Title
CYTOKINE CANDIDATE GENES PREDICT THE DEVELOPMENT OF SECONDARY LYMPHEDEMA FOLLOWING BREAST CANCER SURGERY

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Publication Date
2013

Peer reviewed|Thesis/dissertation

Cytokine Candidate Genes Predict the Development of Secondary Lymphedema Following Breast Cancer Surgery

by

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Nursing

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements: This study was funded by grants from the National Cancer Institute (CA107091 and CA118658). Dr. Christine Miaskowski is an American Cancer Society Clinical Research Professor. Dr. Dhruva is funded through NIH Mentored Patient-Oriented Research Career Development Award (K23 AT005340). Dr. Langford is supported by a Department of Defense Breast Cancer Research Program Postdoctoral Fellowship. Mr. Merriman is supported by an NINR fellowship (F31 NR012604), an ACS Doctoral Degree Scholarship (DSCN-10-087), an Oncology Nursing Society Doctoral Scholarship, and a UCSF Nursing Alumni Association Scholarship. Dr. Baggott is funded by an American Cancer Society Mentored Research Scholar Award (MRSG 12-01-PCSM). This project is supported by NIH/NCRR UCSF-CTSI Grant Number UL1 RR024131. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

CYTOKINE CANDIDATE GENES PREDICT THE DEVELOPMENT OF SECONDARY LYMPHEDEMA FOLLOWING BREAST CANCER SURGERY

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ABSTRACT

The purpose of this study was to determine if variations in pro- and anti-inflammatory cytokine genes were associated with lymphedema (LE) following breast cancer treatment. Breast cancer patients completed a number of self-report questionnaires. LE was evaluated using bioimpedance spectroscopy. Genotyping was done using a custom genotyping array. No differences were found between patients with (n = 155) and without LE (n = 387) for the majority of the demographic and clinical characteristics. Patients with LE had a significantly higher body mass index, more advanced disease and a higher number of lymph nodes removed. Genetic associations were identified for four three genes (i.e., interleukin (IL4) 4 (rs2227284), IL 10 (rs1518111) and nuclear kappa factor beta 2 (NFKB2 (rs1056890)) associated with inflammatory responses. These genetic associations suggest a role for a number of pro- and anti-inflammatory genes in the development of LE following breast cancer treatment.

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1. INTRODUCTION

Lymphedema (LE) is a frequent complication of breast cancer treatment (i.e., surgery, radiation therapy (RT), chemotherapy (CTX)). LE is caused by a disruption in the lymphatic system that results in the accumulation of fluid in the interstitial space.(1) LE manifests as swelling of the affected limb and is associated with chronic pain, disfigurement, reduced mobility, functional impairment, predisposition to infections, and increased health care costs.(2, 3)

The true incidence of breast cancer-related LE is unknown, though estimates range from 6% to 83%.(4) This wide variation is due to differences in diagnostic criteria, measurement techniques, timing of measurements, duration of follow-up, and sample characteristics.(5, 6) In a recent review of 11 prospective cohort studies,(7) the median incidence rate for LE within three years of breast cancer treatment was 20%. In the United States, this rate would mean that more than 500,000 breast cancer survivors are affected by this incurable condition.(8)

Research is often directed at identifying risk factors for LE with the hope of developing interventions to reduce its incidence.(9) In our previous study,(13) we identified both phenotypic and genotypic differences between women who did and did not develop LE following breast cancer treatment. The phenotypic characteristics associated with the occurrence of LE were increased BMI, increased number of lymph nodes removed, higher stage of disease, and having had a sentinel lymph node biopsy (SLNB). In addition, a number of candidate genes in the lymphatic and angiogenesis pathways were associated with LE (i.e., lymphocyte cytosolic protein 2 (LCP2), neuropilin-2 (NRP2), protein tyrosine kinase (SYK), Forkhead box protein C2 (FOXC2), vascular cell adhesion molecule 1 (VCAM1), and vascular endothelial growth factor -C (VEGFC)). While this study was novel in uncovering associations between LE and lymphatic and angiogenic candidate genes, further investigation is warranted to identify additional molecular pathways.

Several studies have suggested that cytokines may be involved in the pathophysiology of LE.(10, 11) Cytokines play a key role in modulating inflammatory responses, which may subsequently lead to lymphatic dysfunction and LE.(10) In a study that used a specific bioassay and performed transcriptional microarray analysis on human skin,(12) a number of cytokine genes (i.e., interleukin (IL) 4, IL6, IL10,

IL13) were up-regulated in LE specimens. In another study that investigated the role of inflammation in the regulation of fibrosis and lymphatic dysfunction,(11) the blockade of T-helper 2 cytokines, including IL-4 and IL-13, prevented T-cell differentiation and its subsequent inflammatory response in a mouse-tail model of LE. This blockade resulted in less fibrosis and improved lymphatic function. Findings from these studies suggest that variations in cytokine genes may account for the differences in the development of LE. Therefore, the purpose of this study was to determine if variations in pro-and anti- inflammatory cytokine genes were associated with the development of LE following breast cancer treatment.

2. METHODS

a. Study Samples and Procedures

Demographic, clinical, and genomic data from a cross-sectional study (i.e., LE Study (NR0101282)) and a longitudinal study (i.e., Breast Symptoms Study (CA107091 and CA118658)) were combined for these analyses. Both studies used the same subjective and objective measures. Both studies were approved by the Committee on Human Research at the University of California, San Francisco (UCSF) and the Clinical Translational Science Institute's (CTSI) Clinical Research Center Advisory Committee.

LE Study – The LE study used a cross-sectional design to evaluate for differences in phenotypic and genotypic characteristics in women with (n=70) and without (n=71) LE. Women who were ≥18 years of age and ≥6 months post-treatment for unilateral breast cancer, with or without upper extremity LE were recruited. Women were excluded for bilateral breast cancer, current upper extremity infection, lymphangitis, preexisting LE, current breast cancer, or contraindications to bioimpedance spectroscopy (BIS) testing. Women were recruited through the National Lymphedema Network website, San Francisco Bay area hospitals, and breast cancer or LE support groups and conferences. Women were evaluated in the Clinical Research Center at UCSF. After obtaining written informed consent, women completed the study questionnaires. Following the completion of the questionnaires, the research staff performed the objective measurements: height, weight, and BIS. A blood sample was drawn for genomic analyses.

Breast Symptoms Study – The Breast Symptoms Study used a longitudinal design to evaluate neuropathic pain and LE following breast cancer surgery.(13-16) Women 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 (≥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, 410 were enrolled in the study (response rate 79.5%), and 398 completed the

preoperative assessment. The major reasons for refusal were: too busy, overwhelmed with the cancer diagnosis, or insufficient time available to do the enrollment assessment prior to surgery.

During the patient's preoperative visit, a clinician explained the study, determined the patient's willingness to participate, and introduced the patient to the research nurse. The research nurse met with the woman, determined eligibility, and obtained written informed consent prior to surgery. After obtaining written informed consent, the patient completed these questionnaires prior to surgery. Following the completion of the questionnaires, the research nurse performed the objective measurements: height, weight, and BIS. A blood sample was drawn for genomic analyses. 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, 6, 8, 10, and 12 months after surgery. In the second through fifth years of the study, patients were seen every four months. During each of the study visits, the women completed the study questionnaires and had the objective measures done by the research nurse.

b. Subjective Measures

A demographic questionnaire obtained information on age, marital status, education, ethnicity, employment status, living situation, and financial status. Functional status was evaluated using the Karnofsky Performance Status (KPS) scale that has well established validity and reliability.(17, 18) 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). Clinical information was obtained from patient interviews and medical record reviews.

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.(19) 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 two 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.(19-23)

c. Objective Measures

Bioimpedance Spectroscopy (BIS) of LE – BIS measurements, of the affected and unaffected arms, were done using the procedures described by Cornish and colleagues.(24-26) Patients were instructed not to exercise or take a sauna within 8 hours of the assessment. In addition, they were asked to refrain from drinking alcohol for 12 hours prior to the assessment. BIS measurements were taken using a single channel BIS device (i.e., SFB7 device; ImpediMed, San Diego, CA in the LE study or the Quantum X Bioelectrical Impedance Device; RJL Systems, Clinton Township, MI in the Breast Symptoms Study). Women removed all jewelry and their skin was prepped with an alcohol wipe prior to surface electrode placement. Patients lay supine on a massage table with their arms 30 degrees from the body and legs not touching for at least 10 minutes prior to the BIS measurements. Electrodes were placed on the dorsum of the wrists adjacent to the ulnar styloid process, the dorsum of the hands just proximal to the third metacarpophalangeal joint, anterior to the ankle joints between the malleoli, and over the dorsum of the feet over the third metatarsal bone just proximal to the third metatarsophalangeal joint. Two 'measurement' electrodes were placed at either end of the 40 cm length over which the circumference measurements were made and the 'drive' electrodes were placed 8 to 10 cm distal to these measurement electrodes. Two readings of resistance were obtained from the affected and unaffected arms and averaged for subsequent analyses.

While cases and non-cases of LE were known in the LE study, for the Breast Symptoms Study, LE cases were determined based on the procedures of Cornish and colleagues(24-26) using all of the data obtained from each woman during her participation in the study. A woman was defined as a LE case if the resistance ratio for the untreated arm/treated arm was >1.139 or >1.066 for those women who had

surgery on the dominant or nondominant side, respectively at any of the BIS assessments.

d. Methods of Analysis for Phenotypic Data

Data were analyzed using SPSS Version 19.(27) Descriptive statistics and frequency distributions were generated on the sample characteristics. Independent sample t-tests, Chi-square analyses, and Mann Whitney U tests were done to evaluate for differences in demographic and clinical characteristics between patients with and without LE.

e. Methods of Analysis for Genomic Data

Gene Selection: Cytokines and their receptors are classes of polypeptides that mediate inflammatory processes.(28) These polypeptides are divided into pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines promote systemic inflammation and include: interferon gamma (IFNG) 1, IFNG 1 receptor (IFNGR1), IL1R1, IL2, IL8, IL17A, nuclear factor kappa beta (NFKB1), NFKB2, and tumor necrosis factor alpha (TNFA).(28, 29) Anti-inflammatory cytokines suppress the activity of pro-inflammatory cytokines and include: IL1R2, IL4, IL10, and IL13. (28, 29) Of note, IFNG1, IL1B, and IL6 possess pro- and anti-inflammatory functions.(29)

Blood collection and genotyping: Genomic DNA was extracted from archived buffy coats using the PUREGene DNA Isolation System (Invitrogen, Carlsbad, CA). Of the 543 patients recruited for this study, DNA was recovered from the archive buffy coat of 407 patients (i.e., 110 with and 297 without LE) who provided a blood sample. Genotyping was performed blinded to LE status and positive and negative controls were included. DNA was quantitated with a Nanodrop Spectrophotometer (ND-1000) and normalized to a concentration of 50 ng/μL (diluted in 10 mM Tris/1 mM EDTA). Samples were genotyped using the GoldenGate genotyping platform (Illumina, San Diego, CA) and processed according to the standard protocol using GenomeStudio (Illumina, San Diego, CA). Signal intensity profiles and resulting genotype calls for each single nucleotide polymorphism (SNP) were visually inspected by two blinded reviewers. Disagreements were adjudicated by a third reviewer.

SNP Selection: A combination of tagging SNPs and literature driven SNPs were selected for analysis. Tagging SNPs were required to be common (i.e., estimated to have a minor allele frequency

≥.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 of <95% or Hardy-Weinberg p-values of <.001 were excluded.

As shown in Table 1, a total of 86 SNPs among the 15 candidate genes (IFNG1: 5 SNPs, IFNGR1: 1 SNP; IL1B: 12 SNPs; IL1R1: 4 SNPs; IL1R2: 3 SNPs; IL2: 5 SNPs; IL4: 3 SNPs; IL6: 9 SNPs; IL8: 3 SNPs; IL10: 8 SNPs; IL13: 4 SNPs; IL17A: 5 SNPs; NFKB1: 11 SNPs; NFKB2: 4 SNPs; TNFA: 9 SNPs) passed all quality control filters and were included in the genetic association analyses. Potential functional roles of SNPs associated with LE were examined using PUPASuite 2.0,(30) a comprehensive search engine that tests a series of functional effects (i.e., non-synonymous changes, altered transcription factor binding sites, exonic splicing enhancing or silencing, splice site alterations, microRNA target alterations).

Statistical Analyses: Allele and genotype frequencies were determined by gene counting. Hardy-Weinberg equilibrium was assessed by the Chi-square or Fisher Exact tests. Measures of linkage disequilibrium ((LD) i.e., D' and r^2) were computed from the patients' genotypes with Haploview 4.2. LD-based haplotype block definition was based on D' confidence interval.(31)

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.(32) 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.(33-35) Homogeneity in ancestry among patients was verified by principal component analysis,(36) 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). One hundred and six AIMs were included in the analysis. 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.

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 relationship between genotype and LE group membership. A backwards stepwise approach was used to create a parsimonious model. Genetic model fit and both unadjusted and covariate-adjusted odds ratios were estimated using STATA version 9.(37)

As was done in our previous studies (15, 38, 39), based on recommendations in the literature,(40, 41) 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. In addition, 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.

3. RESULTS

a. Differences in Demographic and Clinical Characteristics

As shown in Table 2, no differences were found between patients with and without LE for the majority of the demographic and clinical characteristics. Patients with LE had a significantly higher body mass index (BMI) and a lower KPS score, and were more likely to report lung disease. In addition, patients with LE had a higher number of lymph nodes removed, a higher number of positive nodes, more advanced disease at the time of diagnosis, were less likely to have had a SLNB, were more likely to have had an axillary lymph node dissection (ALND), had received CTX prior to or following surgery, and had received RT following surgery.

b. Candidate Gene Analyses for the Development of LE

As summarized in Table 1, no associations with the occurrence of LE were found in the SNPs evaluated for INFG1, INFGR1, IL1R2, IL2, IL8, IL13, IL17, NFKB1, and TNFA. However, the genotype frequency was significantly different between those who did and did not develop LE for six SNPs and three haplotypes spanning six genes (i.e., IL1B, IL1R1, IL4, IL6, IL10, NFKB2). One haplotype (HapB1, p= .018) was identified for IL1B. For the SNP in IL1R1 (rs949963), an additive model fit the data best (p=.021). For the SNP in IL4 (rs2227284), a recessive model fit the data best (p=.010). One SNP (rs2066992) and 1 haplotype (HapB1, p=.022) were identified in IL6. For rs2066992, a dominant model fit the data best (p=.023). Two SNPs (rs151811, rs1518110) and 1 haplotype (HapA1, p=.023) were identified in IL10. For both SNPs, a dominant model fit the data best (p=.014, .010, respectively). For the SNP in NFKB2 (rs1056890), a recessive model fit the data best (p=.049).

c. Regression Analyses of IL4, IL10, and NFKB2 Genotypes and Haplotypes and the Development of LE

In order to better estimate the magnitude (i.e., odds ratio, OR) and precision (95% confidence interval, CI) of genotype on the development of LE, multivariate logistic regression models were fit. As shown in Table 3, in addition to genotype, the phenotypic characteristics included in the regression models were ethnicity (i.e., White, Black, Asian, Hispanic/Mixed ethnic background/Other), BMI, stage of

disease, having a SLNB, and number of lymph nodes removed. Receipt of CTX and RT, while not significant after the inclusion of genomic estimates of and self-reported race/ethnicity,(13) were retained in all of the regression models for face validity.

The only genetic associations that remained significant in the multivariate logistic regression analyses were for IL4 rs2227284, IL10 rs1518111, IL10 rs1518110, and NFKB2 rs1056890 (see Table 3 and Figure 1). In the regression analysis for IL4 rs2227284, carrying two doses of the rare allele (i.e., CC+CA versus AA) was associated with a 69.9% decrease in the odds of developing LE (Figure 1A). In the regression analysis for IL10 rs1518111, carrying one or two doses of the rare allele (i.e., GG versus GA+AA) was associated with 51.0% decrease in the odds of developing LE (Figure 1B). The analyses for the second SNP in IL10, namely rs1518110, revealed that it is a perfect surrogate for IL10 rs1518111. IL10 rs1518111 was selected to represent the two surrogate SNPs. In the regression analysis for NFKB2 rs1056890, carrying two doses of the rare allele (i.e., CC+CT versus TT) was associated with a 3.06-fold increase in the odds of developing LE (Figure 1C).

4. DISCUSSION

This study is the first to evaluate for variations in pro- and anti- inflammatory cytokine genes and the development of LE following breast cancer treatment. In brief, in the bivariate analyses (Table 2), the phenotypic predictors of LE included: a higher BMI, lower KPS score, having lung disease, increased number of lymph nodes removed, increased number of positive lymph nodes, a higher stage of disease at the time of diagnosis, not having a SLND, having an ALND, and receiving CTX or RT. However, in the multivariate analysis (Table 3), KPS score, having lung disease, number of positive nodes removed, and having an ALND were not retained in the final model (Table 3). In addition, when genomic estimates of and self-reported race/ethnicity were included in the multivariate logistic regression analysis,(13) neither receipt of CTX nor receipt of RT remained significant predictors of LE.

The complex molecular pathways that underlie the development of LE following breast cancer treatment are being uncovered. In our previous study,(13) variations in seven genes that play a role in lymphatic development and angiogenesis were associated with the development of LE. In this study, we extend this work and evaluated for variations in pro- and anti-inflammatory cytokine genes and their association with the development of LE.

Consistent with preclinical and clinical studies that identified a role for IL4 in the molecular pathway of LE development,(11, 12) patients who were homozygous for the rare allele in IL4 rs2227284 had a 69% decrease in the odds of developing LE. IL4 is a multifunctional cytokine that is known to induce T-helper 2 (Th2) cell immune responses in asthma and scleroderma. IL4 plays a regulatory role in apoptosis and cell proliferation, as well as in the expression of numerous genes in macrophages, lymphocytes, fibroblasts, endothelial cells, and epithelial cells.(11, 12) In addition, IL4 has the ability to differentially activate macrophages into M2 macrophages rather than M1 macrophages. M2 macrophages function in tissue repair, fibrosis, and the regulation of inflammation. A subset of M2 macrophages produce the chemokine CCL18, which has both direct effects on fibroblasts and indirect effects on T cells that result in fibrotic inflammatory diseases, including hypersensitivity pneumonitis and idiopathic pulmonary fibrosis.(42) In addition, IL4 activated M2 macrophages increase the production of transforming growth factor beta (TGF-β), a tissue activator that leads to fibroblast production and collagen synthesis.(43) It is plausible to hypothesize that dysregulation in the production of IL4 could lead to the

development of soft tissue fibrosis and lymphatic dysfunction associated with LE. This hypothesis is supported by a recent preclinical study that demonstrated that inhibition of Th2 differentiation using IL4 prevented the initiation and progression of LE by decreasing tissue fibrosis and increasing lymphatic function.(11)

IL4 rs2227284 is located in the intronic region of chromosome 5 in a region of the gene that undergoes DNA methylation. While no studies were identified that evaluated for an association between this SNP and the development of LE, in one study of Japanese women, individuals who were homozygous for the rare allele had a decreased risk for the development of rhinoconjunctivitis.(44) In another study of Chinese children who were vaccinated for hepatitis B, the rare allele was associated with a poor humoral response to the vaccine.(45) Taken together, these findings suggest that rs2227284 or a SNP(s) in linkage disequilibrium with rs2227284 may modulate a variety of inflammatory and immune responses. Additional research is warranted to confirm these findings in a larger cohort of breast cancer patients with LE.

In our study, patients who were heterozygous or homozygous for the rare allele in IL10 rs1518111 had a 51% decrease in the odds of developing LE. IL-10 rs1518111 is located in the intronic region of chromosome 1 in a region that undergoes DNA methylation. In addition, this SNP is known to influence active transcription factor binding sites (i.e., PU.1, Pol2). This SNP was associated with ischemic stroke,(46) benign prostate hyperplasia,(47) and Behcet's disease (i.e., a chronic vasculitis that affects the skin, joints, lungs, and central nervous system (48)). These studies suggest that variations in the expression of IL10 may result in increased inflammation and contribute to these diseases. In addition, in a sample of healthy controls who were homozygous for the rare allele in IL-10 rs1518111, mRNA expression and protein production of IL10 were decreased.(49)

Recent evidence has implicated IL10 in the development of LE.(12) In addition to its anti-inflammatory effects, Shi et al.(50) demonstrated, using human dermal fibroblasts, that IL-10 has anti-fibrotic properties and can inhibit excessive deposition of collagen and the transformation of fibroblasts to myofibroblasts. In addition, polymorphisms in several candidate genes in IL10 and the IL10 receptor, that were not evaluated in this study, were associated with the development of LE following infection with filarial parasites.

Patients who were homozygous for the rare allele in NFKB2 rs1056890 had a 3.1-fold increase in the odds of developing LE. NF-κB transcription factors play a role in diverse cellular processes including the regulation of angiogenesis, metastasis, cell proliferation, tumor promotion, suppression of apoptosis, and inflammation.(51) The NF-κB signaling pathway leads to the transcription of pro-inflammatory molecules, such as cytokines and chemokines. Alterations in NF-κB regulation are linked to diseases of chronic inflammation (e.g., Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus). NF-κB2 (p52 and its precursor p100) is one of five subunits that contribute to dimeric NF-κB and is responsible for activating the non-canonical pathway of NF-κB.(52) NF-κB2 functions within an autoregulatory loop in which the precursor protein p100 is processed to become the active NF-κB2 subunit known as p52, which can up-regulate p100 expression. p100 can repress p52 activity, which acts as a negative feedback control loop.(53) This autoregulatory loop is tightly controlled.

In one study, Yang et al. (54) found that p52 transgenic mice that were deficient in the p100 precursor protein developed fatal lung inflammation characterized by diffuse alveolar damage with localized fibrosis. The lung tissue of the mice demonstrated high level induction of the Th1 cytokine IFN-γ and its inducible inflammatory chemokines, which are known to activate macrophages and result in a cycle of inflammatory processes and tissue damage. In addition, the transgenic mice displayed a significant increase in TNF-α which acts synergistically with IFN-γ to activate macrophages and regulate fibroblast proliferation and activation.

NFKB2 rs1056890 is located near genes NF-κB PSD on chromosome 10 and is located in the 3’ UTR region of NFKB2. In one study of Chinese patients with multiple myeloma patients, who were treated bortezomib,(51) individuals who were heterozygous or homozygous for the rare allele had an overall lower response rate and decreased survival. In relationship to LE, one can hypothesize that SNPs in the 3' UTR region of the NFKB2 gene may disrupt the engagement process needed for p100 proteolytic processing or affect its ability to repress p52 activity and disrupt the delicate autoregulatory loop.

Several limitations of this study need to be acknowledged. Although the sample size was relatively large, larger samples may reveal additional significant candidate gene associations. In addition, future studies need to confirm the functional effects of these polymorphisms.

5. CONCLUSION

Despite these limitations, the novel findings from this study suggest that genetic variations in pro- and anti- inflammatory cytokine genes may play a role in the development of secondary LE following breast cancer treatment. Although the pathophysiology of LE is complex and largely undetermined, the identified genetic associations may help with risk assessment and the development of targeted molecular therapy for this incurable condition.

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Table 1. Cytokine genes and single nucleotide polymorphisms analyzed for lymphedema versus no lymphedema

Gene	SNP	Position	Chr	MAF	Alleles	Chi Square	p-value	Model
IFNG1	rs2069728	66834051	12	.101	G>A	0.606	.739	A
IFNG1	rs2069727	66834490	12	.397	A>G	0.369	.831	A
IFNG1	rs2069718	66836429	12	.489	C>T	0.719	.698	A
IFNG1	rs1861493	66837463	12	.278	A>G	0.615	.735	A
IFNG1	rs1861494	66837676	12	.285	T>C	1.192	.551	A
IFNG1	rs2069709	66839970	12	.002	G>T	n/a	n/a	n/a
IFNG1	HapA3					0.685	.710	
IFNG1	HapA5					0.412	.814	
IFNGR1	rs9376268	137574444	6	.262	G>A	0.387	.824	A
IL1B	rs1071676	106042060	2	.189	G>C	2.856	.240	A
IL1B	rs1143643	106042929	2	.385	G>A	2.190	.335	A
IL1B	rs1143642	106043180	2	.080	C>T	0.918	.632	A
IL1B	rs1143634	106045017	2	.187	C>T	2.776	.250	A
IL1B	rs1143633	106045094	2	.393	G>A	2.876	.238	A
IL1B	rs1143630	106046282	2	.110	C>A	0.332	.847	A
IL1B	rs3917356	106046990	2	.457	G>A	0.622	.733	A
IL1B	rs1143629	106048145	2	.380	T>C	2.479	.290	A
IL1B	rs1143627	106049014	2	.386	T>C	3.397	.183	A
IL1B	rs16944	106049494	2	.380	G>A	4.658	.097	A
IL1B	rs1143623	106050452	2	.278	G>C	1.003	.606	A
IL1B	rs13032029	106055022	2	.455	C>T	0.590	.745	A
IL1B	HapA1					3.917	.141	
IL1B	HapA4					2.127	.345	

IL1B	HapA6					2.964	.227	
IL1B	HapB1					8.064	.018	
IL1B	HapB6					1.013	.602	
IL1B	HapB8					1.053	.591	
IL1R1	rs949963	96533648	2	.211	G>A	7.695	.021	A
IL1R1	rs2228139	96545511	2	.054	C>G	0.391	.823	A
IL1R1	rs3917320	96556738	2	.048	A>C	n/a	n/a	n/a
IL1R1	rs2110726	96558145	2	.336	C>T	1.720	.423	A
IL1R1	rs3917332	96560387	2	.184	A>T	2.612	.271	A
IL1R1	HapA1					1.827	.401	
IL1R1	HapA2					2.792	.248	
IL1R1	HapA3					2.683	.261	
IL1R2	rs4141134	96370336	2	.378	T>C	2.388	.303	A
IL1R2	rs11674595	96374804	2	.254	T>C	4.848	.089	A
IL1R2	rs7570441	96380807	2	.411	G>A	2.978	.226	A
IL1R2	HapA1					2.406	.300	
IL1R2	HapA2					2.292	.318	
IL1R2	HapA4					4.803	.091	
IL2	rs1479923	119096993	4	.302	C>T	0.540	.763	A
IL2	rs2069776	119098582	4	.264	T>C	1.245	.536	A
IL2	rs2069772	119099739	4	.247	A>G	0.251	.882	A
IL2	rs2069777	119103043	4	.053	C>T	0.747	.688	A
IL2	rs2069763	119104088	4	.275	T>G	0.770	.680	A
IL2	HapA1					1.805	.406	
IL2	HapA2					0.245	.885	

IL2	HapA3					1.980	.372	
IL4	rs2243248	127200946	5	.087	T>G	1.061	.588	A
IL4	rs2243250	127201455	5	.244	C>T	n/a	n/a	n/a
IL4	rs2070874	127202011	5	.224	C>T	n/a	n/a	n/a
IL4	rs2227284	127205027	5	.366	C>A	FE	.010	R
IL4	rs2227282	127205481	5	.368	C>G	n/a	n/a	n/a
IL4	rs2243263	127205601	5	.127	C>G	3.268	.195	A
IL4	rs2243266	127206091	5	.216	G>A	n/a	n/a	n/a
IL4	rs2243267	127206188	5	.217	G>C	n/a	n/a	n/a
IL4	rs2243274	127207134	5	.239	G>A	n/a	n/a	n/a
IL6	rs4719714	22643793	7	.252	A>T	0.190	.910	A
IL6	rs2069827	22648536	7	.071	G>T	0.771	.680	A
IL6	rs1800796	22649326	7	.123	C>G	n/a	n/a	n/a
IL6	rs1800795	22649725	7	.316	C>G	0.357	.837	A
IL6	rs2069835	22650951	7	.061	T>C	n/a	n/a	n/a
IL6	rs2066992	22651329	7	.124	G>T	FE	.023	D
IL6	rs2069840	22651652	7	.323	C>G	0.585	.746	A
IL6	rs1554606	22651787	7	.343	G>T	2.265	.322	A
IL6	rs2069845	22653229	7	.343	A>G	1.893	.388	A
IL6	rs2069849	22654236	7	.021	C>T	n/a	n/a	n/a
IL6	rs2069861	22654734	7	.072	C>T	1.140	.566	A
IL6	rs35610689	22656903	7	.254	A>G	4.146	.126	A
IL6	HapA1					0.158	.924	
IL6	HapA2					0.285	.867	
IL6	HapB1					7.655	.022	

IL6	HapB2					4.402	.111	
IL6	HapB6					1.555	.460	
IL8	rs4073	70417508	4	.450	T>A	2.672	.263	A
IL8	rs2227306	70418539	4	.371	C>T	2.868	.238	A
IL8	rs2227543	70419394	4	.375	C>T	2.117	.347	A
IL8	HapA1					3.305	.192	
IL8	HapA4					2.564	.278	
IL10	rs3024505	177638230	1	.129	C>T	2.112	.348	A
IL10	rs3024498	177639855	1	.210	A>G	2.556	.279	A
IL10	rs3024496	177640190	1	.413	T>C	0.778	.678	A
IL10	rs1878672	177642039	1	.412	G>C	0.460	.795	A
IL10	rs3024492	177642438	1	.199	T>A	2.986	.225	A
IL10	rs1518111	177642971	1	.299	G>A	FE	.014	D
IL10	rs1518110	177643187	1	.296	G>T	FE	.010	D
IL10	rs3024491	177643372	1	.403	G>T	1.190	.552	A
IL10	HapA1					7.517	.023	
IL10	HapA2					4.372	.112	
IL10	HapA9					3.360	.186	
IL13	rs1881457	127184713	5	.229	A>C	1.229	.541	A
IL13	rs1800925	127185113	5	.243	C>T	1.163	.559	A
IL13	rs2069743	127185579	5	.017	A>G	n/a	n/a	n/a
IL13	rs1295686	127188147	5	.259	G>A	0.654	.721	A
IL13	rs20541	127188268	5	.213	C>T	1.273	.529	A
IL13	HapA1					0.572	.751	
IL13	HapA4					1.067	.586	

IL17A	rs4711998	51881422	6	.337	G>A	3.022	.221	A
IL17A	rs8193036	51881562	6	.321	T>C	0.625	.732	A
IL17A	rs3819024	51881855	6	.366	A>G	0.613	.736	A
IL17A	rs2275913	51882102	6	.359	G>A	0.443	.801	A
IL17A	rs3804513	51884266	6	.019	A>T	n/a	n/a	n/a
IL17A	rs7747909	51885318	6	.215	G>A	0.013	.994	A
NFKB1	rs3774933	103645369	4	.416	T>C	0.568	.753	A
NFKB1	rs170731	103667933	4	.362	A>T	0.480	.786	A
NFKB1	rs17032779	103685279	4	.009	T>C	n/a	n/a	n/a
NFKB1	rs230510	103695201	4	.409	T>A	1.640	.441	A
NFKB1	rs230494	103706005	4	.434	A>G	0.043	.979	A
NFKB1	rs4648016	103708706	4	.007	C>T	n/a	n/a	n/a
NFKB1	rs4648018	103709236	4	.015	G>C	n/a	n/a	n/a
NFKB1	rs3774956	103727564	4	.437	C>T	0.023	.989	A
NFKB1	rs10489114	103730426	4	.015	A>G	n/a	n/a	n/a
NFKB1	rs4648068	103737343	4	.359	A>G	1.605	.448	A
NFKB1	rs4648095	103746914	4	.052	T>C	FE	.853	A
NFKB1	rs4648110	103752867	4	.175	T>A	0.334	.846	A
NFKB1	rs4648135	103755716	4	.061	A>G	FE	.605	A
NFKB1	rs4648141	103755947	4	.174	G>A	1.474	.478	A
NFKB1	rs1609798	103756488	4	.339	C>T	1.789	.409	A
NFKB1	HapA1					1.435	.488	
NFKB1	HapA4					0.934	.627	
NFKB1	HapA9					0.248	.883	
NFKB2	rs12772374	104146901	10	.170	A>G	0.972	.615	A

NFKB2	rs7897947	104147701	10	.215	T>G	0.872	.647	A
NFKB2	rs11574849	104149686	10	.064	G>A	1.036	.596	A
NFKB2	rs1056890	104152760	10	.311	C>T	FE	.049	R
NFKB2	HapA1					1.759	.415	
NFKB2	HapA2					0.899	.638	
NFKB2	HapA3					0.723	.697	
TNFA	rs2857602	31533378	6	.361	T>C	0.223	.894	A
TNFA	rs1800683	31540071	6	.377	G>A	1.527	.466	A
TNFA	rs2239704	31540141	6	.356	G>T	0.175	.916	A
TNFA	rs2229094	31540556	6	.273	T>C	0.452	.798	A
TNFA	rs1041981	31540784	6	.371	C>A	1.116	.572	A
TNFA	rs1799964	31542308	6	.220	T>C	0.271	.873	A
TNFA	rs1800750	31542963	6	.016	G>A	n/a	n/a	n/a
TNFA	rs1800629	31543031	6	.146	G>A	0.121	.941	A
TNFA	rs1800610	31543827	6	.103	C>T	3.613	.164	A
TNFA	rs3093662	31544189	6	.071	A>G	3.566	.168	A
TNFA	HapA1					1.579	.664	
TNFA	HapA6					0.683	.711	
TNFA	HapA8					2.767	.251	

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 patients with (n=155) and without (n=387) lymphedema

Characteristic	No Lymphedema Mean (SD)	Lymphedema Mean (SD)	Statistics
Age (years)	54.9 (11.1)	56.2 (10.8)	NS
Education (years)	16.0 (2.7)	15.8 (2.8)	NS
Age at menopause (years)	47.8 (7.2)	46.7 (9.1)	NS
Body mass index (kg/m$^{2)}$	26.1 (5.6)	28.2 (6.7)	p=.001
Karnofsky Performance Status score	93.3 (9.7)	91.1 (11.1)	p=.028
Comorbidity score	4.0 (2.9)	4.5 (3.3)	NS
Number of nodes removed	5.8 (6.3)	10.9 (9.0)	p<.0001
Number of positive nodes	0.7 (1.7)	1.7 (3.4)	p=.009
	% (n)	% (n)	
Ethnicity			
White	68.8 (265)	72.9 (113)	NS
Black	7.5 (29)	9.7 (15)	
Asian/Pacific Islander	13.0 (50)	7.1 (11)	
Hispanic/Mixed ethnic background/Other	10.6 (41)	10.3 (16)	
Lives alone			
Yes	23.0 (88)	28.9 (44)	NS
No	77.0 (295)	71.1 (108)	
Married/partnered			
Yes	47.4 (182)	52.0 (79)	NS
No	52.6 (202)	48.0 (73)	
Employed			
Yes	51.4 (197)	49.7 (76)	NS
No	48.6 (186)	50.3 (77)	
Handedness			
Right	88.8 (341)	88.9 (136)	NS
Left	8.1 (31)	9.2 (14)	
Both	3.1 (12)	2.0 (3)	
Occurrence of comorbid conditions (% and number of women who reported each comorbid condition from the Self-Administered Comorbidity Questionnaire)			
Heart disease	5.6 (21)	6.0 (9)	NS
High blood pressure	27.0 (103)	34.9 (53)	NS
Lung disease	3.7 (14)	8.1 (12)	p=.04
Diabetes	6.6 (25)	7.4 (11)	NS
Ulcer	3.7 (14)	4.7 (7)	NS
Kidney disease	1.6 (6)	2.0 (3)	NS
Liver disease	2.1 (8)	4.8 (7)	NS
Anemia	7.2 (27)	9.5 (14)	NS
Depression	21.8 (81)	26.7 (39)	NS
Osteoarthritis	19.2 (72)	26.7 (40)	NS
Back pain	29.3 (110)	31.5 (47)	NS
Rheumatoid arthritis	3.5 (13)	4.7 (7)	NS
Diagnosed with mastitis			
Yes	13.1 (50)	11.3 (17)	NS
No	86.9 (332)	88.7 (134)	
Diagnosed with cystic breast disease			
Yes	21.5 (81)	23.3 (34)	NS
No	78.5 (295)	76.7 (112)	

Breastfed			
Yes	49.4 (190)	45.1 (69)	NS
No	50.6 (195)	54.9 (84)	
Surgery on affected breast not related to cancer			
Yes	9.3 (36)	14.8 (23)	NS
No	90.7 (351)	85.2 (132)	
Surgery to the affected arm not related to cancer			
Yes	3.1 (12)	5.2 (8)	NS
No	96.9 (375)	94.8 (147)	
Surgery on the affected hand not related to cancer			
Yes	5.2 (20)	7.1 (11)	NS
No	94.8 (367)	92.9 (144)	
Injury to the affected arm			
Yes	17.3 (67)	22.6 (35)	NS
No	82.7 (320)	77.4 (120)	
Injury to the affected hand			
Yes	17.1 (66)	17.4 (27)	NS
No	82.9 (321)	82.6 (128)	
Side of cancer surgery			
Dominant	49.9 (193)	41.9 (65)	NS
Nondominant	50.1 (194)	58.1 (90)	
Type of surgery			
Breast conservation	75.2 (291)	70.3 (109)	NS
Mastectomy	24.8 (96)	29.7 (46)	
Stage of disease			
Stage 0	18.1 (70)	5.2 (8)	
Stage I	40.1 (155)	32.9 (51)	p<.0001
Stage IIA and IIB	35.4 (137)	48.4 (75)	
Stage IIIA, IIIB, IIIC, and IV	6.5 (25)	13.5 (21)	
Sentinel lymph node biopsy			
Yes	80.9 (313)	69.7 (108)	p=.006
No	19.1 (74)	30.3 (47)	
Axillary lymph node dissection			
Yes	39.3 (152)	69.3 (106)	p<.0001
No	60.7 (235)	30.7 (47)	
Reconstruction at the time of surgery			
Yes	21.6 (68)	22.2 (18)	NS
No	78.4 (247)	77.8 (63)	
Adjuvant chemotherapy			
Yes	36.7 (142)	59.7 (92)	p<.0001
No	63.3 (245)	40.3 (62)	
Adjuvant radiation therapy			
Yes	57.1 (221)	71.0 (110)	p<.0001
No	42.9 (166)	29.0 (45)	
Combinations of treatments			
Only surgery			
Surgery and radiation therapy	23.8 (92)	8.4 (13)	p<.0001
Surgery and chemotherapy	39.5 (153)	32.3 (50)	
Surgery, radiation therapy, and chemotherapy	19.1 (74)	20.6 (32)	
	17.6 (68)	38.7 (60)	
Exercise on a regular basis			
Yes	73.7 (283)	75.2 (115)	NS
No	26.3 (101)	24.8 (38)	

Abbreviations: kg = kilograms, m^2 – meter squared, NS = not significant, SD = standard deviation

Table 3. Multiple logistic regression analyses for IL4, IL10, and NFKB2 genotypes to predict the development of lymphedema

Predictor	Odds Ratio	Standard Error	95% CI	Z	p-value
IL4 genotype	0.31	0.156	0.119, 0.829	-2.34	.019
BMI	1.06	0.022	1.014, 1.102	2.61	.009
Stage of disease					
Stage 0 versus I	3.22	1.927	0.996, 10.404	1.95	.051
Stage 0 versus II	4.27	2.714	1.229, 14.838	2.28	.022
Stage 0 versus III and IV	6.38	4.714	1.500, 27.145	2.51	.012
SLNB	0.41	0.140	0.206, 0.796	-2.62	.009
Number of nodes removed	1.09	0.022	1.047, 1.132	4.24	<.0001
Any chemotherapy	1.11	0.344	0.604, 2.038	0.33	.738
Any radiation therapy	1.23	0.366	0.685, 2.204	0.69	.489
Overall model fit: $\chi^2 = 83.69$, $p < 0.0001$, $R^2 = 0.1865$					
IL10 genotype	0.49	0.139	0.282, 0.857	-2.51	.012
BMI	1.05	0.022	1.012, 1.099	2.53	.011
Stage of disease					
Stage 0 versus I	2.64	1.553	0.836, 8.359	1.65	.098
Stage 0 versus II	3.25	2.027	0.954, 11.039	1.88	.059
Stage 0 versus III and IV	5.78	4.227	1.378, 24.234	2.40	.016
SLNB	0.40	0.138	0.204, 0.786	-2.66	.008
Number of nodes removed	1.08	0.022	1.043, 1.128	4.07	<.0001
Any chemotherapy	1.27	0.399	0.687, 2.354	0.77	.444
Any radiation therapy	1.41	0.422	0.781, 2.531	1.14	.256
Overall model fit: $\chi^2 = 84.06$, $p < 0.0001$, $R^2 = 0.1876$					
NFKB2 genotype	3.06	1.338	1.299, 7.209	2.56	.011
BMI	1.06	0.022	1.015, 1.103	2.69	.007
Stage of disease					
Stage 0 versus I	2.91	1.725	0.912, 9.301	1.80	.071
Stage 0 versus II	3.81	2.406	1.108, 13.135	2.12	.034
Stage 0 versus III and IV	6.23	4.570	1.479, 26.233	2.49	.013
SLNB	0.40	0.137	0.203, 0.783	-2.67	.008
Number of nodes removed	1.08	0.021	1.043, 1.126	4.08	<.0001
Any chemotherapy	1.15	0.361	0.624, 2.129	0.45	.650
Any radiation therapy	1.36	0.406	0.755, 2.439	1.02	.307
Overall model fit: $\chi^2 = 84.16$, $p < 0.0001$, $R^2 = 0.1876$					

For each model, the first three principal components identified from the analysis of ancestry informative markers as well as self-report race/ethnicity (i.e., White, Black, Asian/Pacific Islander, Hispanic/Mixed ethnic background/Other) 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 (IL4 rs2227284: CC + CA versus AA; IL10 rs1518111: GG versus GA + AA; NFKB2 rs1056890:

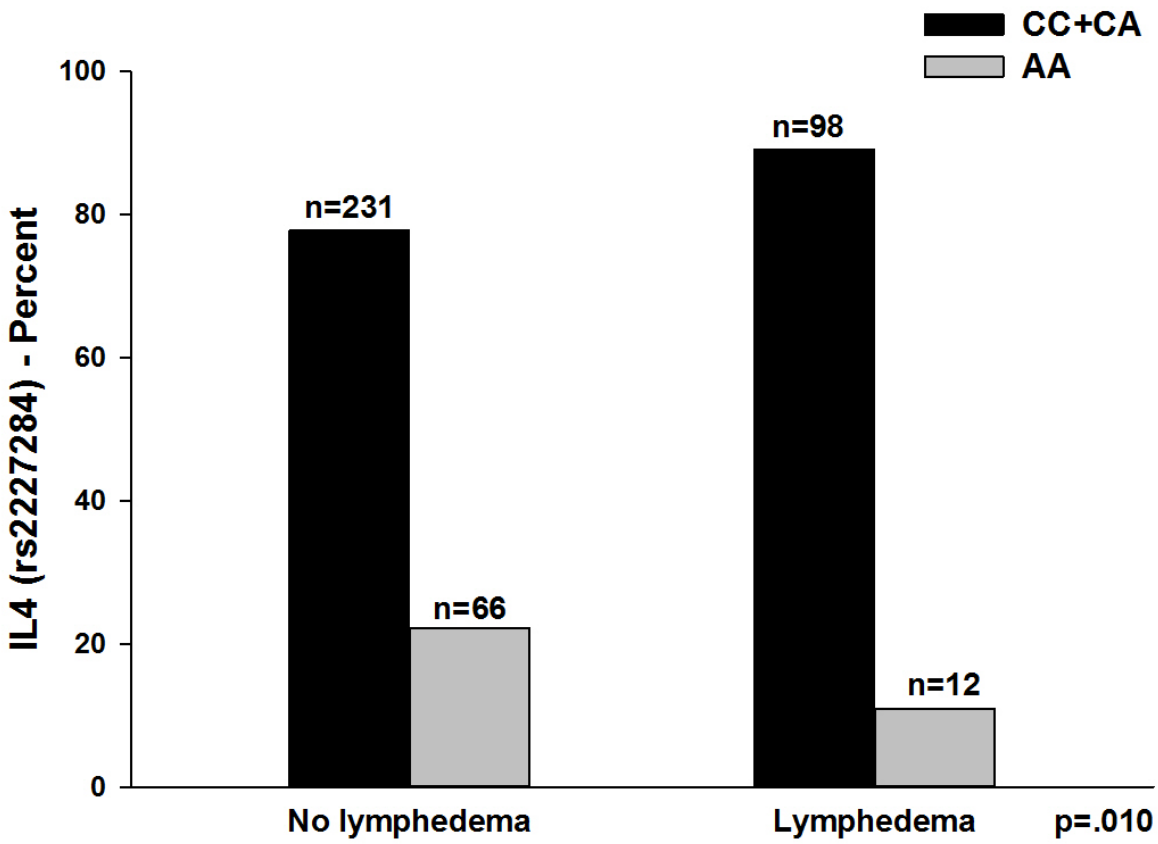

Figure 1A. Differences in the percentages of patient with and without lymphedema who were homozygous or heterozygous for the common allele (CC+CA) or homozygous for the rare allele (AA) for rs2227284 in interleukin 4 (IL4). Values are plotted as unadjusted proportions with the corresponding p-value.

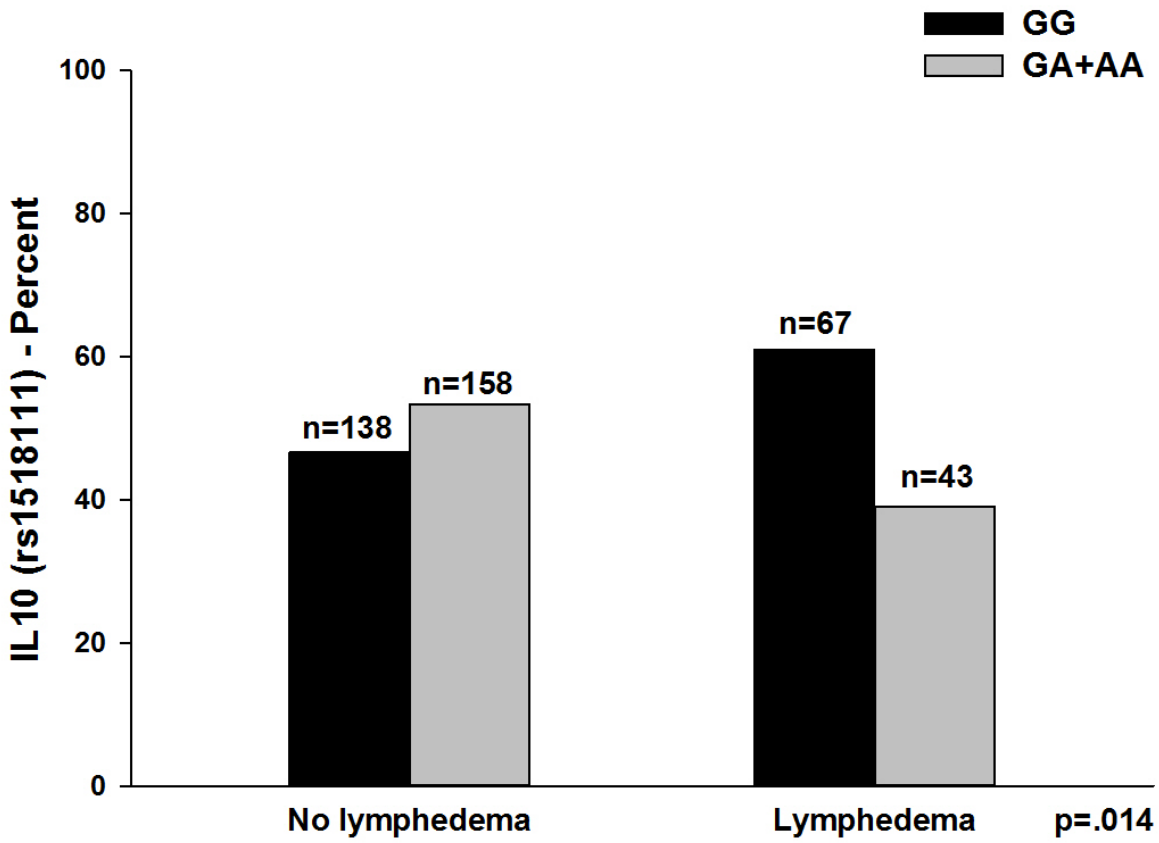

Figure 1B. Differences in the percentages of patients with and without lymphedema who were homozygous for the common allele (GG) or heterozygous or homozygous for the rare allele (GA+AA) for rs1518111 in IL10. Values are plotted as unadjusted proportions with the corresponding p-value.

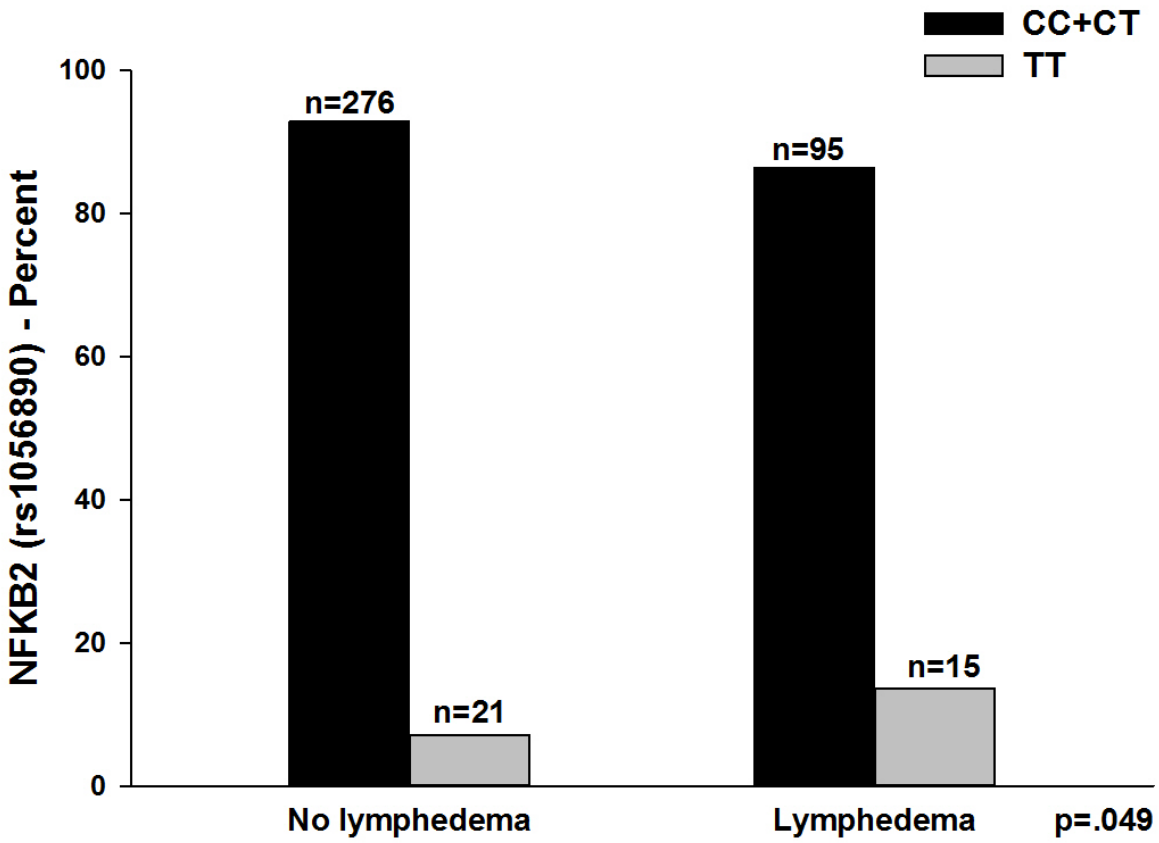

Figure 1C. Differences in the percentages of patients with and without lymphedema who were homozygous or heterozygous for the common allele (CC+CT) or homozygous for the rare allele (TT) for rs1056890 in nuclear factor kappa beta 2 (NFKB2). Values are plotted as unadjusted proportions with the corresponding p-value.

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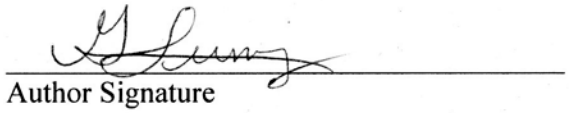

Author Signature

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Date