

**MICROSATELLITE INSTABILITY
IN SQUAMOUS CELL CARCINOMA
AND LICHEN SCLEROSIS
OF THE VULVA**

A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

In

The Department of Environmental Studies

by
Sidney Joseph Marlborough III
B.S., Louisiana State University, 1998
May 2004

ACKNOWLEDGEMENTS

I would like to thank Dr. Vince Wilson, my major professor and advisor, for his guidance, advice, and bent ear throughout this process. I would also like to thank Dr Ralph Portier for all of his advice and interest in my future. My wife, Aimee Marlborough, was supportive and patient and I would like to thank her for her help with my thesis and efforts.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	ii
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
ABBREVIATIONS AND DEFINITIONS.....	vii
ABSTRACT.....	viii
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW.....	1
1.1: Introduction.....	1
1.2: Carcinogenesis.....	2
1.3: Genomic Instability.....	3
1.4: Microsatellite Instability.....	4
1.5: Mismatch Repair.....	10
1.6: Polymerase Chain Reaction.....	12
1.7: Vulvar Squamous Cell Carcinoma.....	15
1.8: Vulvar Lichen Sclerosis.....	16
CHAPTER 2: MATERIALS AND METHODS.....	17
2.1: Reagents.....	17
2.2: Tissue Samples.....	17
2.3: Extraction and Purification.....	18
2.4: Spectrophotometric Determination of DNA Concentration.....	18
2.5: Polymerase Chain Reaction (PCR) Amplification.....	19
2.6: Polyacrylamide Gel Electrophoresis.....	20
2.7: PCR Oligonucleotide Selection.....	20
2.8: BAT-25 and BAT-26.....	21
2.9: D10S208 and D10S587.....	25
CHAPTER 3: RESULTS AND DISCUSSION.....	28
3.1: General Results.....	28
3.2: BAT-25.....	31
3.3: BAT-26.....	33
3.4: D10S208.....	36
3.5: D10S587.....	36
3.6: MSI-H and MSI-L.....	39
3.7: Statistical Analysis.....	42
CHAPTER 4: CONCLUSION.....	43
REFERENCES.....	46

APPENDIX	
A: PROTOCOL FOR PHENOL EXTRACTION	50
B: PROTOCOL FOR ETHANOL PRECIPITATION.....	52
C: PROTOCOL FOR ENDLABELING WITH GAMMA P32.....	53
D: PROTOCOL FOR POLYMERASE CHAIN REACTION (PCR).....	54
E: PREPARATION OF 5% POLYACRYLAMIDE GEL	55
F: FISHER'S EXACT TEST OF BAT-25.....	56
G: FISHER'S EXACT TEST OF BAT-26.....	57
H: FISHER'S EXACT TEST OF D10S208.....	58
I: FISHER'S EXACT TEST OF D10S587.....	59
VITA.....	60

LIST OF TABLES

Table 1.1: Characteristics of the stages of Carcinogenesis (modified from Pitot and Dragan, 1994).....	3
Table 1.2: Oligonucleotide Location and Sequence.....	13
Table 2.1: Microsatellite Primer Annealing Temperatures.....	21
Table 3.1: Results from MSI analysis of all tissue types.....	29
Table 3.2: Assessment of SCC tissue for MSI with BAT-25.....	32
Table 3.3: Assessment of SCC tissue for MSI with BAT-26.....	34
Table 3.4: Assessment of MSI with D10S208.....	37
Table 3.5: Assessment of MSI with D10S587.....	39
Table 3.6: Assessment of MSI-H, MSI-L, and MSS in tissue samples.....	41
Table 3.7: Statistical Comparison Between SCC and Adjacent Normal Samples.....	42

LIST OF FIGURES

Figure 1.1: Adapted Vogelgram, Pathway to Cancer and Genomic Instability.....	5
Figure 1.2: Diagram of Mismatch Repair (Modified from Dunlop MG et al., 1997).....	11
Figure 1.3: Polymerase Chain Reaction.....	14
Figure 2.1: Sequence and Binding Site of the BAT-25 Loci.....	23
Figure 2.2: Sequence and Binding Site of the BAT-26 Loci.....	24
Figure 2.3: Sequence and Binding Site of the D10S587 Loci.....	26
Figure 2.4: Sequence and Binding Site of the D10S208 Loci.....	27
Figure 3.1: Representative analysis of MSI with BAT-25.....	33
Figure 3.2: Representative analysis of MSI with BAT-26.....	35
Figure 3.3: Representative analysis of MSI with D10S208.....	38
Figure 3.4: Representative analysis of MSI with D10S587.....	40

DEFINITIONS AND ABBREVIATIONS

MSI:	Microsatellite Instability
MSS:	Microsatellite Stability
MSI-L:	Low occurrence of Microsatellite Instability
MSI-H:	High occurrence of Microsatellite Instability
SCC:	Squamous Cell Carcinoma
LS:	Lichen Sclerosis
Adjacent tissue:	Tissue sample that was taken adjacent to the SCC
Quisimonomorphic:	Low occurrence of variation in population
PCR:	Polymerase Chain Reaction
Neoplasia:	Abnormal mass of relatively autonomous new growth of tissue
DCC:	Deleted in Colorectal Carcinoma
MCC:	Mutation in Colorectal Carcinoma
APC:	Anaphase-promoting complex
Oligonucleotides:	PCR primers used in amplification of target DNA
Intron:	Non Coding region of the DNA
Exon:	Coding region of the DNA

ABSTRACT

The usefulness of detecting genomic instability in the form of microsatellite instability (MSI) has been examined in a number of cancers. Squamous cell carcinoma (SCC) and adjacent normal and lichen sclerosis (LS) samples were taken from patients with radical vulvectomies. Vulvar SCC samples were compared with adjacent normal samples using four oligonucleotide markers (BAT-25, BAT-26, D10S208 and D10S587) found to be informative with other carcinomas. Using polymerase chain reaction techniques, 30 vulvar SCC DNA samples were examined for MSI. BAT-26 displayed the highest level of MSI and was considered to be the most sensitive marker in studying vulvar SCC.

There was not a statistically significant difference between adjacent normal and adjacent LS samples analyzed. The level of MSI discovered in two of the four loci (BAT-26 and D10S208) were statistically significant when compared to adjacent normal samples and indicates a dysfunction in mismatch repair genes. Genomic instability are pathways of carcinogenesis and is characterized by mismatch repair defects and MSI. Based on the high incidence of MSI in association with BAT-26, it appears that MSI has a role in the genesis of vulvar SCC.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Toxic waste and pollution has been a problem since the beginning of the industrial revolution. Humans are exposed to a mixture of pollutants constantly throughout a normal lifetime. The short-term and long-term effects of these exogenous chemical exposures are unknown or at best, not well understood. Carcinogenesis and mutagenesis can result from these environmental exposures. The number of individuals that have been diagnosed with cancer per capita each year has been increasing since the late seventies (Cancer Facts and Figures - 1997, American Cancer Society: Hoel et al., 1992).

Research is being conducted throughout the world in order to classify new and existing toxic chemicals. Pesticides, cleaning products, and industrial chemicals are constantly being created or reformulated to increase their efficiency and function. These changes in chemical properties also change the associated biological and toxicological properties. We do not fully understand the biological and toxicological mechanisms and hazards of either these new or existing chemicals. Until we have an understanding of these mechanisms, we cannot fully comprehend or mitigate the risk associated with toxic, mutagenic, or carcinogenic substances.

The study of toxicology examines and tries to explain these environmental and human health problems. Toxicology is the “study of adverse effects of chemicals on living organisms” (David and Curtis, 1995). Genetic toxicology focuses on the

mutagenic effect of chemicals on DNA and RNA as well as the consequences of exposure to mutagens. In genetic toxicology, we look at the mechanisms of mutagenesis, characteristics of mutagens and their human health effects. Mutagens are chemicals and compounds that have the ability to alter DNA and can affect its fidelity of replication. Mutagenesis is important in studying genetic diseases, but it is also important in the study of carcinogenesis (George, 1995).

1.2 Carcinogenesis

Carcinogenesis is characterized as an accumulation of mutations in DNA that causes cellular dysfunction as well as uncontrolled growth. These changes in function can lead to a decrease or failure of DNA repair mechanisms, failure of apoptosis (programmed cell death), and/or an increase in cell proliferation. A carcinogen is an agent that causes or induces neoplasia (abnormal mass of relatively autonomous new growth of tissue), although a better definition would be “an agent whose administration to previously untreated animals leads to a statistically significant increased incidence of neoplasms in appropriate untreated animals” (Pitot, 1986).

Recent studies have shown that tumor generation is performed in stages. Carcinogenesis can be the result one or more chemical, physical, biological and genetic insults to cellular structure and function (David and Curtis, 1995). This multistage process can be broken down into three distinct steps: Initiation, Promotion, and Progression as show in table 1.1. The first stage, initiation, results from one or more fixed mutations or genetic alterations. Promotion is a reversible stage that involves the expression of the mutated genome mediated through promoter-receptor interactions. Progression is the final stage that is characterized by instability and tumor growth, and is

enhanced by uncontrolled cell proliferation and lack of the apoptotic pathway (Pitot and Dragan, 1994).

Table 1.1 Characteristics of the stages of Carcinogenesis (modified from Pitot and Dragan, 1994).

Initiation	Promotion	Progression
Simple mutations	Reversible both at the level of gene expression and at the cell level	Irreversible genetic alteration in both cellular and genomic structures resulting from karyotypic instability.
Point mutations in proto-oncogenes and/or deletion or inhibition of tumor suppressor genes	Inhibition of apoptotic pathways. Increased cell proliferation	Preneoplastic lesions and damage that can lead to benign tumor and malignancy

Proto-oncogenes and tumor suppressor genes are critical targets in the initiation and promotion stage. Alterations in these genes increase the risk of tumor development and promotion of neoplasia. Cancer is sometimes characterized as an accumulation of mutations during the initiation and promotion stages, with tumor development during the progression stage.

1.3 Genomic Instability

Cancer progression is a multi-stage process that involves an accumulation of mutations and an expansion of mutant lineages leading to incremental increases in tumor size, a transition from a benign to a malignant state, metastasis and disease pathology (Cahill et al., 1999; Lengauer et al., 1998). A typical characteristic of cancer cells is the phenomenon of genetic instability (Cahill et al., 1999).

Genetic instability can be defined as an increase in the rate of mutation caused by corruptions in checkpoint genes that normally ensure faithful replication of the genome (Wodarz and Krakauer, 2001). The relevance and role of genetic instability for cancer progression remains unclear. The elevated rate of mutation effectively reduces the overall replication rate of the tumor cell population (Lengauer et al., 1998). This is because a high mutation rate results in a reduced number of viable daughter cells. If the mutation rate is above a certain threshold, the tumor population can decrease. Because of this, genetic instability can reduce the fitness of the tumor.

During the cell cycle, the order of events is maintained by controls termed checkpoints. Two checkpoints are sensitive to DNA damage, one that acts before mitosis and a second that acts before DNA replication (Weinert and Lydall, 1993). This is relevant to cancer because checkpoint mutants show genetic instability, and such instability is characteristic of many cancers (Wodarz and Krakauer, 2001).

The loss of genomic stability in cancer cells is characterized by an accumulation of mutations. One theory about cancer investigated in the early 1990's by Bert Vogelstein is that when there is a loss genomic stability the cells will have a greater incidence of mutations (Fearon and Vogelstein, 1990). In his Vogelgram, the progression of normal cells with genomic stability to carcinoma cells with a loss of genomic stability is detailed in figure 1.1 (Lengauer et al., 1998).

1.4 Microsatellite Instability

The protein-coding regions of human DNA only account for about 3% of the human genome. Most polymorphisms (DNA differences present in greater than 1% of the population) occur in the 97% of the human genome that does not encode for proteins;

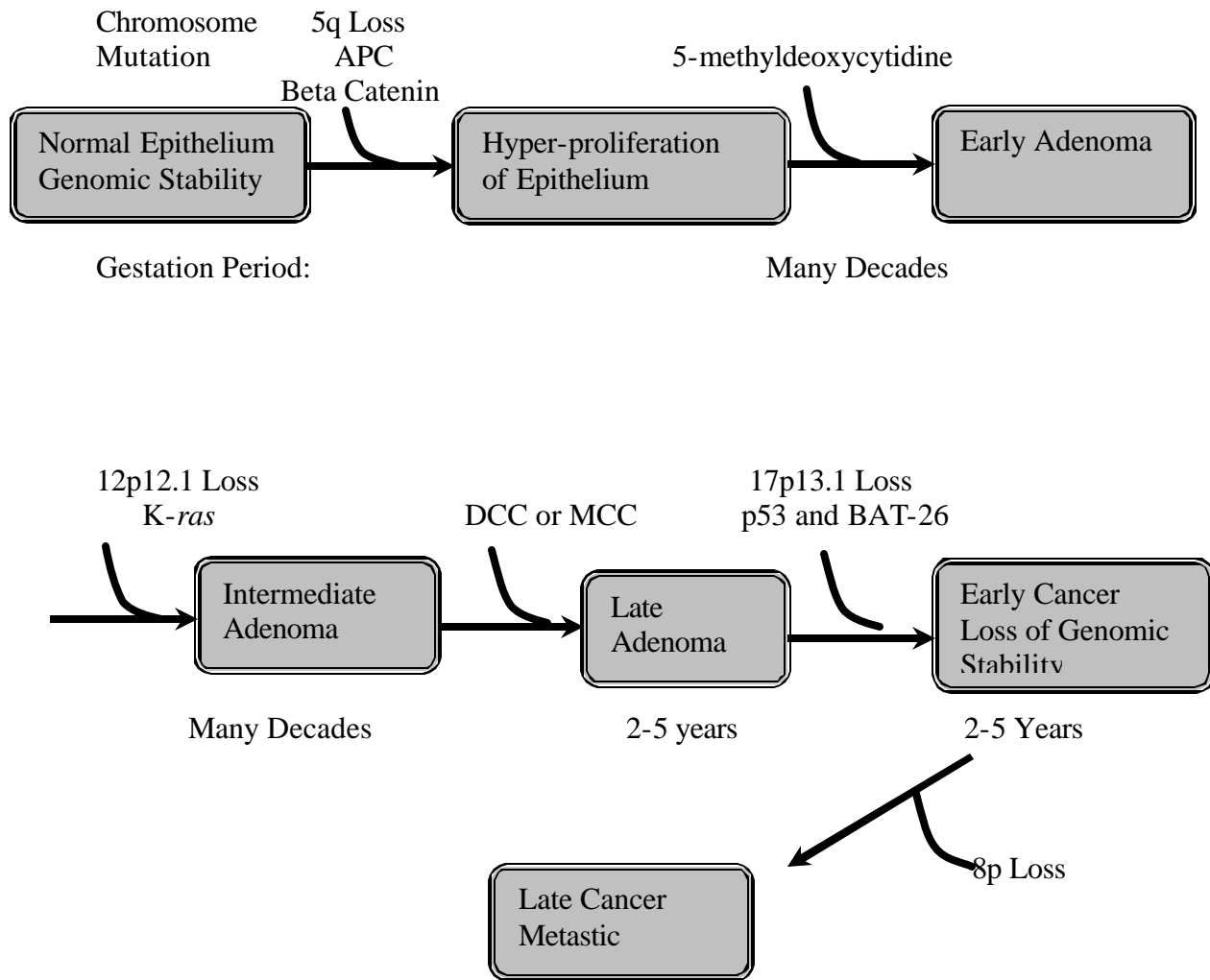


Figure 1.1: Vogelgram, Pathway to Cancer and Genomic Instability (Adapted Fearon and Vogelstein, 1990).

p53 mutations are the most common mutation in human cancer (>50%). During the early and late cancer stages there is a total loss of genomic stability. Deleted in Colorectal Carcinoma (DCC) and Mutation in Colorectal Carcinoma (MCC). Instability with BAT-26 occurs in the early cancer stage at the beginning of genomic instability.

in the past, this area has been referred to as “junk” DNA. The term “junk” DNA is now being considered a misnomer, these non-coding regions may play a vital role in protein synthesis and the normal functionality of the genome (Flam, 1994). However, mutations in noncoding regions such as introns are being found that have multiple effects. These effects include the disruption of splice site function, altering self-splicing RNA (the introns itself), an exon of another gene due to being encoded in the intron, or trans effects on other genes or distant DNA sequences.

Non-coding regions of DNA may be located within genes (introns) or in between genes. Normally, variations in these regions are functionally inconsequential, since the introns do not code for proteins. Because introns are noncoding, there has developed tremendous genetic diversity in these regions. Much of this non-coding DNA consists of highly repetitive segments of DNA with several iterations of specific sequences known as “DNA repeats”. These repeated sequences can occur as a tandem array called Variable Number Tandem Repeats (VNTRs) and they are unique to each person and are the basis for the precise DNA fingerprinting (Edwards et al., 1992).

Microsatellites are stretches of DNA in which a short motif (usually one to five nucleotides long) is repeated several times as a simple tandem repeat. These short sequences are usually 15 to 300 nucleotides in length (Chapelle, 2003). Microsatellites can be found in the human genome as a mononucleotide or dinucleotide (sometimes tri- or tetra-) repeat. A single pair of polymerase chain reaction (PCR) oligonucleotide primers for specific microsatellite loci can produce variably sized DNA fragments depending upon the number of repeats.

The most common microsatellite found in humans is a dinucleotide repeat of cytosine and adenine, (CA)_n. Microsatellites are distributed unevenly throughout the human genome, and are frequently used as landmarks for gene mapping and identification (Chapelle, 2003).

Many of these microsatellites are highly polymorphic (variable throughout human population) and instability of the microsatellite sites has been detected in a number of tumors. Detecting microsatellite instability (MSI) involves comparing the length of microsatellite alleles amplified from tumor DNA with the corresponding allele in normal tissue from the same individual. The addition or deletion of these novel microsatellite alleles in a tissue samples is called MSI.

MSI is caused by a defective mismatch repair system. As a consequence, errors that occur during replication of DNA cannot be repaired and lead to nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences (Slebos et al., 2002). MSI is regarded as an important phenotype of defective DNA mismatch repair and, consequently, as a marker of a high risk for cancer (Hartmann et al., 2002; Shin et al., 2002).

It has been found that mutations in mismatch repair genes are responsible for hereditary nonpolyposis colon cancer (HNPCC) (Loukola et al., 2001), and therefore HNPCC tumors frequently display genetic instability in the form of MSI (Goel et al., 2003; Morifuji et al., 2002; Whitehall et al., 2002; Gryfe et al., 2000). This was the first clue that a mismatch repair defect could be responsible for MSI in carcinomas. The carcinoma tissue that was tested demonstrated new microsatellite alleles in tumor DNA compared to the associated nonneoplastic DNA.

MSI was discovered to be the result of germline mutations in the genes that encode the components of the DNA proofreading complex (Ichikawa et al., 2001; Morifuji et al., 2002). It has been identified in a high percentage of tumors not only in patients with inherited defects in DNA mismatch repair enzymes, but also in a smaller but definite number of oral squamous cell carcinomas as well as other tumors such as endometrial carcinoma (Partridge et al., 2000). The presence of additional microsatellite alleles in cancerous tissue, resulting from the inherent susceptibility of these areas to such alterations and from mutations in the DNA mismatch repair mechanism that would normally correct these errors, may be an important early event in carcinogenesis (Shin et al., 2002; Gryfe et al., 2000).

Cancer arises as a result of the accumulation of genetic changes, which affect many chromosomes and genes. Limited research has been done to show MSI in tissues with squamous cell carcinoma (SCC) and predisposing lesions such as lichen sclerosis (LS). MSI has been seen in tissue associated with lung, colorectal, and other cancers. The focus of this project was to determine if human vulvar SCC and LS display genomic instability in the form of MSI.

Despite numerous studies, the reported rates for MSI for each malignancy differ widely in the literature. The discrepancies may stem from differences in the methodology used by each study, the assay techniques, and the specific reagents and enzymes. Composites of methodologies (Materials and Methods) used in comparable studies were applied to the analysis of microsatellite instability in vulvar SCC in the present study. Oligonucleotide primers were chosen based on previously published works dealing with MSI of the colon and oral SCC.

Oligonucleotide primer selection was based on literature citations with MSI. Four microsatellite loci were chosen for this study based on previous literature reviews and cancers with comparative tissue types. BAT-25, BAT-26, D10S208, and D10S587 were oligonucleotides used in the analysis of vulvar squamous cell carcinoma and associated tissues. BAT-25 and BAT-26 are two mononucleotide markers considered highly informative in the study of MSI (Borland et al., 1998; Pyatt et al., 1999). D10S208 and D10S587 were considered to be highly informative in the study of MSI in oral squamous cell carcinoma (Yshimitsu, 2002). Since vulvar and oral squamous cell carcinoma are cancers of like tissues with comparative functions, it can be assumed that informative markers for oral SCC would be informative for vulvar SCC.

Five microsatellite loci have been recommended by the international meeting of MSI investigators held by the National Cancer Institute in 1997 (Berg et al., 2000). In 1998, the International Workshop on Microsatellite Instability and RER (Replication Error Repair) Phenotypes in Cancer detection and Familial Predisposition published their approval of five microsatellite loci for the determination of MSI in tumors and associated tissues (Pyatt et al., 1999). Of the recommended five-marker panel, three were dinucleotide microsatellites and two mononucleotide microsatellites, BAT-25 and BAT-26. Two of the four loci used in this study were BAT-25 and BAT-26. Both are typical mononucleotide microsatellites, which contain a stretch of 25 and 26 adenines abbreviated (A)₂₅ and (A)₂₆, respectively.

A study by Yamashita et al., in 2001, looked at MSI of chromosome 10 in association with oral SCC. Sixteen microsatellite markers were considered for the analysis of MSI of chromosome 10. Two of the sixteen markers were chosen to examine

MSI in this study. D10S208 was selected because this microsatellite site exhibited the highest incidence of MSI and D10S587 demonstrated a high level of MSI and is associated with the Deleted in Malignant Brain Tumor 1 (DMBT1) tumor suppressor gene (Yamashita et al., 2001).

1.5 Mismatch Repair

Human mismatch repair genes have the ability to repair both mismatched bases and insertion loop errors. To repair mismatched bases, enzymatic mechanisms have to be able to distinguish which base is the correct one. The GATC sequences in DNA are normally methylated (Me) at the 5' position of cytosine. Immediately after DNA replication, the template strand has been methylated, but the newly synthesized strand is not yet methylated (Mitchell et al., 2002). Because of this, the template strand and the new strand can be distinguished thus indicating the original or correct copy.

During semiconservative DNA synthesis, a GT mismatch arises in one of the sister DNA duplexes. This is achieved by recognition of the transient lack of methylation of the newly synthesized strand before postreplicative DNA methylation takes place. The nonmethylated daughter strand containing the incorrect base is enzymatically attacked by mismatch correction enzymes, and the mis-incorporated base is excised (Dunlop et al., 1997). Repair synthesis and daughter-strand methylation at GATC sequences restore the sister DNA duplexes to their native state (Figure 1.2).

Mismatch repair defects have been studied as a mechanism of carcinogenesis equally important to the primary mutation of proto-oncogenes and tumor suppressor

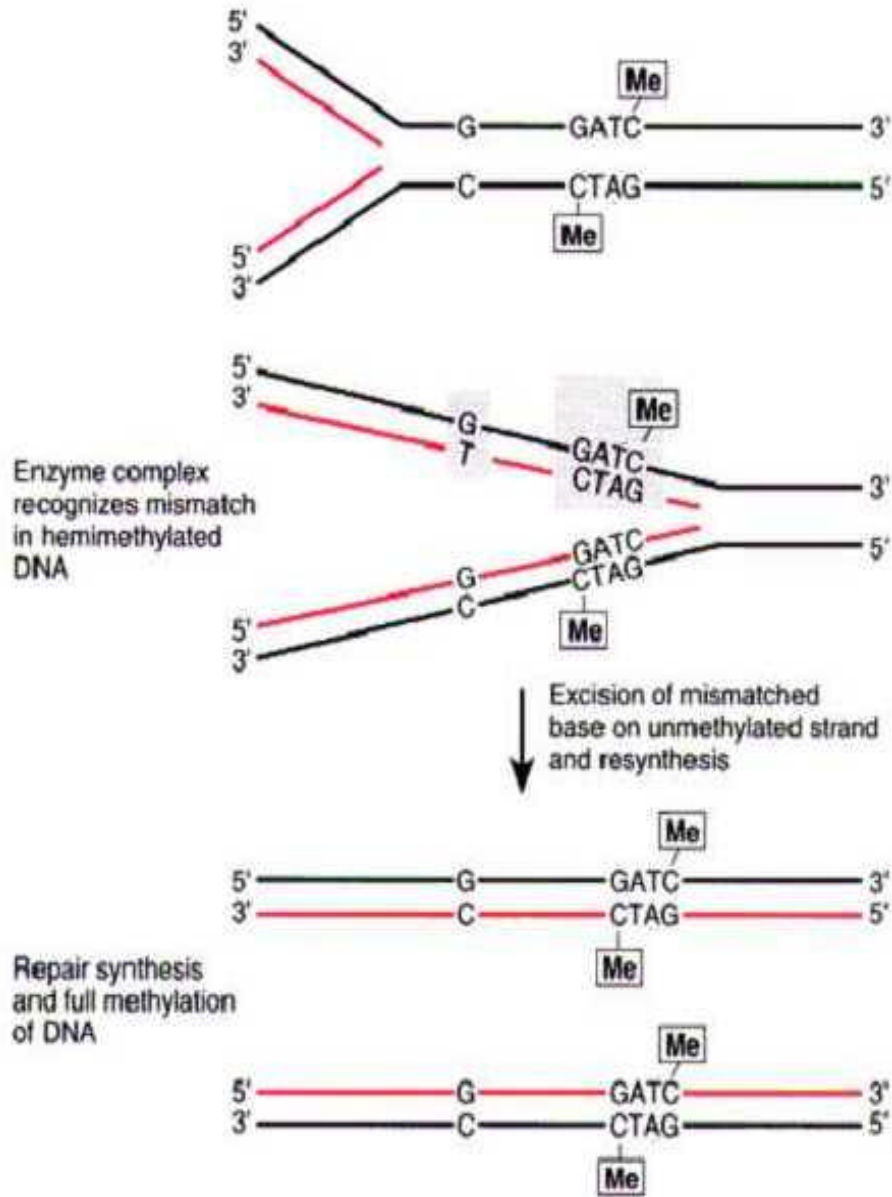


Figure 1.2: Diagram of Mismatch Repair (modified from Dunlop et al., 1997).

genes. Recent *in vitro* evidence shows that cells with defective mismatch repair genes carry a high frequency of errors in microsatellite sequences and would be defined as MSI (Berg et al., 2000). In affected carcinomas, the number of repeat units in the microsatellite site may either increase or decrease, although the composition of the unit itself is unaffected.

1.6 Polymerase Chain Reaction (PCR)

The Polymerase chain reaction (PCR) is a common method of creating copies of specific nucleic acid sequences. Double stranded fragments or sections of DNA are replicated in a mixture of reagents and temperature cycles that are optimized for the sequence being amplified. This amplification of DNA is conducted in a series of cycles with each reaction cycle resulting in the formation of one copy from each DNA template. Each of the new strands and their template are then template for the next cycle. The effect is a theoretical exponential growth rate of selected DNA fragments. PCR requires a template DNA sample, a buffered solution of DNA polymerase, oligonucleotide primers for the template (forward primer) and coding strand (reverse primer), dATP, dCTP, dGTP, dTTP (deoxyribonucleic acid triphosphates/dNTPs), PCR reaction buffer, and magnesium ions ($MgCl_2$) (Promega Corp., 1996).

Oligonucleotide primers are short oligonucleotides (containing about two dozen nucleotides) that are precisely complementary to the sequence at the 3' end of each strand of the DNA to be amplified. These Oligonucleotides will anneal at their complimentary sequence and synthesis begins (as always 5' -> 3') using the original strand as the template. During the extension step of PCR, the DNA polymerase adds dNTPs to the 3'

carbon of the deoxyribose sugar moiety of the primer and then to the 3' end of the developing fragment.

A PCR cycle is broken up into three distinct steps, denaturing, annealing, and extension as seen in figure 1.3. Each step is conducted for between thirty seconds to one minute depending of the sequence being amplified. In step 1, the DNA template is denatured using an extreme temperature of about 95°C. In step 2, the mixture is then cooled to an optimal annealing temperature as defined by the oligonucleotides; this can vary for each primer set. In the third step, the chosen sequence is extended by a step wise addition of dNTPs by the DNA polymerase. *Thermus Aquaticus* (Taq Polymerase) DNA polymerase is a heat stable polymerase used in PCR and is most efficient at 72°C (Watson et al 1992).

For the purposes of the present investigative work, oligonucleotide primer sets to detect MSI in SCC samples were selected based on current colorectal cancer studies and studies of oral SCC (Yshimitsu, 2002; Borland et al., 1998; Pyatt et al., 1999). Four different sets of oligonucleotide primers for PCR were chosen to amplify different sequences in order to test for instability in multiple microsatellite sites (Table 1.2).

Table 1.2 Oligonucleotide location and Sequence

Primer	Location	Microsatellite Repeat
BAT-25	Intron 16, <i>c-Kit</i> protooncogene	(T) ₂₅
BAT-26	intron 5 <i>hMSH2</i> tumor suppressor gene	(A) ₂₆
D10S208	10q	(CA) ₂₄
D10S587	10q25.3-26.1-DMBT1 gene	(AC) ₂₀

Polymerase Chain Reaction (PCR)

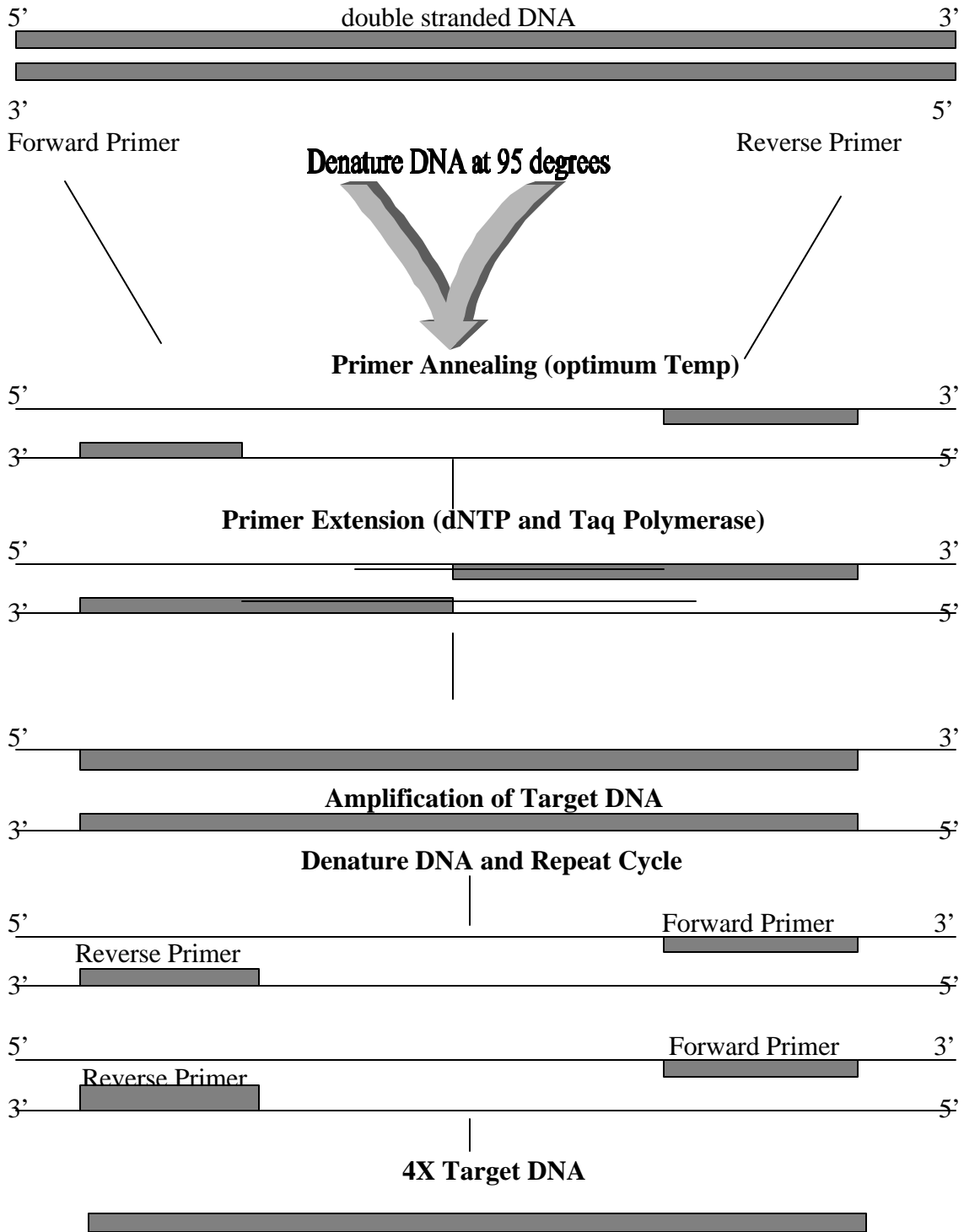


Figure 1.3: Polymerase Chain Reaction

1.7 Vulvar Squamous Cell Carcinoma (SCC)

Vulvar Squamous Cell Carcinoma is a biologically and morphologically diverse disease consisting of tumors that differ in their morphological phenotypes and associated vulvar mucosal disorders (Pinto et al., 1999). Vulvar SCC refers to an abnormal, cancerous growth in the external female genitalia, which includes the labia, the opening of the vagina, the clitoris, and the perineum. SCC represents 90% of the vulvar cancer and occurs mainly in elderly women, but has been diagnosed in young women below the age of 20 (Carlson et al., 1998, Poulsen et al., 2003). Women with this disease are more likely to recover if the cancer is diagnosed and treated early. Although it can affect any part of the female genitalia, it usually affects the labia.

Squamous cells are one of the main types of skin cells. SCC often begins at the edges of the labia majora or labia minora or the area around the vagina. This type of cancer is usually slow growing and may begin with a precancerous condition referred to as vulvar intraepithelial neoplasia, or dysplasia (Carlson et al., 1998). These precancerous cells are present in the surface or epidermal layer of skin. SCC *in situ* (high-grade vulvar intraepithelial lesion), the epithelium is thickened and it consists of atypical cells involving the entire thickness of the epidermis (Abeloff, Clinical Oncology 2nd Ed., 2000).

Vulvar SCC is most common in women over 50 years of age. Vulvar SCC has been associated with sexually transmitted diseases and the human papilloma virus. Additional risk factors for vulvar cancer include having multiple sexual partners, immunodeficiency, cervical cancer, and the presence of chronic vaginal and vulvar inflammations (The Merck Manual of Diagnosis and Therapy, 1999). Vulvar lichen

sclerosis is a known precancerous condition, characterized as chronic squamous cell dermatitis.

1.8 Vulvar Lichen Sclerosis (LS)

Vulvar Lichen Sclerosis (LS) is a painful skin condition that typically affects the vulva and anus. This condition is not contagious and is usually characterized as a chronic dermatosis of the vulva and surrounding tissues. Vulvar lichen sclerosis is typically found in peri-menopausal women with a mean age of 54 years and begins as small white spots starting around the perineum (Wallace, 1971; Carlson et al., 1998; Powell and Wojnarowska, 1999). The symptoms of lichen sclerosis are inflammation, swelling, thinning skin, burning, sores and lesions. If not treated, lichen sclerosis can result in fusing of the skin, atrophy, and narrowing of the vagina.

It is not known what causes lichen sclerosis, but it has been found to be connected to thyroid disease, vitiligo, and autoimmune diseases (loss of skin pigment). These findings show that there is a possibility that LS may be linked to genetic disorders. LS predisposes the skin to cancer, as skin that is scarred by dermatosis is more likely to develop skin cancer. About 1 in 20 women with untreated or misdiagnosed vulvar lichen sclerosis develops SCC (Carlson et al., 1998). In a study of histologically confirmed LS by Carli et al., 1995, it was found that compared to an age-matched control group the cumulative risk of SCC was 14.8%.

CHAPTER 2

MATERIAL AND METHODS

2.1 Reagents

The Taq polymerase (AmpliTaq Gold) was purchased from Perkin-Elmer / Applied Biosystems (Foster City, CA). The AmpliTaq Gold was used in all PCR reactions because of its stability. Enzymes for end-labeling were purchased from New England Biolabs (Beverly, MA). The reagents and DNA samples were stored in a chest freezer at -20°C and were defrosted in an ice bath to ensure that they did not reach room temperature.

Adenosine 5'-[γ - ^{32}P] Triphosphate Gamma was obtained from Amersham Bioscience Corporation (Arlington Heights, IL). All radioactive material was stored and used in a room specific for the use of radioisotopes. ATP [γ - ^{32}P] Gamma, radiolabeled primers, and radioactive PCR products were stored in a locked refrigerator.

2.2 Tissue Samples

Thirty SCC, fifteen adjacent LS, and ten tumor adjacent normal tissue samples were isolated and extracted for analysis. The genomic DNA was isolated from vulvar tissue samples obtained and histologically categorized as SCC, LS, or normal by Dr. Andrew Carlson (Albany Medical College), and shipped to LSU individually packaged and frozen. The vulvar tissue samples were kept in a freezer at -80°C until the genomic DNA could be extracted and isolated. The remaining tissue sample was then repackaged and stored in the freezer at -80°C .

Over a hundred vulvar tissue specimens were obtained from radical vulvectomy patients and supplied to our laboratory for analyses. Dr. Carlson has completed pathological and clinical data on each specimen received. The tissue samples were split three ways, one was frozen at -80°C , the second was fixed in formalin for histologic and eosin examination as well as quantitative and qualitative analysis of multiple immunohistochemical and fluorescent *in situ* hybridization (FISH) markers of cytogenetic and neoplastic progression. The third portion was submitted to short-term culture for cytogenetic karyotyping. The second and third samples have been or will be analyzed by Dr. Carlson's laboratory.

2.3 Extraction and Purification

Two different protocols were followed in order to extract and purify the genomic DNA. The first method used a standard phenol and chloroform extraction with concentration of DNA by ethanol precipitation (Ausubel, et al., 1999). The detailed method for the phenol and chloroform extraction and ethanol precipitation can be found in appendix A and B, respectively. The second method was the use of the Qiagen Kit (Qiagen Inc., Chatsworth, CA) for the extraction and purification of genomic DNA from human tissue.

2.4 Spectrophotometric Determination of DNA Concentration

The DNA products from these extraction and isolation protocols were analyzed for purity and output from their optical density using a Beckman DU-64 Spectrophotometer. The bases of DNA molecules absorb ultraviolet (UV) light at a maximum absorbance wavelength of 260 nm. Because the base pairs are able to absorb UV light at A_{260} (absorbance at 260 nm), the total concentration of DNA in the solution

can be measured. An A₂₆₀ (also known as optical density) of 1.0 equates to 50µg/ml of double stranded DNA in a 1 cm spectrophotometric beam path. This spectrophotometric determination of the concentration can be done if the solution has little or no protein contamination.

Proteins and RNA absorb UV light at a wavelength of A₂₈₀, and so the reading at this wavelength is a measure of protein and RNA contamination. If the concentration is pure, then the ratio of A₂₆₀:A₂₈₀ will be theoretically 1.95. When the ratio is significantly less than 1.95, the sample is too contaminated with either proteins or RNA to use the spectrophotometric method. In this case, a concentration standard method can be employed to estimate the concentration of DNA in a solution by loading a known volume of the sample into an agarose gel, along with an identical volume of DNA with a known concentration.

2.5 Polmerase Chain Reaction (PCR) Amplicfication

Each primer sequence was analyzed using software for the Macintosh called Ogllo 5.0v analysis software (National Biosciences, Inc., Plymouth, MN). The software helped to estimate the proper annealing temperature for each primer and the probability that it might anneal to itself during the PCR reaction forming dimers. These primer dimers can reduce the efficiency of the PCR reaction and decrease total amplification of the targeted sequence.

The PCR reactions were standardized on a 9700 Thermal-Cycler by Perkin-Elmer/ Applied Biosystems (Foster City, CA). Each reaction contained 200 ng of DNA, 1 unit of AmpliTaq Gold, 1.5 mM MgCl₂, 200 mM dNTPs, 1mM of each PCR primer, 1 µl of end labeled forward primer (Appendix C) and 1X PCR buffer in a total volume of

25 μ l. The DNA was denatured at 95°C for 7 minutes then amplification was carried out for 35 cycles of 95°C for 1 minute, 1 minute at 55°C (BAT-26 and D10S208) and 58 °C (BAT-25 and D10S587), and 72°C extension for 1 minute, followed by a 10 minute 72⁰C extension (Appendix D).

2.6 Polyacrylamide Gel Electrophoresis

The 32p labeled PCR amplified products were run on a 7M urea, 5% denaturing polyacrylamide sequencing gel (Appendix E). The gel was vacuum dried on a Model 583 gel dryer from Bio-Rad at 65°C for 20 minutes and then allowed to cool for 10 minutes. The gel was transferred to an exposure cassette with intensifying screens and placed in the -80°C freezer for 4-24 hours depending on the signal strength of the products.

2.7 PCR Oligonucleotide Selection

PCR primers were selected based on literature citations with MSI. BAT-25, BAT-26, D10S208, and D10S587 were the four loci chosen for the analysis of MSI. BAT-25 and BAT-26 were recommended by a reference panel for detecting high frequency MSI in colorectal cancer (Borland et al., 1998) and D10S208 and D10S587 were selected from a paper on oral squamous cell carcinoma by Yshimitsu, 2002.

PCR primers D10S208 and D10S587 were sited as being associated with oral squamous cell carcinoma and are relevant in studying vulvar SCC because of the similarity in cell type and tissue function. Both of these microsatellite markers are dinucleotide repeats of cytosine and adinosine (CA_n). D10S587 is associated with the DMBT1 tumor suppressor gene and D10S208 was found to be the most informative marker in oral squamous cell carcinoma.

Incidence of primer dimers and loops were analyzed using Oligo v5.0 Primer Analysis software (National Biosciences, inc., Plymouth, MN). The PCR primers were purchased from BioServe Biotechnologies, Ltd (Laurel, MD). The sequences of the PCR primers and experimentally determined optimal annealing temperatures for primer sets are displayed in Table 2.1.

Table 2.1 Microsatellite primer anneal temperature

Marker	Product length	Annealing Temp	Sequence
BAT-25	121-126	58°C	5'-TCG CCT CCA AGA ATG TAA GT-3' 5'-TCT GCA TTT TAA CTA TGG CTC-3'
BAT-26	125	55°C	5'-TGA CTA CTT TTG ACT TCA GCC-3' 5'-AAC CAT TCA ACA TTT TTA ACC C-3'
D10S208	181	55°C	5'-AAG TGA CTG TTT TGG GGG AG-3' 5'-CCC ACA ACT CAA TAA ACT CAA ACT C-3'
D10S587	179	58°C	5'- CCC AGA TTC ATG GCT TTC-3' 5'- TTC TGC TGA CAC GGG C-3'

2.8 BAT-25 and BAT-26

MSI status was determined by screening the polyadenosine sequence, BAT-26, which has proved 99.4% efficient at detecting MSI-associated cancer. The BAT-25 and BAT-26 loci are both mononucleotide repeats containing a 25-repeat thymine tract (poly T) and a 26-repeat deoxyadenine tract (poly A), respectively. BAT-26 is a highly sensitive mononucleotide repeat; located within the fifth intron of the *hMSH2* (human MutS homologue) gene and is a specific indicator of generalized MSI as evident in human cancers.

BAT-25 and BAT-26 were selected for this study based on markers picked by two conferences on the study of MSI. Both microsatellite loci are mononucleotide repeats and are considered to be sensitive markers for MSI (Loukola et al., 2001). The BAT-25 locus contains a 25-repeat thymine tract, (T)₂₅, located within intron 16 of the *c-Kit* proto-oncogene, assigned to 4q12 (Figure 2.1) (Morifuji et al., 2002; Ichikawa et al., 2001; Pyatt et al., 1999). The *c-Kit* gene is important for the protein KIT receptor and proper cell proliferation. When damaged, the KIT receptor can be continuously activated, which caused the receptor to signal the cell to divide, without the proper stimulus (London et al., 1996).

The BAT-26 locus contains a 26-repeat adenosine tract, (A)₂₆, located within intron five of the *hMSH2* (human MutS homologue) tumor suppressor gene (Figure 2.2) (Pyatt et al., 1999; Loukola et al., 2001; Shin et al., 2002; Morifuji et al., 2002). The *hMSH2* tumor suppressor gene is a mismatch repair gene necessary for maintenance of DNA fidelity. Studies of MSI have been focused on defective mismatch repair genes in the pathogenesis of malignant carcinoma. Cells with defective mismatch repair mechanisms can not correct genetic errors that occur during cellular replication, e.g., point mutations, deletions, insertions and strand slippage. Thus, the fidelity of DNA replication is reduced.

Loss of control over cell growth and proliferation may occur when there are mutations of proto-oncogenes and tumor suppressor genes. Cells with mutated or deleted mismatch repair genes are not able to repair replication errors during DNA replication, microsatellites are particularly prone to replication errors, and so MSI is the hallmark of

Homo Sapiens c-Kit proto-oncogene, intron 16 sequence

GenBank U63834

Product Length: 125bp

```

80101 CCTCTCTTCC TCACAGGCTC ATACATAGAA AGAGATGTGA CTCCCGCCAT CATGGAGGAT
      GGAGAGAAGG AGTGTCCGAG TATGTATCTT TCTCTACACT GAGGGCGGTA GTACCTCTCA

80161 GACGAGTTGG CCCTAGACTT AGAAGACTTG CTGAGCTTTT CTTACCAGGT GGCAAAGGGC
      CTGCTCAACC GGGATCTGAA TCTTCTGAAC GACTCGAAAA GAATGGTCCA CCGTTTCCCG

      |
      | BAT-25 F 20mer 62°C
      |
80221 ATGGCTTTCC TCGCCTCCAA GAATGTAAGT GGGAGTGATT CTCTAAAGAG TTTTGTGTTT
      TACCGAAAGG AGCGGAGGTT CTTACATTCA CCCTCACTAA GAGATTTCTC AAAACACAAA

      |
      | Microsatellite Site
      |
80281 TGTTTTTTTTG ATTTTTTTTTT TTTTTTTTTT TTTTTTTGAG AACAGAGCAT TTTAGAGCCA
      ACAAAAAAAC TAAAAAATAA AAAAAAATAA AAAAAAACTC TTGTCTCGTA AAATCTCGGT
      |
      |
80341 TAGTTAAAAT GCAGAATGTC ATTTTGAAGT GTGGTAACCA AAAGCAGAGG AAATTTAGTT
      ATCAATTTTA CGTCTTACAG TAAAACCTCA CACCATTGGT TTTTCGTCTCC TTTAAATCAA
      BAT-25 R 21mer      60°C
      |
80401 TCTTCATGTT CCAACTGCTG TCTCTTTGGA ATTCCTGTTC TAATTTATAA GCTGTAAAGT
      AGAAGTACAA GGTTGACGAC AGAGAAACCT TAAGGACAAG ATTAATATT CGACATTTCA

80461 ACAAGCCTGT CTAAATGAGT TTTTCTATGA ATATTCTTTT ATATGCAGTG AAATTCTTTT

80581 AAAACTTTTG GCTTTTAGGA TATAGGATAT GTTCCTAGAG AACAGAATCA TTTTATCAGT

80641 TGAAGTTAGA TCCAAATATT ATATGTGTGG CTTATGAAGT GTCAGGAAAT AAGGGGTCAG

80701 AGGGAGTAAT AAACACTTGG GAGAAGGTTA GGAATGGAAA GAATGATGGA ACCAAAACAA

80761 GGAGCATGGT CTGTGGAAGG GTGAAAGGAG TTCCTTAGGA AGTAAGATTA ACCGAACAGA

80821 ATGAGTTACC AGTCCTACCC TTAAATGTCA TGGGTGACAT TTCCCAACAA TTACCAAACCT

80881 AAGAAAGGAT ATAAGATGGC TGAAATAAAG ACCTTCTTCC GTGTGTCCTT GGGAGATGTC

80941 AGATTGAATT TGCAAAGGCA TATTAGGAAC TCTGTGAAAG GACATTCAA GAGATGCATG

81001 CAAAATGAAT TTTCAGTTTA AACAATATGA TATGACTATT TCTTATGTAT TTCCCTATGA

81061 ATGAAAGCAG TCCTGAGAAG AAAACAGCAT TTATTAGAAT TGCTTTTAAA AGAGATTATA

81121 ATAATTAGAC TCTTGATTAT GCGAACATCA TTCAAGGCGT ACTTTTGATT TTTATTTTTG
  
```

Figure 2.1 Sequence and Binding Site of the BAT-25 Loci (Ichikawa et al., 2001).

Homo Sapiens MSH2 tumor suppressor gene, introns 5 Sequence

GenBank U63834

Product Length: 126 bp

80001 CCAGTGGTAT AGAAATCTTC GATTTTTTAAA TTCTTAATTT TAGGTTGCAG TTTCATCACT
GGTCACCATA TCTTTAGAAG CTAAAAATTT AAGAATTAAA ATCCAACGTC AAAGTAGTGA

80061 GTCTGCGGTA ATCAAGTTTT TAGAACTCTT ATCAGATGAT TCCAACTTTG GACAGTTTGA
CAGACGCCAT TAGTTCAAAA ATCTTGAGAA TAGTCTACTA AGGTTGAAAC CTGTCAAAC

→ | BAT-26 F 21mer 60°C | ←
80121 ACTGACTACT TTTGACTTCA GCCAGTATAT GAAATTGGAT ATTGCAGCAG TCAGAGCCCT
TGACTGATGA AAACGAAGT CGGTCATATA CTTAACCTA TAACGTCGTC AGTCTCGGGA

→ | Microsatellite Site | ←
80181 TAACCTTTTT CAGGTA⁵AAAAA AAAAAAAAAA AAAAAAAAAA | AGGGTTAAAA ATGTTGATTG
ATTGGA⁵AAAAA GTCCATTTTT TTTTTTTTTT TTTTTTTTTT TCCCAATTTT TACAAC⁵TAAAC
→ | BAT-26 R 22mer 62°C

80241 GTTAANNNNN NNNGACAGAT AGTGAAGAAG GCTTAGAAAAG GAGCTAAAAG AGTTCGACAT
CAATTNNNNN NNNCTGTCTA TCACTTCTTC CGAATCTTTC CTCGATTTTC TCAAGCTGTA
←

80301 CAATATTAGA CAAG
GTTATAATCT GTTC

Figure 2.2: Sequence and Binding Site of the BAT-26 Loci

inactivated mismatch repair genes, which can lead to increased risk of oncogenesis (Shin et al., 2002; Morifuji et al., 2002).

Because of the quasimonomorphic (low occurrence of polymorphisms) profile of both loci, BAT-25 and BAT-26 have proven very useful for the identification of MSI (Pyatt et al., 1999). The shortened and unstable alleles can easily be differentiated from alleles of normal size.

2.9 D10S208 and D10S587

The deleted in malignant brain tumors 1 (DMBT1) gene is located on 10q25.3-26.1 (Sasaki et al., 2002;) and the deletion or loss of expression of the DMBT1 gene has been reported in association with brain, esophagus, stomach, colon and lung cancers (Yamashita et al., 2001). D10S2587 is a dinucleotide composed of 20 repeats of adenosine and cystein, (AC)₂₀ (Figure 2.3), microsatellite site contained within the DMBT1 tumor suppressor gene. This locus was chosen because of the connection between the DMBT1 tumor suppressor gene and oral SCC (Yamashita et al., 2001). Oral and vulvar SCC are cancers of related tissue types, both are mucosa tissue.

In a study by Yasuhiro Yamashita in 2001, 16 microsatellite sites located on chromosome 10 were investigated to see the correlation between oral SCC and MSI. Of the 16 loci chosen for the study, D10S208 displayed the highest degree of MSI. D10S208 is a dinucleotide made up of 24 repeats of cystein and adenosine, (CA)₂₄ (Figure 2.4). Although it is not associated with a tumor suppressor gene or proto-oncogene, it was considered highly informative in the study of oral SCC (Yamashita et al., 2001).

Homo Sapiens (D10S587) DNA segment containing (CA) repeat, Associated with DMBT1 tumor suppressor gene, Sequence

GenBank Z24180
Product length=179

```

                                → | D10S587 F 17mer 60°C | ←
1  AGCTCATTGA GAAGAATAAC AGGCCAGAT TCATGGCTTT CACTCAGCCC TTCCTTTCTT
   TCGAGTAACT CTTCTTATTG TCCGGGTCTA AGTACCGAAA GTGAGTCGGG AAGGAAAGAA

                                → |                               | ←
61 TGGGTTGAAA CACACACACA CACACACACA CACACACACA CACACACACT GTCTTACCTT
   ACCCAACTTT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGA CAGAATGGAA

121 CTGTGAGTGG GAGGGTGTCA GTGCCTCTTC AGATGTCTGC TTTTGTGATAT TAACCACTTG
    GAACTCACC  CTCCACAGT  CACGGAGAAG  TCTACAGACG  AAAAACTATA  ATTGGTGAAC

181 CTGGCCCGTG TCAGCAGAAA GGCTCTGTAT AGATGTCGTT TAGCGGGAAT TCCTAAAGCC
    GACCGGGCAC AGTCGCTTTT CCGAGACATA TCTACAGCAA ATCGCCCTTA AGGATTTCTGG
→ | D10S587 R 16mer | ←
   60°C

241 CTCCCCTCCC TCTAAGGCCT CCAGAGACCA CCCTACCTTC TAGGTCACAA ATGAGATGTC
    GAGGGGAGGG AGATTCCGGA GGTCTCTGGT GGGATGGAAG ATCCAGTGTT TACTCTACAG

301 TTCTAGGTCA CAAATGAGAT GTCTGAACCT TTATACCTGC TGGTCTCCAG CT
    AAGATCCAGT GTTTACTCTA CAGACTTGGG AATATGGACG ACCAGAGGTC GA

```

Figure 2.3: Sequence and Binding Site of the D10S587 Loci

Homo Sapiens (D10S208) DNA segment containing (CA) repeat, Sequence

Product Length = 181

Sequence

```

1  AGCTATATTT GAGACCTTTN | D10S208 F 20Mer 58°C | GGGCAATATT TTCAAATTCT
   TCGATATAAA CTCTGGAAAN | AGGTGACTGT TTTGGGGGAG | CCCGTTATAA AAGTTTAAGA

61  GAAATTTCTT ATTTAGAAAT AATTATTGGA TCTCACACAC ACACACACAC ACACACACAC
    CTTTAAAGAA TAAATCTTTA TTAATAACCT AGAGTGTGTG TGTGTGTGTG TGTGTGTGTG

121  Repeat
     ACACACACAC ACACACACAC | AACTTACCT GTGGTTCTTG TTCTCTGAAG GACATNCAGT
     TGTGTGTGTG TGTGTGTGTG | TTTGAATGGA CACCAAGAAC AAGAGACTTC CTGTANGTCA

181  ANTTGAAAAC ATCCCCACAC TCTTGGGTAC ATTCTCCTAA GTGTG
     TTTAACTTTTG TAGGGGTGTG AGAACCCATG TAAGAGGATT CACAC
     → | D10S208 R 22Mer 58°C | ←

```

Figure 2.4 Sequence and binding site of the D10S208 loci

CHAPTER 3

RESULTS AND DISCUSSION

To study the association of MSI with vulvar SCC and adjacent vulvar LS, the presence and frequency, if applicable, of additions or deletions in each of the microsatellite loci were tested. Some of the DNA samples analyzed could not be amplified by all of the microsatellite markers, these samples were considered to be non-informative (NI) and were not considered in calculating the percent instability. NI samples were analyzed a second time to ensure that the results were not due to procedural failure. SCC samples displayed a greater frequency of NI than the adjacent normal and adjacent LS samples, which displayed a comparative frequency.

3.1 General Results

In this study, vulvar SCC, adjacent LS and adjacent normal tissue samples were examined using four microsatellite loci (BAT-25, BAT-26, D10S208 and D10S587) in order to establish that these markers were informative in studying vulvar SCC. The association with MSI is an important marker of genetic instability that characterizes cancers with replication errors and nonfunctional mismatch repair genes. Representative samples that tested positive (+) for MSI were analyzed a second time to ensure reproducibility. Adjacent normal tissue samples as well as blanks were used in each PCR reaction as positive and negative controls and for comparison to establish the presence or the absence of MSI.

It was found that BAT-26 was the most informative marker for MSI and was found to be significantly different than the adjacent normal tissue tested ($p=0.0013$). D10S208 was considered to be informative and displayed a significant difference in

MSI when compared to the adjacent normal tissue tested ($p=0.009$). The adjacent normal and adjacent LS tissue tested indicated no significant difference in MSI and was considered to be microsatellite stable (MSS) or to have a low occurrence of MSI (MSI-L), with only two adjacent LS specimens considered to have a high occurrence of MSI (MSI-H). The results of all of the tests for MSI conducted in this study are found on Table 3.1.

Table 3.1: Results from MSI analysis of all tissue types.

Vulvar Specimens for Microsatellite Instability						
Specimen	Identifier	BAT-25	BAT-26	D10S208	D10S587	Stability
CW 3-3	SCC	-	+	NI	-	MSI L
CW 5-1	SCC	NI	+	+	-	MSI H
CW 12-4	SCC	-	-	-	+	MSI L
CW 16-2	SCC	-	-	+	NI	MSI L
CW 16-3	N	-	-	-	NI	MSS
CW 17-1	SCC	-	-	-	-	MSS
CW 17-5	N	-	-	-	-	MSS
CW 17-6	LS	-	-	+	NI	MSI-L
CW 18-1	SCC	-	-	-	-	MSS
CW 18-5	N	-	-	-	-	MSS
CW 19-1	SCC	-	-	-	-	MSS
CW 19-2	SCC	NI	-	NI	NI	NI
CW 21-2	LS	-	-	-	-	MSS
CW 23-1	SCC	-	+	-	NI	MSI L
CW 23-3	N	-	-	-	-	MSS
CW 24-1	SCC	-	+	-	NI	MSI L
CW 25-1	SCC	-	+	-	NI	MSI L
CW 25-4	LS	-	-	-	-	MSS
CW 26-2	SCC	+	+	+	+	MSI H
CW 28-1	SCC	-	+	-	-	MSI L
CW 28-4	LS	-	-	-	-	MSS
CW 29-1	SCC	-	NI	+	+	MSI H
CW 29-3	N	-	-	-	-	MSS
CW 29-5	LS	-	-	-	-	MSS
CW 29-6	SCC	-	+	-	+	MSI H
CW 30-6	LS	-	NI	-	-	MSS
CW 35-3	SCC	NI	-	NI	NI	NI
CW 39-1	N	-	-	-	-	MSS

(Table 3.1 Continued)

Specimen	Identifier	BAT-25	BAT-26	D10S208	D10S587	Stability
CW 39-2	SCC	-	+	-	+	MSI H
CW 39-3	LS	+	+	+	-	MSI H
CW 40-1	SCC	+	-	-	-	MSI L
CW 40-4	N	-	-	-	-	MSS
CW 40-5	LS	-	-	-	-	MSS
CW 41-1	SCC	-	-	+	-	MSI L
CW 41-8	SCC	-	+	-	-	MSI L
CW 43-1	SCC	-	-	+	+	MSI H
CW 44-1	N	-	+	-	-	MSI L
CW 44-2	SCC	-	+	+	-	MSI H
CW 44-4	LS	-	+	-	+	MSI H
CW 45-4	N	-	NI	-	-	MSS
CW 46-6	LS	-	-	-	-	MSS
CW 47-2	SCC	-	-	+	-	MSI L
CW 47-3	LS	-	NI	-	-	MSS
CW 49-1	SCC	-	NI	-	-	MSS
CW 49-4	LS	-	-	-	-	MSS
CW 49-6	LS	-	-	-	-	MSS
CW 49-7	N	+	-	-	-	MSI L
CW 50-1	SCC	-	+	+	-	MSI H
CW 50-5	LS	-	-	-	-	MSS
CW 52-2	N	-	NI	-	-	MSS
CW 57-1	SCC	NI	+	-	-	MSI L
CW 61-2	LS	-	-	-	-	MSS
CW 61-6	SCC	-	+	+	-	MSI H
CW 63-1	SCC	+	+	+	-	MSI H
CW 64-3	SCC	-	+	-	-	MSI L
CW 70-1	LS	-	-	-	-	MSS
CW 70-3	SCC	-	-	-	-	MSS
CW 70-5	LS	-	-	-	-	MSS
CW 70-7	N	-	NI	-	-	MSS
CW 73-1	N	-	-	-	+	MSI-L
CW 80-2	SCC	NI	+	-	-	MSI L
CW 91-5	LS	-	-	-	-	MSS
CW 97-1	SCC	-	-	-	-	MSS
	Informative Cases	MSI +	% MSI SCC	% MSI LS	% MSI Normal	
BAT-25	27.0	3.0	11.1	5.6	7.7	
BAT-26	30.0	17.0	56.7	12.5	10.0	
D10S208	30.0	11.0	37.9	11.1	0.0	
D10S587	26.0	6.0	23.1	5.9	8.3	

+ Positive for MSI

- Negative for MSI

NI

Non Informative with specified marker

3.2 BAT-25

The BAT-25 locus has been shown to be a sensitive marker of MSI, and is characterized by a shortening or lengthening in the size of the mononucleotide repeat in tumor DNA and is shown on an autoradiograph as a banding of alleles. MSS tumor, adjacent lichen sclerosis, or adjacent normal tissues exhibit little or no polymorphic variation in the size of the poly (T) tracts. This fact is useful in the analysis of MSI, allowing the use non-associated normal DNA samples. Because of these characteristics, shortened, unstable alleles can easily be differentiated from alleles of normal size.

A total of thirty-two primary tumors and thirteen adjacent normal tissues from patients with SCC were screened for MSI using BAT-25, a quasimonomorphic (low occurrence of variation in population) marker used in the study of HNPCC (Morifuji et al., 2002; Loukola et al., 2001; Gryfe et al., 2000). The frequency of MSI was studied in sixteen associated lichen sclerosis tissue samples in order to study the possibility of progression from normal to tumor. Progression of MSI was studied using five individual cases containing at least one sample from each category, normal, LS, and SCC tissue.

Of the thirty-two SCC samples analyzed, three samples were characterized as positive (+) for MSI (Table 3.2). Twelve percent of the SCC samples showed MSI and was not found to be significantly different than the MSI in normal tissue ($p=1.00$). This would indicate that this locus was not a sensitive marker for vulvar SCC. Five of the thirty-two SCC tissue samples were considered non-informative and could not be characterized as either MSI or MSS.

The corresponding adjacent normal tissue samples were negative (-) for MSI in all but one of the eleven cases that were analyzed. The seventeen of the eighteen samples of adjacent LS tissue with matching SCC cases were also negative (-) for MSI. Representative examples of the analysis of MSI with BAT-25 are illustrated in figure 3.1.

Table 3.2 Assessment of SCC tissue for MSI with BAT-25

Specimen	Identifier	BAT-25	Specimen	Identifier	BAT-25
CW 3-3	SCC	-	CW 39-2	SCC	-
CW 5-1	SCC	NI	CW 40-1	SCC	+
CW 12-4	SCC	-	CW 41-1	SCC	-
CW 16-2	SCC	-	CW 41-8	SCC	-
CW 17-1	SCC	-	CW 43-1	SCC	-
CW 18-1	SCC	-	CW 44-2	SCC	-
CW 19-1	SCC	-	CW 47-2	SCC	-
CW 19-2	SCC	NI	CW 49-1	SCC	-
CW 23-1	SCC	-	CW 50-1	SCC	-
CW 24-1	SCC	-	CW 57-1	SCC	NI
CW 25-1	SCC	-	CW 61-6	SCC	-
CW 26-2	SCC	+	CW 63-1	SCC	+
CW 28-1	SCC	-	CW 64-3	SCC	-
CW 29-1	SCC	-	CW 70-3	SCC	-
CW 29-6	SCC	-	CW 80-2	SCC	NI
CW 35-3	SCC	NI	CW 97-1	SCC	-
BAT-25	Informative Cases	MSI +		% MSI	
SCC	27.0	3.0		11.1	
LS	18.0	1.0		5.6	
N	13.0	1.0		7.7	

+ Positive for MSI
 - Negative for MSI
 NI Non Informative with specified marker

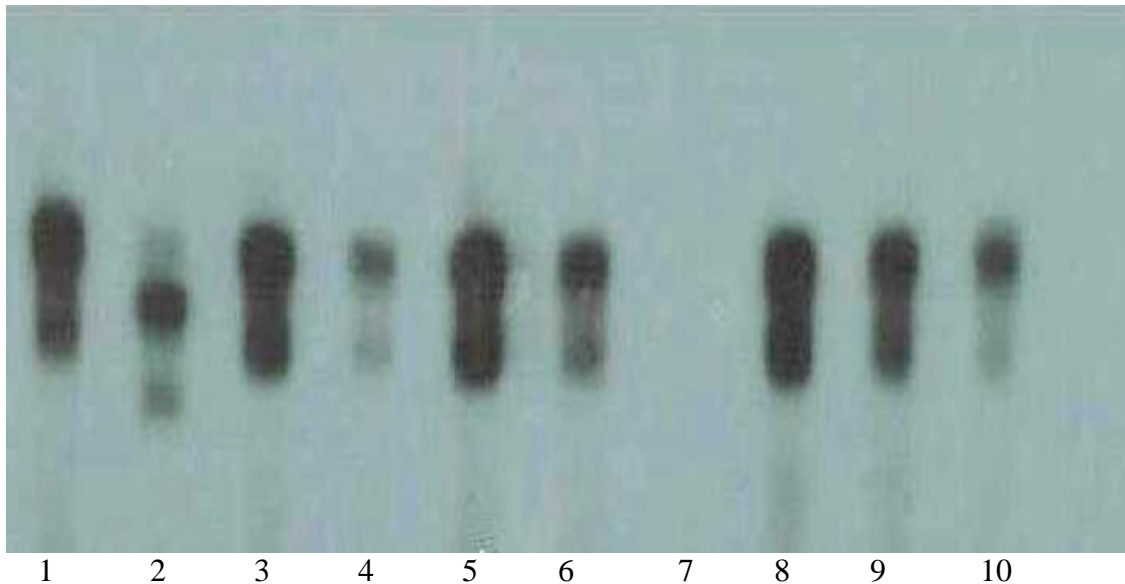


Figure 3.1: Representative samples of the analysis of MSI with BAT-25

Autoradiograph of MSI test. Lane 4 is a blank and was the negative control in this analysis with water instead of the DNA template. Lanes 1 and 2 are positive controls of normal DNA template for comparison to SSC tissue. Lane 9 was positive for MSI and is characterized by added alleles shifting both above and below the target site.

3.3 BAT-26

The BAT-26 locus has demonstrated in previous cancer studies that it can be an extremely sensitive marker for a variety of cancers. MSI in this allele is mainly characterized by a deletion of between five and fifteen bases when compared to normal tissue (Morifuji, 2002). The BAT-26 locus is a quasimonomorphic, poly (A) repeat of 26 adenosines that has been used in numerous studies of MSI and is associated with dissimilar tumors and cancerous tissues.

SCC, adjacent lichen sclerosis, and adjacent normal tissues were analyzed and those that displayed an alteration in the size of the allele were considered to be positive

(+) for MSI. Thirty-one SCC tissue samples were tested using BAT-26, and thirty samples were considered to be informative. Seventeen (56.7%) of the thirty informative cases tested positive (+) for MSI as shown in Table 3.2. Since seventeen cases (56.7%) exhibited an allelic shift, the BAT-26 locus was considered a highly informative marker with respect to vulvar SCC. The high percentage of MSI found in BAT-26 suggests a lack of expression of the mismatch repair genes in the SCC tissue, specifically the hMSH2 tumor suppressor gene.

Table 3.3 Assessment of SCC tissue for MSI with BAT-26

Specimen	Identifier	BAT-26	Specimen	Identifier	BAT-26
CW 3-3	SCC	+	CW 39-2	SCC	+
CW 5-1	SCC	+	CW 40-1	SCC	-
CW 12-4	SCC	-	CW 41-1	SCC	-
CW 16-2	SCC	-	CW 41-8	SCC	+
CW 17-1	SCC	-	CW 43-1	SCC	-
CW 18-1	SCC	-	CW 44-2	SCC	+
CW 19-1	SCC	-	CW 47-2	SCC	-
CW 19-2	SCC	-	CW 49-1	SCC	NI
CW 23-1	SCC	+	CW 50-1	SCC	+
CW 24-1	SCC	+	CW 57-1	SCC	+
CW 25-1	SCC	+	CW 61-6	SCC	+
CW 26-2	SCC	+	CW 63-1	SCC	+
CW 28-1	SCC	+	CW 64-3	SCC	+
CW 29-1	SCC	NI	CW 70-3	SCC	-
CW 29-6	SCC	+	CW 80-2	SCC	+
CW 35-3	SCC	-	CW 97-1	SCC	-
BAT-26	Informative Cases	MSI +		% MSI	
SCC	30.0	17.0		56.7	
LS	16.0	2.0		12.5	
N	10.0	1.0		10.0	

+ Positive for MSI
 - Negative for MSI
 NI Non Informative with specified marker

Aberrant fragments from the PCR amplification were detected in one (10.0%) sample out of the ten adjacent normal tissue samples tested. The adjacent lichen sclerosis samples studied showed an increase in MSI, but were not significantly different than normal tissue. The MSI detected in the SCC tissue using the BAT-26 locus was found to be significantly different from the adjacent normal tissue samples tested ($p=0.0013$). This relationship follows the dogma that from normal to LS to SCC is a progression characterized by increased instability in this microsatellite locus. Representative examples of the analysis of MSI in the three tissue types are illustrated in figure 3.2.

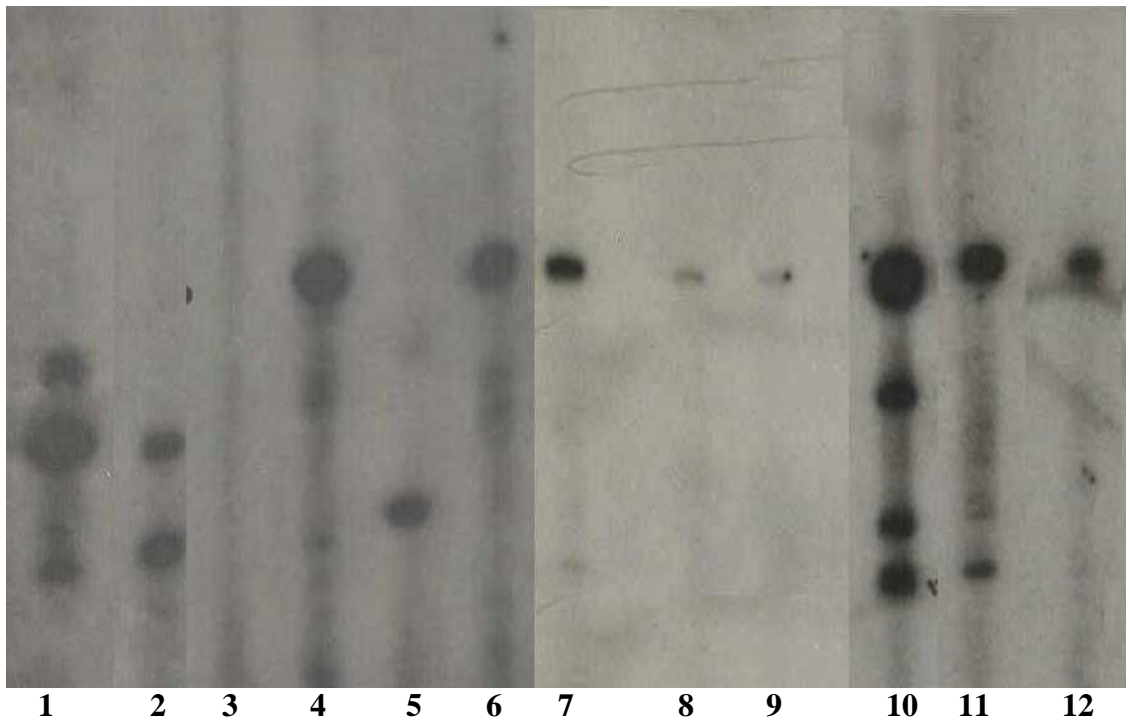


Figure 3.2: Representative samples of the analysis of MSI with BAT-26

Audioradiograph of MSI test. Lane 3 is a blank and was the negative control in this analysis with water instead of DNA template. Lanes 6 and 7 are positive controls of normal DNA template for comparison to SSC tissue. Lane 1, 2, 5, 10, and 11 were positive for MSI and is characterized by a deletion of bases and the separation of alleles.

3.4 D10S208

D10S208 is a dinucleotide microsatellite site consisting of 24 cytosine and adenosine, (CA)₂₄, repeats located on chromosome 10 (10q). Although this locus is not associated with a tumor suppressor gene or proto-oncogene, it was considered to be a highly informative marker by Yasuhiro Yamashita (2001) for MSI in human oral squamous cell carcinoma. Since the oral and vulvar tissues are both mucosal tissue and similar in function, D10S208 was considered for the analysis of vulvar SCC in this study.

Out of the thirty-two primary tumor samples analyzed, thirty samples were considered informative. MSI was found in eleven (37.9%) of the thirty SCC samples tested (Table 3.4). The high percentage of instability of the D10S208 locus in association with the tumor tissue samples analyzed is considered to be evidence that this microsatellite marker can be considered informative with respect to vulvar SCC.

The level of MSI detected in the SCC tissue using the D10S208 locus was found to be significantly different from the normal and lichen sclerosis samples ($p=0.009$). Thirteen adjacent normal and eighteen adjacent lichen sclerosis tissue samples were tested for MSI. Two adjacent LS samples tested positive (+) for MSI, sixteen adjacent LS and 13 adjacent normal samples tested negative for MSI. Representative examples of the analysis of MSI in the three tissue types are illustrated in figure 3.3.

3.5 D10S587

D10S587 is a dinucleotide microsatellite site consisting of 20 adenosine and, cytosine (AC)₂₀, repeats located on chromosome 10 (10q25.3-26.1) (Sasaki et al., 2002). This highly polymorphic marker is mapped around the DMBT1 tumor

suppressor gene. Because of the association with the DMBT1 gene and oral SCC, the D10S587 was considered to have potential in this study of vulvar SCC.

Table 3.4 Assessment of MSI with D10S208

Specimen	Identifier	D10S208	Specimen	Identifier	D10S208
CW 3-3	SCC	NI	CW 39-2	SCC	-
CW 5-1	SCC	+	CW 40-1	SCC	-
CW 12-4	SCC	-	CW 41-1	SCC	+
CW 16-2	SCC	+	CW 41-8	SCC	-
CW 17-1	SCC	-	CW 43-1	SCC	+
CW 18-1	SCC	-	CW 44-2	SCC	+
CW 19-1	SCC	-	CW 47-2	SCC	+
CW 19-2	SCC	-	CW 49-1	SCC	-
CW 23-1	SCC	-	CW 50-1	SCC	+
CW 24-1	SCC	-	CW 57-1	SCC	-
CW 25-1	SCC	-	CW 61-6	SCC	+
CW 26-2	SCC	+	CW 63-1	SCC	+
CW 28-1	SCC	-	CW 64-3	SCC	-
CW 29-1	SCC	+	CW 70-3	SCC	-
CW 29-6	SCC	-	CW 80-2	SCC	-
CW 35-3	SCC	NI	CW 97-1	SCC	-
D10S208	Informative Cases	MSI +		% MSI	
SCC	30.0	11.0		37.9	
LS	18.0	2.0		11.1	
N	13.0	0		0	

+ Positive for MSI
 - Negative for MSI
 NI Non Informative with specified marker

Of the thirty-two samples analyzed, twenty-six were considered to be informative in this study. Six (23.1%) of the twenty-six informative samples tested positive for MSI when compared to adjacent normal samples (Table 3.5). Tumor samples that displayed additional shifted bands when compared to adjacent normal

tissue samples were considered an indication of instability in the microsatellite site and scored positive (+).

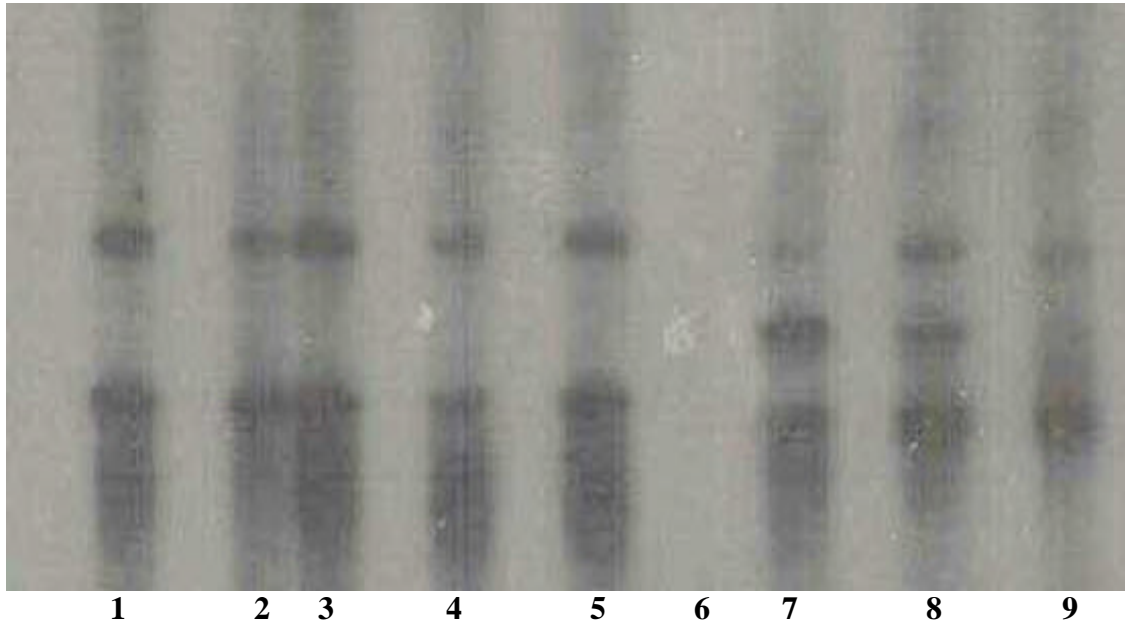


Figure 3.3 Representative examples of D10S208 Analysis.

Autoradiograph of MSI test. Lane 6 is a blank and was the negative control in this analysis with water instead of DNA template. Lane 1 is the positive control of normal DNA template for comparison to SCC tissue. Lane 7 and 8 were positive for MSI and was characterized by the addition of an allele when compared to normal tissue samples.

The level of MSI detected in the SCC tissue using the D10S587 locus was not found to be a statistically significantly different from the adjacent normal tissue samples analyzed ($p=0.395$). Thirteen adjacent normal and eighteen adjacent lichen sclerosis tissue samples were tested for MSI. Twelve out of the thirteen adjacent normal samples tested negative (-) for MSI. Representative examples of the analysis of MSI in the three tissue types are illustrated in figure 3.4.

Table 3.5 Assessment of MSI with D10S587

Specimen	Identifier	D10S587	Specimen	Identifier	D10S587
CW 3-3	SCC	-	CW 39-2	SCC	+
CW 5-1	SCC	-	CW 40-1	SCC	-
CW 12-4	SCC	+	CW 41-1	SCC	-
CW 16-2	SCC	NI	CW 41-8	SCC	-
CW 17-1	SCC	-	CW 43-1	SCC	+
CW 18-1	SCC	-	CW 44-2	SCC	-
CW 19-1	SCC	-	CW 47-2	SCC	-
CW 19-2	SCC	NI	CW 49-1	SCC	-
CW 23-1	SCC	NI	CW 50-1	SCC	-
CW 24-1	SCC	NI	CW 57-1	SCC	-
CW 25-1	SCC	NI	CW 61-6	SCC	-
CW 26-2	SCC	+	CW 63-1	SCC	-
CW 28-1	SCC	-	CW 64-3	SCC	-
CW 29-1	SCC	+	CW 70-3	SCC	-
CW 29-6	SCC	+	CW 80-2	SCC	-
CW 35-3	SCC	NI	CW 97-1	SCC	-
D10S208	Informative Cases	MSI +		% MSI	
SCC	26.0	6.0		23.1	
LS	17.0	1.0		5.9	
N	12.0	1.0		8.3	

+ Positive for MSI
 - Negative for MSI
 NI Non Informative with specified marker

3.6 MSI-H and MSI-L

Tumors occurring in humans that are exhibiting microsatellite instability high (MSI-H) is characterized by defective DNA mismatch repair genes (Morifugi et al., 2003). Disruption of the mismatch repair system in MSI-H cancers is most often caused by methylation of *hMSH1* or *hMSH2* (Cejka et al., 2003; Fleisher et al., 2000). Non-MSI-H cancers can either be classified as microsatellite stable (MSS) cancers or cancers with low-level microsatellite instability (MSI-L). MSI-L cancers are based solely on frequency because they lack the definitive molecular and biological features

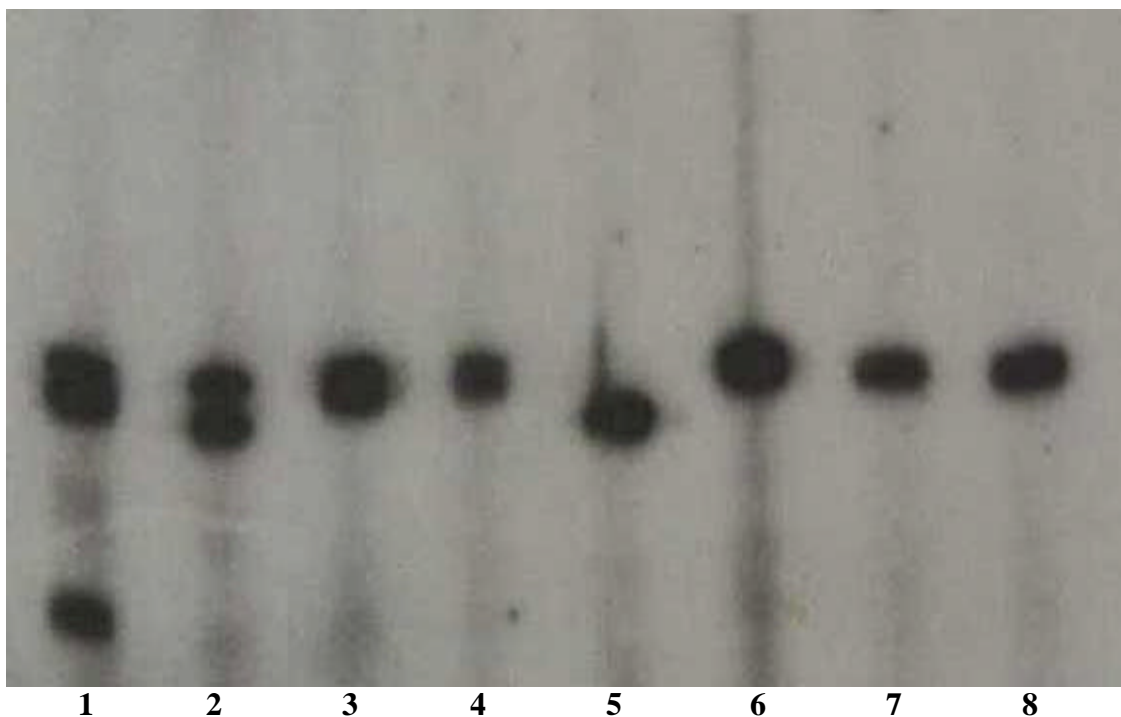


Figure 3.4: Representative examples of D10S587 Analysis.

Autoradiograph of SCC Tissue tested for MSI. Lane 2 is the positive control of normal DNA template for comparison to SCC tissue. Lane 1 was positive for MSI and was characterized by the addition of an allele when compared to normal tissue samples.

observed in MSI-H cancers. The studies reviewed have failed to reveal any significant difference in the pathological features between MSS and MSI-L cancers.

As in previous studies, cases were considered to be MSI-H when two or more of the microsatellite loci displayed variations in the fragment pattern of the allele with the same tissue sample (Goel et al., 2003; Hartmann et al., 2002; Cruz et al., 2000). Cases were labeled MSI-L when the case was positive for MSI with one marker and MSS when all of the markers were negative for MSI. The high frequency of MSI tumors (MSI-H), defined as tumors having instability in more than two markers, were detected in ten of the thirty-two (33%) SCC samples analyzed (Table 3.6). The adjacent normal

Table 3.6: Assessment of MSI-H, MSI-L, and MSS in tissue samples

Case	Identifier	Stability	Case	Identifier	Stability
CW 3-3	SCC	MSI L	CW 40-5	LS	MSS
CW 5-1	SCC	MSI H	CW 41-1	SCC	MSI L
CW 12-4	SCC	MSI L	CW 41-8	SCC	MSI L
CW 16-2	SCC	MSI L	CW 43-1	SCC	MSI H
CW 16-3	N	MSS	CW 44-1	N	MSI L
CW 17-1	SCC	MSS	CW 44-2	SCC	MSI H
CW 17-5	N	MSS	CW 44-4	LS	MSI H
CW 17-6	LS	MSI L	CW 45-4	N	MSS
CW 18-1	SCC	MSS	CW 46-6	LS	MSS
CW 18-5	N	MSS	CW 47-2	SCC	MSI L
CW 19-1	SCC	MSS	CW 47-3	LS	MSS
CW 19-2	SCC	NI	CW 49-1	SCC	MSS
CW 21-2	LS	MSS	CW 49-4	LS	MSS
CW 23-1	SCC	MSI L	CW 49-6	LS	MSS
CW 23-3	N	MSS	CW 49-7	N	MSI L
CW 24-1	SCC	MSI L	CW 50-1	SCC	MSI H
CW 25-1	SCC	MSI L	CW 50-5	LS	MSS
CW 25-4	LS	MSS	CW 52-2	N	MSS
CW 26-2	SCC	MSI H	CW 57-1	SCC	MSI L
CW 28-1	SCC	MSI L	CW 61-2	LS	MSS
CW 28-4	LS	MSS	CW 61-6	SCC	MSI H
CW 29-1	SCC	MSI H	CW 63-1	SCC	MSI H
CW 29-3	N	MSS	CW 64-3	SCC	MSI L
CW 29-5	LS	MSS	CW 70-1	LS	MSS
CW 29-6	SCC	MSI H	CW 70-3	SCC	MSS
CW 30-6	LS	MSS	CW 70-5	LS	MSS
CW 35-3	SCC	NI	CW 70-7	N	MSS
CW 39-1	N	MSS	CW 73-1	N	MSI L
CW 39-2	SCC	MSI H	CW 73-3	COND	MSS
CW 39-3	LS	MSI H	CW 80-2	SCC	MSI L
CW 40-1	SCC	MSI L	CW 91-5	LSC	MSS
CW 40-4	N	MSS	CW 97-1	SCC	MSS
	Stability Class				
Pathology	MSI-H	MSI-L	MSS	NI	Total Cases Tested
SCC	10 (33%)	14 (47%)	6 (20%)	2 (6%)	32
LS	2 (11%)	1 (6%)	15 (83%)	0 (0%)	18
Normal	0 (0%)	3 (15%)	10 (85%)	0 (0%)	13

tissue samples analyzed were either MSI-L or MSS. Two of the adjacent LS were considered to be MSI-H (11%) and one adjacent LS tissue sample was considered to be MSI-L (6%) the other fifteen samples were considered to be MSS.

3.7 Statistical Analysis

Frequencies of allelic loss at individual loci were expressed as the number of cases scoring positive for MSI over the total number of cases informative at that locus. The Fisher’s exact test was used for the comparative analysis of the results (Appendix F G, H, and I). A *p* value of equal to or less than 0.05 was considered to demonstrate the presence of a statistically significant difference between vulvar SCC and adjacent normal tissue samples (Table 3.7). Loci that showed statistically significant differences between vulvar SCC and adjacent normal tissue samples were considered to be informative markers for MSI.

Table 3.7: Statistical comparison between SCC and adjacent normal samples

Primer	SCC	Normal	P Value	P<0.05
BAT-25	11.1 %	7.7 %	1.000	No
BAT-26	56.7 %	10.0 %	0.013	Yes
D10S208	37.9 %	0.0 %	0.009	Yes
D10S587	23.1%	8.3 %	0.395	No

CHAPTER 4

CONCLUSION

Most cancers arise as a result of the accumulation of genetic changes, which affect many chromosomes and genes. Limited research has been done to examine MSI in tissues with squamous cell carcinoma and predisposing lesions such as lichen sclerosis. MSI has been seen in tissue associated with lung, colorectal, and other cancers. The focus of this project was to determine if human vulvar SCC and adjacent LS display genomic instability in the form of MSI.

The major objective of this study was to determine whether a panel of four markers could be associated with vulvar SCC. It was found that three markers, BAT-26, D10S208, and D10S587, were considered informative in examining MSI in vulvar SCC. MSI was found in using all four loci, with BAT-26 being the most informative of the four. A high level of MSI was found in 33% of the SCC tissue tested. The progression from adjacent normal tissue to adjacent LS did not display a relative increase in MSI. Vulvar SCC tissue demonstrated the greatest levels of MSI and the MSI is a characteristic of genetic instability and the cancerous state.

The results of the analysis of MSI in the adjacent normal and adjacent LS found that there were no significant differences between these two tissue types. The adjacent normal and adjacent LS tissue tested indicated no significant difference in MSI and was considered to be MSS or MSI-L, with only two adjacent LS specimens considered to be MSI-H. The difference between tumors with MSI-L and MSS is considered to be ambiguous and not well understood. Several studies find that there is little pathological difference between these two classifications.

In colorectal carcinoma, high-frequency MSI tends to confer a considerable survival advantage over patients with cancers that are not associated MSI-H (Gryfe R et al., 2000). Contrary to this statement, a National Cancer Institute workshop concluded that MSI had not yet been shown conclusively to be an independent predictor of survival (Dietmaier et al., 1999). More research needs to be done to understand the effects MSI has on prognosis.

A comparison of case specific results for each marker was analyzed and found to follow the accepted idea of instability through progression. MSI in adjacent normal tissue and adjacent LS tissue was comparatively similar and statistically different than SCC samples. BAT-26 was noted in the volgalgram in chapter one and was displayed in the progression to genomic instability characterized in early carcinoma. The findings of the current study of MSI with vulvar SCC and adjacent LS correlate to Dr. Volgastien's work on progression of cancer and genomic instability.

BAT-26 is associated with the *hMSH2* tumor suppressor gene and is important in mismatch repair. BAT-26 was considered to have the highest level of MSI and since MSI is a hallmark of mismatch repair problems, it can be assumed that there is a dysfunction in the mismatch repair genes. Genomic instability pathways of carcinogenesis, characterized by mismatch repair defects and MSI, appear to play a role in the genesis of some vulvar SCC types, based on the high incidence of MSI in association with BAT-26.

Further study of the production efficiency of the mismatch repair gene would be informative in association with present findings. The next step would be to study the efficiency of the *hMSH2*, *DMBT1* and *c-Kit* genes associated to the microsatellite

markers analyzed in this study. The study should examine the difference in expression of these three genes in associated with MSI. The SCC samples that display MSI and MSI-H and have not been effected by hyper-methylated inactivation or mutation in the gene that causes negative effects.

A secondary study looking at the expression of associated genes would establish a direct relationship between MSI and progression of genetic instability within the tumor tissue. Findings of this type of study can be correlated with prognosis and help to establish a connection to MSI status.

Vulvar SCC has shown genetic instability in the form of MSI with BAT-26, D10S208, and D10S587. Further study will need to be conducted to asses what this association indicates. The direction the study should take that would be the most informative in linking MSI to effects, would be to look at the interaction between MSI and gene expression. Since three loci have been associated with vulvar SCC and MSI, the expression efficiency of their associated genes would be a good starting point and the most informative based on current research.

REFERENCES

1. Albert de la Chapelle, M.D., Ph.D: Microsatellite Instability. *New England Journal of Medicine* 349:209-210, 2003.
2. Abeloff: *Clinical Oncology* 2nd Ed., Chapter 71, pg 653, 2000.
3. Beers MH, M.D., and Berkow R, M.D.: *The Merck Manual of Diagnosis and Therapy*. Section 18. Gynecology and Obstetrics, Chapter 241. *Gynecologic Neoplasms*, 2004.
4. Berg KD, Glaser CL, Thompson RE, Hamilton SR, Griffin CA, and Eshleman JR: Detection of Microsatellite Instability by Fluorescence Multiplex Polymerase Chain Reaction. *J Mol Diag* Vol. 2, No. 1, 1999.
5. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S: A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for determination of microsatellite instability in colorectal cancer. *Cancer Research* 58: 5248-57, 1998.
6. Carlson JA, Ambros R, Malfetano J, Ross J, Grabowski R, Lamb P, Figge H, Mihm MC Jr.: Vulvar lichen sclerosus and squamous cell carcinoma: a cohort, case control, and investigational study with historical perspective; implications for chronic inflammation and sclerosis in the development of neoplasia. *Hum Pathology* 29(9): 932-48, 1998.
7. Cejka P, Marra G, Hemmerle C, Cannavo E, Storchova Z, and Jiricny J: Differential Killing of Mismatch Repair-Deficient and -Proficient Cells: Towards the Therapy of Tumors with Microsatellite Instability. *Cancer Research*, 63: 8113-8117, 2003.
8. Cruz CP, Claro LP, Leitao CM, and Soares J: Immunohistochemical detection of mismatch repair gene proteins as a useful tool for the identification of colorectal carcinoma with the mutator phenotype. *Pathology* 191: 355-360, 2000.
9. Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R: Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. *Genomics* 12(2): 241-53, 1992.
10. Fearon ER and Vogelstein B: A genetic model for colorectal tumorigenesis. *Cell* 61:759-767, 1990.
11. Flam F.: Hints of a language in junk DNA. *Science* 266: 1320, 1994.

12. Fleisher AS, Esteller M, Harpaz N, Leytin A, Rashid A, Xu Y, Liang J, Stine OC, Yin J, Zou T, Abraham JM, Kong D, Wilson DT, James SP, Herman JG, and Meltzer SJ: Microsatellite Instability in Inflammatory Bowel Disease associated Neoplastic Lesions Is Associated with Hypermethylation and Diminished Expression of the DNA Mismatch Repair Gene, hMLH1. *Cancer Research* 60: 4864-4868, 2000.
13. Goel A, Arnold CN, Niedzwiecki D, Chang DK, Ricciardiello L, Carethers JM, Dowell JM, Wasserman L, Compton C, Mayer RJ, Bertagnolli MM, and Boland CR: Characterization of Sporadic Colon Cancer by Patterns of Genomic Instability. *Cancer Research*, 63: 1608-1614, 2003.
14. Gryfe R, Kim H, Hsieh ETK, Aronson MD, Holowaty EJ, Bull SB, Redston M, and Gallinger S: Tumor Microsatellite Instability and Clinical Outcome in Young Patients with Colorectal Cancer. *New England Journal of Medicine*, 342 (2): 69-77, 2000.
15. Hartmann A, Zanardo L, Bocker-Edmonston T, Blaszyk H, Dietmaier W, Stoehr R, Chevillie JC, Junker K, Wieland W, Knuechel R, Rueschoff J, Hofstaedter F, and Fishel R: Frequent Microsatellite Instability in Sporadic Tumors of the Upper Urinary Tract. *Cancer Research*, 62: 6796-6802, 2002.
16. Hoel DG, Davis DL, Miller AB, Sondik EJ and Swerdlow AJ: Trends in Cancer Mortality in 15 Industrialized Countries. *Journal of the National Cancer Institute* 84(5): 313-320, 1992.
17. Ichikawa A, Sugano K, and Fujita S: DNA Variants of BAT-25 in Japanese, a Locus Frequently Used for Analysis of Microsatellite Instability. *Japanese Journal of Clinical Oncology* 31: 346-348, 2001.
18. Morifuji M, Hiyama E, Murakami Y, Imamura Y, Sueda T and Yokoyama T: Fluorescent-based -Bat-26 analysis for distinct screening of microsatellite instability in colorectal cancers. *International Journal of Oncology* 22: 807-813, 2003.
19. Leung WK, Kim JJ, Kim JG, Graham DY, and Sepulveda AR: Microsatellite Instability in Gastric Intestinal Metaplasia in Patients with and without Gastric Cancer. *Am J Pathol*, 156 (2): 537-543, 2000.
20. Lin D, Wang Y, Scherer SJ, Clark AB, Yang K, Avdievich E, Jin B, Werling U, Parris T, Kurihara N, Umar A, Kucherlapati R, Lipkin M, Kunkel TA, and Edelmann W: An Msh2 Point Mutation Uncouples DNA Mismatch Repair and Apoptosis. *Cancer Research*. 64: 517-522, 2004.

21. London CA, Kisseberth WC, Galli SJ, Geissler EN, Helfand SC: Expression of stem cell factor receptor (c-kit) by the malignant mast cells from spontaneous canine mast cell tumors. *J Comp Pathol.* 115(4): 399-414, 1996.
22. Loukola A, Eklin K, Laiho P, Salovaara R, Kristo P, Jarvinen H, Mecklin J, Launonen V, and Aaltonen LA: Microsatellite Marker Analysis in Screening for Hereditary Nonpolyposis Colorectal Cancer (HNPCC). *Cancer Research* 61: 4545-4549, 2001.
23. Matsumoto N, Yoshida T, and Okayasu I: High Epithelial and Stromal Genetic Instability of Chromosome 17 in Ulcerative Colitis-associated Carcinogenesis. *Cancer Research*, 63: 6158-6161, 2003.
24. Mitchell RJ, Farrington SM, Dunlop MG and Campbell H: Mismatch Repair Genes *hMLH1* and *hMSH2* and Colorectal Cancer: A Huge Review. *Am J Epidemiol*, 156: 885-902, 2002.
25. Miturski R, Bogusiewicz M, Tarkowski R, Ciotta C, Bignami M, Burnouf D, and Jakowicki JA: BAT-26 Microsatellite Instability does not correlate with the Loss of hMLH and hMSH2 Protein Expression in Sporadic Endometrial Cancers. *Oncology Reports*, 10: 1039-1043, 2003.
26. Mori Y, Selaru FM, Sato F, Yin J, Simms LA, Xu Y, Oлару A, Deacu E, Wang S, Taylor JM, Young J, Leggett B, Jass JR, Abraham JM, Shibata D, and Meltzer SJ: The Impact of Microsatellite Instability on the Molecular Phenotype of Colorectal Tumors. *Cancer Research*, 63 (15): 4577-4582, 2003.
27. Partridge M, Pateromichelakis S, Phillips E, Emilion GG, A'Hern RP, and Langdon JD: A Case-Control Study Confirms that Microsatellite Assay Can Identify Patients at Risk of Developing Oral Squamous Cell Carcinoma with a Field of Cacerization. *Cancer Research* 60: 3893-3898, 2000.
28. Pinto AP, Lin M, Mutter GL, Sun D, Villa LL, and Crum CP: Allelic Loss in Human Papillomavirus Positive and Negative Vulvar Squamous Cell Carcinomas. *Am J Pathol* 154: 1009-1015, 1999.
29. Poulsen H, Junge J, Vyberg M, Horn T, Lundvall F: Small vulvar squamous cell carcinomas and adjacent tissues. A morphologic study. *APMIS* 111(9): 835, 2003.
30. Pyatt R, Chadwick RB, Johnson CK, Adebamowo C, Chapelle A and Prior TW: Polymorphic Variation at the BAT-25 and Bat-26 Loci in Individuals of African Origin. *Am J Pathol* 155: 349-353, 1999.

31. Quade BJ, Pinto AP, Howard DR, Peters WA, and Crum CP : Frequent Loss of Heterozygosity for Chromosome 10 in Uterine Leiomyosarcoma in Contrast to Leiomyoma. *Am J Pathol* 154: 945-950, 1999.
32. Ricciardiello L, Goel A, Mantovani V, Fiorini T, Fossi S, Chang DK, Lunedei V, Pozzato P, Zagari RM, Luca LD, Fuccio L, Martinelli GN, Roda E, Boland CR, and Bazzoli F: Frequent Loss of hMLH1 by Promoter Hypermethylation Leads to Microsatellite Instability in Adenomatous Polyps of Patients with a Single First-Degree Member Affected by Colon Cancer. *Cancer Research*, 63: 787-792, 2003.
33. Sasaki H, Betensky RA, Cairncross JG, and Louis DN: DMBT1 Polymorphisms. *Cancer Research* 62: 1790-1796, 2002.
34. Shin K, Park K, Hong HJ, Kim J, Oh J, Choung P, and Min B: Prevalence of microsatellite instability, inactivation of mismatch repair genes, *p53* mutation, and human papillomavirus infection in Korean oral cancer patients. *International Journal of Oncology* 21: 297-302, 2002.
35. Whiteall VLJ, Wynter CVA, Walsh MD, Simms LA, Purdie D, Pandeya N, Young J, Meltzer SJ, Leggett BA, and Jass JR: Morphological and Molecular Heterogeneity within Nonmicrosatellite Instability-High Colorectal Cancer. *Cancer Research*, 62: 6011-6014, 2002.
36. Yamashita Y, Miyakawa A, Mochida Y, Aisaki K, Yama M, Shiba M, Watanabe T, Yokoe H, Uzawa K, Imai Y, and Tanzawa H.: Genetic aberration on Chromosome 10 in human oral squamous cell carcinoma. *International Journal of Oncology* 20: 595-598, 2002.

APPENDIX A

PROTOCOL FOR PHENOL EXTRACTION

This protocol is adapted from the Beginning Molecular Biology Laboratory Manual and from Bruce A. Roe, Department of Chemistry and Biochemistry, The University of Oklahoma, Norman, Oklahoma 73019 for the isolation and extraction of DNA.

DNA Isolation

1. Add 500 μ l grinding buffer to each pencil eraser size tissue sample and vortex for at least 15 minutes.
2. Decant solution and add 500 μ l of lysing buffer and 8 μ l of proteinase K to each sample. Incubate at 37°C for at least 12 hours.
3. Add 500 μ l of phenol to each tube vortex, and centrifuge for 10 minutes.
4. Transfer aqueous top solution (no interphase) to clean labeled tubes and add 500 μ l of chloroform. Vortex and centrifuge for 10 minutes.
5. Repeat step 4, then transfer aqueous top solution to clean labeled tubes.
6. Add 2-3 μ l of sodium acetate to each sample then add 1000 μ l of cold 95% ethanol to each sample and age in -70°C freezer for 15 minutes. Centrifuge samples for 30 minutes and retain just the DNA pellet.
7. Add 700 μ l of 70% ethanol to each sample and put in -70 freezer for 10 minutes then centrifuge for 10 minutes.
8. Pour off ethanol and dry the pellets in a vacuum centrifuge.

DNA Extraction

1. Add an equal volume of TE-saturated phenol to the DNA sample contained in a 1.5 ml micro centrifuge tube and vortex for 15-30 seconds.
2. Centrifuge the sample for 5 minutes at room temperature to separate the phases.
3. Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous: phenol interface. At this stage the aqueous phase can be

extracted a second time with an equal volume of 1:1 TE-saturated phenol: chloroform, centrifuged and removed to a clean tube as above but this additional extraction usually is not necessary if care is taken during the first phenol extraction.

4. Add an equal volume of water-saturated ether, vortex briefly, and centrifuge for 3 minutes at room temperature. Remove and discard the upper, ether layer, taking care to remove phenol droplets at the ether: aqueous interface. Repeat the ether extraction.
5. Ethanol precipitate the DNA by adding 2.5-3 volumes of ethanol-acetate.

Phenol extraction is a common technique used to purify a DNA sample. Typically, an equal volume of TE-saturated phenol is added to an aqueous DNA sample in a micro centrifuge tube. The mixture is vigorously vortexed, and then centrifuged to enact phase separation. The upper, aqueous layer carefully is removed to a new tube, avoiding the phenol interface and then is subjected to two ether extractions to remove residual phenol. An equal volume of water-saturated ether is added to the tube, the mixture is vortexed, and the tube is centrifuged to allow phase separation. The upper, ether layer is removed and discarded, including phenol droplets at the interface. After this extraction is repeated, the DNA is concentrated by ethanol precipitation.

APPENDIX B

PROTOCOL FOR ETHANOL PRECIPITATION

This protocol is adapted from the Beginning Molecular Biology Laboratory Manual and from Bruce A. Roe, Department of Chemistry and Biochemistry, The University of Oklahoma, Norman, Oklahoma 73019 for the concentrating of DNA.

1. Add 2.5-3 volumes of 95% ethanol/0.12 M sodium acetate to the DNA sample contained in a 1.5 ml micro centrifuge tube, invert to mix, and incubate in an ice-water bath for at least 10 minutes. It is possible to place the sample at -20°C overnight at this stage.
2. Centrifuge at 12,000 rpm in a micro centrifuge for 15 min at 4°C, decant the supernatant, and drain inverted on a paper towel.
3. Add 80% ethanol (corresponding to about two volume of the original sample), incubate at room temperature for 5-10 min and centrifuge again for 5 min, and decant and drain the tube, as above.
4. Place the tube in a vacuum centrifuge and dry the DNA pellet for about 5-10 min, or until dry.
5. Always dissolve dried DNA in 10 mM Tris-HCl, pH 7.6-8.0, 0.1 mM EDTA (termed 10:0.1 TE buffer).
6. It is advisable to aliquot the DNA purified in large scale isolations (i.e. 100 µg or more) into several small (0.5 ml) micro centrifuge tubes for frozen storage because repeated freezing and thawing is not advisable.

Typically, 2.5 - 3 µl of an ethanol/acetate solution is added to the DNA sample in a micro centrifuge tube, which is incubated at -20C overnight. To recover the precipitated DNA, the tube is centrifuged, the supernatant discarded, and the DNA pellet is rinsed with a more dilute ethanol solution. After a second centrifugation, the supernatant again is discarded, and the DNA pellet is dried in a vacuum centrifuge.

APPENDIX C

PROTOCOL FOR ENDLABELING WITH GAMMA

- 1) Prepare a 0.7mL tube for each primer to be end-labeled and add the following:
 - a) 5 μ l of Invariant primer dilution (25 pmol/ μ l)
 - b) 1 μ l of T4 Polynucleotide Kinase (New England Biolabs, Inc.)
 - c) 4 μ l of NEB 10X PNK Buffer1
 - d) 32P ATP (>5000Ci/mmol) (14 day half-life)
Add (12 μ l 32P ATP + 1 μ l for each day past the assay date) (34 μ l max)
Add dH₂O to bring total reaction volume to 40 μ l
- 2) Incubate tubes at 37 oC (40-60 minutes)
- 3) Add 2 μ l of 0.5 mM ATP to force reaction and incubate at 37 oC for 4 minutes.
- 4) Add 2 μ l of 0.5 M EDTA to stop reaction
- 5) Prepare G25 columns to purify end-labeled primers
 - a) Use 15 ml capped tubes for spinning columns.
 - b) Spin G25 tubes @ 2200 rpm, 4-5 minutes to drain the buffer, repeat
 - c) Join G25 columns to final collection tubes (labeled), in the 15 ml tubes.
- 6) Recover 32P labeled primer
 - a) Pipette reaction mixture to center of column bed
 - b) Centrifuge 4-5 minutes @ 2200 rpm
 - c) Discard columns in the hot room radioactive waste bin
 - d) Save 15 ml capped tubes for re-use
- 7) Quantitate radioactive incorporation using scintillation counter
- 8) Place a 0.7 ml tube with 1 μ l of labeled primer and push start.
- 9) Determine volumes needed for PCR.

APPENDIX D

PROTOCOL FOR POLYMERASE CHAIN REACTION (PCR)

This protocol was designed to amplify MSI sites in target DNA sequences. All of the solutions in this protocol are prepared in a cold block (0°C), unless noted otherwise. HPLC grade water, obtained from Fisher Scientific, Fair Lawn, NJ, used in this protocol was passed through a 0.045 micron filter to ensure purity.

Each sample was mixed in a 50 µl micro centrifuge tube and contained:

- a) 200 ng of sample DNA
- b) 2.5 µl of 10X PCR Buffer
- c) 1.5 µl MgCl₂ (25 mM Stock)
- d) 1 µl of PCR forward and reverse primer mix (25pmol/µl Stock of each)
- e) 2 µl of dNTP (10 mM each, deoxyribonucleotide triphosphated dA, dC, dG, dT)
- f) 0.2 µl Taq Gold DNA Polymerase (1 unit per reaction)
- h) add pure water to 25 µl

Thermal-Cycler is then programmed at:

96°C for 8 minutes (activation of Taq Gold)

Then,

96°C for 1 minute (denaturation)

55°C for 1 minute (annealing)

72°C for 1 minute (polymerase extension)

For 35 cycles then,

72°C for 10 minutes (final extension)

10°C on hold.

APPENDIX E

PREPARATION OF 5% POLYACRYLAMIDE GEL

- 1) Prepare glass plates by cleaning with ethyl alcohol and large kimwipes
- 2) Siliconize glass plates (internal surfaces) with 550-600 μ l of Sigmacote.
- 3) Prepare a 150 ml beaker with a stir bar and add (total volume is 70 ml):
 - a) 30.3 g of UltrapureTM urea (Sigma)
 - b) 30 ml of dionized water
 - c) 7.2 ml of autoclaved TBE
 - d) 11 ml of 19:1 Acrylamide 40 % (W/V): Bis (Amresco)
- 4) Allow flask to stir on a plate set a #4 (a higher setting incorporates air) until urea is dissolved.
- 5) After solution is thoroughly dissolved add following:
 - a) 500 μ l of 10 % APS (Ammonium Persulfate)
 - b) 50 μ l of TEMED (N,N,N',N'-Tetra-methyl-ethyl-enediamine)
- 6) Place spacer strips on prepared glass plates and tape together.
- 7) Add binder clamps to the sides of the glass plates to ensure a tight fit.
- 8) Mixing the solution thoroughly, pour the gel into the prepared glass plates and allow it to harden (30 minutes to 1 hour).

APPENDIX F

FISHER'S EXACT TEST OF BAT-25

Data source: BAT-25 vs. Normal
(P = 1.000)

Subjects	MSI	Stable	
Normal	1.000	12.000	Counts
	1.300	11.700	Expected Counts
	7.692	92.308	Row %
	25.000	33.333	Column %
	2.500	30.000	Total %

SCC	3.000	24.000	Counts
	2.700	24.300	Expected Counts
	11.111	88.889	Row %
	75.000	66.667	Column %
	7.500	60.000	Total %

The proportion of observations in the different categories which define the contingency table is not significantly different than is expected from random occurrence (P = 1.000).

APPENDIX G

FISHER'S EXACT TEST OF BAT-26

Data source: BAT-26 vs. Normal

(P = 0.013)

Subjects	MSI	Stable	
Normal	1.000	9.000	Counts
	4.500	5.500	Expected Counts
	10.000	90.000	Row %
	5.556	40.909	Column %
	2.500	22.500	Total %

SCC	17.000	13.000	Counts
	13.500	16.500	Expected Counts
	56.667	43.333	Row %
	94.444	59.091	Column %
	42.500	32.500	Total %

The proportion of observations in the different categories which define the contingency table is significantly different than is expected from random occurrence (P = 0.013).

APPENDIX H

FISHER'S EXACT TEST OF D10S208

Data source: D10S208 vs. Normal
(P = 0.009)

Subjects	MSI	Stable	
Normal	0.000	13.000	Counts
	3.405	9.595	Expected Counts
	0.000	100.000	Row %
	0.000	41.935	Column %
	0.000	30.952	Total %

SCC	11.000	18.000	Counts
	7.595	21.405	Expected Counts
	37.931	62.069	Row %
	100.000	58.065	Column %
	26.190	42.857	Total %

The proportion of observations in the different categories which define the contingency table is significantly different than is expected from random occurrence (P = 0.009).

APPENDIX I

FISHER'S EXACT TEST OF D10S587

Data source: D10S587 vs. Normal
(P = 0.395)

Subjects	MSI	Stable	
Normal	1.000	11.000	Counts
	2.211	9.789	Expected Counts
	8.333	91.667	Row %
	14.286	35.484	Column %
	2.632	28.947	Total %

SCC	6.000	20.000	Counts
	4.789	21.211	Expected Counts
	23.077	76.923	Row %
	85.714	64.516	Column %
	15.789	52.632	Total %

The proportion of observations in the different categories which define the contingency table is not significantly different than is expected from random occurrence (P = 0.395).

VITA

Sidney Joseph Marlborough, III, was born on October 4, 1973 in a taxi crossing the Greater New Orleans Mississippi River Bridge, and life has been an uphill climb since. He graduated eighth grade from Holy Name of Mary in Old Algiers on the west bank. He then went on to San Diego for the next four years and finished high school at Orange Glen High School in Escondido, California. His freshman year of college was completed at Louisiana State University in 1992. After the grueling years of studying, working and dragon slaying (drinking, partying, and gaming), he finally graduated in 1998 with a Bachelor of Science in environmental management systems.

Upon completion he spent three years of mind numbing hell at the state's Department of Environmental Quality where he found out how far a man can go. From this experience he ran to the nearest consulting company as well as back to school to begin a masters program under the world renowned tutelage of Doctor Vince Wilson. Once starting this project he dove in, figuratively, and found out that genetics was not an exact science (who knew?). Finally graduating from LSU with a Master of Science in environmental sciences (toxicology), he shall go on into the world and try and pay off the student loans and other bills. He is joined in this effort by his plucky sidekick (wife) and mini me (daughter) adventuring onward fighting injustice and evil as well as looking for a good Chinese food. We hear there is a great one at the end of the Universe, See You There.