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ASSOCIATIONS BETWEEN GENETIC AND EPIGENETIC VARIATIONS IN CYTOKINE GENES AND MILD PERSISTENT BREAST PAIN IN WOMEN FOLLOWING BREAST CANCER SURGERY

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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, three SNPs (i.e., interleukin 6 (*IL6*) rs2069840, C-X-C motif chemokine ligand 8 (*CXCL8*) rs4073, tumor necrosis factor (*TNF*) rs1800610) and two *TNF* CpG sites (i.e., c.-350C, c.-344C) were associated with pain group membership. These findings suggest that variations in *IL6*, *CXCL8*, and *TNF* are associated with the development and maintenance of mild persistent breast pain. CpG methylation within the *TNF* promoter may provide an additional mechanism through which *TNF* 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.

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Keywords

cytokine genes; breast cancer; DNA methylation; persistent pain; post-surgical pain; post-mastectomy pain; epigenetics; interleukin 6; C-X-C motif chemokine ligand 8; tumor necrosis factor

1. Introduction

Persistent pain in women following breast cancer surgery is common and associated with altered mood and sleep patterns, decreased quality of life, and disability [1, 2]. Persistent postsurgical pain may result from ongoing nociceptor activation and/or nerve injury [3]. During the early postoperative period, inflammatory mediators produce sensitization at the affected area. These reversible changes in sensitivity discourage stimulation which serves as a protective mechanism that facilitates healing. Sustained activation of nociceptors may lead to maintenance of central sensitization and maladaptive phenotypic changes that alter the normal stimulus-response relationship and produce persistent pain [4].

Persistent alterations within nociceptors include changes in gene expression [5]. Evidence suggests that ongoing activation of inflammatory cells plays a role in the establishment of persistent pain [6]. In addition, peripheral nerve injury elicits an inflammatory reaction that prompts the aggregation of immune cells and increases the local concentration of pro-inflammatory cytokines [7]. These mediators participate in the initiation and maintenance of persistent pain after nerve injury by generating ectopic activity [8], altering neuronal connectivity [9], and reducing the number of inhibitory neurons [10].

Despite a clear connection between immune mechanisms and persistent pain [7], few studies were identified that evaluated for associations between polymorphisms in cytokine pathways and cancer-related pain [11–14]. Findings from these studies are difficult to interpret because pain was characterized using a dichotomized rating, the samples were small, and the number of polymorphisms evaluated was not comprehensive.

Emerging evidence suggests that acquired adaptations to genetic regulation (termed “epigenetics”) are pervasive in biology [15]. Deoxyribonucleic acid (DNA) methylation is an epigenetic mechanism that regulates gene expression [15]. Acquisition of methylation at CpG dinucleotides provides an adaptive capacity for the organism to adjust to sustained changes in its environment. The methylation status of CpG sites within gene promoters has emerged as a promising biomarker for risk stratification and detection of human disease [16].

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 [17]. Three distinct classes were identified using patients’ ratings of worst pain in their breast. A fourth pain group was designated for those women who did not experience breast pain. The largest subgroup of women identified was the mild breast pain class (n= 173, 43.5%) which had a mean worst pain severity score of 2.5 (on a 0 to 10 numeric rating scale (NRS)). Of note, mild levels of persistent postsurgical pain are

associated with diminished perceptions of overall health and reduced physical and social functioning [18]. Therefore, using data from women who were classified into the no breast pain and mild breast pain classes, the purposes of this study were to: 1) evaluate for associations between single nucleotide polymorphisms (SNPs) contained within cytokine genes and pain group membership and 2) determine the methylation status of CpG sites contained within the promoter of cytokine genes that harbored gene variations associated with pain group membership.

2. Methods

2.1. Patients and settings

This longitudinal study is part of a larger study of women who underwent breast cancer surgery [11, 17, 19]. Patients were recruited from a Comprehensive Cancer Center, two public hospitals, and four community practices. Patients were included if they: were female; 18 years of age; underwent unilateral breast cancer surgery; were able to read, write, and understand English; and gave written informed consent. Patients were excluded if they had bilateral breast cancer surgery and/or had distant metastasis. Of the 516 patients approached, 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.

2.2. Subjective measures

The Breast Symptoms Questionnaire (BSQ) and Post Surgical Pain Questionnaire, evaluated persistent and acute postoperative pain, respectively. Part 1 of the BSQ obtained information on the occurrence of pain and the occurrence of other symptoms in the breast scar area. Additional symptoms that were based on the work of Tasmuth and colleagues [20, 21]. Patients completed Part 2 of the BSQ if they had pain in the breast scar area. Patients rated the intensity of their average and worst pain using a 0 (no pain) to 10 (worst imaginable pain) NRS. The NRS is a valid and reliable measure of pain intensity [22].

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 intensity were rated on a 0 (no pain) to 10 (worst imaginable pain) NRS. Pain relief was rated on a 0% (no relief) to 100% (complete relief) scale. Satisfaction with pain treatment was rated on a 0 (not satisfied at all) to 10 (extremely satisfied) NRS. Patients completed this questionnaire during the month 1 study visit.

2.3. Study procedures

The Committee on Human Research at the University of California, San Francisco and the Institutional Review Boards at our other sites approved this study. During the patient's preoperative visit, a clinician explained the study to the patient and determined her willingness to participate. For women who were willing to participate, clinicians introduced the patient to the research nurse. The research nurse determined the woman's eligibility, obtained written informed consent prior to surgery, had the patients complete the enrollment questionnaires (Assessment 0).

Patients were contacted two weeks after surgery to schedule the first post-surgical appointment. Patients were seen 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 visits, the women completed the study questionnaires and provided information on new and ongoing treatments. The blood sample was collected at the time of enrollment or during one of the monthly study visits. Patients' medical records were reviewed for disease and treatment information.

2.4. Characterization of the persistent breast pain phenotype

The characterization of the persistent breast pain phenotype used in the current study was described previously [17]. At each assessment, patients were asked, "Are you experiencing pain in your affected breast?" If the patient reported pain, she rated her "current pain at its worst" using a 0 (no pain) to 10 (worst pain) NRS. Prior to performing 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%). These patients 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 [23]. 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 established guidelines [24–26]. 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 between the GMM classes. 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 for 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 significantly 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 in the models that evaluated the associations between genotype and pain group membership and CpG methylation level and pain group membership.

2.5. Genotype determination

Genomic 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 nanogram/microliter (ng/μL) (diluted in 10 mM Tris/1 mM EDTA). A combination of tagging SNPs and literature driven SNPs (i.e., reported as being associated with altered function and/or symptoms) were chosen for this study. Tagging SNPs needed to be common (i.e., had a minor allele frequency (MAF) of .05 in public databases (e.g., HapMap)).

The genotyping was performed blinded to clinical status and incorporated positive and negative controls. Samples were genotyped using a custom built array on the Golden Gate platform (Illumina, San Diego, CA) and processed with a 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. Quality control filtering of SNPs was performed. SNPs with call rates <95%, Hardy-Weinberg $P < .001$, and/or a MAF of <5% were excluded.

As shown in Supplementary Table 1, 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; IL1 receptor 1 (*IL1R1*): 4 SNPs; *IL1R2*: 3 SNPs; *IL2*: 3 SNPs; *IL4*: 2 SNPs; *IL6*: 9 SNPs; C-X-C motif chemokine ligand 8 (*CXCL8*): 3 SNPs; *IL10*: 7 SNPs; *IL13*: 4 SNPs; *IL17A*: 5 SNPs; nuclear factor kappa beta-1 (*NFKB1*): 11 SNPs; *NFKB2*: 4 SNPs; tumor necrosis factor (*TNF*): 9 SNPs) passed all quality control filters and are included in subsequent analyses. These candidate genes were selected based on a review of the literature on the role of inflammatory mediators in the development of persistent pain [27–29]. PUPASuite 2.0 was used to evaluate potential functional roles for SNPs associated with persistent pain [30].

2.6. 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*, *CXCL8*, *TNF*) was determined using bisulfite sequencing. Genomic DNA from each patient was normalized to a concentration of 75 ng/μl to 125 ng/μl (diluted in 10 mM Tris/1 mM EDTA). Approximately 1.5 micrograms (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 the template for polymerase chain reaction (PCR) of the promoter regions using primers complementary to the bisulfite converted DNA sequences (Figure 1A, Figure 2A, Figure 3A). The promoter region of *TNF* was assessed using previously reported primers [31]. Two regions of the *IL6* promoter and one region of the *CXCL8* promoter were assessed with primers designed using MethPrimer (<http://www.urogene.org/methprimer/>). Primers were designed to assess methylation of CpG sites within the proximal promoter for *CXCL8*. Specific CpG sites within the *IL6* promoter were

selected for bisulfite sequencing based on prior evidence of differential methylation (i.e., c.-1162C [32], c.-727C (unpublished data)). Primer sequences and annealing temperatures for each amplicon are provided in Supplementary Table 2.

Each 25 μ l reaction contained 2 μ l 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 [33].

2.7. Statistical analyses

2.7.1. Candidate gene analysis—Gene counting was used to determine allele and genotype frequencies. Hardy-Weinberg equilibrium was assessed by the Chi Square test. Measures of linkage disequilibrium (i.e., D' and r^2) were done using Haploview 4.2. Linkage disequilibrium (LD)-based haplotype block definition was based on D' confidence interval method [34].

Haplotype analyses were conducted in order to localize the association signal within each gene and to determine if the identified haplotypes improved the strength of the association with the phenotype. Haplotypes were constructed using the program PHASE version 2.1 [35]. The haplotype construction procedure was done five times using different seed numbers each time. Only haplotypes that were inferred with probability estimates of $\geq .85$, across all five iterations, were retained for downstream analyses. Haplotypes were evaluated assuming a dosage model.

2.7.2. 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 of < 0.05 for blood samples collected within 6 months following surgery ($n=120$). Bland-Altman analysis 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) [36]. The 95% limits of agreement are provided in Supplementary Table 3. All CpG methylation estimates are expressed as mean percentages $\pm$ standard deviations.

2.7.3. Multivariate logistic regression analyses—Ancestry informative markers (AIMs) were used to minimize confounding due to population stratification [37–39]. These AIMs were determined by principal component analysis [40] using Helix Tree (Golden Helix, Bozeman, MT). The number of principal components (PCs) was sought that

distinguished the major racial/ethnic groups in the sample by visual inspection of scatter plots of orthogonal PCs. 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 for use as covariates in all of the regression models to adjust for potential confounding due to population substructure (i.e., race/ethnicity). One hundred and six AIMs were used in the analysis.

Three genetic models were assessed for each SNP: additive, dominant, and recessive. 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. The backwards stepwise approach was employed 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 all of our previous studies (e.g., [11, 41]), based on recommendations in the literature [42, 43], the implementation of rigorous quality controls for genomic data, the non-independence of SNPs/haplotypes in LD, as well as the exploratory nature of the analyses, adjustments were not made for multiple testing. The significant SNPs identified in the bivariate analyses were evaluated further using regression analyses. These analyses controlled for differences in phenotypic characteristics identified in our prior study (Table 1) [44], potential confounding due to population stratification, as well as variation in other SNPs/haplotypes within the same gene. Only the SNPs that remained significant are reported in this paper. Therefore, the significant independent associations reported in this paper are unlikely to be due solely to chance. Unadjusted (bivariate) associations are reported for all of the SNPs that passed quality control criteria in Supplementary Table 1 to allow for subsequent comparisons and meta-analyses.

3. Results

3.1. Demographic and clinical characteristics of the pain classes

As summarized in Table 1, patients in the mild pain class were younger and more likely to be premenopausal. In addition, these patients had a lower KPS score; had had a higher number of breast biopsies; and were more likely to report pain, strange sensations, and hardness in their breast prior to surgery. Patients in the mild pain class were more likely to have had a higher number of lymph nodes removed; to have had reconstruction at the time of surgery; to have had higher levels of postoperative pain; and to have had a re-excision or mastectomy in the 6 months following surgery [17].

3.2. Candidate gene analysis for pain group membership

As summarized in Supplementary 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 7 SNPs and 2 haplotypes among 5 genes (*IFNG1*: 1 SNP; *IL6*: 1 SNP, 1 haplotype; *CXCL8*: 3 SNPs, 1 haplotype; *IL10*: 1 SNP; *TNF*: 1 SNP). 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 2, in addition to genotype and genomic estimates of and self-reported race/ethnicity, the phenotypic characteristics included in all of the regression models were: the presence of preoperative pain in the affected breast, hardness in the affected breast preoperatively, and occurrence of re-excision or mastectomy within the first 6 months following surgery. The genetic associations that remained significant were for *IL6* rs2069840, *CXCL8* rs4073, and *TNF* rs1800610 (Table 2).

In the regression analysis for *IL6* rs2069840, patients homozygous the rare G allele (i.e., CC +CG versus GG) were 79% less likely to be in the mild breast pain class. In the regression analysis for *CXCL8* rs4073, patients homozygous for the rare A allele (i.e., TT + TA versus AA) were 60% less likely to be in the mild breast pain class. In the regression analysis for *TNF* rs1800610, patients who were heterozygous or homozygous for the rare T allele (i.e., CC versus CT+TT) were 63% less likely to be in the mild breast pain class.

3.3. 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 any of the *IL6* CpG sites examined between the two breast pain groups (Figure 1B). 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.

Six CpG sites were assayed in the *CXCL8* promoter (i.e., c.-116C, c.-106C, c.-31, c.+46C, c.+125, c.+171C). All CpG sites were largely unmethylated. No statistically significant differences were found in the percentage of methylation at each of the *CXCL8* sites examined between the two breast pain groups (Figure 2B).

Eleven CpG sites were assayed in the *TNF* 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 3B). Of note, these three CpG sites are moderately correlated (Figure 3C).

In order to better estimate the magnitude (i.e., odds ratio, OR) and precision (i.e., 95% confidence interval, CI) of *TNF* 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 3, two CpG sites (i.e., *TNFC*-350C, *TNFC*-344C) remained significant in the final models.

4. 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. While cytokines modulate nociceptive signaling during acute and chronic inflammation and following tissue injury [45], significant inter-individual variability exists in the development, intensity, and resolution of postoperative pain. In this study, three SNPs (i.e., *IL6*rs2069840, *CXCL8*rs4073, *TNFR*rs1800610) were associated with pain group membership after adjusting for phenotypic characteristics.

IL6 is produced by a variety of cells at sites of tissue injury and inflammation. IL6 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 [46]. In addition to its pro-inflammatory effects, IL6 exerts anti-inflammatory effects by promoting the release of TNF α and IL1 antagonists to attenuate pro-inflammatory signaling [47]. Our results suggest that the rare G allele of rs2069840 decreases the risk for mild persistent breast pain after breast cancer surgery. Recent work found that the rare G allele of rs2069840 is associated with lower plasma IL6 concentrations in survivors of myocardial infarction [48] and in patients with leprosy [49]. In addition, serum concentrations of IL6 in the immediate postoperative period were positively associated with the extent of tissue damage that occurred during surgery [50]. Preoperative administration of pentoxifylline, a phosphodiesterase inhibitor that inhibits the synthesis of IL6 [51], lowered serum IL6 concentrations and reduced analgesic consumption following cholecystectomy [52]. Our findings are consistent with these studies and suggest that the rare G allele of rs2069840, which was less frequent in the mild persistent breast pain group, is associated with decreased serum concentrations of IL6 which prevents the development of mild persistent breast pain following surgery.

CXCL8 is a chemokine and a prominent mediator of the innate immune response to inflammatory stimuli [53]. Our results suggest that the rare A allele of rs4073 decreases the risk for the development of mild persistent breast pain following breast cancer surgery. This SNP is located in the proximal promoter of *CXCL8* and is known to be functional. The common T allele is associated with increased *CXCL8* gene and protein expression *in vitro* [53, 54]. Increased levels of CXCL8 were associated with increased pain following disc herniation [55] and postherpetic neuralgia [56]. Our findings are consistent with these studies and suggest that the common T allele of rs4073, which was more frequent in the mild persistent breast pain group, is associated with increased serum concentrations of CXCL8 which promotes the development of mild persistent breast pain following surgery.

TNF α is a pleiotropic pro-inflammatory cytokine that promotes the production of other pro-inflammatory cytokines following nerve injury [57] and plays a prominent role in the development and maintenance of persistent pain [58]. Our results suggest that the rare T

allele of rs1800610 decreases the risk for the development of mild persistent breast pain following breast cancer surgery. *TNF* rs1800610 is located in a non-coding region. The functional consequences of rs1800610 were investigated *in vitro* and during physiologic stimulation in patients with rheumatoid arthritis and healthy controls [59, 60]. In these studies, no differences were found in *TNF* precursor mRNA production between the rs1800610 alleles. Additional studies are needed to determine whether this SNP is in LD with other variations in the *TNF* locus that affect cytokine production or if tissue-specific influences of this SNP on *TNF* 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. Ongoing activation of inflammatory cells, and 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 [61, 62]. In this study, the c.-350C and c.-344C sites of *TNF* had higher levels of methylation in the mild breast pain class compared to the no breast pain class. The effect sizes calculated for percentage methylation level at the *TNF* 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 *TNF* transcription occurs at transcription factor binding sites within the proximal *TNF* promoter [63–65]. The *TNF* proximal promoter contains putative binding sites for several transcription factors that alter *TNF*α production in response to lipopolysaccharide [65, 66]. 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 [67]. For example, nitric oxide released in the course of an inflammatory response results in Sp1 binding to the *TNF* promoter and the initiation of *TNF* gene transcription in human leukocytes [68]. It is unclear how cytosine methylation at the Sp1 binding site within the *TNF* promoter may affect *TNF* 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 [69], suggests that methylation may be directly responsible for modulation of *TNF* gene expression at this site. 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.

In this sample, our group previously identified associations between variations in two cytokine genes (i.e., *IL1R1*, *IL13*) and preoperative pain in the affected breast [11]. 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 [70, 71]. Subsequently, mechanical trauma during surgery produces significant tissue injury that releases inflammatory mediators and intense nociceptive activity in peripheral nociceptors. 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 [72]. In the current study, we evaluated associations between the mild breast pain class and the same cytokine genes. Three SNPs (i.e., *IL6* rs2069840, *CXCL8* rs4073, *TNF* rs1800610) in three different genes predicted membership in the mild pain class.

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 [27–29]. 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 pro-inflammatory cytokines must be balanced by an adequate anti-inflammatory response. Aberrant release of cytokines from immune cells 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 the balance between pro- and anti-inflammatory cytokine production and response which may ultimately predispose an individual to the development of persistent pain.

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 *TNFSNP* associations. Third, methylation was evaluated using peripheral leukocytes which may not reflect changes in the tissues at the site of injury. Finally, DNA methylation was the only epigenetic mechanism evaluated in this study. The expression of *IL6*, *CXCL8*, and *TNF* may be modulated by other epigenetic mechanisms and/or DNA methylation outside of the regions evaluated in this study.

In conclusion, findings from this study suggest a role for polymorphisms within the *IL6*, *CXCL8*, and *TNF* genes and changes in methylation in the *TNF* 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Variations in *IL6*, *CXCL8*, and *TNF* are associated with the development and maintenance of mild persistent breast pain.
- CpG methylation within the *TNF* promoter may be an additional mechanism through which TNF alters the risk for mild persistent breast pain after breast cancer surgery.
- These genetic and epigenetic variations may help to identify patients who are at greater risk for mild levels of persistent breast pain following breast cancer surgery.

Figure 1.

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*.

Abbreviations: na, not assayed

Figure 2.

Deoxyribonucleic acid (DNA) methylation at the promoter region of the C-X-C motif chemokine ligand 8 (*CXCL8*) gene. (A) Scaled schematic representation of the 5' untranslated region of the *CXCL8* 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_000584.3. Primer binding sites are shown (arrows). (B) DNA methylation levels of the promoter region for *CXCL8*.

Figure 3.

Deoxyribonucleic acid (DNA) methylation at the promoter region of the tumor necrosis factor alpha (TNF) gene. (A) Scaled schematic representation of the 5' untranslated region of the TNF 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 TNF. 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. 5' 3'

Table 1

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

	No Pain n=126	Mild Pain n=173	Statistics
Demographic Characteristics	Mean (SD)	Mean (SD)	
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)	X ² =3.07; p=0.381
Black	7.1 (9)	8.8 (15)	
Asian/Pacific Islander	10.3 (13)	12.9 (22)	
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; p=0.473
\$20,000 to \$99,000	48.1 (51)	43.8 (64)	
\$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

	No Pain n=126	Mild Pain n=173	Statistics
Demographic Characteristics	Mean (SD)	Mean (SD)	
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	17.5 (22)	20.8 (36)	Z=-0.07; p=0.945
Stage 1	41.3 (52)	35.8 (62)	
Stage IIA and IIB	35.7 (45)	35.8 (62)	
Stage IIIA, IIIB, IIIC, and IV	5.6 (7)	7.5 (13)	
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)	

	No Pain n=126	Mild Pain n=173	Statistics
Demographic Characteristics	Mean (SD)	Mean (SD)	
Type of surgery			
Breast conserving	84.1 (106)	77.5 (134)	FE; p=0.186
Mastectomy	15.9 (20)	22.5 (39)	
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; m² = meters squared

Adapted from Miaskowski et al. [1].

Table 2

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=228)	<i>IL6</i> rs2069840	0.21	0.12	0.07, 0.63	-2.78	0.005
	Breast pain preoperatively	28.75	18.85	7.96, 103.91	5.12	<0.001
	Hardness in the breast preoperatively	3.42	1.97	1.10, 10.59	2.13	0.033
	Re-excision/mastectomy	3.17	1.27	1.45, 6.95	2.88	0.004
	Overall model fit: $\chi^2 = 81.67$, $p < 0.0001$; pseudo $R^2 = 0.2637$					
No pain versus mild pain (n=228)	<i>CXCL8</i> rs4073	0.40	0.16	0.18, 0.87	-2.32	0.020
	Breast pain preoperatively	29.95	19.48	8.37, 107.16	5.23	<0.001
	Hardness in the breast preoperatively	3.02	1.71	1.00, 9.13	1.96	0.050
	Re-excision/mastectomy	2.52	0.97	1.19, 5.34	2.41	0.016
	Overall model fit: $\chi^2 = 78.55$, $p < 0.0001$; pseudo $R^2 = 0.2536$					
No pain versus mild pain (n=228)	<i>TNFR</i> rs1800610	0.37	0.17	0.15, 0.89	-2.22	0.026
	Breast pain preoperatively	26.52	17.02	7.54, 93.33	5.11	<0.001
	Hardness in the breast preoperatively	4.14	2.39	1.34, 12.81	2.46	0.014
	Re-excision/mastectomy	2.72	0.86	1.08, 4.78	2.16	0.030
	Overall model fit: $\chi^2 = 78.15$, $p < 0.0001$; pseudo $R^2 = 0.2523$					

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; *CXCL8*rs4073 TT+TA versus AA; *TNFR*rs1800610: CC versus CT+TT), pain in the affected breast prior to surgery, hardness in the affected breast prior to surgery, and re-excision or mastectomy was performed within 6 months after the initial surgery for breast cancer.

Abbreviations: CI = confidence interval; CXCL8 = C-X-C motif chemokine ligand 8; GMM = growth mixture model; IL6 = interleukin 6; TNF = tumor necrosis factor alpha

Table 3

Multiple logistic regression analyses for TNF CpG promoter methylation

GMM Class Comparison	Predictor	Odds Ratio	Standard Error	95% CI	Z	p-value
No pain versus mild pain (n=117)	TNFC-180C methylation	83.21	163.05	1.79, 3872.62	2.26	0.024
	Breast pain preoperatively	31.09	32.84	3.92, 246.48	3.25	0.001
	Hardness in the breast preoperatively	2.87	2.17	0.65, 12.65	1.40	0.162
	Re-excision/mastectomy	2.30	1.26	0.79, 6.72	1.53	0.126
	Overall model fit: $\chi^2 = 44.06$, $p < 0.0001$; pseudo $R^2 = 0.2810$					
No pain versus mild pain (n=116)	TNFC-174C methylation	215.90	543.46	1.55, 29987.25	2.14	0.033
	Breast pain preoperatively	28.37	30.07	3.55, 226.47	3.16	0.002
	Hardness in the breast preoperatively	4.13	3.17	0.92, 18.57	1.85	0.065
	Re-excision/mastectomy	2.39	1.32	0.81, 7.05	1.58	0.113
	Overall model fit: $\chi^2 = 44.72$, $p < 0.0001$; pseudo $R^2 = 0.2870$					

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 1% increments (i.e., *TNFA* c.-180C, *TNFA* c.-174C), pain in the affected breast prior to surgery, presence of hardness in the affected breast prior to 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; TNF = tumor necrosis factor alpha