originally obtained from a published source, but amplification lacked specificity. Table 2.1 lists primer names, sequences, product base pair size, annealing temperatures, and sources.

MethPrimer (Li and Dahiya 2002) was used to design new primers for the p15 amplification site (http://www.ucsf.edu/urogene/methprimer/). MethPrimer, which is based on Primer3, is a program designed for the production of MSP primers. It takes a DNA sequence as its input and searches the sequence for CpG islands (Li and Dahiya

2002). Primers are then picked within the CpG island based on four factors (Li and Dahiya 2002).

1. Primers should contain at least one CpG site at the most 3'-end. By default one of the three bases at the end must be a CpG 'C'. This can be edited by the user within the program by changing the number of bases from the end that the CpG 'C' will be located.

2. Multiple CpG sites in both primers are preferred.

3. Primers in the M pair and U pair should contain the same CpG sites within their sequence. For example if the forward M primer is ATT TAG TTT CGT TTA AGG TTC GA, the forward U primer must also contain the two CpG sites (underlined) as in the M pair, but with the 5 methyl Cytosine replaced by 'T'.

4. Two sets of primers should preferably have similar *Tm* values, thus allowing the two PCR reactions for each sample to be carried out in the same PCR machine under the same annealing conditions.

2.6 Methylation Specific PCR Amplification

The PCR reaction mixture contained .625U Taq polymerase, 1X PCR buffer, 3.0mM $MgCl_2$, dNTP's (each at 1.25mM), primers (1µM final), .01% cresol red redi-load, and bisulfite-modified DNA (50ng) in a final volume of 25µl. 0.2ml 96 well, thin wall reaction plates were used for each PCR. Primer attributes are described in table 2.1. PCR amplification was carried out using a GeneAmp PCR System 9700 (Applied Biosystems). Initial denaturation at 95C for 9 minutes is required to activate the thermostable Amplitaq Gold prior to amplification. Nested amplification parameters were similar to the final PCR with the annealing temperature being the exception. PCR reactions were carried out for 30 cycles (1 minute at 95C, 1 minute at the annealing temperature listed below, 1 minute at 72C), followed by a 7 minute final extension at 72C.

Table 2.1 Primer Data.

Primer set	Sense primer, 5' → 3'	Antisense primer, 5' → 3'	Size, bp	Anneal temp., C	Reference
p16-T	GAAGAAAGAGGAG GGGTTGG	CTACAAACCCTCTAC CCACC	279	55	(Palmisano, Divine et al. 2000)
p16-M	TTATTAGAGGGTGG GGCGGATCGC	GACCCCGAACCGCGA CCGTAA	156	62	(Herman, Graff et al. 1996)
p16-U	TTATTAGAGGGTGG GGTGGATTGT	CAACCCCAAACCACA ACCATAA	151	62	(Herman, Graff et al. 1996)
MGMT-T	GGATATGTTGGGAT AGTT	CCAAAAACCCCAAAC CC	289	55	(Palmisano, Divine et al. 2000)
MGMT-U	TTTGTGTTTTGATGT TTGTAGGTTTTTGT	AACTCCACACTCTTC CAAAAACAAAACA	93	62	(Esteller, Toyota et al. 2000)
MGMT-M	TTTCGACGTTCGTA GGTTTTCGC	GCACTCTTCCGAAAA CGAAACG	81	62	(Esteller, Toyota et al. 2000)
GSTP1-T	GGATTTTAGGGCGT TTTTTT	CCGAACCTTATAAAA ATAATCCC	154	57	Wilson and Hutchinson Design
GSTP1-U	GATGTTTGGGGTGT AGTGGTTGTT	CCACCCCAATACTAA ATCACAACA	97	67	(Esteller, Corn et al. 1998)
GSTP1-M	TTCGGGGTGTAGCG GTCGTC	GCCCCAATACTAAAT CACGACG	91	67	(Esteller, Corn et al. 1998)
p15-U	TGTGATGTGTTTGT ATTTTGTGGTT	CCATACAATAACCAA ACAACCAA	199	62	Methprimer Design
p15-M	GCGTTCGTATTTTG CGGTT	CGTACAATAACCGAA CGACCGA	200	62	Methprimer Design

2.7 Agarose Gel Electrophoresis

PCR product (11.5ul) containing sucrose loading dye and .01% cresol red was directly loaded onto a 2% TBE agarose gel using a multipipetor. The TBE gels contained 0.5 µg per mL Ethidium Bromide solution for UV visualization. The cresol red loading dye acts as a visual detector and adds weight to the sample so that it will sink to the bottom of the agarose gel wells. Gels were run on a BioRad Sub Cell GT 2 at 150 Volts for 70 minutes. The gels were then removed and placed on a BioRad Gel Doc imager and exposed to ultra violet light for analysis and image capture.

CHAPTER 3

RESULTS

3.1 Introduction

Four tumor suppressor genes that are commonly associated with inactivation by epigenetic instability leading to subsequent human cancer were analyzed by methylation specific polymerase chain reaction (MSP.) Vulvar SCC, adjacent LS, adjacent normal tissue, and unassociated normal tissues from radical vulvectomies were examined to establish an etiological model of disease. To determine associations between tumor suppressor inactivation and human vulvar cancer the following genes were studied: p16, p15, GSTP1, and MGMT.

3.2 MSP Standards

Water blanks were included in each MSP reaction to eliminate the possibility of reagent contamination. Universally unmethylated and methylated genomic DNA standards were also included in each to assure specificity of MSP product. Unmethylated standards were those that exhibited amplification with the unmethylated (U) primer from the analyzed gene, therefore a band will appear at the appropriate size in the agarose gel. Amplification does not occur in the presence of the methylated (M) primers. Methylated standards are those that exhibit amplification in the presence of the M primers and not the U primers.

3.3 Samples

NV specimens were collected from one individual exhibiting a normal histological evaluation. Samples designated CW are those from individuals with

histological types associated with cancer (SCC), lichen sclerosis (LS), and normal adjacent tissues. The sub-grouping (last number in the sequence) is an identifier used to discriminate multiple tissues taken from one patient.

3.4 Interpreting MSP Results

Those samples that were unmethylated were confirmed if amplification occurred with the unmethylated (U) primer only. Therefore, a single band of the appropriate size was seen in the unmethylated lane upon running the agarose gel. Positive methylation of the four genes analyzed was confirmed if both primers M and U amplified the template DNA resulting in a band in both the unmethylated and methylated lanes. This 'partial methylation' is observed in many tumor types (Herman, Graff et al. 1996). Two DNA samples would not amplify with any of the DNA primers so they were withdrawn from the study. Complete non-amplifications were most likely due to a small tissue amounts prior to genomic DNA isolation. Representative samples that tested positive for methylation were analyzed a second time to ensure reproducibility.

Those samples that amplified in one or more genes, but would not amplify in all four were analyzed two times to insure that template was incorporated into the MSP reaction. This lack of amplification could be due to many factors. One is that both alleles in the DNA of the withdrawn sample could have been permanently deleted. The other explanation is that degradation could have occurred in the sample that inhibited amplification of the template. SCC samples exhibited this phenomenon most prevalently, which tends to point to the first explanation. Although interesting further study using different methods would be required to justify allelic deletion.

3.5 Statistical Analysis

A contingency table shows numerically the results of an experiment in which the outcome is a categorical variable. Commonly, two groups of subjects are studied and there are two possible outcomes. In this case the groups analyzed were SCC vs. normal and SCC vs. LS, with possible outcomes being methylated and unmethylated. 2x2 contingency tables such as this, are best analyzed by a procedure very commonly called the Fisher exact test (Zar 1999). A *p* value which is equal to or less than 0.05 is considered to demonstrate a statistically significant difference between sets of samples.

3.6 p16 Gene

MSP amplification of the p16 gene was achieved by nested PCR. The outside PCR yielded a product that was 279 base pair. p16 methylated primers and p16 unmethylated primers produced products that were of 156 base pair and 151 base pair in length, respectively.

MSP analysis of the 157 samples analyzed for p16 revealed methylation in thirty one samples. Methylation was found in nineteen of seventy three (26%) cases of vulvar SCC. Lichen sclerosis also seemed informative in p16 yielding methylation in twelve of fifty five (22%) cases. None of the eighteen normal adjacent tissues or the nine normal unassociated tissues revealed methylation.

The methylation of SCC specimens using the p16 primer was found to be significantly higher than normal tissues ($p=0.0013$). The occurrence of methylation in adjacent lichen sclerosis tissues were also found to be significantly different from normal tissues ($p=0.0085$). This relationship showing increased methylation from normal to LS

suggests that epigenetic silencing of p16 is an early event in vulval neoplasia. Representative examples of p16 MSP are illustrated in figure 3.1.

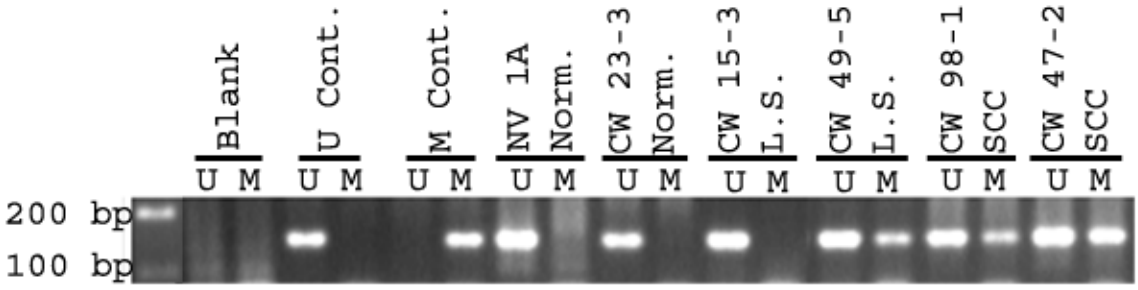

Figure 3.1 Representative samples of MSP analysis of the p16 gene.

Five cases (patient specimens CW40, CW41, CW44, CW49, and CW70) were analyzed for progression of normal vulva to LS, and finally to SCC. In case 40 there was no progression of methylation from normal to SCC. Cases CW41, CW44, and CW70 exhibited methylation in SCC, but no methylation in normal or LS samples. Patient CW49 showed no methylation in normal tissues, but methylation in both LS and SCC samples. Several of these samples were run numerous times for validation. Table 3.1 illustrates the results of these analyses for the five complete cases.

Table 3.1 Progression from normal to SCC for p16

Specimen	Identifier	p16 Status
CW 40-4	N	-
CW 40-5	LS	-
CW 40-1	SCC	-
CW 40-2	SCC	-
CW 41-2	N	-
CW 41-4	LS	-
CW 41-1	SCC	+
CW 44-1	N	-
CW 44-3	LS	-
CW 44-4	LS	-
CW 44-6	LS	-
CW 44-2	SCC	+
CW 49-7	N	-
CW 49-4	LS	+
CW 49-5	LS	+
CW 49-6	LS	+

(Table 3.1 Continued)

Specimen	Identifier	p16 Status
CW 49-1	SCC	+
CW 49-3	SCC	+
CW 70-7	N	-
CW 70-1	LS	-
CW 70-4	LS	-
CW 70-5	LS	-
CW 70-6	LS	-
CW 70-3	SCC	+
CW 70-2	SCC	+

\+ Methylated
– Unmethylated

3.7 p15 Gene

Amplification of the p15 promoter region was accomplished using a non-nested MSP reaction. The bioinformatics software MethPrimer was used to design appropriate primers that would provide consistent amplification. The unmethylated and methylated p15 amplification products were 199 and 200 base pair respectively.

MSP analysis of the p15 gene revealed thirty nine cases of methylation out of a total one hundred thirty nine samples analyzed. Of the SCC samples analyzed, twenty two of the sixty four (34%) exhibited methylation. In LS samples fifteen of forty eight (31%) were positive for methylation. Two of the twenty five (8%) normal samples were methylated. Of the normal tissues exhibiting methylation all ten unassociated normal samples were unmethylated. Representative results are shown in Figure 3.2.

p15 methylation of SCC carcinoma tissues was significantly different than normal tissues (p=0.0085). The associated LS tissue was also found to be significantly different than that of normal tissues (p=0.0217). As with p16 the methylation trend from SCC to LS suggests and early silencing event of the p15 marker in vulval neoplasia. It is noteworthy that the methylation in the normal samples was observed in the cancer associated normal tissues only.

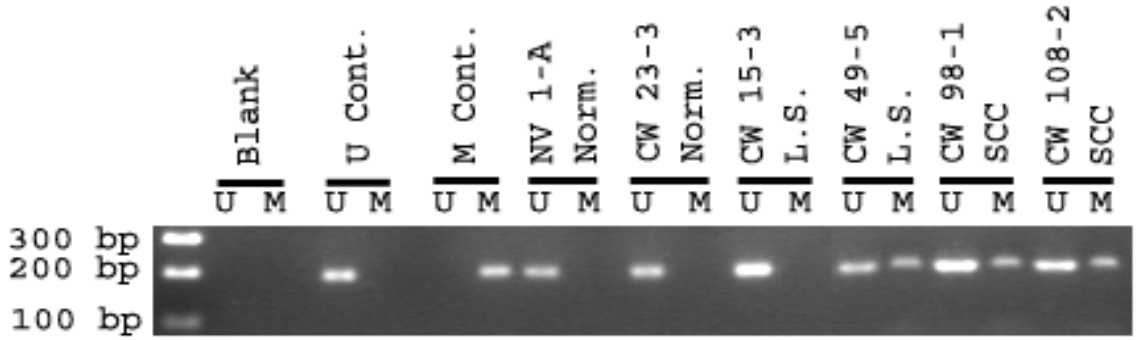

Figure 3.2 Representative samples of MSP analysis of the p15 gene.

Four cases that contained all three histological types were analyzed for p15 promoter methylation. None of the specimens analyzed for the patients CW30, CW44, CW49, or CW70 displayed of methylation in the adjacent normal tissues. Interestingly, patient CW 49 SCC tissue did not harbor a methylated p15 gene, but did display methylation in one of two LS specimens. The methylation data for these four samples is reported in table 3.3.

Table 3.2 Progression from normal to SCC for p15

Specimen	Identifier	p15 Status
CW 30-2	N	-
CW 30-3	LS	-
CW 30-4	LS	-
CW 30-5	LS	-
CW 30-1	SCC	-
CW 44-1	N	-
CW 44-6	LS	-
CW 44-3	LS	+
CW 44-4	LS	+
CW 44-2	SCC	+
CW 49-7	N	-
CW 49-4	LS	-
CW 49-5	LS	+
CW 49-1	SCC	-

Table 3.2 (Continued)

Specimen	Identifier	p15 Status
CW 49-3	SCC	-
CW 70-7	N	-
CW 70-1	LS	-
CW 70-5	LS	-
CW 70-6	LS	-
CW 70-3	SCC	+
CW 70-2	SCC	+

\+ Methylated
– Unmethylated

3.8 GSTP1 Gene

Amplification of the GSTP1 promoter sequence was accomplished using the nested MSP approach. The outside primers resulted in a 154 base pair DNA fragment, while the U primer and M primer yielded a 97 and 91 base pair fragment.

Sample analysis of the GSTP1 promoter following MSP revealed a total of fourteen methylated samples of a total one hundred forty four analyzed. Four (6%) of the sixty nine samples tested for SCC yielded methylation. Samples with LS histology were found methylated in seven of forty eight total cases (15%). Two (7%) of the twenty eight total normal samples analyzed were methylated. The ten normal associated samples that were analyzed contained no methylation.

SCC tissue at the GSTP1 promoter were not found to be statistically significant from the normal tissue samples analyzed (p=1.0000). When LS tissues were compared to the normal tissues again no significant difference in the two were found (p=0.4717). Furthermore, when excluding the normal adjacent tissues from the analysis the same trend was observed. There was no statistically significant difference observed between the SCC and normal unassociated groups (p=1.0000) or the LS and normal unassociated groups (p=0.0591). GSTP1 proves to be a poor indicator of cancer due to low

methylation in cancer cells. Representative examples of the analysis of methylation from the three tissue types are illustrated in figure 3.3.

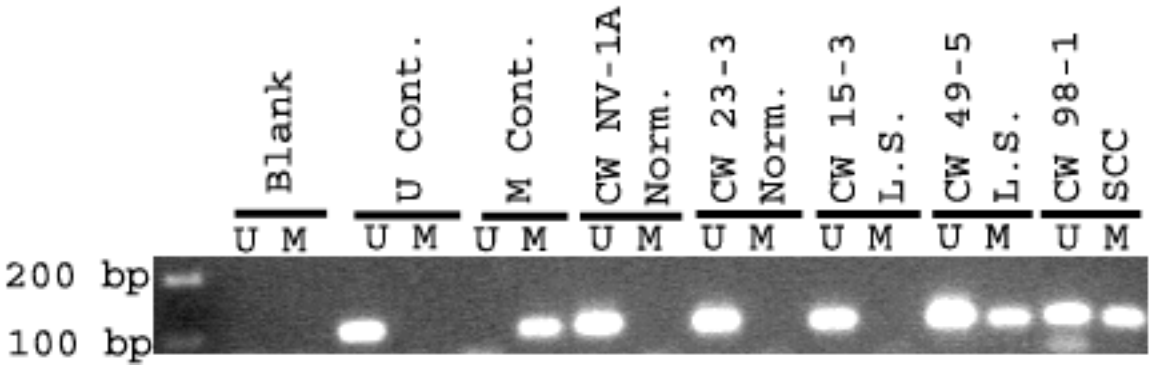

Figure 3.3 Representative samples of MSP analysis of the GSTP1 gene.

No apparent trend in progression from normal to SCC tissues was observed in four of the specimens that were examined that had all three histological types. The methylation data for these four samples is reported in Table 3.4.

Table 3.3 Progression from normal to SCC for GSTP1

Specimen	Identifier	GSTP1 Status
CW 41-2	N	-
CW 41-4	LS	-
CW 41-1	SCC	-
CW 44-1	N	-
CW 44-6	LS	-
CW 44-3	LS	-
CW 44-4	LS	-
CW 44-2	SCC	-
CW 49-7	N	-
CW 49-5	LS	+
CW 49-1	SCC	-
CW 49-3	SCC	-
CW 70-7	N	+
CW 70-1	LS	-
CW 70-5	LS	-
CW 70-6	LS	+
CW 70-3	SCC	-
CW 70-2	SCC	-

MGMT Gene

Identical nested PCR to that of the John's Hopkins group led by James G. Herman (Palmisano, Divine et al. 2000) was employed in the study that resulted in a 289 base pair outside fragment and a 93 and 81 unmethylated and methylated fragment. SCC exhibited the highest methylation with half of the seventy samples that were analyzed displaying methylation. LS tissues were similar with twenty three of the forty nine aberrantly methylated (47%). Of the twenty seven normal tissues analyzed twelve were methylated (44%). Of the normal unassociated tissues (normal associated) in this study five of the ten were methylated (50%) This corroborates with studies from other labs that indicate a high degree of methylation in normal tissues (Eads, Lord et al. 2001). The data for MGMT failed to reveal any significant difference in the methylation status between histological types. Representative examples of the analysis of methylation from the three tissue types are illustrated in figure 3.4.

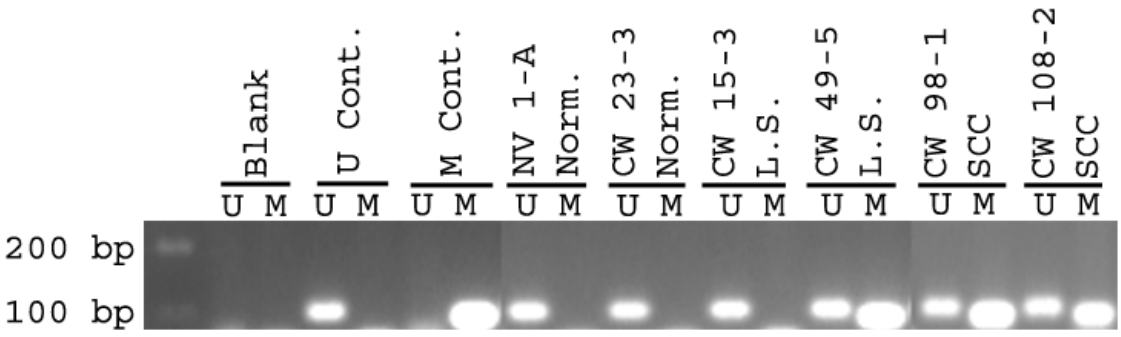

Figure 3.4 Representative samples of MSP analysis of the MGMT gene.

Four cases were analyzed that displayed all three histological types, as presented in Table 3.4. With the MGMT gene no correlation could be seen from normal tissue to progression of SCC.

Table 3.4 Progression from normal to SCC for MGMT

Specimen	Identifier	MGMT Status
CW 41-2	N	-
CW 41-4	LS	-
CW 41-1	SCC	+
CW 44-1	N	+
CW 44-6	LS	+
CW 44-3	LS	+
CW 44-4	LS	+
CW 44-2	SCC	-
CW 49-7	N	-
CW 49-5	LS	+
CW 49-1	SCC	-
CW 49-3	SCC	-
CW 70-7	N	-
CW 70-1	LS	-
CW 70-5	LS	+
CW 70-6	LS	+
CW 70-3	SCC	-
CW 70-2	SCC	+

3.10 Overall Results

It was found that p16 and p15 were promising in detecting aberrant methylation in both SCC and LS samples. Of the two p16 was most advantageous exhibiting a significant difference in methylation between SCC and normal tissues (normal adjacent and unassociated normal) (p=0.0014). A significant difference in methylation between LS and normal tissues were also observed (p=0.0075). p15 was also informative displaying a significant difference in SCC and normal tissues (p=0.0045) and LS and normal tissues (p=0.0028).

No significant difference was found in the methylation status of either GSTP1 or MGMT genes. GSTP1 was found to have a low occurrence of methylation in all tissues. MGMT was unusual in that the overall methylation was unexpectedly high in all tissues tested. The results of all methylation tests conducted in this study are listed below in table 3.5.

Table 3.5 Results from methylation analysis from all tissues examined.

Specimen	Identifier	p16	p15	MGMT	GSTP1
NV-1A	N	-	-	-	-
NV-1B	N	ND	-	-	-
NV-2C	N	-	-	-	-
NV-3A	N	-	-	-	-
NV-3B	N	-	-	+	-
NV-3C	N	-	-	+	-
NV-4A	N	-	-	+	-
NV-4B	N	-	-	+	-
NV-4C	N	-	-	+	-
NV-5	N	-	-	-	-
CW A-1	SCC	ND	ND	ND	-
CW C-1	SCC	-	ND	-	ND
CW 1-1	SCC	ND	ND	ND	-
CW 2-2	LS	-	ND	ND	-
CW 2-3	SCC	+	ND	ND	-
CW 5-1	SCC	-	ND	ND	-
CW 6-5	LS	ND	-	ND	ND
CW 7-1	SCC	-	ND	ND	ND
CW 10-1	SCC	-	ND	-	ND
CW 11-1	SCC	-	-	-	-
CW 12-1	SCC	ND	-	-	ND
CW 12-2	SCC	-	+	-	-
CW 12-4	SCC	-	-	-	-
CW 13-4	N	-	-	+	+
CW 13-5	N	-	ND	ND	ND
CW 14-1	SCC	-	-	+	-
CW 14-2	SCC	-	-	+	-
CW 15-1	SCC	+	+	+	-
CW 15-2	SCC	-	+	+	-
CW 15-3	LS	-	-	-	-
CW 16-3	N	-	-	+	-
CW 17-1	SCC	-	-	-	+
CW 17-2	LS	-	-	-	+
CW 17-4	LS	-	-	-	-
CW 17-6	LS	-	-	-	-
CW 18-1	SCC	-	+	-	-
CW 18-2	SCC	-	ND	ND	-
CW 18-3	SCC	+	-	-	-
CW 18-4	SCC	-	ND	-	ND
CW 18-6	SCC	+	ND	-	ND
CW 19-1	SCC	-	-	-	-
CW 19-2	SCC	+	ND	-	ND
CW 21-1	SCC	-	+	-	-
CW 21-2	LS	-	-	-	-
CW 21-3	LS	-	+	+	-
CW 21-4	LS	-	+	-	-

(Table 3.5 Continued)

Specimen	Identifier	p16	p15	MGMT	GSTP1
CW 22-3	LS	-	ND	ND	ND
CW 22-4	LS	+	+	+	-
CW 23-3	N	-	-	-	-
CW 25-1	SCC	+	-	-	-
CW 25-3	LS	-	-	-	-
CW 27-2	SCC	-	-	ND	-
CW 28-1	SCC	-	+	+	-
CW 28-5	LS	-	ND	ND	ND
CW 29-2	SCC	+	ND	ND	-
CW 29-4	LS	-	-	ND	-
CW 29-5	LS	ND	-	ND	ND
CW 29-6	SCC	+	+	+	-
CW 29-7	LS	-	-	-	ND
CW 30-1	SCC	ND	-	ND	ND
CW 30-2	N	-	-	-	-
CW 30-3	LS	-	-	+	-
CW 30-4	LS	-	-	+	-
CW 30-5	LS	-	-	-	-
CW 31-3	LS	-	-	-	-
CW 32-1	SCC	-	-	+	+
CW 34-1	LS	-	ND	-	-
CW 34-2	SCC	-	-	-	+
CW 34-3	LS	-	-	+	+
CW 35-1	LS	-	ND	-	-
CW 35-2	LS	-	-	+	-
CW 35-3	SCC	-	+	+	-
CW 35-5	LS	-	+	+	-
CW 35-6	LS	+	-	-	-
CW 35-7	LS	-	-	-	+
CW 39-2	SCC	-	-	+	ND
CW 40-1	SCC	-	-	-	-
CW 40-2	SCC	-	-	-	-
CW 40-4	N	-	-	+	-
CW 40-5	LS	-	ND		ND
CW 41-1	SCC	+	-	+	-
CW 41-2	N	-	-	-	-
CW 41-4	LS	-	ND	-	-
CW 43-1	SCC	+	ND	-	-
CW 43-4	SCC	+	+	-	-
CW 44-1	N	-	-	+	-
CW 44-2	SCC	+	+	-	-
CW 44-3	LS	-	+	+	-
CW 44-4	LS	-	+	+	-
CW 44-6	LS	-	-	+	-
CW 45-4	N	-	+	-	-
CW 45-5	LS	-	+	-	-

(Table 3.5 Continued)

Specimen	Identifier	p16	p15	MGMT	GSTP1
CW 46-3	LS	+	-	+	-
CW 46-4	LS	+	-	-	-
CW 46-6	LS	-	-	-	-
CW 47-2	SCC	+	-	+	-
CW 47-3	LS	+	-	+	-
CW 47-4	LS	-	-	-	-
CW 49-1	SCC	+	-	-	-
CW 49-3	SCC	+	-	-	-
CW 49-4	LS	+	-	ND	ND
CW 49-5	LS	+	+	+	+
CW 49-6	LS	+	ND	ND	ND
CW 49-7	N	-	-	-	-
CW 50-1	SCC	ND	ND	+	ND
CW 50-2	SCC	-	-	-	-
CW 50-4	SCC	+	+	-	-
CW 50-5	LS	+	+	+	-
CW 50-6	SCC	-	-	+	-
CW 50-7	SCC	-	+	+	-
CW 52-1	LS	+	+	+	-
CW 52-2	N	-	-	-	-
CW 52-4	LS	-	-	+	-
CW 52-6	LS	+	+	-	-
CW 53-1	LS	-	+	+	-
CW 53-2	LS	+	+	-	-
CW 57-1	SCC	-	+	+	-
CW 57-3	N	-	-	+	-
CW 60-1	N	-	-	-	-
CW 61-2	LS	-	-	+	-
CW 61-4	LS	ND	ND	-	ND
CW 61-6	SCC	-	+	+	-
CW 62-1	N	-	-	-	-
CW 63-1	SCC	-	-	+	-
CW 63-2	SCC	-	-	-	-
CW 63-5	SCC	-	-	+	-
CW 64-3	SCC	-	-	+	-
CW 66-3	LS	-	+	-	+
CW 66-4	LS	-	-	+	ND
CW 67-2	N	-	ND	ND	ND
CW 70-1	LS	-	-	-	-
CW 70-2	SCC	+	+	+	-
CW 70-3	SCC	+	+	-	-
CW 70-4	LS	-	ND	-	-
CW 70-5	LS	-	-	+	-
CW 70-6	LS	-	-	+	+
CW 70-7	N	-	-	-	+
CW 72-1	SCC	-	-	+	-

(Table 3.5 Continued)

Specimen	Identifier	p16	p15	MGMT	GSTP1
CW 73-1	N	-	+	-	-
CW 73-4	N	-	ND	ND	ND
CW 78-1	SCC	-	-	+	-
CW 78-2	SCC	-	-	+	-
CW 78-3	SCC	-	+	+	-
CW 78-4	SCC	-	+	+	-
CW 78-5	SCC	-	+	-	-
CW 79-1	SCC	-	-	+	-
CW 79-2	SCC	-	-	+	-
CW 79-3	N	-	-	+	-
CW 80-2	SCC	-	-	-	-
CW 89-1	SCC	-	-	-	-
CW 89-2	SCC	-	-	-	-
CW 89-3	LS	-	-	-	-
CW 91-1	SCC	-	+	-	-
CW 91-2	SCC	-	ND	-	-
CW 91-5	LS	-	+	+	-
CW 93-1	SCC	-	+	+	-
CW 93-2	SCC	-	-	-	-
CW 96-1	SCC	-	-	+	-
CW 96-2	SCC	-	-	+	-
CW 97-1	SCC	-	+	+	-
CW 97-5	SCC	ND	ND	ND	-
CW 98-1	SCC	+	-	+	+
CW 108-1	N	-	-	+	-
CW 108-2	LS	-	-	+	+
CW 109-1	SCC	ND	-	-	ND
CW 109-2	SCC	-	-	+	-
CW 110-1	SCC	-	-	+	-
CW 110-3	SCC	-	-	+	-

Samples designated + were found to be methylated.
Samples designated – were found to be unmethylated.
Samples designated ND were not determined.

This study found significant differences in the methylation patterns of the two genes analyzed. The statistically significant differences of p16 and p15 were observed between SCC and normal samples as well as between LS and normal samples. These statistically significant relationships of increased methylation among tissues are very powerful. The data suggest that that epigenetic silencing of p16 and p15 is an early

event in vulvar neoplasia. Tables 3.6, 3.7, and 3.8 summarize the significant differences between the various histological types.

Table 3.6 Statistical comparison of SCC and total normal samples

Gene	SCC	Normal	P Value	P<0.05
p16	26.0 %	0 %	0.0013	Yes
p15	34.4 %	7.4 %	0.0085	Yes
GSTP1	5.8 %	7.1 %	1.0000	No
MGMT	50.0 %	44.4 %	0.6567	No

Table 3.7 Statistical comparison of LS and total normal samples

Gene	LS	Normal	P Value	P<0.05
p16	21.8 %	0 %	0.0065	Yes
p15	31.3 %	7.4 %	0.0217	Yes
GSTP1	14.6 %	7.1 %	0.4717	No
MGMT	46.9 %	44.4 %	1.0000	No

Table 3.8 Statistical comparison of SCC and LS samples

Gene	SCC	LS	P Value	P<0.05
p16	26.0 %	21.8 %	0.6785	No
p15	34.4 %	31.3 %	0.8397	No
GSTP1	5.8 %	14.6 %	0.1221	No
MGMT	50.0 %	46.9 %	0.8525	No

Table 3.9 Statistical comparison of SCC and unassociated normal samples for those samples with methylated adjacent normal samples

Gene	SCC	Normal	P Value	P<0.05
p16	26.0%	0 %	0.1084	No
p15	34.4 %	0 %	0.0279	Yes
GSTP1	5.8 %	0 %	1.0000	No
MGMT	50.0 %	50 %	1.0000	No

Samples were compared among the four genes that were tested. Two of the possible sixty one SCC sample displayed methylation in three genes (3.3%). Nineteen SCC samples had methylation in two or three genes (27.5%), and fifty two of the SCC samples had methylation in one or more genes (68.4%).

One LS sample (CW 49-5) was found that displayed aberrant methylation in all four genes. Four samples presented methylation in three genes or more out of a total

forty nine for 7.1%. Fourteen of the LS samples had methylation in two or more genes (35.4%) and thirty three had methylation in one or more samples (56.9%).

When combining SCC and LS tissues five were methylated in three genes (4.4%), thirty six were methylated in two genes or more (28.6%), and eighty five had methylation in one or more genes (61.6%). This data is summarized in Figure 3.5 which depicts methylation analysis of histological type and the number of genes methylated per specimen.

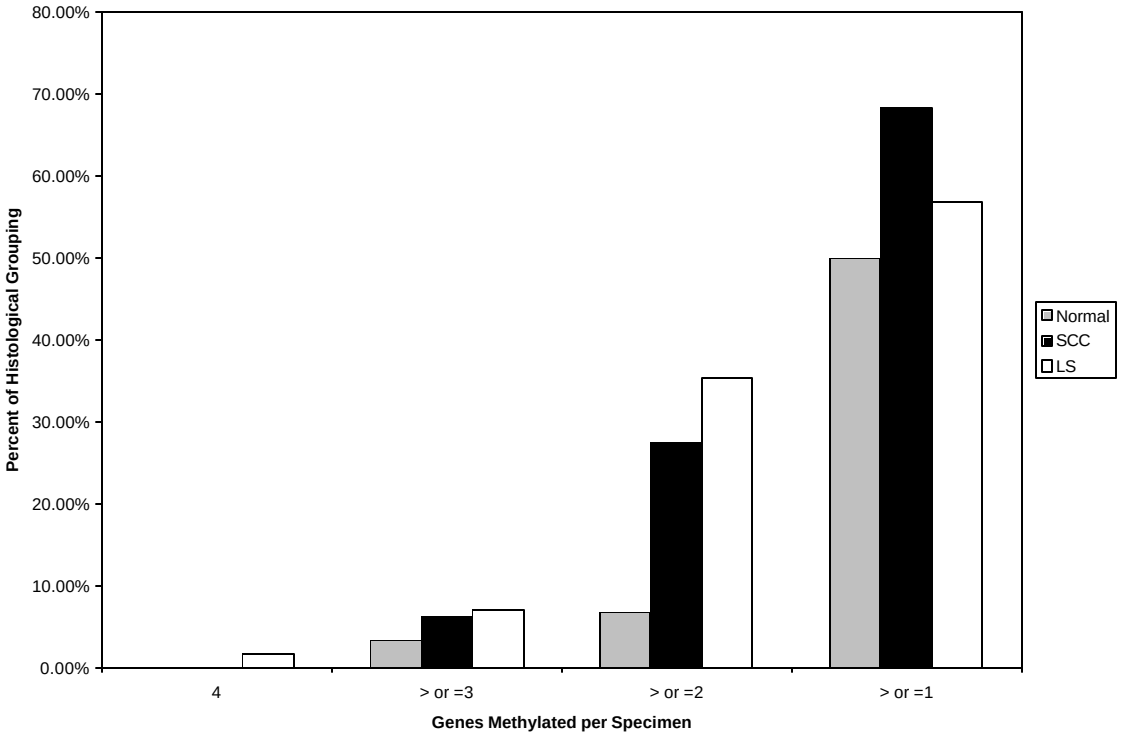

Figure 3.5 Methylation by histological type using all genes.

Finally for normal adjacent samples, one sample exhibited methylation in 3 genes out of a total thirty samples (3.3%), and fifteen samples were methylated in one and two genes (50.0%).

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 General Discussion

By studying the molecular change in methylation patterns associated with transitions from normal to lichen sclerosis and finally to squamous cell carcinoma in human vulva an etiological model of disease was formed. Four genes were chosen that are commonly inactivated in human neoplasia; p16, p15, and Glutathione S-Transferase P1 (GSTP1) and O^6-methylguanine-DNA Methyltransferase (MGMT). The two genes that appear to be most promising for early prognosis of vulval squamous cell carcinoma are p16 and p15. Overall, the methylation results observed in the present study were not abnormal from those seen in other studies.

4.2 p16 and p15 Genes

The p16 and p15 genes are both cyclin dependent kinase inhibitors (CDKs) which regulate cell division. Hypermethylation of CpG islands in the tumor suppressor gene p16 occurs frequently in various types of human malignancies (Xing, Nie et al. 1999; Kim, Nelson et al. 2001; Gasco, Sullivan et al. 2002; Kresty, Mallery et al. 2002; Holst, Nuovo et al. 2003). Inactivation of the p16 gene has been observed in esophageal squamous cell carcinoma (Xing, Nie et al. 1999), vulvar squamous cell carcinoma (Gasco, Sullivan et al. 2002), and others. The frequency of p16 mutations in primary tumors also vary among reports from 0-50%, and differed significantly between different ethnic groups if not due to experimental variation (Xing, Nie et al. 1999). Coincident inactivation of p16 in vulvar SCC was found previously at an occurrence of 60% in 36

cases (Gasco, Sullivan et al. 2002). p15 is highly homologous to p16, particularly in exon 2, where they share 91% sequence identity (Hannon and Beach 1994), indicating their origination by a gene duplication event (Xing, Nie et al. 1999). The p15 gene is also commonly hypermethylated in human neoplasms (Yeh, Chang et al. 2003), however, hypermethylation in both p16 and p15 is uncommon (Issa 2004).

This study demonstrated p16 and p15 were found to exhibit a high frequency of promoter methylation in vulvar SCC. As a percentage of SCC tissues analyzed methylation was found p16 and p15 at 26% and 34%, respectively. Lichen sclerosis (LS) tissues were also informative yielding 22% methylation in p16 and 31% in p15. These values for SCC and LS tissues were both found to be statistically different from normal tissues analyzed. This relationship showing increasing methylation from normal to LS and SCC suggests that epigenetic silencing of p16 is an early event in vulvar neoplasia. Both p16 and p15 data present a clear picture with exceptionally low methylation patterns in normal tissues. These data correlate well with data from others studying SCC and other cancers. p15 exhibited methylation in the cancer associated normal tissues only. This could be an indicator that the associated normal tissues for this marker are undergoing methylation leading to cell cycle disruption early. Another explanation is that there is a cancer field effect present in neighboring tissues.

4.3 GSTP1 Gene

GSTP1 is a member of the GST superfamily of four genes that provide important defense against oxidative damage to DNA and other cellular macromolecules (Gilliland, Harms et al. 2002). GSTP1 is a vital gene that plays an important role in protecting cells from cytotoxic and carcinogenic agents (Zhong, Tang et al. 2002). Published data has

demonstrated that GSTP1 methylation correlates highly with many primary tumors including: prostate carcinoma (83%) breast cancer (31%), renal cancer (20%), lung cancer (9%), colon cancer (4%), and others (Esteller, Corn et al. 1998). Primary tumors reported by Esteller, et al. 1998 not exhibiting significant methylation include endometrial carcinoma, melanoma, head and neck carcinoma, and others. Hepatocellular Carcinomas exhibited a background level in normal tissues of four out of a total forty samples (10%) (Zhong, Tang et al. 2002).

GSTP1 was not found to have a statistically significant difference between SCC, LS, or normal tissues in this study. A high methylation rate was found in normal tissues adjacent to SCC and LS tissues. Although this was not statistically significant from SCC and LS it still raised concern. Further, other groups found similar trends of high GSTP1 methylation in normal tissues. Zhong, Tang et al. 2002 found 10% of hepatocellular carcinomas methylated in normal tissues. Since none of the ten unassociated tissues were found to be methylated, the methylation in the normal adjacent tissues may be due to the proximal association with SCC or possibly a cancer field effect (Dong, Ip et al. 2002).

4.4 MGMT Gene

An association between the DNA repair gene MGMT and cancer has been recognized in brain, colon lymphoma, and non-small cell lung cancer (Esteller, Toyota et al. 2000). Loss of MGMT is associated with increased risk of carcinogenesis and increased sensitivity to methylating agents (Gerson 2004).

In this study the DNA repair gene MGMT had very high methylation status in all three histological cell types, all very near 50%. There was no statistically significant difference in any of the three histological types. Ranges in methylation of published data

have been broad, from 2% in bladder cancer (Maruyama, Toyooka et al. 2001) to 60% in esophageal carcinoma (Eads, Lord et al. 2001). A high level background in normal tissues has been observed. The studies of esophageal carcinoma at The University of Southern California, Keck School of Medicine, Norris Comprehensive Cancer Center, Los Angeles California included normal stomach tissue (NE) samples that exhibited a high propensity for methylation (Eads, Lord et al. 2001). The Los Angeles group found thirty one of fifty one normal samples (60%) that displayed methylation. The group statistically analyzed the data using Fisher's Exact Test to compare the esophageal carcinoma tissues to normal stomach tissues and found that there was no significant difference (no *p* value reported.) Other groups have found a high incidence of methylation in normal tissues. The James G. Herman (the architect of MSP) lab group from John's Hopkins Comprehensive Cancer Center, Baltimore Maryland found similar results when studying aberrant methylation in sputum of lung cancer patients. The group originally studied ten cases of matched SCC and sputum normal tissues. Of these, three cases of the normal sputum were methylated (Palmisano, Divine et al. 2000).

Among samples that displayed methylation in SCC there were no cases that displayed methylation in all four genes. Two samples displayed methylation in three genes (3.3%) and the trend increased to sixty eight percent exhibiting methylation in at least one gene. LS tissues displayed a somewhat similar trend with one sample displaying methylation in all four genes and three exhibiting methylation in three genes. As with SCC the methylation trend increased as the number of genes methylated decreased. Fifty seven percent of LS samples had at least one gene methylated. Normal samples exhibiting methylation in three or more genes was found in one sample. Fifteen

normal samples were methylated in one and two genes increasing the total percentage of normal methylation to fifty percent. If excluding the MGMT gene this number the number of normal genes methylated drops dramatically to approximately thirty percent. The methylation of the panel of genes selected indicates an apparent trend among cases from SCC to LS to normal tissues.

4.5 Conclusion

In recent years cancer has begun to be understood not only as a genetic disease, but also as an epigenetic one (Baylin and Herman 2000). It appears that these pathways are interrelated by a complex network involving many distinct processes. Changes in DNA methylation along with chromatin modifications are at the heart of epigenetic changes. DNA methylation patterns are tremendously altered in neoplasia and are often comprised of genomic level loss of methylation and both gains and losses of regional methylation. In particular aberrant promoter hypermethylation is associated with inappropriate gene silencing at every step in tumor progression (Jones and Baylin 2002). Aberrant DNA methylation of promoter sequences of genes has been associated with numerous types of human cancers including squamous cell carcinoma. Understanding the evolution of abnormal methylation patterns could provide an insight into human cancer development. This insight could lead to early diagnosis and provide us with possible targets for molecular gene therapy. One such approach is treatment with 5-aza-cytidine to prevent hypermethylation of tumor suppressor genes. Furthermore, increased understanding may lead to other pharmaceutical interventions that could be more effective and less invasive than those currently employed.

Valuable data was obtained that contributes to the range of information being collected on: aberrant DNA methylation, gene silencing, and for the progression of vulvar disease from normal to LS to SCC. Further studies of these genes and their functions would contribute to these data. Other tumor suppressors and proto-oncogenes should be studied to increase the overall knowledge of silenced genes associated with vulvar cancer. Primarily proteins that the four genes encode should be studied. It is known that post transcriptional events are also important in cell function. The protein expression of the normal genes and expressed mutant genes could be monitored by the use of antibodies specific. Protein data would strengthen the results to an even greater degree and would give unarguable conclusion to the fates of these genes.

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APPENDIX A

PHENOL AND CHLOROFORM EXTRACTION PROCEDURE

Preparation of Genomic DNA from Mammalian Tissue

DNA Isolation

1. Excise an immediately mince tissue quickly and freeze in liquid nitrogen.
2. Grind 200 mg to 1g tissues with prechilled mortar and pestle, or crush with hammer to fine powder.
3. Add 500 µl grinding buffer to the tissue in a 1.5 ml Eppendorf tube and vortex for several minutes.
4. Spin the samples in a microfuge for 15 minutes.
5. Decant the solution and add 500 µl of lysis buffer and add 10 µl (6 Units) of proteinase K to each sample. Digest at 37° for 12 hours.
6. Add 500 µl of phenol to each tube. Vortex briefly and centrifuge for 10 minutes.
7. Making certain not to disturb the interphase, transfer aqueous top solution to a clean labeled tube and add 500 µl of chloroform. Vortex briefly and centrifuge for 10 minutes.
8. If a white precipitate is present at the aqueous/organic interface, reextract the organic phase and pool aqueous phases.
9. Carefully remove the top aqueous phase containing the DNA using a 200 ul pipettor and transfer to a new tube.

APPENDIX B

ETHANOL PRECIPITATION OF DNA

1. Add 1/10 volume of 3M sodium acetate, pH 5.2, to the solution of DNA. Mix by vortexing briefly or by flicking the tube several times with your finger.
2. Add 2 to 2.5 volumes of ice cold 100% ethanol. Mix by vortexing and place in crushed dry ice for 5 minutes or longer. Alternately, the tubes can be placed at -80° for 1 hour or at -20° overnight.
3. Spin 5 minutes in a microcentrifuge at high speed and remove the supernatant.
4. Add 1 ml of room temperature 70% ethanol. Invert the tube several times and microcentrifuge as in step 6.
5. Remove the supernatant. Dry the pellet in a desiccator under vacuum or in a hood.
6. Dissolve the dry pellet in an appropriate volume of water or TE buffer (10mM Tris-HCL, 1mM EDTA), pH8.0

APPENDIX C

BISULFITE TREATMENT OF DNA

This protocol was adapted from Frommer et.al.1992.

1. Dilute DNA (up to 2 mg) into 50 ml with distilled H_2O.
2. Add 5.5 ml of 2M NaOH.
3. Incubate at 37°C for 10 minutes (to create single stranded DNA).
4. Add 30 ml of 10 mM hydroquinone (Sigma) to each tube, freshly prepared by adding 55 mg of hydroquinone to 50 ml of water.
5. Add 520 ml freshly prepared 3M Sodium bisulfite (Sigma S-8890), prepared by adding 1.88 gm of sodium bisulfite per 5 ml of H_2O, and adjusting pH to 5.0 with NaOH.
6. Assure that reagents are mixed with DNA.
7. Layer with mineral oil.
8. Incubate at 50°C for 16 hours (avoid incubations of much longer duration as methylated C will start converting to T).
9. Remove oil.
10. Add 1 ml of DNA wizard cleanup (Promega A7280) to each tube and add mixture to miniprep column in kit.
11. Apply vacuum (manifold makes this convenient).
12. Wash with 2 ml of 80% isopropanol.
13. Place column in clean, labeled 1.5 ml tube.
14. Add 50 ml of heated water (60-70°C).
15. Spin tube/column in microfuge for 1 minute.
16. Add 5.5 ml of 3 M NaOH to each tube, and incubate at room temperature for 5 minutes.
17. Add 1 ml glycogen as carrier (we use Boehringer glycogen, undiluted).
18. Add 33 ml of 10 M NH_4Ac, and 3 volumes of ethanol.
19. Precipitate DNA as normal (overnight at -20°C, spin 30 mins), wash with 70% ethanol, dry pellet and resuspend in 20 ml water.
20. Treat DNA like RNA (keep cold, minimize freeze/thaws, store at -20°C)

APPENDIX D

METHYLATION SPECIFIC POLYMERASE CHIAN REACTION

This protocol was adapted from other protocols to allow for consistent results using the primers for this experiment only. Primers are all unique in that annealing is optimal at different temperatures. Other factors such as $MgCl^2$ concentration must be optimized for the primers and type of PCR that is to be utilized.

1. Prepare a master mix (on ice) containing the following for each sample to be analyzed. Add 10% to each total volume to account for pipetting errors.
2. For MSP use a 25uL reaction containing the following.
   a. 2.5 µl 10X taq gold buffer
   b. 3.0 µl of 25mM MgCl2
   c. 0.125 µl taq gold polymerase
   d. 2.5 µl Redi-Load
   e. 1.0 µl 10mM total dNTP's
   d. Primers at .5 µM final concentration.
3. Add water filtered in a 0.2 µM filter to achieve final volume of master mix and vortex briefly.
4. Distribute 24 µl in to each 200 µl tube. Add 50 ng of DNA to each reaction (If 1 µg DNA was dilute to 40 µl after bisulfite treatement use 2ul.)
5. If not using a thermal cycler with a heated lid cover the samples with mineral oil.

*Notes on nested PCR

If 'nested' PCR is to be used, the above steps should be using the outside primers. Subsequent PCR's should be set up with 23 µl of the master mix in each tube. 4 µl of product from the first PCR will be used for each sample. 2 µl will be used with the methylated primers and 2 µl will be used with the unmethylated primers.

Thermal Cycling Parameters

1. **Initial Denaturation.** For Amplitaq Gold Polymerase this is performed at 95° for 9 minutes. This is to activate the thermostable properties.

2. **Cycling.** Amplification involves 20 to 40 cycles. For the first step in a 'nested' PCR 30 cycles are used, followed by the inside PCR at 30 or 35 cycles. a denaturing step at 95 for 1 minute **Anealing.** Annealing should be at the temperature that has been found to be optimal for the primers in use for 1 minute.

Extension. Occurs with most reactions at 72°. This should be a one minute step.

2. **Final Extension.** Seven minutes at 72°.

APPENDIX E

AGAROSE GEL ELECTROPHORESIS

1. Prepare a 2 – 3% agarose gel by adding the appropriate amount of agarose to TBE buffer. The total amount of gel should be determined by the size of the gel apparatus, and the thickness required for sufficient loading of DNA product.
2. Microwave the gel in a Pyrex flask until boiling. Continue to heat until all agarose particles are melted.
3. Let the gel cool at room temperature until it is approximately 60°.
4. Add ethidium bromide to a final concentration of 0.5 mg / ml.
5. Pour into the gel casting apparatus and let cool till the gel reaches room temperature.
6. Place the gel and the casting apparatus into the gel unit. Fill with TBE until the gel is covered by 1mm of TBE.
7. If DNA rediload is used with the PCR reaction then the product can be loaded directly into the gel. If not add approximately 1 - 2 µl of bromophenol blue loading dye.
8. Cover and run at 5V per centimeter of gel until the loading dye travels 4 – 5 cm.
9. Visualize with UV on a gel viewing apparatus.

APPENDIX F

p16 BISULFITE TREATED SEQUENCE

Accession Number NM_058197

```
                                        (Outside Forward Primer) GA AGAAAGAGGA
 61 AGGATTTGAG GGATAGGGTC GGAGGGGGTT TTTTCGTTAG TATCGGAGGA AGAAAGAGGA
    TCCTAAACTC CCTATCCCAG CCTCCCCCAA AAAAGCAATC ATAGCCTCCT TCTTTCTCCT


                    TTATTAGA GGGTGGGGCG GATCGC (Methylated Forward)➔
    GGGGTTGG➔    TTATTAGA GGGTGGGGTG GATTGT (Unmethylated Forward)➔
121 GGGGTTGGTT GGTTATTAGA GGGTGGGGCG GATCGCGTGC GTTCGGCGGT TGCGGAGAGG
    CCCCAACCAA CCAATAATCT CCCACCCCGC CTAGCGCACG CAAGCCCCCA ACGCCTCTCC


181 GGGAGAGTAG GTAGCGGGCG GCGGGGAGTA GTATGGAGTC GGCGGCGGGGA GTAGTATGG
    CCCTCTCATC CATCGCCCGC CGCCCCTCAT CATACCTCAG CCGCCGCCCCT CATCATACC


241 AGTTTTCGGT TGATTGGTTG GTTACGGTCG CGGTTCGGGG TCGGGTAGAGG AGGTGCGGG
    TCAAAAGCCA ACTAACCAAC CAATGCCAGC GCCAAGCCCC AGCCCATCTCC TCCACGCCC
                           ←AATGCCAGC GCCAAGCCCC AG (Methylated Reverse)
                           ←AATACCAAC ACCAAACCCC AAC (Unmethylated Reverse)


301 CGTTGTTGGA GGCGGGGGCG TTGTTTAACG TATCGAATAG TTACGGTCGGA GGTCGATTT
    GCAACAACCT CCGCCCCCGC AACAAATTGC ATAGCTTATC AATGCCAGCCT CCAGCTAAA


361 AGGTGGGTAG AAGGTTTGTA GCGGGAGTAG GGGATGGCGG GCGATTTTGGA GGACGAAGT
    TCCACCCATC TTCCAAACAT CGCCCTCATC CCCTACCGCC CGCTAAAACCT CCTGCTTCA
  ←CCACCCATC TCCCAAACAT C (Outside Reverse Primer)
```

APPENDIX G

p15 BISULFITE TREATED SEQUENCE

Accession Number S75756

```
            TT AGTTGAAAAC GGAATTTTTT GTC (Methylated Forward)➔
             T AGTTGAAAAT GGAATTTTTT GTTG (Unmethylated Forward)➔
 61 TTTTTGGTTT AGTTGAAAAC GGAATTTTTT GTCGGTTGGT TTTTTATTTT GTTAGAGCGA
    AAAAACCAAA TCAACTTTTG CCTTAAAAAA CAGCCAACCA AAAAATAAAA CAATCTCGCT


121 GGCGGGGTAG TGAGGATTTC GCGACGCGTT CGTATTTTGC GGTTAGAGCG GTTTTGAGTT
    CCGCCCCATC ACTCCTAAAG CGCTGCGCAA GCATAAAACG CCAATCTCGC CAAAACTCAA


181 CGGTTGCGTT CGCGTTAGGC GTTTTTTTTT AGAAGTAATT TAGGCGCGTT CGTTGGTTTT
    GCCAACGCAA GCGCAATCCG CAAAAAAAAA TCTTCATTAA ATCCGCGCAA GCAACCAAAA


241 TGAGCGTTAG GAAAAGTTCG GAGTTAACGA TCGGTCGTTC GGTTATTGTA CGGGGTTTTA
    ACTCGCAATC CTTTTCAAGC CTCAATTGCT AGCCAGCAAG CCAATAACAT GCCCCAAAAT
      ←GCAATC CTTTTCAAGC CTCAATTG (Methylated Reverse)
    ←TCACAATC CTTTTCAAAC CTCAATTA (Unmethylated Reverse)
```

APPENDIX H

GSTP1 BISULFITE TREATED SEQUENCE

Accession Number AY324387

```
                (Outside Forward Primer) GGATTTT AGGGCGTTTT TTT➔    G
1681 AGGTTTTTTC GGTTAGTTGC GCGGCGATTT CGGGGATTTT AGGGCGTTTT TTTGCGGTCG
     TCCAAAAAAG CCAATCAACG CGCCGCTAAA GCCCCTAAAA TCCCGCAAAA AAACGCCAGC

        TTCGGGG TGTAGCGGTC GTC (Methylated Forward)➔
     ATGTTTGGGG TGTAGTG (Unmethylated Forward)➔
1741 ACGTTCGGGG TGTAGCGGTC GTCGGGGTTG GGGTCGGCGG GAGTTCGCGG GATTTTTTAG
     TGCAAGCCCC ACATCGCCAG CAGCCCCAAC CCCAGCCGCC CTCAAGCGCC CTAAAAAATC

1801 AAGAGCGGTC GGCGTCGTGA TTTAGTATTG GGGCGGAGCG GGGCGGGATT ATTTTTATAA
     TTCTCGCCAG CCGCAGCACT AAATCATAAC CCCGCCTCGC CCCGCCCTAA TAAAAATATT
                ←GCAGCACT AAATCATAAC CCCG (Methylated Reverse)
                ←ACAACACT AAATCATAAC CCCACC (Unmethylated Reverse)
                                                ←CCCTAA TAAAAATATT

1861 GGTTCGGAGG TCGCGAGGTT TTCGTTGGAG TTTCGTCGTC GTAGTTTTCG TTATTAGTGA
     CCAAGCCTCC AGCGCTCCAA AAGCAACCTC AAAGCAGCAG CATCAAAAGC AATAATCACT
     CCAAGCC (Outside Reverse Primer)

1921 GTACGCGCGG TTCGCGTTTT CGGGGATGGG GTTTAGAGTT TTTAGTATGG GGTTAATTCG
     CATGCGCGCC AAGCGCAAAA GCCCCTACCC CAAATCTCAA AAATCATACC CCAATTAAGC
```

VITA

Kirk Robert Hutchinson was born in Baton Rouge, Louisiana, on June 26, 1978. His loving parents were living in Baton Rouge at the time, where his mother was student teaching and his father was in the last year of his Ph.D. Perhaps his longing to be an eternal student was inherited.

Upon completion of his final degree, Kirk's father moved this small family to Winnsboro, Louisiana, where Kirk would enjoy growing up in a town with less than six thousand residents. Living in a quiet town, Kirk was able to enjoy many outdoor activities such as fishing with his parents. Kirk attended Winnsboro public schools until the beginning of his senior year. It was this year that his father would have the opportunity to advance with his position through Louisiana State University. The still rather small, but close family moved to Saint Joseph, Louisiana, on the bank of the Mississippi River in Tensas Parish. It was in Saint Joseph that despite a rough year (one in which Kirk would barely skate through trigonometry), Kirk would graduate from Tensas Academy. Perhaps it was the move in general, or the fact that there could be a city that was one-sixth the size of Winnsboro that was so shocking. In fact it was said that when Kirk joined the class at Tensas Academy he was the sixteenth student, therefore completing the "Sweet Sixteen." Yes this does refer to a graduating class of sixteen.

It would seem like Kirk did not have a choice in deciding his academic career, for he did enroll in Louisiana State University in Baton Rouge. Kirk began his academic career in environmental management systems, but would change his mind and his major at whim. His first four years were very enjoyable, filled with late nights and partying. In fact Kirk partied with a focus and tenacity, which was never quite evident in his studies. It was the hand of God that pushed Kirk through his Bachelor of Science allowing him to graduate in December 2000.

Kirk, still unsure of his future dreams enrolled again at Louisiana State University as a non-matriculating student. It was at the end of this semester that Kirk obtained a Research Associate position in The Department of Agronomy. After a few months in this position he decided that soils were definitely not of interest to him. He then applied to The Department of Environmental Studies. The decision was probably based more on the fact that he would have two environmental titles in future degrees more than anything (two is better than one.) Floating through the department Kirk stumbled upon Dr. Vincent Wilson. After a brief visit he chose Dr. Wilson as his major professor. The choice, although Kirk did not know it at the time, was a wise one. Kirk began to enjoy the molecular research that Dr. Wilson had exposed him to in the laboratory. In fact he enjoyed molecular methods enough to change positions from The Agronomy Department to The Department of Biological Sciences. Kirk will be graduating at the end of the summer semester 2004, still lacking good study habits, but learning them slowly.

He was joined with his gorgeous wife Karlye in December of 2003. Their future remains unknown. Karlye is considering graduate school at the moment and Kirk, with his genetic predisposition to graduate school, is also unable to break away from the academic world. Kirk will be applying to graduate schools to focus further on the cancer research that was introduced to him by Dr. Wilson.