Published in final edited form as:

Breast Cancer Res Treat. 2013 April ; 138(3): 917–924. doi:10.1007/s10549-013-2445-x.

Prognostic Value of Single Nucleotide Polymorphisms of Candidate Genes Associated with Inflammation in Early Stage Breast Cancer

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Abstract

To examine the role of germline genetic variations in inflammatory pathways as modifiers of time to recurrence (TTR) in patients with early stage breast cancer (BC), DNA from 997 early stage BC patients was genotyped for 53 tagging single nucleotide polymorphisms (SNPs) in 12 genes involved in inflammation. SNPs were analyzed separately for Caucasians versus African Americans and Hispanics. Cox proportional hazards models were used to evaluate the association between SNPs in the inflammatory genes and time to recurrence (TTR), adjusted for clinical and pathologic covariates. In univariable analyses of Caucasian women, the homozygous genotype of 12 SNPs, including 6 *NFKB1* SNPs, 4 *IL4* SNPs, and 2 *IL13* SNPs, were significantly associated with a decrease in TTR compared with the heterozygous and/ or corresponding homozygous genotype ($P < 0.05$). The significant *NFKB1* and *IL4* SNPs were in an area of high linkage disequilibrium ($D' > 0.8$). After adjusting for stage, age, and treatment, carriage of the homozygous genotypes for *NFKB1*rs230532 and *IL13*rs1800925 were independently associated with a shorter TTR ($P = 0.001$ and $p = 0.034$, respectively). In African-American and Hispanic patients, expression of *NFKB1*rs3774932, *TNFR*rs1799964, and *IL4*rs3024543 SNPs were associated with a shorter TTR in univariable model. Only *NFKB1* rs3774932 ($P = 0.02$) and *IL4*rs3024543 ($P = 0.03$) had independent prognostic value in the multivariable model. These data support the existence of host genetic susceptibility as a component in recurrence risk mediated by pro-inflammatory and immune factors, and suggest the potential for drugs which modify immune responses and inflammatory genes to improve prognosis in early stage BC.

Keywords

gene polymorphisms; inflammation; breast cancer

Introduction

Breast cancer (BC) remains the most frequent malignant neoplasm in North American women (1). Although earlier diagnosis and new and improved treatments have changed the overall prognosis in women diagnosed with early stage BC, 30–40% of women experience a recurrence of their cancer within 3 to 5 years of diagnosis.

The authors indicated no potential conflicts of interest.

The prognosis of early stage BC is influenced by a number of well-established factors, including tumor stage (2), axillary lymph node status (3), tumor grade (4), and pathologic markers such as estrogen receptor (ER), progesterone receptor (PR), and HER2/neu oncoprotein expression (5-6). Hierarchical clustering of gene expression data from tumor samples of BC patients has identified specific genes that can be used to divide patients into prognostic subgroups on the basis of expression of the above markers (7,8). Furthermore, specific patterns of gene expression within subgroups that include inflammatory and immune response gene signatures can further stratify patients in terms of overall and disease-free survival, irrespective of the type of therapy (9-12). An inflammatory tumor environment can result in tumor promotion through enhanced proliferation and survival of malignant cells and the subversion of innate and adoptive immune responses (reviewed in 13).

Several investigators have attempted to correlate germline single nucleotide polymorphisms (SNPs) of inflammatory cytokine genes with BC risk, with mixed results (14-15). A recent large meta-analysis showed no correlation between the expression of a large number of cytokine SNPs and the risk of developing BC (16), but only a few small studies have specifically examined whether SNPs in inflammatory genes are associated with BC prognosis (reviewed in 17). To address this gap in knowledge, we utilized a large cohort of early stage BC patients and examined the role of 53 tagging SNPs in 12 genes involved in the inflammatory pathway in predicting time to recurrence (TTR).

Materials and Methods

Study population

Detailed clinical information, including patient age, disease stage, nuclear grade, ER and PR status, and primary treatment including surgery, radiation, chemotherapy, and/or endocrine therapy, was retrospectively abstracted from the medical records of a cohort of 1390 BC patients diagnosed between 1985 and 2002 who were enrolled in the Early Stage Breast Cancer Repository at the MD Anderson Cancer Center. (18). Of these 1390 patients, 1089 had blood or normal lymph node samples available for genotyping. Fifty-nine patients were excluded from study analysis because of assay failure (n=54), insufficient clinical information (n=5), or lack of information on race (n=33). The study analysis included 997 patients (739 Caucasians, 141 African Americans and 117 Hispanics). The study was approved by the Institutional Review Board of MD Anderson Cancer Center.

SNP selection and genotyping

We reviewed the existing literature and selected 53 SNPs from 12 genes associated with inflammation in BC (IL6, IL8, TNF, IL4, IL4R, IL13, PTGS2 [COX2], TGFB1, IL10, IL1B, and *IL1RN*). All selected SNPs were within genes or linkage disequilibrium (LD) blocks containing these genes and were identified using data from the HapMap Project (www.hapmap.org, version 23). All SNPs met the following criteria: minor allele frequency of ≥ 0.05 , Illumina design score >0.4 , and $r^2 \geq 0.8$ for binning. We genotyped samples using Illumina Golden Gate technology (San Diego, CA, USA) as part of a larger array of 1,514 SNPs in total. Genomic DNA was extracted from blood or buccal samples using the QIAamp DNA Blood Maxi kit (QIAGEN) and from paraffin-embedded (FFPE) nodal tissue using the PicoPure DNA extraction method (Applied Biosystems, Foster City, CA, USA.) The call rates from the FFPE samples and blood or buccal samples were 92% and 99% respectively. Blinded duplicate samples were included in the array platform and the duplication concordance was 100%. All genotyping clustering was performed using GenomeStudio data analysis software (Illumina).

Statistical analyses—We analyzed SNPs associated with TTR in Caucasian separately from African American and Hispanic patients because of racial differences in the frequency of SNPs involving multiple genes. TTR was defined as the time between the date of first treatment and date of BC loco-regional or distant metastatic recurrence. The relationship between each SNP and clinical variable and TTR was evaluated separately using a univariate Cox proportional hazards regression model. (19). To build a Cox proportional hazards model with high-dimensional covariates, we applied the CoxBoost algorithm to the SNPs that had prognostic potential suggested by the univariable analyses ($P \leq 0.05$) and then used a backwards elimination procedure ($\alpha=0.05$) (20). Interaction terms between significant SNPs from univariable analyses and hormone receptor status (ER+ or PR+ versus ER- and PR-) were constructed to test for interaction among Caucasian patients. Interactions between SNPs and hormone receptor status were assessed using the likelihood-ratio test ($P < 0.05$) comparing the model including an interaction term with the reduced model without the interaction term adjusted for age, stage and treatment. Stratified multivariable analysis was performed where effect modification was observed. Linkage mapping was performed using Haploview version 4.2 (21) for the linkage disequilibrium (LD) blocks, assuming 1 Mb = 1 cM, using the SNPs which had prognostic significance in the univariable model. All other analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC).

Results

Univariable Analysis of clinical and pathologic variables and Breast Cancer Recurrence

There were 182 recurrences with a median time to follow-up of 18 years. Table 1 lists the clinical and pathologic covariates associated with TTR in univariable analysis. Variables significantly associated with a prolonged TTR included clinical stage I disease, ER or progesterone receptor (PR) positivity, patient age ≥ 50 years, and use of hormonal therapy alone.

Univariable analyses of SNPs associated with TTR in Caucasian women

In the univariable analysis of the 53 SNPs in 739 Caucasian patients, 6 SNPs of *NFKB1*, 4 SNPs of *IL4*, and 2 SNPs of *IL13* were associated with significant differences in TTR ($P < 0.05$). After multiple testing adjustments those 12 SNPs were significant at a false discovery rate (FDR) of 0.12 (Table 2). As shown in Figure 1, there was significant LD between the *NFKB1* SNPs (SNPs 1-6, Figure 1). Similarly, the four *IL4* SNPs (9-12, Figure 1) were in a block of high LD, but LD was not demonstrated for *IL13* SNPs rs1800925 and rs1295686.

Multivariable analyses of SNPs associated with TTR in Caucasian women

In multivariable analysis, SNPs *NFKB1* rs230532 (IVS2-910G>A) and *IL13* rs1800925 (-1069C>T), were independent predictors of a shorter TTR adjusted for age, stage and treatment (Table 3). There was evidence of a significant interaction between SNPs *NFKB1* rs3774932 ($p_{\text{interaction}} = 0.02$) and rs230532 ($p_{\text{interaction}} = 0.01$) and hormone receptor status. In a stratified multivariable model adjusted for age, stage and treatment, carriers of the SNPs *NFKB1* rs230532 (TT vs AA+TT) and *NFKB1* rs3774932 (AA vs GG) genotypes had a shorter TTR only in the ER+ or PR+ subgroup (Table 4).

Prognostic impact of SNPs in non-Caucasian women

To determine if the frequency and prognostic impact of inflammatory SNPs found in Caucasians were the same or different as those in non-Caucasian patients, we performed similar analyses using 53 SNPs in the 141 African American and 117 Hispanic patients combined (Tables 5 and 6). Univariable analysis revealed that carriage of the less frequent

homozygous genotype of *NFKB1*rs3774932, was associated with a significantly shorter TTR ($P=0.03$) in these ethnic groups (Table 5). A significant trend for shorter TTR was observed for two additional polymorphisms not found in Caucasians, *TNF*rs1799964 ($P=0.07$) and *IL4R*rs3024543. ($P=0.09$). Multivariable analysis including the three SNPs that were associated with TTR in the univariable model and adjusted for stage and hormonal therapy revealed that only SNPs *NFKB1*rs3774932 ($p=0.02$) and *IL4R*rs3024543 ($p=0.03$) were independent predictors for shorter TTR (Table 6).

Discussion

To our knowledge, this retrospective study is one of the first to examine the prognostic impact of 53 germline polymorphisms from 12 inflammatory genes in a large well characterized homogeneous cohort of patients with early stage BC. After adjusting for prognostic clinical parameters, *NFKB1*rs230532 and *IL13*rs1800925 SNPs were independent predictors of a shorter TTR in Caucasians, while *NFKB1*rs3774932 and *IL4R*rs3024543 SNPs were predictive of a shorter TTR in African Americans and Hispanics.

NFKB encodes nuclear factor kappa B, (NFkB) a family of proteins consisting of five heterodimeric transcription factors (22,23) which serve as a master regulators for a plethora of genes involved in inflammation, cell proliferation, apoptosis inhibition, bone remodeling angiogenesis, chemokine production and metalloproteinase production (24,25). Despite the abundance of information in regards to the function of NFkB in tumors there are no published studies assessing the putative role of *NFKB* germline polymorphisms and prognosis in BC. A study by Kurt et al. demonstrated that peripheral T lymphocytes from women with BC demonstrated an impaired ability to translocate NFkB p65 (Rel-A) following activation by anti-CD3 and Interleukin-2 (IL2) (26).

We were unable to evaluate the association between SNP expression and TTR by molecular subtypes (8) because patients in our BC cohort were treated between 1985 and 2000, prior to the routine assessment of HER2/neu status. However, when stratified by ER and PR receptor status, Caucasian patients with ER+ or PR+ disease had a shorter TTR if they carried *NFKB1* genotypes rs230532 and rs3774932. This is an interesting finding in view of the fact that inflammation in general has been shown to be associated with more aggressive ER+ BC (27). Moreover, it has recently been demonstrated that NFkB activation can maintain immunosuppressive function of type 2 tumor associated macrophages (TAM) (28) which have been found to comprise up to 50% of breast tumor mass (29). Although NFkB activation has been demonstrated more frequently in ER negative tumors (30), recent evidence has demonstrated antagonistic cross-talk between the NFkB and ER pathways (31). Additional evidence suggests that NFkB activation occurs in a subset of ER+ tumors and cell lines with poor response to endocrine therapy and that incubation of an NFkB inhibitor with resistant lines could restore endocrine sensitivity (32,33) Hence, our findings may have relevance with respect to predicting those ER+ patients who are resistant to endocrine therapy.

As confirmed by this study, variations in allele frequencies and specific SNPs associated with inflammation have been observed for different races (34). Erdei et al. measured polymorphisms in cytokine genes along with secreted cytokines in New Mexican Hispanic women with BC compared to age-, gender-, and smoking-matched women without incident BC (controls) and found a higher frequency of SNPs rs2069705, rs2243248 and rs1800925 located in the promoter regions of the interferon gamma (*IFN*γ), *IL4* and *IL13* genes, respectively (35). The authors hypothesized that immune dysregulation might account for more aggressive tumors found in Hispanics compared to Whites.

IL13 and IL4 are members of the TH2 cytokine family along with IL10, IL6 and TGF β , and are involved in immune suppression, antibody production, and activation of other pro-inflammatory molecules (36). IL4 and IL13 are crucial immune factors which signal through the IL4 receptor (IL4R). A recent large prospective study in patients with diffuse large B-cell lymphoma demonstrated a favorable impact of the interleukin-4 receptor allelic variant I75 (rs1805010) on overall survival (37). Preclinical studies in mice have shown that CD4-positive T cells infiltrating breast tumors secrete high levels of IL13, which promotes tumor growth through immune suppression by dendritic cells, an effect that could be prevented by administration of IL13 antagonists (38).

There was close linkage between all of the significant *NFKB1* and *IL4* SNPs in Caucasians. Most of these SNPs were intronic, except for *IL4* SNP rs2070874 which was located near the 5'UTR. Hence the latter SNP is more likely to influence transcription, whereas the mechanisms of the intronic SNP is less clear. It is more likely that either SNP could be in LD with another functional SNP in the *NFKB1* or *IL4* gene region or that one or more of the SNPs could be in some other regulatory region of the gene. Preclinical studies suggest that the presence of SNPs in the regulatory or coding regions of cytokine genes can result in functional alterations in the transcriptional regulation of these genes or the proteins they encode (39). Dominant homozygous polymorphisms within the promoter regions of their respective genes induce higher circulating levels of cytokines and other growth factors, resulting in increased oncogenesis and inflammation resulting in immune suppression (40).

In univariable analysis there was a significant trend ($p=0.07$) for carriage of *TNF* SNP rs1799964 and a shorter TTR in African American and Hispanic patients. This SNP was not an independent predictor of prognosis when stratified for other clinical covariates in the multivariable model. The product of the *TNF* gene, *TNF α* , is a pro-inflammatory cytokine induced under hypoxic conditions (41). Chronic production of *TNF α* in benign and malignant BC tissues is associated with a poor outcome (42). Previous investigators have demonstrated that carriage of several *TNF* SNPs significantly correlated with shorter disease-free survival and overall survival (43,44). In one study, The *TNF* type II homozygous genotype correlated with higher levels of *TNF α* in serum and a worse prognosis than the *TNF* type I genotype.(44) Whether the SNP rs1799964 identified in our study is associated with increased serum levels of *TNF α* is currently unknown and needs further study.

A significant weakness of our study was that serum cytokine levels were not measured, making it difficult to associate specific cytokine SNPs with immune function. In a case control study, 13 cytokines were measured in 40 New Mexican Hispanic breast cancer patients compared to 40 controls (34). Of the 13 cytokines observed, only 5: IL1 β , IL-5, *TNF α* , IL6, and IL2 showed elevated levels in BC patients compared to controls. A significant problem with cytokine measurements in association with disease states is that levels can vary significantly based on the response to chemotherapy (45), and other factors associated with inflammation. Additional large studies which assess SNP expression with cytokine function in different ethnic groups with early stage BC are needed.

Another possible weakness is that the low recurrence rate along with stratification by race and other variables may have resulted in our study being underpowered. Larger cohort studies that allow for the evaluation of breast cancer molecular subtypes and interactions between polymorphisms and other clinical and environmental factors are needed to validate and extend our findings. Despite these limitations, our data, as well as data from several smaller studies has demonstrated that the presence of specific polymorphisms of genes for molecules and cytokines involved in inflammation and tumor immunity may have prognostic value in BC patients. These findings could have important implications for the

use of blocking antibodies and other molecules that target the protein products generated through over expression of the significant gene polymorphisms involved (46) in an attempt to improve the disease free survival of patients with early stage BC.

Acknowledgments

The authors wish to acknowledge Ricardo Mottu and Katharina Alvarez for manuscript preparation.

(Financial support: Supported by Grants R03 CA123553 (A.M.B.), Susan Komen Disparities Career Award (A.M.B.), R01 CA089608 (M.L.B., P.T.), K07 CA160753 (M.P.) and CA16672 (MD Anderson Cancer Center) from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

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Figure 1. Linkage Analysis of NFKB1 and IL4 SNPs found to be prognostically significant in univariable analysis. All of the *NFKB1* SNPs as well as the four *IL4* SNPs were in a single block of LD ($D' > 0.9$)

Table 1

Clinical and pathologic variables associated with time to recurrence (TTR) in early stage BC

Parameter	No. Of Patients (%)	HR (95% ci) ^a	P-value
Stage:			
1	308 (30)	1(reference)	
2	722 (70)	1.82 (1.34, 2.48)	0.00
Black's Nuclear Grade:			
1	99 (10)	1	
2	541 (53)	0.87 (0.66, 1.14)	0.29
3	347 (37)	1.90 (0.68, 1.64)	0.81
Hormone receptor status:			
ER– and PR–	246 (24)	1	
ER+ or PR+	750 (73)	0.76 (0.58, 1.00)	0.05
NA ^b	34 (3)		
Age:			
50	436 (42)	1	
>50	594 (58)	0.57 (0.45, 0.73)	0.00
Treatment:			
None	231 (23)	1	
Chemotherapy only	333 (32)	1.27 (0.93, 1.73)	0.14
Endocrine therapy only	259 (25)	0.68 (0.46, 0.99)	0.04
Chemotherapy + Endocrine therapy	202 (20)	0.73 (0.48, 1.11)	0.14
NA ^b	5 (---)		
BMI:			
Underweight/normal	429 (42)	1	
Overweight	297 (29)	1.05 (0.78, 1.40)	0.71
Obese	279 (27)	0.91 (0.66, 1.25)	0.57
NA	25 (2)		
Race:			
Caucasian	739 (72)	1	
African-American	141 (14)	1.17 (0.83, 1.67)	0.37
Hispanic	117 (11)	1.08 (0.72, 1.60)	0.72
NA	33 (3)		

^b Abbreviations used: NA: not available; ER:estrogen receptor; PR: Progesterone receptor; BM: body mass index; HR: hazard ratio; CI: confidence interval

Table 2Univariable Analysis of Inflammatory SNPs associated with shorter TTR in Caucasian Breast Cancer Patients^a

Gene	rs number	Risk allele	No. of patients (events)	HR (95% CI)	P value (global testing)
<i>NFKB1</i>	3774932a	GG ^b	191 (54)	1 (reference)	0.01
		AG	380 (76)	0.68 (0.48,0.97)	
		AA	144 (48)	1.19 (0.08,1.76)	
<i>NFKB1</i>	230533	AG + AA	400 (86)	1	0.01
		GG ^b	323 (94)	1.50 (1.11,2.01)	
<i>NFKB1</i>	4648058	CG + CC	384 (87)	1	0.05
		GG ^b	328 (91)	1.34 (1.00,1.80)	
<i>NFKB1</i>	230521	GG ^b	260 (74)	1	0.01
		CG	356 (71)	0.66 (0.47,0.91)	
		CC	107 (35)	1.07 (0.72,1.61)	
<i>NFKB1</i>	230496	AA ^b	252 (70)	1	0.03
		AG	340 (68)	0.65 (0.48,0.90)	
		GG	82 (24)	0.94 (0.60,1.48)	
<i>NFKB1</i>	230532	AT + AA	401 (88)	1	0.01
		TT ^b	319 (92)	1.47 (1.09,1.97)	
<i>IL 4</i>	2070874	AG + AA	187 (36)	1	0.03
		GG ^b	521 (140)	1.49 (1.03,2.15)	
<i>IL4</i>	2243268	AC + CC	188 (37)	1	0.04
		AA ^b	536 (143)	1.45 (1.01,2.08)	
<i>IL4</i>	2243267	CG + CC	181 (36)	1	0.04
		GG ^b	499 (134)	1.48 (1.02,2.13)	
<i>IL4</i>	2243270	AG + GG	204 (41)	1	0.05
		AA ^b	540 (129)	1.42 (1.01,2.02)	
<i>IL13</i>	1800925	AG + AA	181 (36)	1	0.04
		GG ^b	499 (134)	1.48 (1.02,2.13)	
<i>IL13</i>	1295686	AG + AA	204 (41)	1	0.05
		GG ^b	520 (139)	1.42 (1.01,2.02)	

^a Abbreviations: HR: hazard ratio; CI: confidence interval; ref: referent SNP to which others were compared.^b Most frequent homozygous allele

Table 3

Multivariable Analysis of Inflammatory SNPs associated with shorter TTR in Caucasian Breast Cancer Patients

Gene	SNP	Genotype	HR ^a (95% CI)	P value (global testing)
<i>NFKB1</i>	230532	AT+AA TT	1(reference) 1.41(1.02,1.95)	0.04
<i>IL13</i>	1800925	AG + AA GG	1 1.47(1.04,2.07)	0.03
Clinical parameters				
Stage:1	_____	_____	1	
2	_____	_____	1.83 (1.34, 2.48)	0.02
Age: 50	_____	_____	1	
> 50	_____	_____	0.57(0.45,0.73)	0.00

^a Abbreviations: HR: hazard ratio; CI: confidence interval

Table 4

Interaction between SNPs, hormone receptor (ER/PR) status and time to breast cancer recurrence.

SNPs	Genotype	ER- and PR- HR (95% CI) N=174	ER+ or PR+ HR (95% CI) N=545	P _{interaction}
rs3774932	GG	1.0 (reference)	1.0	0.02
	AG	0.64 (0.34, 1.24)	0.90 (0.58, 1.40)	
	AA	0.49 (0.20, 1.20)	1.82 (1.14, 2.91)	
rs230532	AA+ AT	1.0 (reference)	1.0	0.01
	TT	0.72 (0.43, 1.38)	1.76 (1.23, 2.52)	

ER (estrogen receptor), PR (progesterone receptor), HR (hazard ratio) and 95% confidence interval (CI) adjusted for age, stage and treatment

Table 5

Univariable Analysis of Inflammatory SNPs associated with shorter TTR in African-American and Hispanic Breast Cancer Patients

Gene	SNPs	Genotype	No. of patients (events)	HR ^a (95% CI)	P value (global testing)
<i>NFKB1</i>	3774932	AA + AG	232 (58)	1(reference)	0.03
		GG	120 (38)	2.52 (0.91,2.89)	
<i>TNF</i>	1799964	CC + CT	232 (58)	1	0.07
		TT	9 (4)	2.52 (0.91,6.95)	
<i>IL4R</i>	3024543	GG	400 (86)	1	0.09
		AG + AA	323 (94)	1.57 (0.93,2.64)	

^aAbbreviations: HR: hazard ratio; CI: confidence interval

Table 6

Multivariable Analysis of Inflammatory SNPs and Clinical Parameters associated with shorter TTR in African-American and Hispanic Breast Cancer Patients

Gene	SNP	Genotype	HR ^a (95% CI)	Pvalue (global testing)
<i>NFKB1</i>	3774932	AA+AG GG	1 (reference) 2.52 (0.91,2.89)	0.02
<i>IL4R</i>	3024543	GG AG+AA	1 1.57 (0.93,2.64)	0.03
Clinical parameter				
Stage 1	_____	_____	1	
2	_____	_____	1.91 (1.01, 3.63)	0.05

^a Abbreviations: HR: hazard ratio; CI: confidence interval