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Research Paper

Correlation between *CCL4* gene polymorphisms and clinical aspects of breast cancer

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Abstract

Breast cancer is a major cause of cancer mortality amongst women. Chemokine (C-C motif) ligand 4 is encoded by the *CCL4* gene; specific *CCL4* gene polymorphisms are related to the risks and prognoses of various diseases. In this study, we examined whether *CCL4* gene single nucleotide polymorphisms (SNPs) predict the risk and progression of breast cancer. Between 2014 and 2016, we recruited 314 patients diagnosed with breast cancer and a cohort of 209 healthy participants (controls) without a history of cancer. Genotyping of the *CCL4* rs1634507, rs10491121 and rs1719153 SNPs revealed no significant between-group differences for these polymorphisms. However, amongst luminal A and luminal B subtypes, compared with patients with the AA genotype, those carrying the AG genotype at SNP rs10491121 were less likely to develop lymph node metastasis. In addition, compared with AA carriers, those carrying the AG + GG genotype at SNP rs10491121 were at lower risk of developing distant metastasis, while the presence of the AT genotype at SNP rs1719153 increased the likelihood of pathologic grade (G3 or G4) disease. Variations in the *CCL4* gene may help to predict breast cancer progression and metastasis.

Key words: single nucleotide polymorphism, breast cancer, chemokine C-C motif ligand 4 (CCL4), genotype

Introduction

Breast cancer is the second leading cause of cancer deaths amongst women worldwide. Nearly million women worldwide are diagnosed with breast cancer annually and more than 500,000 die from this disease [1]. Besides age, reproductive and gynecologic factors, other risk factors such as family history and environmental factors including tobacco and alcohol consumption, as well as overall amount of physical activity, can greatly modify the risk of developing breast cancer [2]. In addition, gynecologic diseases including polycystic ovarian syndrome and adenomyosis have been found to enhance the risk of breast cancer [3, 4].

Mammography screening and genetic testing have limited sensitivity and specificity for estimating breast cancer risk [2]. It is uncertain as to whether single nucleotide polymorphism (SNP) genotyping could more accurately predict breast cancer risk and guide disease management [5, 6]. Susceptibility to breast cancer appears to be influenced by certain SNPs, as well as clinicopathologic status [7]. *BRCA1* and *BRCA2* gene mutations increase the risk of breast

cancer [8, 9]. Fascin-1 (FSCN1) and high-mobility group box protein 1 (HMGB1) genetic polymorphisms have also been identified as predictive biomarkers for breast cancer [10].

Chemokine (C-C motif) ligand 4 (CCL4) is a protein that is encoded by the *CCL4* gene and acts as a chemoattractant for natural killer cells, monocytes and various other immune cells in the site of inflamed or damaged tissue. *CCL4* polymorphisms influence gene expression, protein function and susceptibility to various diseases, including hepatocellular carcinoma, oral cancer, and psoriasis [11-14]. *CCL4* belongs to a cluster of genes located in the chromosomal region 17q11-q21. The CCL4 protein acts as the chemokine being secreted under mitogenic signals and antigens and attracting monocytes, dendritic cells, natural killer cells and other effector cells into the site of inflamed or damaged tissue [15, 16]. On the other hand, the *CCL4* gene polymorphisms has been associated with risk and development in oral cancer and hepatocellular carcinoma [12, 17]. Despite the well-known impact of chemokines on cancer progression and the recognition that *CCL4* gene SNPs play important roles in a variety of human diseases, little is known about the association between these SNPs and the susceptibility to breast cancer and its progression. In this study, we evaluated the predictive capacity of three *CCL4* SNPs as candidate biomarkers for breast cancer risk.

Materials and Methods

Participants

Between 2014 and 2016, we collected 314 blood specimens from patients (cases) diagnosed with breast cancer at Dongyang People's Hospital. A total of 209 healthy, cancer-free individuals served as controls. Written informed consent was obtained from all participants before study entry. The Ethics Committee of Dongyang People's Hospital granted study approval. Pathohistologic diagnosis used the World Health Organization breast tumor classification and tumors were graded using the Scarff-Bloom-Richardson method [18]. Breast cancer cases were categorized by estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 status and grouped under 1 of 4 subtypes: Luminal A (ER-positive $^{[+]}$ and/or PR$^+$, HER2-negative $^{[-]}$, Ki-67 <14%); Luminal B (ER$^+$ and/or PR$^+$, HER2$^-$, Ki-67 ≥14%; or ER$^+$ and/or PR$^+$, HER2$^+$); HER2-enriched (ER$^-$, PR$^-$, HER2$^+$); or as triple-negative breast cancer (TNBC; ER$^-$, PR$^-$, HER2$^-$) [19-21]. A standardized questionnaire at study entry collected sociodemographic data and electronic medical records provided clinicopathologic information.

Selection of *CCL4* polymorphisms

The *CCL4* SNPs selected for this study were identified from multi-allelic copy number variation (CNV) profiles encompassing the q12 region of chromosome 17 containing *CCL4* genes. Nonsynonymous SNPs rs1634507, rs10491121 and rs1719153 were extracted from a search of the National Center for Biotechnology Information (NCBI) dbSNP database.

Genomic DNA extraction

The QIAamp DNA Blood Mini Kit (Qiagen, Inc., Valencia, CA, USA) purified genomic DNA from peripheral blood leukocytes. The DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA; pH 7.8), quantified by OD_{260}, then stored at –20°C for further analysis.

Real-time PCR

The ABI StepOne™ real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA) assessed sequencing of allelic discrimination for the *CCL4* SNP. The TaqMan assay used Software Design Specification version 3.0 software (Applied Biosystems) to analyze the discrimination data. Primers and probes consisted of rs1634507 "AGTTTTCTTGACCTCATGAATGCTG-[G/T]TGAGGCTTTATCCCTCTCTCAGGAA" (product ID: C_7451708_10), rs10491121 "CCTATCCCCT TCCTGAATTAAGTCC-[A/G]AATATAGTCAGTCT TTGAGTGTGGA" (product ID: C_11626804_10) and rs1719153 "TAGGGACTGTTGCACCGAGTTTCAC-[A/T]GTTAAGGAAACAGAGGCACAGAGAG" (product ID: C_12120537_10). PCRs were performed in a total volume of 10 µL containing Master Mix (5 µL), probes (0.25 µL) and genomic DNA (10 ng). The real-time PCR reaction included an initial denaturation step at 95°C for 10 min, then 40 amplification cycles of 95°C for 15 secs and 60°C for 1 min [19, 22].

Statistical analysis

Between-group differences were considered significant if *p*-values were less than 0.05. Chi-square analysis tested for Hardy-Weinberg equilibrium in the SNP genotype distributions. The Mann-Whitney U-test and Fisher's exact test were utilized for between-group demographic comparisons. Multiple logistic regression models adjusted for confounding variables estimated adjusted odds ratios (AORs) and 95% confidence intervals (CIs) for associations between genotype frequencies and the risk of breast cancer or clinicopathologic characteristics. Haplotype frequencies were analyzed using Haploview [23]. All data were analyzed with the software program

Statistical Analytic System version 9.1 and are reported as the sample mean ± the standard deviation (SD).

Results

All study participants were Chinese Han (Table 1). The majority were nonsmokers and did not consume alcohol. There was a significantly higher proportion of younger age participants in the control group compared with the breast cancer cohort (p<0.05). Most patients (77.1%) had stage I/II breast cancer; 22.9% had stage III/IV disease (Table 1). In an analysis of hormone receptor status, tumors were mostly ER$^-$ (69.7%), PR$^-$ (54.1%), or HER2$^+$ (63.1%) (Table 1).

Table 1. Demographic and clinicopathologic characteristics among healthy cancer-free controls and patients with breast cancer.

Variable	Controls N=209 (%)	Patients N=314 (%)	*p* value
Age (years)	Mean ± SD	Mean ± SD	
	38.5±16.7	53.1±11.4	***p*<0.05**
Tobacco smokers			
No	202 (96.7)	313 (99.7)	
Yes	7 (3.3)	1 (0.3)	***p*<0.05**
Alcohol consumption			
No	203 (97.1)	295 (93.9)	
Yes	6 (2.9)	19 (6.1)	*p*>0.05
Clinical stage			
I/II		242 (77.1)	
III/IV		72 (22.9)	
Tumor size			
≤T2		298 (94.9)	
>T2		16 (5.1)	
Lymph node status			
N0+N1		247 (78.7)	
N2+N3		67 (21.3)	
Distant metastasis			
M0		304 (96.8)	
M1		10 (3.2)	
Histological grade			
G1+G2		218 (69.4)	
G3+G4		96 (30.6)	
ER status			
Positive		95 (30.3)	
Negative		219 (69.7)	
PR status			
Positive		144 (45.9)	
Negative		170 (54.1)	
HER2 status			
Positive		198 (63.1)	
Negative		116 (36.9)	

The Mann-Whitney U-test and Fisher's exact test were used to compare values between controls and patients with breast cancer. *p < 0.05 was statistically significant. T2 = tumor >20 mm but ≤50 mm in greatest dimension; N0 = lymph node-negative; N1 = cancer has spread to 1–3 lymph node(s); N2 = 4–9 lymph nodes; N3 = ≥10 positive lymph nodes; M0 = noninvasive cancer; M1 = cancer has metastasized to organs or lymph nodes away from the breast; G1 = well differentiated (low grade); G2 = moderately differentiated (intermediate grade); G3 = poorly differentiated (high grade); G4 = undifferentiated (high grade); ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2.

Polymorphism frequencies are shown in Table 2. All genotypes were in Hardy-Weinberg equilibrium (p > 0.05). In both study groups, the most frequent genotypes for SNPs rs10491121, rs1634507 and rs1719153 were homozygous for A/A, homozygous for G/G and homozygous for A/A. Analyses that adjusted for potential confounders found no significant between-group differences for the polymorphism frequencies.

Table 2. Distribution frequencies of *CCL4* genotypes among healthy cancer-free controls and patients with breast cancer.

Variable	Controls N=209 (%)	Patients N=314 (%)	OR (95% CI)
rs10491121			
AA	64 (41)	79 (34.2)	1.00 (reference)
AG	92 (59)	152 (65.8)	1.338 (0.88-2.035)
GG	53 (45.3)	83 (51.2)	1.269 (0.787-2.044)
AG+GG	145 (69.4)	235 (74.8)	1.313 (0.89-1.938)
rs1634507			
GG	101 (54.9)	135 (49.5)	1.00 (reference)
GT	83 (45.1)	138 (50.5)	1.244 (0.855-1.810)
TT	25 (19.8)	41 (23.3)	1.227 (0.701-2.148)
GT+TT	108 (51.7)	179 (57)	1.240 (0.873-1.762)
rs1719153			
AA	101 (55.5)	149 (52.7)	1.00 (reference)
AT	81 (44.5)	134 (47.3)	1.121 (0.771-1.630)
TT	27 (21.1)	31 (17.2)	0.778 (0.438-1.382)
AT+TT	108 (51.7)	165 (52.5)	1.036 (0.73-1.470)

The odds ratios (ORs) with their 95% confidence intervals (CIs) were estimated by logistic regression analysis. The adjusted ORs (AORs) with their 95% CIs were estimated by multiple logistic regression analysis that controlled for tobacco smoking, alcohol consumption and age.

A comparison of clinicopathologic characteristics and *CCL4* genotypes revealed no significant differences (Table 3). Similarly, an analysis of *CCL4* genotypic frequencies amongst breast cancer subtypes failed to identify any significant differences between patients and controls (Table 4). However, among luminal A and luminal B subtypes, patients carrying the AG genotype at SNP rs10491121 were less likely to develop lymph node metastasis compared with AA genotype carriers (AOR, 0.298; 95% CI: 0.1-0.885) (Table 5). In addition, patients with the rs10491121 AG + GG genotype were at lower risk of developing distant metastasis compared with AA genotype carriers (AOR, 0.106; 95% CI: 0.011-1.038). Moreover, the presence of the TT haplotype at the SNP rs1719153 (AOR 3.316; 95% CI: 1.12-9.815) increased the likelihood of developing pathologic grade (G3+G4) disease (Table 5).

Figure 1 represents the reconstructed linkage disequilibrium plot of the genotyped polymorphisms in our study population. In one haploblock, rs1634507 and rs10491121 displayed 98% linkage disequilibrium. *CCL4* SNPs rs1634507 and rs1719153 expressed 95% linkage disequilibrium; rs10491121 and rs1719153 expressed 97% linkage disequilibrium (Fig. 1).

Table 3. Odds ratios and their confidence intervals for clinical status and *CCL4* genotypic frequencies in patients with breast cancer.

Genotype	Patients N=314 (%)		OR (95% CI)
	Clinical stage		
	Stage I/II	**Stage III/IV**	
rs10491121			
AA	55 (25)	24 (25.5)	1.00 (reference)
AG+GG	165 (75)	70 (74.5)	0.972 (0.558-1.694)
rs1634507			
GG	98 (44.5)	37 (39.4)	1.00 (reference)
GT+TT	122 (55.5)	57 (60.6)	1.237 (0.757-2.024)
rs1719153			
AA	109 (49.5)	40 (42.6)	1.00 (reference)
AT+TT	111 (50.5)	54 (57.4)	1.326 (0.815-2.157)
	Tumor size		
	≤T2	**>T2**	
rs10491121			
AA	76 (25.5)	3 (18.8)	1.00 (reference)
AG+GG	222 (74.5)	13 (81.2)	1.483 (0.412-5.347)
rs1634507			
GG	130 (43.6)	5 (31.2)	1.00 (reference)
GT+TT	168 (56.4)	11 (68.8)	1.702 (0.577-5.021)
rs1719153			
AA	144 (48.3)	5 (31.2)	1.00 (reference)
AT+TT	154 (51.7)	11 (68.8)	2.057 (0.698-6.065)
	Lymph node status		
	N0+N1	**N2+N3**	
rs10491121			
AA	68 (86.1)	11 (13.9)	1.00 (reference)
AG+GG	215 (91.5)	20 (8.5)	0.575 (0.262-1.260)
rs1634507			
GG	121 (89.6)	14 (10.4)	1.00 (reference)
GT+TT	162 (90.5)	17 (9.5)	0.907 (0.403-1.911)
rs1719153			
AA	136 (91.3)	13 (8.7)	1.00 (reference)
AT+TT	147 (89.1)	18 (10.9)	1.281 (0.605-2.713)
	Distant metastasis		
	M0	**M1**	
rs10491121			
AA	74 (93.7)	5 (6.3)	1.00 (reference)
AG+GG	230 (97.9)	5 (2.1)	0.322 (0.91-1.142)
rs1634507			
GG	130 (96.3)	5 (3.7)	1.00 (reference)
GT+TT	174 (97.2)	5 (2.8)	0.747 (0.212-2.635)
rs1719153			
AA	144 (96.6)	5 (3.4)	1.00 (reference)
AT+TT	160 (97)	5 (3)	0.9 (0.255-3.172)
	Histologic grade		
	G1+G2	**G3+G4**	
rs10491121			
AA	58 (73.4)	21 (26.6)	1.00 (reference)
AG+GG	160 (68.1)	75 (31.9)	1.295 (0.732-2.288)
rs1634507			
GG	99 (73.3)	36 (26.7)	1.00 (reference)
GT+TT	119 (66.5)	60 (33.5)	1.387 (0.848-2.267)
rs1719153			
AA	109 (73.2)	40 (26.8)	1.00 (reference)
AT+TT	109 (66.1)	56 (33.9)	1.4 (0.862-2.274)

The odds ratios (ORs) with their 95% confidence intervals (CIs) were estimated by logistic regression analysis. The adjusted odds ratios (AORs) with their 95% CIs were estimated by multiple logistic regression analysis that controlled for smoking, consumption and age.

T2 = tumor >20 mm but ≤50 mm in greatest dimension; N0 = lymph node-negative; N1 = cancer has spread to 1–3 lymph node(s); N2 = 4–9 lymph nodes; N3 = ≥10 positive lymph nodes; M0 = noninvasive cancer; M1 = cancer has metastasized to organs or lymph nodes away from the breast; G1 = well differentiated (low grade); G2 = moderately differentiated (intermediate grade); G3 = poorly differentiated (high grade); G4 = undifferentiated (high grade).

Table 4. Distribution frequencies of *CCL4* genotypes in breast cancer subtypes.

Variable	Control N= 209(%)	Patients N= 220(%) Lumina A + Lumina B	OR (95% CI)	Variable	Control N= 209(%)	Patients N= 94(%) HER2 overexpression + TNBC	OR (95% CI)
rs10491121				**rs10491121**			
AA	64 (53.8)	55 (46.2)	1.00 (reference)	AA	64 (76.2)	20 (23.8)	1.00 (reference)
AG	92 (45.8)	109 (54.2)	1.379 (0.875-2.173)	AG	92 (74.2)	32 (25.8)	1.113 (0.585-2.118)
GG	53 (48.6)	56 (51.4)	1.23 (0.731-2.069)	GG	53 (72.6)	20 (27.4)	1.208 (0.588-2.478)
AG+GG	145 (46.8)	165 (53.2)	1.324 (0.867-2.023)	AG+GG	145 (73.6)	52 (26.4)	1.148 (0.634-2.078)
rs1634507				**rs1634507**			
GG	101 (50.8)	98 (49.2)	1.00 (reference)	GG	101 (77.7)	29 (22.3)	1.00 (reference)
GT	83 (46.6)	95 (53.4)	1.18 (0.787-1.768)	GT	83 (69.7)	36 (30.3)	1.511 (0.855-2.668)
TT	25 (48.1)	27 (49.8)	1.113 (0.604-2.050)	TT	25 (78.1)	7 (21.9)	0.975 (0.383-2.482)
GT+TT	108 (47)	122 (53)	1.164 (0.796-1.702)	GT+TT	108 (74.4)	72 (25.6)	1.387 (0.805-2.388)
rs1719153				**rs1719153**			
AA	101 (48.1)	109 (51.9)	1.00 (reference)	AA	101 (75.9)	32 (24.1)	1.00 (reference)
AT	81 (46.3)	94 (53.7)	1.075 (0.719-1.607)	AT	81 (69.8)	35 (30.2)	1.364 (0.778-2.391)
TT	27 (61.4)	17 (38.6)	0.583 (0.3-1.134)	TT	27 (84.4)	5 (15.6)	0.584 (0.208-1.643)
AT+TT	108 (49.3)	111 (50.7)	0.952 (0.652-1.391)	AT+TT	108 (73)	40 (27)	1.169 (0.682-2.002)

The odds ratios (ORs) with their 95% confidence intervals (CIs) were estimated by logistic regression analysis. The adjusted odds ratios (AORs) with their 95% CIs were estimated by multiple logistic regression analysis that controlled for smoking, consumption and age.
HER2 = human epidermal growth factor receptor 2; TNBC = triple-negative breast cancer.

Table 5. Odds ratios and their confidence intervals for clinical status and *CCL4* genotypic frequencies in breast cancer subtypes.

Variable		Luminal A + Luminal B N=220 (%)			HER2 overexpression + TNBC N=94 (%)		
		Clinical Stage			**Clinical Stage**		
		Stage I/II	**Stage III/IV**	**OR (95% CI)**	**Stage I/II**	**Stage III/IV**	**OR (95% CI)**
	rs10491121						
AA		40 (72.7)	15 (27.3)	1.00 (reference)	19 (79.2)	5 (20.8)	1.00 (reference)
AG		93 (85.3)	16 (14.7)	0.459 (0.207-1.017)	27 (62.8)	16 (37.2)	2.252 (0.704-7.206)
GG		40 (71.4)	16 (28.6)	1.067 (0.465-2.445)	23 (85.2)	4 (14.8)	0.661 (0.155-2.813)
AG+GG		133 (80.6)	32 (19.4)	0.642 (0.316-1.302)	50 (71.4)	20 (28.6)	1.52 (0.499-4.627)
	rs1634507						
GG		77 (78.6)	21 (21.4)	1.00 (reference)	29 (78.4)	8 (21.6)	1.00 (reference)
GT		74 (77.9)	21 (22.1)	1.041 (0.525-2.062)	28 (65.1)	15 (34.9)	1.942 (0.712-5.294)
TT		22 (81.5)	5 (18.5)	0.833 (0.282-2.464)	12 (85.7)	2 (14.3)	0.604 (0.112-3.272)
GT+TT		96 (78.7)	26 (21.3)	0.993 (0.519-1.899)	40 (70.2)	17 (29.8)	1.541 (0.586-4.051)
	rs1719153						
AA		85 (78)	24 (22)	1.00 (reference)	32 (80)	8 (20)	1.00 (reference)
AT		74 (78.7)	20 (21.3)	0.957 (0.49-1.871)	25 (62.5)	15 (37.5)	2.4 (0.879-6.556)
TT		14 (82.4)	3 (17.6)	0.759 (0.201-2.86)	12 (85.7)	2 (14.3)	0.667 (0.124-3.597)
AT+TT		88 (79.3)	23 (20.7)	0.926 (0.486-1.764)	37 (68.5)	17 (31.5)	1.838 (0.701-4.821)
		Tumor size			**Tumor size**		
		≤T2	**>T2**	**OR (95% CI)**	**≤T2**	**>T2**	**OR (95% CI)**
	rs10491121						
AA		53 (96.4)	2 (3.6)	1.00 (reference)	23 (95.8)	1 (4.2)	1.00 (reference)
AG		106 (97.2)	3 (2.8)	0.75 (0.122-4.626)	38 (88.4)	5 (11.6)	3.026 (0.332-27.548)
GG		54 (96.4)	2 (3.6)	0.981 (0.133-7.225)	24 (88.9)	3 (11.1)	2.875 (0.279-29.677)
AG+GG		160 (97)	5 (3)	0.828 (0.156-4.395)	62 (88.6)	8 (11.4)	2.968 (0.352-25.054)
	rs1634507						
GG		95 (96.9)	3 (3.1)	1.00 (reference)	35 (94.6)	2 (5.4)	1.00 (reference)
GT		92 (96.8)	3 (3.2)	1.033 (0.203-5.248)	37 (86)	6 (14)	2.838 (0.537-15.01)
TT		26 (96.3)	1 (3.7)	1.218 (0.122-12.201)	13 (92.9)	1 (7.1)	1.346 (0.112-16.13)
GT+TT		118 (96.7)	4 (3.3)	1.073 (0.235-4.914)	50 (87.7)	7 (12.3)	2.45 (0.48-12.501)
	rs1719153						
AA		106 (97.2)	3 (2.8)	1.00 (reference)	38 (95)	2 (5)	1.00 (reference)
AT		91 (96.8)	3 (3.2)	1.165 (0.229-5.913)	34 (85)	6 (15)	3.353 (0.634-17.738)
TT		16 (94.1)	1 (5.9)	2.208 (0.216-22.548)	13 (92.9)	1 (7.1)	1.462 (0.122-17.482)
AT+TT		107 (96.4)	4 (3.6)	1.321 (0.289-6.044)	47 (87)	7 (13)	2.83 (0.555-14.423)
		Lymph node status			**Lymph node status**		
		N0+N1	**N2+N3**	**OR (95% CI)**	**N0+N1**	**N2+N3**	**OR (95% CI)**
	rs10491121						
AA		46 (83.6)	9 (16.4)	1.00 (reference)	22 (91.7)	2 (8.3)	1.00 (reference)
AG		103 (94.5)	6 (5.5)	**0.298 (0.1-0.885)***	37 (86)	6 (14)	1.784 (0.331-9.619)
GG		48 (85.7)	8 (14.3)	0.852 (0.303-2.397)	27 (100)	0 (0)	0.917 (0.813-1.034)
AG+GG		151 (91.5)	14 (8.5)	0.474 (0.193-1.166)	64 (91.4)	6 (8.6)	1.031 (0.194-5.489)
	rs1634507						
GG		87 (88.8)	11 (11.2)	1.00 (reference)	34 (91.9)	3 (8.1)	1.00 (reference)

Variable		Luminal A + Luminal B N=220 (%)			HER2 overexpression + TNBC N=94 (%)		
GT		87 (91.6)	8 (8.4)	0.727 (0.279-1.896)	38 (88.4)	5 (11.6)	1.491 (0.331-6.712)
TT		23 (85.2)	4 (14.8)	1.375 (0.401-4.721)	14 (100)	0 (0)	0.919 (0.835-1.011)
GT+TT		110 (90.2)	23 (10.5)	0.863 (0.363-2.049)	52 (91.2)	5 (8.8)	1.09 (0.244-4.861)
	rs1719153						
AA		99 (90.8)	10 (9.2)	1.00 (reference)	37 (92.5)	3 (7.5)	1.00 (reference)
AT		84 (89.4)	10 (10.6)	1.179 (0.468-2.968)	35 (87.5)	5 (12.5)	1.762 (0.392-7.929)
TT		14 (82.4)	3 (17.6)	2.121 (0.52-8.658)	14 (100)	0 (0)	0.925 (0.847-1.01)
AT+TT		98 (88.3)	13 (11.7)	1.313 (0.55-3.136)	49 (90.7)	5 (9.3)	1.259 (0.283-5.605)
		Distant metastasis			**Distant metastasis**		
		M0	**M1**	**OR (95% CI)**	**M0**	**M1**	**OR (95% CI)**
	rs10491121						
AA		52 (94.5)	3 (5.5)	1.00 (reference)	22 (91.7)	2 (8.3)	1.00 (reference)
AG		109 (100)	0 (0)	**0.945 (0.887-1.007)***	40 (93)	3 (7)	0.825 (0.128-5.317)
GG		55 (98.2)	1 (1.8)	0.315 (0.032-3.127)	26 (96.3)	1 (3.7)	0.423 (0.036-4.985)
AG+GG		164 (99.4)	1 (0.6)	**0.106 (0.011-1.038)***	66 (94.3)	4 (5.7)	0.667 (0.114-3.893)
	rs1634507						
GG		95 (96.9)	3 (3.1)	1.00 (reference)	35 (94.6)	2 (5.4)	1.00 (reference)
GT		95 (100)	0 (0)	0.969 (0.936-1.004)	39 (90.7)	4 (9.3)	1.795 (0.31-10.408)
TT		26 (96.3)	1 (3.7)	1.218 (0.122-12.201)	14 (100)	0 (0)	0.946 (0.876-1.022)
GT+TT		121 (99.2)	1 (0.8)	0.262 (0.027-2.556)	53 (93)	4 (7)	1.321 (0.229-7.602)
	rs1719153						
AA		106 (97.2)	3 (2.8)	1.00 (reference)	38 (95)	2 (5)	1.00 (reference)
AT		94 (100)	0 (0)	0.972 (0.942-1.004)	36 (90)	4 (10)	2.111 (0.364-12.24)
TT		16 (94.1)	1 (5.9)	2.208 (0.216-22.548)	14 (100)	0 (0)	0.95 (0.885-1.02)
AT+TT		110 (99.1)	1 (0.9)	0.321 (0.033-3.137)	50 (92.6)	4 (7.4)	1.52 (0.264-8.738)
		Histological grade			**Histological grade**		
		G1+G2	**G3+G4**	**OR (95% CI)**	**G1+G2**	**G3+G4**	**OR (95% CI)**
	rs10491121						
AA		45 (81.8)	10 (18.2)	1.00 (reference)	13 (54.2)	11 (45.8)	1.00 (reference)
AG		95 (87.2)	14 (12.8)	0.663 (0.274-1.608)	16 (37.2)	27 (62.8)	1.994 (0.724-5.495)
GG		40 (71.4)	16 (28.6)	1.8 (0.734-4.417)	9 (33.3)	18 (66.7)	2.364 (0.761-7.343)
AG+GG		135 (81.8)	30 (18.2)	1 (0.453-2.206)	25 (35.7)	45 (64.3)	2.127 (0.831-5.446)
	rs1634507						
GG		81 (82.7)	17 (17.3)	1.00 (reference)	18 (48.6)	19 (51.4)	1.00 (reference)
GT		81 (85.3)	14 (14.7)	0.824 (0.381-1.781)	16 (37.2)	27 (62.8)	1.599 (0.654-3.906)
TT		18 (66.7)	9 (33.3)	2.382 (0.916-6.196)	4 (28.6)	10 (71.4)	2.368 (0.628-8.926)
GT+TT		99 (81.1)	23 (18.9)	1.107 (0.554-2.212)	20 (35.1)	37 (64.9)	1.753 (0.754-4.074)
	rs1719153						
AA		90 (82.6)	19 (17.4)	1.00 (reference)	19 (47.5)	21 (52.5)	1.00 (reference)
AT		80 (85.1)	14 (14.9)	0.829 (0.39-1.76)	13 (32.5)	27 (67.5)	1.879 (0.759-4.655)
TT		10 (58.8)	7 (41.2)	**3.316 (1.12-9.815)***	6 (42.9)	8 (57.1)	1.206 (0.354-4.115)
AT+TT		90 (81.1)	21 (18.9)	1.105 (0.557-2.195)	19 (35.2)	35 (64.8)	1.667 (0.723-3.841)

The odds ratios (ORs) with their 95% confidence intervals (CIs) were estimated by logistic regression analysis. The adjusted odds ratios (AORs) with their 95% CIs were estimated by multiple logistic regression analysis that controlled for smoking, consumption and age. * $p<0.05$.

HER2 = human epidermal growth factor receptor 2; TNBC = triple-negative breast cancer; T2 = tumor >20 mm but ≤50 mm in greatest dimension; N0 = lymph node-negative; N1 = cancer has spread to 1–3 lymph node(s); N2 = 4–9 lymph nodes; N3 = ≥10 positive lymph nodes; M0 = noninvasive cancer; M1 = cancer has metastasized to organs or lymph nodes away from the breast; G1 = well differentiated (low grade); G2 = moderately differentiated (intermediate grade); G3 = poorly differentiated (high grade); G4 = undifferentiated (high grade).

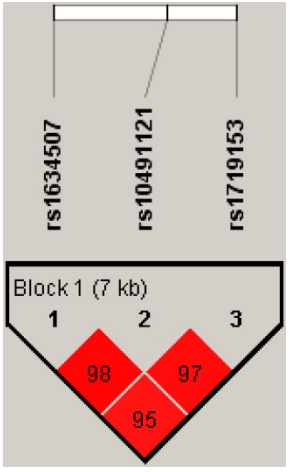

Figure 1. Linkage disequilibrium patterns of three single nucleotide polymorphisms in the *CCL4* gene.

Discussion

CCL4, also known as macrophage inflammatory protein-1β (MIP-1β), belongs to the pro-inflammatory CC subfamily. MIP proteins recruit pro-inflammatory cells and thus play a crucial role in acute and chronic inflammatory responses in various conditions including asthma, granuloma formation, wound healing, arthritis, multiple sclerosis, pneumonia, and psoriasis [16]. Accumulating evidences indicated CCL4 expression associated with cancer progression such as oral cancer and hepatocellular carcinoma [12, 17]. We have previously suggested that *CCL4* gene polymorphisms influence susceptibility to oral cancer and hepatocellular carcinoma and affect their progression [11, 12]. We found that *CCL4* rs1634507

G/T polymorphism increased a risk in oral-cancer susceptibility, but *CCL4* rs10491121 A/G polymorphism decreased a risk in hepatocellular carcinoma. Now, the findings from this study indicate that *CCL4* SNPs may serve as candidate biomarkers for susceptibility to breast cancer.

The 5-year relative survival rate for breast cancer has gradually increased since the early 1990s; between 2007 and 2011 it was ~89.2%. As breast cancer prognosis depends upon the disease stage at the time of diagnosis, increasing screening rates and making genetic testing more widely available increase the chances of early diagnosis [24, 25]. Our study is the first to examine the expression of SNPs rs1634507, rs10491121 and rs1719153 and their possible association with the development of breast cancer. Our investigation into possible associations between these *CCL4* SNPs, clinicopathologic markers, and disease susceptibility failed to find any significant differences between patients and healthy controls. Moreover, *CCL4* SNPs did not differ significantly according to breast cancer clinical aspects. Amongst luminal A and luminal B subtypes, patients carrying the AG haplotype at SNP rs10491121 were less likely to develop lymph node metastasis compared with patients with the AA haplotype, while patients carrying the AG + GG haplotype at rs10491121 were less likely to develop distant metastasis. The presence of the AT haplotype at the SNP rs1719153 increased the likelihood of developing pathologic grade (G3+G4) disease.

Linkage disequilibrium is expressed across the human genome. Thus, loci can be used as genetic markers to locate adjacent variants that participate in the detection and treatment of disease. Haplotype analyses clarify genetic contribution to disease susceptibility [26, 27]. We observed 98% linkage disequilibrium between rs1634507 and rs10491121, 95% linkage disequilibrium between rs1634507 and rs1719153, and 97% between rs10491121 and rs1719153. These results suggest that these *CCL4* haplotypes play an important role in breast cancer development.

This is the first study to demonstrate a correlation between *CCL4* polymorphisms and breast cancer risk. *CCL4* may prove to be a diagnostic marker and therapeutic target for breast cancer therapy.

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Competing Interests

The authors have declared that no competing interest exists.

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