DNA METHYLATION AND THE IMMUNE SYSTEM IN BREAST CANCER

by

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ABSTRACT

Breast cancer is the cancer with the highest incidence in women and the most commonly diagnosed. It is estimated that 268,000 cases are diagnosed each year in the U.S. of which about 12.8% of women are affected during their lifetime. Many of these patients tend to have high rates of recurrence, high rates of distant metastasis and a poor survival rate across the various types of breast cancer. Hence, there is a need for improved and rapid diagnosis of breast cancer and more targeted treatment methods.

The tumor microenvironment is associated with cancer cell proliferation, survival and metastasis. It includes a variety of cells including immune cells, inflammatory cells and tumor cells. Recent cancer studies on the interactions between the immune system and tumors has led to speculation that the tumor microenvironment plays a role in tumor recurrence and therapeutic resistance. Tumor growth and progression have been associated with immunosuppressive changes in immune cells, specifically in tumor infiltrating and peripheral blood mononuclear cells (PBMC's). There is a lack of consensus regarding how the tumor microenvironment affects the immune system with regard to impairing the anti-tumor immune response. Of the many epigenetic mechanisms, DNA methylation has been shown to regulate various immune responses. Therefore, the hypothesis was DNA methylation plays a role in the shift of immune cells from an anti-tumor response to an immunosuppressive response. To test this hypothesis, I investigated the methylation patterns of inflammatory genes in peripheral blood mononuclear cells of women with or without breast cancer.

A panel of genes of inflammatory and immune genes were screened using blood samples from normal patients and patients with invasive breast cancer. To further

investigate methylation changes in specific CpG sites in these genes, promoter and enhancer regions were analyzed by methylation specific restriction enzyme PCR and restriction enzymes MSPI and HPAII. Five genes were found to have (TNF-alpha, TYK2, IL-4R, IL17RA and IL-17C) significant differences in methylation level in patients with invasive cancer compared to normal patients.

Our data supports the hypothesis that altered DNA methylation in immune and inflammatory genes occurs in invasive disease. Epigenetic changes in these genes may play a role in the shift of immune cells from an anti-tumor response to an immunosuppressive response. Further study is needed to determine if these changes in methylation load have an impact on anti-tumor immune responses.

Chapter 1

INTRODUCTION

1.1 Breast Cancer

Over the last few decades, many developments have been made in improving the screening and treatment of breast cancer. However, it is still the most frequently diagnosed and chief cause of cancer death in women (6). In the United States of America, it is the most commonly diagnosed cancer in women with an estimated 276,480 cases of invasive breast cancer diagnosed annually in approximately 12.8% of women (1). It is also the second most deadly cancer in American women with 42,170 cases predicted to result in death. However, the overall survival rate has improved due to the knowledge of risk factors and recommendations for screening resulting in early detection and advancements in treatment. In 2020, the number of women diagnosed was 81% and the 5-year survival rate for women with this disease was reported at 91%. (2) However, breast cancer has distinct clinical behaviors and therapeutic responses due to its heterogeneity. Therefore, treatment and survival vary significantly depending on the type of breast cancer.

Breast cancer is traditionally classified based on tumor size, grade, histology, regional lymph node infiltration, and protein marker expression through molecular profiling. Immunohistochemical research of protein markers is also another categorization for receptor groups which are progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor receptor 2 (Her2). Triple negative breast cancer (TNBC) is a type of breast cancer characterized by the lack of expression of

ER/PR and HER2. (3) Molecular profiling helps provide a more specific classification method.

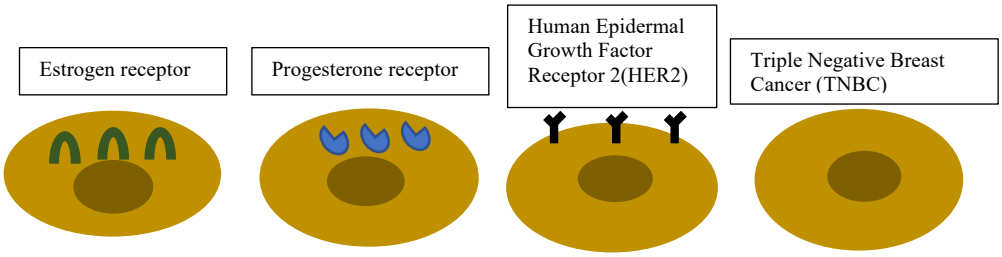

Figure 1. Classification of breast cancer. Estrogen receptor positive, progesterone receptor positive, human epidermal growth factor receptor (Her2) and Triple negative breast cancer (TNBC).

Breast cancer is a heterogenous disease which can be classified into luminal (hormone-receptor positive), human epidermal growth factor 2(Her2) and triple negative (TN) based on their native differences in transcriptional expressions or hormone and growth factor receptors. The lack of effective targeted treatment further complicates the diagnosis and therapeutics (4). The risk factors for breast cancer are lifestyle changes associated with diet, age, ethnic differences, obesity, and genetic mutations, among others. The mortality rates of breast cancer are declining especially in developed countries due to early detection and treatment modalities. However, there is a lack of good treatment for late stage and metastatic disease (2).

1.1.2 Tumor microenvironment

The tumor microenvironment is important in immune activation and response to treatment. In breast cancer, it can either be anti-tumorigenic or pro-tumorigenic

depending on the types of immune cells present and their interaction with the tumor cells. Immune cells in the tumor microenvironment may include mast cells, neutrophils, macrophages, dendritic cells, monocytes, natural killer cells and lymphocytes as shown in Figure 2. A summary of cells in the tumor microenvironment is described in Figure 2.

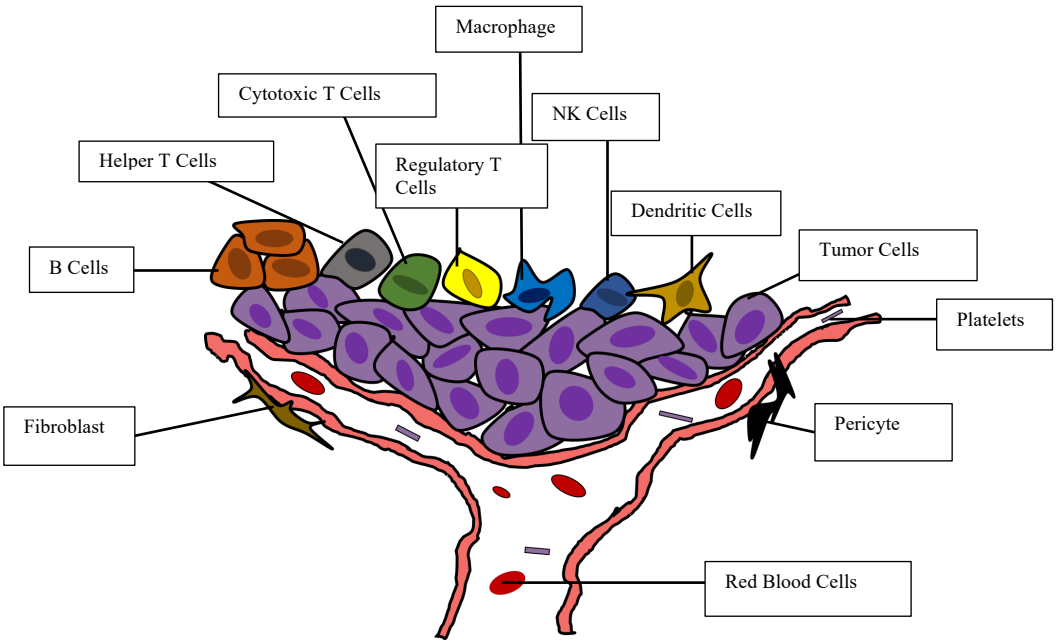

Figure 2. The tumor microenvironment. Immune cells in the tumor microenvironment include regulatory T cells, cytotoxic T cells, helper T cells, B cells, dendritic cells, natural killer cells, macrophages and tumor cells. There are also additional parts like fibroblasts and pericytes, among others.

The immune cells in the tumor microenvironment may drive the growth of the tumor or regress it, which may result in positive or negative outcomes. Plasmacytoid dendritic cells secrete high levels of interferons which has been associated with poor prognosis (2). The plasmacytoid dendritic cells have also been found to expand regulatory T cell (T-reg) population, which is a positive sign for immunosuppression (15). Another immune cell, natural killer cell attacks tumor cells without antigenic

stimulation and has been associated with positive clinical characteristics and good survival in patients with Triple negative breast cancer (15). Immune cells in breast cancer phenotypes, specifically ER positive breast tumors, have shown a high proportion of natural killer cells (NK) and neutrophils but lower proportions of cytotoxic T cells (CD8+) and Helper T cells (CD4+). Cytotoxic T lymphocytes express the transmembrane glycoprotein CD8 and are associated with positive clinical outcome and treatment response in breast cancer (19). Cytotoxic T cell (CD8+) have the ability to attack and kill cancer cells. Helper T lymphocytes express the transmembrane glycoprotein CD4 and are composed of a variety of subsets (50). T helper 1 cells (Th1) mainly express interferon gamma (IFNγ) and T helper 2 cells (Th2) mainly express interleukin 4(IL-4). Cell mediated immunity is also activated by T-helper 1 cells with humoral immunity activated by T helper 2 cells (19). Another group of T helper cell are FOXP3+ T-regs which are associated with immune suppression and poor overall survival in breast cancer. In ER negative breast tumors, the CD8+ T cells often make up a lot of the tumor infiltrating lymphocytes (15). There is also a list of immune cells; T regs, TAM2 and activated mast cells which are all linked to a negative prognosis (19). In Her2 positive breast cancer, a high proportion of dendritic cells, mast cells, T-lymphocytes and neutrophils have been linked to poor prognosis, disease relapse and metastasis (50).

Recent cancer studies with PBMC's have focused on gene expression and circulating free DNA in efforts to create a panel of biomarker profiles for treatment. PBMCs are a very important tool for researchers and clinicians in delineating, testing immune responses, and finding treatments and cures for diseases like cancer. (13)

PBMC's can also provide an imprint of the tumor microenvironment which may present a non-invasive method to understand tumor induced immune changes.

1.1.3 Inflammation

Inflammation is the body's response to tissue damage and plays a role in tumorigenesis. Immune cells produce an inflammatory response when they encounter cancer cells in the body causing them to differentiate through various signaling pathways i.e. NFkB pathway (26). During an inflammatory response, immune cells such as neutrophils, mast cells, macrophages, T and B lymphocytes and natural killer cells collect in the tumor microenvironment (27). Most of the documented data is on the role of T-lymphocytes and antitumor immunity in breast cancer. Tumor-infiltrating lymphocytes are linked to breast cancer through activated T helper 1 cells secreting TNF-alpha, IFN-gamma, IL-2, TGF-beta and activated T-helper 2 cells secreting IL-13, IL-10, IL-6, IL-4 and IL-5 inflammatory cytokines (28). Chronic inflammation contributes to cancer development though various mechanisms such as DNA methylation. Studies have shown that chronic inflammation increases DNA methylation during tumor growth (18). Chronic inflammation can create an immunosuppressive microenvironment characterized by proinflammatory mediators, immune checkpoint pathway activation in effector T cells and infiltration of immune suppressor cells that lead to tumor growth and progression (36). Immunosuppression contributes to cancer growth. The methylation status of particular genes identified during this process can be used as potential biomarkers. However, in order for this to be implemented, studies need to be conducted to scrutinize DNA methylation patterns and their effects on gene expression and inflammation.

1.1.4 Tumor Immunoediting

Cancer immunoediting is the process by which the immune system can promote tumor growth and development through three phases: Elimination, equilibrium and escape (37). In these phases, as shown in Figure 3, the tumor immunogenicity is edited and immunosuppressive mechanisms for disease progression are acquired. Most cancers are able to evade detection by the immune system after they undergo immunoediting. Elimination involves the detection and destruction of damaged cancer cells. The elimination phase has innate and adaptive immunity mechanisms working together to destroy tumor cells. Immune cells in this phase include T cells, Natural killer cells and dendritic cells. If the tumor cells are not destroyed in the elimination phase, they enter the equilibrium phase where outgrowth is prevented by immune mechanisms like T-cells, IFN- γ and IL12. The goal is to keep tumor cells in a state of functional dormancy. However, unique tumor variants that emerge can no longer be detected by immune cells or may proliferate at such a high rate the immune system cannot keep tumor growth in check. These tumor cells move into the escape phase where they can no longer be blocked by immunity. Adaptive immunity is used to constrain the growth of clinically undetectable tumor cells and edit their immunogenicity. Tumor cells that are able to break the dormancy of this phase move to the escape stage where edited tumors of low immunogenicity grow unrestrained leading to an immunosuppressive tumor microenvironment. The editing of the tumor cells allows them to exploit immune checkpoints like PDL-1/PD-1, CTLA-4, TIM3, LAG-3 and TIGIT for survival. Immunotherapy involves the use of immune-checkpoint inhibitors to relieve tumor-induced immunosuppression. High expression of immune checkpoints in tumor

microenvironment plays significant role in inhibiting anti-tumor immunity, which is associated with poor prognosis and cancer progression. Generally, escape from immune control is important as one of the hallmarks of cancer (38) and is associated with cancer progression and poor prognosis.

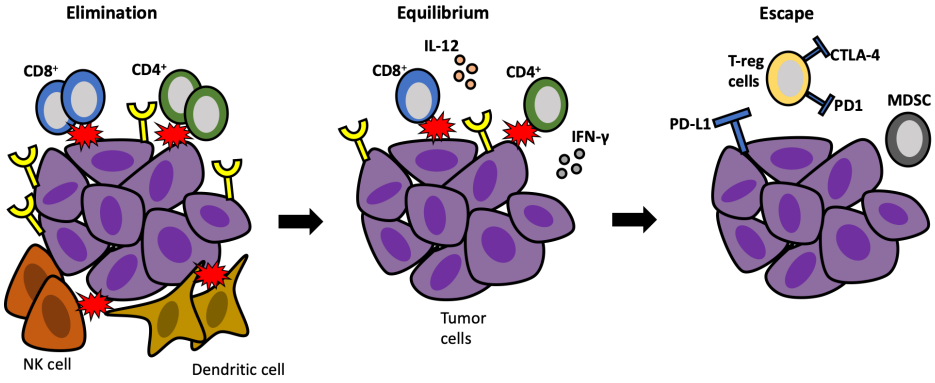

Figure 3: Cancer Immunoediting. This is the process by which the immune system can promote tumor growth and development through three phases: Elimination, equilibrium and escape (37). In these phases, the tumor immunogenicity is edited and immunosuppressive mechanisms for disease progression are acquired.

1.1.5 Epigenetics

Epigenetics, as described by Waddington in the 1940's, is a branch of biology which investigates the interactions between genes and their products which bring the phenotype into being (83). Over the years, research in epigenetics has evolved and is now used to regulate gene expression without altering the DNA sequence (83). The human body has over two hundred different cells types which contain almost the same genomic sequence. Epigenetic regulation is therefore one of the most important regulatory events in gene expression. The important epigenetic regulations within a cell are RNA modifications, histone modifications and the most common, DNA methylation. The

correlation between these epigenetic modifications them to act like a complex machinery with composers, proofreaders and eliminators. The effector proteins act as composers or writers that modify DNA. The proofreaders identify these changes leading to transcriptional activation or repression of the modified regions. The eliminators play the last role of removing these modifications in the DNA and alter transcription (84).

1.1.6 CpG Islands

Changes in the immune system during immunoediting may be regulated by methylation of CpG islands, which are regulatory regions located near the promoters of housekeeping genes throughout the genome (3). CpG islands are stretches of DNA roughly 500 base pairs with a higher frequency of cytosine and guanine residues than the rest of the genome linked (5). A majority of gene promoters reside within CpG Islands and are most often not methylated (32). CpG sites together form CpG islands and their location and preservation are critical in understanding and identifying the epigenetic effects occurring in DNA. Methylation of CpG islands can result in the silencing of gene expression (33). In early embryonic embryonic development, CpG islands undergo differential methylation (34). The methylation of CpG islands and control of gene expression can explain the display of tissue-specific patterns of DNA methylation. CpG islands located in intragenic and gene body regions can have tissue-specific patterns of methylation. However, CpG islands associated with transcription start sites rarely show tissue specific methylation patterns, instead, regions called CpG island shores located 2kb from CpG islands have highly conserved patterns of tissue-specific methylation and hence show reduced gene expression (35). The role of CpG islands in regulating gene

expression is still being studied. Both methylation patterns, hypermethylation, which leads to silencing of regulatory genes resulting in uncontrolled growth while hypomethylation leads to activation of genes required for unabated cellular replication (7). In normal breast cells, as shown in Figure 4, candidate tumor suppressor gene CTCF prevents DNA methylation modification. However, disrupting CTCF leads to methylation of tumor suppressor genes such as p53, BRCA1, BRCA2 results in metastasis and growth of tumors.

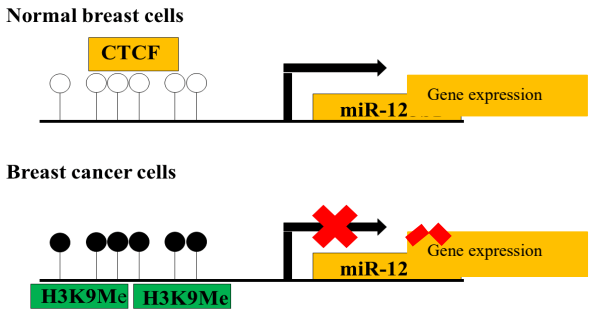

Figure 4: Lack of CTCF binding at the CpG island in human cancers. Normal breast cells have CTCF present which prevents the recruitment of epigenetic silencing components such as DNA methylation leading to expression. Breast cancer cells have a loss of CTCF which leads to methylation of the CpG island and repression of gene expression through histone H3k9Me modification.

1.2 DNA Methylation

DNA methylation is the earliest epigenetic alteration found in humans (6). It is catalyzed by a family of DNA methyltransferases that use S-adenosyl methionine (SAM) as the methyl donor and is dependent on both Ca2+ ion and a reducing environment (10). It was first discovered in 1948 by Rollin Hotchkiss while looking at a modified cytosine in a preparation of calf thymus using paper chromatography (24). DNA

methyltransferases (DNMT's) are a highly regulated family of cytosine methylases that are important for normal mammalian development and can lead to mutations by silencing tumor suppressor genes (8). DNMT 1, DNMT 3a and DNMT3b are examples of these DNA -modifying enzymes that play a role in epigenetic gene regulation and methylation patterns. (25) DNMT 1 is the most abundant and primarily binds to hemi methylated DNA (DNA with only one methylated strand). It is mainly responsible for maintaining methylation patterns during DNA replication (9,10). Unlike DNMT1, DNMT 3a and 3b enzymes are *de novo* DNA methyltransferases which have affinity for both hemi methylated and unmethylated DNA (10). They are important in early development, especially with methylation that occurs after embryo transplantation (10). The DNMT3 group also includes a catalytically inactive member, DNMTL which is also important for development (10).

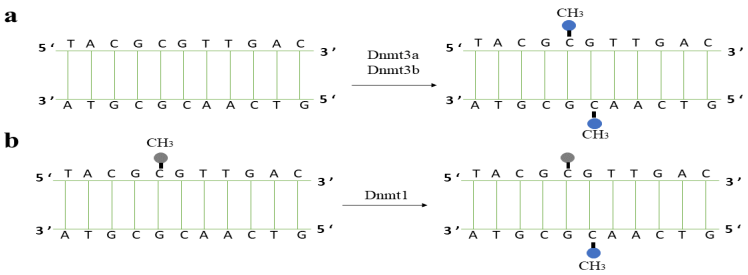

Figure 5: DNA methylation pathways. DNA methyltransferases (DNMT's) catalyze the transfer of a methyl group from S-adenosyl methionine (SAM) to the 5-carbon position of cytosine residue to form 5-methylcytosine(5mC). (a)DNMT3a and DNMT3b enzymes are *de novo* DNMT's that transfer methyl groups(blue) onto the naked DNA. (b) Dnmt1 enzyme is the most abundant DNMT and is responsible for maintaining methylation patterns during replication. During semiconservative replication, the original DNA methylation pattern is maintained by the parental DNA strand (grey)

DNA methylation can play an essential role in understanding the expression of inflammatory genes. During chronic inflammation, myeloid suppressor cells, CD4+ T cells and Regulatory T cells(T-regs) act in an immune suppressive function to repress CD8+ cytotoxic T cells and promote tumor growth. (9) The polarity of the immune cells during chronic inflammation promotes a proangiogenic and protumor microenvironment. Other recent studies have shown that epigenetic changes in inflammatory genes IFNG, IL4 and IL13 regulate T helper 1 and T helper 2 cells. (8) In human naïve T cells, the IFNG promoter is hypermethylated and becomes demethylated during the differentiation to T helper 1 cells. IL4 and IL13 are partially methylated in the promoter region specific to the T helper 2 cells with IL4 promoter being highly methylated in naïve and T helper 1 cells. Identification of DNA methylation levels of certain genes could be used as potential biomarkers for early diagnosis. In cancer, the use of epigenetics such as DNA methylation is well established for diagnosis and prognosis of tumors. (8) DNA methylation can be used in developing non-invasive biomarkers that allow for early detection.

1.3 DNA methylation in Peripheral blood mononuclear cells (PBMC's)

Recent cancer studies on the interactions between the immune system and tumors has led to speculation that the tumor microenvironment plays a role in tumor recurrence and therapeutic resistance. Immune cells, specifically peripheral blood mononuclear cells in the tumor microenvironment have been shown to be associated with progression of tumor growth. The tumor microenvironment is important in immune activation and

response to treatment (15). PBMC's have a single round nucleus with a heterogenous cell population consisting of lymphocytes, dendritic cells, and monocytes (12). They are important in surveillance for infectious threats with both innate and adaptive immune response functions (16). Therefore, PBMC's are a very important tool for researchers and clinicians in understanding the immune system, testing immune responses, and finding treatments and cures for diseases like cancer (13). Study of PBMC's may also be able to yield valuable information about tumor status. Identification of alterations occurring in lymphocytic subsets in tumor tissues and peripheral blood subsets from precancerous patients are currently providing ways to find clinicopathological and prognostic variables for cancer treatment (14). Epigenetic changes specifically DNA methylation are important prognostic biomarkers. In breast cancer, global methylation of PBMC's has shown their potential in providing disease specific epigenetic signatures needed for prognosis and treatment (16). Methylation changes and pattern identification can provide ways to greatly improve cancer treatment. Cancer tumor immunoediting is an important process whereby the immune system constrains and promotes tumor development is one of the effects of the tumor microenvironment (17). DNA methylation has also been shown to be strongly associated with PBMC response to Toll-like receptor ligands (TLR) (Akira et al). TLR3 agonist polyinosinic - polycytidylic acid (poly I:C), an artificial surrogate for double-stranded RNA (dsRNA) virus, has been used in simulating viral infections (19). Hence, DNA methylation study linked to the immune and inflammatory responses of PBMC's can contribute in identifying epigenetic markers for pathogenesis of viral diseases (16). The clinical potential of DNA methylation in cancer therapy is a

broad field for more research. Further studies may help us understand other roles of this epigenetic mechanism in cancer tumorigenesis.

1.4 Importance of DNA methylation

DNA methylation has been shown to be essential in cellular differentiation, embryonic development, genomic imprinting but most importantly mediation of gene expression. It is the most common epigenetic modification for gene expression in eukaryotes. Recent studies have shown that methylation near gene promoters vary considerably depending on cell type and occur at specific locations within the genome with more methylation showing low to no transcription levels (9). In diseases like cancer, errors in DNA methylation have devastating consequences (9). Cancer is the uncontrollable division and growth of cells caused by biological and internal factors leading to mutations that can start anywhere in the body (9). Over the years, researchers and clinicians have tried to understand the role of DNA methylation in carcinogenesis to improve cancer treatment. Two types of epigenetic abnormalities, Cancer-associated hypomethylation and Cancer linked-hypermethylation commonly affect DNA sequences (10). Genomic hypermethylation has been observed to occur mainly at CpG islands in promoter regions of the DNA with hypomethylation seen in other parts of the genome with repeated DNA sequences in cancer (11). The biological significance of DNA hypermethylation in carcinogenesis is due to the transcriptional silencing of genes important for prevention of cancer. However, an experiment involving DNA methylation inhibitors *in vivo* and *in vitro* with analysis of DNA methyltransferase-deficient mice showed the importance of DNA

induced hypomethylation in oncogenesis (11). An increased frequency of cancer-linked DNA hypomethylation in cancer can be correlated with karyotypic instability and activation of tumor-promoting genes by cis and trans effects that may favor oncogenesis. Therefore, understanding the relationship between DNA hypermethylation and hypomethylation is very important in understanding tumorigenesis and creating cancer therapies involving DNA methylation. While much focus has been on understanding the role of DNA methylation in tumor cells, a role for methylation changes in regulation of tumor immune responses has recently become realized.

Chapter 2

HYPOTHESIS AND AIMS

Our hypothesis for this study was DNA methylation plays a role in inducing a shift of immune cells from an anti-tumor response to an immunosuppressive response. To test this hypothesis, we investigated the methylation patterns of inflammatory genes in peripheral blood mononuclear cells of women with or without breast cancer. The aim was to investigate a preliminary screen of inflammatory genes, Peripheral Blood Mononuclear Cells (PBMCs) of women with or without breast cancer. For this aim, we used qPCR to investigate the patterns of methylation in model inflammatory genes from a preliminary screen and from literature (71,72). The working hypothesis was that there are differences in methylation patterns of immune genes in PBMCs from women with and without breast cancer. The completion of this aim will lead to a better understanding of the role of DNA methylation in immune genes and how they can potentially help with treatment of breast cancer.

Chapter 3

MATERIALS AND METHODS

3.1 Sample collection and IRB approval

Blood samples were collected from women undergoing treatment at the Helen F. Graham Cancer Center and Research Institute (Delaware, USA) under an IRB approved protocol and with consent from patients. The Breast Imaging Reporting and Data System or BI-RADS was used in describing mammograph findings and results. Samples with screening mammograms in categories 1-2 were considered normal. Samples with screening mammograms in categories 3-5 were considered invasive and were confirmed by pathological examination. Patient samples were collected prior to surgery or treatment using vacutainer EDTA collection tubes. Patient data was recorded, and blood samples were aliquoted into cryogenic vials and stored at -80°C. Patient samples with previous cancer history or immunosuppressive medication treatment were excluded from the study.

3.2 DNA Isolation

Genomic DNA was isolated from whole peripheral blood samples of 60 patients (30 normal and 30 invasive). DNA was isolated using a Qiagen QI Amp DNA blood mini kit per the manufacturer's instructions. Genomic DNA was eluted in 200ul buffer and quantified on the Infinite® 200 PRO Nano Quant (Tecan Life Sciences) by analyzing absorbance at 260nm and 280nm in duplicate.

3.3 PCR amplification

The total PCR reaction mix used was 25µL; 12.5µL GoTaq Green Master Mix (Promega), 1µL of the forward primer, 1µL of the reverse primers, 2-3µL(100ng) of the DNA template and nuclease free water. The PCR program was a 40cycle run consisting of 94°C denaturation for 60seconds, annealing 63°C for 90seconds and elongation 72°C for 1minute with a 10minute extension step at 72°C. A 5µL loading dye (New England Biolabs, Purple 6X gel loading dye) was added before agarose gel electrophoresis. 1g agarose (IBI scientific standard agarose #18B1004, 100gm) was mixed with 100mL 1X TAE (Corning 1L 10X TAE Buffer Liquid, pH 8.3 ± 0.1 RNase-/DNase- and protease-free). SYBR safe was added and the gel was poured into a gel tray with the well comb and left to solidify at room temperature. The DNA samples were run at 100 volts for 45 minutes.

3.4 PCR Primer Design

Primers were designed using NCBI Primer BLAST and the Meth Primer application around target regions in promoters and enhancers of genes to be amplified (IL4R, IL17RA, IL17C and TYK2). Ideally, primers chosen had a GC content of 50-60%, melting temperature between 60-63°C and a self-complementarity score below 5. (Table 1). Amplicons were designed to include restriction sites for the methylation sensitive and insensitive enzymes HPAII and MSPI which cleave at the CCGG regions (in bold).

Table1 Primers and their sequences. Table 1 shows primers and their sequences that were designed using NCBI Primer BLAST and the Meth Primer application around target regions in promoters and enhancers of genes to be amplified. It also shows genes that were identified from the literature (de Souza et al, 2019; Suárez-Álvarez, 2013) for further study. The genes were IL4R, IL17RA, IL17C and TYK2, IL4 CpG 1 and CpG3, TNFα and IFNγ.

Primer	Sequence
IL4R promoter	Restriction site: 4 Forward: ACCACTCTCACTTGGAAGCC Reverse: GGATCCCCTTGGTCTCGGAA
	Restriction site: 2 Forward: GCCCCACTGGTCTAAGAGGT Reverse: TTCGAACACAGGCCCCATAG
	Restriction site: 13 Forward: CCCGGACGGCGAATGGAG Reverse: CCGGGCGATACCTTCGCA
IL17RA promoter	Restriction site: 1 Forward: CGCGTGCTAAGAAGGAGACT Reverse: GGGGACACTTGAGTTCCTGG
	Restriction site: 2 Forward: AACGACTCTCTTAGGTGCGG Reverse: ATGGGACAGCAGTCACTTCC
	Restriction site: 4, 5, 6 Forward: AAAATCGACAGTTGCTGCGG Reverse: TCCCTACCCAGTCCTTCCG
IL17C promoter	Restriction site: 3 Forward: ACTGAACCACCCTTGGCAC Reverse: GTTTCTGCTTGCGTATCCGTC
	Restriction site: 4 Forward: AGCAGACGGAGAAGAACTGT Reverse: GTTTCTGCTTGCGTATCCGT
	Restriction site: 5 Forward: GAGGAAGTGGTGCCGGAATTT Reverse: ATGTGGAGAGGCTCACCGT
TYK2 promoter	Restriction site: 4 Forward: CCGGGTTTAGAGGTTTTGGG Reverse: ACAGGGTCGGAGTGAAGTTT
	Restriction site: 5 Forward: CACTCCGACCCTGTCTTCAAA Reverse: GCTTTCGCGGAGCCTACTTC
	Restriction site: 6 Forward: CGGCCGGGTTTAGAGGTTT Reverse: TGAAGACAGGGTCGGAGTGA
IL4R enhancer	Restriction site: 1 Forward: GGCAAGCTCTGGTAAGTCAC Reverse: AATCAGGGCCACTTGTCATT
	Restriction site: 1 Forward: GTGGCAAGCTCTGGTAAGTC Reverse: AATCTCCATAAGGCCAGGTGA
IL4 CpG1	Forward: 5'AACTGCTTCCCCCTCTGTTC -3' Reverse: 3' CTTGGAGGCAGCAAAGA -3'
IL4 CpG3	Forward: 5' TCCATAATGAACCTCAAATACCTC -3' Reverse: 3' AAGGGCAGCTTTAGTGCAAG -3'
TNF-α	Forward: 5' GGCAGGTTCTCTTCCTCTCA -3' Reverse: 3' GGCACTCACCTCCTCCCTCT -3'
IFNG	Forward: 5' TTAAGCCAAAGAAGTTGAAATCAG -3' Reverse: 3' ACACCCAATGCCACAAAAC 3'

3.5 Real-time quantitative PCR

Quantitative PCR (qPCR) was performed using the RT^2 SYBR® Green ROX qPCR Master Mix (Qiagen, Hilden, Germany) per the manufacturer's instructions on an ABI 7500 Fast Real-Time PCR System by the comparative Ct method, using an annealing temperature of 61°C. Threshold cycle (Ct) values were used in calculating the percentages of methylated(M) and unmethylated (UM) DNA.

3.6 Restriction enzyme digest (MSPI and HPAII)

Restriction enzyme digest was performed using enzymes HPAII (cat# R0171M) and MSPI (cat#R0106M) from New England Biolabs according to the manufacturer's instructions. Both restriction enzymes recognize and cut CCGG sites. HPAII is methylation sensitive and digests unmethylated DNA. MSPI is methylation insensitive and digests both methylated and unmethylated regions of DNA. The digestions for both HPAII and MSPI were performed at 37°C for 60 minutes followed by a heat inactivation step for HPAII at 80°C for 20mintutes.

3.7. Statistical analysis

Statistical analysis was done on GraphPad Prism 8. Analysis of the data was shown using dot plots with the mean (dotted line) and 95% confidence (error bars). The formula below was used in calculating the methylation index using ct values from the real-time qPCR reaction data.

$$\text{Methylation Index} = \frac{2^{-dCt\ value\ of\ HpaII}}{2^{-dCt\ value\ of\ MspI}}$$

Chapter 4

RESULTS

Patient Characteristics

60 subjects were included in our study with 30 being normal and 30 being invasive cancer patients. Table 2 provides a summary of clinical data and patient characteristics. 5 patients were <50 (16.67%), and 25patients were > 50 (83.34), with a median age of 57(range 47-79). This is close to the average age of diagnosis of breast cancer overall. Grading information was available for 17 out of 30 tumors. The majority of the tumors were grade 2(8patients,47%). 5 tumors were grade 3 (29.4%) and 4 tumors were grade 1(23.5%). The mean tumor size was 1.61cm with a range of 0.3-5.5cm. 14 patients (73.6%) had tumors smaller than 2cm and 5 patients (26%) had tumors larger than 2cm. The average BMI was 31.1.

Table 2: Clinicopathological data and patient characteristics

Clinicopathological characteristics Normal patients			IDC patients		
Characteristic	#	%	Characteristic	#	%
# of patients	30		# of patients	30	
Age			Age		
Mean(range)	57(46-74)		Mean(range)	57(47-79)	
<50	8	26.6	<50	5	16.67
>50	22	73.4	>50	25	83.34
Grade			Grade		
1	--	--	1	4	23.5
2	--	--	2	8	47
3	--	--	3	5	29.4
Tumor size			Tumor size		
Mean(range)cm	--	--	Mean(range)cm	1.61(0.3-5.5)	
<2cm	--	--	<2cm	14	73.6
>2cm	--	--	>2cm	5	26
Race			Race		
White	24	80	White	26	86.67
Black	6	20	Black	4	13.34

4.1 Identification of target sites

To test the hypothesis that PBMCs undergo methylation changes in patients with invasive cancers, a preliminary screen was conducted using a commercial qPCR-based methylation array (Appendix 1). The Epitect methyl II DNA restriction kit provided by Qiagen (Chatsworth, CA) was used as a screening tool for the study by looking at CpG sites in promoters and enhancers of 22 genes using PBMCs from 8 women without breast cancer and 8 women with invasive disease. Of these 22 genes, 4 genes were shown to have differential methylation (IL-4R, IL17RA, IL17C and TYK2) and were chosen for further study. Additionally, other genes which undergo methylation changes in chronic inflammation were identified from the literature (71,72).

4.2 MSRE-qPCR of candidate genes using DNA from screening cohort

In order to investigate differences in methylation at specific CpG sites, we employed a technique that includes quantitative PCR in combination with a methylation specific restriction digest. Methylation Sensitive Restriction Enzymatic digest quantitative PCR (MSREqPCR) is based on digestion of genomic DNA with enzymes that are differently affected by the presence of a methyl group in their target sequence, resulting in differential digestion and amplification. MSRE qPCR is more sensitive than bi-sulfite sequencing and can detect methylation patterns in small subpopulations (73). MSRE-PCR was used for analysis of gene-specific DNA methylation. (24). Primers targeting specific enzymatic sites were based on previous studies (71) or were designed by our group**.** As the specific target and restriction sites in the screening array are unknown, we chose to target CpG sites encompassing the restriction site CCGG which

was found in the promoter and enhancer regions of candidate genes. This site is specifically recognized by the restriction enzymes HPAII and MSPI. HPAII is a methylation sensitive enzyme and will not cut methylated DNA. MSPI will cut regardless of methylation status. Amplicons were designed to include restriction sites for the methylation sensitive and insensitive enzymes HPAII and MSPI which cleave at the CCGG regions (in bold). Primers were designed around restriction sites shown in Table 3. To ensure specificity and determine appropriate melting temperature, we performed conventional PCR and analysis of products using agarose gel electrophoresis. As shown in Figure 6, 14 primers showed specific bands and were chosen for MSRE-qPCR. Our initial screen MSRE-qPCR was carried out in a small subset of patients using DNA from the same samples included in the array screening. Our results identified hypomethylation in 15 target sites. (Table 5)

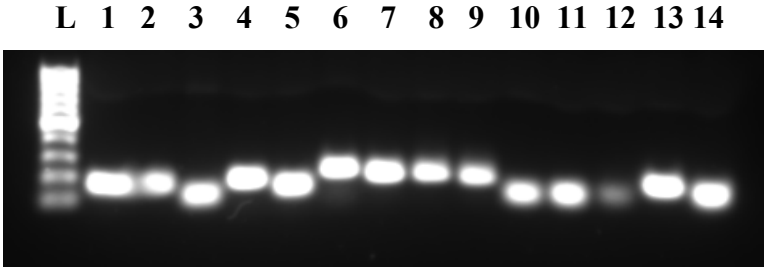

Figure 6: Agarose gel electrophoresis of primers for promoter and enhancer regions. Primers were designed around restriction sites shown to ensure specificity and determine appropriate melting temperature. We performed conventional PCR and analysis of products using agarose gel electrophoresis. L=100bp DNA ladder, 1: IL4R promoter RS4, 2: IL4R promoter RS2, 3:IL4R promoter RS1,3, 4: IL17RA promoter RS1, 5:IL17RA promoter RS2, 6: IL17RA promoter RS4,5,6, 7:IL17C promoter RS4, 8:IL17C promoter RS3, 9:IL17C promoter RS5, 10:TYK2 promoter RS5, 11:TYK2 promoter RS6, 12:TYK2 promoter RS4, IL4R Enhancer RS1, IL4R Enhancer RS1,2. All 14 primers showed specific bands and were chosen for MSRE-qPCR.

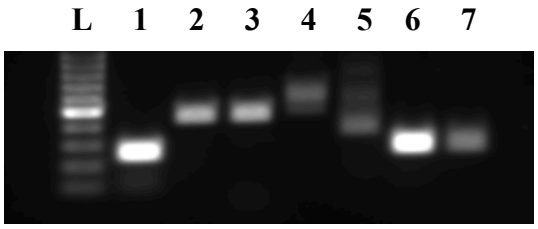

Figure 7: Validation of primers from chronic inflammation study. Genes which undergo methylation changes in chronic inflammation were identified from the literature. To ensure specificity and determine appropriate melting temperature, we performed conventional PCR and analysis of products using agarose gel electrophoresis. L=100bp DNA ladder, 1LESC02(positive ctrl), 2:IL4CpG1, 3:IL4CpG3, 4:IL10, 5:cs17, 6: TNFα, 7: IFNγ. 4 primers showed specific bands and were chosen: IL4CpG1, IL4CpG3, TNFα and IFNγ for MSRE-qPCR.

Table 3 shows genes and methylation changes. The genes were IL4R, IL17RA, IL17C, TYK2, IL4, TNFα and IFNγ. Methylation changes in the genes are shown on the right of the table.

Gene	Methylation change
Interleukin 4 receptor (IL4R)	Hypomethylation
Interleukin 17 receptor A (IL17RA)	Hypomethylation
Interleukin 17C (IL17C)	Hypomethylation
Tyrosine Kinase 2 (TYK2)	Hypomethylation
Interleukin 4 (IL4)	--------
Tumor Necrosis Factor-α	Hypermethylation
Interferon gamma (IFNG)	-------

Interleukin 4-Receptor (IL4R)

Interleukin 4 Receptor (IL4R) is a type 1 transmembrane protein that binds both IL4 and IL13 (61).Three sets of primers were designed for the promoter region of IL4R to amplify the CpG sites; IL4R promoter RS1,2,3,4 and two sets of primers were designed for the enhancer regions of IL-4R; IL-4R Enhancer RS 1 and 2.

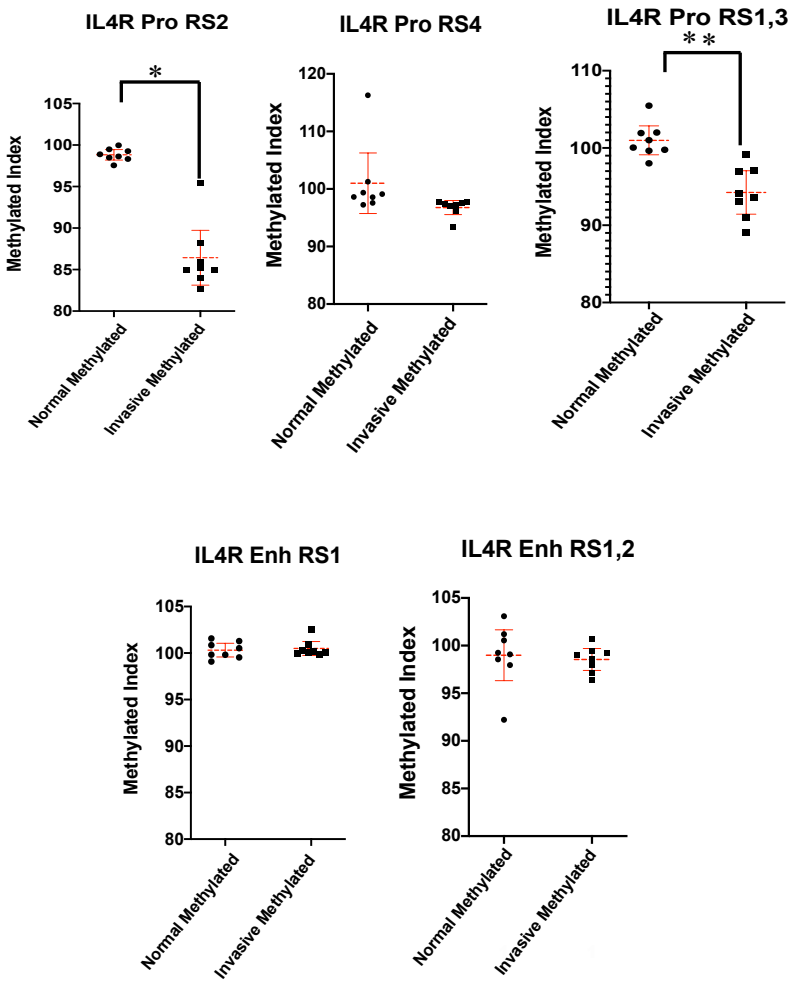

Figure 8: Methylation changes in IL-4R promoter and enhancer regions. These 2 primers showed decreases (hypomethylation) in PBMCs obtained from women with invasive cancers compared to normal samples. These 2 primers showed decreases (hypomethylation) in PBMCs obtained from women with invasive cancers compared to normal samples.

Interleukin 17 Receptor A (IL-17RA)

Interleukin-17 Receptor A(IL-17RA) is a common signaling and co-receptor subunit for members of the IL-17 family. (66) Three sets of primers were designed for the promoter region of IL-17RA to amplify the CpG sites; IL-17RA promoter RS1, IL-17RA promoter RS 2 and IL-17-RA promoter 4,5,6.

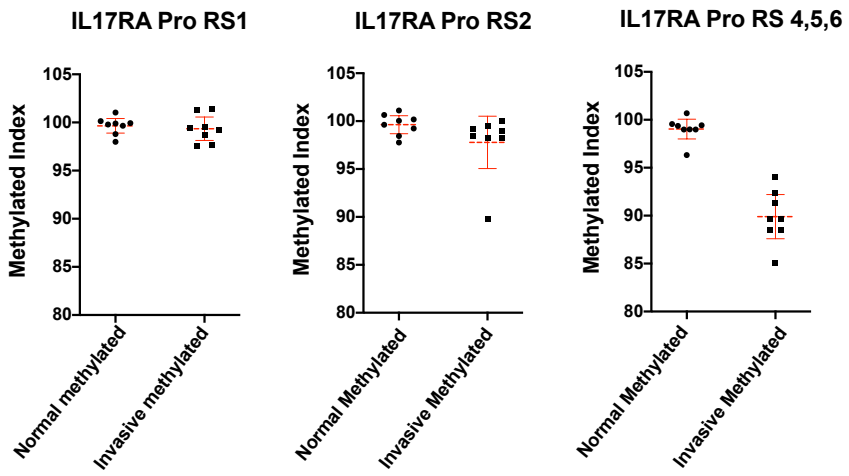

Figure 9: Methylation changes in IL-17RA promoter region. All primers showed no significant increase in expression (hypomethylation) in PBMCs obtained from women with invasive cancers compared to normal samples.

Interleukin 17C (IL-17C)

Interleukin-17C (IL-17C) is a member of the 1L17 family and selectively induces inflammatory responses like the expression of proinflammatory cytokines. (70). Three sets of primers were designed for the promoter region of IL17C; IL-17C promoter RS3, IL-17C promoter RS4 and IL-17C promoter RS5.

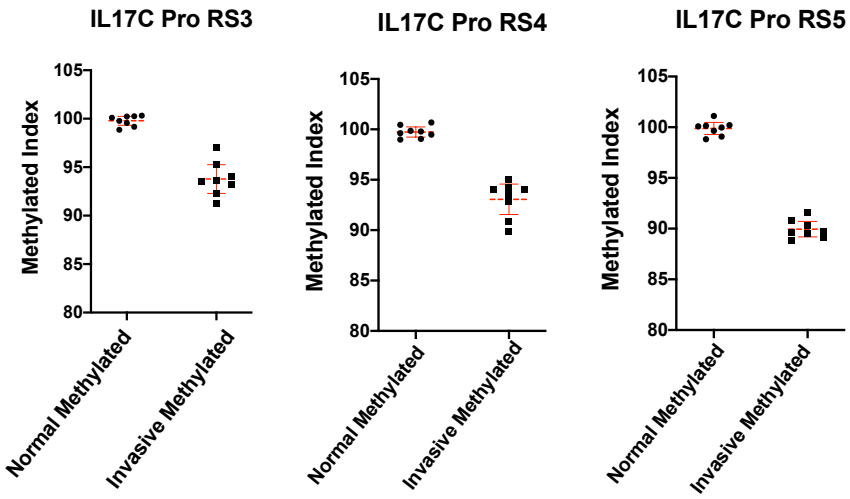

Figure 10: Methylation changes for IL-17C promoter region. All primers showed no significant decreases (hypomethylation) in PBMCs obtained from women with invasive cancers compared to normal samples.

Tyrosine Kinase 2(TYK2)

Tyrosine kinase 2 (TYK2) is an intracellular enzyme which plays a role in intracellular signaling and activation of the immune system. (40) Three sets of primers were designed for the promoter region of TYK2; TYK2 promoter RS4, TYK2 promoter RS5 and TYK2 promoter RS6.

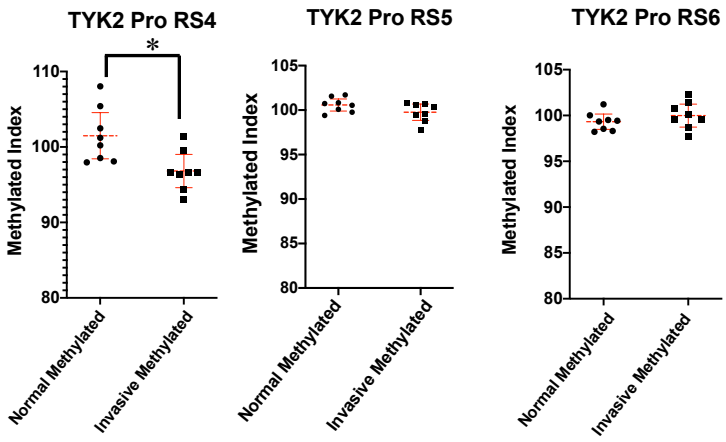

Figure 11: Methylation changes for TYK2 promoter region. TYK2 promoter RS 4 primer showed a decrease (hypomethylation) in PBMCs obtained from women with invasive cancers compared to normal samples.

These 4 primers showed decreases (hypomethylation) in PBMCs obtained from women with invasive cancers compared to normal samples. (p-value: $*$ 0.005,$**$0.0005)

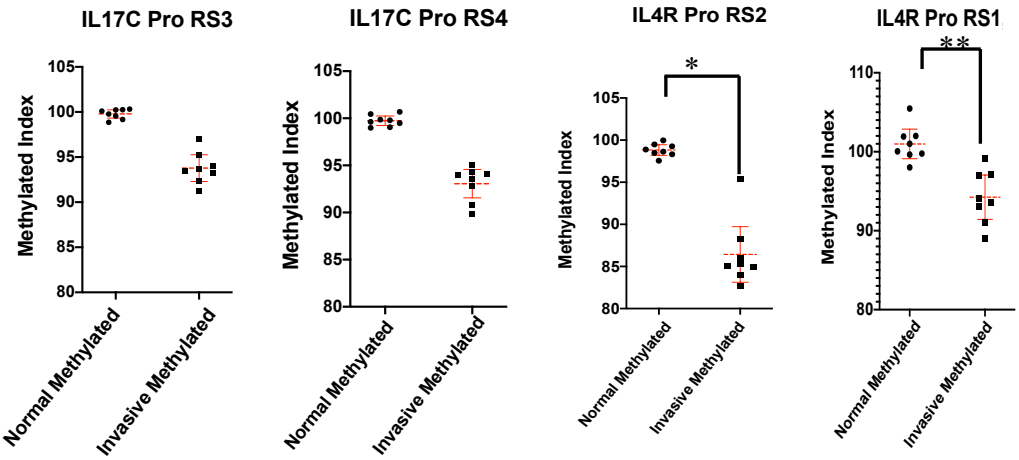

Figure 12: Analysis of promoter and enhancer regions for four genes: IL4R, IL17RA, IL17C and TYK2. Figure 12 shows differential methylation observed in 4 primers. (IL4R Pro RS1, IL4R Pro RS2, IL17C Pro RS3 and IL17C Pro RS4). These 4primers showed decreases (hypomethylation) in PBMCs obtained from women with invasive cancers compared to normal samples. (p-value:$*$ 0.005, $**$0.0005)

4.3 Methylation index of genes shown to have differences in promoter and enhancer regions

Primers were designed around the promoter and enhancer regions of 4 genes identified in the preliminary screen from the array. They were designed to amplify regions containing CpG sites that had restriction sites for restriction enzymes HPAII and MSPI. A qPCR run with 16 patient samples used in the array showed significant differences in 4 out of 15 CpG sites between normal and invasive samples. The 4 primers were run with the 60 patient samples (30 normal, 30 invasive) to validate the differences.

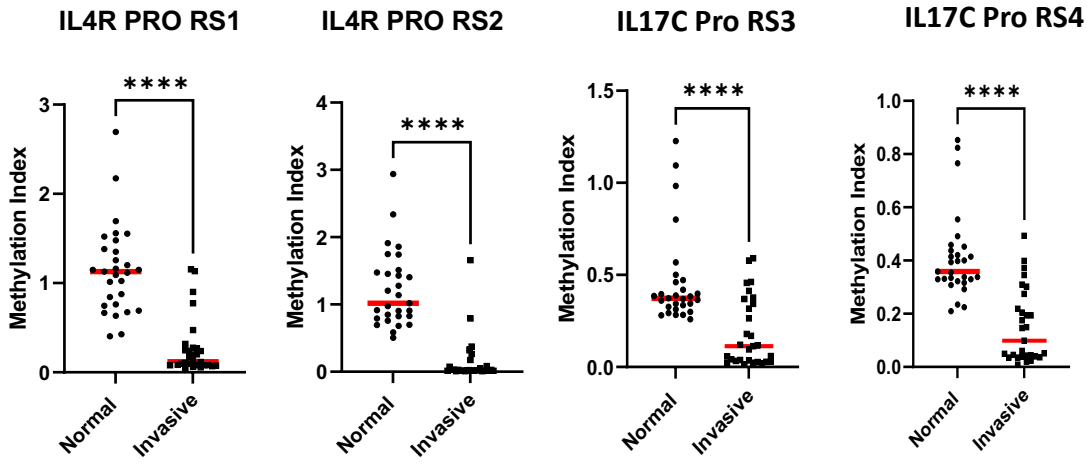

Figure 13: Analysis of primers with changes in promoter and enhancer regions: Samples were run using qPCR and ct values were recorded. Analysis of the data shows dot plots with the mean (dotted line) and 95% confidence (error bars) for 6 primers. Hypomethylation was observed in all the invasive samples showing an increased gene expression.

4.4 Methylation index of genes obtained from previous studies

For the second part of my study, in order to validate our screening tool, a set of genes which undergo methylation changes in chronic inflammation were identified from

the literature (71,72) were run with a large cohort of 60 patient samples, 30 normal and 30 invasive. The genes were IL4CpG 1, IL4 CpG 3, TNF-α and IFNγ. The genes were run under similar conditions as the genes in the previous group with restriction enzymes HPAII and MSPI. As shown in Figure 13, changes in methylation were observed in all 4 genes.

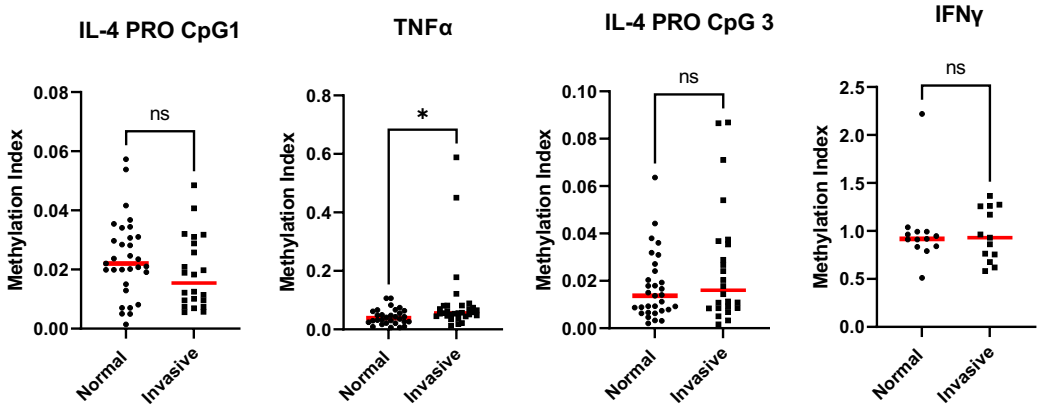

Figure 14: Methylation fraction of 60 patient samples with 4 genes found to be methylated in other types of cancer. Four genes found to show methylation changes in chronic inflammatory conditions were tested using restriction enzymes MSPI and HPAII. Samples were run using qPCR and CT values were recorded. Analysis of the data shows dot plots with the mean (dotted line) and 95% confidence (error bars) for the 4 genes between 30 normal and 30 invasive patient samples. Sample showed no change in IL4 pro CpG1, IL4 CpG3 and IFN-γ in invasive samples compared to normal. TNF-α showed hypermethylation in invasive samples compared to normal.

Chapter 5

DISCUSSION

The goal of this study was to show whether DNA methylation induces a shift of immune cells, peripheral blood mononuclear cells (PBMCs) from an anti-tumor response to an immunosuppressive response in normal and invasive breast cancer. PBMCs (lymphocytes and monocytes) are peripheral blood cells having a round nucleus and play a significant role in the immune system (36). In this study, we screened potential blood DNA samples collected from a cohort to identify CpG sites in the promoter and enhancer regions of inflammatory genes that showed differences in methylation between women with invasive breast cancer and women without. We used MSRE qPCR to investigate the patterns of methylation in model inflammatory genes from a preliminary screen and from literature (71,72). The results for our study showed hypomethylation in IL17C, IL4R and hypermethylation in TNF-α. Additionally IL4 (CpG1, CpG 3) and IFN-γ showed no changes in methylation.

5.1 Overview of Interleukin17 (IL-17C)

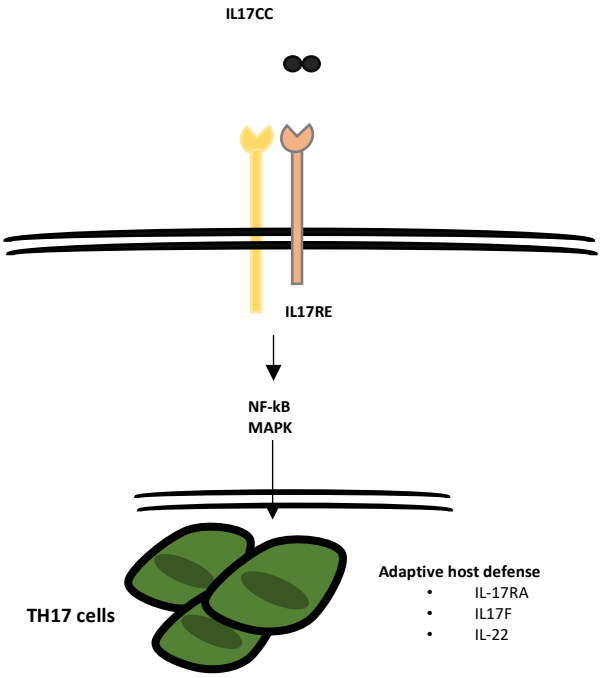

Figure 15: Signaling pathway for IL17C and its receptor IL17RE. IL17C signals through the receptor IL17RE to mediate inflammatory effects.IL17RE through signaling activates other pathways involved in the expression of proinflammatory cytokines and chemokines like Nuclear factor kappa light chain enhancer of activated B cells (NFkB) and Mitogen activated protein kinase (MAPK)

Interleukin-17C (IL-17C) is a proinflammatory cytokine which plays a role in inflammatory response through its receptor IL17RE (70). It is a member of the IL17 family located on chromosome 16q24, is about 1.1kb and shares amino acids with IL17RA (64). IL17C promotes Th17cell response through IL17Receptor E expression on CD4+Th17 cells (71). IL17C/IL17RE pathway have helped present an important therapeutic strategy for Th17 induced autoimmune disorders. IL17C is produced by CD4+ T lymphocytes, dendritic cells and macrophages (75). It is also synergistically produced by IL17RA (76). However, studies have shown that IL17C mRNA is absent in CD4+ cells which are the main source for IL-17RA (64). Hence, it shows a unique role of IL17C in the IL17 family. In our methylation study, IL17C showed changes, specifically hypomethylation in samples for patients with invasive disease compared to normal patient samples. Previous studies have shown increased expression of IL17C in the tumor microenvironment (70). The IL-17C pathway shows a boost in Th17 cell function and stimulation of the adaptive immune system in T-cells. Th17 cells also produce IL-17, IL-6, IL-21, IL-22 and TNF-alpha (86). Therefore, Th17 cells lead to an adaptive immune response which infers that T-cells are responding and expressed in invasive breast cancer.

5.2 Overview of Interleukin4 receptor (IL4R) and Interleukin 4 (IL4)

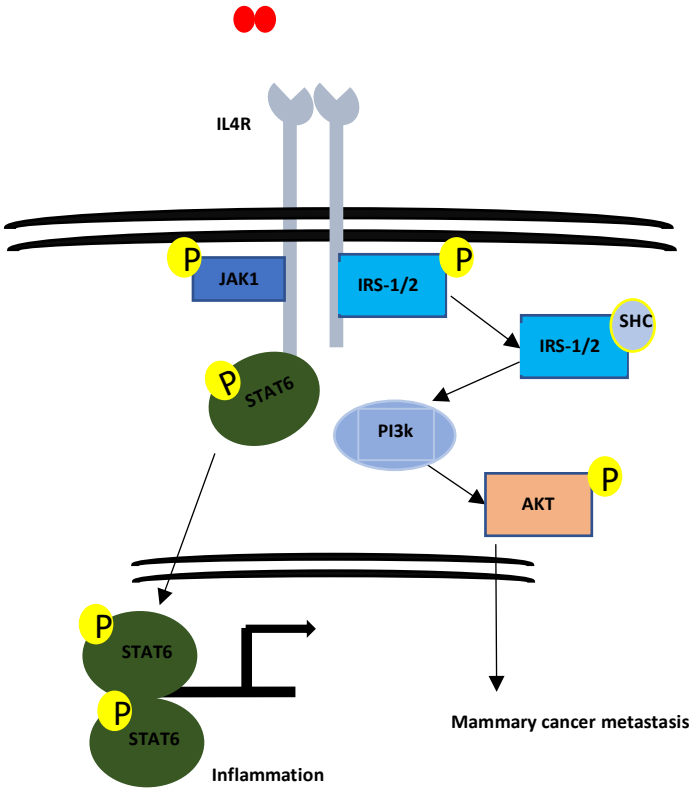

Figure 16: Signaling pathway for IL4/IL4R. IL4 receptor mediates JAK/STAT6 pathway leading to inflammation. It also mediates the Akt, Erk, and mTOR pathway in mammary cancer cells. It has been identified that IL4/IL4R signaling is a potent driver of mammary cancer metastasis.

Interleukin 4 Receptor (IL4R) is a type 1 transmembrane protein that binds both IL4 and IL13. It is coupled with the JAK 1/2/3-STAT6 pathway for signaling. Upregulation of IL4R facilitates class-switch to increase the synthesis of IgE in B cells. (79) It is also expressed in CD23-positive B cells. IL4 has been shown to be upregulated by its natural ligand, IL4R. Therefore, an increase in IL4R correlates with an increase in IL4. It has been shown that IL4 stimulates IL4R on lymphocytes at the protein and

messenger RNA levels. Interleukin 4(IL4) is a type 2 cytokine secreted by mast cells, eosinophils and basophils and functions as a potent regulator of immunity (77). In B cells, IL4 acts as a co-mitogen and plays an important role in leukocyte survival conditions such as Th2 cell mediated immunity, IgE class switching and tissue repair. (76) It also plays a role in B cell activation. It also has anti-inflammatory characteristics which enable it to downregulate the production of inflammatory cytokines like TNF-α. Studies have shown that higher levels of IL4 are secreted in the tumor microenvironment contributing to proliferation and metastasis in breast cancer. (53) However, our results show no methylation change in IL4 but a change in IL4R (hypomethylation) when between patients with invasive breast cancer compared to patients with non-invasive disease. Hypomethylation of IL4R will lead to the presence of more receptors compared to the ligands present which may lead to lack of stimulation of some receptors present However, this may not affect the overall effect of IL4R. Future studies with IL4R and IL4 will help provide more data in supporting this finding.

5.3 Overview of Tumor necrosis factor-α (TNF-α)

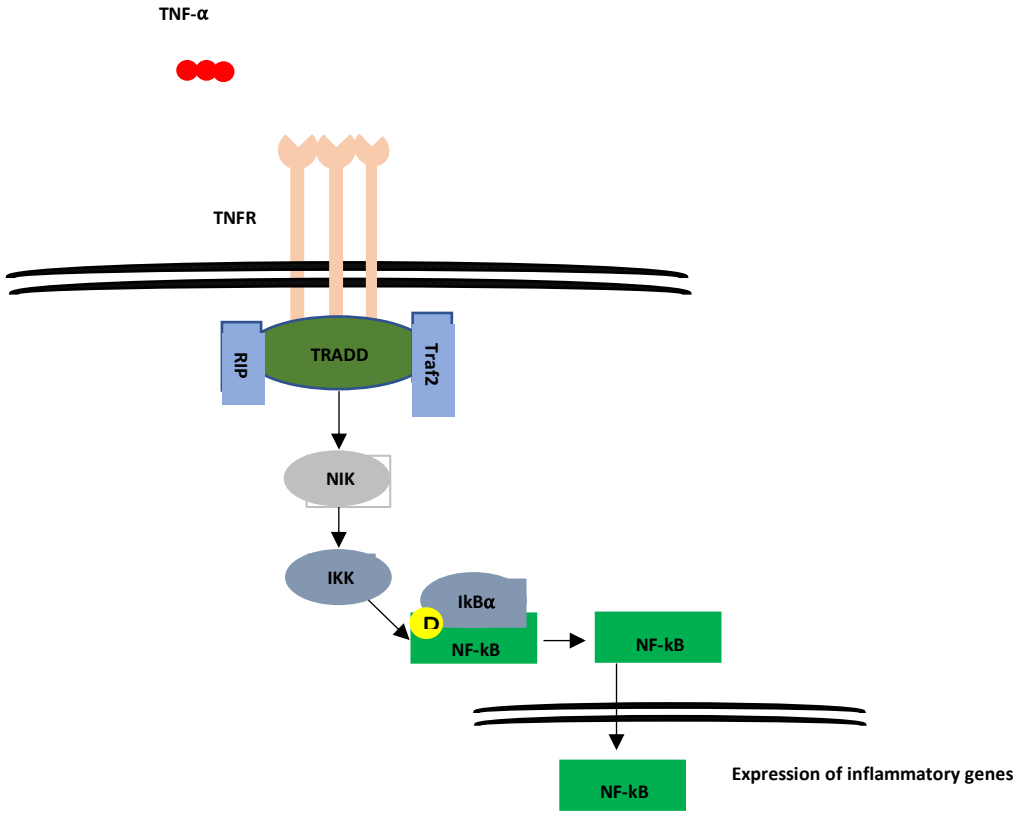

Figure 17: Signaling pathway for TNF-α.TNF-α binds to its receptor TNFR, resulting in recruitment of TRADD, TRAF2 and RIP. This leads to a signaling cascade resulting in the activation of the IKK complex. The IKK complex forms IkBα which binds to NFkB and then is transported to the nucleus leading to expression of several inflammatory genes.

TNF-α is a cytokine involved in inflammation. It is mainly synthesized by macrophages, lymphocytes and mast cells. TNF-α activates the transcription factor Nuclear factor Kappa-light-chain enhancer of activated B cells (NFKB). TNF-α bind to the TNF-α receptor leading to transcription of several proinflammatory cytokines and chemokines like interleukin 6 (IL-6) and IL-1Beta. (78) TNF-α binding also activates the mitogen-activated protein kinase (MAPK) pathway which also leads to activation of transcription factors, involved in cell differentiation and proliferation. TNF-α is one of the essential

proinflammatory cytokines found in the tumor microenvironment in breast cancer secreted by stromal cells (75). In our methylation study, our results showed changes, specifically hypermethylation between patients with invasive breast cancer compared to patients with non-invasive disease. TNF-α leads to the expression of inflammatory genes through the NFKB pathway which play an important role in invasive disease (78).

5.4 Overview of Interferon gamma (IFN- γ)

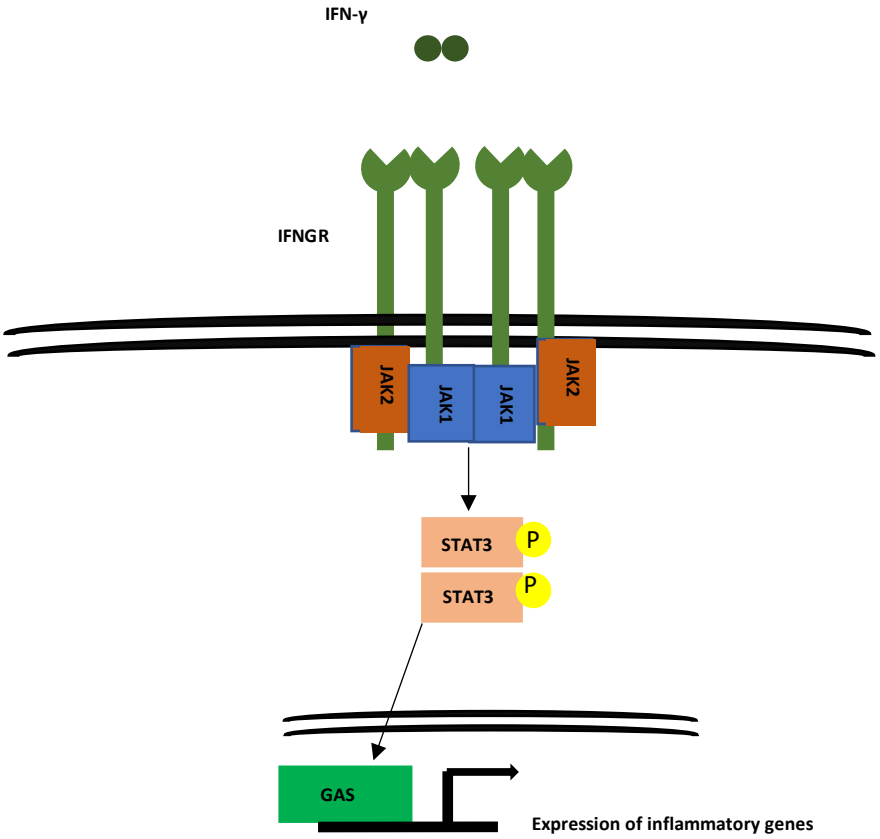

Figure 18: Signaling pathway for IFN-γ. IFN-γ binding leads to the activation of the JAK/STAT3 signaling pathway. STAT3 is phosphorylated and activated GAS which leads to the expression of inflammatory genes.

Interferon gamma (IFN- γ) is a proinflammatory cytokine which plays a role in inducing immune responses. It is secreted and activated by T-cells and natural killer cells (NK cells). Immune cells with adaptive immunity characteristics have also been shown to produce IFN- γ in the tumor microenvironment. IFN- γ signals through the IFN- γ receptor which is mediated through the Janus family of protein tyrosine kinases leading to the expression of inflammatory genes. (66) It also regulates T-helper cells 1 and 2 balance. A previous study on the tumor microenvironment with IFN- γ showed both pro and anti-effects. (57) Other previous studies with this gene have shown upregulation in breast cancer. However, our results show no changes in methylation between patients with invasive breast cancer compared to patients with non-invasive disease. This observation refutes our hypothesis that methylation changes are occurring.

DNA hypermethylation and hypomethylation are independent in their role in affecting gene expression and functions associated with cancer. Our study showed hypomethylation in IL-4R and IL-17C and hypermethylation in TNF. Our study also showed no changes in IFNγ, IL4 and IL-17RA. IL-17C is also synergistically produced by IL17RA (76). However, since IL-17C mRNA has been shown to absent in CD4+ cells which are the main source for IL-17A (64), it could explain the difference in methylation changes observed between both genes. Understanding the changes occurring in these genes and how it affects their functions in invasive cancer is important in validating these genes as prognostic biomarkers.

Chapter 6

CONCLUSION

This study showed changes in some immune genes between the normal and invasive patient samples by examining their CpG sites was due to the epigenetic mechanism, DNA methylation. As a result, we suggest that the changes in these genes can play important roles in understanding these immune genes and how they can be used as targets for future immunotherapeutic treatments for breast cancer.

Immunotherapy deals with boosting the immune system to fight cancer. One type of immunotherapy is with the use of immune checkpoints, particularly antibody mediated interventions targeting cytotoxic T lymphocytes antigen-4 (CTLA-4) and programmed death receptor-1(PD-1) with ligand PD-L1 have been shown to reverse immunosuppressive changes. These immune checkpoints have been widely validated and accepted as predictive biomarkers. Immune checkpoint inhibitor targeting PD-1 or PD-L1 have also been shown to be very important and have improved the outcome of patients with many types of cancer. However, a major problem with these current immunotherapies is the identification of these biomarkers that will help predict clinical responses for these checkpoint blockades. Our study was able to show changes of specific inflammatory genes for example, IL4R that could possibly serve as a future biomarker after further study. IL-4R is a receptor for IL-4 and IL-3 which are both central cytokines in type 2 immunity (82). We were able to show hypomethylation in the IL4R gene in our invasive patient samples. One limitation of our study was that the effect of methylation on gene expression was not determined. A previous study showed that IL4R gene was highly expressed in advanced stage breast cancer (84). This study agrees with our findings, however, the specific effect of DNA methylation on

expression of these genes would have to be carried in future studies to validate our results. Methylation changes can occur differently in genes in different cell types and this could affect their function. This is because regulation of genes in cell types occurs differently. This is due to the fact that many factors can affect the expression of genes in these immune cells, for example, cell differentiation which can affect their function and expression. In order to understand the effects of DNA methylation exclusively on gene expression, we can perform studies using qPCR to detect gene expression of these genes and then use a DNA methylation reversal agent, 5-azacitidine, after which the gene expression levels can be compared. The changes in the methylation levels will show DNA methylation played a role in the expression of the gene. Another experiment will be to use flow cytometry to sort out specific cell types and compare the levels of expression in demethylated vs methylated genes in subsets of cells. This could help us understand the different functions of these immune genes in the different cells. This will help show if methylation had an effect on the expression of these genes. Some other major conclusions from our results were that they supported our hypothesis that there are methylation changes in immune genes in cancer. Hypomethylation was observed in genes that are found in Th2 and Th17 immune responses that are less effective in controlling tumor growth. There were no changes observed in IFN-gamma which is a driver in cytotoxic immune responses. Hypermethylation was observed in TNF-a. TNF-a is required for immune priming of CD8+ T cells during tumor surveillance. Our findings also indicated that methylation changes are occurring in immune genes that are involved in less effective anti-tumor immune responses.

Overall there were some important strengths. One strength of this study was that it provided another way for studying these immune genes in breast cancer which with further studies can be validated as prognostic biomarkers and help with immunotherapeutic treatment. There were also some other limitations of our study. Our evidence was able to provide some conclusive differences between normal and invasive patient samples in some of the immune genes. A possible reason for this could be due to the lack of generalizable demographic differences like the race and ethnicity. The percentage of white patients used for our study was 77% compared to black patients which accounted for only 17%. The ethnicity of 45% of the patients in the study were also unknown. Finally, the study had a small sample size with only 60 patient samples.

Chapter 7

FUTURE DIRECTIONS

Our study helped show that DNA methylation plays a role in inducing immunosuppressive changes in some immune genes. To further validate and provide more conclusive evidence, a number of experiments have to be performed. In order to support our findings, we need a larger cohort of patients. Our study was done to examine if immunosuppression could be induced by methylation changes in the tumor. Other studies can be conducted on other immune genes to help understand them better. It will also help to understand the impact of these methylation changes on gene expression. The epigenetic changes detected shows that they could, with further study, eventually become biomarkers for the prognosis and hopefully future immunotherapeutic treatments for breast cancer. Finally, trying to understand what subsets of cells these changes are occurring in and if they are indicative of T cell differentiation or global changes in gene expression is also necessary. The long-term goal after understanding the changes in these genes will be to further evolve them as prognostic biomarkers to include the best combination of methylation markers and clinical variables to validate and implement them in clinical use.

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Appendix A

MSRE REAL TIME QUANTITATIVE PCR ANALYSIS

RTqPCR analysis was done using the formulas provided by the Epitect Methyl II PCR Array kit. “UM” shows the fraction of the genomic DNA containing no methylated CpG sites in the amplified region of the gene. “M” shows the fraction of genomic DNA containing 2 or more methylated CpG sites in the amplified region of the gene. The number of CpG sites in the targeted region varies based on the fraction of methylated DNA.

Unmethylated (UM) DNA fraction:

$$F_{UM} = \frac{CMd}{CMo - CMsd} = \frac{2^{-CT(Md)}}{2^{-CT(Mo)} - 2^{-CT(Msd)}}$$

Hypermethylated (HM) DNA fraction:

$$F_{HM} = \frac{CMs}{CMo - CMsd} = \frac{2^{-CT(Ms)}}{2^{-CT(Mo)} - 2^{-CT(Msd)}}$$

Intermediately Methylated (IM) DNA fraction:

$F_{IM} = 1 - F_{HM} - F_{UM}$

Methylated (M) DNA fraction:

$F_M = F_{HM} + F_{IM}$

Where no-enzyme (Mo), Methylation sensitive enzyme (Ms), Methylation dependent enzyme (Md) and a Double digest (both methylation sensitive and methylation dependent enzymes) (Msd)

Table 4: Array kit profile panel of 22 genes

Biological groups	Genes	Description
Cytokines	IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)
	IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
	IL17C	Interleukin 17C
	INHA	Inhibin, alpha
Chemokines	CCL25	Chemokine (C-C motif) ligand 25
	CXCL14	Chemokine (C-C motif) ligand 14
	CXCL3	Chemokine (C-X-C motif) ligand 3
	CXCL5	Chemokine (C-X-C motif) ligand 5
	CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein)
Cytokine Receptor and Associated proteins	IL10RA	Interleukin 10 receptor, alpha
	IL13RAI	Interleukin 13 receptor, alpha 1
	IL15	Interleukin 15
	IL17RA	Interleukin 17 receptor A
	IL4R	Interleukin 4 receptor
	IL6R	Interleukin 6 receptor
	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)
Inflammatory Response and Autoimmunity genes	ATF2	Activating transcription factor 2
	FADD	Fas (TNFRSF6)- associated via death domain
	GATA3	GATA binding protein 3
	IL13	Interleukin 13
	IL7	Interleukin 17C
	TYK2	Tyrosine kinase 2
Control assays	EP_SEC	SEC-sensitive enzyme control assay
	EP_DEC	DEC-dependent enzyme control assay

Restriction Digestion

The restriction digestions were performed using the Epitect Methyl II DNA restriction kit provided by Qiagen (Chatsworth, CA) per the manufacturer's instructions. The digested DNA samples were analyzed using the Human Inflammatory Response and Autoimmunity Epitect Methyl II signature PCR Array (22) kit (cat#: 335212 EAHS-521ZA). A reaction mix without enzymes was set up in a microcentrifuge tube 1ug genomic DNA, 5x restriction digestion buffer and RNase/DNase free water up to 120uL.(table 4) Four digestion reactions were set up: no-enzyme (Mo), Methylation sensitive enzyme (Ms), Methylation dependent enzyme (Md) and a Double digest (both methylation sensitive and methylation dependent enzymes) (Msd) as shown in table 5 to make a final volume of 30μL for each digest. The DNA was then quantified using real-time PCR. This array kit profiles the promoter methylation status of a panel of 22 genes.

Appendix B

SCREENING OF 16 PATIENT SAMPLES WITH 22 SPECIFIC INFLAMMATORY ARRAY GENES USING MSRE DIGEST

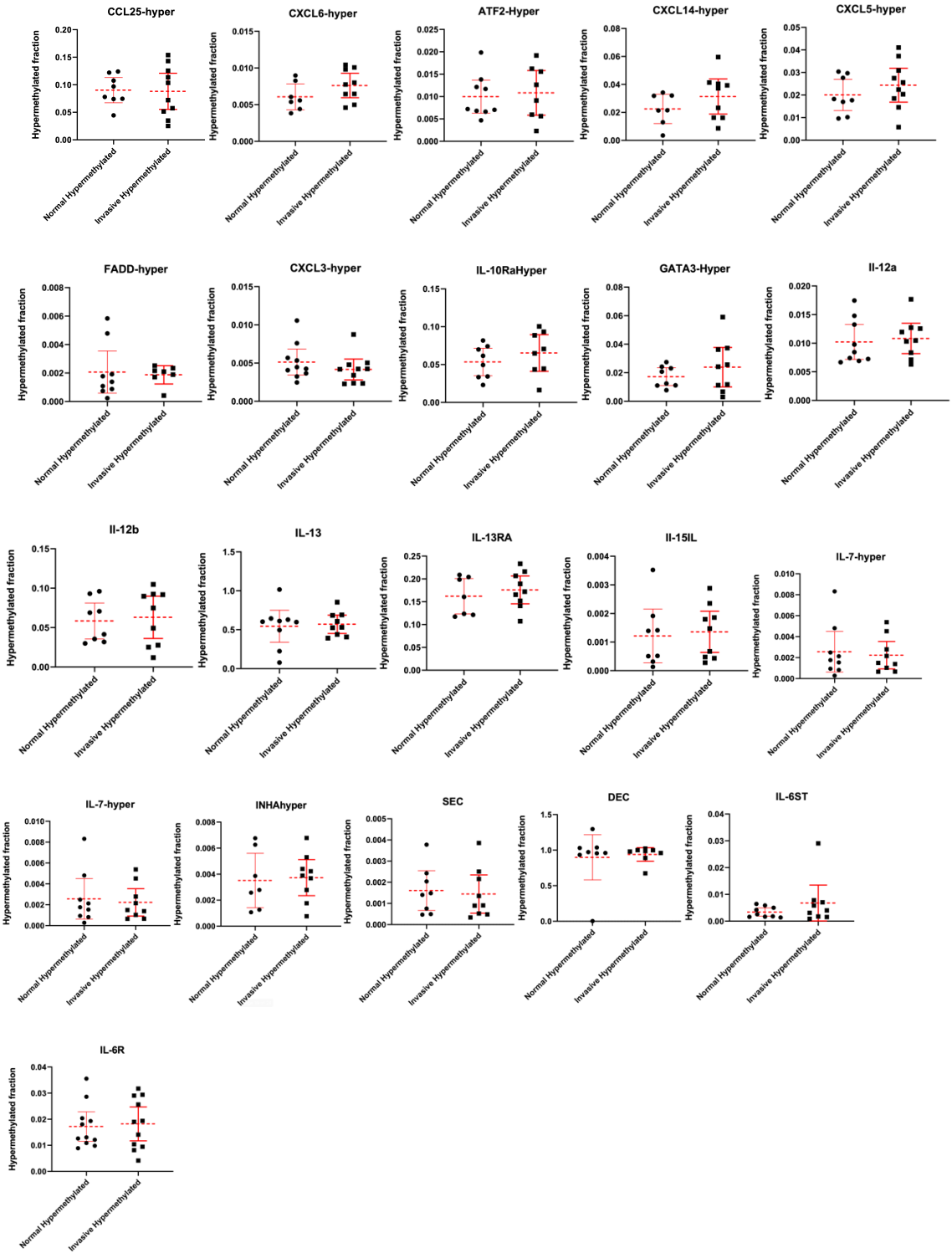

Figure 19: Analysis of the data from the inflammatory array genes for the 16 patient samples showing the percent hypermethylation. Analysis of the data shows dot plots with the mean (dotted line) and 95% confidence (error bars) for the 20 inflammatory array genes. Two controls, SEC and DEC are controls for DNA cutting efficiency. Four genes, IL4, IL17RA, IL17C and TYK2 showed significant differences compared to the other genes.

Appendix C

FOUR INFLAMMATORY ARRAY GENES WITH DIFFERENCES IN HYERMETHYLATION BETWEEN NORMAL AND INVASIVE SAMPLES

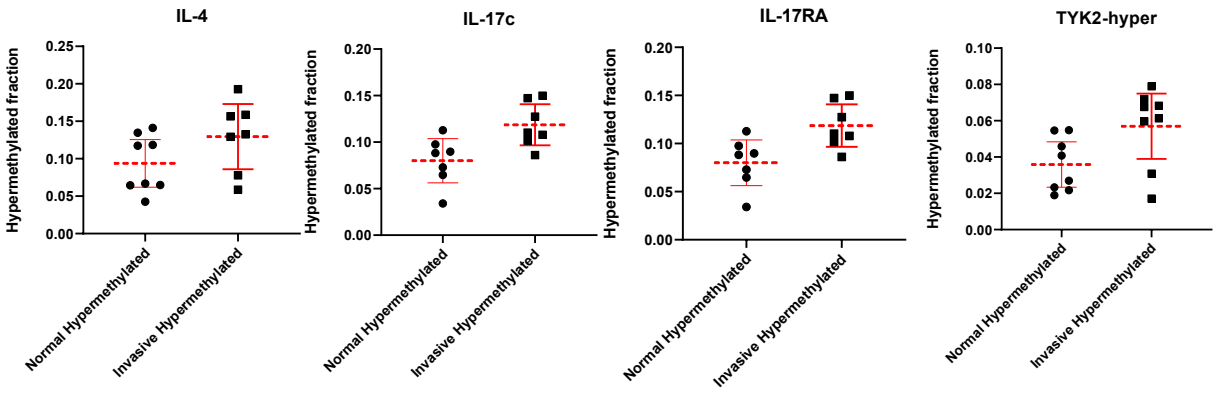

Figure 20: Analysis of the data from 4 inflammatory array genes for the 16 patient samples showing the percent hypermethylation. Analysis of the data shows dot plots with the mean (dotted line) and 95% confidence (error bars) for 4 genes (IL4, IL17RA, IL17C and TYK2) that showed significant differences after removing outliers.

Appendix D

FOUR INFLAMMATORY ARRAY GENES WITH DIFFERENCES IN METHYLATION BETWEEN NORMAL AND INVASIVE SAMPLES

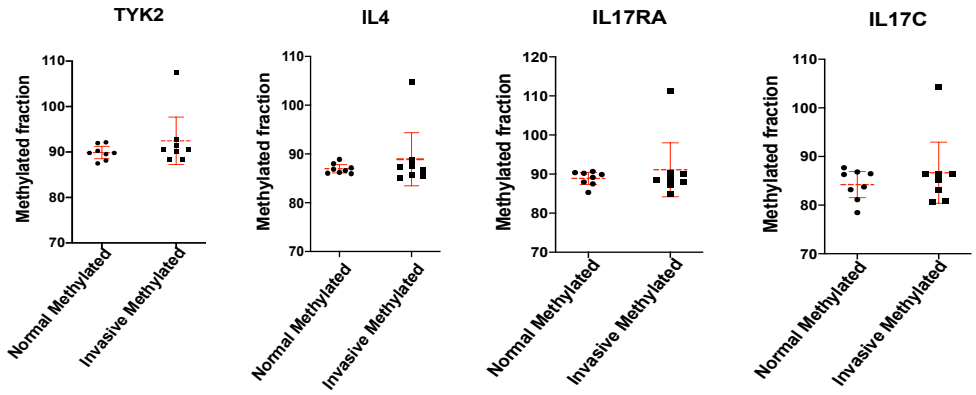

Figure 21: Analysis of data from 4 inflammatory array genes for 16 patient samples showing percent methylation. Samples were run using qPCR and CT values of the samples were recorded. Analysis of the data shows dot plots with the mean (dotted line) and 95% confidence (error bars) for 4 genes (IL4, IL17RA, IL17C and TYK2) that showed significant differences between 16 normal and invasive patient samples. The four genes are from a human inflammatory response and autoimmunity methyl II signature pcr array kit. Data shows no significant changes in methylation fraction in the four genes analyzed.

Appendix E

IRB APPROVAL

Institutional Review Board
FWA00006557

Helen F. Graham Cancer Center & Research Institute
West Pavilion - Suite 2350
4701 Ogletown Stanton Road
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MEMORANDUM

DATE: December 2, 2020

TO: Jennifer Sims-Mourtada, PhD
Oncology Research
Christiana Hospital

FROM: Heidi Derr

RE: ***CCC# 37012*** **- A blood based diagnostic for cancer: (DDD# 603618)**

This is to officially inform you that the Continuing Review for your protocol, which was received on **12/02/2020,** was reviewed by Expedited Review and approved by Jerry Castellano, Pharm.D, CIP, Corporate Director of the Christiana Care Health System Institutional Review Board (IRB00000480), on **12/02/2020**.

Approval was extended for a period of one year, through **12/01/2021.**

Our records indicate this study is OPEN.

If you have any questions or concerns, please contact the IRB Office.
Thank you.

This approval verifies that the IRB operates in Accordance with applicable ICH, federal, local and institutional regulations, and with all GCP Guidelines that govern institutional IRB operation.