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Associations between genetic and epigenetic variations in cytokine genes and persistent pain after breast cancer surgery

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Associations between Genetic and Epigenetic Variations in Cytokine Genes and Persistent Breast Pain after Breast Cancer Surgery

by

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DISSERTATION

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Dedication

To my grandmother

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Chapter 2, "Epigenetic Regulation and Measurement of Epigenetic Changes," is a reprint of the material as it appears in *Biological Research for Nursing.* The coauthors listed in this publication directed and supervised the research that forms the basis for this dissertation.

Associations between Genetic and Epigenetic Variations in Cytokine Genes and Persistent Breast pain after Breast Cancer Surgery

Kimberly E. Stephens

Abstract

Persistent pain following breast cancer surgery is a significant clinical problem. Both inherited and acquired inflammatory factors (e.g., cytokines) appear to play a role in the development and maintenance of persistent pain. However, less is known about the molecular mechanisms of inflammation associated with the development of persistent pain following breast cancer surgery. Growth mixture modeling was used to identify persistent breast pain phenotypes based on pain assessments obtained prior to and monthly for six months following breast cancer surgery. The purpose of this dissertation is to evaluate for differences in demographic and clinical characteristics as well as to evaluate the associations between single nucleotide polymorphisms contained within candidate cytokine genes and pain group membership. In addition, methylation of the promoter region of the genes that harbored gene variations associated with pain group membership were evaluated in the no pain and mild pain classes. Different subsets of phenotypic characteristics (i.e., age, strange sensations prior to surgery, reconstruction performed at the time of surgery, re-excision/mastectomy done within six months and worst postoperative pain intensity) and genes (i.e., interleukin (IL) 1 receptor 2, IL4, IL10, IL13 and IL6, tumor necrosis factor alpha (TNFA)) were associated with the distinct phenotypes (i.e., mild persistent pain, severe persistent pain) and suggest that different mechanisms of heritable susceptibility may exist. In addition, CpG methylation

within the TNFA promoter may provide an additional mechanism through which TNFA may alter the risk for mild persistent breast pain after breast cancer surgery. Coupled with phenotypic variations, these genetic and epigenetic variations may help to identify individuals who are predisposed to the development of persistent breast pain following breast cancer surgery, differentiate biological mechanisms, and facilitate the development of novel therapies.

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Chapter 1

Introduction

Introduction

Persistent pain in women following breast cancer surgery is common with an estimated prevalence of between 21% and 55% [3,8,19,29,30,37,38,40,41]. Persistent pain is associated with alterations in mood [38] and sleep patterns [4,14], as well as decreased quality of life [3,29,37] and disability [19,40]. Persistent postsurgical pain may result from ongoing nociceptor activation and/or nerve injury [22]. During the early postoperative period, numerous inflammatory mediators are released that produce peripheral and central sensitization in and around the affected area [42]. These reversible changes in sensitivity to innocuous and noxious stimuli discourage stimulation of the surgical incision which serves as a protective mechanism to facilitates healing. However, sustained activation of nociceptors may lead to the maintenance of central sensitization and phenotypic changes that permanently alter the normal stimulus-response relationship and produce persistent pain. Persistent alterations within nociceptors include changes in gene expression as well as changes in receptor and ion channel distributions within the neuronal membranes [34].

A large body of evidence supports the contribution of the immune system to the development and maintenance of acute and persistent pain [23]. Cytokines are key modulators of the immune response and have an essential role in the sensitization of sensory neurons [17,23,35,36,45]. These small pleiotropic proteins with redundant and sometimes opposing functions are produced on demand by a variety of cell types to mediate communication between cells. While cytokines are not involved directly in nociceptive pain, the effects of pro- and anti-inflammatory cytokines on immune cells are known to modulate nociceptive signaling in acute and chronic inflammation and

following tissue injury and nerve lesions [35,43]. Evidence suggests that ongoing activation of inflammatory and glial cells [24] as well as spinal inhibitory mechanisms [46] play a role in the establishment of persistent pain. Peripheral nerve injury elicits an inflammatory response that prompts the aggregation of immune cells that increases the local concentration of proinflammatory cytokines [23]. These mediators participate in the initiation and maintenance of persistent pain after nerve injury by generating ectopic activity [9], altering neuronal connectivity [13], and reducing the number of inhibitory neurons [10].

While substantial advances have been made in our knowledge of immunomodulation of nociceptive pathways, our current understanding is insufficient to explain variations in pain sensitivity among individuals with persistent pain. Genetic and epigenetic variations within genes encoding inflammatory mediators may cause specific proteins to be available in greater quantities or at times when the protein would otherwise not be present. This altered protein production may modulate nociception and/or alter feedback loops that result in the enhancement of pain and the establishment of persistent pain states.

The human genome contains large amounts of sequence that vary among individuals. These variations contribute to phenotypic variations within the population including differences in how individuals sense and perceive noxious stimuli. Single nucleotide polymorphisms (SNPs) are sites in which the genomic sequence differs by a single nucleotide base (i.e., alleles) at a frequency of at least 1%. SNPs are the most common type of variation with an estimated 10 million sites in the human genome [18]. Genetic association studies evaluate for associations between the presence of specific

SNP alleles and a phenotype (e.g., disease, symptom) in affected and unaffected individuals to identify genetic risk factors [31,32].

Because pain is a complex trait, the effect of common single nucleotide polymorphisms within a single gene on pain sensitivity would likely be small. In addition to the independent contribution of variations within individual genes, the interactions among variations in several genes and the environment in which these genes are expressed may contribute to substantial inter-individual differences in pain perception. Acquired adaptations to genetic regulation are referred to as epigenetics [2,12]. DNA methylation is a key epigenetic mechanism that regulates gene expression in various cell types [6,33]. DNA methylation refers to the covalent addition of a methyl group to one of the DNA nucleotides. In humans, methylation occurs at the 5 carbon position of the cytosine nucleotide when it lies immediately 5' to a guanine nucleotide. Since phosphate groups link nucleotides within the DNA molecule, the Cytosine-Guanine sequence is commonly referred to as the CpG dinucleotide. CpG dinucleotides are aggregated in the promoter region of many genes.

DNA methylation influences the ability of transcription factors and other DNA binding proteins to recognize a nucleotide sequence contained within the promoter that regulates gene expression [15,33]. When DNA methylation occurs in the promoter, gene expression is decreased [6]. Conversely, active gene transcription occurs in the absence of DNA methylation [6,39]. DNA methylation is an essential process for normal development and cellular differentiation [33] and plays a critical role in genome stability and maintenance including genomic imprinting, X-chromosome inactivation, and

suppression of repetitive elements [1,44]. In addition, deregulation of DNA methylation processes are associated with several diseases including the development of cancer [15].

Acquisition of methylation at CpG dinucleotides during a cell's lifetime provides an adaptive capacity for the cell, tissue, and ultimately the organism to adjust to sustained changes in its internal and external environment. A gene containing polymorphisms associated with a specific phenotype may accrue epigenetic adaptation(s) within its promoter in order to modulate gene expression as a dynamic mechanism for improving homeostasis in the setting of a sustained change of environment. However, these attempts at homeostasis could be maladaptive when environmental contexts shift (i.e., surgery for cancer). A mounting body of evidence suggests that epigenetics has the potential to develop biological markers that may be used to predict which individuals would be more susceptible for poorer outcomes following a given exposure, further elucidate biological mechanisms, and facilitate the development of novel therapies.

Pain is a multidimensional experience that results from a complex set of interactions among genetic, environmental, and psychosocial factors that together modulate neuronal processing of nociceptive stimuli. A promising model to study persistent pain in humans is postsurgical pain. Surgery introduces a predictable source of tissue injury that allows for a prospective study design. Breast cancer surgery is associated with a high incidence of persistent postsurgical pain [28]. This model of persistent pain may increase the probability of detecting genetic and epigenetic associations within a relatively homogenous group of individuals. However, within this relatively homogeneous group, several different pain phenotypes have been reported (e.g., phantom breast pain, scar pain) [21]. Scar pain refers to allodynia and spontaneous

pain that occurs at the incision site. Surgical incisions may sever or injure peripheral nerves and result in the formation of neuromas. Neuromas contain entrapped axons and may include other nerve components (i.e., Schwann cells, nerve fascicles). Evidence suggests that abnormal afferent activity originates from neuromas to produce chronic neuropathic pain [16]. Phantom breast pain refers to a painful sensation of a breast that is still present following mastectomy [16]. Evidence suggests that phantom pain is the result of the disruption of normal afferent activity to the spinal cord [7]. In addition, neuromas form as the severed nerve attempts to regenerate [7]. Spontaneous and evoked pain may be the result of aggregation of sodium channels within the neuroma [5].

Recent advances in statistical modeling enable these more homogenous groups of patients to be identified. Standard statistical analyses use means and assume that covariates influence the outcome for all individuals in a similar way over time. Unique groups of individuals may exist within a sample that show similar patterns of change in a particular outcome over time that may not be apparent if measured at a single time point. These subgroups with distinct pain trajectories may be masked through the use of sample means. Growth mixture modeling (GMM) is a statistical approach that groups individuals into latent classes based on similarities in an outcome over time and results in greater homogeneity of members within each latent class [26,27]. GMM may be especially useful in determining different postsurgical pain phenotypes because individuals may differ in the development and resolution of pain over time as a result of genetic and environmental risk factors.

Using data from a large, longitudinal study, GMM identified subgroups of women with distinct persistent breast pain trajectories prior to and for six months following

breast cancer surgery [25]. Three distinct classes were identified using patients' ratings of worst postoperative pain in their affected breast (i.e, mild, moderate, severe pain). A fourth pain group was identified of women who did not experience breast pain preoperatively or at any of the postoperative assessments. The purpose of this dissertation study is to evaluate for differences in demographic and clinical characteristics as well as evaluate the associations between SNPs contained within candidate cytokine genes and pain group membership. In addition, methylation signatures of the promoter region of the genes that harbored gene variations associated with pain group membership were evaluated in the no pain and mild pain classes.

Chapter two of this dissertation is a review of the literature on the best characterized forms of epigenetic regulation (i.e., histone modification, DNA methylation, noncoding RNA expression) and the most common methods used to measure these epigenetic changes. Future studies may identify associations that contribute to interindividual variability in the severity of patients' symptoms. Understanding how to measure these epigenetic processes will assist in the elucidation of mechanisms that produce specific phenotypic changes and identify therapeutic targets.

Chapter three of this dissertation is a study that uses data from the patients who were classified into the no pain and severe pain classes (i.e., the extreme pain phenotype) to: 1) evaluate for differences in demographic and clinical characteristics and 2) evaluate the associations between SNPs contained within cytokine genes and pain group membership. An evaluation of associations between extreme pain phenotypes may increase the effect size that can be detected in genetic association studies [20].

Chapter four of this dissertation evaluates for associations between the no pain and mild pain classes. The largest subgroup of women identified in the GMM analysis was the mild breast pain class (n= 173, 43.5%). Mild levels of persistent postsurgical pain are associated with diminished perceptions of overall health and reduced physical and social functioning [11]. Therefore, using data from the patients who were classified into the no pain and mild pain classes, the purposes of this study were to: 1) evaluate for differences in demographic and clinical characteristics; 2) evaluate the associations between SNPs contained within cytokine genes and pain group membership; 3) determine the methylation status of CpG sites contained within the promoter region of cytokine genes that harbor gene variations associated with pain group membership; and 4) determine if methylation of CpG sites in the promoter was associated with changes in gene expression.

Chapter five summarizes the findings of the dissertation with respect to the GMM pain phenotype, genotype, and epigenotype, describes implications for clinical practice, and future research directions. An evaluation of similarities and differences in the cytokine genes associated with the "mild breast pain" and "severe breast pain" phenotypes may identify similar and/or novel mechanisms for these two distinct pain phenotypes.

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Chapter 2

Epigenetic Regulation and Measurement of Epigenetic Changes

Abstract

Epigenetic mechanisms provide an adaptive layer of control in the regulation of gene expression that enables an organism to adjust to a changing environment. Epigenetic regulation increases the functional complexity of deoxyribonucleic acid (DNA) by altering chromatin structure, nuclear organization, and transcript stability. These changes may additively or synergistically influence gene expression and result in long-term molecular and functional consequences independent of the DNA sequence that may ultimately define an individual's phenotype. This paper (1) describes histone modification, DNA methylation, and expression of small noncoding ribonucleic acid (RNA) species; (2) reviews the most common methods used to measure these epigenetic changes; and (3) presents factors that need to be considered when choosing a specific tissue to evaluate for epigenetic changes.

The consensus sequence of the human genome was published 10 years ago, and information on variation within disease susceptibility genes has increased. Genome search methods (e.g., candidate gene association studies, family-based studies, genome-wide association studies) have uncovered many genetic variants associated with a trait or disease phenotype. However, for any given study, any combination of these variants accounts for only a relatively small proportion of the total phenotypic variance observed in a sample. Therefore, alternative sources of phenotypic variation in heritable traits must exist.

Epigenetics (*epi* meaning above; that is, above the level of the DNA) is defined as changes in phenotype or gene expression caused by a mechanism other than alterations in the underlying DNA sequence (Berger, Kouzarides, Shiekhattar, & Shilatifard, 2009). These changes may remain for the duration of the cell's life, persist through cell division, and be transmitted in a specific cell lineage for multiple generations. Epigenetics provides an adaptive layer of control in the regulation of gene expression to enable the organism to adjust to a changing environment. This additional layer of control is influenced by developmental stage, tissue type, environmental conditions, and disease status of the individual (De Bustos et al., 2009; Keshet et al., 2006; Mikkelsen et al., 2007). Epigenetic mechanisms can alter chromatin structure, nuclear organization, and transcript stability. These changes, alone or in combination, influence gene expression and result in long-term molecular and functional consequences.

For example, in a seminal article by Weaver et al. (2004), maternal care of newborn rats was associated with epigenetic changes that resulted in altered stress reactivity in adult offspring. Inbred newborn rats reared by mothers who provided higher

rates of licking and grooming and arched-back nursing behaviors displayed fewer fear behaviors than pups reared by mothers with lower rates of these maternal care behaviors. This association persisted after pups were cross-fostered to adoptive mothers with different maternal care styles and suggested that the variation in maternal care style was responsible for individual differences in the long-term stress responses of these pups. Subsequent investigation found that maternal care differentially altered the expression of the glucocorticoid receptor gene involved in the stress response pathway. Epigenetic mechanisms, specifically the methylation of the promoter region of this gene and acetylation of histone proteins, altered the ability of a transcription factor to bind to the promoter that resulted in decreases in gene expression.

In addition to critical developmental periods, epigenetic alterations may accumulate throughout the individual's lifetime. Recent studies have investigated the relationship between aberrant epigenetic events on health and predisposition to chronic diseases such as cancer. Cancer development is characterized by the progressive loss of normal physiologic regulation of cellular proliferation which results in uncontrolled cell growth. One of the most recognized epigenetic states that promotes oncogenesis is the inactivation of tumor suppressor genes by hypermethylation of CpG islands located in the promoter of the gene (Rodríguez-Paredes & Esteller, 2011). For example, the Breast Cancer 1 (BRCA1) gene encodes the breast cancer type 1 susceptibility protein that participates in DNA repair (Friedenson, 2007). An increase of DNA methylation of the CpG island located in the promoter region of BRCA1 prevents expression of BRCA1 (Esteller et al., 2000). As a result, damaged DNA is not properly repaired which increases the risk of cancer. While cancer has been the focus of the majority of epigenetic studies,

epigenetic alterations have also been described in other diseases including asthma (Ober & Vercelli, 2011), autism (Nguyen, Rauch, Pfeifer, & Hu, 2010), and diabetic neuropathy (Bell et al., 2010).

As the field of epigenetics matures, linkages will emerge between environmentally induced epigenetic changes and health and disease. These associations may prove useful clinically as biomarkers to establish disease risk, monitor disease progression and guide interventions (Rodriguez-Paredes & Esteller, 2011). For example, cancer-specific hypermethylation of CpG dinucleotides in saliva has been associated with oral cancer and may be useful as a tool to predict the incidence of oral cancer (Viet & Schmidt, 2008).

Future studies may identify associations that contribute to interindividual variability in the severity of patients' symptoms. Epigenetic processes may program cells for alterations in biological pathways in advance of the development of a clinically significant phenotype. Therefore, understanding how to measure these epigenetic processes will assist in the elucidation of mechanisms that produce specific phenotypic changes and identify therapeutic targets.

Any evaluation of epigenetic changes should be interpreted within the context of other genomic analyses because it is the interaction between genetic variations and epigenetic regulatory events that may ultimately define an individual's phenotype. The purpose of this paper is to describe three of the best characterized forms of epigenetic regulation: histone modification, DNA methylation, and noncoding ribonucleic acid (RNA) expression. In addition, we describe the most common methods used to measure

these epigenetic changes. Finally, we discuss the factors to consider when choosing a specific tissue to evaluate for epigenetic changes.

Histone Modifications

Histone modification is defined as a posttranslational modification to one of the amino acid side chains in a histone protein. Epigenetic regulation of chromatin structure through histone modifications enables cells to alter gene expression by modifying a transcription factor's ability to access DNA (Felsenfeld & Groudine, 2003). Almost every human cell contains approximately 6.4 billion nucleotides divided among 46 chromosomes (i.e., one pair of 22 autosomes, two sex chromosomes). As a linear structure, DNA is too long to fit within the nucleus of a cell. As illustrated in Figure 1, a precise combination of proteins produces hierarchical levels of protein–DNA structures that are responsible for organizing, stabilizing, and compacting DNA within the nucleus (Luger & Hansen, 2005).

In a low-ordered structure, chromatin is organized into discrete units called nucleosomes. A nucleosome is made up of eight histone proteins and a 150-nucleotide sequence of DNA. The N-terminal regions of the histones extend from the nucleosome and are referred to as histone tails. These histone tails, composed of amino acids, are subject to a variety of posttranslational modifications (Figure 2; Mersfelder & Parthun, 2006). The functional consequences of these modifications include changes in gene transcription, DNA repair, DNA replication, and chromatin condensation through alterations in the chemical properties and physical conformation of histones (Kouzarides, 2007).

The functional consequence of any single modification depends on the type of modification (e.g., acetylation, phosphorylation) and the site at which it occurs (e.g., amino acid, location in the histone tail). Modifications may act individually, sequentially, and/or in combination to define a "histone code." This histone code can modify access to specific DNA sequences and produce distinct regulatory events (Jenuwein & Allis, 2001). An individual modification may exclude the possibility of other modifications occurring in the same location. Histone modifications can influence the binding of proteins to sites on adjacent amino acid side chains (Fischle et al., 2005) or alter an enzyme's ability to recognize a neighboring site (Clements et al., 2003).

Histone modifications are thought to function through two different mechanisms. First, covalent modification of amino acid side chains may result in either nucleosome–nucleosome interactions or alterations in the contact between the histone and DNA (Grewal & Moazed, 2003). For example, one of the best-studied histone modifications is acetylation of lysine (Shogren-Knaak et al., 2006). Histone tails contain several lysine residues. Lysine has an aliphatic backbone with a terminal amino group. At physiologic pH, lysine has a positive charge that is strongly attracted to negatively charged DNA. These ionic qualities favor the winding of DNA around histones, which results in a condensed chromatin structure. When histones are acetylated by acetyltransferases, the positive charge is removed from lysine and DNA loosens from the histone. Histone deacetylases remove the acetyl group from lysine, which restores the positive charge and promotes a closed chromatin structure.

Second, histone modifications may actively recruit or repel nonhistone gene regulatory proteins (Bhaumik, Smith, & Shilatifard, 2007). Specialized structural

domains within these recruited proteins recognize histone modifications (Higgs, Vernimmen, Hughes, & Gibbons, 2007) and enable these proteins to preferentially associate with modified histone tails. Some modifications may inhibit binding of these proteins, promoting a closed chromatin structure, which represses transcription (Jacobs & Khorasanizadeh, 2002; Margueron, Trojer, & Reinberg, 2005).

Various regions of the eukaryotic genome assume plastic and dynamic chromatin structures. The extent of compaction dictates the expression pattern of the genes contained within a specific segment of DNA. Regions of highly condensed chromatin contain genes that are refractory to expression. As chromatin structures become less condensed, the binding sites for transcriptional proteins are more accessible and the genes within these regions can be transcribed (Hu, Kireev, Plutz, Ashourian, & Belmont, 2009). Histone modifications are dynamic events that occur rapidly in response to cellular signaling and alter compaction of a specific region of DNA. The status of modifications both in the 5' regulatory region and throughout the length of the gene alters access of DNA-binding proteins to DNA. As a result, gene expression varies from silent to active depending on the amount and types of histone modifications that are present (Barski et al., 2007).

Measurement of Histone Modifications

Chromatin immunoprecipitation (ChIP)-based methods are used to measure the presence of histone modifications in specific gene regions (Park, 2009). First, cells from a tissue sample are treated with an agent, such as formaldehyde, to cross-link DNA to nearby histone proteins so that the nucleotide sequence associated with the protein can be determined. Then, cells are lysed and chromatin is broken into small fragments through

sonication. These fragments consist of short lengths of DNA and covalently bound proteins. Antibodies for the modification of interest (e.g., acetylated histone core protein X) are added and bind to their respective epitopes. The Fc region of the antibody is used to precipitate the protein–DNA fragment complex out of solution. Once the antibody–protein–DNA complexes are isolated, the cross-linking is reversed and the proteins are separated from the DNA. The DNA is assayed with sequencing (ChIP-Seq) or microarray (ChIP-Chip) technologies to determine the nucleotide sequence where the protein was bound.

Any modification to histone proteins can be examined with ChIP as long as an antibody is available. When ChIP is combined with microarray technology, the entire genome can be interrogated for a specific histone modification in a DNA–protein interaction. However, the quality of the data depends on several factors including the specificity and affinity of the antibody used and the effectiveness of the precipitation reaction. Many histone modifications do not have an antibody available. In addition, chromatin proteins may block an antibody's access to histone modifications, which would produce false negative findings. Though beyond the scope of this review, several variations of ChIP were developed to overcome many of these limitations (Mito, Henikoff, & Henikoff, 2005; O'Neill & Turner, 2003; van Steensel, Delrow, & Henikoff, 2001).

DNA Methylation

DNA methylation refers to the covalent addition of a methyl group to one of the DNA nucleotides. DNA methylation influences the ability of transcription factors and other DNA-binding proteins to recognize a nucleotide sequence that regulates gene

expression. This process is associated with repression of gene transcription (Jones & Baylin, 2007). While DNA methylation is believed to occur in all organisms, the specific nucleotide and the position of the nucleotide that becomes methylated differs among species (Hattman, 2005). In mammals, methylation occurs predominantly on cytosine at the carbon 5 position of the pyrimidine ring to form 5-methylcytosine (Ehrlich & Wang, 1981).

DNA methylation is an essential process for normal development and cellular differentiation (Robertson, 2005). All nucleated cells within an organism contain DNA that is identical in sequence. DNA methylation enables cells to differentiate and maintain lineage by either downregulating or turning off promoters for genes that are not to be expressed by particular tissues. As these cells replicate, DNA methylases enable cells to retain their differentiated identity by transmitting their DNA methylation pattern during mitosis.

DNA methylation plays a number of roles in promoting genome stability and maintenance including genomic imprinting, X-chromosome inactivation, and suppression of repetitive elements (Avner & Heard, 2001; Wilkins, 2005). Changes in the methylation patterns of DNA during a cell's lifetime provide an adaptive ability for the organism to adjust to changes in the environment. In addition, deregulation of DNA methylation processes is associated with several diseases including the development of cancer (Jones & Baylin, 2007). For example, compared to normal somatic cells, cancer cells are relatively hypomethylated (Robertson, 2005). Loss of normal DNA methylation results in genomic instability and decreased growth controls which permit adaptation and metastasis of tumor cells.

In humans, methylation occurs on the cytosine nucleotide when it lies immediately 5' to a guanine nucleotide. Since phosphate groups link nucleotides within the DNA molecule, the cytosine–guanine sequence is commonly referred to as the CpG dinucleotide. The CpG sequence is paired to the GpC sequence on the complementary strand and methylation is symmetrical. Therefore, cytosine paired with a guanine on the opposite strand is also methylated.

CpG dinucleotides are aggregated in the promoter region of many genes and are referred to as CpG islands. These CpG islands are defined as sequences comprising greater than 50% guanine and cytosine with a ratio of CpG to GpC on the same strand of at least 0.6 (Gardiner-Garden & Frommer, 1987). A common form of DNA damage is the hydrolysis of the cytosine nucleotide. Hydrolysis of cytosine results in the mutation of cytosine to uracil and the release of ammonia (i.e., deamination of cytosine). Both methylated and unmethylated cytosines are subject to spontaneous deamination under physiologic conditions. Deamination of unmethylated cytosine to uracil is repaired accurately by DNA repair mechanisms, leaving the CpG dinucleotide intact. However, deamination of methylated cytosine results in the creation of thymine, which DNA repair mechanisms do not recognize. Over time, this DNA damage-and-repair cycle appears to have resulted in a nonrandom decreased prevalence of the CpG dinucleotide in the human genome. The CpG dinucleotides that have resisted mutation are positioned in regions of DNA where a change in sequence would be deleterious to the organism. For example, within the promoter region of a gene, mutation of a cytosine may affect recognition by sequence-specific transcription factors. Altered expression patterns of that gene may change the organism's phenotype and fitness for survival.

If DNA methylation occurs in the promoter region, gene expression is decreased (Egger, Liang, Aparicio, & Jones, 2004). Conversely, active gene transcription occurs in the absence of DNA methylation (Egger et al., 2004; Tazi & Bird, 1990). DNA methylation represses gene expression through two mechanisms (Singal & Ginder, 1999). First, methylation of regulatory regions of a gene may impede transcription factors from physically binding to the DNA (Hark et al., 2000). Transcription factors bind to specific sequences of DNA in response to extracellular signals and internal programs. Following binding, transcription machinery is recruited to the site and the gene is expressed. When DNA methylation occurs, gene expression is repressed because transcription cannot be initiated.

Second, when cytosines within the promoter region are methylated, methyl-CpG-binding proteins have a greater affinity for the promoter sequence than transcription factors. Binding of the methyl-CpG-binding proteins to CpGs within the promoter physically impedes the binding of transcription factors so the gene cannot be expressed. In addition, the methyl-CpG-binding proteins recruit additional proteins, which bind to each other and form a complex (Bird, 2002; Jin, Jiang, Rauch, Li, & Pfeifer, 2005). These complexes have histone deacetylase activity and alter the structure of chromatin into a closed conformation (Boyes & Bird, 1991). Therefore, transcription factors are not able to gain access to the promoter region, and transcription of that gene is repressed.

Measurement of DNA Methylation Status

The common approaches used to evaluate DNA methylation are high-performance liquid chromatography (HPLC), bisulfite sequencing, and CpG island microarrays. HPLC measures the total amount of methylation present in the DNA.

Bisulfite sequencing and CpG island microarrays allow for locus-specific assessment of methylation (Laird, 2010).

High-Performance Liquid Chromatography (HPLC)

HPLC is one of the most common methods used to separate proteins and small molecules and is a form of column chromatography (Snyder & Dolan, 2006). In addition to collecting fractions enriched for specific molecules, HPLC generates a chromatogram that measures the number of molecules in a given fraction. This quantitative property enables HPLC to determine the total amount of methylated cytosine in a DNA sample (Kuo, McCune, Gehrke, Midgett, & Ehrlich, 1980). In order to use HPLC to quantify the level of methylated cytosine, DNA must be denatured and digested into single nucleotides (Ramsahoye, 2002). Then, HPLC is used to separate nucleotides based on size. The height of each peak on the chromatogram corresponds to the amount of that nucleotide present in the genomic DNA. In this way, HPLC provides a high-throughput measure of total DNA methylation in a sample and can be used to determine differences in methylation between cell types or tissues. However, since the DNA is fully digested into nucleotides, HPLC cannot reveal the location of methylation in the genome. Therefore, other methods are required to determine the methylation status of specific genes or alleles.

Locus-specific Methods for Detection of DNA Methylation

Hybridization- and sequencing-based applications are used to reveal the location of methylated and unmethylated cytosines at a specific locus following methylation-dependent changes to the DNA. The methyl group of 5-methylcytosine is located within the major groove of DNA. Therefore, it does not interfere with base pairing. As a result,

traditional hybridization-based molecular biology techniques are not adequate to evaluate the methylation status of cytosines. Current DNA methylation detection techniques rely on the introduction of methylation-dependent changes to the DNA sequence. These techniques include immunoprecipitation, digestion with restriction endonucleases, and sodium bisulfite treatment. The gold standard for assaying DNA methylation within a specific locus is bisulfite sequencing (Clark, Harrison, Paul, & Frommer, 1994; Frommer et al., 1992).

Bisulfite sequencing. Bisulfite sequencing provides accurate data regarding 5-methylcytosine content at the single nucleotide level of resolution. Through this process, sodium bisulfite is used to convert unmethylated cytosine to uracil (Hayatsu, Wataya, Kai, & Iida, 1970). Methylated cytosine is protected from conversion and remains cytosine. Then the region of interest can be amplified by polymerase chain reaction (PCR) and sequenced through conventional means. The locations of all methylated cytosines can then be determined because they are the only loci where cytosines remain. Following bisulfite treatment, the two DNA strands are no longer complementary. Therefore, one pair of primers specific for bisulfite-converted DNA must be designed for each strand that will be sequenced.

PCR primers for bisulfite-converted DNA templates must be designed carefully to achieve accurate results (Li & Dahiya, 2002). Following PCR amplification, uracil is amplified as thymine. Methylated cytosines are amplified as unmethylated cytosines. Primers should not include CpG dinucleotides, and thymine must be substituted in place of all cytosines that are not located within a CpG dinucleotide. If CpGs are included within the primer sequence, the methylation status of the cytosines prior to bisulfite

treatment may affect hybridization of the primer to the target sequence, introducing measurement bias.

Bisulfite sequencing has several limitations (Laird, 2010). The success of this method is dependent on complete conversion of unmethylated cytosine into uracil. Incomplete conversion may produce false positive results if the unconverted nucleotides are located within the region of interest. Because most cytosine will be converted to thymine during PCR, the complexity of the DNA sequence is reduced (i.e., DNA is comprised of three nucleotides instead of four). Therefore, optimizing PCR may be more difficult than in nonbisulfite-converted DNA samples. Additionally, prolonged incubation with sodium bisulfite may degrade DNA, and single nucleotide polymorphisms within the region of interest may complicate interpretation of the results.

Furthermore, there are several limitations related to the current state of bioinformatics (Laird, 2010). The current generation of sequencing technology limits the length of the region that can be examined. Sequencing generates large data files for short reads. Therefore, extensive computing resources and bioinformatics tools are required to align and analyze data on the genomic scale. Data processing and analysis are complicated due to the need for a bisulfite-converted reference genome. As new sequencing technologies are developed, locus-specific amplification may not be required and whole-genome coverage will become feasible.

CpG island microarray. In order to interrogate larger areas of DNA including genome-wide coverage and overcome many of the limitations of bisulfite sequencing, investigators routinely use CpG island microarrays. Microarrays provide high-throughput assessment of methylation status of up to hundreds of thousands of CpG sites in parallel

using hybridization (Figure 3). For a detailed review on CpG island microarray see the paper by Gitan, Shi, Chen, Yan, & Huang (2002).

Advantages of microarray analysis are that only small amounts of genomic DNA are required, and whole genome coverage is possible. The widespread availability of software programs to analyze and interpret array data renders this method more feasible than large amounts of sequencing output. However, compared to sequencing, microarrays have lower resolution, and the areas of the genome that need to be examined must be known so specific probes can be developed. Designing highly specific probes is critical to the success of this technology. Imperfect base-pairing between the probe and target sequences produces erroneous results (Gitan et al., 2002). In addition, interrogation of areas contained within repetitive sequences is limited by the ability to develop probes that are unique to the specific locus of interest.

Small RNA Expression

The role of RNA as an intermediate carrier of genetic information is well established. Noncoding RNAs were initially believed to consist of ribosomal RNAs and transfer RNAs that are dedicated to protein synthesis. However, the development of specialized methods to detect and quantify small RNAs has led to the discovery of novel species. In the mammalian genome, three classes of small, noncoding RNAs are known to exist (i.e., microRNA [miRNA], endogenous small-interfering RNA [endo-siRNA], and piwi-interacting RNA [piRNA]) (Kim, Han, & Siomi, 2009). These classes of RNAs are believed to regulate gene expression posttranscriptionally by targeting messenger RNAs and silencing transposons through heterochromatin formation. These small RNAs are generally classified by nucleotide length, biogenesis, and their associated proteins

(Kim, Han, & Siomi, 2009). Of note, these newly discovered species of noncoding RNAs are conceptualized to function in an epigenetic manner. However, the inclusion of transient gene expression as an epigenetic mechanism is under debate.

While the role of endo-siRNAs in physiologic processes is unknown, current evidence suggests that miRNAs play critical roles in the development of the organism (Ambros, 2003; Chen, Li, Lodish, & Bartel, 2004) and in cell differentiation (Calin et al., 2002; Calin et al., 2004). In addition, miRNAs are associated with oncogenesis (Calin et al., 2002, 2004) and human disease (Perera & Ray, 2007). Evidence suggests that piRNAs suppress gene expression by influencing the activity of methyltransferases (Aravin et al., 2008) and alter DNA methylation patterns in a sequence-specific manner (Watanabe et al., 2011). Additional information on small noncoding RNAs can be found in a recent review by Wery, Kwapisz, and Morillon (2011).

Due to the potential impact of tissue-specific and temporal expression of miRNA genes, highly sensitive methods that are capable of assessing large numbers of genes are required to verify the roles of miRNAs identified using *in silico* approaches. The most common methods used to measure small noncoding RNA are quantitative real-time polymerase chain reaction (qPCR), microarrays, and sequencing.

Choice of Cell Type for Evaluation of Epigenetic Regulation

All nucleated cells within an organism contain identical copies of DNA. However, distinct cell types display a variety of structural and functional differences related to differences in gene expression that define their phenotype. Epigenetic mechanisms regulate gene expression transcriptionally and posttranscriptionally. The dynamic cell-type specific changes that occur through epigenetic mechanisms during

development and in disease states must be considered when using any of the methods described previously.

Many of the tissues of interest for epigenetic profiling (e.g., cerebral spinal fluid, cardiac muscle) require invasive procedures that limit their accessibility for clinical investigations. Epigenetic profiles in surrogate tissues can be used to assess and monitor epigenetic status in more remote tissues. Peripheral blood contains several types of circulating cells, including peripheral blood mononuclear cells (PBMCs), that are exposed to molecular signals produced by disease processes in remote tissues. Importantly, peripheral blood samples are easy and inexpensive to obtain.

An assessment of global epigenetic expression within PBMCs may identify a set of genes that are sensitive to the trait/condition of interest. These expression patterns provide an "epigenetic signature" that can be used to measure changes over time. These signatures may identify novel mechanistic pathways involved in the pathophysiology of a condition. PBMCs have been used to evaluate epigenetic changes in a variety of conditions including heart failure (Voellenkle et al., 2010) and transplant rejection (Anglicheau et al., 2009). These studies underscore the central role of neuroimmunomodulation in these disease states. The sensitivity of PBMCs as mediators of inflammatory processes may help investigators to identify additional biomarkers that are capable of determining changes in health status.

Conclusions

Epigenetic regulation increases the functional complexity of DNA by providing mechanisms for modifying cellular processes. A comprehensive understanding of health and disease involves an evaluation of both the DNA sequence and epigenetic modulation

of gene expression. Several forms of epigenetic regulation exist, and a variety of methods can be employed to characterize them. The choice of method depends on a variety of factors including the number of samples that will be analyzed, the amount of DNA available, the nature of the samples, and the resources available. The progressive development of improved techniques and the refinement of current methods will enable a more comprehensive understanding of additional types of epigenetic regulation and their interactions with genetic variation.

Epigenetic control mechanisms may additively or synergistically interact to precisely respond to internal and external environmental cues. Determining the hierarchical relationships involved in epigenetic regulation and identifying mechanisms that are differentially expressed in different cell types will increase our understanding of gene expression and protein regulation. Comparative studies of epigenetic mechanisms involved in physiologic processes may reveal transcriptional and translational changes in genes that can be used to develop biomarkers and therapeutic targets for specific diseases. In combination with genomics, an improved understanding of epigenetic regulation of gene expression may provide a more comprehensive understanding of human health and the trajectories of various diseases.

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Figure Legends

Figure 1. Chromatin organization**.** Chromatin is arranged into nucleosomes as its first level of organization. A nucleosome is made up of histone proteins and double-stranded deoxyribonucleic acid (DNA) that wraps around a histone octomer 1.7 times in a left-handed coil. Hydrogen bonds hold the phosphodiester backbone of DNA to the amino acid side chains of the histones. Each nucleosome is separated from the next by a short segment of DNA. The conformation resembles a "beads-on-a-string" structure. Further condensation of chromatin structure is facilitated by histone "tails" that extend from the nucleosome and help to stack nucleosomes by linking them to neighboring nucleosomes. These circular stacks of nucleosomes form a solenoid that results in a chromatin fiber that measures 30 nanometers in diameter. As the final chromatin structure, chromosomes are thought to be the result of a mesh formed through fibers connected by cross-linked proteins or as a result of hierarchical packaging of chromatin around a central axis mediated by structural maintenance of chromosome proteins. Reprinted by permission from Macmillan Publishers Ltd.: Felsenfeld & Groudine, 2003.

Figure 2. Posttranslational modifications of the N-terminal region of histone H4.
The majority of the known histone modifications affect the amino acids that make up the unstructured N-terminal region of the histone core protein. Covalent modifications of the human H4 histone protein include phosphorylation (P), acetylation (Ac), and methylation (Me). Single letter abbreviations for amino acid residues: A = alanine; G = glycine; H = histidine; K = lysine; L = leucine; R = arginine; S = serine.

Figure 3. CpG island microarray. Following amplification of the region of interest by polymerase chain reaction (PCR), array-based methods rely on hybridization of a sample of deoxyribonucleic acid (DNA) to oligonucleotides contained within the array. Sodium bisulfite treatment of DNA produces a change in the nucleotide sequence of all unmethylated cytosines. Amplification of a specific locus of interest is performed, and a "tag" is incorporated into the PCR products using modified PCR primers that feature one of two unique sequence tails (i.e., one primer detects the methylated state and has one specific tag, while the other primer detects the unmethylated state and has a different tag). This procedure results in fluorescently labeled PCR products with a nucleotide sequence specific to its methylation status. A set of two oligonucleotides is synthetically made with their sequences complementary to the unmethylated and methylated sequence of the labeled PCR products and is attached to the surface of a glass slide. These immobilized oligonucleotides serve as a target for the labeled PCR products and are referred to as probes. The labeled PCR products are incubated with an array of immobilized probes to permit complementary sequences to hybridize to a given probe. A high-resolution camera captures the position of emitted fluorescence. The difference in signal intensities between the paired methylated and unmethylated alleles is used to calculate the percentage of methylation for the sequence associated with the probe. Modified from "Methylation-specific oligonucleotide microarray: A new potential for high-throughput methylation analysis," by R. S. Gitan, H. Shi, C.-M. Chen, P. S. Yan, & T. H.-M. Huang, *Genome Research, 12*, p. 159. Copyright 2002 by Cold Springs Harbor Laboratory Press.

Figure 1

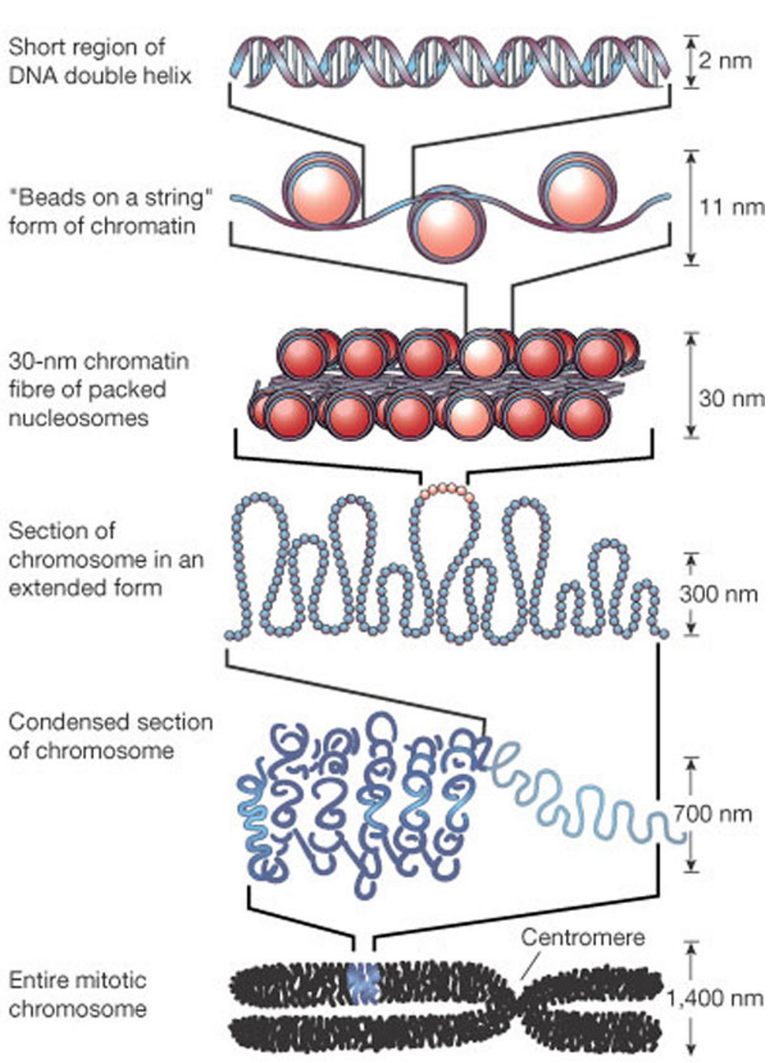

Short region of DNA double helix
2 nm
"Beads on a string" form of chromatin
11 nm
30-nm chromatin fibre of packed nucleosomes
30 nm
Section of chromosome in an extended form
300 nm
Condensed section of chromosome
700 nm
Centromere
Entire mitotic chromosome
1,400 nm

Figure 2

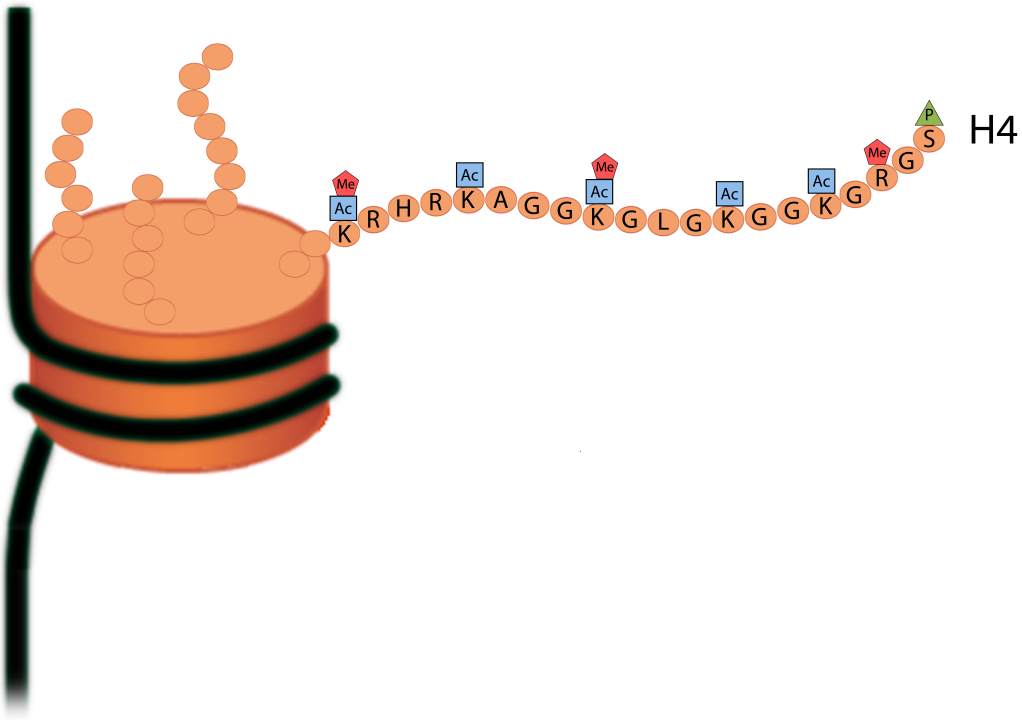
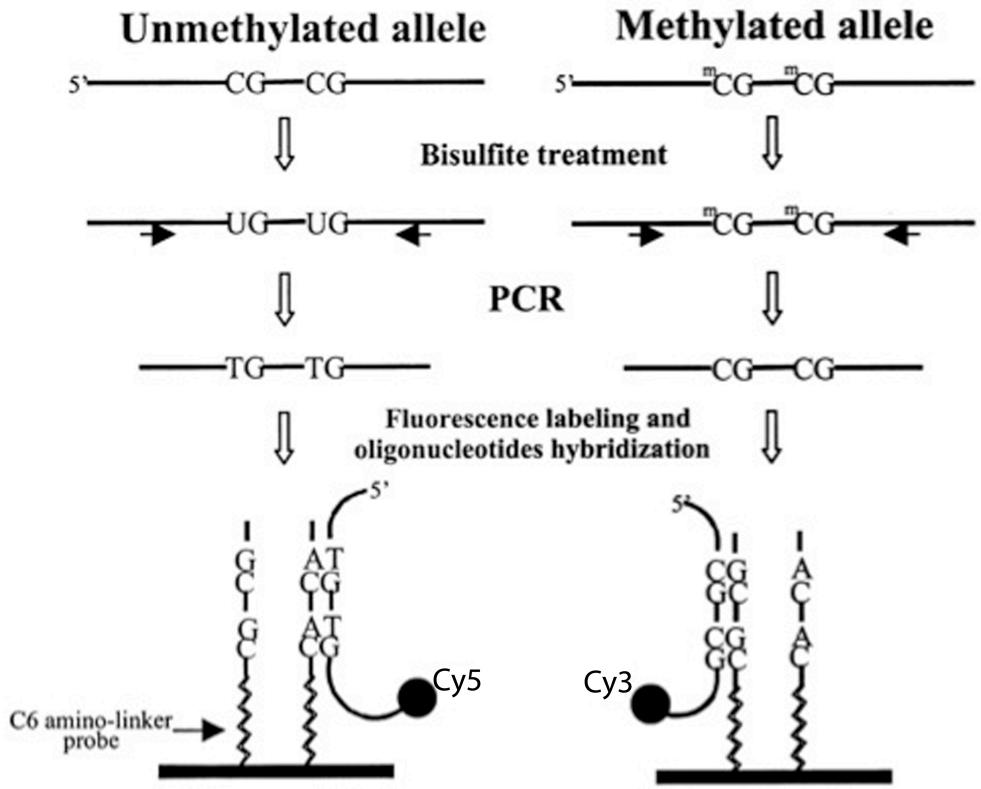


Figure 3


Unmethylated allele
Methylated allele
5'
CG CG
Bisulfite treatment
UG UG
PCR
TG TG
CG CG
Fluorescence labeling and
oligonucleotides hybridization
5'
Cy5
Cy3
C6 amino-linker
probe

Chapter 3

Associations between cytokine gene variations and severe persistent breast pain in women following breast cancer surgery

Abstract

Persistent pain following breast cancer surgery is a significant clinical problem. While immune mechanisms may play a role in the development and maintenance of persistent pain, few studies have evaluated for associations between persistent breast pain following breast cancer surgery and variations in cytokine genes. In this study, associations between previously identified extreme persistent breast pain phenotypes (i.e., no pain versus severe pain) and single nucleotide polymorphisms (SNPs) spanning 15 cytokine genes were evaluated. In unadjusted analyses, the frequency of 13 SNPs and 3 haplotypes in 7 genes differed significantly between the no pain and severe pain classes. After adjustment for severity of worst postoperative pain, three SNPs (i.e., interleukin (IL) 1 receptor 2 rs11674595; IL4 rs2243248; IL13 rs1800925) and one haplotype (i.e., IL10 haplotype A8) were associated with pain group membership. These findings suggest a role for cytokine gene polymorphisms in the development of persistent pain following breast cancer surgery. Coupled with phenotypic variations, these genetic markers may help to identify patients who are predisposed to the development of severe persistent breast pain following breast cancer surgery.

Introduction

Persistent pain in women following breast cancer surgery is common, with an estimated prevalence of between 21% and 55%.[1-9] Persistent pain is associated with depressed mood,[7] sleep disturbance,[10, 11] decreased quality of life,[1, 4, 6] and disability.[3, 8] Persistent postsurgical pain may result from ongoing nociceptor activation and/or nerve injury.[12] During the early postoperative period, release of numerous inflammatory mediators produce peripheral sensitization in and around the surgical site.[13] These reversible changes in sensitivity to innocuous and noxious stimuli discourage stimulation of the surgical incision and facilitate healing. However, sustained activation of nociceptors may lead to the maintenance of central sensitization and phenotypic changes that alter the normal stimulus-response relationship and produce persistent pain. Evidence suggests that ongoing activation of inflammatory and glial cells[14] and spinal inhibitory mechanisms[15] play a role in the establishment of persistent pain. In addition, peripheral nerve injury prompts the aggregation of immune cells that increases the local concentration of proinflammatory cytokines.[16] These mediators participate in the initiation and maintenance of persistent pain by generating ectopic activity,[17] altering neuronal connectivity,[18] and reducing the number of inhibitory neurons.[19]

While several studies have identified phenotypic characteristics that predispose patients to the development of persistent pain following breast cancer surgery,[20-24] less is known about the molecular mechanisms associated with this significant clinical problem. In fact, despite the strong evidence that persistent activation of immune mechanisms results in persistent pain,[16] only four studies have evaluated for associations between polymorphisms in cytokine genes and cancer-related pain.[25-28] Three of these studies[26-28]

assessed pain intensity prior to the initiation of cancer treatment. Associations were found between severe pain (i.e., pain rated >6 on a 0 to 10 numeric rating scale (NRS)) and interleukin (IL) 1 beta (IL1B) rs1143627,[26] IL8 rs4073,[26, 27] and tumor necrosis factor alpha (TNFA) rs1800629.[28] However, findings from these studies are difficult to interpret because the pain phenotype was characterized using only a dichotomized pain severity rating, had modest sample sizes, and the number of polymorphisms evaluated was not optimal. Recent work from our group evaluated for associations between variations in cytokine genes and pain in the affected breast of women prior to breast cancer surgery.[25] Associations were found between the presence of preoperative pain and IL1 receptor 1 (IL1R1) rs2110726 and IL13 rs1295686. Of note, no studies were found that evaluated for associations between cytokine gene polymorphisms and persistent postsurgical pain.

In this same sample of women assessed for pain prior to breast cancer surgery,[25] growth mixture modeling (GMM) was used to identify subgroups of women with distinct persistent breast pain trajectories prior to and for six months following breast cancer surgery.[29] In brief, three distinct classes were identified using patients' ratings of worst pain in their breast (i.e, mild, moderate, severe; Figure 1). A fourth pain class was identified of women who did not experience breast pain preoperatively or at any of the postoperative assessments. An evaluation of associations between extreme pain phenotypes may increase the effect size that can be detected in genetic association studies.[30] Therefore, using the extreme pain phenotypes identified in this GMM analysis, the purposes of this study were to evaluate for differences in demographic and clinical

characteristics, as well as for variations in cytokine genes, between the no pain and severe pain classes.

Materials and Methods

Patients and Settings

This study is part of a larger, longitudinal study that evaluated for neuropathic pain and lymphedema in a sample of women who underwent breast cancer surgery.[25, 29] Patients were recruited from Breast Care Centers located in a Comprehensive Cancer Center, two public hospitals, and four community practices. Patients were eligible to participate if they: were an adult woman (≥18 years) who would undergo breast cancer surgery on one breast; were able to read, write, and understand English; agreed to participate; and gave written informed consent. Patients were excluded if they were having breast cancer surgery on both breasts and/or had distant metastasis at the time of diagnosis. A total of 516 patients were approached to participate and 410 were enrolled in the study (response rate 79.5%). The major reasons for refusal were: too busy, overwhelmed with the cancer diagnosis, or insufficient time available to do baseline assessment prior to surgery.

Subjective Measures

The demographic questionnaire obtained information on age, education, ethnicity, marital status, employment status, living situation, and financial status. The Karnofsky Performance Status (KPS) scale is widely used to evaluate functional status in patients with cancer and has well established validity and reliability.[31, 32] Patients rated their functional status using the KPS scale that ranged from 30 (I feel severely disabled and

need to be hospitalized) to 100 (I feel normal; I have no complaints or symptoms). Patients were asked to indicate if they exercised on a regular basis (yes/no format).

The Self-Administered Comorbidity Questionnaire (SCQ) is a short and easily understood instrument that was developed to measure comorbidity in clinical and health service research settings.[33] The questionnaire consists of 13 common medical conditions that were simplified into language that could be understood without any prior medical knowledge. Patients were asked to indicate if they had the condition using a "yes/no" format. If they indicated that they had a condition, they were asked if they received treatment for it (yes/no; proxy for disease severity) and did it limit their activities (yes/no; indication of functional limitations). Patients were given the option to add three additional conditions not listed on the instrument. For each condition, a patient can receive a maximum of 3 points. Because there are 13 defined medical conditions and 2 optional conditions, the maximum score totals 45 points if the open-ended items are used and 39 points if only the closed-ended items are used. The SCQ has well-established validity and reliability and has been used in studies of patients with a variety of chronic conditions.[33-37]

Preoperative and persistent, as well as acute postoperative pain ratings were evaluated using the Breast Symptoms Questionnaire (BSQ) and Postsurgical Pain Questionnaire, respectively. The BSQ consists of two parts. Part 1 obtained information on the occurrence of pain and the occurrence of other symptoms in the breast scar area (i.e., swelling, numbness, strange sensations, hardness). The additional symptoms that were assessed were identified in studies by Tasmuth and colleagues.[38, 39] If the patient had pain in the breast scar area, they completed Part 2 of

the BSQ. Patients were asked to rate the intensity of their average and worst pain using a 0 (no pain) to 10 (worst imaginable pain) NRS. A NRS is a valid and reliable measure of pain intensity.[40]

The Postsurgical Pain Questionnaire evaluated pain intensity in the first 24 to 48 hours after surgery. Average and worst pain were rated using a 0 (no pain) to 10 (worst imaginable pain) NRS. This questionnaire was completed during the month 1 study visit.

Study Procedures

The study was approved by the Committee on Human Research at the University of California, San Francisco and by the Institutional Review Boards at each of the study sites. During the patient's preoperative visit, a clinician explained the study to the patient and determined her willingness to participate. For those women who were willing to participate, the clinician introduced the patient to the research nurse. The research nurse met with the women, determined eligibility, and obtained written informed consent prior to surgery. After obtaining written informed consent, patients completed the enrollment questionnaires (Assessment 0).

Patients were contacted two weeks after surgery to schedule the first postsurgical appointment. The research nurse met with the patients either in their home or in the Clinical Research Center at 1, 2, 3, 4, 5, and 6 months after surgery. During each of the study visits, the women completed the study questionnaires, and provided information on new and ongoing treatments. Over the course of the study, patients' medical records were reviewed for disease and treatment information.

Characterization of the persistent breast pain phenotype

Characterization of the persistent breast pain phenotype used in this study was described previously.[29] At each assessment, patients were asked, "Are you experiencing pain in your affected breast?" If the patient reported pain, she was asked to rate her "current pain at its worst" using a 0 (no pain) to 10 (worst pain) NRS. Prior to conducting GMM analyses, patients who reported no pain in their affected breast for all 6 assessments (i.e., enrollment and 2, 3, 4, 5, and 6 months) were identified (N=126; 31.7%) and were not included in the GMM analysis. For the remaining 272 women, the six ratings of worst breast pain were used in the GMM analysis to assign each individual into a latent class. Pain ratings obtained at the 1-month follow-up assessment were excluded from the model. The high prevalence of pain at the month 1 assessment reduced the variability in pain ratings among the patients. This reduced variability prohibited the determination of latent classes when month 1 ratings were included in the GMM.

A single, unadjusted growth curve that represented the "average" change trajectory was estimated for the sample. Then, the number of latent growth classes that best fit the data were identified using guidelines recommended in the literature.[41-43] Model fit was assessed statistically by identifying the model with the lowest Bayesian Information Criterion (BIC). The parametric bootstrapped likelihood ratio test (BLRT) was used to evaluate whether a model with K classes fit the data better than a model with K-1 classes. In addition to using the BLRT to compare models, the Vuong-Lo-Mendell-Rubin Likelihood Ratio Test (VLMR) for the "K" versus "K-1" class models were examined. When the VLMR test is non-significant, it provides evidence that the K-class model is not better than the K-1 class model. The fourth index used to evaluate model fit was entropy, with >.80 being preferred.[44, 45] Finally, the best fitting model was visually

inspected by plotting observed against model-predicted values to determine whether the predicted trajectories followed the empiric trajectories for the classes, and to evaluate whether the predicted plots "made sense" theoretically and clinically.[41-43] The GMM analyses were done using MPlus 6.1.[45]

Descriptive statistics and frequency distributions for the no breast pain and severe breast pain classes were generated for demographic and clinical characteristics using Stata version 12.1 (StataCorp, College Station, TX). Independent sample t-tests, Mann-Whitney U tests, Chi square tests, and Fisher's Exact tests were used to evaluate for differences in demographic and clinical characteristics between the two breast pain classes. Logistic regression analysis was performed to evaluate the association between phenotypic characteristics and pain group membership. All phenotypic characteristics that were identified in the bivariate analyses as being different between the pain classes were evaluated for inclusion in the multivariate analysis based on a review of the literature. A backwards stepwise approach was used to create the most parsimonious model. Only predictors with a p-value of $<.05$ were retained in the final model. These predictors were used in the logistic regression analyses to evaluate the associations between genotype and pain group membership.

Genotype determination

Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood mononuclear cells using the PUREGene DNA Isolation System (Invitrogen, Carlsbad, CA). DNA was available from 310 of the 398 patients. DNA samples were quantitated with a Nanodrop Spectrophotometer (ND-1000; Nanodrop Products, Wilmington, DE) and normalized to a concentration of 50 ng/μL (diluted in 10 mM Tris/1 mM EDTA).

Genotyping was performed blinded to clinical status and positive and negative controls were included. Samples were genotyped using the Golden Gate genotyping platform (Illumina, San Diego, CA) and processed according to the standard protocol using GenomeStudio (Illumina, San Diego, CA). Two blinded reviewers visually inspected signal intensity profiles and resulting genotype calls for each single nucleotide polymorphism (SNP). Disagreements were adjudicated by a third reviewer.

A combination of tagging SNPs and literature driven SNPs (i.e., reported as being associated with altered function and/or symptoms) were selected for analysis. Tagging SNPs were required to be common (i.e., defined as having a minor allele frequency (MAF) of ≥.05) in public databases (e.g., HapMap). In order to ensure robust genetic association analyses, quality control filtering of SNPs was performed. SNPs with call rates <95%, Hardy-Weinberg $p < .001$, and/or a MAF of <5% were excluded. A total of 82 SNPs from 15 inflammatory cytokine genes (i.e., interferon gamma (IFNG): 5 SNPs; IFNG receptor 1 (IFNGR1): 1 SNP; IL1B: 12 SNPs; IL1R1: 4 SNPs; IL1 receptor 2 (IL1R2): 3 SNPs; IL2: 3 SNPs; IL4: 2 SNPs; IL6: 9 SNPs; IL8: 3 SNPs; IL10: 7 SNPs; IL13: 4 SNPs; IL17A: 5 SNPs; nuclear factor kappa beta-1 (NFKB1): 11 SNPs; NFKB2: 4 SNPs; TNFA: 9 SNPs) passed all quality control filters and are included in subsequent analyses. Potential functional roles of SNPs associated with persistent breast pain were examined using PUPASuite 2.0,[46] a comprehensive search engine that examines for a series of putative functional effects (i.e., non-synonymous changes, altered transcription factor binding sites, exonic splicing enhancing or silencing, splice site alterations, microRNA target alterations).

Statistical analysis

Allele and genotype frequencies were determined by gene counting. Hardy-Weinberg equilibrium was assessed by the Chi-square test. Measures of linkage disequilibrium (i.e., D' and r^2) were computed from the patients' genotypes with Haploview 4.2. Linkage disequilibrium (LD)-based haplotype block definition was based on the D' confidence interval method.[47]

For SNPs that were members of the same haploblock, haplotype analyses were conducted in order to localize the association signal within each gene and to determine if haplotypes improved the strength of the association with the phenotype. Haplotypes were constructed using the program PHASE version 2.1.[48] In order to improve the stability of haplotype inference, the haplotype construction procedure was repeated five times using different seed numbers with each cycle. Only haplotypes that were inferred with probability estimates of ≥.85, across the five iterations, were retained for downstream analyses. Haplotypes were evaluated assuming a dosage model (i.e., analogous to the additive model).

Ancestry informative markers (AIMs) were used to minimize confounding due to population stratification.[49-51] Homogeneity in ancestry among patients was verified by principal component analysis[52] using Helix Tree (Golden Helix, Bozeman, MT). Briefly, the number of principal components (PCs) was sought which distinguished the major racial/ethnic groups in the sample by visual inspection of scatter plots of orthogonal PCs (i.e., PC 1 versus PC2, PC2 versus PC3). This procedure was repeated until no discernible clustering of patients by their self-reported race/ethnicity was possible (data not shown). The first three PCs were selected to adjust for potential confounding due to

population substructure (i.e., race/ethnicity) by including the three covariates in all regression models. One hundred and six AIMs were included in the analysis.

For association tests, three genetic models were assessed for each SNP: additive, dominant, and recessive. Barring trivial improvements (i.e., delta <10%), the genetic model that best fit the data, by maximizing the significance of the p-value, was selected for each SNP. Logistic regression analysis, that controlled for significant covariates, as well as genomic estimates of and self-reported race/ethnicity, was used to evaluate the associations between genotype and pain group membership. A backwards stepwise approach was used to create the most parsimonious model. Except for genomic estimates of and self-reported race/ethnicity, only predictors with a p-value of <.05 were retained in the final model. Genetic model fit and both unadjusted and covariate-adjusted odds ratios were estimated using Stata version 12.1.

As was done in our previous studies,[25, 53] based on recommendations in the literature,[54, 55] the implementation of rigorous quality controls for genomic data, the non-independence of SNPs/haplotypes in LD, and the exploratory nature of the analyses, adjustments were not made for multiple testing. Significant SNPs identified in the bivariate analyses were evaluated further using regression analyses that controlled for differences in phenotypic characteristics, potential confounding due to population stratification, and variation in other SNPs/haplotypes within the same gene. Only those SNPs that remained significant are included in the final presentation of the results. Therefore, the significant independent associations reported are unlikely to be due solely to chance. Unadjusted associations are reported for all SNPs passing quality control criteria in Table 1 to allow for subsequent comparisons and meta-analyses.

Results

Differences in demographic and clinical characteristics between pain classes

Of the 398 women who completed the presurgical assessment, 126 (31.7%) were classified into the no breast pain class and 46 (11.6%) were classified into the severe breast pain class. Differences in demographic and clinical characteristics among the four breast pain classes at the time of enrollment are described in detail elsewhere.[29] Differences in demographic and clinical characteristics between the no breast pain and severe breast pain classes are provided in Table 2.

Women who were classified into the severe breast pain class were significantly younger, had fewer years of education, and were more likely to have an annual household income below $20,000 than women in the no breast pain class. In terms of ethnicity, post-hoc analyses revealed that the representation of Whites was greater in the no breast pain class (73%) than in the severe breast pain class (41%) (Bonferroni-corrected p-value = 0.006).

In terms of preoperative clinical characteristics, women in the severe breast pain class reported a higher number of comorbidities (i.e., SCQ score), lower functional status (i.e., KPS score), and were more likely to have a history of back pain and rheumatoid arthritis than women in the no breast pain class. Forty-three percent of women in the severe breast pain class, compared to 2.4% in the no breast pain class, reported pain in the affected breast prior to surgery. Women in the severe breast pain class were more likely to report swelling, numbness, strange sensations, and hardness in their affected breast prior to surgery compared to women in the no breast pain class.

Differences between the no breast pain and severe breast pain classes were found in a number of surgical and postoperative characteristics. Compared to the no breast pain class, women in the severe pain class had a greater number of lymph modes removed, reported higher average and worst postoperative pain scores, and were more likely to have re-excision or mastectomy within 6 months after surgery.

Regression analysis for phenotypic characteristics

Based on a review of the literature that identified associations between specific phenotypic characteristics and persistent pain, the following characteristics were evaluated in the logistic regression analysis: age,[20] ethnicity,[21] KPS score,[56-58] SCQ score,[59, 60] history of back pain,[2] whether strange sensations were present in the affected breast prior to surgery,[3] number of lymph nodes removed,[61, 62] severity of worst postoperative pain,[7, 23, 24, 38, 63] and whether re-excision or mastectomy was performed within six months after surgery.[9] The only predictor that remained significant in the final regression model of clinical and demographic characteristics was the severity of worst postoperative pain. For each one-unit increase in worst postoperative pain, the odds of being in the severe pain class increased 1.82-fold (95% confidence interval (CI): 1.47, 2.25; $p<0.001$).

Regression analyses for candidate genes

As summarized in Table 1, no associations were found between pain group membership and SNPs in IFNGR1, IL1B, IL2, IL6, IL8, IL17A, NFKB2, and TNFA. However, the genotype frequency was significantly different between the no breast pain and severe breast pain classes for 13 SNPs and 3 haplotypes among 7 genes (IFNG: 2

SNPs, 1 haplotype; IL1R1: 1 SNP; IL1R2: 1 SNP; IL4: 1 SNP; IL10: 3 SNPs, 1 haplotype; IL13: 4 SNPs, 1 haplotype; NFKB1: 1 SNP).

In order to better estimate the magnitude (i.e., odds ratio, OR) and precision (i.e., CI) of genotype on pain group membership, multivariate logistic regression models were fit. In addition to genotype, the phenotypic variables included in the regression models were genomic estimates of and self-reported race/ethnicity (i.e., White, Black, Asian, Hispanic/Mixed ethnic background/other) and severity of worst postoperative pain. As shown in Table 4, the genetic associations that remained significant were for IL1R2 rs11674595, IL4 rs2243248, IL10 haplotype A8, and IL13 rs1800925 (see Table 4 and Figures 2 and 3).

In the regression analysis for IL1R2 rs11674595 (Figure 2A), individuals who were homozygous for the rare "C" allele (i.e., TT + CT versus CC) had a 28.3-fold increase in the odds of belonging to the severe breast pain class (95% CI: 2.37, 338.58, p=0.008).

In the regression analysis for IL4 rs2243248 (Figure 2B), carrying one or two doses of the rare "G" allele (i.e., TT versus TG + GG) was associated with a 6.1-fold increase in the odds of belonging to the severe breast pain class (95% CI: 1.08, 34.93, p=0.041).

In the regression analysis for IL10 haplotype A8, each dose of this haplotype decreased the odds of belonging to the severe breast pain class by 75% (95% CI: 11%, 93%, p=0.032). The IL10 haplotype A8 is composed of seven SNPs (i.e., rs3024505 "C" allele, rs3024498 "G" allele, rs3024496 "C" allele, rs1878672 "G" allele, rs1518111 "A" allele, rs1518110 "T" allele, rs3024491 "T" allele) (Figure 3).

In the regression analysis for IL13 rs1800925 (Figure 2C), carrying one or two doses of the rare "T" allele (i.e., CC versus CT + TT) was associated with a 3.6-fold increase in the odds of belonging to the severe breast pain class (95% CI: 1.01, 12.87, p=0.049).

Discussion

This study is the first to evaluate for associations between variations in cytokine genes and the development of persistent breast pain in women following breast cancer surgery. Consistent with previous reports (for review see Anderson and Kehlet[20]), differences in a number of demographic and clinical characteristics were found between the no pain and severe breast pain classes in the bivariate analyses. However, as shown in Table 3, the severity of worst postoperative pain was the only phenotypic characteristic that remained significant in the multivariate analysis. Of note, compared to the no pain class (4.2±2.6), the mean worst postoperative pain intensity score reported by patients in the severe pain class (7.9±2.5) represented not only a statistically significant ($p<0.001$), but a clinically meaningful, difference (d=1.20) in pain intensity scores. While the postoperative pain scores for patients in the no pain class are in the moderate range, based on work by Dihle and colleagues,[64] worst pain scores for patients in the severe pain class are in the severe range.

Findings from this study suggest that inadequately treated postoperative pain is a significant risk factor for the development of severe persistent breast pain in women following breast cancer surgery. One can hypothesize that sensitized and injured peripheral nerves produce intense and prolonged afferent ectopic activity that is transmitted to dorsal horn neurons in the central nervous system.[65] This excessive

ectopic activity may alter the morphological and biochemical properties of the pre- and post-synaptic membranes and change the excitability of the dorsal horn neurons. Prolonged central sensitization leads to permanent alterations in the structures responsible for processing nociceptive stimuli.[66] Prolonged stimulation of peripheral nociceptors by postoperative pain of high intensity maintains a hyperexcited state in dorsal horn neurons.[67]

Several review articles[23, 24, 63] have concluded that severe postoperative pain is a well-established risk factor for the development of phantom breast pain and other neuropathic pain syndromes following breast cancer surgery. In two studies that asked women to recall the intensity of their postoperative pain one year after surgery,[7, 38] the development of persistent pain in the breast area was associated with higher postoperative pain intensity scores after controlling for demographic and clinical characteristics. In a prospective study designed to identify predictors of persistent pain after breast cancer surgery,[5] pain ratings of ≥5 out of 10 at both 2 and 10 days postoperatively were associated with more intense pain three months after surgery. In addition to a higher intensity of acute postoperative pain, these three studies found that younger age,[38] more invasive surgery,[5] more advanced disease,[38] recurrence,[38] and receipt of radiation therapy[7] were significant predictors of persistent pain. Among these characteristics, only age was a significant predictor in bivariate analysis in our study. Type of surgery, stage of disease, and receipt of radiation therapy did not differ between the breast pain classes. None of the patients in our study were diagnosed with recurrent disease. The methods used to characterize the persistent pain phenotype may explain these inconsistent findings. In previous studies, that assessed pain only once after surgery, patients who

reported pain of any severity were classified into a persistent pain group. This categorization may result in a more heterogeneous pain phenotype than the one obtained in our study using GMM.

Pro- and anti-inflammatory cytokines are known to modulate nociceptive signaling during acute and chronic inflammation and following tissue injury and nerve lesions.[68] However, significant interindividual variability exists in the development and resolution of postsurgical pain. In this study, three SNPs and one haplotype in four cytokine genes were associated with pain group membership after adjusting for severity of worst postoperative pain.

Findings from this study suggest that the rare "C" allele of IL1R2 rs11674595 increases the risk for the development of severe persistent breast pain. To date, no associations were reported between any SNP in IL1R2 and a pain phenotype. IL1R2 rs11674595 is located in a non-coding though evolutionarily conserved region and its impact on IL-1R2 production is unknown. However, IL1R2 encodes for the IL-1 type II receptor that inhibits inflammatory signaling by titrating IL-1β away from binding to IL-1R1.[69] Upon binding to IL-1R1, IL-1β initiates signaling cascades that promote the production and subsequent release of nitric oxide, bradykinin, and prostaglandins.[70-72] These mediators alter the biophysical properties and kinetics of ion channels and receptors present in neuronal membranes to augment nociceptor excitability.[73]

IL-4, an anti-inflammatory cytokine, promotes hypoalgesia by suppressing the actions of activated macrophages, prevents the expression of cyclooxygenase 2 and inducible nitric oxide synthase, and inhibits the synthesis of pro-inflammatory cytokines.[74, 75] In addition, IL-4 induces μ- and δ-opioid receptor transcription which

promotes hypoalgesia through the endogenous opioid system.[76, 77] Our results suggest that the rare G allele of IL4 rs2243248 increases the risk for the development of severe persistent pain. Consistent with this finding, Illi et al[53] found that the same G allele was associated with the presence of a symptom cluster composed of clinically meaningful levels of depression, pain, sleep disturbance, and fatigue in patients who underwent primary or adjuvant radiotherapy and their family caregivers.

IL4 rs2243248 is located in the 5' untranslated region of the gene. While one study found that this SNP did not alter IL-4 cytokine levels,[78] the functional effects of IL4 rs2243248 are not known. However, IL4 rs2243248 was found to be in strong LD with rs2243250.[79, 80] The T allele of IL4 rs2243250 is associated with increased transcriptional activity of IL-4 *in vitro*.[81] IL4 rs2243250 was genotyped in the present study. However, this SNP deviated from Hardy Weinberg expectations and was not evaluated for an association with the pain phenotype. Additional studies are needed to determine whether rs2243248 is in LD with other variations in the IL4 locus that affect cytokine production.

IL-10 reduces the bioavailability of proinflammatory cytokines by downregulating expression of IL-1, IL-6, and TNF alpha (α) in activated macrophages.[82, 83] Activated macrophages are the major source of proinflammatory cytokines in the periphery following tissue injury and inflammation. In addition, IL-10 alters the production of antagonists of proinflammatory cytokines by decreasing the expression of IL-1 receptors,[84] increasing the production of soluble TNFα receptors,[85] and preventing the degradation of IL1RN mRNA.[86] The increased availability of an antagonist for the proinflammatory cytokines further attenuates proinflammatory cytokine signaling and

dampens their positive feedback loops. Findings from this study suggest that the IL10 haplotype A8 decreases the risk for the development of severe persistent pain. To date, no associations were reported between any of the SNPs contained in IL10 haplotype A8 and a pain phenotype.

Although the SNPs that comprise this haplotype are located in introns or in the 3' untranslated region of IL10, the functional significance of some of these SNPs were evaluated previously.[81, 87, 88] These studies provide conflicting evidence for a potential effect of the IL10 haplotype A8 on IL10 expression. IL10 rs3024498 is located in the 3' untranslated region of IL10 and falls in a putative transcription factor binding site region. The rare G allele of IL10 rs3024498 was associated with elevated serum IL-10 levels in patients with tuberculosis.[89] IL10 rs1518111 is located in a non-coding region of IL10. The rare A allele of IL10 rs1518111 is associated with decreased mRNA expression of IL-10.[88] Further investigation is necessary to determine how SNPs contained within this haplotype alter IL-10 gene expression and protein production.

Current evidence supports anti-inflammatory actions for IL-13 in the context of neuropathic pain.[90, 91] IL-13 is secreted by Th2 lymphocytes and opposes the actions of pro-inflammatory cytokines released from activated immune cells following neuronal injury.[92] Our results suggest that the rare T allele of IL13 rs1800925 is associated with increased risk for the development of severe persistent breast pain. IL13 rs1800925 is a functional polymorphism that is located within a Nuclear Factor of Activated T cells (NFAT) binding site of the IL13 promoter. [93, 94] Functional studies demonstrated that the C to T substitution increases the affinity of NFAT for the IL13 promoter[93] and results in enhanced IL13 gene transcription and IL-13 secretion from Th2 lymphocytes.[94]

Although the currently known functional effects of IL13 rs1800925 may seem counterintuitive, speculation about the function of the IL13 rs1800925 polymorphism is based on a limited number of *in vitro* studies. Cytokines participate in a complex series of cascades and feedback loops.[95] A change in the availability of IL-13 may alter feedback loops and impact pain severity. Therefore, the effects of IL-13 on the initiation and maintenance of persistent pain may be context dependent. Pro-inflammatory properties of IL-13 were identified in allergy.[94, 96] In addition, the rare T allele of rs1800925 was associated with an increased risk of asthma[93, 97, 98] Finally, in a recent study,[99] women who reported pain one month after fine needle biopsy or resection of their breast tumor had elevated plasma levels of IL-13 compared to women without pain. Further investigation is necessary to determine how rs1800925 alters IL13 gene expression and protein production in the context of persistent postsurgical pain.

Study limitations need to be acknowledged. First, no direct measurements of serum cytokines were done to provide additional data on the mechanisms that underlie the development of persistent breast pain. Second, future studies with a larger sample size may increase the power to detect differences in other cytokine genes. Finally, a number of clinical characteristics identified in bivariate analyses may be significant predictors of severe persistent breast pain in larger samples.

In conclusion, our findings suggest that polymorphisms in cytokine genes play a role in the development of severe persistent breast pain in women following breast cancer surgery. The genes and SNPs found in this study may help to identify individuals who are predisposed to the development of persistent, postsurgical breast pain. Future studies are warranted to confirm our findings and to determine if these associations are present in

other persistent postsurgical pain syndromes and to determine the mechanism underlying these associations.

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Figure Legends

Figure 1: Observed and estimated worst breast pain severity trajectories for patients in the mild, moderate, and severe breast pain latent classes. Differences between the no pain and severe pain classes (black) were evaluated. The mild and moderate pain classes are shown in grey. Observed trajectories are shown with dashed lines and the estimated trajectories are shown with solid lines. This figure was adapted from "Identification of distinct patient subgroups and risk factors for persistent breast pain following breast cancer surgery," by Miaskowski et al.[29]

Figure 2: Differences in the percentages of patients in the no breast pain and severe breast pain latent classes who were: A) homozygous for the common allele or heterozygous (TT+TC) or homozygous for the minor allele (CC) for rs11674595 in IL1R2; B) homozygous for the common allele (TT) or heterozygous or homozygous for the minor allele (TG+GG) for rs2243248 in IL4; and C) homozygous for the common allele (CC) or heterozygous or homozygous for the minor allele (CT+TT) for rs1800925 in IL13.

Figure 3: IL10 linkage disequilibrium-based heatmap and haplotype analysis. In the figure embedded in the top row of the table, an ideogram of interleukin 10 (IL10) is presented above the white bar that represents the physical distance along human chromosome 1 (position 206,940,948 to 206,945,839; genome build 37.10, NG_012088.1). Exons are represented as boxes. Gray lines connecting the exons represent introns. The direction of transcription is from right to left. Reference sequence identifiers (rsID) for each single nucleotide polymorphism (SNP) are plotted both in

terms of their physical distance (i.e., the white bar at the top of the figure) and also equidistantly in order to render the pairwise linkage disequilibrium (LD) estimates that were calculated and visualized with Haploview 4.2. The gene structure for IL10 (i.e., hg18 NM_000572) was rendered with FancyGene 1.4. The correlation statistics (r^2 and D') are provided in the heatmap. LD-based haplotype block definition was based on D' confidence interval [46]. The haploblock is indicated in a bolded triangle and its component SNPs are rendered in bold font. Pairwise D' values (range: 0-1, inclusive) were rendered in color, with dark red diamonds representing D' values approaching 1.0. When the r^2 values (range of 0-100, inclusive) are not equal to 0 or 100, they are provided in a given diamond. The haplotypes observed in the haploblock are listed in each row, starting with the nucleotide composition across the seven SNPs that compose the haplotype (i.e., rs3024505, rs3024498, rs3024496, rs1878672, rs1518111, rs1518110, rs3024491) and the count frequency (%) of each haplotype observed in the no breast pain and severe breast pain classes.

[#] The haplotype (i.e. CGCGATT) identified in the bivariate analyses (Table 1) remained significant after controlling for relevant confounders.

n=number of individuals; s = number of alleles.

Table 1 – Differences in cytokine genes/single nucleotide polymorphisms between the no pain and the severe pain classes

Gene	SNP	Position	Chr	MAF	Alleles	Chi square	p-value	Model
IFNG1	rs2069728	66834051	12	.110	G>A	4.05	0.132	A
IFNG1	rs2069727	66834490	12	.384	A>G	FE	0.025	R
IFNG1	rs2069718	66836429	12	.494	C>T	10.09	0.006	A
IFNG1	rs1861493	66837463	12	.266	A>G	1.92	0.383	A
IFNG1	rs1861494	66837676	12	.273	T>C	1.92	0.383	A
IFNG1	rs2069709	66839970	12	.003	G>T	n/a	n/a	n/a
IFNG1	HapA3					1.92	0.383	
IFNG1	HapA5					6.58	0.037	
IFNGR1	rs9376268	137574444	6	.254	G>A	0.79	0.672	A
IL1B	rs1071676	106042060	2	.189	G>C	1.15	0.564	A
IL1B	rs1143643	106042929	2	.383	G>A	0.06	0.970	A
IL1B	rs1143642	106043180	2	.082	C>T	0.65	0.722	A
IL1B	rs1143634	106045017	2	.187	C>T	1.15	0.564	A
IL1B	rs1143633	106045094	2	.392	G>A	0.26	0.877	A
IL1B	rs1143630	106046282	2	.115	C>A	4.02	0.134	A
IL1B	rs3917356	106046990	2	.450	G>A	0.36	0.837	A
IL1B	rs1143629	106048145	2	.389	T>C	1.91	0.384	A
IL1B	rs1143627	106049014	2	.397	T>C	1.91	0.384	A
IL1B	rs16944	106049494	2	.386	G>A	2.19	0.334	A
IL1B	rs1143623	106050452	2	.277	G>C	0.37	0.830	A
IL1B	rs13032029	106055022	2	.448	C>T	0.24	0.886	A
IL1B	HapA1					1.11	0.573	
IL1B	HapA4					0.06	0.970	
IL1B	HapA6					1.15	0.564	
IL1B	HapB1					1.64	0.441	
IL1B	HapB6					2.46	0.292	
IL1B	HapB8					0.22	0.894	
IL1R1	rs949963	96533648	2	.223	G>A	2.36	0.307	A
IL1R1	rs2228139	96545511	2	.053	C>G	1.00	0.461	A
IL1R1	rs3917320	96556738	2	.047	A>C	n/a	n/a	n/a
IL1R1	rs2110726	96558145	2	.317	C>T	0.57	0.753	A
IL1R1	rs3917332	96560387	2	.187	A>T	FE	0.037	D
IL1R1	HapA1					3.05	0.218	
IL1R1	HapA2					5.94	0.051	
IL1R1	HapA3					4.91	0.086	
IL1R2	rs4141134	96370336	2	.362	T>C	0.08	0.959	A
IL1R2	rs11674595	96374804	2	.258	T>C	FE	0.041	R
IL1R2	rs7570441	96380807	2	.408	G>A	1.17	0.557	A
IL1R2	HapA1					1.93	0.381	
IL1R2	HapA2					FE	0.630	

IL1R2	HapA4					2.27	0.322	
IL2	rs1479923	119096993	4	.308	C>T	2.40	0.301	A
IL2	rs2069776	119098582	4	.184	T>C	n/a	n/a	n/a
IL2	rs2069772	119099739	4	.241	A>G	2.80	0.246	A
IL2	rs2069777	119103043	4	.047	C>T	n/a	n/a	n/a
IL2	rs2069763	119104088	4	.277	T>G	2.33	0.313	A
IL2	HapA1					0.88	0.645	
IL2	HapA2					2.33	0.313	
IL2	HapA3					2.80	0.246	
IL4	rs2243248	127200946	5	.086	T>G	FE	0.033	D
IL4	rs2243250	127201455	5	.269	C>T	n/a	n/a	n/a
IL4	rs2070874	127202011	5	.245	C>T	n/a	n/a	n/a
IL4	rs2227284	127205027	5	.387	C>A	n/a	n/a	n/a
IL4	rs2227282	127205481	5	.390	C>G	n/a	n/a	n/a
IL4	rs2243263	127205601	5	.124	C>G	5.26	0.072	A
IL4	rs2243266	127206091	5	.237	G>A	n/a	n/a	n/a
IL4	rs2243267	127206188	5	.237	G>C	n/a	n/a	n/a
IL4	rs2243274	127207134	5	.261	G>A	n/a	n/a	n/a
IL4	HapA1					1.79	0.409	
IL4	HapA3					0.69	0.709	
IL4	HapX1					1.33	0.513	
IL6	rs4719714	22643793	7	.255	A>T	2.10	0.350	A
IL6	rs2069827	22648536	7	.069	G>T	0.67	0.715	A
IL6	rs1800796	22649326	7	.134	C>G	n/a	n/a	n/a
IL6	rs1800795	22649725	7	.285	C>G	1.23	0.540	A
IL6	rs2069835	22650951	7	.061	T>C	n/a	n/a	n/a
IL6	rs2066992	22651329	7	.049	G>T	5.40	0.067	A
IL6	rs2069840	22651652	7	.333	C>G	5.40	0.067	A
IL6	rs1554606	22651787	7	.319	G>T	3.59	0.166	A
IL6	rs2069845	22653229	7	.319	A>G	0.21	0.900	A
IL6	rs2069849	22654236	7	.024	C>T	n/a	n/a	n/a
IL6	rs2069861	22654734	7	.056	C>T	1.81	0.405	A
IL6	rs35610689	22656903	7	.259	A>G	1.30	0.521	A
IL6	HapA1					5.40	0.067	
IL6	HapA5					3.31	0.192	
IL6	HapA8					0.06	0.971	
IL8	rs4073	70417508	4	.455	T>A	0.76	0.684	A
IL8	rs2227306	70418539	4	.366	C>T	2.69	0.260	A
IL8	rs2227543	70419394	4	.368	C>T	2.39	0.303	A
IL8	HapA1					0.76	0.684	
IL8	HapA4					2.43	0.296	
IL10	rs3024505	177638230	1	.129	C>T	5.00	0.082	A
IL10	rs3024498	177639855	1	.204	A>G	FE	0.015	D
IL10	rs3024496	177640190	1	.421	T>C	3.90	0.142	A

IL10	rs1878672	177642039	1	.416	G>C	FE	0.029	D
IL10	rs3024492	177642438	1	.190	T>A	n/a	n/a	n/a
IL10	rs1518111	177642971	1	.303	G>A	0.52	0.771	A
IL10	rs1518110	177643187	1	.301	G>T	0.54	0.765	A
IL10	rs3024491	177643372	1	.408	G>T	FE	0.035	D
IL10	HapA1					0.51	0.776	
IL10	HapA2					4.42	0.110	
IL10	HapA8					6.39	0.041	
IL13	rs1881457	127184713	5	.210	A>C	FE	0.043	D
IL13	rs1800925	127185113	5	.233	C>T	FE	0.007	D
IL13	rs2069743	127185579	5	.019	A>G	n/a	n/a	n/a
IL13	rs1295686	127188147	5	.265	G>A	FE	0.014	D
IL13	rs20541	127188268	5	.212	C>T	FE	0.017	A
IL13	HapA1					8.70	0.013	
IL13	HapA4					5.96	0.051	
IL17A	rs4711998	51881422	6	.346	G>A	0.82	0.663	A
IL17A	rs8193036	51881562	6	.327	T>C	0.87	0.646	A
IL17A	rs3819024	51881855	6	.372	A>G	1.22	0.544	A
IL17A	rs2275913	51882102	6	.361	G>A	1.24	0.538	A
IL17A	rs3804513	51884266	6	.023	A>T	n/a	n/a	n/a
IL17A	rs7747909	51885318	6	.217	G>A	2.47	0.291	A
NFKB1	rs3774933	103645369	4	.409	T>C	2.35	0.309	A
NFKB1	rs170731	103667933	4	.358	A>T	1.34	0.512	A
NFKB1	rs17032779	103685279	4	.011	T>C	n/a	n/a	n/a
NFKB1	rs230510	103695201	4	.410	T>A	0.24	0.885	A
NFKB1	rs230494	103706005	4	.434	A>G	0.97	0.615	A
NFKB1	rs4648016	103708706	4	.010	C>T	n/a	n/a	n/a
NFKB1	rs4648018	103709236	4	.018	G>C	n/a	n/a	n/a
NFKB1	rs3774956	103727564	4	.435	C>T	1.60	0.450	A
NFKB1	rs10489114	103730426	4	.018	A>G	n/a	n/a	n/a
NFKB1	rs4648068	103737343	4	.363	A>G	0.70	0.705	A
NFKB1	rs4648095	103746914	4	.052	T>C	FE	1.000	A
NFKB1	rs4648110	103752867	4	.170	T>A	3.63	0.163	A
NFKB1	rs4648135	103755716	4	.061	A>G	FE	0.733	A
NFKB1	rs4648141	103755947	4	.180	G>A	FE	0.041	R
NFKB1	rs1609798	103756488	4	.337	C>T	1.13	0.567	A
NFKB1	HapA1					0.18	0.916	
NFKB1	HapA9					1.91	0.385	
NFKB2	rs12772374	104146901	10	.168	A>G	0.29	0.866	A
NFKB2	rs7897947	104147701	10	.221	T>G	2.96	0.228	A
NFKB2	rs11574849	104149686	10	.070	G>A	FE	1.000	A
NFKB2	rs1056890	104152760	10	.305	C>T	1.66	0.436	A
TNFA	rs2857602	31533378	6	.341	T>C	0.01	0.993	A
TNFA	rs1800683	31540071	6	.390	G>A	0.18	0.916	A

TNFA	rs2239704	31540141	6	.335	G>T	0.14	0.932	A
TNFA	rs2229094	31540556	6	.278	T>C	0.74	0.692	A
TNFA	rs1041981	31540784	6	.386	C>A	0.09	0.954	A
TNFA	rs1799964	31542308	6	.224	T>C	1.07	0.587	A
TNFA	rs1800750	31542963	6	.016	G>A	n/a	n/a	n/a
TNFA	rs1800629	31543031	6	.149	G>A	3.31	0.191	A
TNFA	rs1800610	31543827	6	.100	C>T	2.51	0.285	A
TNFA	rs3093662	31544189	6	.074	A>G	FE	0.751	A
TNFA	HapA1					1.27	0.530	
TNFA	HapA5					1.10	0.577	
TNFA	HapA6					0.14	0.931	

Abbreviations: A = additive model; Chr = chromosome; D = dominant model; Hap = haplotype; IFNG = interferon gamma; IL = interleukin; MAF = minor allele frequency; n/a = not assayed because SNP violated Hardy-Weinberg expectations ($p<0.001$) or because MAF was <.05; NFKB = nuclear factor kappa beta; R = recessive model; SNP= single nucleotide polymorphism; TNFA = tumor necrosis factor alpha

Table 2 - Differences in demographic and clinical characteristics between the breast pain classes prior to surgery

Demographic Characteristics	No Pain n=126 Mean (SD)	Severe Pain n=46 Mean (SD)	Statistics
Age (years)	58.6 (11.4)	52.4 (9.4)	t=3.30; p=0.001
Education (years)	15.8 (2.8)	14.3 (2.9)	t=2.99; p=0.003
	% (N)	% (N)	
Ethnicity			
White	73.0 (92)	41.3 (19)	
Black	7.1 (9)	21.7 (10)	
Asian/Pacific Islander	10.3 (13)	21.7 (10)	X^2=16.03; p=0.001
Hispanic/mixed ethnic background/other	9.5 (12)	15.2 (7)	
Lives alone	20.8 (26)	29.5 (13)	FE; p=0.298
Marital status			
Married/partnered	41.3 (52)	58.1 (25)	FE; p=0.076
Single/separated/widowed/divorced	58.7 (74)	41.9 (18)	
Currently working for pay	52.0 (65)	34.8 (16)	FE; p=0.057
Total annual household income			
< $10,000 to $19,999	8.5 (9)	39.5 (15)	Z=-4.26; p<0.001
$20,000 to $99,000	48.1 (51)	44.7 (17)	
≥ $100,000	43.4 (46)	15.8 (6)	
Clinical Characteristics	Mean (SD)	Mean (SD)	
Body mass index (kg/m^2)	27.1 (7.0)	28.6 (6.3)	t=-1.29; p=0.197
Karnofsky Performance Status score	96.2 (8.7)	87.6 (14.9)	t=3.66; p=0.001
Self-Administered Comorbidity Scale score	4.0 (2.3)	5.6 (3.2)	t=-3.01; p=0.004
Number of breast biopsies	1.4 (0.7)	1.6 (1.1)	Z=-1.80; p=0.072
	% (N)	% (N)	
Occurrence of comorbid conditions (% and number of women who reported each comorbid condition from the Self-Administered Comorbidity Questionnaire)			
Heart disease	4.0 (5)	2.2 (1)	FE; p=1.000
High blood pressure	34.9 (44)	45.7 (21)	FE; p=0.217
Lung disease	2.4 (3)	6.5 (3)	FE; p=0.344
Diabetes	7.1 (9)	17.4 (8)	FE; p=0.079
Ulcer	3.2 (4)	2.2 (1)	FE; p=1.000
Kidney disease	1.6 (2)	0.0 (0)	FE; p=1.000
Liver disease	3.2 (4)	4.3 (2)	FE; p=0.659

Anemia	7.9 (10)	13.0 (6)	FE; p=0.374
Depression	16.7 (21)	34.8 (16)	FE; p=0.020
Osteoarthritis	17.5 (22)	19.6 (9)	FE; p=0.823
Back pain	22.2 (28)	41.3 (19)	FE; p=0.020
Rheumatoid arthritis	1.6 (2)	13.0 (6)	FE; p=0.005
Diagnosed with mastitis	11.2 (14)	7.0 (3)	FE; p=0.564
Diagnosed with fibrocystic disease	18.6 (22)	11.4 (5)	FE; p=0.347
Ever breast fed	48.0 (60)	41.3 (19)	FE; p=0.491
Surgery to affected breast unrelated to cancer	7.9 (10)	6.5 (3)	FE; p=1.000
Post-menopausal	71.0 (88)	62.8 (27)	FE; p=0.343
Received neoadjuvant chemotherapy	17.5 (22)	17.4 (8)	FE; p=1.000
On hormonal replacement therapy prior to surgery	19.0 (24)	6.7 (3)	FE; p=0.058
Stage of disease			
Stage 0	17.5 (22)	13.0 (6)	
Stage 1	41.3 (52)	34.8 (16)	Z=-1.50;
Stage IIA and IIB	35.7 (45)	39.1 (18)	p=0.1334
Stage IIIA, IIIB, IIIC, and IV	5.6 (7)	13.0 (6)	
Pain in breast prior to surgery	2.4 (3)	43.2 (19)	FE; p<0.001
Swelling in affected breast	3.2 (4)	23.9 (11)	FE; p<0.001
Numbness in affected breast	2.4 (3)	17.4 (8)	FE; p=0.001
Strange sensations in affected breast	12.7 (16)	28.3 (13)	FE; p=0.022
Hardness in affected breast	7.9 (10)	30.4 (14)	FE; p=0.001
Surgical Characteristics	Mean (SD)	Mean (SD)	
Number of lymph nodes removed	4.3 (4.7)	8.0 (9.0)	t=-2.61; p=0.012
Number of drains placed during surgery	0.4 (0.7)	0.5 (0.6)	t=-0.92; p=0.359
	% (N)	% (N)	
Type of surgery			
Breast conserving	84.1 (106)	82.6 (38)	FE; p=0.818
Mastectomy	15.9 (20)	17.4 (8)	
Reconstruction at the time of surgery	15.9 (20)	13.0 (6)	FE; p=0.811
Surgical drain placed at time of surgery	31.7 (40)	45.7 (21)	FE; p=0.106
Surgical drain placed in breast at time of surgery	65.7 (23)	43.8 (7)	FE; p=0.220
Postoperative Characteristics	Mean (SD)	Mean (SD)	
Number of postoperative complications	0.2 (0.5)	0.3 (0.6)	t=-1.17; p=0.246
Severity of average postoperative pain	2.8 (2.1)	6.5 (2.2)	t=-9.66; p<0.001

Severity of worst postoperative pain	4.2 (2.6)	7.9 (2.5)	t=-8.13; p<0.001
	% (N)	% (N)	
Had a postoperative complication	18.3 (23)	26.1 (12)	FE; p=0.287
Received radiation therapy during the 6 months	56.3 (71)	41.3 (19)	FE; p=0.087
Received adjuvant chemotherapy during the 6 months	31.7 (40)	39.1 (18)	FE; p=0.369
Received hormonal therapy during the 6 months	45.2 (57)	30.4 (14)	FE; p=0.115
Received biological therapy during the 6 months	8.7 (11)	6.5 (3)	FE; p=0.762
Received complementary therapy during the 6 months	23.8 (30)	23.9 (11)	FE; p=1.000
Had breast reconstruction during the 6 months	4.8 (6)	2.2 (1)	FE; p=0.676
Had re-excision or mastectomy during the 6 months	18.3 (23)	39.1 (18)	FE; p=0.008

Abbreviations: FE = Fisher's Exact; SD = standard deviation; kg = kilogram; m^2 = meters squared

Table 3 – Multiple logistic regression model using phenotypic characteristics to predict pain class membership

GMM Class Comparison	Predictor	Odds Ratio	Standard Error	95% CI	Z	p-value
No breast pain versus severe breast pain (n=147)	Age	0.99	0.03	0.94, 1.04	-0.43	0.670
	Self-reported race/ethnicity					
	White versus Black	2.40	1.81	0.55, 10.50	1.16	0.244
	White versus Asian/Pacific Islander	2.16	1.54	0.53, 8.75	1.08	0.282
	White versus Hispanic/mixed/other	2.59	2.27	0.47, 14.40	1.09	0.276
	KPS score	0.99	0.02	0.95, 1.03	-0.36	0.721
	SCQ score	1.08	0.12	0.86, 1.35	0.66	0.508
	History of back pain	1.89	1.12	0.59, 6.04	1.08	0.281
	Strange sensations in affected breast	1.82	1.20	0.50, 6.64	0.90	0.366
	Number of lymph nodes removed	1.03	0.04	0.95, 1.12	0.77	0.440
	Severity of worst postoperative pain	1.53	0.20	1.19, 1.97	3.32	0.001
	Re-excision/mastectomy after surgery	2.62	1.46	0.88, 7.82	1.73	0.083
	Overall model fit: $\chi^2 = 62.87$, $p < 0.0001$; $R^2 = 0.3613$					

Abbreviations: CI = confidence interval; GMM = growth mixture model; KPS = Karnofsy Performance Status; SCQ = Self-administered Comorbidity Questionnaire

Table 4 – Multiple logistic regression analyses for IL1R2, IL4, IL10, and IL13 candidate gene markers

GMM Class Comparison	Predictor	Odds Ratio	Standard Error	95% CI	Z	p-value
No breast pain versus severe breast pain (n=110)	IL1R2 genotype	28.30	35.83	2.37, 338.58	2.64	0.008
	Severity of worst postoperative pain	1.81	0.29	1.32, 2.49	3.68	<0.001
	Overall model fit: $\chi^2 = 48.18$, $p < 0.0001$; $R^2 = 0.4005$					
No breast pain versus severe breast pain (n=110)	IL4 genotype	6.13	5.44	1.08, 34.93	2.04	0.041
	Severity of worst postoperative pain	1.75	0.26	1.31, 2.36	3.75	<0.001
	Overall model fit: $\chi^2 = 44.11$, $p < 0.0001$; $R^2 = 0.3666$					
No breast pain versus severe breast pain (n=110)	IL10 Haplotype A8	0.25	0.16	0.07, 0.89	-2.14	0.032
	Severity of worst postoperative pain	1.80	0.28	1.32, 2.45	3.72	<0.001
	Overall model fit: $\chi^2 = 44.91$, $p < 0.0001$; $R^2 = 0.3733$					
No breast pain versus severe breast pain (n=110)	IL13 genotype	3.60	2.34	1.01, 12.87	1.97	0.049
	Severity of worst postoperative pain	1.76	0.27	1.30, 2.37	3.68	<0.001
	Overall model fit: $\chi^2 = 43.65$, $p < 0.0001$; $R^2 = 0.3628$					

Notes: Multiple logistic regression analysis of the no pain and the severe pain GMM classes based on rating of worst breast pain. For each model, the first three principal components identified from the analysis of ancestry informative markers as well as self-report race/ethnicity were retained in all models to adjust for potential confounding due to race or ethnicity (data not shown). Predictors evaluated in each model included genotype (IL1R2 rs11674595: TT + TC versus CC; IL4 rs2243248: TT versus TG + GG; IL10 haplotype A8 composed of rs3024505-rs3024498-rs3024496-rs1878672-rs1518111-rs1518110-rs3024491: zero, one, or two doses of the C-G-C-G-A-T-T haplotype; IL13 rs1800925: CC versus CT + TT) and severity of worst postoperative pain.

Abbreviations: CI = confidence interval; GMM = growth mixture model; IL1R2 = interleukin 1 receptor, type II; IL4 = interleukin 4; IL10 = interleukin 10; IL13 = interleukin 13

Figure 1

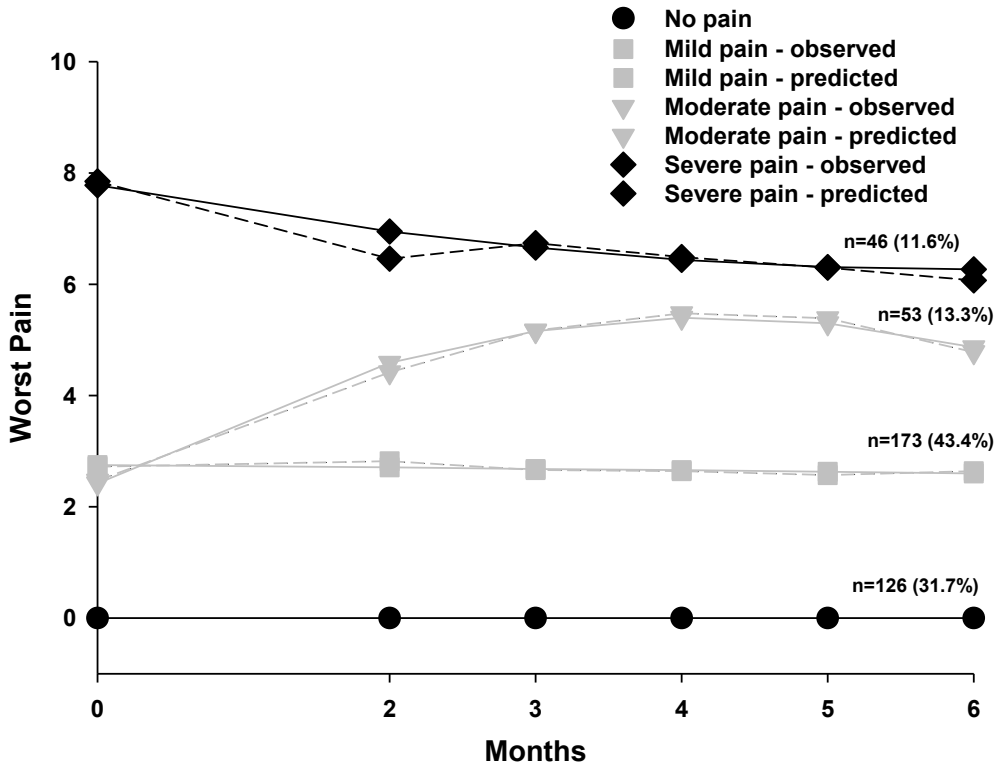

No pain
Mild pain - observed
Mild pain - predicted
Moderate pain - observed
Moderate pain - predicted
Severe pain - observed
Severe pain - predicted
n=46 (11.6%)
n=53 (13.3%)
n=173 (43.4%)
n=126 (31.7%)
Worst Pain
Months
0
2
4
6
8
10
0
2
3
4
5
6

Figure 2

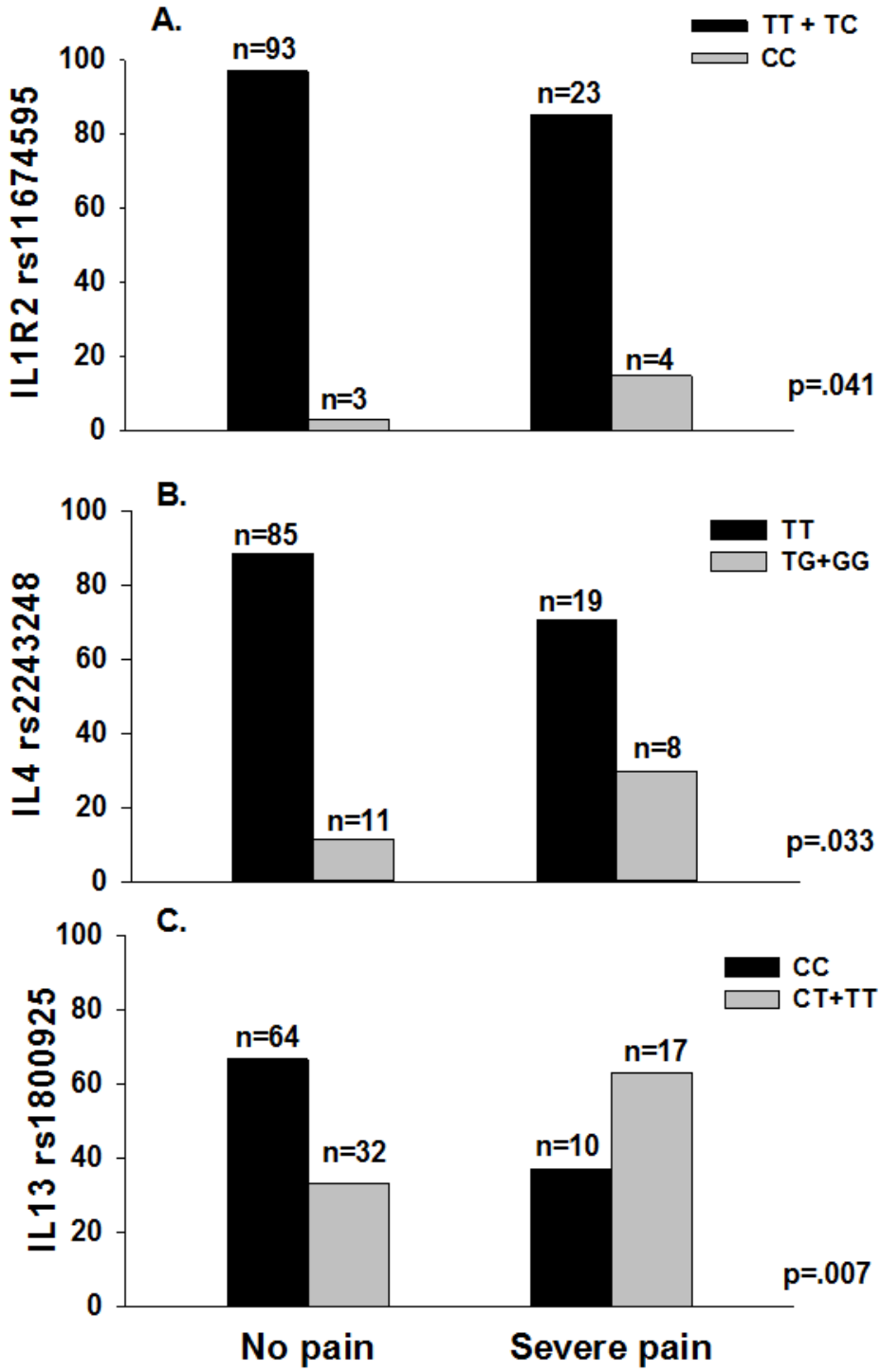
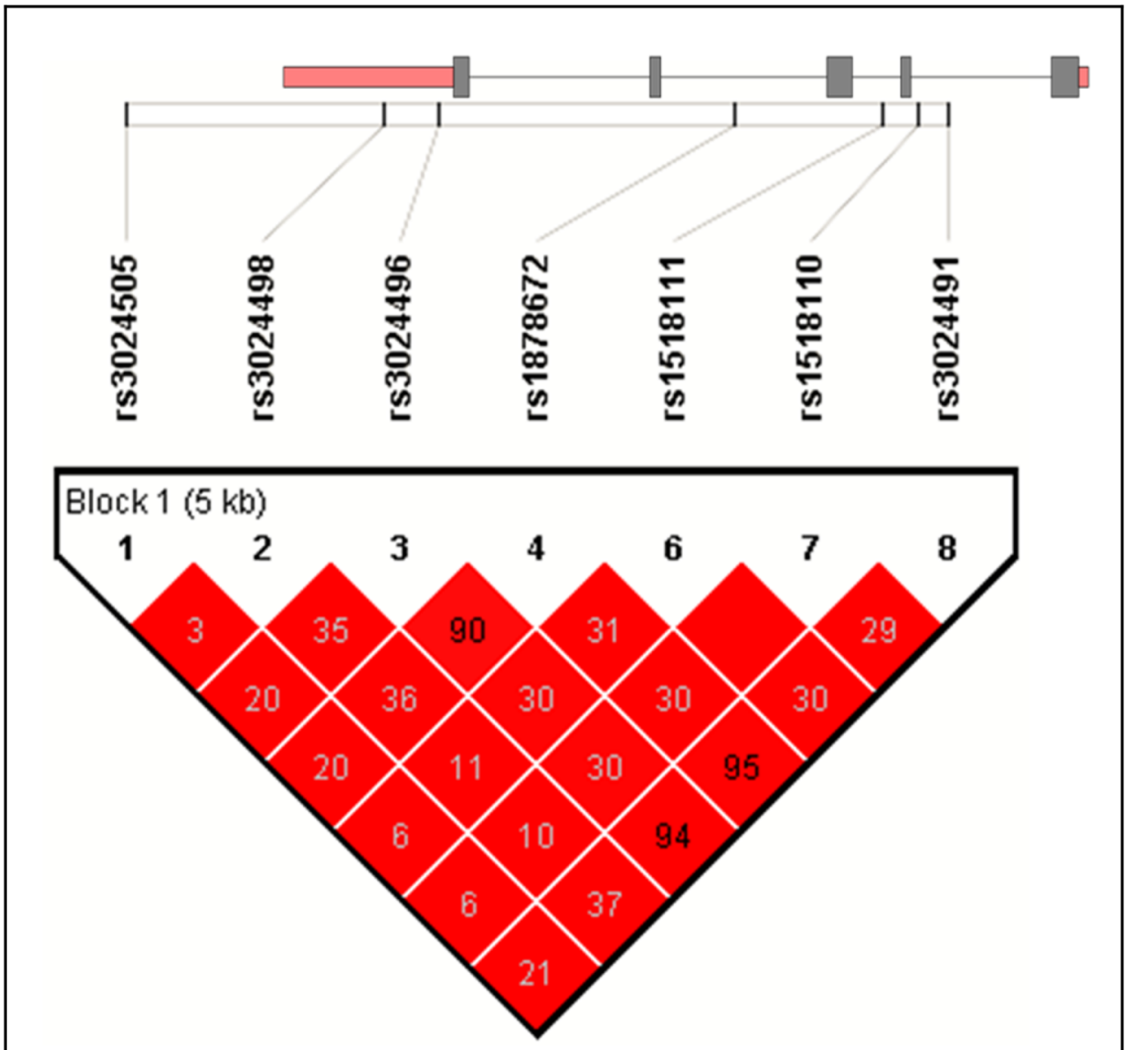

A.
TT + TC
CC
IL1R2 rs11674595
100
80
60
40
20
0
n=93
n=3
n=23
n=4
p=.041
B.
TT
TG+GG
IL4 rs2243248
n=85
n=11
n=19
n=8
p=.033
C.
CC
CT+TT
IL13 rs1800925
n=64
n=32
n=10
n=17
p=.007
No pain
Severe pain

Figure 3



Haplotype	No Pain (n=142, s=284)	Severe Pain (n=32, s=64)
CATCGGG	43 (30.3%)	12 (37.5%)
CATCATG	32 (22.5%)	11 (34.4%)
CATGATG	1 (<0.01%)	0 (0.0%)
CACCGGG	0 (0.0%)	0 (0.0%)
CACCATG	1 (<0.01%)	1 (0.01%)
CACCATT	0 (0.0%)	0 (0.0%)
CACGATT	11 (8.7%)	2 (6.3%)
CGCGATT	33 (23.2%)	3 (9.4%)
TAGGATT	21 (14.8%)	3 (9.4%)

Chapter 4

Association between genetic and epigenetic variations in cytokine genes and mild persistent breast pain in women following breast cancer surgery

Abstract

Persistent pain following breast cancer surgery is a significant problem. Both inherited and acquired mechanisms of inflammation appear to play a role in the development and maintenance of persistent pain. In this longitudinal study, growth mixture modeling was used to identify persistent breast pain phenotypes based on pain assessments obtained prior to and monthly for 6 months following breast cancer surgery. Associations between the "no pain" and "mild pain" phenotypes and single nucleotide polymorphisms (SNPs) spanning 15 cytokine genes were evaluated. The methylation status of the CpG sites found in the promoters of genes associated with pain group membership was determined using bisulfite sequencing. In the multivariate analysis, two SNPs (i.e., interleukin 6 (IL6) rs2069840, tumor necrosis factor alpha (TNFA) rs1800610) two TNFA CpG sites (i.e., c.-350C, c.-344C) were associated with pain group membership. Negative correlations were found between the percentage methylation at three CpG sites (i.e., c.-350C, c.-344C, c.-342C) and TNFA gene expression. These findings suggest that variations in IL6 and TNFA are associated with the development and maintenance of mild persistent breast pain. CpG methylation within the TNFA promoter may provide an additional mechanism through which TNFA alters the risk for mild persistent breast pain after breast cancer surgery. These genetic and epigenetic variations may help to identify individuals who are predisposed to the development of mild levels of persistent breast pain following breast cancer surgery.

Summary: The associations between cytokine gene variations and epigenetic modifications and mild persistent breast pain in women following breast cancer surgery

were evaluated. The results suggest that cytokines play a role in the development of mild persistent postsurgical pain.

Keywords: cytokine genes, breast cancer, DNA methylation, persistent pain, post-surgical pain, gene expression, post-mastectomy pain, epigenetics, interleukin 6, tumor necrosis factor alpha

Introduction

Persistent pain in women following breast cancer surgery is common with a prevalence rate of between 20% and 55% [7,24,46,63,66,85,88,90,100,102]. Persistent pain is associated with alterations in mood [90] and sleep patterns [15,36], as well as decreased quality of life [7,63,85] and disability [46,100].

Persistent postsurgical pain may result from ongoing nociceptor activation and/or nerve injury [54]. During the early postoperative period, numerous inflammatory mediators are released that produce peripheral and central sensitization in and around the affected area [103]. These reversible changes in sensitivity to innocuous and noxious stimuli discourage stimulation of the surgical incision which serves as a protective mechanism that facilitates healing. However, sustained activation of nociceptors may lead to the maintenance of central sensitization and maladaptive phenotypic changes that alter the normal stimulus-response relationship and produce persistent pain.

Persistent alterations within nociceptors include changes in gene expression as well as changes in receptor and ion channel distributions within the neuronal membranes [80]. Evidence suggests that ongoing activation of inflammatory and glial cells, [58] as well as changes in spinal inhibitory mechanisms, [110] play a role in the establishment of persistent pain. In addition, peripheral nerve injury elicits an inflammatory reaction that prompts the aggregation of immune cells and increases the local concentration of proinflammatory cytokines [56]. These mediators participate in the initiation and maintenance of persistent pain after nerve injury by generating ectopic activity [26], altering neuronal connectivity [35], and reducing the number of inhibitory neurons [30].

Despite a clear connection between immune mechanisms and persistent pain [56], only four studies were identified that evaluated for associations between polymorphisms in cytokine pathways and cancer-related pain [57,71-73]. Three of these studies [71-73] assessed pain intensity prior to the initiation of cancer treatment. Associations were found between severe pain (i.e., pain rated >6/10 on a numeric ratings scale (NRS)) and IL1B rs1143627 [71], IL8 rs4073 [71,72], and TNFA rs1800629 [73]. However, findings from these studies are difficult to interpret because the pain phenotype was characterized using only a dichotomized pain severity rating, the samples were small, and the number of polymorphisms evaluated was not comprehensive. In a more recent study that evaluated for associations between variations in cytokine genes and pain in the affected breast of women prior to breast cancer surgery [57], associations were found between the presence of preoperative pain and IL1 receptor 1 (IL1R1) rs2110726 and IL13 rs1295686. Of note, no studies were found that evaluated for associations between cytokine gene polymorphisms and persistent postsurgical pain.

Emerging evidence suggests that acquired adaptations to genetic regulation (termed "epigenetics") are pervasive in biology [75]. DNA methylation is an epigenetic mechanism that regulates gene expression in numerous cell types [75]. Acquisition of methylation at CpG dinucleotides during a cell's lifetime provides an adaptive capacity for the cell, tissues, and ultimately the organism to adjust to sustained changes in its internal and external environment. Methylation of CpG dinucleotides aggregated in a gene's promoter region affects the ability of DNA binding proteins (e.g., transcription factors) to recognize a nucleotide sequence that regulates gene expression [40]. The methylation status and pattern of methylation of CpG sites within gene promoters have

emerged as promising biomarkers for risk stratification and detection of human disease [47].

Recent work from our group used growth mixture modeling (GMM) to identify subgroups of women with distinct persistent breast pain trajectories prior to and for six months following breast cancer surgery [59]. GMM is useful in identifying distinct pain phenotypes following surgery because individuals differ in the development and resolution of pain over time as a result of genetic and environmental risk factors. In our sample, three distinct classes were identified using patients' ratings of worst pain in their breast (i.e, mild, moderate, severe) (Figure 1). A fourth pain group was designated for those women who did not experience breast pain pre-operatively or at any of the postoperative assessments. Following this phenotypic characterization, we evaluated for associations between the extreme pain phenotypes identified through GMM (i.e., "no pain" and "severe pain" classes) and single nucleotide polymorphisms (SNP)s contained within cytokine genes [86]. In the multivariate analysis, the only characteristic that remained associated with pain group membership was the severity of worst postoperative pain. After adjustment for severity of worst postoperative pain, three SNPs (i.e., IL1R2 rs11674595; IL4 rs2243248; IL13 rs1800925) and one haplotype (i.e., IL10 haplotype A8) were associated with pain group membership.

However, the largest subgroup of women identified in this GMM analysis was the mild breast pain class (n= 173, 43.5%). This class had a mean preoperative worst pain severity score of 2.5 (on a 0 to 10 NRS) that did not change for the first 6 months after surgery. Mild levels of persistent postsurgical pain are associated with diminished perceptions of overall health and reduced physical and social functioning [32]. An

evaluation of similarities and differences in the cytokine genes associated with the "mild breast pain" and "severe breast pain" phenotypes may identify similar and/or novel mechanisms for these two distinct pain phenotypes.

Therefore, using data from the women who were classified into the no breast pain and mild breast pain classes, the purposes of this study were to: 1) evaluate for differences in demographic and clinical characteristics; 2) evaluate the associations between SNPs contained within cytokine genes and pain group membership; 3) determine the methylation status of CpG sites contained within the promoter region of cytokine genes that harbored gene variations associated with pain group membership; and 4) determine if methylation of promoter CpG sites was associated with changes in messenger ribonucleic acid (mRNA) expression.

Methods

Patients and settings

This longitudinal study is part of a larger study that evaluated for neuropathic pain and lymphedema in a sample of women who underwent breast cancer surgery [57,59,60]. Patients were recruited from Breast Care Centers located in a Comprehensive Cancer Center, two public hospitals, and four community practices. Patients were eligible to participate if they: were adult women ($\geq$18 years) who would undergo breast cancer surgery on one breast; were able to read, write, and understand English; agreed to participate; and gave written informed consent. Patients were excluded if they were having breast cancer surgery on both breasts and/or had distant metastasis at the time of diagnosis. A total of 516 patients were approached to participate and 410 were enrolled in the study (response rate 79.4%). The major reasons for refusal were: being too busy,

overwhelmed with the cancer diagnosis, or insufficient time available to do the baseline assessment prior to surgery.

Subjective measures

The demographic questionnaire obtained information on age, education, ethnicity, marital status, employment status, living situation, and income. The Karnofsky Performance Status (KPS) scale is widely used to evaluate functional status in patients with cancer and has well established validity and reliability [44,45]. Patients rated their functional status using the KPS scale that ranged from 30 (I feel severely disabled and need to be hospitalized) to 100 (I feel normal; I have no complaints or symptoms). Patients were asked to indicate if they exercised on a regular basis (yes/no format).

The Self-Administered Comorbidity Questionnaire (SCQ) is a short and easily understood instrument that was developed to measure comorbidity in clinical and health service research settings [79]. The questionnaire consists of 13 common medical conditions that were simplified into language that could be understood without any prior medical knowledge. Patients were asked to indicate if they had the condition using a "yes/no" format. If they indicated that they had a condition, they were asked if they received treatment for it (yes/no; proxy for disease severity) and did it limit their activities (yes/no; indication of functional limitations). Patients were given the option to add three additional conditions not listed on the instrument. For each condition, a patient can receive a maximum of 3 points. Because there are 13 defined medical conditions and 2 optional conditions, the maximum score totals 45 points if the open-ended items are used and 39 points if only the closed-ended items are used. The SCQ has well-established

validity and reliability and has been used in studies of patients with a variety of chronic conditions [5,9,53,79,83].

Persistent and acute postoperative pain were evaluated using the Breast Symptoms Questionnaire (BSQ) and Post Surgical Pain Questionnaire, respectively. The BSQ consists of two parts. Part 1 obtained information on the occurrence of pain and the occurrence of other symptoms in the breast scar area (i.e., swelling, numbness, strange sensations, hardness). The additional symptoms that were assessed were identified in studies by Tasmuth and colleagues [91,92]. If the patient had pain in the breast scar area, they completed Part 2 of the BSQ. Patients were asked to rate the intensity of their average and worst pain using a NRS that ranged from 0 (no pain) to 10 (worst imaginable pain). The NRS is a valid and reliable measure of pain intensity [37].

The Postsurgical Pain Questionnaire evaluated pain intensity, pain relief, and satisfaction with pain treatment in the first 24 to 48 hours after surgery. Average and worst pain were rated using a 0 (no pain) to 10 (worst imaginable pain) NRS. Pain relief was rated on a 0% (no relief) to 100% (complete relief) rating scale. Satisfaction with pain treatment was rated on a 0 (not satisfied at all) to 10 (extremely satisfied) NRS. This questionnaire was completed during the month 1 study visit.

Study procedures

The study was approved by the Committee on Human Research at the University of California, San Francisco and by the Institutional Review Boards at each of the study sites. During the patient's preoperative visit, a clinician explained the study to the patient and determined her willingness to participate. For those women who were willing to participate, the clinician introduced the patient to the research nurse. The research nurse

met with the women, determined eligibility and obtained written informed consent prior to surgery. After obtaining written informed consent, patients completed the enrollment questionnaires (Assessment 0).

Patients were contacted two weeks after surgery to schedule the first post-surgical appointment. The research nurse met with the patients either in their home or in the Clinical Research Center at 1, 2, 3, 4, 5, and 6 months after surgery. During each of the study visits, the women completed the study questionnaires and provided information on new and ongoing treatments. A blood sample was collected at the time of enrollment or during one of the monthly study visits. Over the course of the study, patients' medical records were reviewed for disease and treatment information.

Characterization of the persistent breast pain phenotype

Characterization of the persistent breast pain phenotype used in this study was described previously [59]. At each assessment, patients were asked, "Are you experiencing pain in your affected breast?" If the patient reported pain, she was asked to rate her "current pain at its worst" using a 0 (no pain) to 10 (worst pain) NRS. Prior to conducting the GMM analysis, patients who reported no pain in their affected breast for all 6 assessments (i.e., enrollment and 2, 3, 4, 5, and 6 months) were identified (N=126; 31.7%) and were not included in the GMM analysis. The remaining 272 women's ratings of worst breast pain were used in the GMM analysis. GMM was used to assign each individual into a latent class based on similarities in worst pain ratings at enrollment and at 2, 3, 4, 5, and 6 months after surgery. Pain ratings obtained at the 1-month follow-up assessment were excluded from the model because it reduced the variability in pain

ratings among the patients. Attempts to determine the latent classes failed when the month 1 ratings were included in the GMM analysis.

The GMM methods are described in detail elsewhere [19]. In brief, a single growth curve that represented the "average" change trajectory was estimated for the total sample. Then the number of latent growth classes that best fit the data was identified using published guidelines [42,62,95]. Descriptive statistics and frequency distributions for the no breast pain and mild breast pain classes were generated for demographic and clinical characteristics using Stata version 12.1 (StataCorp, College Station, TX). Independent sample t-tests, Mann-Whitney U tests, and Chi square and Fisher's Exact tests were used to evaluate for differences in demographic and clinical characteristics between the two breast pain classes. Adjustments were not made for missing data in comparisons of the classes identified with the GMM. Therefore, the cohort for each analysis was dependent on the largest set of available data across groups. A p-value of <0.05 was considered statistically significant.

Logistic regression analysis was performed to evaluate the associations between phenotypic characteristics and pain group membership. Based on a review of the literature, all phenotypic characteristics that were identified in the bivariate analyses as being different between the pain classes were evaluated for inclusion in the multivariate analysis. A backwards stepwise approach was used to create the most parsimonious model. Only predictors with a p-value of $<.05$ were retained in the final model. These same predictors were used to evaluate the associations between genotype and pain group membership and CpG methylation level and pain group membership.

Genotype determination

Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood mononuclear cells using the PUREGene DNA Isolation System (Invitrogen, Carlsbad, CA). DNA was available from 310 of the 398 patients. DNA samples were quantitated with a Nanodrop Spectrophotometer (ND-1000; Nanodrop Products, Wilmington, DE) and normalized to a concentration of 50 ng/μL (diluted in 10 mM Tris/1 mM EDTA). Genotyping was performed blinded to clinical status and positive and negative controls were included. Samples were genotyped using the Golden Gate genotyping platform (Illumina, San Diego, CA) and processed according to the standard protocol using GenomeStudio (Illumina, San Diego, CA). Two blinded reviewers visually inspected signal intensity profiles and resulting genotype calls for each SNP. Disagreements were adjudicated by a third reviewer.

A combination of tagging SNPs and literature driven SNPs (i.e., reported as being associated with altered function and/or symptoms) were selected for analysis. Tagging SNPs were required to be common (defined as having a minor allele frequency (MAF) of ≥.05) in public databases (e.g., HapMap). In order to ensure robust genetic association analyses, quality control filtering of SNPs was performed. SNPs with call rates <95%, Hardy-Weinberg $P < .001$, and/or a MAF of <5% were excluded.

A total of 82 SNPs from 15 cytokine genes (i.e., interferon gamma (IFNG): 5 SNPs; IFNG receptor 1 (IFNGR1): 1 SNP; IL1B: 12 SNPs; IL 1 receptor 1 (IL1R1): 4 SNPs; IL1R2: 3 SNPs; IL2: 3 SNPs; IL4: 2 SNPs; IL6: 9 SNPs; IL8: 3 SNPs; IL10: 7 SNPs; IL13: 4 SNPs; IL17A: 5 SNPs; nuclear factor kappa beta-1 (NFKB1): 11 SNPs; NFKB2: 4 SNPs; TNFA: 9 SNPs) passed all quality control filters and are included in

subsequent analyses. Potential functional roles of SNPs associated with persistent pain were examined using PUPASuite 2.0 [11].

CpG methylation determination

The percentage of CpG methylation in promoter regions of genes harboring SNPs associated with the persistent pain phenotype in multivariate analyses (i.e., IL6, TNFA) was determined using bisulfite sequencing. Genomic DNA from each patient was normalized to a concentration of 75ng/µl to 125 ng/µl (diluted in 10 mM Tris/1 mM EDTA). Approximately 1.5 µg of genomic DNA was treated with sodium bisulfite and purified using the Methyl Detector Kit (Active Motif, Carlsbad, CA). One duplicate DNA sample was included within each bisulfite conversion group (n=10) to serve as a technical replicate.

Bisulfite treated DNA was used as a template for polymerase chain reaction (PCR) of the promoter regions using primers complementary to the bisulfite converted DNA sequences (Figure 2A and Figure 3A). The promoter region of TNFA was assessed using previously reported primers [111]. Two regions of the IL6 promoter were assessed with primers designed using MethPrimer (http://www.urogene.org/methprimer/). Specific CpG sites within the IL6 promoter were selected for bisulfite sequencing based on prior evidence of differential methylation (i.e., c.-1162C [61], c.-727C (unpublished data)). Primer sequences and annealing temperatures for each amplicon are provided in Supplementary Table 1.

Each 25 µl reaction contained 2 µl of bisulfite treated DNA and 2.5 units PfuTurbo Cx DNA Polymerase (Agilent, Santa Clara, CA). Optimal efficiency of PCR was achieved through touchdown thermocycling and titration of reaction components to

minimize the formation of non-specific products. Excess primers and nucleotides were removed from the PCR products using ExoSAP-IT (USB Corp., Cleveland, OH). The PCR products were sequenced directly using the reverse primer with BigDye terminator sequencing chemistry (Applied Biosystems, Carlsbad, CA).

Quantitation of methylation at each CpG site was estimated from sequence trace files (i.e., .abi files) using Epigenetic Sequencing Methylation Analysis (ESME) software version 3.2.4 (Epigenomics AG, Berlin). ESME performs quality control, aligns the sequence trace file to the expected bisulfite converted genomic reference sequence, normalizes the signal intensities, corrects for incomplete bisulfite conversion, and calculates methylation levels for each CpG site by comparing the cytosine to thymine peaks [50]. ESME can discriminate between levels of methylation that differ by as little as 20% [68].

Statistical analyses

Candidate gene analysis

Allele and genotype frequencies were determined by gene counting. Hardy-Weinberg equilibrium was assessed by the Chi-square test. Measures of linkage disequilibrium (i.e., D' and r^2) were computed from the patients' genotypes with Haploview 4.2. Linkage disequilibrium (LD)-based haplotype block definition was based on D' confidence interval method [22].

For SNPs that were members of the same haploblock, haplotype analyses were conducted in order to localize the association signal within each gene and to determine if haplotypes improved the strength of the association with the phenotype. Haplotypes were constructed using the program PHASE version 2.1 [87]. In order to improve the stability

of haplotype inference, the haplotype construction procedure was repeated five times using different seed numbers with each cycle. Only haplotypes that were inferred with probability estimates of ≥.85, across the five iterations, were retained for downstream analyses. Haplotypes were evaluated assuming a dosage model (i.e., analogous to the additive model).

DNA methylation analysis

The association between the percentage of methylation at each CpG site and the persistent breast pain phenotype was evaluated by t-test at a p-value < 0.05 for blood samples collected within 6 months following surgery (n=120). Bland-Altman analysis [3] was used to assess the level of agreement between technical replicates and to determine the quality control replicate range methylation estimates (i.e., measurement noise). The 95% limits of agreement are provided in Supplementary Table 2. All CpG methylation rates are expressed as a mean percentage ± standard deviation.

Multivariate logistic regression analyses

Ancestry informative markers (AIMs) were used to minimize confounding due to population stratification [29,33,93]. Homogeneity in ancestry among patients was verified by principal component analysis [67] using Helix Tree (Golden Helix, Bozeman, MT). Briefly, the number of principal components (PCs) was sought which distinguished the major racial/ethnic groups in the sample by visual inspection of scatter plots of orthogonal PCs (i.e., PC1 versus PC2, PC2 versus PC3). This procedure was repeated until no discernible clustering of patients by their self-reported race/ethnicity was possible (data not shown). The first three PCs were selected to adjust for potential confounding due to population substructure (i.e., race/ethnicity) by including the three

covariates in all regression models. One hundred and six AIMs were included in the analysis.

For association tests, three genetic models were assessed for each SNP: additive, dominant, and recessive. Barring trivial improvements (i.e., delta <10%), the genetic model that best fit the data, by maximizing the significance of the p-value, was selected for each SNP. Logistic regression analysis, that controlled for significant covariates, as well as genomic estimates of and self-reported race/ethnicity, was used to evaluate for associations between genotype and pain group membership or CpG methylation level and pain group membership. A backwards stepwise approach was used to create the most parsimonious model. Except for genomic estimates of and self-reported race/ethnicity, only predictors with a p-value of <.05 were retained in the final model. (Epi)genetic model fit and both unadjusted and adjusted odds ratios were estimated using STATA version 12.1.

As was done in our previous studies [34,57], based on recommendations in the literature [31,76], the implementation of rigorous quality controls for genomic data, the non-independence of SNPs/haplotypes in linkage disequilibrium, and the exploratory nature of the analyses, adjustments were not made for multiple testing. Significant SNPs identified in the bivariate analyses were evaluated further using regression analyses that controlled for differences in phenotypic characteristics, potential confounding due to population stratification, and variation in other SNPs/haplotypes within the same gene. Only those SNPs that remained significant were included in the final presentation of the results. Therefore, the significant independent associations reported are unlikely to be due solely to chance. Unadjusted (bivariate) associations are reported for all SNPs passing

quality control criteria in Table 1 to allow for subsequent comparisons and meta-analyses.

Gene expression analysis

To determine whether methylation levels in the TNFA gene promoter were associated with gene expression, TNFA promoter methylation levels and TNFA mRNA expression were measured. Because RNA was not available from the patients in the primary cohort, RNA and DNA were isolated from circulating leukocytes from 44 women with breast cancer who participated in a longitudinal study that is evaluating symptoms associated with chemotherapy (CTX) administration. These patients were ≥18 years of age; had a diagnosis of breast, colorectal, gynecological, or lung cancer; had received CTX within the preceding four weeks; were scheduled to receive at least two cycles of CTX; were able to read, write, and understand English; and gave written informed consent. Patients were recruited from the Comprehensive Cancer Center at the University of California, San Francisco. Eligible patients were approached by a research staff member in the infusion unit to discuss participation in the study. After obtaining written informed consent at the subsequent enrollment visit (corresponding to the beginning of the patient's next cycle of CTX), patients completed the study instruments.

Genomic DNA was isolated using the PureGene DNA isolation kit (Gentra Systems) as described previously and normalized to a concentration of 100 ng/μl (diluted in 10 mM Tris/μ1 mM EDTA). Two micrograms of genomic DNA were treated with sodium bisulfite and purified using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) following manufacturer's instructions. Successful conversion was confirmed by direct sequencing of the TNFA promoter as previously described.

Quantitation of methylation of CpGs within the TNFA promoter was estimated from sequence trace files using the ESME software.

Total RNA extraction was performed using the PAXgene Blood miRNA kit (Qiagen, Valencia, CA) with one protocol modification (i.e., DNAse incubation was increased to 30 minutes). Quantification and integrity of total RNA were determined using the Agilent RNA 6000 Pico kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). First-strand cDNA synthesis from 125 ng total RNA was performed using the miScript II Reverse Transcription Kit (Qiagen, Valencia, CA) and the miScript HiFlex Buffer according to manufacturer's instructions. Samples were diluted (10X) and used for real-time PCR analysis.

TNFA mRNA was estimated by quantitative real-time PCR. Each 20 µl PCR reaction contained 2 µl of the diluted cDNA or standards, 10 µl of 2X QuantiTect SYBR Green Master Mix, and 2 µl of 10X QuantiTect Primer Assay (Assay name: Hs_TNF_3_SG). Quantitative PCR was performed on a BioRad CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Amplification of 18S ribosomal RNA was used to normalize the data for differences in the amount of starting material between the samples, the efficiency of RNA extraction and reverse transcription, and the variation of cDNA loaded in each reaction. Five-point standard curves of a 5-fold dilution series from a pooled sample of cDNA were used to calculate PCR efficiency. Melting curve analysis identified the formation of the expected PCR product. Samples were run in quintuplicate and a standard curve was included on each plate. Threshold cycle (C_t) values were determined by the Bio-Rad CFX Manager software, version 3.0, using the C_t determination mode "single threshold." The relative normalized

expression for each sample was determined using the Pfaffl method [64]. Measurements that exceeded 0.4 standard deviations were considered to be outliers and excluded from the analysis. Associations between the relative normalized mRNA expression and percent CpG methylation were evaluated using the Pearson product-moment correlation coefficient. Because CpG methylation is understood to decrease gene expression, a one-sided test was employed.

Results

Differences in demographic and clinical characteristics between the pain groups

Of the 398 women who completed the enrollment assessment, 126 (31.7%) were classified into the no breast pain class and 173 (43.4%) were classified into the mild breast pain class. Differences in demographic and clinical characteristics between the pain classes at the time of enrollment are provided in Table 2. Women who were classified into the mild breast pain class were significantly younger and less likely to have gone through menopause than women classified in the no breast pain class.

In terms of pre-operative clinical characteristics, women in the mild breast pain class had lower functional status scores and had more breast biopsies performed than women in the no breast pain class. For women in the mild breast pain class, 41.1% of women reported pain in the affected breast prior to surgery compared to 2.4% in the no breast pain class. Women in the mild breast pain class were more likely to report strange sensations and hardness in their affected breast prior to surgery compared to women in the no breast pain class.

In terms of surgical and postoperative characteristics, women in the mild breast pain class had a greater number of lymph nodes removed and were more likely to have

reconstruction or a surgical drain placed at the time of surgery than women in the no breast pain class. Women in the mild breast pain class reported higher average and worst postoperative pain intensity scores and were more likely to have had re-excision or mastectomy within 6 months after surgery than women in the no breast pain class.

Regression analysis for phenotypic characteristics

Based on a review of the literature that identified associations between specific phenotypic characteristics and persistent pain, the following characteristics were evaluated in the logistic regression analysis: age [1], KPS score [8,48,98], menopausal status, whether strange sensations were present in the affected breast prior to surgery [46], number of breast biopsies, number of lymph nodes removed [28,39], reconstruction performed at the time of surgery [21,46,104], severity of worst postoperative pain [41,69,90,91,99], and whether re-excision or mastectomy was performed within six months after surgery [102]. The predictors that remained significant in the final logistic regression model of demographic and clinical characteristics were age, presence of strange sensations in the affected breast prior to surgery, reconstruction performed at the time of surgery, and re-excision or mastectomy performed within six months after surgery ($p<0.0001$).

Candidate gene analysis for pain group membership

As summarized in Table 1, no associations with pain group membership were found for the SNPs in IFNGR1, IL1B, IL1R1, IL1R2, IL2, IL4, IL13, IL17A, NFKB1, and NFKB2. However, the genotype frequency was significantly different between the no pain and mild pain classes for 6 SNPs and 2 haplotypes among 4 genes (IL6: 1 SNP, 1 haplotype; IL8: 3 SNPs, 1 haplotype; IL10: 1 SNP; TNFA: 1 SNP) (Table 1).

In order to better estimate the magnitude (i.e., odds ratio, OR) and precision (i.e., 95% confidence interval, CI) of genotype on pain group membership, multivariate logistic regression models were fit. As shown in Table 3, in addition to genotype and genomic estimates of and self-reported race/ethnicity, the phenotypic characteristics included in the regression models were age, presence of strange sensations in the affected breast preoperatively, reconstruction at the time of surgery, and occurrence of re-excision or mastectomy. The genetic associations that remained significant were for IL6 rs2069840 and TNFA rs1800610 (Table 3 and Figure 4).

In the regression analysis for IL6 rs2069840 (Figure 4A), carriers of two doses of the rare G allele (i.e., CC+CG versus GG) were 76% less likely to be in the mild breast pain class. In the regression analysis for TNFA rs1800610, carriers of one or two doses of the rare T allele (i.e., CC versus CT+TT) were 63% less likely to be in the mild breast pain class.

DNA methylation analysis

Eleven CpG sites were examined in the IL6 promoter. The distal CpG sites (i.e., c.-1162C, c.-1159C, c.-1157C, c.-1132C, c.-1124C, c.-1120C) were highly methylated (i.e, >90%) and the proximal CpG sites (i.e., c.-729C, c.-727C, c.-691C, c.-673C, c.-637C) were largely unmethylated (i.e., <15%). No statistically significant differences were found in the percentage of methylation at each IL6 CpG site examined between the two breast pain groups (Figure 2). Of note, the presence of the rare C allele in rs1800796 disrupted the CpG site at c.-637C (i.e., from CpG to CpC). However, when the common G allele was present, the c.-637C CpG site was unmethylated in both pain groups.

Eleven CpG sites were assayed in the TNFA promoter. The distal CpG sites (i.e., c.-484C, c.-425C, c.-419C) were highly methylated (i.e., >70%) and the proximal CpG sites (i.e., c.-350C, c.-344C, c.-342C, c.-327C, c.-300C, c.-253C, c.-230C, c.-219C) were largely unmethylated (i.e., <30%). Compared to the no breast pain class, a significantly higher percentage of the c.-350C, c.-344C, and c.-342C sites were methylated in the mild breast pain class (all $p<0.05$, Figure 3).

In order to better estimate the magnitude (i.e., odds ratio, OR) and precision (i.e., 95% confidence interval, CI) of TNFA promoter methylation on pain group membership, multivariate logistic regression models were fit. In addition to methylation percentage, genomic estimates of and self-reported race/ethnicity, the same phenotypic characteristics were included as was done for the candidate gene analyses. As shown in Table 4, only two phenotypic characteristics (i.e., reconstruction at the time of surgery, re-excision or mastectomy within 6 months after surgery) and two CpG sites (i.e., TNFA c.-350C, TNFA c.-344C) remained significant in the final models.

In the regression analysis for TNFA c.-350C, every 5% increase in methylation was associated with a 1.33-fold increase in the odds of belonging to the mild breast pain class. In the regression analysis for TNFA c.-344C, every 5% increase in methylation was associated with a 1.47-fold increase in the odds of belonging to the mild breast pain class.

Differences in TNFA gene expression by CpG methylation status

In order to determine if the TNFA methylation status associated with pain group membership was functional (i.e., influenced gene expression), percentage methylation at TNFA c.-350, c.-344C, and c.-342C sites was correlated with TNFA gene expression in an independent sample. The expression of TNFA mRNA was inversely correlated with

the mean methylation level at each site (i.e., c.-350C (r=-0.20, p=0.114), c.-344C (r=-0.25, p=0.060), c.-342C (r=-0.28; p=0.042)). While TNFA c.-342C was not significant in the multivariate models, methylation at this site was negatively correlated with TNFA mRNA levels

Discussion

This study is the first to evaluate for associations between genetic and epigenetic variations in cytokine genes and the development of mild persistent breast pain in women following breast cancer surgery. As shown in Table 3, four phenotypic characteristics were associated with membership in the mild pain class. As noted in a recent review [1], younger age is a well established predictor of more severe postoperative pain as well as persistent pain after breast cancer surgery. In addition, consistent with a previous report [46], women who reported strange sensations in their affected breast prior to surgery were more likely to have mild persistent breast pain than women who did not report strange sensations. Our study provides prospective evidence of an association between the presence of preoperative strange sensations and the development of mild persistent breast pain following breast cancer surgery.

Findings on the impact of reconstruction at the time of surgery on persistent pain following mastectomy are inconsistent [21,46,104]. In two recent studies [21,46] no differences were found in the proportion of patients who had reconstruction done at the time of surgery and persistent pain at least 6 months postoperatively. An earlier study [104] found that immediate reconstruction was associated with a lower occurrence of persistent pain than when reconstruction was delayed. Only one study [102] evaluated the risk for persistent pain after a second surgery on the same breast. Women who had prior

breast surgery had an increased risk of developing persistent pain following surgery for breast cancer. However, the reason for the earlier surgery was not disclosed and may have been for reasons unrelated to cancer. Our findings suggest that reconstruction at the time of surgery or re-operation within 6 months of the initial surgery increases the risk for persistent breast pain of mild intensity. Reasons for these inconsistent findings may be related to differences in the assessment of the specific location of the pain (i.e., breast pain versus pain anywhere in the operative site) [1,21,24,102]; differences in the characterization of the persistent pain phenotype [46,85,102]; and/or prospective versus retrospective evaluation of the persistent pain phenotype [21,46,85,102].

Taken together, the four risk factors identified in our study suggest that more extensive or repeated injury to the breast in younger women results in mild persistent pain following breast cancer surgery. These findings are in contrast in our previous report that compared the no pain to the severe pain class in which the only phenotypic characteristic that predicted pain group membership was the severity of worst postoperative pain. While one might expect that more extensive or repeated injury to the breast might predispose to membership in the severe pain class, these associations were not found in our previous study [86]. One potential explanation for the lack of associations with other predictors is that the severe pain class was relatively small (i.e., n=46, 11.6% of the sample). Additional research is warranted to replicate our GMM classes and confirm our risk factors for mild and severe persistent breast pain.

The effects of pro- and anti-inflammatory cytokines on immune cells are known to modulate nociceptive signaling in acute and chronic inflammation and following tissue injury and nerve lesions [107]. However, significant inter-individual variability exists in

the development, intensity, and resolution of postoperative pain. In this study, two SNPs (i.e., IL6 rs2069840, TNFA rs1800610) were associated with pain group membership after adjusting for phenotypic characteristics. IL6 encodes for the IL-6 protein and is produced by a variety of cells at sites of tissue injury and inflammation. IL-6 promotes the development of persistent pain through prolonged or permanent sensitization of nociceptors and spinal cord neurons; sympathetic sprouting and microglia activation; and modulation of nociceptive mediators that produce long-term potentiation (e.g., increased nitric oxide production, altered NMDA receptor stimulation, altered synthesis of substance P and nerve growth factor) [16]. In addition to its pro-inflammatory effects, IL-6 exerts anti-inflammatory effects by promoting the release of TNFα and IL-1 antagonists to attenuate pro-inflammatory signaling [94].

Our results suggest that the rare G allele of IL6 rs2069840 decreases the risk for mild persistent breast pain after breast cancer surgery. Recent work found that the rare G allele of IL6 rs2069840 is associated with lower plasma IL-6 concentrations in survivors of myocardial infarction [65] and in patients with leprosy [84]. In addition, serum concentrations of IL-6 in the immediate postoperative period were positively associated with the extent of tissue damage that occurred during surgery [12]. In one study [108], the preoperative administration of pentoxifylline, a phosphodiesterase inhibitor that inhibits the synthesis of IL-6 [52], lowered serum IL-6 concentrations and reduced analgesic consumption following cholecystectomy. Our findings are consistent with these studies and suggest that the rare G allele of IL6 rs2069840, which was less frequent in the mild persistent breast pain group, is associated with decreased serum

concentrations of IL-6 which prevents the development of mild persistent breast pain following surgery.

TNFα is a pleiotropic pro-inflammatory cytokine that promotes the production of other proinflammatory cytokines following nerve injury [82] and plays a prominent role in the development and maintenance of persistent pain [49]. Our results suggest that the rare T allele of TNFA rs1800610 decreases the risk for the development of mild persistent breast pain following breast cancer surgery. TNFA rs1800610 is located in a non-coding region. The functional consequences of TNFA rs1800610 have been investigated *in vitro* and during physiologic stimulation in patients with rheumatoid arthritis and healthy controls [43,101]. In these studies, no differences were found in TNFA precursor mRNA production between the rs1800610 alleles. Further studies are needed to determine whether this SNP is in LD with other variations in the TNFA locus that affect cytokine production or if tissue-specific influences of this SNP on TNFA gene expression occur.

A gene containing polymorphisms associated with a specific phenotype may accrue epigenetic adaptation(s) that modulate gene expression. These epigenetic changes provide a dynamic mechanism to improve homeostasis in the setting of a sustained change in the environment. However, these attempts at homeostasis may be maladaptive when environmental contexts shift precipitously (e.g., surgery for cancer). Ongoing activation of inflammatory and glial cells, as well as inadequate compensation by spinal inhibitory mechanisms, may promote the establishment of persistent pain following neuronal injury, at least in part, through changes in gene expression moderated by epigenetic processes. However, evidence of altered epigenetic processes associated with

persistent pain is limited [17,25]. High levels of methylation in promoters are associated with repression of gene transcription [70]. In this study, the c.-350C and c.-344C sites of TNFA had higher levels of methylation in the mild breast pain class than in the no breast pain class. The effect sizes calculated for percentage methylation level at the TNFA c.-350C and c.-344C sites were 0.63 and 0.66, respectively, by Cohen's *d* which indicates a moderate to strong effect on pain group membership.

Regulation of TNFA transcription occurs at transcription factor binding sites within the proximal TNFA promoter [20,74,109]. The TNFA proximal promoter contains putative binding sites for several transcription factors that alter TNFα production in response to lipopolysaccharide [96,109], an inflammatory agent. The c.-350C site lies within the putative binding site of the specificity protein 1 (Sp1) transcription factor (i.e., 5′-CCGCCC-3′; c.-350C site underlined). The binding of Sp1 at this locus may be affected by methylation at c.-344C and c.-342C. Sp1 is a member of the specificity protein family of transcription factors that enhances or represses gene transcription in response to physiologic and pathologic stimuli [89]. For example, nitric oxide released in the course of an inflammatory response results in Sp1 binding to the TNFA promoter and the initiation of TNFA gene transcription in human leukocytes [105]. It is unclear how cytosine methylation at the Sp1 binding site within the TNFA promoter may affect TNFA gene expression. However, the observation that cytosine methylation within the putative Sp1 binding site impaired binding of nuclear proteins to the bromodomain containing 7 (BRD7) gene promoter in human nasopharyngeal carcinoma cells [51], suggests that methylation may be directly responsible for modulation of TNFA gene expression at this site.

Consistent with a previous report [27], we found a modest, inverse relationship between TNFA promoter methylation (i.e, c.-350C, c.-344C, c-342C) and TNFA mRNA transcript levels. Additional studies are needed to determine how methylation at these sites alters cytokine hemostasis and whether Sp1 binding occurs at these sites and is impacted by CpG methylation.

Taken together, our results suggest that different mechanisms may be involved in the development of preoperative breast pain [57] as well as for the development of mild and severe levels of persistent breast pain in women following breast cancer surgery. Prior studies that examined associations between preoperative breast pain or persistent postoperative pain of mild and severe intensity and variations in cytokine genes are lacking. In this same sample of patients, our group identified associations between variations in two cytokine genes (i.e., IL1R1 rs2110726, IL13 rs1295686) and the presence of preoperative pain in the affected breast [57]. Presurgical pain in cancer patients may be associated with sensitization of peripheral nerves by inflammatory mediators released from cells within the tumor and/or nerve injury due to compression by the tumor [10,55]. In addition, patients who reported preoperative pain in our study had undergone significantly more biopsies which may have increased the degree of tissue injury in the affected breast [57]. Upon binding to IL-1R1, IL-1β initiates signaling cascades that promote the production and subsequent release of inflammatory mediators that alter the biophysical properties and kinetics of ion channels and receptors embedded in neuronal membranes to augment nociceptor excitability [4,14,18,78]. Future research is needed to determine the differential effects of IL1R1 following acute tissue injury. Evidence supports a role for IL-10 and IL-13 in the regulation of acute inflammatory

responses following tissue damage [106]. Both IL-10 and IL-13 prevent the translocation of NFκB to the cell nucleus by suppressing the hydrolysis of an NFκB inhibitor. However, IL-13 displays more potent effects on hyperalgesia and cytokine regulation than IL-10 during the acute inflammatory response following acute exposure to ultraviolet B radiation [77]. Therefore, variations in IL13 may alter the inflammatory response following acute injury which may provide a rationale for the genetic association observed in the previous study [57].

Subsequently, mechanical trauma during surgery produces significant tissue injury that results in the release of inflammatory mediators and intense nociceptive activity in peripheral nociceptors. Previously, we evaluated the associations between the severe breast pain class and the same cytokine genes evaluated in this study [86]. In addition to different phenotypic characteristics, variations in different cytokine genes were found to predict pain group membership. Three SNPs (i.e., IL1R2 rs11674595; IL4 rs2243248; IL13 rs1800925) and one haplotype (i.e., IL10 haplotype A8) among four genes predicted membership in the severe pain class. Evidence supports shared biologic actions for IL-4, IL-10, and IL-13 that include limiting inflammatory hyperalgesia by inhibiting the expression of proinflammatory cytokines (e.g., TNFα, IL-6, IL-1β) [13,77,97,106]. Evidence of a role of IL1R2 and the intensity of persistent pain is lacking. However, IL-1R2 sequesters IL-1β from its signaling receptor IL-1R1. IL-1R2 may alter nociceptor excitability through the actions of IL-1β.

The different subsets of genes associated with these distinct pain phenotypes suggest different underlying mechanisms for these pain conditions. Strong evidence supports the involvement of immune cells in the development and maintenance of

persistent pain states [2,6,81]. Sequential production and release of pro- and anti-inflammatory cytokines orchestrate the inflammatory response following tissue injury. Pro-inflammatory cytokines induce their production through positive feedback and act synergistically to amplify inflammatory signals. The local release of proinflammatory cytokines must be balanced by an adequate antiinflammatory response. Aberrant release of cytokines from immune cells and glia may affect the cascade of events that are initiated by tissue injury, lead to alterations in gene expression and processing of afferent signals. Therefore, variations within cytokine genes may alter this balance between pro and anti-inflammatory cytokine production and response which may ultimately predispose an individual to the development of persistent pain.

In the current study, we evaluated the associations between the mild breast pain class and 15 cytokine genes. Two SNPs (i.e, IL6 rs2069840, TNFA rs1800610) in two different genes predicted membership in the mild pain class. Both IL6 and TNFA have prominent proinflammatory roles. Our findings suggest that variations in proinflammatory responses are associated with the development of mild persistent pain. Proinflammatory cytokines have an important role in repair processes following nerve injury. These mediators are the first substances to be released following peripheral nerve injury, recruit immune cells to the site of tissue damage, and sensitize local nociceptors to protect the affected area from further damage [6]. Activated astrocytes participate in the maintenance of persistent pain [38]. A recent study found that intrathecal administration of TNFα activated astrocytes is sufficient to induce mechanical allodynia in mice [23]. Therefore, variations in TNFA that increase the bioavailability of TNFα centrally may prolong astrocyte activation and produce persistent postoperative pain. In addition, our

findings that variations in IL1R2, IL4, IL10, and IL13 were associated with persistent breast pain of severe intensity suggest that severe pain may be a consequence of alterations in the negative feedback inflammatory mechanisms. Because we were unable to interrogate all genes associated with the cytokine pathways, future studies are needed to confirm this hypothesis.

Findings from our study provide evidence that persistent breast pain of mild intensity has a number of characteristics that differentiate these patients from patients who have no pain following breast cancer surgery. Consistent with our findings, a prior study found that mild levels of persistent postsurgical pain were associated with diminished perceptions of overall health and reduced physical and social functioning [32]. Because the no pain and mild pain groups may be treated similarly in a clinical setting, future studies should investigate whether other differences between these groups exist (e.g., functional, social, psychological). In addition, future genetic association studies should use a pain phenotype that incorporates pain intensity ratings within the pain phenotype.

Study limitations must be acknowledged. First, no direct measurements of serum cytokines were done to provide additional data on the mechanisms that underlie the development of mild persistent pain. Second, future studies are needed to validate the IL6 and TNFA SNP associations. Third, DNA methylation was the only epigenetic mechanism evaluated in this study. The expression of IL6 and TNFA may be modulated by other epigenetic mechanisms (e.g., histone modification, non-coding RNAs) and/or DNA methylation outside of the regions evaluated in this study. Finally, additional

studies are needed to replicate the TNFA promoter methylation findings in this study as well as the correlations between TNFA methylation level and gene expression.

In conclusion, findings from this study suggest a role for polymorphisms within the IL6 and TNFA genes and changes in methylation in the TNFA gene promoter in the development of mild persistent breast pain in women following breast cancer surgery. The genes, SNPs, and methylation sites identified in this study may help to identify individuals who are predisposed to the development of mild persistent postsurgical breast pain. Future studies are needed to confirm our findings and determine if these associations are seen in other persistent postsurgical pain syndromes.

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Figure legends

Figure 1: Observed and estimated worst breast pain severity trajectories for patients in the mild, moderate and severe breast pain latent classes. Differences between the no pain and mild pain classes (black) were evaluated. The moderate and severe pain classes are shown in grey. Observed trajectories are shown with dashed lines and the estimated trajectories are shown with solid lines. This figure was adapted from Miaskowski et al [59].

Figure 2: Deoxyribonucleic acid (DNA) methylation at the promoter region of the interleukin 6 (IL6) gene. (A) Scaled schematic representation of the 5' untranslated region of the IL6 gene showing the distribution of CpG sites (vertical lines) and regions (horizontal bars) amplified using polymerase chain reaction. Nucleotide positions in relation to predicted translation start site are provided from GRCh37.p9 Primary Assembly; NM_000600.3. Two regions of the IL6 gene promoter were assessed. Region I assessed the percent methylation of c.-1162C, c.-1159C, c.-1157C, c.-1132C, c.-1124C and c.-1120C. Region II assessed the percent methylation of c.-729C, c.-727C, c.-691C, c.-673C, and c.-637C. The c.-1064C locus was not assayed. The association of the methylation level at c.-1120C and pain group membership was not evaluated because this site was 100% methylated for all samples. Locations of the amplified regions of the bisulfite-modified genome are shown as horizontal bars above the promoter. (B) DNA methylation levels of the promoter regions for IL6. Data are shown as the group mean and standard deviation. (C) Heat map showing the pairwise shared variance (r^2) of methylation levels between CpG sites. The value of r^2 multiplied by 100 is provided

within each box. The degree of shared variance was color-coded to indicate high (red) to none (white) with intermediate values rendered in pink.

Abbreviations: na, not assayed

Figure 3: Deoxyribonucleic acid (DNA) methylation at the promoter region of the tumor necrosis factor alpha (TNFA) gene. (A) Scaled schematic representation of the 5' untranslated region of the TNFA gene showing the distribution of CpG sites (vertical lines) and region (horizontal bars) amplified using polymerase chain reaction. Nucleotide positions in relation to predicted translation start sites are provided from GRCh37.p9 Primary Assembly; NM_000594.3. Primer binding sites are shown (arrows). (B) DNA methylation levels of the promoter region for TNFA. Data are shown as the group mean and standard deviation. Asterisks indicates statistical significance at $p<0.05$. (C) Heat map showing the pairwise shared variance (r^2) of methylation levels between CpG sites. The value of r^2 multiplied by 100 is provided within each box. The degree of shared variance was color-coded to indicate high (red) to none (white) with intermediate values rendered in pink.

Figure 4: Differences in the percentages of patients in the no breast pain and mild breast pain latent classes who were: A) homozygous for the common allele or heterozygous (CC+CG) or homozygous for the rare allele (GG) for rs2069840 in the interleukin 6 (IL6) gene; B) homozygous for the common allele (CC) or heterozygous or homozygous for the rare allele (CT+TT) for rs1800610 in the tumor necrosis factor alpha (TNFA) gene.

Table 1 – Differences in single nucleotide polymorphisms in cytokine genes between the no pain and the mild pain groups

Gene	SNP	Position	Chr	MAF	Alleles	Chi Square	p-value	Model
IFNG1	rs2069728	66834051	12	.110	G>A	3.18	0.204	A
IFNG1	rs2069727	66834490	12	.384	A>G	4.80	0.091	A
IFNG1	rs2069718	66836429	12	.494	C>T	2.74	0.254	A
IFNG1	rs1861493	66837463	12	.266	A>G	5.62	0.060	A
IFNG1	rs1861494	66837676	12	.273	T>C	6.11	0.047	A
IFNG1	rs2069709	66839970	12	.003	G>T	n/a	n/a	n/a
IFNG1	HapA3					5.82	0.055	
IFNG1	HapA5					4.60	0.100	
IFNGR1	rs9376268	137574444	6	.254	G>A	1.02	0.601	A
IL1B	rs1071676	106042060	2	.189	G>C	0.53	0.769	A
IL1B	rs1143643	106042929	2	.383	G>A	0.42	0.811	A
IL1B	rs1143642	106043180	2	.082	C>T	0.44	0.802	A
IL1B	rs1143634	106045017	2	.187	C>T	0.59	0.744	A
IL1B	rs1143633	106045094	2	.392	G>A	0.34	0.845	A
IL1B	rs1143630	106046282	2	.115	C>A	0.26	0.878	A
IL1B	rs3917356	106046990	2	.450	G>A	0.99	0.609	A
IL1B	rs1143629	106048145	2	.389	T>C	2.05	0.358	A
IL1B	rs1143627	106049014	2	.397	T>C	1.46	0.481	A
IL1B	rs16944	106049494	2	.386	G>A	1.71	0.425	A
IL1B	rs1143623	106050452	2	.277	G>C	0.36	0.833	A
IL1B	rs13032029	106055022	2	.448	C>T	0.34	0.843	A
IL1B	HapA1					0.03	0.985	
IL1B	HapA4					0.35	0.838	
IL1B	HapA6					0.58	0.749	
IL1B	HapB1					1.41	0.495	
IL1B	HapB6					0.81	0.667	
IL1B	HapB8					0.46	0.796	
IL1R1	rs949963	96533648	2	.223	G>A	2.78	0.249	A
IL1R1	rs2228139	96545511	2	.053	C>G	4.54	0.103	A
IL1R1	rs3917320	96556738	2	.047	A>C	n/a	n/a	n/a
IL1R1	rs2110726	96558145	2	.317	C>T	1.39	0.499	A
IL1R1	rs3917332	96560387	2	.187	A>T	0.90	0.639	A
IL1R1	HapA1					0.71	0.702	
IL1R1	HapA2					2.29	0.318	
IL1R1	HapA3					0.87	0.648	
IL1R2	rs4141134	96370336	2	.362	T>C	1.36	0.508	A
IL1R2	rs11674595	96374804	2	.258	T>C	2.85	0.241	A
IL1R2	rs7570441	96380807	2	.408	G>A	1.33	0.515	
IL1R2	HapA1					1.33	0.515	
IL1R2	HapA2					0.07	0.790	

IL1R2	HapA4					1.82	0.403	
IL2	rs1479923	119096993	4	.308	C>T	0.53	0.766	A
IL2	rs2069776	119098582	4	.184	T>C	n/a	n/a	n/a
IL2	rs2069772	119099739	4	.241	A>G	4.46	0.108	A
IL2	rs2069777	119103043	4	.047	C>T	n/a	n/a	n/a
IL2	rs2069763	119104088	4	.277	T>G	0.42	0.811	A
IL2	HapA1					2.99	0.224	
IL2	HapA2					0.47	0.791	
IL2	HapA3					4.46	0.108	
IL4	rs2243248	127200946	5	.086	T>G	1.10	0.576	A
IL4	rs2243250	127201455	5	.269	C>T	n/a	n/a	n/a
IL4	rs2070874	127202011	5	.245	C>T	n/a	n/a	n/a
IL4	rs2227284	127205027	5	.387	C>A	n/a	n/a	n/a
IL4	rs2227282	127205481	5	.390	C>G	n/a	n/a	n/a
IL4	rs2243263	127205601	5	.124	C>G	3.29	0.193	A
IL4	rs2243266	127206091	5	.237	G>A	n/a	n/a	n/a
IL4	rs2243267	127206188	5	.237	G>C	n/a	n/a	n/a
IL4	rs2243274	127207134	5	.261	G>A	n/a	n/a	n/a
IL4	HapA1					2.48	0.289	
IL4	HapA3					2.75	0.253	
IL4	HapX1					2.86	0.240	
IL6	rs4719714	22643793	7	.255	A>T	0.42	0.810	A
IL6	rs2069827	22648536	7	.069	G>T	0.16	0.923	A
IL6	rs1800796	22649326	7	.134	C>G	n/a	n/a	n/a
IL6	rs1800795	22649725	7	.285	C>G	0.34	0.845	A
IL6	rs2069835	22650951	7	.061	T>C	n/a	n/a	n/a
IL6	rs2066992	22651329	7	.049	G>T	n/a	n/a	n/a
IL6	rs2069840	22651652	7	.333	C>G	FE	0.005	R
IL6	rs1554606	22651787	7	.319	G>T	0.73	0.693	A
IL6	rs2069845	22653229	7	.319	A>G	0.73	0.693	A
IL6	rs2069849	22654236	7	.024	C>T	n/a	n/a	n/a
IL6	rs2069861	22654734	7	.056	C>T	2.96	0.227	A
IL6	rs35610689	22656903	7	.259	A>G	2.84	0.241	A
IL6	HapA1					1.14	0.565	
IL6	HapA5					7.87	0.020	
IL6	HapA8					1.57	0.456	
IL8	rs4073	70417508	4	.455	T>A	FE	0.044	R
IL8	rs2227306	70418539	4	.366	C>T	FE	0.025	R
IL8	rs2227543	70419394	4	.368	C>T	FE	0.027	R
IL8	HapA1					4.85	0.089	
IL8	HapA4					6.15	0.046	
IL10	rs3024505	177638230	1	.129	C>T	8.09	0.018	A
IL10	rs3024498	177639855	1	.204	A>G	1.73	0.422	A
IL10	rs3024496	177640190	1	.421	T>C	3.62	0.163	A

IL10	rs1878672	177642039	1	.416	G>C	3.63	0.162	A
IL10	rs3024492	177642438	1	.190	T>A	n/a	n/a	n/a
IL10	rs1518111	177642971	1	.303	G>A	3.13	0.209	A
IL10	rs1518110	177643187	1	.301	G>T	2.84	0.242	A
IL10	rs3024491	177643372	1	.408	G>T	3.14	0.208	A
IL10	HapA1					3.82	0.148	
IL10	HapA2					4.98	0.083	
IL10	HapA8					1.57	0.456	
IL13	rs1881457	127184713	5	.210	A>C	1.72	0.423	A
IL13	rs1800925	127185113	5	.233	C>T	3.66	0.160	A
IL13	rs2069743	127185579	5	.019	A>G	n/a	n/a	n/a
IL13	rs1295686	127188147	5	.265	G>A	4.09	0.130	A
IL13	rs20541	127188268	5	.212	C>T	2.70	0.259	A
IL13	HapA1					4.09	0.130	
IL13	HapA4					2.57	0.276	
IL17A	rs4711998	51881422	6	.346	G>A	1.72	0.425	A
IL17A	rs8193036	51881562	6	.327	T>C	0.85	0.652	A
IL17A	rs3819024	51881855	6	.372	A>G	0.81	0.668	A
IL17A	rs2275913	51882102	6	.361	G>A	1.70	0.427	A
IL17A	rs3804513	51884266	6	.023	A>T	n/a	n/a	n/a
IL17A	rs7747909	51885318	6	.217	G>A	0.82	0.663	A
NFKB1	rs3774933	103645369	4	.409	T>C	0.19	0.908	A
NFKB1	rs170731	103667933	4	.358	A>T	0.44	0.803	A
NFKB1	rs17032779	103685279	4	.011	T>C	n/a	n/a	n/a
NFKB1	rs230510	103695201	4	.410	T>A	1.80	0.406	A
NFKB1	rs230494	103706005	4	.434	A>G	1.40	0.496	A
NFKB1	rs4648016	103708706	4	.010	C>T	n/a	n/a	n/a
NFKB1	rs4648018	103709236	4	.018	G>C	n/a	n/a	n/a
NFKB1	rs3774956	103727564	4	.435	C>T	1.35	0.509	A
NFKB1	rs10489114	103730426	4	.018	A>G	n/a	n/a	n/a
NFKB1	rs4648068	103737343	4	.363	A>G	1.72	0.424	A
NFKB1	rs4648095	103746914	4	.052	T>C	0.45	0.505	A
NFKB1	rs4648110	103752867	4	.170	T>A	3.83	0.147	A
NFKB1	rs4648135	103755716	4	.061	A>G	0.06	0.811	A
NFKB1	rs4648141	103755947	4	.180	G>A	2.07	0.355	A
NFKB1	rs1609798	103756488	4	.337	C>T	2.59	0.273	A
NFKB1	HapA1					1.56	0.457	
NFKB1	HapA9					0.46	0.796	
NFKB2	rs12772374	104146901	10	.168	A>G	0.15	0.928	A
NFKB2	rs7897947	104147701	10	.221	T>G	1.91	0.385	A
NFKB2	rs11574849	104149686	10	.070	G>A	1.12	0.572	A
NFKB2	rs1056890	104152760	10	.305	C>T	1.76	0.414	A
TNFA	rs2857602	31533378	6	.341	T>C	1.66	0.435	A
TNFA	rs1800683	31540071	6	.390	G>A	0.12	0.943	A

TNFA	rs2239704	31540141	6	.335	G>T	1.24	0.538	A
TNFA	rs2229094	31540556	6	.278	T>C	0.96	0.620	A
TNFA	rs1041981	31540784	6	.386	C>A	0.08	0.962	A
TNFA	rs1799964	31542308	6	.224	T>C	1.89	0.388	A
TNFA	rs1800750	31542963	6	.016	G>A	n/a	n/a	n/a
TNFA	rs1800629	31543031	6	.149	G>A	0.84	0.658	A
TNFA	rs1800610	31543827	6	.100	C>T	FE	0.014	D
TNFA	rs3093662	31544189	6	.074	A>G	0.98	0.613	A
TNFA	HapA1					0.43	0.806	
TNFA	HapA5					1.70	0.428	
TNFA	HapA6					2.23	0.328	

Note: IFNG rs1861494 was excluded from further consideration as the distribution of alleles did not follow a Mendelian mode (i.e., additive, dominant, recessive).

Abbreviations: A = additive model, Chr = chromosome, D = dominant model, Hap = haplotype, IFNG = interferon gamma, IL = interleukin, MAF = minor allele frequency, n/a = not assayed because SNP violated Hardy-Weinberg expectations ($p<0.001$) or because MAF was <.05, NFKB = nuclear factor kappa beta, R = recessive model, SNP= single nucleotide polymorphism, TNFA = tumor necrosis factor alpha

Table 2 - Differences in demographic and clinical characteristics between the breast pain classes prior to surgery

Demographic Characteristics	No Pain n=126 Mean (SD)	Mild Pain n=173 Mean (SD)	Statistics
Age (years)	58.6 (11.4)	53.4 (11.5)	t=3.84; p<0.001
Education (years)	15.8 (2.8)	16.0 (2.6)	t=-0.79; p=0.430
	% (N)	% (N)	
Ethnicity			
White	73.0 (92)	63.7 (109)	
Black	7.1 (9)	8.8 (15)	X^2=3.07;
Asian/Pacific Islander	10.3 (13)	12.9 (22)	p=0.381
Hispanic/ mixed ethnic background/other	9.5 (12)	14.6 (25)	
Lives alone*	20.8 (26)	24.6 (42)	FE; p=0.486
Marital status			
Married/partnered	41.3 (52)	39.5 (68)	FE; p=0.811
Single, separated, widowed, divorced	58.7 (74)	60.5 (104)	
Currently working for pay*	52.0 (65)	50.9 (87)	FE; p=0.906
Total annual household income			
< $10,000 to $19,999	8.5 (9)	14.4 (21)	Z=-0.717;
$20,000 to $99,000	48.1 (51)	43.8 (64)	p=0.473
≥ $100,000	43.4 (46)	41.8 (61)	
Clinical Characteristics	Mean (SD)	Mean (SD)	
Body mass index (kg/m^2)	27.1 (7.0)	25.9 (5.3)	t=1.62; p=0.107
Karnofsky Performance Status score	96.2 (8.7)	93.6 (9.3)	t=2.42; p=0.016
Self-Administered Comorbidity Scale score	4.0 (2.3)	4.0 (3.0)	t=-0.02; p=0.981
Number of breast biopsies	1.4 (0.7)	1.6 (0.8)	Z=-2.45; p=0.014
	% (N)	% (N)	
Occurrence of comorbid conditions (% and number of women who reported each comorbid condition from the Self-Administered Comorbidity Questionnaire)*			
Heart disease	4.0 (5)	2.9 (5)	FE; p=0.747
High blood pressure	34.9 (44)	24.3 (42)	FE; p=0.052
Lung disease	2.4 (3)	3.5 (6)	FE; p=0.738
Diabetes	7.1 (9)	5.8 (10)	FE; p=0.640
Ulcer	3.2 (4)	4.0 (7)	FE; p=0.765
Kidney disease	1.6 (2)	0.6 (1)	FE; p=0.575
Liver disease	3.2 (4)	1.7 (3)	FE; p=0.460
Anemia	7.9 (10)	5.2 (9)	FE; p=0.348
Depression	16.7 (21)	22.5 (39)	FE; p=0.243
Osteoarthritis	17.5 (22)	14.5 (25)	FE; p=0.522
Back pain	22.2 (28)	27.2 (47)	FE; p=0.348

Rheumatoid arthritis	1.6 (2)	2.9 (5)	FE; p=0.703
Diagnosed with mastitis*	11.2 (14)	12.3 (21)	FE; p=0.856
Diagnosed with fibrocystic disease*	18.6 (22)	22.4 (38)	FE; p=0.465
Ever breast fed*	48.0 (60)	47.1 (81)	FE; p=0.907
Surgery to affected breast unrelated to cancer*	7.9 (10)	14.5 (25)	FE; p=0.101
Post-menopausal*	71.0 (88)	58.3 (98)	FE; p=0.027
Received neoadjuvant chemotherapy*	17.5 (22)	18.6 (32)	FE; p=0.879
On hormonal replacement therapy prior to surgery*	19.0 (24)	16.9 (29)	FE; p=0.648
Stage of disease Stage 0 Stage 1 Stage IIA and IIB Stage IIIA, IIIB, IIIC, and IV	 17.5 (22) 41.3 (52) 35.7 (45) 5.6 (7)	 20.8 (36) 35.8 (62) 35.8 (62) 7.5 (13)	Z=-0.07; p=0.945
Pain in breast prior to surgery*	2.4 (3)	41.1 (69)	FE; p<0.001
Swelling in affected breast*	3.2 (4)	6.4 (11)	FE; p=0.286
Numbness in affected breast*	2.4 (3)	3.5 (6)	FE; p=0.738
Strange sensations in affected breast*	12.7 (16)	34.7 (60)	FE; p<0.001
Hardness in affected breast*	7.9 (10)	21.4 (37)	FE; p=0.002
Surgical Characteristics	Mean (SD)	Mean (SD)	
Number of lymph nodes removed	4.3 (4.7)	6.0 (7.1)	t=-2.45; p=0.015
Number of drains placed during surgery	0.4 (0.7)	0.5 (0.7)	t=-1.33; p=0.184
	% (N)	% (N)	
Type of surgery Breast conserving Mastectomy	 84.1 (106) 15.9 (20)	 77.5 (134) 22.5 (39)	FE; p=0.186
Reconstruction at the time of surgery*	15.9 (20)	29.1 (50)	FE; p=0.009
Surgical drain placed in breast at time of surgery*	65.7 (23)	64.2 (43)	FE; p=1.000
Post-surgical Characteristics	Mean (SD)	Mean (SD)	
Number of postoperative complications	0.2 (0.5)	0.2 (0.5)	t=-0.49; p=0.625
Severity of average postoperative pain	2.8 (2.1)	3.7 (2.2)	t=-3.46; p<0.001
Severity of worst postoperative pain	4.2 (2.6)	5.0 (2.6)	t=-2.60; p=0.010
	% (N)	% (N)	
Received radiation therapy during the 6 months*	56.3 (71)	54.3 (94)	FE; p=0.814
Received adjuvant chemotherapy during the 6 months*	31.7 (40)	34.7 (60)	FE; p=0.621
Received hormonal therapy during the 6 months*	45.2 (57)	41.6 (72)	FE; p=0.556
Received biological therapy during the 6 months*	8.7 (11)	12.7 (22)	FE; p=0.351
Received complementary therapy during the 6 months*	23.8 (30)	28.3 (49)	FE; p=0.427
Had breast reconstruction during the 6 months*	4.8 (6)	10.4 (18)	FE; p=0.087
Had re-excision or mastectomy during the 6 months*	18.3 (23)	31.2 (54)	FE; p=0.016

* denotes the percentage of patients with the demographic or clinical characteristic
Abbreviations: FE = Fisher's Exact; SD = standard deviation; kg = kilogram; m^2 = meters squared
Adapted from Miaskowski et al. [59].

Table 3 – Multiple logistic regression analyses for candidate gene polymorphisms

GMM Class Comparison	Predictor	Odds Ratio	Standard Error	95% CI	Z	p-value
No pain versus mild pain (n=233)	IL6 genotype	0.24	0.12	0.09, 0.65	-2.79	0.005
	Age	0.84	0.06	0.73, 0.96	-2.57	0.010
	Strange breast sensations preoperatively	3.08	1.22	1.42, 6.68	2.85	0.004
	Reconstruction at time of surgery	2.50	0.92	1.22, 5.16	2.49	0.013
	Re-excision/mastectomy	2.79	1.03	1.35, 5.77	2.77	0.006
	Overall model fit: $\chi^2 = 48.76$, $p < 0.0001$; $R^2 = 0.1544$					
No pain versus mild pain (n=233)	TNFA genotype	0.37	0.15	0.17, 0.80	-2.51	0.012
	Age	0.83	0.06	0.72, 0.95	-2.65	0.008
	Strange breast sensations preoperatively	3.45	1.36	1.59, 7.49	3.14	0.002
	Reconstruction at time of surgery	2.47	0.92	1.20, 5.11	2.44	0.015
	Re-excision/mastectomy	2.23	0.81	1.10, 4.52	2.21	0.027
	Overall model fit: $\chi^2 = 46.82$, $p < 0.0001$; $R^2 = 0.1483$					

Notes: Multiple logistic regression analysis of the no pain and the mild pain GMM group on the worst pain rating. For each model, the first three principal components identified from the analysis of ancestry informative markers as well as self-report race/ethnicity were retained in all models to adjust for potential confounding due to race or ethnicity (data not shown). Predictors evaluated in each model included genotype (IL6 rs2069840: CC + CG versus GG; TNFA rs1800610: CC versus CT + TT), age at time of enrollment in 5-year increments, presence of strange sensations in the affected breast prior to surgery, reconstruction performed at the time of surgery, and re-excision or mastectomy was performed within 6 months after the initial surgery for breast cancer.

Abbreviations: CI =confidence interval; GMM = growth mixture model; IL6 = interleukin 6; TNFA = tumor necrosis factor alpha

Table 4 – Multiple logistic regression analyses for TNFA CpG promoter methylation

GMM Class Comparison	Predictor	Odds Ratio	Standard Error	95% CI	Z	p-value
No pain versus mild pain (n=120)	TNFA c.-350C methylation	1.33	0.12	1.11, 1.59	3.08	0.002
	Reconstruction at time of surgery	2.69	1.33	1.02, 7.09	2.00	0.046
	Re-excision/mastectomy	2.86	1.49	1.02, 7.96	2.01	0.045
	Overall model fit: $\chi^2 = 22.01$, p=0.0089; $R^2 = 0.1377$					
No pain versus mild pain (n=119)	TNFA c.-344C methylation	1.47	0.17	1.17, 1.85	3.31	0.001
	Reconstruction at time of surgery	3.05	1.54	1.13, 8.23	2.21	0.027
	Re-excision/mastectomy	2.92	1.52	1.05, 8.11	2.05	0.040
	Overall model fit: $\chi^2 = 25.03$, p=0.0029; $R^2 = 0.1576$					

Notes: Multiple logistic regression analysis of the no pain and the mild pain GMM group on the worst pain rating. For each model, the first three principal components identified from the analysis of ancestry informative markers as well as self-report race/ethnicity were retained in all models to adjust for potential confounding due to race or ethnicity (data not shown). Predictors evaluated in each model included CpG methylation level in 5% increments (i.e., TNFA c.-350C and c.-344C), age at time of enrollment, presence of strange sensations in the affected breast prior to surgery, reconstruction performed at the time of surgery, and re-excision or mastectomy was performed within 6 months after the initial surgery for breast cancer.

Abbreviations: CI =confidence interval; GMM = growth mixture model; TNFA = tumor necrosis factor alpha

Supplementary Table 1 – Primer sequences used for bisulfite sequencing reactions

Gene	Gene region	Gene location*	Chromosomal location#	Primer sequences	T_m (°C)
TNFA	Proximal promoter (-529 to -131)	4641 – 4669 5012 – 5039	6:31542990-31543018 6:31543362-31543389	Fwd 5'-GTT TTT AAA AGA AAT GGA GGT AAT AGG TT-3' Rev 5'-TCC CTC TTA ACT AAT CCT CTA CTA TCC T-3'	52.7 55.6
IL6	Distal promoter (-1344 to -1065)	3772 – 3796 4024 – 4041	7:22765538-22765562 7:22765790-22765807	Fwd 5'- GTT GTG TAA GGG TTT GGT TTT AGT T -3' Rev 5'- CTC CCT CTC CCT ATA AAT CTT AAT TTA A -3'	54.1 52.3
IL6	Proximal promoter (-804 to -559)	4533 – 4557 4312 – 4335	7:22766078-22766101 7:22766299-22766323	Fwd 5'- TAT TTG GGA GAG GGT AGG GTA GTA -3' Rev 5'- CCC CCA AAC CTA AAA TTA TAA AAA A -3'	56.0 50.3

* TNF: NG_007462.1 ;IL6: NG_011640.1

GRCh37.p9 Primary Assembly: TNF: NC_000006.11; IL6: NC_000007.13

Abbreviations: TNF, tumor necrosis factor alpha; IL6, interleukin 6; T_m, annealing temperature; Fwd, forward; Rev, reverse

Supplementary Table 2 – Bland Altman analysis of control DNA to assess the repeatability in the measurement of CpG methylation levels.

Gene	Gene location	Average % methylation	LLA	95% CI of LLA	ULA	95% CI of ULA
IL6	c.-1162C	95.5%	-0.048	(-.072 , -.024)	0.059	(.035 , .083)
IL6	c.-1159C	97.0%	-0.055	(-.077 , -.034)	0.041	(.020 , .063)
IL6	c.-1157C	98.0%	-0.031	(-.044 , -.018)	0.026	(.014 , .039)
IL6	c.-12C	100.0%	0	(0, 0)	0	(0, 0)
IL6	c.-1124C	100.0%	0	(0, 0)	0	(0, 0)
IL6	c.-1120C	100.0%	0	(0, 0)	0	(0, 0)
IL6	c.-729C	2.2%	-0.089	(-.129 , -.049)	0.091	(.051 , .131)
IL6	c.-727C	9.1%	-0.154	(-.228 , -.081)	0.174	(.101 , .248)
IL6	c.-691C	0.2%	-0.016	(-.023 , -.009)	0.015	(.008 , .022)
IL6	c.-673C	1.9%	-0.047	(-.069 , -.026)	0.047	(.026 , .069)
IL6	c.-637C	1.2%	-0.053	(-.081 , -.025)	0.073	(.045 , .101)
TNFA	c.-484C	86.7%	-0.119	(-.159 , -.079)	0.089	(.049 , .129)
TNFA	c.-425C	70.7%	-0.129	(-.179 , -.078)	0.134	(.084 , .185)
TNFA	c.-419C	70.5%	-0.133	(-.182 , -.084)	0.121	(.072 , .170)
TNFA	c.-350C	24.6%	-0.194	(-.263 , -.125)	0.164	(.095 , .233)
TNFA	c.-344C	11.1%	-0.197	(-.270 , -.123)	0.186	(.113 , .260)
TNFA	c.-342C	12.6%	-0.086	(-.118 , -.054)	0.079	(.047 , .111)
TNFA	c.-327C	2.3%	-0.104	(-.141 , -.068)	0.075	(.039 , .112)
TNFA	c.-300C	19.2%	-0.135	(-.189 , -.081)	0.140	(.086 , .194)
TNFA	c.-253C	14.0%	-0.131	(-.190 , -.072)	0.177	(.118 , .236)
TNFA	c.-230C	14.4%	-0.169	(-.237 , -.101)	0.185	(.117 , .252)
TNFA	c.-219C	7.8%	-0.149	(-.211 , -.086)	0.175	(.113 , .237)

Notes: Bisulfite conversions were performed with groups of 10 DNA samples. Identical control DNA was included as one of 10 DNA samples included in each bisulfite conversion group. The percent methylation of the control DNA from each group was paired. The upper and lower limits of agreement and 95% confidence intervals were calculated from the mean difference of these pairs. Genome build: GRCh37.p9 Primary Assembly: TNF: NM_000594.3; IL6: NM_000600.3

Abbreviations: CI, confidence interval; DNA, deoxyribonucleic acid; IL6, interleukin 6; LLA, lower limit of agreement; TNFA, tumor necrosis factor alpha; ULA, upper limit of agreement

Figure 1

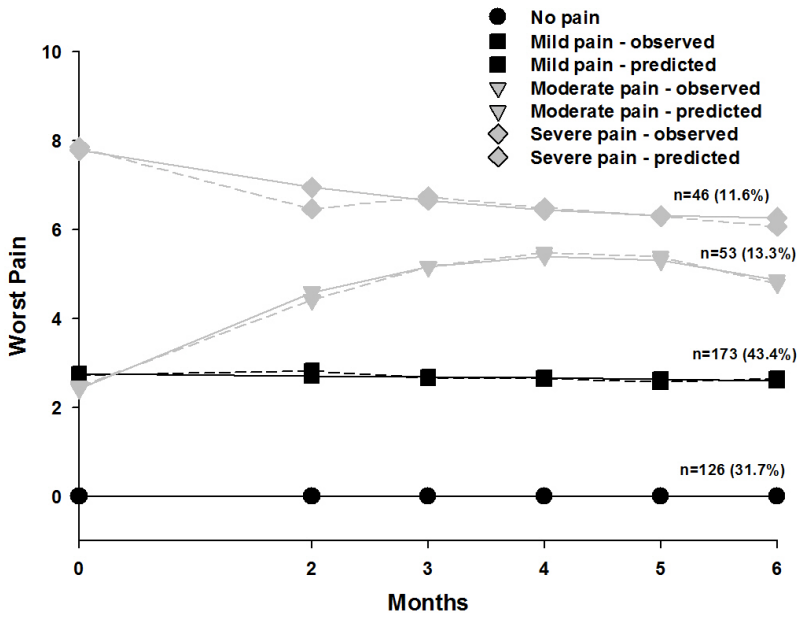

No pain
Mild pain - observed
Mild pain - predicted
Moderate pain - observed
Moderate pain - predicted
Severe pain - observed
Severe pain - predicted
n=46 (11.6%)
n=53 (13.3%)
n=173 (43.4%)
n=126 (31.7%)
Worst Pain
Months
0
2
4
6
8
10
0
2
3
4
5
6

Figure 2

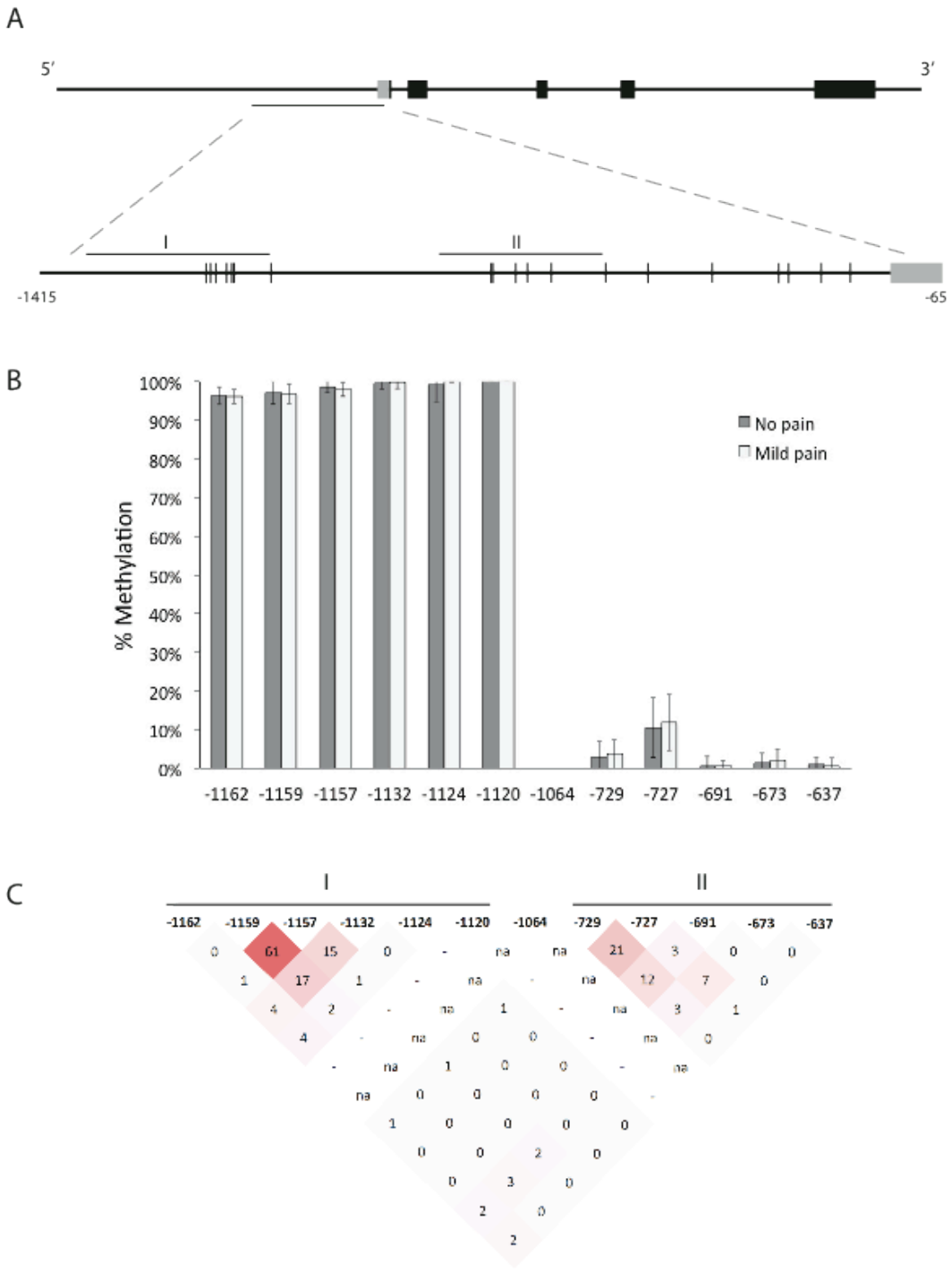

A
5′
3′
I
II
-1415
-65
B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Methylation
No pain
Mild pain
-1162 -1159 -1157 -1132 -1124 -1120 -1064 -729 -727 -691 -673 -637
C
I
II
-1162 -1159 -1157 -1132 -1124 -1120 -1064 -729 -727 -691 -673 -637

Figure 3

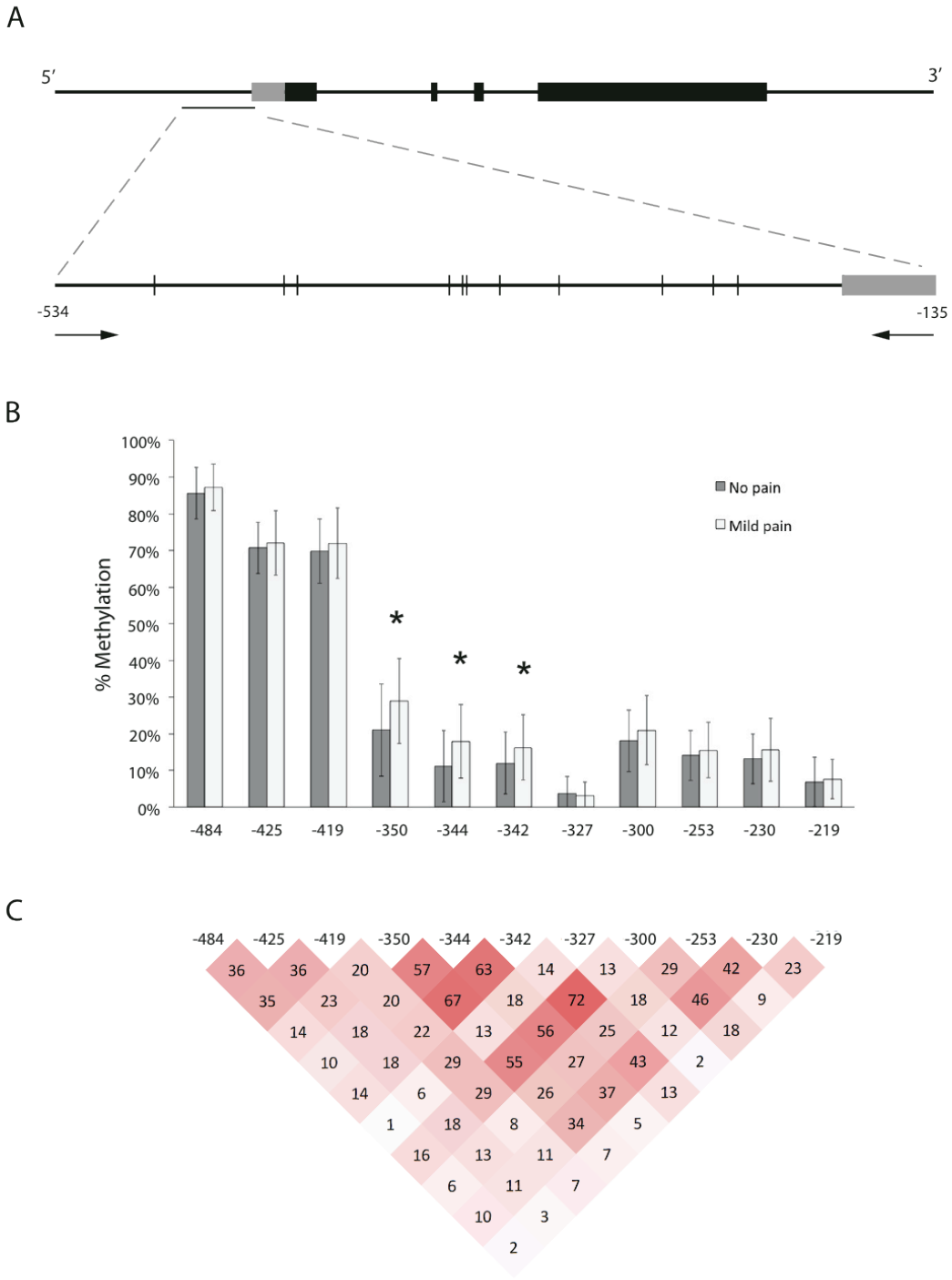
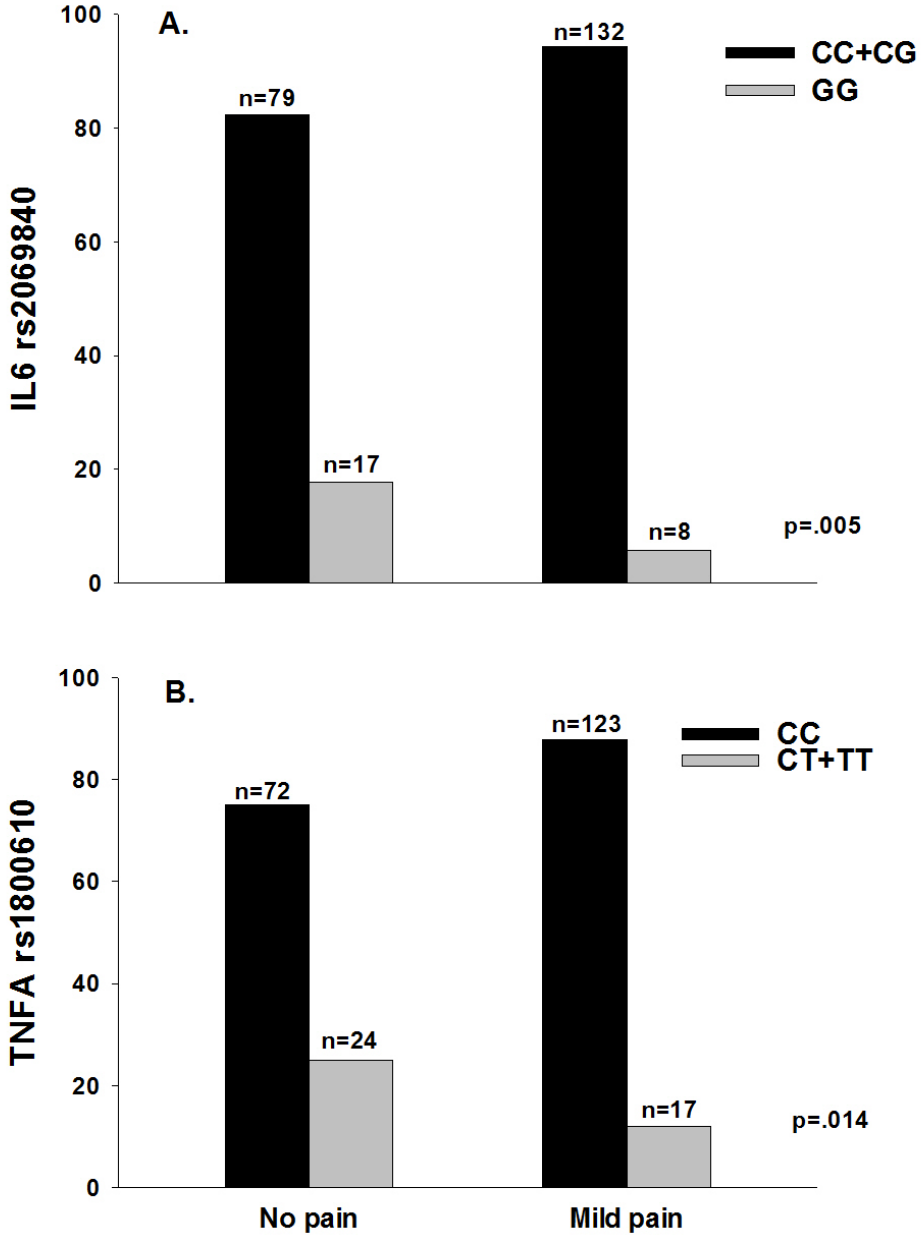

A
5′
3′
-534
-135
B
% Methylation
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
No pain
Mild pain
*
*
*
-484
-425
-419
-350
-344
-342
-327
-300
-253
-230
-219
C
-484 -425 -419 -350 -344 -342 -327 -300 -253 -230 -219
36 36 20 57 63 14 13 29 42 23
35 23 20 67 18 72 18 46 9
14 18 22 13 56 25 12 18
10 18 29 55 27 43 2
14 6 29 26 37 13
1 18 8 34 5
16 13 11 7
6 11 7
10 3
2

Figure 4


A.
IL6 rs2069840
100
80
60
40
20
0
n=79
n=17
n=132
n=8
p=.005
CC+CG
GG
B.
TNFA rs1800610
n=72
n=24
n=123
n=17
p=.014
CC
CT+TT
No pain
Mild pain

Chapter 5

Conclusions

Findings from this dissertation research provide evidence that different non-genetic and genetic risk factors exist for the development of persistent breast pain of mild and severe intensity following breast cancer surgery. In addition, findings suggest that the development of mild persistent breast pain after breast cancer surgery is associated with changes in epigenetic regulation.

Evaluation of demographic and clinical characteristics

In the overall study sample, four subgroups of women were identified with distinct persistent breast pain trajectories prior to and for six months following breast cancer surgery (i.e., none, mild, moderate, severe)[1]. Differences in demographic, pre-operative, intra-operative, and postoperative characteristics were found among these classes. In terms of demographic characteristics, patients in the mild, moderate, and severe pain classes were significantly younger than patients in the no pain class. In addition, patients in the severe pain class were more likely to be non-White, had fewer years of education, and reported less income than patients in the no pain and mild pain classes. In terms of preoperative characteristics, patients in the severe pain class had a lower Karnofsy Performance Status (KPS) score, higher ratings of average and worst breast pain prior to surgery, decreased range of motion in their affected arm, and were more likely to report a history of rheumatoid arthritis compared with the patients in the no pain class. A higher percentage of patients in all three pain classes reported numbness and hardness in the affected breast prior to surgery compared to patients in the no pain class. Patients in the moderate and severe pain classes reported significantly higher depression, trait anxiety, sleep disturbance and fatigue scores, and poorer attentional

function scores compared to the no pain class. In addition, patients in the moderate and severe pain classes reported lower quality of life scores than patients in the no pain class.

In terms of intraoperative characteristics, patients in the severe pain class had a higher number of lymph nodes removed and were more likely to have had an axillary lymph node dissection compared to patients in the no pain class. In terms of postoperative characteristics, patients in the mild, moderate, and severe pain classes reported higher average and worst pain intensity scores in the immediate postoperative period compared to those in the no pain class. Patients in the severe pain class were more likely to have had a re-excision or mastectomy in the 6 months following surgery compared to patients in the no pain class. Finally, compared to the mild pain and the severe pain classes, a higher percentage of patients in the moderate pain class had radiation therapy in the 6 months following surgery.

The study presented in chapter three evaluated for associations among variations in 15 cytokine genes between the no breast pain and the severe breast pain classes. Several demographic and clinical characteristics differed statistically in the bivariate analyses. However, the only phenotypic characteristic that predicted pain group membership in the multivariate analysis was the severity of worst postoperative pain. This finding agrees with several review articles[2-4] that concluded that severe postoperative pain is a well-established risk factor for the development of phantom breast pain and other neuropathic pain syndromes following breast cancer surgery.

This finding is also consistent with our current knowledge of the development of persistent pain. Sensitized and injured peripheral nerves produce intense and prolonged afferent ectopic activity that is transmitted to dorsal horn neurons in the central nervous

system[5-7]. This excessive ectopic activity may alter the morphologic and biochemical properties of the pre- and post-synaptic membranes and change the excitability of dorsal horn neurons. Prolonged central sensitization leads to permanent alterations in the structures responsible for processing nociceptive stimuli[8]. Prolonged stimulation of peripheral nociceptors by postoperative pain of high intensity maintains a hyperexcited state in dorsal horn neurons[9]. One might expect that more extensive or repeated injury to the breast might predispose to membership in the severe pain class. However, these associations were not found. One potential explanation for the lack of associations with other predictors is that the severe pain class was a relatively small sample (i.e., n=46, only11.6% of the total sample).

The study presented in chapter four evaluated for variations in 15 cytokine genes between the no breast pain class and the mild breast pain class. In terms of demographic and clinical characteristics, results from this study suggest that age, presence of strange sensations in the affected breast prior to surgery, whether reconstruction was performed at the time of surgery, and whether re-excision or mastectomy was performed within 6 months after surgery are risk factors for the development of mild persistent breast pain following breast cancer surgery. Findings from this study suggest that more extensive or repeated injury to the breast in younger women can result in mild persistent pain following breast cancer surgery.

These findings are consistent with current evidence that suggests that younger age is a well established predictor of more severe postoperative pain as well as persistent pain after breast cancer surgery[10]. In addition, a previous report[11] found that women who reported strange sensations in their affected breast prior to surgery were more likely to

have mild persistent breast pain than women who did not report strange sensations. Prior studies that evaluated the impact of reconstruction at the time of surgery on persistent pain following mastectomy report inconsistent findings[11-13]. Findings from this dissertation study suggest that reconstruction at the time of surgery or re-operation within 6 months of the initial surgery increases the risk for persistent breast pain of mild intensity. Reasons for these inconsistent findings may be related to differences in the assessment of the specific location for the pain (i.e., breast pain versus pain anywhere in the operative site)[10,12,14,15]; differences in the characterization of the persistent pain phenotype[11,15,16]; and/or preoperative versus retrospective evaluation of the persistent pain phenotype[11,12,15,16].

The different subsets of phenotypic characteristics associated with the distinct breast pain phenotypes provide different risk factors for the development of mild and severe breast pain following breast cancer surgery. A mounting body of evidence supports the association between the intensity of postoperative pain and the development of persistent pain following breast cancer surgery. However, limited evidence exists that supports an association between phenotypic characteristics and the development of mild persistent pain. Additional research is warranted to confirm the risk factors identified in this dissertation research.

Genetic associations

Pro- and anti-inflammatory cytokines are known to modulate nociceptive signaling during acute and chronic inflammation and following tissue injury and nerve lesions[17]. However, significant interindividual variability exists in the development and resolution of postsurgical pain. Results of this dissertation study suggest that different

mechanisms may be involved in the development of preoperative breast pain[18] as well as in the development of mild and severe levels of persistent breast pain in women following breast cancer surgery.

In this sample, associations were found previously between variations in two cytokine genes (i.e., IL1R1 rs2110726, IL13 rs1295686) and the presence of preoperative pain in the affected breast[18]. Presurgical pain in cancer patients may be associated with sensitization of peripheral nerves by inflammatory mediators released from cells within the tumor and/or nerve injury due to compression by the tumor[19,20]. In addition, patients in our study who reported preoperative pain had undergone significantly more biopsies which may have increased the degree of tissue injury in the affected breast[18]. Upon binding to IL-1R1, IL-1β initiates signaling cascades that promote the production and subsequent release of inflammatory mediators that alter the biophysical properties and kinetics of ion channels and receptors embedded in neuronal membranes to augment nociceptor excitability[21-24]. Future research is needed to determine the differential effects of IL-1R1 following acute tissue injury. Evidence supports a role for IL-10 and IL-13 in the regulation of the acute inflammatory response following tissue damage[25]. Both IL-10 and IL-13 prevent the translocation of NFκB to the cell nucleus by suppressing the hydrolysis of an NFκB inhibitor. However, IL-13 was shown to have more potent effects on hyperalgesia and cytokine regulation than IL-10 during the acute inflammatory response following acute exposure to ultraviolet B radiation[26]. Therefore, variations in IL13 may alter the inflammatory response following acute injury which may partially explain the genetic association observed in this study.

Subsequently, mechanical trauma during surgery produces significant tissue

injury that results in the release of inflammatory mediators and intense nociceptive activity in peripheral nociceptors. In addition to different phenotypic characteristics that predicted pain group membership in this dissertation study, variations within different cytokine genes predicted membership in the mild and severe pain classes.

In the study presented in chapter three, after adjustment for severity of worst postoperative pain, three single nucleotide polymorphisms (SNPs) (i.e., interleukin (IL) 1 receptor 2 rs11674595, IL4 rs2243248, IL13 rs1800925) and one haplotype (i.e., IL10 haplotype A8) differed between the no pain and severe pain classes. Findings from this study suggest that the rare "C" allele of IL1R2 rs11674595, the rare “G” allele of IL4 rs2243248, and the rare “T” allele of IL13 rs1800925 increase the risk for the development of severe persistent breast pain. In addition, the IL10 haplotype A8 was associated with a decreased risk for the development of severe persistent pain. The IL10 haplotype A8 is composed of seven SNPs (i.e., rs3024505 “C” allele, rs3024498 “G” allele, rs3024496 “C” allele, rs1878672 “G” allele, rs1518111 “A” allele, rs1518110 “T” allele, rs3024491 “T” allele).

Evidence supports shared biologic actions of IL-4, IL-10, and IL-13 that include being produced mainly by Th2 lymphocytes and limiting inflammatory hyperalgesia by inhibiting the expression of proinflammatory cytokines (e.g., TNFα, IL-6, IL-1β)[25-28]. The rare G allele of IL4 rs2243248 was associated with the presence of a symptom cluster composed of clinically meaningful levels of depression, pain, sleep disturbance, and fatigue in patients who underwent primary or adjuvant radiotherapy and their family caregivers[29]. The functional consequences of IL4 rs2243248 are not known. However, IL4 rs2243248 was found to be in strong linkage disequilibrium (LD) with

rs2243250[30,31]. The "T" allele of IL4 rs2243250 is associated with increased transcriptional activity of IL-4 *in vitro*[32]. IL-4 promotes hypoalgesia by suppressing the actions of activated macrophages, prevents the expression of cyclooxygenase-2 and inducible nitric oxide synthase, and inhibits the synthesis of pro-inflammatory cytokines[24,33]. In addition, IL-4 induces μ- and δ-opioid receptor transcription which promotes hypoalgesia through the endogenous opioid system[34,35].

Current evidence supports anti-inflammatory actions for IL-13 in the context of neuropathic pain[36,37]. Findings from this dissertation research suggest that the rare T allele of IL13 rs1800925 is associated with increased risk for the development of severe persistent breast pain. IL13 rs1800925 is a functional polymorphism that is located within a Nuclear Factor of Activated T cells (NFAT) binding site of the IL13 promoter[38,39]. Functional studies demonstrated that the C to T substitution increases the affinity of NFAT for the IL13 promoter[38] and enhances IL13 gene transcription and IL-13 secretion from Th2 lymphocytes[39]. Although findings from this dissertation research may seem counterintuitive, the effects of IL-13 on the initiation and maintenance of persistent pain may be context dependent. Pro-inflammatory properties of IL-13 have been identified in allergy[39,40]. In addition, the rare T allele of rs1800925 has been associated with an increased risk of asthma[38,41,42]. Finally, findings from this dissertation research are consistent with a recent study[43] in which women who reported pain one month after fine needle biopsy or resection of their breast tumor had elevated plasma levels of IL-13 compared to women without pain.

In the study presented in chapter four, two SNPs in two genes (i.e., IL6 rs2069840, tumor necrosis factor alpha (TNFA) rs1800610) differed between the no pain

and mild pain classes. The rare "G" allele of IL6 rs2069840 and the rare "T" allele of TNFA rs1800610 decreased the risk for mild persistent breast pain after breast cancer surgery. These findings are consistent with prior studies and suggest that the rare G allele of IL6 rs2069840 is associated with decreased serum concentrations of IL-6 which prevents the development of mild persistent breast pain following surgery[44,45]. Prior studies that investigated the functional consequences of TNFA rs1800610 have found no differences in TNFA precursor mRNA production between the rs1800610 alleles[46,47]. Additional studies are needed to determine whether this SNP is in LD with other variations in the TNFA locus that affect cytokine production and/or if tissue-specific expression of TNFA occurs with this SNP.

The different subsets of genes associated with these distinct phenotypes suggest different mechanisms of genetic susceptibility. Strong evidence supports the involvement of immune cells in the development and maintenance of persistent pain states[48,49]. Sequential production and release of pro- and anti-inflammatory cytokines orchestrate the inflammatory responses following tissue damage. Induction of pro-inflammatory cytokines occurs through positive feedback and synergistic interactions that amplify inflammatory signals. The local release of pro-inflammatory cytokines must be balanced by an adequate anti-inflammatory response. Aberrant release of cytokines from immune cells and glia may affect the cascade of events that are initiated by tissue injury and lead to alterations in gene expression and processing of afferent signals. Therefore, variations within cytokine genes may alter the balance between pro and anti-inflammatory cytokines which may ultimately predispose an individual to the development of persistent pain.

Both IL6 and TNFA have prominent pro-inflammatory roles. Findings from this dissertation study suggest that variations in pro-inflammatory responses may be associated with the development of mild persistent pain. Pro-inflammatory cytokines (e.g., TNFa, IL-6) have an important role in repair processes following nerve injury. These mediators are the first substances to be released following peripheral nerve injury, recruit immune cells to the site of tissue damage, and sensitize local nociceptors to protect the affected area from further damage[49]. The associations between variations in IL1R2, IL4, IL10, and IL13 and persistent breast pain of severe intensity suggest that severe pain may be a consequence of alterations in the negative feedback inflammatory mechanisms. However, all of the genes associated with the cytokine pathways were not interrogated in this dissertation research.

Epigenetic associations

In addition to the independent contribution of variations within individual genes, the interactions among variations in several genes and the environment in which these genes are expressed may contribute to substantial inter-individual differences in pain perception. Ongoing activation of inflammatory and glial cells, as well as inadequate compensation by spinal inhibitory mechanisms, may promote the establishment of persistent pain following neuronal injury, at least in part, through changes in gene expression moderated by epigenetic processes. However, evidence of altered epigenetic processes associated with persistent pain is limited[50,51]. As described in chapter two, the best characterized forms of epigenetic regulation are histone modification, DNA methylation, and noncoding RNA expression. Evaluation of changes in these epigenetic

mechanisms may identify associations that contribute to interindividual variability in pain severity.

Findings from the study presented in chapter four show an association between increased CpG methylation within the TNFA promoter and pain group membership. In adjusted analyses, the c.-350C and c.-344C sites of TNFA had higher levels of methylation in the mild breast pain class than in the no breast pain class. The effect sizes calculated for percentage methylation level at the TNFA c.-350C and c.-344C sites were 0.63 and 0.66, respectively, by Cohen's *d* which indicates a moderate to strong effect on pain group membership.

Regulation of TNFA transcription occurs at transcription factor binding sites within the proximal TNFA promoter[52-54]. The c.-350C site lies within the putative binding site of the specificity protein 1 (Sp1) transcription factor (i.e., 5′-CCGCCC-3′; c.-350C site underlined). Binding of Sp1 at this locus may be affected by methylation at c.-344C and c.-342C. Sp1 is a member of the specificity protein family of transcription factors that enhances or represses gene transcription in response to physiologic and pathologic stimuli[55]. It is unclear how cytosine methylation at the Sp1 binding site within the TNFA promoter may affect TNFA gene expression. However, one possible explanation for how cytosine methylation at the Sp1 binding site may affect TNFA expression is that the presence of a methyl group may impede the Sp1 from physically binding to the region[56]. Alternatively, cytosine methylation may recruit methyl-binding proteins that prevent Sp1 binding[57].

Gene expression studies performed herein in an independent sample of cancer patients confirmed an inverse relationship between the percentage of methylation in the

TNFA gene promoter and TNFA mRNA expression[58]. An increase in promoter methylation that was observed in the mild pain class may seem counterintuitive. However, cytokines participate in complex cascades and are modulated by external signals. It is possible that increased TNFA methylation may be acquired in patients with persistent pain as a response to other molecular mechanisms that produce nociceptor excitability (i.e., TNFA methylation may attempt to decrease nociceptive excitability). Additional research is needed to determine the temporal associations between promoter methylation and persistent pain.

Implications for Future Research

The findings from this dissertation research provide directions for future research. First, the persistent breast pain phenotype identified in this dissertation research warrants replication. In addition, the risk factors for mild and severe persistent breast pain need to be confirmed. This dissertation study evaluated a persistent breast pain phenotype that was identified using pain ratings obtained prior to surgery through the first six months following breast cancer surgery. Future research needs to evaluate whether these models change if the assessments are extended through the first 1 to 2 years. This research would provide novel data on the maintenance and resolution of persistent postoperative breast pain after breast cancer surgery.

A larger cohort of patients would likely increase the sample size of the severe breast pain class. This larger sample size may identify additional demographic and clinical characteristics associated with severe persistent breast pain. In addition, with the larger sample size significant covariates could be included within the growth mixture modeling. Future research should include assessments of preexisting pain conditions and

analgesic use as characteristics that may affect the development of persistent pain as well as the intensity of pain. In addition, findings from this study support prior evidence[59] that important differences exist between patients with no pain and mild persistent pain exist. Because these groups may be treated similarly in a clinical setting, future studies should investigate whether other clinically meaningful differences between these groups exist (e.g., functional, social, psychological). The demographic and clinical characteristics identified from these future studies may enable clinicians to identify patients who are at higher risk for the development of persistent pain.

This dissertation research is the first study to evaluate for associations between cytokine gene polymorphisms and persistent postsurgical pain. Validation of the genetic associations identified in this dissertation research is required in an independent sample. Future research may evaluate for associations between persistent breast pain among all of the cytokine genes as well as in the genes participating downstream and upstream in the pathway in which the genes evaluated for in this dissertation research occur. In addition, future studies may determine the mechanisms by which the SNPs identified in this dissertation research alter gene expression and protein production in the context of persistent postsurgical pain (e.g., gene expression studies, reporter gene constructs).

This dissertation research is the first study to evaluate for associations between DNA methylation of cytokine gene promoters and persistent postsurgical pain. Validation of the epigenetic associations found in this dissertation research is required in an independent sample. Future studies should determine if methylation levels of TNFA and other cytokine gene promoters impact transcription factor binding in these regions and how methylation alters gene expression and cytokine homeostasis. Future research

may determine whether differential methylation levels exist in cytokine genes that did not harbor significant genetic associations with mild persistent pain. In addition, other forms of epigenetic regulation (e.g., histone modification, nucleosome positioning) should be evaluated to determine their associated effects on gene expression in persistent pain alone or in combination with DNA methylation. Longitudinal assessment of epigenetic regulation in leukocytes may help elucidate mechanisms associated with the development and maintenance of persistent pain. Finally, these genetic and epigenetic associations should be examined in other persistent pain syndromes (e.g., post-herpetic neuralgia, diabetic neuropathy) to determine whether observed alterations to epigenetic regulation are specific to postoperative pain or may be generalized to other pain conditions.

Implications for Clinical Practice

Because of the lack of effective treatment options for persistent pain syndromes, it is important that clinicians have the ability to identify patients at risk for the development of persistent pain. The novel breast pain phenotype used in this dissertation research suggests that clinicians may be able to identify patients at risk for persistent breast pain through preoperative screening and during the initial postoperative period.

Findings from this dissertation research suggest that younger patients, patients with strange sensations present in their affected breast, patients who receive reconstruction at the time of surgery, and patients who undergo re-excision or mastectomy within 6 months after surgery are at higher risk for the development of persistent breast pain of mild intensity. In addition, results of this dissertation research underscore the importance of adequate treatment of acute postoperative pain to prevent the development of severe persistent postsurgical pain[2,4,60]. Clinicians may use these

demographic and clinical characteristics to identify patients at higher risk of persistent postoperative pain, follow these patients closely for resolution of postoperative pain, and provide tailored therapies as they become available. In addition, clinicians should aggressively treat pain during the postoperative period and following repeat surgeries.

The novel genetic variations identified in this dissertation research may contribute to the elucidation of nociceptive processes involved in the development of persistent pain states and identify inter-individual differences in the development of persistent pain that may be used as biomarkers for risk stratification and identification of effective treatment options. The epigenetic findings presented in chapter four describe differential methylation of the TNFA promoter. In combination with the genetic variations, these findings may allow the molecular mechanisms that underlie persistent pain states to be further elucidated and promote understanding of how tissue injury and a patient's environment may alter gene expression. This knowledge may be used to individualize treatment regimens to improve the patients' long-term outcomes.

Conclusions

Collectively, the risk factors identified in this dissertation research suggest that inadequately managed postoperative pain, more extensive or repeated injury to the breast in younger women results in persistent pain following breast cancer surgery of mild or severe intensity. Strong evidence supports the involvement of immune cells in the development and maintenance of persistent pain[48,49]. Aberrant release of cytokines from immune cells and glia may affect the cascade of events that are initiated by tissue injury and lead to alterations in gene expression and processing of afferent signals. The genetic and epigenetic variations identified in this dissertation research may affect cytokine

production and predispose patients to the development of persistent postsurgical pain of mild or severe intensity. Findings from this dissertation and subsequent research may ultimately lead to the better identification of individuals who are predisposed to the development of persistent pain following breast cancer surgery, differentiate biological mechanisms, and facilitate the development of novel therapies.

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