Prevalence of Human Papillomaviruses (HPV) in Triple Negative (TN) Breast Cancer

By

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Thesis

Submitted to the College of Health and Human Services

Eastern Michigan University

in partial fulfillment of the requirements

for the degree of

MASTERS OF SCIENCE

in

Clinical Research Administration

Thesis Committee:

Stephen A. Sonstein, PhD, Chair

November 2011

Ypsilanti, Michigan

Dedication

I dedicate this thesis to my wife, Laura, who provided unconditional love and encouragement throughout the course of my studies.

I also would like to recognize the support I received from my loving children, Joshua, Jacob, Bethany, Joseph, Jeremiah and Jonah.

Acknowledgements

I would like to acknowledge Mercy Health Partners Northern Region and the Mercy Adult IRB for allowing this research to take part and for their oversight.

My sincere gratitude is extended to the Dolores Douglas Foundation for providing the funding for this research. The Dolores Douglas Foundation provided a $20,000 grant to complete this research.

Without Dr. Mohammad AlNsour's idea for the study, instruction and continuing guidance this study would not have been possible. I am very thankful for his time and guidance and I would like to thank and acknowledge him as my research mentor, colleague, and friend.

Dr Stephen Strobel, Pathologist for Mercy Saint Vincent's Hospital provided technical expertise for this study. His time and knowledge was greatly appreciated.

Lastly, I would like to recognize my thesis advisor, Dr Stephen Sonstein, for his guidance and support during this project and my course work at Eastern Michigan University.

Abstract

Human papillomavirus (HPV) has been identified in breast carcinogenesis. Paraffin embedded tissue collected between January 2005 and December 2010 from the Mercy Specimen Bank in Toledo, Ohio were sampled in order to look for the presence of HPV by p16 determination.

Forty cases of triple negative breast cancer were identified. When tested for p16 by immunohistochemical (IHC) staining about 87% were positive, mostly with intense, diffuse staining, suggesting the possibility of HPV as a causative mechanism for these tumors. The intensity of p16 staining correlated with younger age, mimicking the demographic distribution of other HPV infections in communities.

Although HPV DNA was not confirmed by *In Situ* hybridization, its low viral load rendered it difficult to confirm. However, the possibility that the elevated p16 occurred as a result of HPV infection during carcinoma development cannot be ruled out and should be furthered studies.

Table of Contents

Table of Contents (Continued)

List of Tables

Chapter 1: Introduction and Background

Cancer

Cancer develops through a multistep process in which normal, healthy cells in the body go through stages that eventually change them to abnormal cells that multiply out of control.

Cells proliferate for growth and to replace worn-out cells. Normal cells in the body communicate with each other and regulate cell division, known as cell proliferation. When cancer occurs, cells escape the normal controls on proliferation.

Part of this multistep process includes acquiring damage, known as mutations, to the cell's genetic instructions, the cell's DNA, which normally regulates cell proliferation. A series of permanent mutations in tumor suppressor genes and proto-oncogenges are needed before cancer develops. Buildup of mutations in these genes can result in uncontrolled cell proliferation. In some cases, further damage can lead to cells that can break away from the primary tumor and form cancers at other sites in the body, known as metastasis.

Carcinogenesis

Carcinogenesis is the process that leads to genetic mutations induced by physical or chemical agents. This process is divided into three stages: initiation, promotion, and progression. Initiation involves an irreversible genetic change, usually a mutation in a single gene. Promotion is generally associated with increased proliferation of initiated cells, causing increases in the population of initiated cells. Progression is the accumulation of more genetic mutations that lead to the malignant or invasive form of cancer.

Environmental factors that cause DNA damage are of great interest to preventing cancer. Environmental agents that can cause DNA damage include ionizing radiation, ultraviolet light, chemical agents and biological agents. Viruses, like Human Papillomaviruses (HPV) fall under biological agents.

Tumor cells differ from normal cells in that they exhibit uncontrolled growth. Because features that distinguish tumor from normal cells may be key to understanding cancerous cell behavior and may ultimately lead to therapies that can target tumor cells, considerable effort has been directed at identifying the phenotypic characteristics of laboratory grown transformed cells (*In Vitro*) and of tumor cells derived from natural host sources (*In Vivo)*.

Proliferation is a process consisting of multiple subroutines that collectively bring about cell division. At the heart of the process of cell proliferation is the cell cycle. This cycle consists of processes that must be completed in sequence. The cell cycle is divided into four phases labeled: G1, S, G2, and M. Two key events in cell division are the replication of DNA (S phase) and chromosome separation (M). The cell cycle consists of oncogenes and tumor suppressor genes that regulate the cell cycle.

Normal cells that are not dividing enter the resting phase, or G0, and the presence of growth factors triggers the replication of DNA, leading the cell to exit G0 and enter into the replication phases (G1 then S phases). This process is frequently misregulated in cancer cells resulting in uncontrolled proliferation.

Movement through the cell cycle is tightly regulated and typically controlled by two classes of cell cycle proteins, cyclin dependent kinases (CDKs) and cyclins. CDKs physically associate to form a protein kinase that drives the cell cycle forward. The accumulation of cyclin proteins occurs through cell cycle dependent induction of gene transcription; on the other hand,

elimination of cyclins occurs through a carefully regulated degradation process. Cyclins A, B and E play key roles in cell proliferation and are most abundant during late G1 and G2 phases of the cell cycle.

Cyclin D1 is synthesized during G1 just before the restriction point and plays an important role in regulation. Cyclin E is most abundant during late G1 and early S and is essential for exit from G1 and progression into S phase. Elevated levels of these two G1 cyclins can result in uncontrolled proliferation. Both cyclin D1 and E are overexpressed in some tumor types, suggesting that the cyclins and other components of the cell cycle could be useful targets in cancer treatments.

Cyclin D1/CDK(4,6) complex activity is essential for pushing cells into replication. A major substrate of the cyclin D1/CDK4,6 complex is the retinoblastoma (Rb) tumor suppressor protein. When the Rb tumor suppressor protein is phosphorylated by this kinase complex it is inactivated. This inactivation is believed to be decisive in the stimulation of resting cells to undergo proliferation.

Cyclin E/CDK2 plays a role, later in the cell cycle, by pushing proliferating cells from G1 into S phase. Cyclin E has been found to be over expressed in some breast cancers where it may enhance the proliferative of tumor cells.

Superimposed on the functions of the cell cycle is a complex network of both positive and negative regulatory pathways. Important negative regulators are the cyclin dependent kinase inhibitors. When a cell is infected with HPV, the virus expresses certain “early” oncogenes, such as E6 and E7. E7 viral oncogene binds the tumor suppressor gene Rb, the potent suppressor of the G1-S cell cycle transition, and inactivates it. Rb also inhibits p16 transcription through a negative feedback loop; as a result of Rb inactivation, p16 levels are paradoxically high in these

highly proliferative tumors. Diffuse p16 immunoreactivity is useful diagnostically for delineation of HPV-related tumors. HPV E6 protein also inactivates p53 in these cancers, further compromising the G1-S cell cycle checkpoint, as well as inhibiting of programmed cell death (apoptosis).

Breast Cancer

Breast cancer is the formation of a malignant tumor as a result of mutations in this multistep process involving cells in the breast.

A basic knowledge of the breast structure and development is necessary for understanding breast cancer. Clinically the breast is divided into four quadrants: upper inner, upper outer, lower inner and lower outer quadrants. Cancer occurs most often in the upper outer quadrant.

The breast is composed of lobes that radiate from the nipple. Each lobe is surrounded by fat and connective tissue and is divided into lobules. The lobule is the basic structural unit of the breast and is lined by epithelial cells. The ducts coalesce into major ducts; each lobe containing one major duct terminating at the nipple.

The most common type of breast cancer originates in epithelial cells. About 85% of breast carcinomas originate within the epithelial cells of the ducts known as ductal carcinoma, the remaining 15% begin in the cells that line the lobules known as lobular carcinoma.

Hormone Receptors

Hormone receptors are proteins found within and on the surface of certain cells throughout the body, including breast cells. These receptor proteins are a source of communication for the cell. The receptors act like an on-off switch for activities in the cell. If the

right substance comes along that fits the receptor the switch is turned on and a particular activity in the cell begins. By attaching to hormone receptors, estrogen and/or progesterone contribute to the growth and function of breast cells, for example high estrogen leads to breast cell proliferation.

Although estrogen does not appear to directly cause the DNA mutations that trigger the development of breast cancer, estrogen does stimulate cell proliferation therefore, if breast cells already possess a DNA mutation that increases the risk of developing cancer, these cells will proliferate in response to estrogen stimulation. Estrogen induced cell production leads to an increase in the total number of mutated cells that exist. These cells are at increased risk of becoming cancerous, so the chances that cancer will develop are increased.

HER2/neu Receptor

Human epidermal growth factor receptor (HER2) also known as proto-oncogene neu is a cell membrane tyrosine kinase receptor and is normally involved in the signal transduction pathway of a cell leading to cell growth and differentiation.

Inappropriate signaling may occur as a result of receptor overexpression or dysregulation of the receptor's activity. Overexpression of HER2 could lead to increased or uncontrolled cell proliferation, a cell resistance to program cell death (apoptosis); enhanced cancer cell motility or blood vessel formation (angiogenesis).

Approximately twenty to thirty percent of breast cancers have an amplification or over expression of HER2 gene.

Triple Negative Breast Cancer

Approximately ten to twenty percent of breast cancer diagnosis are classified as triple negative, which refers to the cancer being estrogen receptor negative (ER-), progesterone receptor negative (PR-) and HER2 negative. Women diagnosed with triple negative breast cancer are more likely to be younger premenopausal women when compared to women with hormone positive breast cancer.

Prevalence of Breast Cancer

Breast cancer is the second leading cause of cancer death for women in the United States (US), after lung cancer. Approximately 39970 women and 450 men will die from the disease this year (American Cancer Statistic 2011). Excluding skin cancer, breast cancer is the most commonly diagnosed cancer among women in the US.

The specific etiology and mechanism related to breast carcinogenesis remains poorly understood. Risk factors associated with breast cancer can be classified into three categories: family genetic factors, hormonal and reproductive factors, and environmental factors (chemical or viral).

Human Papillomavirus

Human Papillomaviruses (HPV) are a group of small double stranded DNA viruses that infects mainly the anogenital epithelium and more recently identified in the head and neck area. There have been over 200 genotypes of this virus identified. There are two classifications or subtypes of HPV; Low risk and high risk, based on their influence to cause cervical cancer. High risk HPV are primarily associated with cervical cancer but also linked with other anogenital cancers such as cancers of the oral cavity, and the oropharynx.

HPV is the etiologic agent for most cases of cervical cancer.

Prevalence of Human Papillomavirus

Currently it is estimated that 20 million people are currently infected with HPV in the United States, most asymptomatically. The peak age range of infection is 15 to 30 years of age. Most HPV viruses are sexually transmitted. HPV is considered the most common sexually transmitted infection.

Most women infected with high-risk genital HPV do not develop cancer; the infection usually is resolved within two years. Persistent infection with high risk HPV genotypes are associated with increased risk for developing high-grade dysplasia or cervical carcinoma, vulvar or vaginal carcinoma.

Human Papillomavirus and Breast Cancer

The relationship between breast cancer and HPV is suggested by preclinical as well as clinical evidence. Human mammary cells are immortalized when infected with HPV, and when these immortalized cells are injected in certain transgenic mice, breast cancer develops, showing that HPV infection can cause breast cancer. Once a cell is infected with HPV, the virus can expresses early oncogenes, such as E6 and E7. E7 viral oncogene binds and inactivates the Rb gene, leading to uncontrolled growth. Rb normally inhibits p16 transcription through a negative feedback loop between p16 and Rb; as a result of Rb inactivation, p16 levels are high, leading to increased proliferation. HPV E6 gene inhibits p53; leading to the cells escaping programmed cell death (apoptosis).

p16 as a Biomarker for Human Papillomavirus

Looking for a surrogate marker, epidemiological studies replaced the laborious and expensive HPV detection and typing with analyzing for p16 protein expression.

Immunohistochemical detection of p16 has been shown to be a reliable marker for squamous dysplasia in the uterine cervix, where the protein is frequently over expressed as a result of infection with high risk HPV. Analyses of head and neck squamous cell carcinomas have revealed frequent infections by HPV genotype 16 in tonsillar carcinomas. The National Comprehensive Cancer Network (NCCN) clinical practice guidelines on oncology recommend p16 by immunohistochemistry (IHC) in the workup of head and neck cancer.

The Molecular Pathology Laboratory Network (MPLN) recommends p16 by IHC as the optimal test method for determining HPV infections in head and neck squamous cancer. P16 and HPV positivity show a strong favorable prognostic outcome for patients treated with chemo-radiation therapy and surgery. Tumor HPV and p16 status (positive or negative) are the strongest predictors of survival in head and neck squamous carcinoma patients treated with radiotherapy.

Statement of the Problem

The prevalence of HPV in cervical and head and neck cancer, particularly the same high risk subtypes, suggest the potential etiologic role for the virus in breast cancer. Molecular researchers have provided important data on the interaction of HPV oncoproteins with the interfering of the cell cycle leading to these cancers. Clinically the associations of HPV infected head and neck cancers have shown a favorable outcome prognosis compared to other causative agents (tobacco or chemical). The more favorable prognosis and treatment response by HPV positive tumors means that HPV detection is required to better plan the patients treatment. Also, identification of HPV as a causative agent for certain subtype of breast cancer will aid in the development of future preventive and therapeutic strategies against breast cancer. An accurate and cost effective test is required to effectively screen high risk patients as a routine assessment.

Purpose of Study

The purpose of this study is to answer the question; What is the prevalence of HPV infection in diagnosed triple negative (ER negative, PR negative and HER2 negative) breast cancer tissue from paraffin embedded tissue collected between January 1, 2005 and December 31, 2010 from the Mercy specimen bank (Mercy St Vincent Medical Center and Mercy St Charles Medical Center).

We would like to determine if HPV infections can be identified by using p16 antigen Immunohistochemical staining in triple negative breast cancer, as you can in HPV cervical cancers and most HPV head and neck cancers. Also, are there any pathological correlates associated with triple negative breast cancer and other HPV infected cancers?

Significance

It is well accepted that HPV positive status is an important prognostic factor associated with a favorable outcome in cervical and head and neck cancers. Although the HPV testing is largely confirmatory, evidence suggest that these patients are associated with better response to treatment and associated with improved survival rate. If HPV is associated with triple negative breast cancer there is no reason to believe that these patients too could benefit with a more favorable outcome or may need less intensive therapy than other patients with breast cancer. Also, identification of HPV as a causative agent may direct future treatment to prevent and treat triple negative breast cancer patients.

Research Question

The current research addressed the following research questions:

- What is the prevalence of HPV infection in diagnosed triple negative (ER negative, PR negative and HER2 negative) breast cancer tissue from paraffin embedded tissue collected between January 1, 2005 and December 31, 2010 from the Mercy specimen bank (Mercy St Vincent Medical Center and Mercy St Charles Medical Center)?
- Can Immunohistochemical staining be used to identify human HPV in triple negative breast cancer? (as you can in HPV cervical cancers and most HPV head and neck cancer)
- Can we identify any pathological correlates associated with triple negative breast cancer?

Chapter 2: Review of Related Literature

The etiology of breast cancer remains largely unknown. The possibility that viruses may have an etiological role in breast cancer was initiated in 1936 by John Bittner. He observed that mouse milk contains an unknown factor which caused mammary tumors in their pups when they grew to maturity. This unknown fact was identified as mouse mammary tumor virus.

Human Papillomavirus (HPV) has been investigated in laboratories around the globe over the past twenty years for its possible association with breast cancer. My literature review is summarized in table 1. Twenty-five of the thirty-two (78%) studies reviewed found high risk HPV subtypes in breast cancer tissue. This stimulated our interest in the possibility that high risk HPV may play a role in the etiology of breast cancer within the patient population at the Mercy Health System in Toledo, Ohio.

High risk HPV subtypes 16, 1 8 and 33 have been identified in breast cancer from populations including Italy, China, Japan, Norway, United States of America, Brazil, Austria, Taiwan, Germany, Greece, Turkey, Korea, France, Mexico, Syria and the United Kingdom. The prevalence of HPV positive breast cancer in these studies ranged from 4.5% in Mexico to 86.2% in the United States of America.

The oncogenic mechanisms by which HPV induces cervical cancer as well as head and neck cancers have been intensely studied. HPV associated in these cancers can be used as a model for breast cancer, summarized in table 2. The biology of HPV in breast cancer is similar to both cervical cancer and head and neck cancers. One major difference is that the viral load (amount of HPV DNA incorporated in cancer cells) is much higher in cervical cancer and head and neck cancer than in breast cancer.

Koilocytes are epithelial cells characterized by perinuclear haloes surrounding condensed nuclei and are commonly present in cervical and head and neck intraepithelial neoplasia. Koilocytosis is accepted as a characteristic of HPV infection. James Lawson has shown that human papillomavirus associated koilocytes were present in breast skin and cancer tissue of patients with ductal carcinoma *In Situ* (DCIS) and invasive ductal carcinomas (IDCs).

Epidemiological studies predicted that cancer was a multistep process with at least five rate-limiting steps before a tumor could arise. It is now known that these rate-limiting steps are genetic mutations that dysregulate the activities of genes that control cell growth, regulate cell death, and maintain genetic stability. It is clear that the successive accumulation of mutations in key genes is the force that drives tumorigenesis. Each successive mutation is thought to provide the developing tumor cell with important growth advantages that allow these cells to outgrow their neighboring "normal" cells.

Much effort by scientists has focused on identifying these genes and understanding how they alter cell growth. Early efforts in this area were lead by virologists studying retrovirus induced tumors in animal models. These studies led to the realization that oncogenes are abnormal forms of genes that have important functions in regulating cell growth. In subsequent studies, these newly identified oncogenes were introduced into normal cells in an effort to reproduce tumorigenesis *in vitro*. It was found that at least two oncogenes acting cooperatively needs to be present to give rise to cells with the fully transformed phenotype. This observation provides important insights into tumorigenesis. The multistep nature of tumorigenesis can be viewed as mutations in different genes with each event providing a selective growth advantage. Oncogenes work cooperatively to change the regulation of cell growth at multiple levels.

Most studies showing an association between HPV and breast cancer have relied on polymerase chain reaction (PCR) as a method of detection HPV, as shown in table 1. Trying to find clinical and pathological features associated with HPV related breast cancer has been difficult and published research has shown inconsistent results. These inconsistencies could be associated with the relatively small number of cases examined in each study, or from the nature of the PCR test itself. Namely, PCR requires the digestion of the tumor tissue before DNA extraction. This can lead to contamination (from positive controls) and the incorporation of other tissue besides mammary epithelial cells. There may be presence of endothelial cells, leukocytes, fibroblast cells, or adipocytes. In Situ methods of HPV identification has the ability to localize the response signal and is resistant to contamination. We elected to have our samples evaluated at ARUP Laboratories. The In Situ hybridization method used at ARUP laboratories uses formalin fixed tissue sections on glass slides with a labeled DNA probe of specific DNA sequence (common to a subtype of HPV) binding to the tissue and evaluated through microscopic evaluation.

At the 2006 European Research Organization on Genital Infection and Neoplasia a subgroup was asked to report on how to apply molecular biomarkers to identify HPV oncogene expression through direct detection of viral mRNA transcripts or through detection of the cellular proteins such as p16.

The group concluded that four main areas were being followed to identify appropriate markers: (1) detection or measurement of proteins E6 and E7 HPV mRNA transcripts, (2) alterations of the methylation pattern of genes, (3) chromosomal gains and losses, and (4) detection of cellular proteins that are over expressed by HPV-infected cells like p16.

Several properties of p16 make this protein a promising biomarker for identification of HPV related cancers. The expression is directly linked to the HPV oncogene action because continuous expression of protein E7 is necessary to maintain a cancerous phenotype in HPV cancers. The expression of p16 seems to be independent of the HPV subtype causing the infection. Also, p16 is not found expressed in normal basal cells or in other cells with proliferative capacity.

Three categories have been used to describe p16 staining: no staining, focal staining of single cells (HPV independent), and diffuse staining (HPV associated lesion). Focal staining is defined as noncontinuous staining of isolated cells or small cell clusters. Diffuse staining is defined as a continuous staining of cells in the basal and parabasal layers. The focal staining pattern is considered negative for HPV associated cancer.

p16 is a cyclin dependent kinase inhibitor that inhibits Rb phosphorylation and blocks cell cycle progression at the G1 to S check point. The loss of p16 expression by deletion, mutation, or hypermethylation is common in head and neck squamous cell carcinomas. Functional inactivation of Rb by HPV protein E7 results in over expression of p16, which makes it a surrogate marker for HPV. Similarly, HPV protein E6 can inactivate p53; thus, there is a lower incidence of *p53 mutations* in HPV positive tumors.

Chapter 3: Research Design and Methodology

Sample Selection

The target population for this study consisted of triple negative breast cancer patients that had permanently fixed embedded tissues of established breast cancer. Paraffin embedded tissue had to be collected between January 1, 2005 and December 31, 2010 and located in the Mercy Saint Vincent's Tissue Repository.

Human Subjects Protection

Prior to initiation this research, the Investigators submitted an application under the Exempt Research status examining pathologic samples previously collected to the Mercy Saint Vincent Medical Center Adult Institutional Review Board (MSVMCAIRB) for review and approval. The MSVMCAIRB approved the study for initiation April 21, 2011. An amendment to the submission was approved September 1, 2011 regarding the intention to do further testing with polymerase chain reaction (PCR) or In Situ Hybridization (ISH) due to preliminary results showing expression of P16 but failing to show HPV through Immunohistochemical staining. (IRB 0211201 Appendix B)

The Investigators also submitted a Request for Approval of Research Involving Human Subjects to the Eastern Michigan University College of Health and Human Services Review Committee (HSRC) for review and approval. The HSRC approved the study for initiation on November 9, 2011 (MS#1050 Appendix A).

A waiver of informed consent was approved by both IRBs.

Data Collection

All cases meeting the inclusion criteria were identified by assigning a serial number used throughout this research. The only subject information collected were patient's age at diagnosis and year of diagnosis. Results were recorded as positive or negative for all samples screened for HPV using Immune Histochemistry and In Situ Hybridization. Intensity of p16 expression was determined by Immune Histochemistry and was recorded.

Methods:

Permanently fixed, paraffin embedded tissues of forty established breast cancer patients in the Mercy Health Partner's Pathology Tissue Repository were analyzed. The samples were de-identified of personnel health information prior to analysis and the only clinical characteristic recorded as age of the patient.

The inclusion criteria for this study were:

- Subjects had to have a diagnosis of triple negative breast cancer
- Diagnosed between 1/1/2005 and 12/31/2010
- Have adequate pathological sample for testing available
- And, reside in the Mercy Health Partner's Pathology Tissue Repository at St. Vincent department of pathology or Mercy St Charles Hospital department of pathology.

All cases meeting inclusion criteria were identified (by pathology sample number) and assigned a serial number used throughout the trial. All subject identifiers were deleted.

The second phase of the study was screening for p16.

p16 Antigen Immunohistochemistry

Immunohistochemistry for p16-expression was performed according to the manufacturer's recommendations as provided by CINtec® Histology kit. The CINtec® Histology Kit is an immunohistochemistry quantitative assay for the detection of the p16 antigen on paraffin embedded formalin fixed tissue sections.

The P16 antigen kit was designed to perform a two step immunohistochemical staining procedure for formalin fixed, paraffin embedded cervical tissue biopsy specimens. A primary monoclonal mouse antibody clone E6H4 was directed to human p16 protein for the detection of the P16 antigen. Positive and negative controls were used to confirm assay.

Interpretation of p16 Antigen Results

Interpretation of the results was performed by Stephen L. Strobel MD, Mercy Laboratories Consulting Pathologist.

A negative rating is assigned if the breast tissue specimen shown either a negative staining reaction in the tumor nuclear or cytoplasmic regions. A positive rating was assigned if the P16 stained slides shown a strong nuclear and cytoplasmic staining. p16 positive staining was limited to the tumor cells.

Histograms were generated in which the percentage of staining intensity was determined for each of four grades (0 = no staining, 1 = mild, 2 = moderate, 3 = intense) within each core. Both nuclear and cytoplasmic staining was considered. The original staining data was then transformed into cumulative percents. For this, 1 + is the percent of cells with mild or greater staining, 2 + is the percent of cells with moderate or intense staining, and 3 + is the percent of cells with intense staining. The percentage of tumor cell staining for p16 was also recorded

Human Papillomavirus Screening by In Situ Hybridization

All samples were screened for HPV DNA using the HPV *In Situ* Hybridization (ISH) process at ARUP Laboratories, ARUP Laboratories is a national clinical and anatomic pathology reference laboratory and an enterprise of the University of Utah and its Department of Pathology.

10% neutral buffered formalin fixed, paraffin embedded triple negative breast tumor tissue were sent to ARUP Laboratories for 200899 testing. At ARUP, 4-mm sections of paraffin embedded breast tumor tissue were placed on positive charged glass slides and then processed to remove the paraffin wax from the tissue, subjected to protease digestion, and then hybridized with a probe cocktail that detects high-risk HPV types (HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66).

Chapter 4: Presentation of Analysis of Data

Study Population

The individual characteristics of the study population and their breast cancer are summarized in Table 3. Breast cancer tissue from 40 patients who underwent surgery for triple negative breast was obtained between January 1, 2007 and December 31, 2010 at Mercy Saint Vincent or Mercy Saint Charles Hospitals in Toledo Ohio.

Table 4 summarizes the patient tumor characteristics. The mean patient age was 57.3 years of age with a range of 38 to 92 years. (7 patients from 2007; 16 from 2008; 10 from 2009 and 7 from 2010). All patients had a histology type of invasive ductal carcinoma.

p16 Positive Detection

Thirty-five of the 40 specimens were positive for the p16 antigen. Five had an intensity score of 0 (no p16 antigen detected), 1 had an intensity of 1+, 11 had an intensity of 2 and 23 had an intensity of 3+, Summarized in table 4.

The intensity of p16 staining and the percentage of cells that are positive were recorded; also the product of the two variables was tabulated and used as a marker of "degree of P16 positivity" for the studied tumors.

p16 Negative Detection

Five out of 40 specimens were negative for the p16 antigen.

Human Papillomavirus Detection

All specimens were determined negative for high risk HPV through testing by ISH at the ARUP Laboratories. However, ARUP warns that a negative result does not rule out the presence of an HPV genotype absent from the test, a low level infection, or specimen sampling error.

There is question about the sensitivity of this test for low level of infection, which is feared to be the case in breast cancer.

Chapter 5: Summary, Conclusion and Inferences

In this study, we were able to identify forty specimens with triple negative breast cancer, diagnosed between 2005 and 2010 in the Mercy health system, north region.
When tested for p16 by IHC, about 87% were positive, mostly with intense, diffuse staining, strongly suggesting the possibility of HPV as a causative mechanism for these tumors.
The intensity of p16 staining correlated with younger age, mimicking the demographic distribution of other HPV infections in the community.

However, trying to further prove the presence of HPV in the breast cancer cells proved to be more challenging, the IHC staining for E6 and E7 failed due to methodological issues. Testing the tumors for HPV by ISH was negative across the board, suggesting that either the ISH test is not sensitive enough to detect the low level of HPV expression typically expected in breast cancer, or that other viruses or unknown factors are causing the p16 over expression. This should be the focus of future studies.

Most of previous studies have relied on PCR to detect the presence of HPV in tumors, commercial testing is not very well validated in fixed, paraffin embedded tissues, especially in the breast, and PCR is generally very expensive. Confirmatory PCR testing of the study samples will be very helpful, and will hopefully be done in the near future.

If HPV positivity is confirmed, then it will be very helpful to know the HPV status for future triple negative breast cancer patients, this could be done inexpensively through testing p16 by IHC. We hope this will fuel future preventive and therapeutic trials. If HPV is confirmed to be a causative agent in breast carcinogenesis Gardasil, which vaccinates against high risk HPV,

could work for preventing breast cancer. Epidemiological studies showing a lower incidence of breast cancer in Gardasil vaccinated individuals would be needed.

Table 1: Summary of Human Papillomavirus and Breast Cancer Research

Author(s)	Year	Subjects Ethnicity	Tissue type	number patients	% HPV positive	PCR	ISH
Di Lonardo et al[1]	1992	Italy	Paraffin embedded tissue	17	29.41		
Bratthauer et al[2]	1992	USA		13	0	PCR/SOUTHERN BLOT	
Wrede et al[3]	1992	UK		80	0	X	
Czerwenka et al[4]	1996	Austria		20	0		
Gopalkrishna et al[5]	1996	India	Fresh	30	0		
Yu et al[6]	1999	China	Paraffin embedded tissue	72	41.67	PCR/SOUTHERN BLOT	
Yu et al[6]	1999	Japan	Paraffin embedded tissue		11.11		
Hennig et al[7]	1999	Norway	Paraffin embedded tissue	41	46.34		
Liu et al[8]	2001	USA	Fresh tissue	17	35.29		
Li et al[9]	2002	China	Paraffin embedded tissue	28	23.17	PCR/S0UTHERN BLOT	
Damin et al[10]	2004	Brazil	Paraffin embedded tissue	101	24.75	x	
Widschwendter et al[11]	2004	Austria	Fixed	11	63.64		
Tsai et al[12]	2005	Taiwan	Fresh tissue	69	12.9	X	
de Villiers et al[13]	2005	Germany	Paraffin embedded tissue	29	86.21	X	
Kan et al[14]	2005	Australia	Paraffin embedded tissue	50	48	X	
Kroupis et al[15]	2006	Greece	Frozen tissue	107	15.9	X	
Gumus et al[16]	2006	Turkey	Fresh tissue	50	74	X	
Choi et al[17]	2007	Korea	Paraffin embedded tissue	123	6.5	X	
Lindel et al[18]	2007	Sweden	Paraffin embedded tissue	81	0	X	
Grenier et al[19]	2007	France		27	14	X	
Mendizabal-Ruiz et al[20]	2008	Mexico	Paraffin embedded tissue	67	4.47	X	
Khan et al[21]	2008	Japan	Paraffin embedded tissue	124	21	X	
de Cremoux et al[22]	2008	France	Fresh tissue	50	0	X	
Akil et al[23]	2008	Syria	Paraffin embedded tissue	113	61.06	X	
de Leo'n et al[24]	2009	Mexico	Paraffin embedded tissue	51	29.41	X	
Heng et al[25]	2009	Australia	Paraffin embedded tissue	26	8	X	
Lawson et al[26]	2009	UK	Formalin Fixed	21	38.01	X	
Hachana et al[27]	2009	Tunisia	Paraffin embedded tissue	123	0	X	X
Hedau et al[28]	2011	India	Fresh tissue/Blood	252	0	conventianalPCR and Real time PCR	
Aguayo et al[29]	2011	Chili	Paraffin embedded tissue	46	8.7		
Antonsson et al[30]	2011	Australia	Fresh frozen	54	50	X	
Baltzell et al[31]	2011	USA	Paraffin embedded tissue	70	8.57	X (2+)	X X(4+)

Table 2: Human Papillomavirus Association with Several Cancers

	Cervical Cancer	Head and Neck Cancer	Breast Cancer
HPV Positive	>95%	25% Mouth 35% Throat	4.5-86.21%
Major HPV Types	High Risk 16, 18	High Risk 16, 18, 31	High Risk 16 and 18
Koilocyte Positive	Approximately 40%	Approximately 40%	Approximately 30%
HPV E6 protein inhibits	HPV E6 Inhibits	HPV E6 Inhibits	HPV E6 Inhibits
p53 Apoptosis	Apoptosis via p53	Apoptosis via p53	Apoptosis via p53

Table 3: Individual Patient and Tumor Characteristics

				Receptors			P16			HPV (ISH)	
Case	Year	Age	Histology	ER	PR	HER2	Intensity 0-3+	Area %	Product (intensity & area)	16	18
1	2007	74	DC	Neg	Neg	Neg	2+	50	1	Neg	Neg
2	2007	65	DC	Neg	Neg	Neg	3+	>90	2.7	Neg	Neg
3	2007	53	DC	Neg	Neg	Neg	3	>90	2.7	Neg	Neg
4	2007	61	DC	Neg	Neg	Neg	3	80	2.4	Neg	Neg
5	2007	55	DC	Neg	Neg	Neg	1-2	50	1	Neg	Neg
6	2007	56	DC	Neg	Neg	Neg	3	>90	2.7	Neg	Neg
7	2007	92	DC	Neg	Neg	Neg	0	-	0	Neg	Neg
8	2008	57	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg
9	2008	54	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg
10	2008	62	DC	Neg	Neg	Neg	2	75	1.5	Neg	Neg
11	2008	44	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg
12	2008	52	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg
13	2008	59	DC	Neg	Neg	Neg	1	25	0.5	Neg	Neg
14	2008	52	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg
15	2008	38	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg
16	2008	79	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg

Table 3: Individual Patient and Tumor Characteristics (Continued)

				Receptors			P16			HPV (ISH)	
Case	Year	Age	Histology	ER	PR	HER2	Intensity 0-3+	Area %	Product (intensity & area)	16	18
17	2008	47	DC	Neg	Neg	Neg	2-3	90	2.7	Neg	Neg
18	2008	39	DC	Neg	Neg	Neg	2	90	1.8	Neg	Neg
19	2008	54	DC	Neg	Neg	Neg	2	75	1.5	Neg	Neg
20	2008	59	DC	Neg	Neg	Neg	2	Stroma + 80	1.6	Neg	Neg
21	2008	61	DC	Neg	Neg	Neg	2-3	80	2.4	Neg	Neg
22	2008	62	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg
23	2008	65	DC	Neg	Neg	Neg	0	Stroma only,0	0	Neg	Neg
24	2009	53	DC	Neg	Neg	Neg	2	50-60	1.2	Neg	Neg
25	2009	72	DC	Neg	Neg	Neg	2-3	60	1.8	Neg	Neg
26	2009	61	DC	Neg	Neg	Neg	0	Stroma only,0	0	Neg	Neg
27	2009	73	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg
28	2009	85	DC	Neg	Neg	Neg	1-2	30-40	0.8	Neg	Neg
29	2009	56	DC	Neg	Neg	Neg	2	50-60	1.2	Neg	Neg
30	2009	47	DC	Neg	Neg	Neg	3	90	2.7	Neg	Neg
31	2009	52	DC	Neg	Neg	Neg	2	50	1	Neg	Neg
32	2009	77	DC	Neg	Neg	Neg	1+ only DCIS	Invasive (-) 0, weak	0	Neg	Neg
33	2009	49	DC	Neg	Neg	Neg	3+	90	2.7	Neg	Neg

Table 3: Individual Patient and Tumor Characteristics (Continued)

				Receptors			P16			HPV Type	
Case	Year	Age	Histology	ER	PR	HER2	Intensity 0-3+	Area %	Product (intensity & area)	16	18
34	2010	51	DC	Neg	Neg	Neg	-	0	0	Neg	Neg
35	2010	55	DC	Neg	Neg	Neg	1-2+	20-30	0.6	Neg	Neg
36	2010	55	DC	Neg	Neg	Neg	3+	>90	2.7	Neg	Neg
37	2010	42	DC	Neg	Neg	Neg	3+	>90	2.7	Neg	Neg
38	2010	42	DC	Neg	Neg	Neg	2-3+	>90	2.7	Neg	Neg
39	2010	40	DC	Neg	Neg	Neg	3+	>90	2.7	Neg	Neg
40	2010	42	DC	Neg	Neg	Neg	3+	>90	2.7	Neg	Neg

Table 4: Summary of Patient Tumor Characteristics

Patient/ Tumor data	All Subjects Number	%	P16 positive Number	%	P16 Negative Number	%
Number of patients	40	100	35	87.5	5	12.5
Age of Patients	57.3		55.6		64.2	
range	38-92		38-85		51-92	
Histology						
Ductal Carcinoma	40		35		5	
Tumor Receptors						
ER negative	40	100	35	87.5	5	12.5
PR negative	40	100	35	87.5	5	12.5
HER2 negative	40	100	35	87.5	5	12.5
P16 Antigen Distribution						
0			-		5	12.5
1+			1	2.5		
2+			11	27.5		
3+			23	57.5		
Intensity			77.29		0	
range			25-90		0	
HPV infection (ISH)						
Negative			na	na	40	100
Positive			0	0	na	na
Type 16			-		40	100
Type 18			--		40	100

Table 5: Average p16 Expression by Age Group

Age Range	Product (intensity & Area)	Average Area (%)
30-39	2.25	90.0
40-49	2.70	90.0
50-59	1.72	66.3
60-69	1.67	59.3
70-79	1.64	58.0
80-89	0.80	40.0
90-99	0.00	0.0

References

1) Di Lonardo A, Venuti A, Marcante ML: Human papillomavirus in breast cancer. Breast Cancer Res Treat 1992, 21:95-100.

2) Bratthauer GL, Tavassoli FA, O'Leary TJ: Etiology of breast carcinoma: no apparent role for papillomavirus types 6/11/16/18. Pathol Res Pract 1992,188(3):384-6.

3) Wrede D, Luqmani YA, Coombes RC, Vousden KH: Absence of HPV16 and 18 DNA in breast cancer. Breast Cancer Research 1992, 65(6):891-4.

4) Czerwenka K, Heuss F, Hosmann JW, Manavi M, Lu Y, Jelincic D, Kubista E: Human papillomavirus DNA: a factor in the pathogenesis of mammary Paget's disease? Breast Cancer Res Treat 1996, 41:51-57. Hedau et al. BMC Cancer 2011, 11:27.

5) Gopalkrishna V, Singh UR, Sodhani P, Sharma JK, Hedau ST, Mandal AK, Das BC: Absence of human papillomavirus DNA in breast cancer as revealed by polymerase chain reaction. Breast Cancer Res Treat 1996, 39(2):197-202.

6) Yu Y, Morimoto T, Sasa M, Okazaki K, Harada Y, Fujiwara T, Irie Y, Takahashi E, Tanigami A, Izumi K: HPV33 DNA in premalignant and malignant breast lesions in Chinese and Japanese populations. Anticancer Res 1999, 19(6B):5057-5061.

7) Hennig EM, Suo Z, Thoresen S, Holm R, Kvinnsland S, Nesland JM (1999) Human papillomavirus 16 in breast cancer of women treated for high grade cervical intraepithelial neoplasia (CIN III). Breast Cancer Res Treat 53:121–135.

8) Liu Y, Klimberg VS, Andrews NR, Hicks CR, Peng H, Chiriva-Internati M, Henry-Tollman R, Hermonat PL: Human papillomavirus DNA is present in a subset of unselected breascancers. J Hum Virol 2001, 4(6):329-334.

9) Li T, Lu ZM, Guo M, Wu QJ, Chen KN, Xing HP, Mei Q, Ke Y (2002) p53 codon 72 polymorphism (C/G) and the risk of human papillomavirus-associated carcinomas in China. Cancer 95: 2571–2576.

10) Damin AP, Karam R, Zettler CG, Caleffi M, Alexandre CO: Evidence for an association of human papillomavirus and breast carcinomas. Breast Cancer Res Treat 2004, 84(2):131-137.

11) Widschwendter A, Brunhuber T, Wiedemair A, Mueller-Holzner E, Marth C: Detection of human papillomavirus DNA in breast cancer of patients with cervical cancer history. J Clin Virol 2004, 31(4):292-297.

12) Tsai JH, Tsai CH, Cheng MH, Lin SJ, Xu FL, Yang CC (2005) Association of viral factors with non-familial breast cancer in Taiwan by comparison with non-cancerous, fibroadenoma, and thyroid tumor tissues. J Med Virol 75:276–281.

13) de Villers EM, Sandstrom R, Hausen H, Buck, C: Presence of papillomavirus sequences in condylomatous lesions of te mamillae and invasive carcinoma of the breast. Breast Cancer Reses 2005, 7☹1)R1-R11.

14) Kan CY, Iacopetta BJ, Lawson JS, Whitaker NJ: Identification of human papillomavirus DNA gene sequences in human breast cancer. Br J Cancer 2005, 93(8):946-948.

15) Kroupis C, Markou A, Vourlidis N, Dionvssiou-Asteriou A, Lianidou ES: Presence of high-risk human papillomavirus sequences in breast cancer tissues and association with histopathological characteristics. Clin Biochem 2006, 39:727-731.

16) Gumus M, Yumuk PF, Salepci T, Aliustaoglu M, Dane F, Ekenel M, Basaran G, Kaya H, Barisik N, Turhal NS: HPV DNA frequency and subset analysis in human breast cancer patients normal and tumoral tissue samples. J Exp Clin Cancer Res 2006, 25(4):515-521.

17) Choi YL, Cho EY, Kim JH et al (2007) Detection of human papillomavirus DNA by DNA chip in breast carcinomas of Korean women. Tumour Biol 28:327–332.

18) Lindel K, Forster A, Altermatt HJ, Greiner R, Gruber G: Breast cancer and human papillomavirus (HPV) infection: no evidence of a viral etiology in a group of Swiss women. Breast 2007, 16:172-177.

19) Grenier J, Soria JC, Mathieu MC et al: Differential immunohistochemical and biological profile of squamous cell carcinoma of the breast. Anticancer Res.2007 27:547-555.

20) Mendizabal-Ruiz AP, Morales JA, Ramirez-Jirano LJ, Padilla-Rosas M, Moran- Moguel MC, Montoya-Fuentes H: Low frequency of human papillomavirus DNA in breast cancer tissue. Breast Cancer Res Treat 2009, 114:189-194.

21) Khan NA, Castillo A, Koriyama C, Kijima Y, Umekita Y, Ohi Y, Higashi M, Sagara Y, Yoshinaka H, Tsuji T, Natsugoe S, Douchi T, Eizuru Y, Akiba S: Human papillomavirus detected in female breast carcinomas in Japan. Br J Cancer 2008, 99(3):408-414.

22) de Cremoux P, Thioux M, Lebigot I, Sigal-Zafrani B, Salmon R, Sastre- Garau X: No evidence of human papillomavirus DNA sequences in invasive breast carcinoma. Breast Cancer Res Treat 2008, 109:55-58.

23) Akil N, Yasmeen A, Kassab A et al: High-risk human papillomavirus infection in breast cancer in Syrian women and their association with Id-1 expression: a tissue microarray study. BR J Cancer 2008,99:404-407.

24) de Leo'n DC, Montiel DP, Nemcova J et al: Human papillomavirus (HPV) in breast tumors: prevalence in a group of Mexican patients. BMC 2009, 9:26.

25) Heng B, Glenn WK, Ye Y, Tran B, Delprado W, et al: Human papilloma virus is associated with breast cancer. BJC 2009, 101:1345-1350.

26) Lawson JS, Glenn WK, Heng B, Ye Y, et al: Kiolcytes indicates a role for human papilloma virus in breast cancer. BJC 2009, 101:1351-1356.

27) Hachana M, iadi S, Amara K et al: No evidence of human papillomavirus DNA in breast carcinoma in Tunisian patients. The Breast 2010,19:541-544.

28) Hedau S, Kumar U, Hussain, Shukla S: Breast cancer and human papillomavirus infection: No evidence of HPV etiology of breast cancer in Indian women. BMC Cancer 2011, 11:27-6.

29) Auguayo F, Khan N, Koriyama C, et al: Human papillomavirus and Epstein-Barr virus infection in breast cancer from chili. IAC 2011, 6:7-13.

30) Antonsson A, Spurr T, Chen A, et al: High prevalence of human papillomaviruses in fresh frozen breast samples. J Med Virol 2011, 83:2157-2163.

31) Baltzell K; Buehring GC, Krishnamurthy S, Kuerer, H, Shen, HM, Sison J: Limited evidence of human papillomavirus on breast tissue using molecular in situ methods. Cancer. 2011 Aug 5;