SOMATIC ACQUISITION OF TGFBR1*6A IN CERVICAL CANCER

THESIS

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By

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ABSTRACT

Cervical cancer is the 2nd most common cancer affecting women worldwide (Chen 1999). This is alarming because cervical cancer is completely treatable and many times preventable. Previous epidemiological study in 1999 found that six of their 16 cervical cancer patients had blood genotypes with a mutation on the gene coding for the type-one receptor for transforming growth factor β (Chen 1999). This three alanine deletion in a nine alanine repeat mapped to the 9q22 chromosome on the exon 1 coding region of TGFBR1 and has now been described as TGFBR1*6A or *6A (Pasche 1998). The more common allele is known as TGFBR1*9A or *9A. The cytokine involved in TGFBR1*6A is TGFβ and the transforming growth factor β family is commonly known as a tumor suppressor gene pathway Further study has found the *6A polymorphism was of higher incidence in cancer patients of many types: colorectal, breast, ovarian, and head & neck; and metanalyses have shown that TGFBR1*6A is a tumor susceptibility allele (Pasche 2004). It has been hypothesized that if presence of *6A predisposes the patient to cancer, it may occur more often in the tumors themselves (Pasche 2005). This is referred to as, somatic acquisition, the mutational act of a cancer patient who has the wild-type

9A/9A blood genotype, and their tumor mutates to acquire the *6A polymorphism. This process has previously been shown in head & neck and colorectal cancers (Pasche 2005). Cervical cancer is similar to these cancers because it also is of epithelial origin. Therefore, we investigated the incidence of somatically acquired TGFBR1*6A in cervical cancer tumors of blood genotyped 9a/9a homozygotes. 119 tumor samples were genotyped which had been patient matched from the Midwestern cervical cancer cohort who had previously been identified as having a blood genotype of 9A/9A homozygote by our laboratory. Seven of the 119 (5.88%) patients showed evidence of somatic acquisition after PCR amplification and genotyping. Furthermore, one of the 119 (0.84%) was found to have a tumor genotype of 6A/6A.

Dedicated to my wonderfully huge family and a very supportive group of close friends.

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CHAPTER I

INTRODUCTION

Cervical cancer is the 2nd most common cancer affecting women worldwide (Chen 1999). This is alarming because cervical cancer is completely treatable and many times preventable. Previous epidemiological study in 1999 found that six of their 16 cervical cancer patients had blood genotypes with a mutation on the gene coding for the type-one receptor for transforming growth factor β (Chen 1999). This three alanine deletion in a nine alanine repeat mapped to the 9q22 chromosome on the exon 1 coding region of TGFBR1 and has now been described as TGFBR1*6A or *6A (Pasche 1998). The more common allele is known as TGFBR1*9A or *9A. The cytokine involved in TGFBR1*6A is TGFβ and the transforming growth factor β family is commonly known as a tumor suppressor pathway. Further study has found the *6A polymorphism was of higher incidence in cancer patients of many types: colorectal, breast, ovarian, and head & neck; and metanalysis have shown that TGFBR1*6A is a tumor susceptibility allele (Pasche 2004). It has been hypothesized that if presence of *6A predisposes the patient to cancer, it may occur more often in the tumors themselves (Pasche 2005). This is referred to as, somatic acquisition, the mutational act of a cancer patient who has the wild-type 9A/9A blood genotype, and their tumor mutates to acquire the *6A polymorphism. This process has

previously been shown in head & neck and colorectal cancers (Pasche 2005). Cervical cancer is similar to these cancers because it also is of epithelial origin. Therefore, we investigated the incidence of somatically acquired TGFBR1*6A in cervical cancer tumors of blood genotyped 9a/9a homozygotes. 119 tumor samples were genotyped which had been patient matched from the Midwestern cervical cancer cohort who had previously been identified as having a blood genotype of 9A/9A homozygote by our laboratory. I hypothesize that there is a significant incidence of somatically acquired TGFBR1*6A in cervical cancer tumors of blood genotyped 9a/9a homozygotes.

CHAPTER II

Background

2.1 Cervical Cancer

One in 145 women in the United States will be diagnosed with cervical cancer in her lifetime. Furthermore, nearly 4000 women in the United States will die of complications of cervical cancer this year (SEER 2008). Our incidence rate is 8.4 women per 100 000 across all races, however when looking at individual races, Hispanic women have the highest incidence rate at 13.2 per 100 000 (SEER 2008).The median age for diagnosis of cervical cancer in our country is 47 years old, with cases over the age of 55 accounting for the majority of the mortality associated with the disease (Waggoner 2003).Cervical cancer is a cancer affecting women young and old.

Cervical cancer survival rates in our country are an example of the recent advances that have been made in preventative medicine. The median age of death for cervical cancer patients is 57 years old in the United States and the five year survival rate for patients with cervical cancer is 71.2%, when compared to the rest of the U.S. population (SEER 2008). However, the survival rate of patients with early stage cervical cancer exceeds 95%. This rate is highly

associated with early detection of disease and shows the benefits of screening tools and preventive medicine (Canavan 2000).

Worldwide, the incidence of cervical cancer is very different than in the United States and other countries with effective preventive health programs. The disease is the second most common malignant disease worldwide for women (Waggoner 2003). It was approximated that 493 000 new cases of cervical cancer and 273 000 deaths attributed to disease during the year 2002 in the world (Sankaranarayanan 2006). These numbers represent the burden of disease worldwide, but do not give an accurate description of the distribution of cervical cancer. 83% of the new cases and 85% of the deaths occur in developing countries (Sankaranarayanan 2006). The disparity between developed and developing countries is vast and can be attributed to access to health care and lack of preventive medicine. The Pap smear is the most cost-effective screening program in the world to date; perhaps with increased resources and infrastructure the incidence gap between developing and developed countries can diminish (Moore 2006).

2.1.1 Histopathology

Cervical cancer is slowly developing cancer, which allows ample time for early detection. Therefore, staging must be set forth for not only the developed cancers, but also its precursors. Cervical neoplasms are defined as precancerous or cancerous. After this designation, they can be staged. Precancerous lesions are referred to as cervical intraepithelial neoplasms or CINs. The stages of CINs range from I to III, depending on the cellular morphology and arrangement in the tissues. Grade I CINs are mild dysplasias, with nuclear atypia and abnormal mitotic figures in the outermost layers of the cervical epithelium (van Hamont 2008). CIN Is rarely progress to cancer, and are the majority of abnormalities detected during the standard screening method, the

Papanicolaou Smear. Cellular changes exhibited by CIN IIs are more similar to malignant cells, with more atypical cells at the lower layers of the epithelium. Those atypic cells have changes in nucleo-cytoplasmic ratio, variation in nuclear size as well as hyperchromasia (Kumar 2008). As the CIN lesion progresses, there is decreased organization in the tissue and progressive loss of differentiation until when there is no surface differentiation of cells compared to the lower levels of the squamous epithelium and it is totally replaced by immature atypical cells (van Hamont 2008). The progression of cellular changes in the cervical epithelial tissue can be seen in FIGURE 2.1 (van Hamont 2008). The majority CIN lesions that progress to cervical carcinomas are squamous cell carcinomas, the approximately 25% remaining are adenocarcimomas, adenosquamous carcinomas, undifferentiated carcinomas or other more rare histological types (Waggoner 2003).

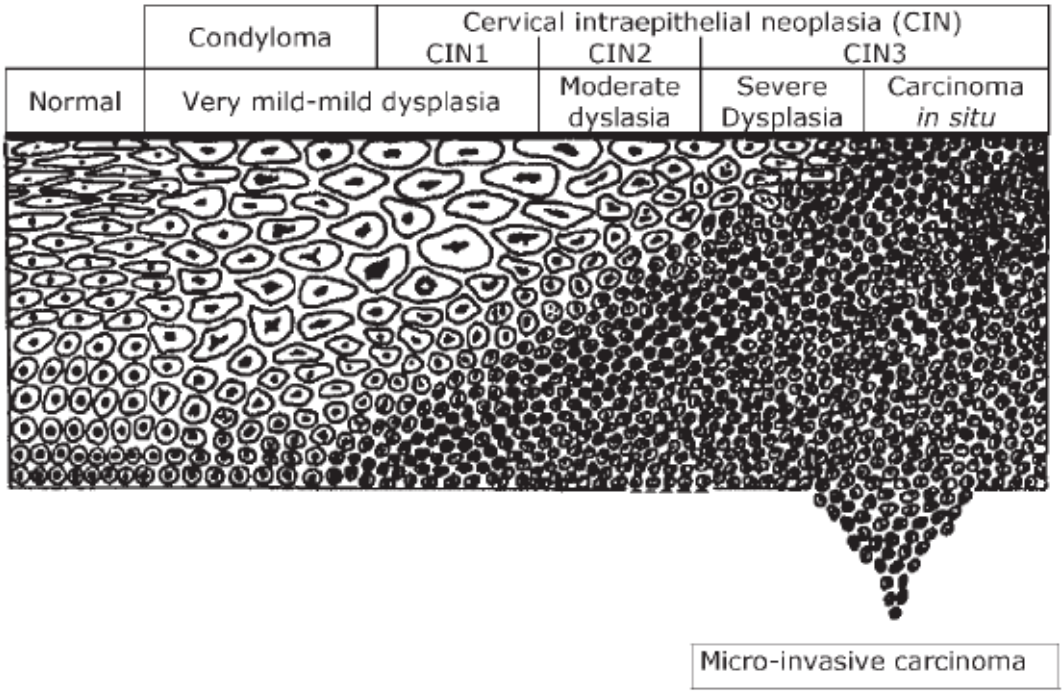

FIGURE 2.1: Illustration of the histologic changes in the development of CIN and cervical cancer (Reprinted with permission from van Hamont 2008).

After diagnosis of cervical cancer, the patient is staged. Staging assists clinicians design the appropriate course of treatment. The staging criterion is set forth by the International Federation of Gynaecology and Obstetrics, commonly referred to as FIGO. TABLE 2.1 (Moore 2006) shows the different classifications. The localized nature of cervical cancer is important at staging, because is often locally destructive before it is metastatic (Canavan 2000). Staging is determined at primary diagnosis and irrespective of recurrence or progression, the initial stage should not ever be changed (Waggoner 2003).

Table 1. FIGO Staging Classification: Cervical Carcinoma

Stage 0	Carcinoma in situ
Stage IA1	Invasive carcinoma, confined to cervix, diagnosed only by microscopy. Stromal invasion ≤ 3 mm in depth and ≤ 7 mm in horizontal spread.
Stage IA2	Invasive carcinoma, confined to cervix, diagnosed only by microscopy. Stromal invasion > 3 mm and ≤ 5 mm in depth and ≤ 7 mm in horizontal spread.
Stage IB1	Invasive carcinoma, confined to cervix, microscopic lesion > IA2 or clinically visible lesion ≤ 4 cm in greatest dimension.
Stage IB2	Invasive carcinoma, confined to cervix, clinically visible lesion > 4 cm in greatest dimension.
Stage IIA	Tumor extension beyond cervix to vagina but not to lower third of vagina. No parametrial invasion..
Stage IIB	Tumor extension beyond cervix. Parametrial invasion but not to pelvic sidewall and not to lower third of vagina.
Stage IIIA	Tumor extension to lower third of vagina but not to pelvic sidewall.
Stage IIIB	Tumor extension to pelvic sidewall or causing hydronephrosis or nonfunctioning kidney.
Stage IVA	Tumor invasion into bladder or rectum.
Stage IVB	Distant metastasis.

FIGO, International Federation of Gynecology and Obstetrics.
Data from Creasman.[14]

TABLE 2.1: FIGO Staging Classification: Cervical Carcinoma (Permission Pending from Moore 2006).

The arrangement of tissues on the cervix may contribute to female susceptibility of cancer. The squamocolumnar junction or SJC is an area of rapidly dividing cells and squamous metaplasia (Canavan 2000). This is commonly referred to as the T-zone and is likely the site of oncogenic transformation, because the detection frequency of cervical cancer and its precursors (Rohan 2004). It could also be because basal cells are both actively proliferating and exposed, especially in women of child-bearing age when the squamocolumnar junction is visible on the ectocervix (Canavan 2000). As tissues mature, the SJC will recede within the endocervical canal; nonetheless it is necessary to continue to monitor the area during screenings (Canavan 2000).

2.1.2 Risk Factors of Cervical Cancer

Epidemiological studies have determined several risk factors related to cervical cancer, many of which are related to sexual practices. Numerous studies have investigated the relationship between the sexually transmitted disease human papillomavirus and all levels of cervical dysplasias. There is evidence that the risk of CIN lesion is directly proportional to the number of HPV infections (Franco 2001). Another highly associated risk factor, long term use of oral contraceptives, may be a spurious correlation. It is suspected that use of oral contraceptives requires regular cytologic screenings, which makes the user more likely to have their disease detected (Franco 2001). This puts the population of individuals that use oral contraceptives for more than five years at an unauthentic level of risk. High parity has also been discovered to have a linear relationship with increased incidence of cervical cancer (Franco 2001). HIV, history of diagnosis with sexually-transmitted diseases, number of sexual partners, and early age of first sexual encounter have all been associated with increased risk of cervical cancer.

Smoking is also an important predictor of cervical cancer, with several speculations of its involvement in carcinogenesis. Canavan *et al.* believe that nicotine is not the direct cause of cervical cancer, but perhaps puts women at higher risk by lowering their immune system. However, a 2003 article by Steven Waggoner indicated that smoking may be an independent risk factor after identifying tobacco-specific carcinogens in cervical mucus and epithelium of smokers. These compounds can bind to DNA, initiating inflammatory complexes and damaging cellular DNA; therefore producing malignant transformation in the cervical tissues (Waggoner 2003). Smoking may also be a confounder, because women who smoke are also more likely to participate in other risk factors; like history of sexually transmitted diseases, lower socioeconomic status, and more than two lifetime sexual partners (Canavan 2000).

Human papillomavirus or HPV is understood to be a very important agent in cervical carcinogenesis. Hybridization techniques have detected HPV in nearly 95% of cancerous lesions on the cervix (Kumar 2004). The host-virus interaction is of sexual origin, which explains the majority of risk factors that relate to patient sexual health and practices.The relative risk for HPV infection and cervical cancer is of very high magnitude, even higher than estimated for the relationship between lung cancer and smoking (Franco 2001). There are over 100 varieties of the virus; differentiated into subgroups by the location of isolation. Each strain HPV is differentiated by sequence relatedness (Rohan 2004). Those strains of HPV described as 'high risk' to cervical cancer are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (Canavan 2000). However, Gardisil®, the only vaccine on the market for HPV, protects against the two strains (16 and 18) that cause approximately 70% of all cases of cervical cancer (Castle 2007). Widespread use of the vaccine against HPV is expected to make a large difference in the number of incident cases of cervical cancer.

2.1.3 Screening tools for Cervical Cancer

Cervical cancer was the leading cause of cancer deaths in women in the United States several decades ago. Screening tests have led to a one-third decline in our death rate over the last two decades (Moore 2006). Cervical cancer is now the seventh leading cause of cancer mortality in the United States (Franco 2001).The population level screening tools of both the Papanicolaou smear and the carcinogenic HPV DNA test have made great strides in reducing the burden of disease in developed countries.

The traditional method of screening for cancer is the Papanicolaou smear, which is commonly referred to as the 'Pap smear'. It is very effective in detection of early cellular abnormalities and precancerous lesions of the cervix. This cytology screening has limitations and often is prone to false negative results. Because the rate of false negatives is believed to be around 20-45%, The U.S. Preventive Services Task Force recommends that all women receive a Pap smear at onset of sexual activity or at age of 18 years; by instituting annual screenings after this time the rate of false negatives is believed to be decreased (Canavan 2000). Despite the low sensitivity of the Pap smear, it is still considered one of the best screening methods in the world (Moore 2006).

A second and more recent method of screening for cervical cancer is the HPV DNA test. The HPV DNA test genotypes cells from the cervix for human papillomavirus and gives information about the carcinogenicity of the infection (van Hamont 2008). Often patients are infected with several strains of the HPV virus, this and the viral load associated are very important for characterization of a patient's cancer risk (van Hamont 2008). It has been suggested that this tool may not be an effective stand alone screening method in developed

countries, where there is a high prevalence of HPV infection in women of reproductive age (Franco 2001). When combined with a Pap smear, the provider can incorporate the patient's HPV status, and complete a risk profile for the patient FIGURE 2.2 (Castle 2007).

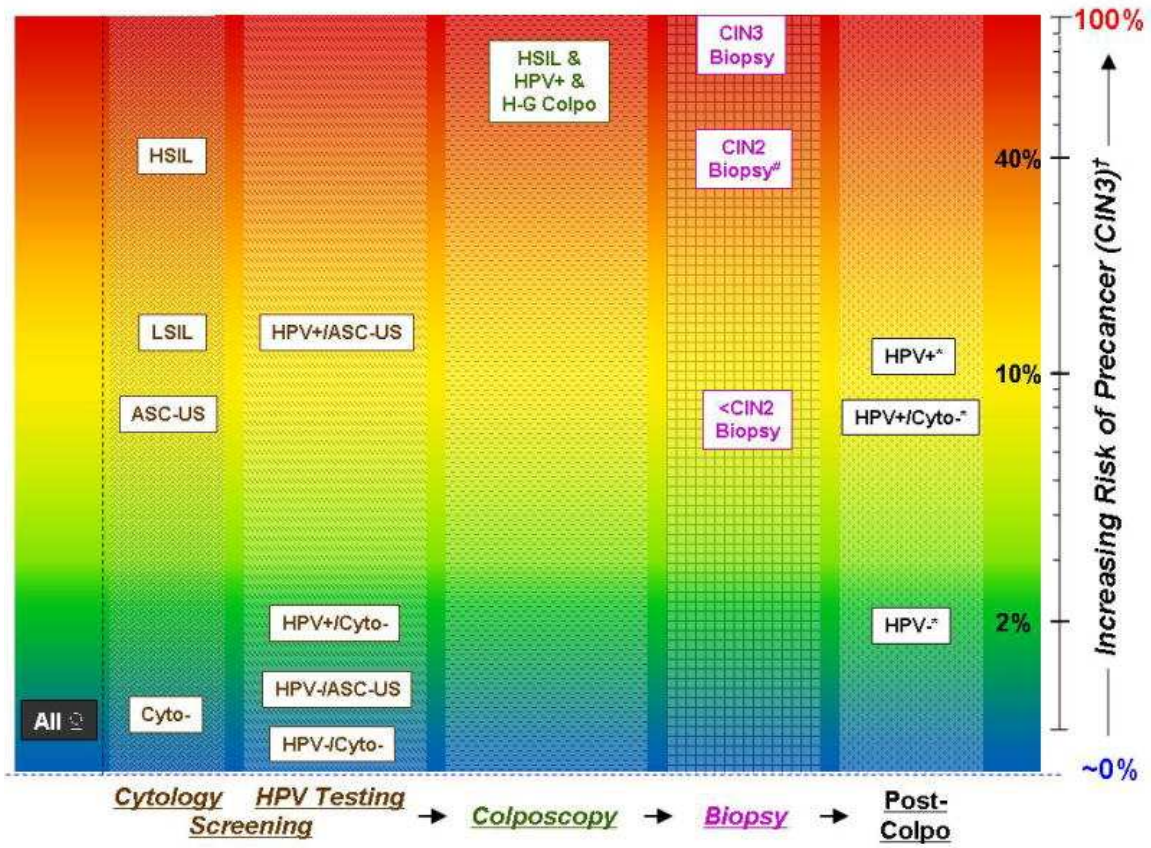

FIGURE 2.2: A graphical representation: the risk of cervical precancer at different stages and results of screening and clinical management for cervical cancer prevention. The risks for each stage and result are approximate risks for CIN3 within a screening interval. The axis to the left of the figure represents increasing risk, from nearly 0% (blue) to 100% (red), of cervical precancer on a log scale. Each stage of screening and clinical management is represented by a different pattern, with the arrows indicating the sequence of the stages. #Less than half of the cases of CIN2 on biopsy are subsequently diagnosed as CIN3 on excisional tissue (precancer). †Within a screening interval. *Test results at the next follow-up visit (≥6 months) (Reprinted with permission from Castle 2007).

As previously described, the majority of cervical cancer health burden is found in developing countries. However, it is estimated that it may be extremely cost-effective to institute a onetime cancer screening in these developing nations. By performing a visual inspection of the cervix with acetic acid or using cervical cell samples to test for HPV on women around 35 years old, their lifetime risk of cancer is estimated to be reduced by 25-36% (Moore 2006). Because the prevalence of HPV infection is lower in developing countries, the HPV DNA screening will be more effective as a stand alone screening tool (Franco 2001). Low cost and easy to institute screening methods may be the key to reducing the disparity of cervical cancer burden between developed and developing countries.

2.1.4 Clinical Response after Diagnosis

After staging and diagnosis of cervical cancer, clinicians must direct future medical care to remove the cancer, or prevent cancerous growth. The clinical response varies depending on the grade of lesion. If the patient were to present with low grade squamous intraepithelial lesion (LSIL or CIN I), the reaction may be to just continue monitoring, because a large proportion of LSILs regress and Pap smears may return to normal. For these same patients, frequent cytologic monitoring is recommended and they may be referred for colposcopic examination (van Hamont 2008). A colposcopy involves washing the cervix with acetic acid and observing the cells with the naked eye (Rock 2000). Around 50% of cervical cancer patients are diagnosed with stage I disease, making surgical removal of cancer a treatment option. Presence of high grade squamous intraepithelial lesion suggests the use of a cone biopsy of the cervix or cervical conization (Moore 2006). Another ablative option is the loop electrosurgical excision procedure or LEEP; which involves an electric current that removes the cervical lesion. Cone biopsies, cervical conization and LEEP are all invasive methods to remove cervical lesions which

protect fertility while also providing tissues which can be used for pathological examination (Wright 2007). Initial investigation of the abnormal cervix should ascertain degree of dessemination, the tissue health of the rectovaginal area, and status of local lymph nodes; this is because often cervical cancer is locally destructive before it metastasizes (Canavan 2000).

More radical treatment options are available for cervical cancer which has been designated above stage Ia. Pelvic radiotherapy is used to destroy cancer cells, but can also affect healthy cells in the pelvic region. Another option, radical hysterectomy, involves the removal of the uterus and cervix. Hysterectomy and radiotherapy are effective tools for treating cervical cancer, and give patients a five year survival rate of 80-90% (Canavan 2000). For patients with cancer above stage IIb, chemotherapy is an option as well as radiation therapy (Canavan 2000). When discussing late stage cervical cancer it is impossible not to consider the benefits of annual screening tools, which are very effective at catching the asymptomatic early stages of cervical cancer.

2.1.5 Molecular Mechanisms

There are many ideas on how cancerous changes occur in the body; for example, the Multi-Hit model is the leading theory on cancer development. It is based on the idea that it takes more than one 'hit' or cellular change to develop cancer, and a series of offenses on the DNA cause carcinogenesis. Inflammation is usually a self-limiting reaction to stressors (possibly HPV), which involves production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both ROS and RNS can bind to DNA, leading to mutations on genes for MAPK, p53, p16 or perhaps TGFβ cellular signaling (Kundu 2008). Conversely, Infectious pathways involve a pathogen which replicates itself within the cell, and as an accidental consequence, cancer

development occurs (Butel 2000). The Multi-Hit theory ties together two potential mechanisms of cervical cancer development- inflammatory and infectious pathways. If the multi-hit theory holds true, the HPV infection would create a window of opportunity for subsequent 'hits' which lead to cellular disregulation and perhaps cancer (Rohan 2004).

Knudson's Two-Hit hypothesis is another theory of oncogenesis that has a mutational component. The theory was developed to explain the genotypic change of a cell from heterozygous to homozygous for the non-wild-type allele (Delaval 2007). Another description of this phenomenon is 'loss of heterozygosity'. The allelic loss often results in deletion of tumor suppressor genes (Marte 2006). Knudson first discovered the loss of tumor suppressor genes in retinoblastoma; however this process may apply to all cells heterozygous for tumor suppressor deletions. For example, DNA which codes for TGFβ, a tumor suppressor gene, may be affected by this loss of heterozygosity.

When discussing molecular mechanisms of cancer, the concept of heredity undoubtedly comes up. Familial risk is a core component of cancer research; all medical histories include the history of cancer in a patient's blood relatives. Many different types of cancer have been established as having a strong hereditary component, in particular breast and ovarian cancer (Hemminki 1999). The strong implication of human papillomavirus in cervical cancer has distracted from the discussion of cervical cancer, however recent research has shown that the cervical cancer heredity may involve HPV. Research using the Swedish Family-Cancer Database has found that host susceptibility may play a role in cervical cancer development (Hemminki 1999). They also calculated the Familial Relative Risk or FRR of cervical cancer, for both carcinoma *in situ* and invasive carcinomas. They calculated excess cancer risk from family using a binomial equation and the found rates of between 1.8 and 2.0. These rates are similar to the

rates found in other cancers identified as having a hereditary component, ovarian, breast and colon cancer (Hemminki 1999). While they suggest that the family component may be artifactual because families also share the same environmental risk factors, we suggest that it may be both. The environmental risk factors may induce mutations in tumor suppressor genes, like TGFβ, and the problem may be compounded by also being passed down through bloodlines. This is the basis of those who investigate the type one receptor polymorphism of the tumor suppressor TGFβ, referred to as TGFBR1*6A.

2.2 Transforming Growth Factor β Pathway

TGFβ is a regulatory cytokine involved with epithelial, endothelial, and neural tissue expansion, as well as cell growth for hematopoietic and mesenchymal cells (Massague 1998). Nearly every cell in the body responds to TGFβ and it is involved in a vast amount of cellular processes, with effects that depend on both the type and stage of the cell (Pasche 2001). In developing organisms, TGFβ is involved in tissue development and expansion. However, in mature tissues, TGFβ keeps cells in a state of homeostasis by moving through its pathway regulating different tumor-suppressive processes; proliferation, differentiation, cytostasis and apoptosis (Massague 1998). Given the wide array of cellular processes that transforming growth factor β is involved in, it is understandable that we are still beginning to understand its interaction with carcinogenesis and cell cycle control.

The TGFβ superfamily contains more than 35 structurally related secreted polypeptides (Leivonen 2007). This family contains several growth factors, bone morphogenic proteins (BMPs) and activins. There are three TGFβ isoforms; TGFβ-1, TGFβ-2, and TGFβ-3, all of which are expressed ubiquitously, even while they are each encoded by a distinct gene. There are also

three receptors for TGFβ; referred to as TβR-I, TβR-II, and TβR-III. Each receptor has a specific role in the TGFβ pathway. Smad proteins facilitate signal propagation. This family of transcription factors involves receptor-activated Smads 2 and 3, commonly known as R-Smads, and co-activator Smad 4 (Pasche 2001). Another protein, SARA, short for Smad anchor for receptor activation facilitates the signal transmission from cell membrane to nucleus. All of these components are involved in the complex process of transforming growth factor β cell regulation.

The three TGFβ ligands- I, II, and III, are all secreted as biologically inactive precursors referred to as latent-TGFβ or L-TGFβ. L-TGFβ changes conformation by proteolytic cleavage and becomes the mature molecule of about 25 kDa (Khalil 1999). This activation of the ligand permits the TGFβs to bind to the receptors, initiating the signaling cascade. All three of the ligands are homologous, irrespective of size differences (Khalil 1999). However, the availability of the different isoforms is dependent on the tissues that are present. TGFβ-1 is found in endothelial and neuronal cells, TGFβ-2 in epithelial and neuronal cells and TGFβ-3 in the less common mesenchymal cells (Massague 1998).

Three different serine/threonine complexes in the cytoplasmic region make up the TGFβ receptor family. TβR-I is a glycoprotein of 55 kDa, with the unique feature of a highly conserved 30 amino acid region which is referred to as the GS domain (Massague 1998). The GS domain is phosphorylated by the type-two receptor and is required for activation of signaling beyond the receptor complex. TβR-II is a 70 kDa glycoprotein which is the only receptor that can bind to TGFβ (Massague 1998). The last type of receptor is TβR-III, is a proteoglycan which is not involved in the signaling pathway, but is responsible for introducing the TGFβ ligand to TβR-II

(Markowitz 1996). The three types of receptors form the beginning of the essential TGFβ pathway.

Smad transcription factors are an integral part of the TGFβ signaling pathway. They possess DNA-binding activity, which along with additional DNA-binding cofactors, they are able to bind to target genes (Massague 2008). The specific gene that the R-Smad and Smad4 complex binds to depends on the regulatory regions and the sequence element combinations (Massague 2008). The Smad proteins are made up of two conserved globular domains; MH1 and MH2, which are joined together by a linker region (Massague 2000). Every section of the Smad proteins have a specific function; the linker region is made of many regulatory sites, MH1 is flanked by an amino group and has DNA-binding control, and MH2 has a carboxy-terminal and mediates transcriptional activity (Massague 2006). TβR-I phosphorylates the Smad proteins in the TGFβ pathway, and depending on the core components of the Smad, different transcription processes are activated.

The process in which TGFβ transmits its signal from cytoplasm to nucleus is complex and best shown by FIGURE 2.3 (Massague 2006). It begins with the large latent TGFβ, which changes conformation to become the mature TGFβ protein. The type-two receptor (TβR-II) binds to the ligand after it has been recruited by the type-three receptor. Once the type-two receptor is bound to the ligand, it phosphorylates to the GS domain of the type-one receptor (TβR-I). This generates the ligand-induced heteromeric complex of usually two type-one and two type-two TGFβ receptors. Receptor formation phosphorylates causing Smad activation. The receptor-associated Smads, Smad2 and Smad3, are transiently associated with the receptor complex by linking through the SARA which facilitates the recruitment. The R-Smads then associate with

Smad4, which is necessary for transcription initiation. The R-Smad and Smad4 complex determines the particular genes that they are to stimulate or repress transcription upon.

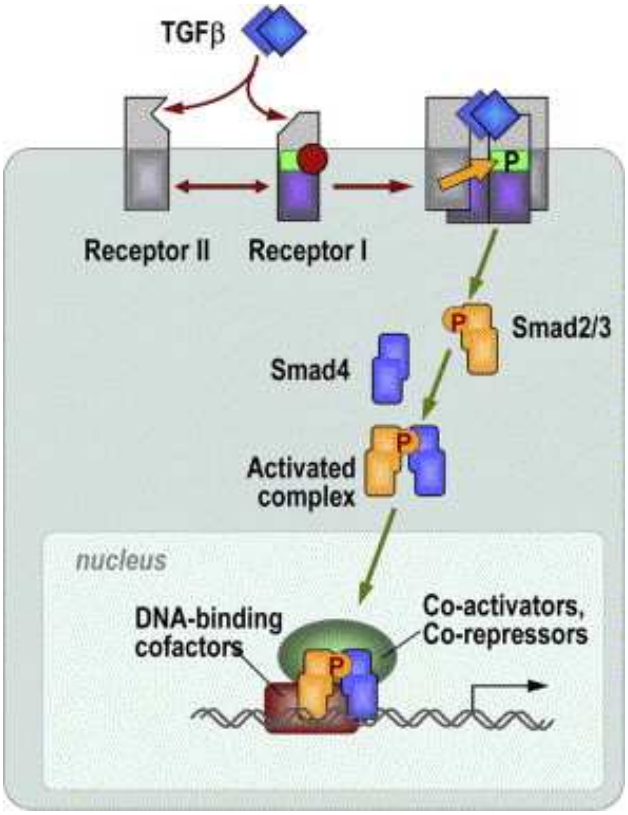

FIGURE 2.3: The Transforming Growth Factor β Signaling Pathway (Reprinted with permission from Massague 2006).

2.2.1 Deregulation of the TGFβ Pathway in Cancer

From the cytoplasm, to the cell membrane receptor, to the nucleus; TGFβ signaling is both specific and prone to mutations, because of its complexity and ubiquitousness. The mechanisms of carcinogenesis related to TGFβ have been investigated extensively. There are

countless explanations for how TGFβ is involved in carcinogenesis and nearly all relate back to deregulation of some part of the pathway. This is shown by TGFβ acting as a tumor suppressor in normal cells, but in most malignant cells there is resistance to the same TGFβ inhibitory effects (Pasche 2001). Inactivation of the TGFβ signaling cascade occurs by many mechanisms; missense mutations, nonsense mutations, frame-shifts, deletions or loss of entire regions of their chromosome. In cancer, there appears to be two directions in which TGFβ can affect tumor development; first, it can be by decreasing signaling in the TGFβ pathway or second, by altered signaling which when increased leads to metastasis and tumor progression (Pasche 2001). These are known as the 'two faces' of TGFβ in carcinogenesis and has been described the difference of TGFβ acting as either an oncogene or a broken tumor suppressor gene (Roberts 2003). The TGFβ pathway is complex and it is integral to cellular homeostasis, therefore it is no surprise that the many components, Smads, ligands or receptors, would be vulnerable to inactivation or manipulation.

Smad signaling is an essential part of the transforming growth factor β pathway as it regulates transcription which leads to different cellular processes. Smad4 inactivation has been related to one-half of all pancreatic carcinomas (Massague 2004). The mutation that leads to Smad4 inactivation has been documented in a large percentage of metastatic colon cancers and a smaller proportion of localized colon cancers as well as other cancer types (Siegel 2003). Furthermore, several phenomena regarding Smad4 mutations have been identified in familial juvenile polyposis (Siegel 2003). A Smad2 inactivating mutation has been correlated to a small proportion of colorectal cancers (Massague 2000). Comparatively, no mutations related to Smad3 have been related to human cancers. Regardless of how or where the mutation occurs,

they appear to have non-overlapping tumor suppressive properties, independent of TGFβ (Massague 2000).

Receptors for TGFβ have been recognized as a particular target for protein inactivation which may lead to increased cancer incidence. The type-I and type-II receptors are particularly vulnerable because they work in an obligatory fashion; therefore, inactivation of one receptor disables the tumor suppressor pathway. A mutation in the type-two receptor, TβR-II, has been identified as leading to inactivation of the pathway when associated with microsatellite instability in colon cancer, gastric cancer, and malignant gliomas. Additionally, sporadic colon cancer, squamous cell carcinoma of the head and neck and ovarian cancers have been linked to inactivating mutations of the type two receptor unrelated to microsatellite instability, however not breast cancers (Lynch 2001). Germline mutations of TβR-II have been linked to a certain type of colon cancer; hereditary non-polyposis (Kaklamani 2003). Furthermore, specific mutations of the type-two receptor may result in absence of TGFβ; this has been established in retinoblastomas, small-cell lung cancers, and some B and T cell lymphomas (Markowitz 1996). Mutations in the type-one receptor are much less common, with the exception of TGFBR1*6A, which is so common it is referred to as a polymorphism. A TβR-I mutation resulting in inactivation has been recognized in one third of a cohort of ovarian cancer patients. However, a missense mutation on the same receptor has shown conflicting results in breast cancer (Massague 2000).

2.2.2 TGFβ Deregulation in Cervical Cancer

The ubiquitousness of TGFβ and its involvement in a vast amount of cellular processes make its involvement in cell dysfunction likely in cervical cancer, another cancer of epithelial

cells. Research evidence shows serum levels of TGFβ differ based on the type of cervical cancer and level of disease progression, with TGFβ elevation in the more advanced carcinomas and adenocarcinomas secreting more TGFβ than squamous cell carcinomas (Chopra 1998, Santin 1997). Also, there is indication that the levels of TGFβ1 in the extracellular stroma are elevated compared to intracellular epithelial expression (Pasche 2001). Pasche suggests that this may show that tumor progression is indirectly promoted because of the loss of epithelial TGFβ1. Furthermore, TβR-I and TβR-II expression is reduced nearly 80% in premalignant CIN lesions (De Geest 1994). This indicates a loss of sensitivity to TGFβ early in the development of cancer.

In vivo assays have shown several interactions between TGFβ and HPV-related cervical cancer. The HPV genome contains coding regions E6 and E7, of which TGFβ inhibits transcription and blocks G1 to S phase cell cycle progression (Woodworth 1990). Region E7 of HPV also can bind to SMADs 2, 3, and 4, which are involved in progression of the TGFβ signal transduction (Lee 2002). Several investigators have found that CIN lesions are increasingly unresponsive to TGFβ as they progress to more serious carcinomas, showing an important biologic relationship between cervical cancer and the transforming growth factor β pathway (Chopra 1999).

2.2.3 TGFBR1*6A

Many epidemiological and mechanistic studies have been examining TGFBR1*6A or *6A, a common polymorphism of the type-one TGFβ receptor (TβR-1), which has been found to transduce TGFβ growth inhibitory signals less effectively than TGFBR1*9A (Pasche 1999). The *6A allele consists of a deletion of three alanines within a nine-alanine (*9A) repeat at the 3'-end of the exon 1 coding sequence (Pasche 1998). FIGURE 2.4 shows the difference between the *6A and *9A genotype. Epidemiological studies suggest it may act as a tumor susceptibility allele

because it increases cancer risk by approximately 24% (Pasche 1999). This is particularly remarkable because this polymorphism is found in a large proportion of the normal population as well, approximately 13.7% (Kaklamani 2003). In-vivo studies using mink lung epithelial cell lines devoid of TβR-I were used to determine the signaling differences of the two TGFBR1 polymorphisms. This in-vivo study was central to the study of *6A and concluded that the *6A polymorphism was a less effective mediator of the TGFβ tumor suppressive properties than the wild-type *9A (Pasche 1999). From that study and we know understand that when the TGFβ pathway and closely associated signaling cascades are disrupted by TGFBR1*6A, cells demonstrate loss of TGFβ inhibition, increased proliferation, and enhanced cell invasion.

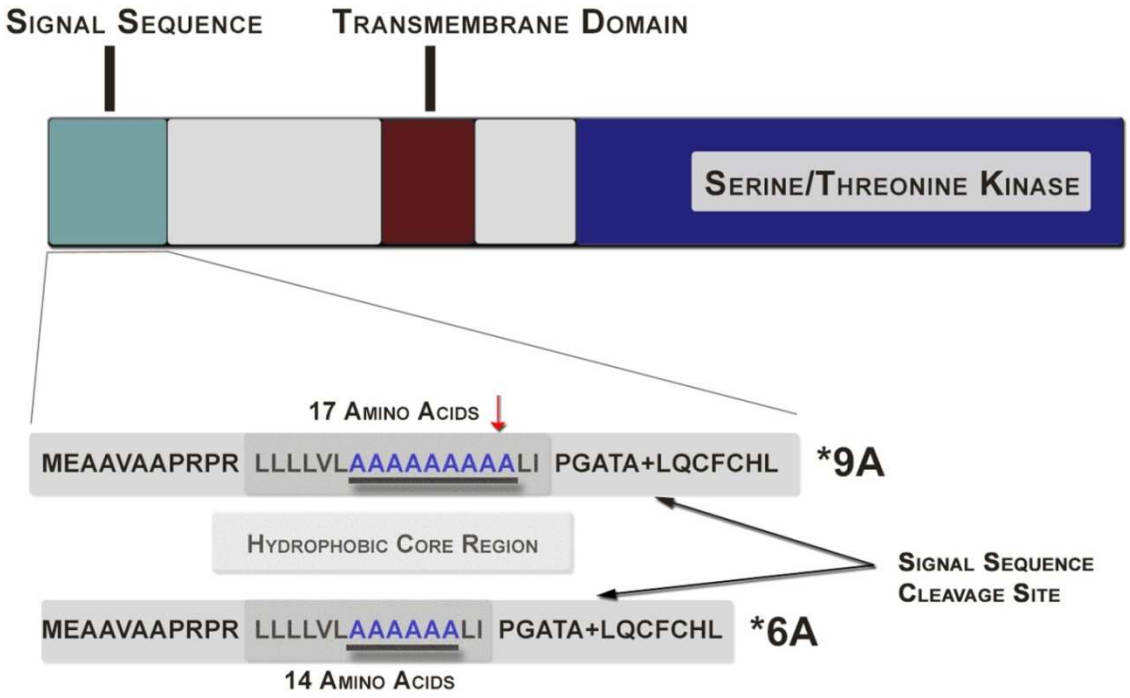

FIGURE 2.4: The three alanine deletion in the exon coding for TGFBR1. Illustration by permission of Dr. Thomas Knobloch.

The difficulty with research related to TGFBR1*6A is achieving statistical power during analysis. There have been many small scale studies which look at the rates in normal populations and also populations of patients affected by cancer.To counter this problem, several *6A researchers have collaborated to create metanalyses. These metanalyses provide greater numbers of cancer patients than individual studies are capable of obtaining, by pooling together, the metanalyses give a large population-level perspective of TGFBR1*6A and cancer. Two metanalyses have been conducted and many inferences have been from their results.The first metanalysis was of seven *6A case control studies, and found a significant association of *6A with cancer (Kaklamani 2003). Furthermore, they examined the differences between in carrier status; if a patient was homozygous for *6A (6A/6A) they found them to be at a higher risk for cancer than heterozygotes or *9A homozygotes (Kaklamani 2003). Their findings established *6A as a high frequency, low penetrane tumor susceptiblity allele (Kaklamani 2003). The second and larger metanalysis involved the previous seven studies, as well as five more previously unpublished *6A case-control studies. This large cohort of over 4000 cancer patients and nearly 3500 set a precedent in *6A investigations. The rate of *6A in normal blood controls was established at 13.7%, including both 6A/6A homozygotes and 6A/9A heterozygotes (Pasche 2004). They also validated their previous statement that *6A is found at higher rates in cancer patients than normal controls. This metanalysis of twelve studies also confirmed that the TGFBR1*6A polymorphism is the most common tumor susceptibility allele that had been reported (Pasche 2004). By establishing these rates of blood genotyped *6A in large populations of both normal donors and cancer patients, future studies can use this data as a reference point.

Understanding how and why cancer incidence is affected by TGFBR1*6A is an ongoing discussion. Many strides have been made in the last five years, but exact mechanisms still have

not been ascertained. Most notably, a 2005 paper published in *JAMA* by several TGFBR1*6A experts, including Dr. Weghorst and Dr. Knobloch, reached several new conclusions about the polymorphism. Through modeling and understanding of signal sequence cleavage, they established that the signal sequence cleavage site is outside of the polyalanine repeat of TGFBR1 (Pasche 2005). This finding shows that there is no difference between *6A and *9A mature proteins. If there had been differences in the mature proteins, the reduced TGFβ signaling by *6A could have been explained by structural differences in the mature type-one receptor (Pasche 2005). Pasche *et al.* went on to suggest that signal sequence, and not receptor mediated differences, are responsible, due to the fact that the mature receptors are the same for TGFBR1*6A and *9A. Because of this knowledge, we now understand why there were no dissimilarities between *6A and *9A in TGFβ binding and receptor turnover (Pasche 2005). The most recent hypothesis for functional differences between the polymorphisms relates to in-vivo studies of RhoA activation. Rosman *et al.* suggest that the expression of TGFBR1*6A induces increased RhoA activation which directly affects ERK activation. This secondary signaling event is suspected to be triggered by the *6A signal sequence protein (Rosman 2008). If this hypothesis is true, the TGFβ pathway may not be involved in phenotypic differences between *6A and *9A and how that relates to increased cancer risk.

2.2.4 *6A in Cervical Cancer

TGFBR1*6A in cervical cancer was first studied by Chen *et al* in 1999, they found that 6 out of 16 (37.5%) of their cervical cancer patients from The Netherlands had the 9 base pair deletion now known as *6A. They hypothesized that the variant may be related to increased cancer susceptibility and that the risk was increased when the patient was a homozygote for the *6A allele. During their study, they investigated the effect of the polyalanine repeat; by western

immunoblotting, they discovered that the majority of the receptor protein was found in the membrane fraction. Furthermore, they found no significant differences in the quantity of type-one receptors with respect to the three alanine deletion (Chen 1999). After establishing this large rate in the Dutch population, Chen *et al.* began looking at two other populations affected by cervical cancer- a U.S. and a Jamaican. Their cohort from the United States was found to have an allelic frequency of 18.8%, which was much higher than the allelic frequency of 4.2% established by Pasche in 1998 when looking at normal blood donors. However, there was an insufficient number of subjects to establish significance in the Jamaican population of cervical cancer patients. The Jamaican population was of African-American descent and had a small sample size, which may contribute to its insignificance. Interestingly, the prevalence of *6A in the control groups was consistent within all three populations (Chen 1999)

In 2007, the Weghorst laboratory began studying the heredity of TGFBR1*6A in cervical cancer after their conclusive findings in head & neck cancer (Knobloch 2001). They established the Midwestern cervical cancer cohort through a collaborative gift from Dr. Janet Rader at Washington University at St. Louis. The material transfer agreement between her laboratory, The Ohio State University Department of Gynecologic Oncology and Dr. Christopher Weghorst included 315 blood DNA samples from patients with invasive cervical cancer. Genotyping revealed that there was 60 of 315 (19.0%) patients that were carriers of the *6A polymorphism. 55 patients were heterozygous 6A/9A and 5 were homozygous 6A/6A. An allelic frequency of 10.3% and a 95% confidence interval (7.98%, 12.7%) were calculated. These findings corroborate with the rates already calculated in other cancers, however are much lower than the rate found in the Dutch cervical cancer population, which may be explained by the small number of participants in the study by Chen in 1999.This Midwestern study is the largest cohort

of cervical cancer patients to be evaluated for *6A significance and is important for further cervical cancer studies of the *6A polymorphism.

2.2.5 *6A in Colorectal Cancer

Colorectal cancer is a leading cause of cancer deaths in the United States, and despite in place screening practices, decline has not been what public health officials had anticipated. Twin studies suggest that approximately 35% of colorectal cancers are inherited (Xu 2007). This known genetic component makes a likely case for involvement of TGFBR1*6A in colorectal cancer. Normal intestinal epithelium responds to the tumor-suppressor effects of TGFβ, but in some cases of colorectal cancer the epithelial cells become resistant to TGFβ. Furthermore, late stages of colorectal cancer have shown that TGFβ may in fact act as a tumor promoter (Xu 2007). The interaction between TGFβ and colorectal cancer may be related to the low-penetrance tumor susceptibility allele TGFBR1*6A. Case-control studies of populations affected by colorectal cancer have shown that the carriers of *6A are at an increased risk of developing the disease with a significant odds ratio of 1.20 (Pasche 2004). Furthermore, a large cohort of colorectal cancers were genotyped in the 2005 *JAMA* paper; of 157 colorectal carcinomas, 30 (19.1%) were determined to be 6A/9A heterozygotes (Pasche 2005). A recent paper by Xu and Pasche showed that mismatch repair gene mutation negative colorectal cancer patients had a higher incidence than the patients who were positive for the mismatch repair gene. This led to conclusions that other downstream effects of *6A may be involved colorectal cancer development. Another study by Valle *et al.* in 2008 focused on allele specific expression (ASE) of several single nucleotide polymorphisms and their relationship to TGFBR1*6A in colorectal cancer. A higher degree of ASE may indicate other subtle changes in genotype may be more significant than in those with a lower degree of ASE (Valle 2008). Rates of blood 6A/9A

heterozygosity were higher in the colorectal cancer patients showing ASE, and they suggest that the same mutation that causes the *6A polymorphism may be also involved in ASE (Valle 2008). Colorectal cancer and its relationship to TGFBR1*6A is still being explored, but the strong epidemiological and mechanistic studies show *6A may play an important role in disease progression of colorectal cancer.

2.2.6 *6A in Head & Neck Cancers

The Weghorst laboratory has previously investigated incidence of TGFBR1*6A in head & neck cancer patients. Their study in 2001 showed a significant incidence of *6A in the tumor samples. Eight of the 30 tumor samples (27%) had a polymorphic sequence change to *6A (Knobloch 2001). They also showed that there was no relationship between age, tumor stage or anatomic location in the head and neck cancer patients. The Weghorst Laboratory also contributed to the 2005 *JAMA* paper, and found that 49 of 226 (21.7%) squamous cell carcinomas of the head and neck were carriers of *6A (Pasche 2005). It needs to be noted that the majority of TGFBR1*6A epidemiologic studies in cancer patient populations use blood tissues to establish rates of *6A, but these studies used tumor tissues for genotyping.

2.2.7 *6A in Various Cancers

Several other cancers have shown slight relationships with TGFBR1*6A; breast, prostrate, lung, and ovarian cancer have all been investigated. Conflicting epidemiological studies of breast cancer patients leads us to believe that the disease may play a role in specific populations or types of breast cancer. For example, a study by Song *et al.* showed no overall increased risk of breast cancer for carriers of TGFBR1*6A, however a group of patients they studied with low-risk familial breast cancer had a significant odds ratio of 1.3. There was also

significance shown in the 2005 *JAMA* paper, with 25 of 104 (24.0%) breast carcinomas showing heterozygosity for *6A (Pasche 2005). Also, a meta-analysis of breast cancer patients showed a significant increased risk, with an odds ratio of 1.38 (Pasche 2004), while a nested study in the Nurses' Health study showed no significance (Cox 2007). Similarly, ovarian cancer has inconsistent results when studied in case-control studies. As study by Baxter *et al.* showed non-significant increase in risk in all types of ovarian cancer, but when separated out, endometrioid cancers showed a very significant increased risk when the patient was a carrier of *6A. Comparatively, another study showed no significance; even when the ovarian cancer patients were stratified by race (Spillman 2005). Prostate cancer patients and lung cancer patients have been investigated and both have shown no significant relationship with TGFBR1*6A (Kaklamani 2004, You 2007). The difference in risk between cancer types is evidence of the incomplete penetrance of TGFBR1*6A and warrants further study incorporating patient larger cohorts and more stratification to focus on specific disease relationships (Pasche 2005).

2.2.8 Somatic Acquisition

Several laboratories were researching the TGFBR1*6A polymorphism, and suspected that the high levels in cancer patients could not be explained by germline mutations alone. Tumor genotypes consistently showed higher frequency of *6A than germline, inferring that there was a mutation taking place after conception. These mutations were then called somatic acquisition because of the tumor's nature to acquire the less common polymorphism. As previously mentioned, a TGFBR1*6A paper was published in *JAMA* in the fall of 2005. This paper dealt directly with somatic acquisition in three cancer types; breast, colorectal and head & neck. The theory behind somatic acquisition is that if presence of *6A predisposes the patient to cancer, it may occur more often in the tumors themselves (Pasche 2005).

The *JAMA* paper by Pasche *et al.* was conducted in a specific sequence; after enrollment of cancer patients, tumor samples were genotyped and any 6A/9A heterozygotes were then matched with a blood sample. The blood sample is evidence of the germline genotype inherited from parents, and any differences between it and the tumor genotype would show somatic acquisition. For example, if a patient was determined to have a 6A/9A tumor sample, but then showed a genotype of 9A/9A when the blood was processed, it would show that the tumor had somatically acquired the *6A polymorphism. This process was conducted in heterozygote tumors from the three cancer types. Somatic acquisition was evident in four of 226 (1.8%) head and neck cancer carcinomas, none of the breast cancer carcinomas, four of 157 (2.5%) colorectal cancer carcinomas, and 13 of 44 (29.5%) colorectal metastases to the liver, however there was no evidence of somatic acquisition in the breast carcinomas (Pasche 2005). FIGURE 2.5 illustrates the process of evaluating somatic acquisition. These findings are very supportive of their theory that presence of *6A predisposes the patient to cancer, it may occur more often in the tumors themselves, hence somatic acquisition. Furthermore, they hypothesized that selective growth advantage is fixed in malignant cells, which is supported by the larger rate of somatic acquisition in colorectal metastases than colorectal carcinomas (Pasche 2005). This study laid the groundwork for my investigation into the same phenomenon in cervical cancer, which will be described below.

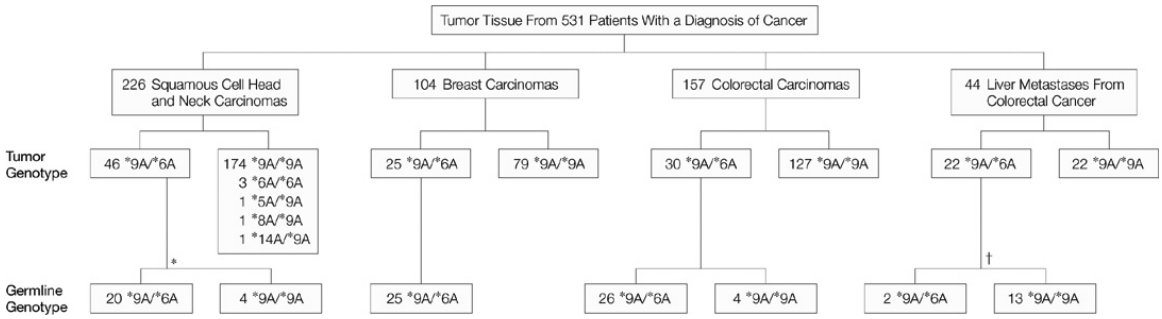

FIGURE 2.5: Summary and illustration of the process used in identifying somatic acquisition in tumor samples used in *JAMA* paper (Permission Pending from Pasche 2005).

CHAPTER III

MATERIALS AND METHODS

3.1 Sample Acquisition

Through the collaborative gift from Washington University-St. Louis, we received 131 patient-matched tumor DNA samples which were previously identified by our laboratory as having the wild-type 9A/9A blood genotype. These patients had all signed forms of informed consent and investigation of their samples was performed under Project Number 2008E0063 at The Ohio State University, for which the Institutional Review Board had determined it to be exempt research. The tumor DNA was stored at -20°c over the course of the study. The DNA was of 5ng/ml concentration we had 10ul of DNA per tumor sample.

3.2 Primer Design

During the amplification of tumor DNAs, two different primer sets were used; both contained a fluorescent end-labeled sense primer. The 6-FAM or 6-Carboxyfluorescein is a primer end label which fluoresces to a blue color when viewed during capillary electrophoresis. FIGURE 3.1 shows the primer design used during DNA amplification. Primers were changed after contamination was suspected during tumor genotyping.

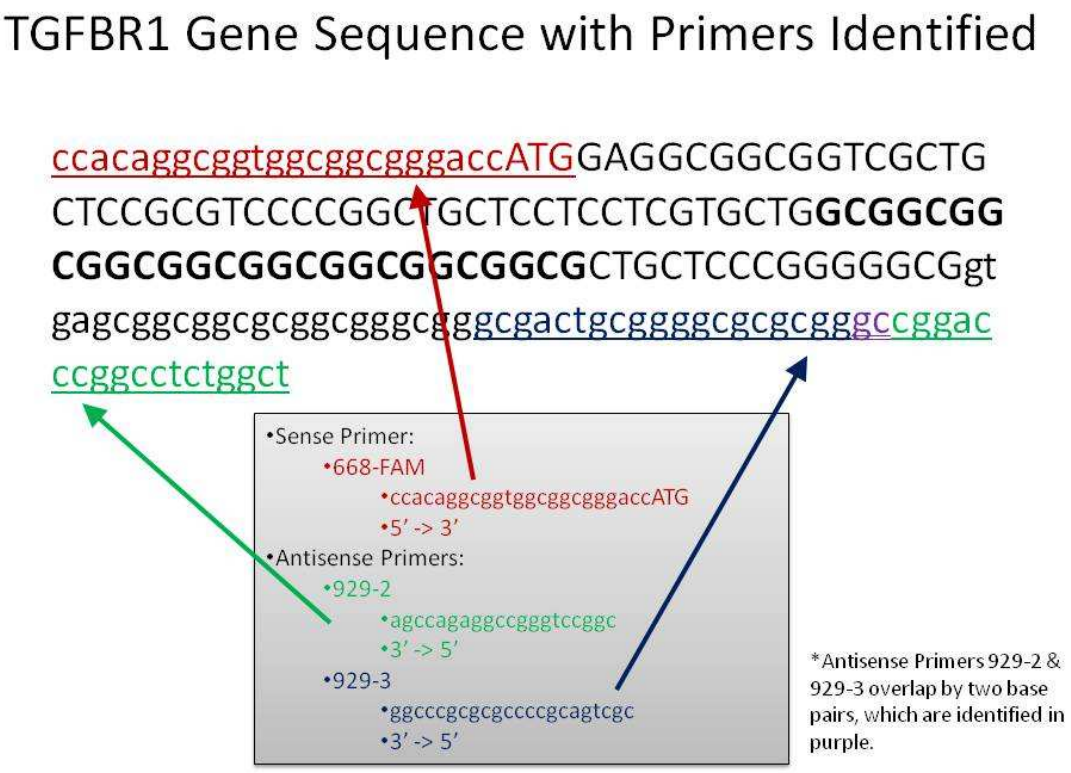

FIGURE 3.1: Primer design used during PCR. Note: 929-2 goes outside of the original primer, 929-3 and the two antisense primers overlap by two basepairs.

To avoid questionable genotyping results the primer pair was moved outside of the original pair, so that the contamination could not be used as template. TABLE 3.1 shows the different size products from each primer pair.

Sense Primer	Antisense Primer	*9A Fragment Size	*6A Fragment Size
668-FAM	929-2	162 base pairs	153 base pairs
668-FAM	929-3	180 base pairs	171 base pairs

TABLE 3.1: Fragment size of amplified TGFBR1 region based on primer set used.

3.3 Polymerase Chain Reaction

Polymerase chain reaction amplification (PCR) of the *TGFBR1* exon was performed on the tumor tissues. Invitrogen® Platinum PCR SuperMix was used; it is a commercially standardized mixture of necessary PCR components. By using the SuperMix, we reduce the chance for error and improve reproducibility because the mix is pre-validated. It contains the *Taq* DNA polymerase and Platinum *Taq* antibody, which provides the enzyme that synthesizes the new strand of DNA from the template DNA provided by the tumor or blood sample being amplified. It also includes Tris-HCL which is necessary to maintain the correct pH conditions throughout the reaction, KCL which encourages primer annealing to the template, and MgCl2

for promoting DNA/DNA interactions and forming complexes with dNTPs which are the actual substrates for Taq Polymerase, the SuperMix contains equal amounts of each dNTP, and finally the stabilizing compounds. The master mix had Invtirogen® PCRX enhancer, which contains betaine, a compound that is beneficial when amplifying GC-rich microsatellites, like *TGFBR1*. Betaine isostabilizes the bonds between all base pairs and by using TAA salts, the melting points are equal irrespective of whether it is a G-C triple or T-A double bond (Rees 1993). Equal amounts of each sense and antisense primer pair were also added to guide amplification of the TGFBR1 template from both the sense and antisense ends of exon 1.

Composition of the polymerase chain reaction buffer was consistent throughout the sample analyses, with the exception of the primer set which was changed during the analysis. For each DNA sample, 17.4 units/ml complexed recombinant *Taq* DNA polymerase with Platinum *Taq* Antibody, 17.4 mM Tris-HCl (pH 8.4), 43.5 mM KCl, 1.31 mM $MgCl_2$, 174.1 µM dGTP, dATP, dTTP and dCTP, as well as, 13.7% Invitrogen® PCRX enhancer, 0.316 pM of each Sense and Antisense primers. TABLE 3.2 summarizes the components used in the PCR master mix. The Applied Biosystems® Veriti 96 well Thermocycler was used for all tumor sample amplification. Before acquisition of the new thermocycler, the blood DNA samples were amplified using a Perkin Elmer® Cetus DNA Thermocycler.

Amount/Concentration	Component	Purpose
17.4 units/ml	*Taq* DNA Polymerase and antibodies	Enzyme for DNA synthesis
17.4 mMol	Tris-HCl (pH 8.4)	Maintains pH conditions
43.5 mMol	KCl	Encourages primer annealing
1.31 mMol	$MgCl_2$	Promoting DNA/DNA interactions
174.1 µMol	dGTP, dATP, dTTP, dCTP	Substrates for transcription
13.70%	PCRX Enhancer	Isostabilizes different bonds

TABLE 3.2: Polymerase Chain Reaction components and their purpose.

Polymerase chain reaction is the process of amplifying small sections of single stranded DNA (usually up to 10kb). The process works by employing several components under specific conditions and is summarized in TABLE 3.3. The first step of PCR is denaturation of the double-stranded DNA into single-stranded, this is done at 95°C for two minutes, during which the DNA polymerase is also heat activated. Following the extended denaturation is a shorter denaturation step of 95°C for 0:50 seconds, which is cycled through repeatedly. This first step is when the entire genome is denatured requiring extra time; during the second step, we will only be denaturing the short target *TGFBR1* exon. Step three is the annealing phase, for 0:40 seconds at 63°C, which is when primers are laid down on the single-stranded template. Stable bonds

between the primer and template are formed only when the primer matches the sequence on the template. The fourth step is at 72°C for 0:40 seconds, providing the optimal temperature for DNA polymerase to synthesize the rest of the complement to the *TGFBR1* exon target sequence. This step is referred to as extension or elongation because of the exponential growth of the target sequence as the steps are cycled through. For our PCR, we cycle from step two, the shorter denaturation step, to the fourth step of elongation 35 times. This amount of cycles is necessary to assure sufficient product to be used for genotyping. After the thirty-fifth cycle, there is a final extension step, step seven, which lasts for 7:30 minutes at 82°C, in order to ensure that any remaining single-stranded DNA is extended. The final step in the *TGFBR1* PCR is to keep the DNA product at 4°C until removed from the thermocycler.

PCR Conditions	Temperature	Duration	Description
Step 1	95°C	2:00	Activation
Step 2	95°C	0:50	Denaturation
Step 3	63°C	0:40	Annealing
Step 4	72°C	0:40	Extension
Step 5	Cycle back to step 2, 35 times		
Step 6	82°C	7:30	Final Extension
Step 7	4°C	Forever	Prevention

TABLE 3.3: PCR Conditions for amplification of tumor DNA samples.

3.4 Preparation for Genotyping

Samples were run in 25 µl volumes; this amount of PCR product was used for two specific purposes. It was used to both validate efficacy of the PCR reaction and prepare dilutions of product for genotyping. From each blood or tumor PCR product, 10 µl was ran on an electrophoresis gel. Gel electrophoresis separates DNA products by size using electromotive force, which causes the negatively charged DNA to move toward the positive anode. A 2.5% agarose gel creates a matrix which differentiates the DNA products based on mass, larger particles move slower thus not traveling as far as smaller particles. The agarose gels are stained with ethridium bromide and the band sizes are imaged by illuminating in UV light. FIGURE 3.2 is a example of the gel electrophoresis' which were performed during the tumor genotyping process. It would be optimal to distinguish differences in bands and band sizes for the different tumor or blood PCR products and obtain genotype of each sample. However, the size of *TGFBR1* product is very small- less than 200 base pairs (varying depending on the primer set used), and the indistinct product makes it impossible to determine *6A or *9A genotype. For this reason, we incorporate the fluorescent end label onto the Sense primer, which enables us to contract the services of the Plant-Microbe Genomics Facility at The Ohio State University.

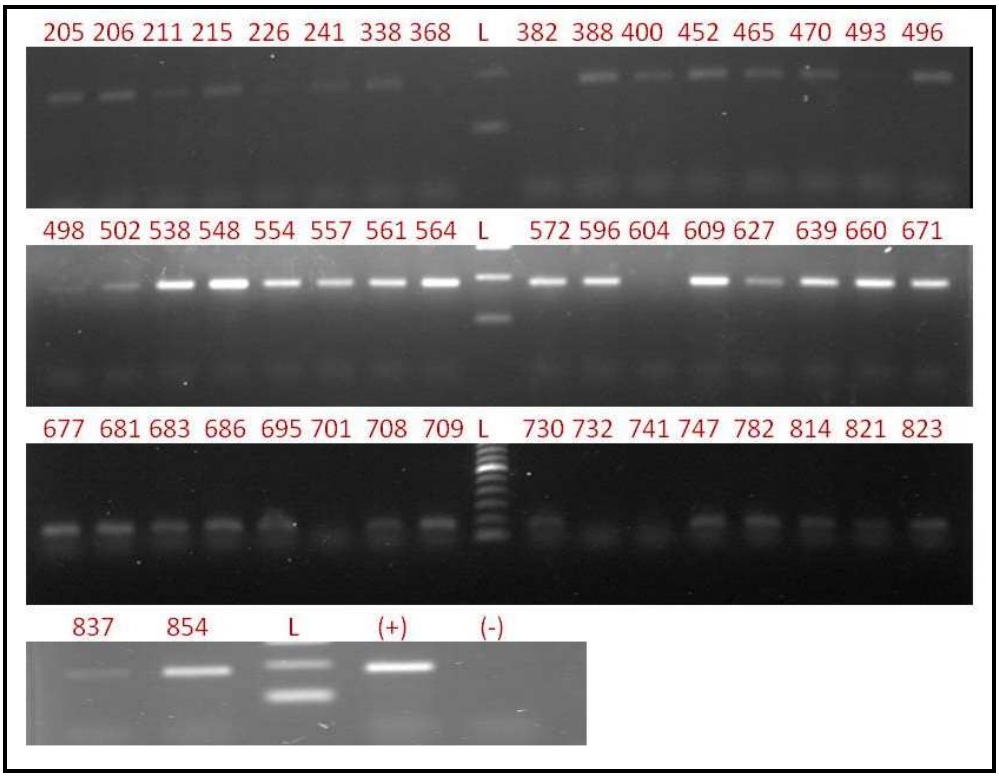

FIGURE 3.2: Example of gel electrophoreses used to check PCR of DNA samples. 'L' denotes the DNA ladder, (+) and (-) indicate positive and negative controls, and number shown is the patient identifier used during the investigation. Note: Primer-dimers present underneath product band, evidence that the reaction was not limited by primers. Also, the variation of band intensity between samples, showing need for serial dilutions when genotyping.

The remainder of the PCR product from the tumor samples was used to create serial dilutions in preparation for genotyping. For the tumor DNA genotyping, the PCR product was diluted and sent for genotyping in triplicate. By doing multiple dilutions, it is a built-in validation tool for human or mechanical error. Three dilutions of PCR product in sterile RNAase and DNAase free water were made for each tumor DNA sample; 1:25, 1:50, and 1:75. We chose these specific dilutions in order to capture the range of success of each PCR, which provides us with reliable genotyping results.

3.5 Genotyping

Genotyping is conducted through the fee for service setup at the Plant-Microbe Genomics Facility at The Ohio State University. They are a research core facility which is routinely utilized by our lab as an economic solution for accessing the machinery necessary to get dependable genotyping results. The Plant-Microbe Genomics Facility or PMGF uses the 3730 DNA Analyzer by Applied Biosystems®.

The DNA Analyzer separates and visualizes DNA fragments that have been labeled with fluorescent dye by means of capillary electrophoresis. This method of electrophoresis employs a group of 48 capillary tubes which are filled with electrolyte loading buffer and the medium separates different sizes of DNA products based on friction and electric charge. Electroosmosis causes the electrolyte –filled buffer to move through the capillary, carrying the DNA fragments. Capillary electrophoresis (CE) detects the migrating DNA product by shining a light source through a portion of the tubing and detecting the light emitted from the other side. The fluorescent dye, 6-FAM, that we labeled the PCR product with is used to show the movement of

the product. CE is highly reliable and efficient. It only requires a small amount of sample and is very sensitive to size differentials, which is beneficial because of the small nine base pair difference in the *TGFBR1**6A to the *9A polymorphism. The samples are subsequently analyzed with the GeneMapper® software in order to determine the size and pattern of the DNA fragments. GeneMapper® uses the relation between DNA fragment size and fluorescence intensity provided by 6-FAM and a fluorescent ladder included in the CE loading buffer to determine relative fluorescent units (RFU). RFUs are useful to show signal strength of the fluorescence, which translates to reliability of the genotype.

3.6 Data Analysis

Output from the GeneMapper® software is an electropherogram. This is a graph showing the relationship between relative fluorescent units on the Y-axis and fragment size on the X-axis. By contrasting these, we are able to determine the genotype of the DNA product. If you refer back to TABLE 3.1, the different sizes of products created by the primer pairs correlates to the position of the peak on the X-axis. By knowing the size of fragment, we can identify the genotype of the tumor or blood DNA sample. A heterozygote with genotype 6A/9A would have and electropherogram with two peaks, one at each fragment size. Homozygotes with genotypes of 6A/6A or 9A/9A identify by having a single peak at the corresponding fragment size. This is illustrated in FIGURE 3.3, showing examples of all three genotypes.

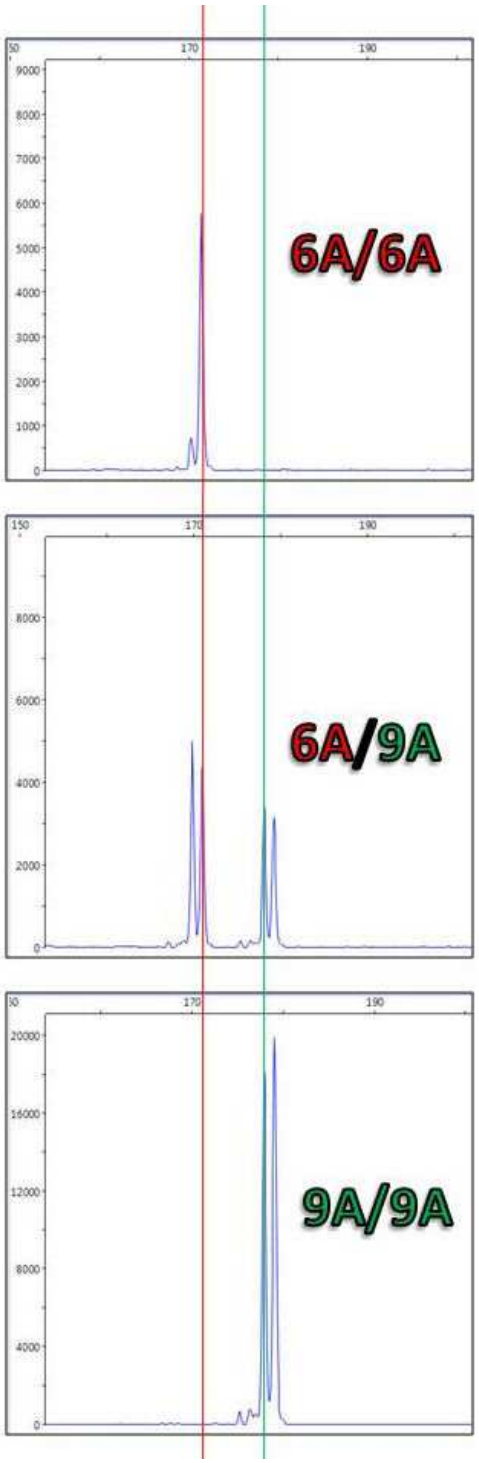

FIGURE 3.3: Example of each different genotype on the electropherogram. The red line indicates where a *6A polymorphism would be shown, and the green line shows *9A.

By analyzing the electropherograms of each tumor DNA sample, which was processed in triplicate, we get a reliable understanding of the tumor's genotype. Each electropherogram was read and genotype verified. There were 132 tumor DNA samples which were processed, eight were not amplifiable, and four more did not produce readable electropherograms, for a total of twelve tumor DNA samples that were not analyzed. One of the tumor DNAs was sent in duplicate, giving us a total of 119 unique genotyped tumor DNA samples from the cervical cancer patients with a 9A/9A blood genotype. Any *6A mutation seen in the tumor DNA sample electropherograms was evidence of somatic acquisition. The proportion of *6A polymorphisms in the sample of 119 allowed us to calculate a rate of somatic acquisition. Confidence intervals were calculated using standard errors and were appropriately adjusted for small sample sizes. Allelic frequency was determined by the ratio of *6A alleles to the total number of alleles in the population.

$$\text{Standard Error} = \sqrt{\frac{rate(1-rate)}{n}}$$

$$\text{95\% Confidence Interval} = rate \pm t_{\alpha,n-1} \times Standard\ Error$$

CHAPTER IV

RESULTS

4.1 Analysis of Somatic Acquisition in Midwestern Cohort

Following PCR amplification of *TGFBR1,* there were 250 patients identified as having a wild-type 9A/9A blood genotype in the Midwestern cohort. We received tumor DNA samples from the 131 of them that had tumor DNA available for analysis. We received one tumor DNA in duplicate, patient number 18P. Tumor DNA samples for patients identified as 13P, 21P, 27P, 52P, 147P, 182P, 226P, 382P, 502P, 521P, 604P and 732P did not have reliable DNA for amplification by polymerase chain reaction. There were a total of twelve ineffectual tumor DNA samples.

There were 119 useful tumor DNA samples from the Midwestern cohort, all of which had a 9A/9A blood genotype. 112 of the 119 (94.11%) had the same 9A/9A tumor genotype as their blood genotype. However, there were seven individuals (5.88%) with a mutation in their tumor genotype compared to their normal blood genotype. Six of the 119 (5.04%) cervical cancer patients had a tumor genotype of 6A/9A. One (0.84%) of the normal blood genotyped individuals had a tumor genotype of 6A/6A. The presence of any tumor DNA mutation in normal

blood genotyped individuals is evidence of TGFBR1*6A somatic acquisition. Allelic frequency of somatically acquired *6A polymorphism was estimated at 3.4% (95% CI: 1.6%, 10.2%). FIGURE 4.1 illustrates the process and results of the tumor and blood DNA genotyping.

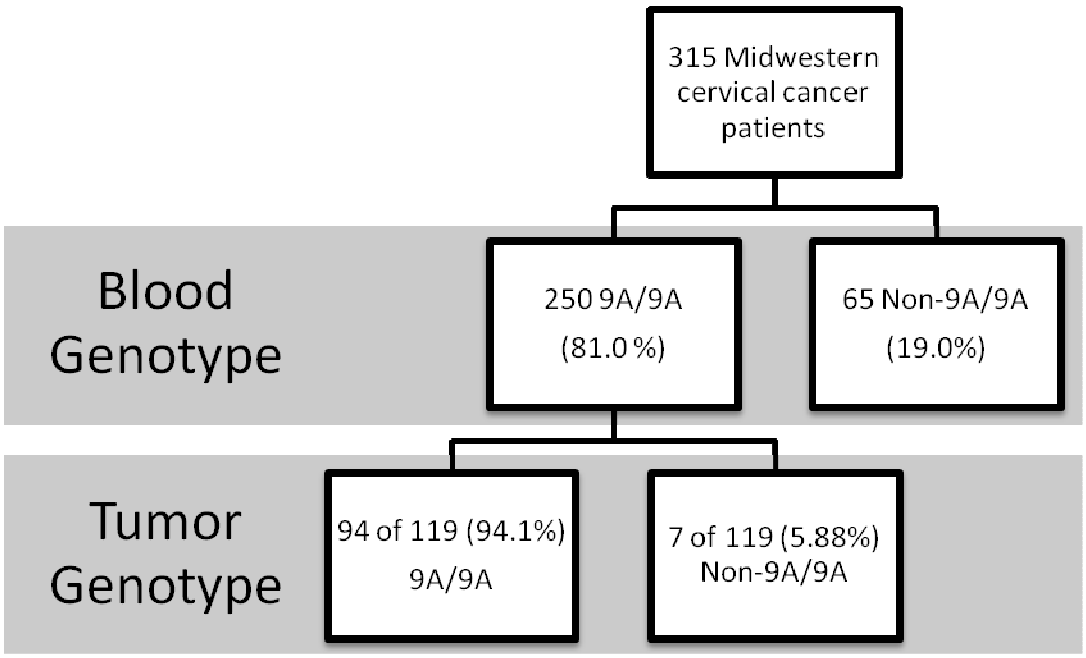

FIGURE 4.1: Schematic showing process and results of tumor DNA genotyping.

Patients identified as 5P, 16P, 22P, 55P, 61P, 84P, 198P were all genotyped as having blood genotypes of 9A/9A, and their tumor tissues had a differing genotype, containing some amount of *6A. The amount of *6A polymorphism in the tumor genotype differs from patient to patient. An early somatic acquisition event would produce a larger amount of *6A, but a more recent event could have only a small amount of *6A. The tumor genotype of patient 16P shows no evidence of *9A, this could have several explanations. The absence of the wild-type polymorphism that made up the entirety of its blood genotype shows either a mutational event that has occurred on both alleles, or an act of haploinsufficiency. FIGURE 4.2 shows the electropherograms of the seven patients with somatically acquired *6A in their tumor tissues.

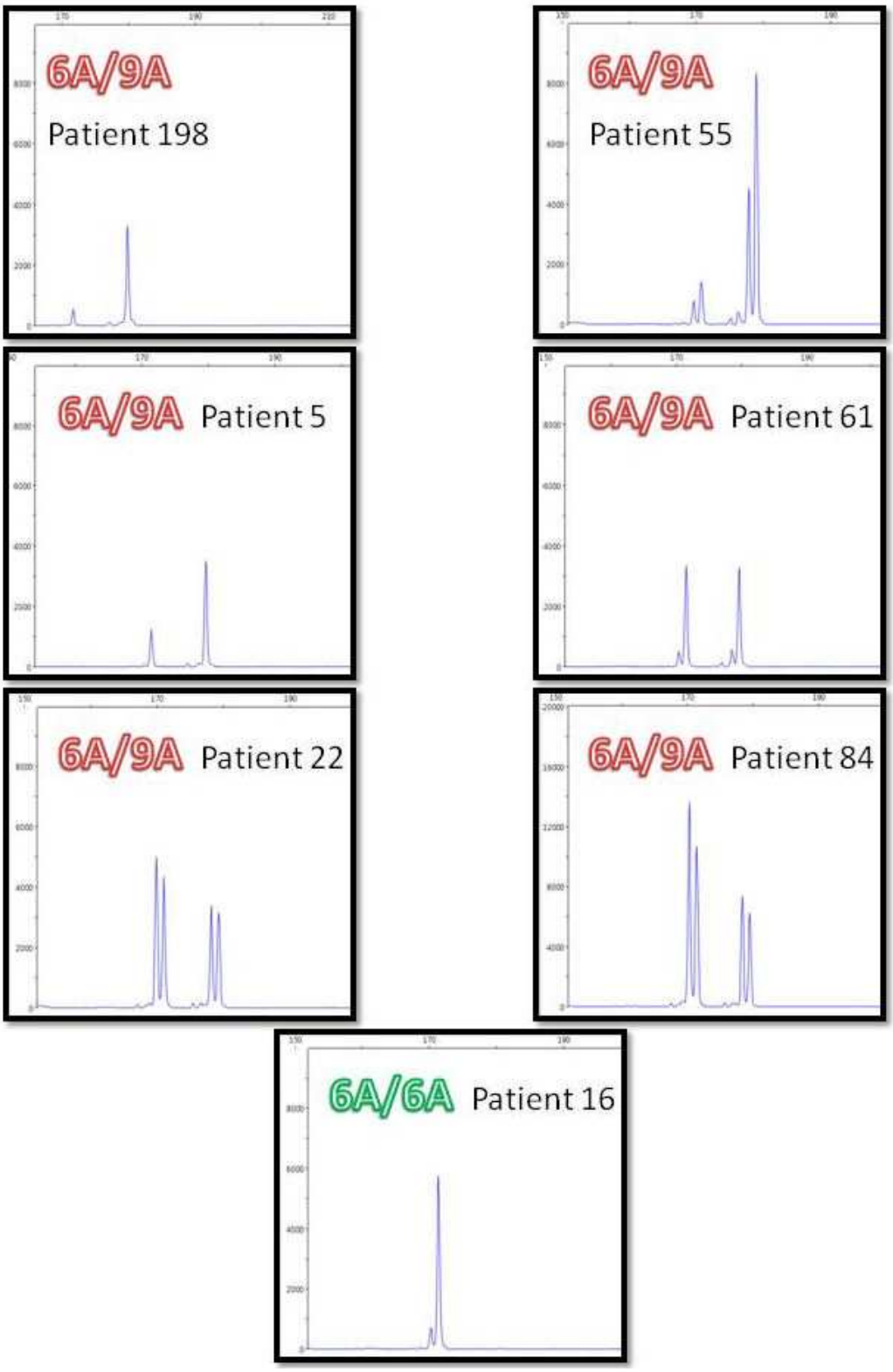

FIGURE 4.2: Electropherograms of patients exhibiting *6A somatic acquisition.

CHAPTER V

DISCUSSION

The Midwestern cervical cancer patient cohort was determined to have a *6A allelic frequency of 10.3%, elevated above the established frequency in normal blood donor populations of 4.2% (Pasche 1998). Evidence of somatic acquisition was ascertained by any presence of the *6A polymorphism in the tumor DNA genotype of blood *9A homozygotes. By genotyping only the members of the Midwestern cohort who were blood homozygotes for *9A, we calculated the most conservative estimate for somatic acquisition in cervical cancer tumors. There were 119 tumor DNA samples that were able to be genotyped; 112 had the same blood and tumor genotype (94.12%). However, the remaining seven tumor samples were found to have acquired the *6A polymorphism, evidence of somatic acquisition within the tumor (5.88%). Six of the tumor samples were heterozygous (6A/9A), but one tumor sample was homozygous (6A/6A). This rate of somatic acquisition is the highest seen in localized carcinomas.

The *6A polymorphism is interesting in many aspects. It is a three alanine deletion in the polyalanine sequence nine alanines in length on the gene that codes for TβR-I. Base pair triplet [GCG] codes for alanine and the nine base pairs that are deleted show a sequence of [GCGGCGGCG]. The three alanine deletion is not found in any other polyalanine sequence,

showing that no mutator phenotype mechanism is affecting GCG repeats (Pasche 2005). Also, the nine basepair deletion is larger than most mutational deletions. Furthermore, the evidence showing that the exact same deletion is occurring in tumors as is found in blood genotypes lends evidence to the theory that *6A gives cells a growth advantage by affecting tumor suppressive effects. The levels of *6A somatically acquired by tumor cells infers that the *6A mutation is background mutational activity which flourishes after loss of TGFβ inhibition. This is supported further by Pasche *et al.*'s JAMA publication data showing that colorectal cancer metastases to the liver had the highest levels of somatic acquisition.

Recent advances have been made investigating the mechanism behind TGFBR1*6A and its affect on tumor suppressive effects. After establishing that the mature receptors of both the *6A and *9A are the same, it is believed that the signal sequence triggers a secondary signaling event (Pasche 2005). Rosman *et al.* examined the differences in *6A signaling using MCF-7 breast cancer cells. They found that in the absence of TGFβ, these cells continued to show differential signaling between *6A and *9A, inferring that the *6A phenotype is independent of TGFβ signaling (Rosman 2008). By using Affymetrix® GeneChip arrays, they found decreased expression of ARHGAP5 and FN1 in *6A transfected cells, compared to *9A. It was then established through functional assays that the down-regulation of ARHGAP5 is associated with increased RhoA activation. This RhoA hyperactivation has been observed to result in an increase in ERK activation, which is directly involved in inhibition of cell motility (Rosman 2008). These findings can be directly applied to our findings of somatic acquisition of TGFBR1*6A in cervical cancer. The migratory effects that are initiated by ERK activation could be contributing to the increased levels of somatic acquisition in the metastases to the liver that was found in the 2005

JAMA paper (Pasche 2005). Furthermore, this is a novel explanation of how the *6A signal sequence could be affecting tumor suppressive effects.

Another recent theory on the differential expression of TGFBR1*6A in cancer was developed by researchers here at The Ohio State University, by Valle *et al.* They suggested that the effect of *6A may be related to allele-specific expression (Valle 2008). Allele-specific expression, or ASE, results in lowered or extinguished expression of specific alleles, for example here, TGFBR1*6A. By looking at three different single nucleotide polymorphisms (SNPs) in the 3' untranslated region of TGFBR1, they found 29 cases of colorectal cancer which exhibited high ASE of at least one SNP. By knowing this, they were able to genotype the patients with colorectal cancer for TGFBR1*6A, and found that there were more patients heterozygous for *6A (14/29) with ASE than patients without ASE (22/108) (Valle 2008). This is indirect evidence that suggests ASE in TGFBR1 may contribute to development of cancer. If this is applied to cervical cancer, it suggests that the increased risk associated with being a carrier of *6A may be related to this incomplete penetrance of *6A, as well as contributing to the genetic predisposition to cervical cancer. However, the presence of ASE in tumors remains to be investigated, and may provide information into the act of somatic acquisition of *6A in cervical cancer tumors.

The increased incidence of *6A in the Midwestern cohort of cervical cancer patients over the normal population demonstrates that those carrying the polymorphism are at a greater risk of cervical cancer than those who have a normal 9A/9A blood genotype. This idea of increased risk has clinical relevance; by understanding a person's TGFBR1*6A status, we may be able to create a risk profile when combined with their Pap smear and carcinogenic HPV status. Whether the *6A polymorphism is diagnostic is yet to be known, however personalized

medicine has many therapeutic advantages. And, when discussing our genetic code, knowledge is power.

The idea of personalized medicine is also relevant when discussing somatic acquisition. Tumor genotype may be a prognostic tool for cervical cancer patients. As seen in colorectal cancer, metastases have higher rates of somatic acquisition. It may be that a patient with tumors that have somatically acquired *6A has a greater risk of metastasis. What we do know is that some mutational change in the tumor has caused it to acquire the *6A genotype which gives the tumor a growth advantage, but what we don't understand is what causes that initial mutation. It has been suggested by Knobloch *et al.* that the when the stem cell acquires the *6A polymorphism, it then creates a 'patch' of daughter cells with a growth advantage which then may develop into cancer (Knobloch 2007). This concept of field cancerization makes it very important to understand the stressor or mechanism which causes the initial mutation.

The goals of this study were to expand the body of knowledge related to TGFBR1*6A into cervical cancer. By showing a higher germline incidence of *6A in our population of cervical cancer patients than the established rate in normal blood donors, we have supported the claim that carriers of *6A are at an increased risk for cancer. Based on the population of cervical cancer patients we have investigated, we find there is a significant incidence of somatic acquisition of *6A in tumors of blood genotyped 9A/9A individuals. Furthermore, the evidence of somatic acquisition in cervical cancer tumors of blood genotyped 9A/9A patients, is another carcinoma of epithelial tissues which is affected by the *6A polymorphism.

CHAPTER VI

FUTURE WORK

This study of the TGFBR1*6A has expanded the knowledge of the gene polymorphism into cervical cancer. Somatic acquisition is a difficult concept to show with certainty because of the cellular level of examination. The line of differentiation between the tumor tissue and normal germline tissue is small and ever-changing. By only examining the 9A/9A blood homozygotes, we use the best possible scenario for showing somatic acquisition. The 9A/9A blood homozygotes should not have any tissue in the body, tumor or otherwise, with a differing polymorphism like *6A. Therefore, when a tumor exhibits a small amount of *6A genotype, we know that this is somatic acquisition. In comparison, a 6A/9A blood heterozygote would not be as easy to confirm somatic acquisition in; their tumor genotype would already contain *6A. This is an obvious direction of research, by establishing a ratio of gene dosage of blood genotype to tumor genotype, somatic acquisition could be ascertained in 6A/9A blood heterozygotes. Our research has established the presence of somatic acquisition in cervical cancer, but expanding on this would contribute to the body of knowledge related to TGFBR1*6A in cervical cancer.

As described in the Discussion section, the recent hypotheses related to the mechanism of *6A in cancer development, have not been characterized in cervical cancer. It

would be beneficial to understand the mechanism of allele-specific expression (ASE) in cervical cancer patients and their tumors. By expanding beyond the previously examined colorectal cancer, we can understand the contribution of ASE in cervical cancer development (Valle 2008). Furthermore, the suggestion that a secondary signaling event by the *6A signal sequence resulting in RhoA activation has only been investigated in breast cancer cells (Rosman 2008). By characterizing the hyperactivation of ERK and RhoA in cervical cancer, we may better understand the mechanism by which *6A affects cervical cancer development. These two investigations are important to both expanding the body of knowledge related cervical cancer, but also examining the suggested phenomena in a different medium.

Gene dosing and gene stability are important factors when investigating microsatellite repeats. The local nature of TGFBR1*6A in cervical cancer makes it vital to examine these occurrences. Alternative explanations for TGFBR1*6A incidence involve chromosomal level gains and losses of the gene locus containing TGFBR1. Previously characterized in head and neck cancers, comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) analyses showed no evidence that this could alternately explain *6A incidence in cancer patient populations. However, to fully understand the mechanism of somatic acquisition or the incidence of *6A in blood genotypes, we would need to repeat these analyses in cervical cancer. Comparative genomic hybridization involves whole genome scanning. This permits the identification of chromosomal imbalances (gains, losses or amplification of DNA sequences) in entire tumor genomes (Balsara 1999). Loss of Heterozygosity shows signs of somatic deletion. The somatic deletion is of the tumor suppressor gene, which causes cell proliferation. Once gene

loci have been mapped, we can check for deletion of tumor suppressor genes on the chromosome 9q22. Genomic instability is an appropriate investigation for GCG-rich genes. This allows us to check if the 9-bp deletion is specific to TGFBR1. If other GCG-rich genes in cervical cancer were affected, it is a sign of genomic instability.

APPENDIX I

SUMMARY OF MIDWESTERN TUMOR SAMPLES

Proband	Blood Genotype	Tumor Genotype
001P	9a/9a	9a/9a
002P	9a/9a	9a/9a
003P	9a/9a	9a/9a
004P	9a/9a	9a/9a
005P	9a/9a	**6a/9a**
006P	9a/9a	9a/9a
009P	9a/9a	9a/9a
011P	9a/9a	9a/9a
012P	9a/9a	9a/9a
013P	9a/9a	Not Amplifiable
014P	9a/9a	9a/9a
016P	9a/9a	**6a/6a**
017P	9a/9a	9a/9a
018P	9a/9a	9a/9a
021P	9a/9a	Not Amplifiable
022P	9a/9a	**6a/9a**
027P	9a/9a	Not Amplifiable
032P	9a/9a	9a/9a
036P	9a/9a	9a/9a
039P	9a/9a	9a/9a
045P	9a/9a	9a/9a
046P	9a/9a	9a/9a
052P	9a/9a	Not Amplifiable
054P	9a/9a	9a/9a
055P	9a/9a	**6a/9a**
059P	9a/9a	9a/9a
061P	9a/9a	**6a/9a**
064P	9a/9a	9a/9a

Proband	Blood Genotype	Tumor Genotype
068P	9a/9a	9a/9a
069P	9a/9a	9a/9a
079P	9a/9a	9a/9a
080P	9a/9a	9a/9a
081P	9a/9a	9a/9a
084P	9a/9a	**6a/9a**
114P	9a/9a	9a/9a
123P	9a/9a	9a/9a
124P	9a/9a	9a/9a
126P	9a/9a	9a/9a
129P	9a/9a	9a/9a
147P	9a/9a	Not Amplifiable
148P	9a/9a	9a/9a
150P	9a/9a	9a/9a
153P	9a/9a	9a/9a
155P	9a/9a	9a/9a
160P	9a/9a	9a/9a
168P	9a/9a	9a/9a
170P	9a/9a	9a/9a
171P	9a/9a	9a/9a
172P	9a/9a	9a/9a
176P	9a/9a	9a/9a
177P	9a/9a	9a/9a
179P	9a/9a	9a/9a
182P	9a/9a	Not Amplifiable
185P	9a/9a	9a/9a
187P	9a/9a	9a/9a
188P	9a/9a	9a/9a
193P	9a/9a	9a/9a
198P	9a/9a	**6a/9a**
205P	9a/9a	9a/9a
206P	9a/9a	9a/9a
209P	9a/9a	9a/9a
211P	9a/9a	9a/9a
215P	9a/9a	9a/9a
216P	9a/9a	9a/9a
226P	9a/9a	Not Amplifiable
241P	9a/9a	9a/9a
278P	9a/9a	9a/9a
338P	9a/9a	9a/9a
368P	9a/9a	9a/9a

Proband	Blood Genotype	Tumor Genotype
381P	9a/9a	9a/9a
382P	9a/9a	Not Amplifiable
388P	9a/9a	9a/9a
392P	9a/9a	9a/9a
400P	9a/9a	9a/9a
452P	9a/9a	9a/9a
455P	9a/9a	9a/9a
465P	9a/9a	9a/9a
470P	9a/9a	9a/9a
482P	9a/9a	9a/9a
483P	9a/9a	9a/9a
493P	9a/9a	9a/9a
496P	9a/9a	9a/9a
498P	9a/9a	9a/9a
502P	9a/9a	Not Amplifiable
521P	9a/9a	Not Amplifiable
538P	9a/9a	9a/9a
544P	9a/9a	9a/9a
548P	9a/9a	9a/9a
554P	9a/9a	9a/9a
557P	9a/9a	9a/9a
560P	9a/9a	9a/9a
561P	9a/9a	9a/9a
564P	9a/9a	9a/9a
566P	9a/9a	9a/9a
570P	9a/9a	9a/9a
572P	9a/9a	9a/9a
575P	9a/9a	9a/9a
577P	9a/9a	9a/9a
578P	9a/9a	9a/9a
592P	9a/9a	9a/9a
595P	9a/9a	9a/9a
596P	9a/9a	9a/9a
604P	9a/9a	Not Amplifiable
609P	9a/9a	9a/9a
627P	9a/9a	9a/9a
639P	9a/9a	9a/9a
660P	9a/9a	9a/9a
671P	9a/9a	9a/9a
677P	9a/9a	9a/9a
679P	9a/9a	9a/9a

Proband	Blood Genotype	Tumor Genotype
681P	9a/9a	9a/9a
682P	9a/9a	9a/9a
683P	9a/9a	9a/9a
686P	9a/9a	9a/9a
695P	9a/9a	9a/9a
700P	9a/9a	9a/9a
701P	9a/9a	9a/9a
708P	9a/9a	9a/9a
709P	9a/9a	9a/9a
730P	9a/9a	9a/9a
732P	9a/9a	Not Amplifiable
741P	9a/9a	9a/9a
747P	9a/9a	9a/9a
782P	9a/9a	9a/9a
804P	9a/9a	9a/9a
814P	9a/9a	9a/9a
821P	9a/9a	9a/9a
823P	9a/9a	9a/9a
837P	9a/9a	9a/9a
847P	9a/9a	9a/9a
854P	9a/9a	9a/9a

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