Original Study

Association of SNP-SNP Interactions Between *RANKL, OPG, CHI3L1,* and *VDR* Genes With Breast Cancer Risk in Egyptian Women

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Abstract

Genetic susceptibility for breast cancer (BC) is still poorly understood. We investigated the association of single nucleotide polymorphism (SNP)-SNP interactions of 6 SNPs in *RANKL, OPG, CHI3L1,* and *VDR* genes with BC risk in 115 BC patients and 120 controls using logistic regression models. A stronger combined effect of SNPs via gene–gene interaction may predict BC risk. Our data have implications in genetic counseling, BC screening, and prognosis.

Background: Genetic susceptibility for breast cancer (BC) is still poorly understood. A combination of multiple low-penetrant alleles of cancer-related genes and gene–gene interactions (epistasis) contributes to BC risk. Genetic variants in receptor activator of nuclear factor κB ligand (*RANKL*), osteoprotegerin (*OPG*), chitinase-3–like protein 1 (*CHI3L1*), and vitamin D receptor (*VDR*) genes are implicated in breast carcinogenesis; however, the influence of their epistatic effects on BC susceptibility has not yet been studied. We investigated the association of single nucleotide polymorphism (SNP)-SNP interactions and haplotypes of 6 SNPs in these 4 genes with the genetic predisposition of BC in Egyptian women. **Patients and Methods:** Data of 115 BC patients and 120 cancer-free controls were studied. Association tests were conducted using logistic regression models. **Results:** Individual SNPs showed weak statistical significance with BC susceptibility. The interactions between RANKL-rs9533156 and OPG-rs2073618; OPG-rs2073618 with CHI3L1-rs4950928, VDR-rs2228570 and VDR-rs1544410; OPG-rs2073617 and VDR-rs1544410; VDR-rs2228570 and VDR-rs1544410 were strongly associated with increased BC risk after adjustment for multiple comparisons. No SNPs were in strong linkage disequilibrium. The TCTCTG-rs9533156-rs2073618-rs2073617-rs4950928-rs2228570-rs1544410 haplotype was significantly associated with increased BC risk (adjusted odds ratio = 8.33; 95% confidence interval, 1.32-52.46; $P = .025$) compared with controls. TCCCTG haplotype stratified BC patients according to estrogen receptor/progesterone receptor status. TCTCTA was positively associated, and TCTCTG and TGTCTG haplotypes inversely correlated with bone metastasis. Bioinformatic analysis revealed 13 proteins commonly interacting with our 4 genes; the most significant was signal transducer and activator of transcription 5B. **Conclusion:** Our results suggested that a stronger combined effect of SNPs in *RANKL, OPG, CHI3L1,* and *VDR* genes via gene–gene interaction may help predict BC risk and prognosis.

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Introduction

Breast cancer (BC) is the most common cancer and is the leading cause of cancer death among women worldwide (25% of cancer cases and 15% of cancer deaths); it is the deadliest cancer among women in less developed countries.[1] In Egypt, BC ranks the second most common cancer after liver cancer, accounting for 15.4% of all cancers, and is the most frequent cancer among women (32.04% of cancer cases).[2]

BC is a multifactorial disease resulting from combined effects of genetic and environmental risk factors. To date, the full genetic

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basis of BC susceptibility is poorly understood. High risk of developing BC is attributable to germ-line mutations in high-penetrance genes such as breast cancer (*BRCA*) 1 and *BRCA2;* however, such mutations are rare (much less than 1%) in the general population. They only account for 5% to 10% of all female BC cases and 15% to 20% of all familial BC cases.[3] By comparison, variants in low-penetrance genes (ie, single nucleotide polymorphisms, SNPs) are more common and have been shown by genome-wide association studies to incrementally contribute to BC risk; however, their individual contributions are relatively small.[4-7] Hence, combinations of variants of multiple low-penetrance loci/genes (ie, genetic interactions) across the genome have been proposed to contribute to overall genetic BC risk.[8]

Epistasis, or gene−gene interaction, describes how genes/loci interact to affect phenotypes and is a component of the genetic framework of complex diseases, including BC.[9-11] However, the current tests for genetic interactions from genome-wide association studies data mainly focus on small sets of genes or SNPs with known association with BC, given that many genetic interactions, particularly among novel variants, remain understudied.[10] In addition, many independent studies have demonstrated the epistatic effects of 2 or more SNPs with reproducible weak single-locus effects on BC susceptibility.[12-14] However, these studies have extensively focused on assessing SNP-SNP interactions in DNA repair, modification, and metabolism-related pathway genes. Thus, identification of interactions between SNPs in other cancer-related genes may shed more light into the unexplained heritability of BC.

To estimate the BC risk conferred by SNP-SNP interactions, we studied 6 SNPs from 4 cancer-related genes: receptor activator of nuclear factor κB ligand (*RANKL*), osteoprotegerin (*OPG*), chitinase-3−like protein 1 (*CHI3L1*), and vitamin D receptor (*VDR*). Beyond regulation of bone remodeling, the receptor activator of nuclear factor κB (RANK)/RANKL/OPG system is expressed by breast normal and tumor cells and is implicated in breast carcinogenesis. RANKL, binding with its receptor RANK, is involved in progesterone-mediated cell proliferation, tumor formation, and bone metastasis in BC.[15,16] OPG, a decoy receptor for RANKL, blocks tumor necrosis factor−related apoptosis ligand (TRAIL)-induced apoptosis, enhances tumor growth and metastasis, and may indirectly promote tumor progression by affecting angiogenesis in BC.[17] The *CHI3L1* gene encodes for YKL-40 glycoprotein, a proinflammatory cytokine secreted by neutrophils and activated macrophages that is involved in inflammatory processes of tumors such as cell proliferation, differentiation, and angiogenesis as well as remodeling of extracellular matrix.[18] In addition, high-tumor-tissue YKL-40 expression has been shown to be closely linked with the recurrence and metastasis of BC.[19] Active vitamin D [$1,25(OH)_2D_3$] binds to VDR, a ligand-dependent transcription factor that attenuates mammary gland formation via regulating transcription of a number of growth-regulatory genes in mammary and BC cells, suggesting tumor suppressive functions of vitamin D.[20] Reduced vitamin D status—in particular the interaction between vitamin D levels and genetic variants of *VDR*—has been associated with the development and/or poor prognosis of BC.[21,22]

RANKL-rs9533156, OPG-rs2073618, OPG-rs2073617, CHI3L1-rs4950928, VDR FokI-rs2228570, and VDR BsmI-rs1544410 SNPs have individually demonstrated weak or nonsignificant statistical effects on BC susceptibility in different studies.[23-29] However, the influence of their combination and SNP-SNP interactions on BC risk is not known.

Therefore, this study investigated the potential SNP-SNP interactions of these selected SNPs in *RANKL, OPG, CHI3L1,* and *VDR* genes and the possible association of their allele combinations (haplotypes) with the genetic predisposition of BC in Egyptian women.

Patients and Methods

Subject Population

This case−control retrospective study was conducted in the Medical Biochemistry Department, Faculty of Medicine, Cairo University, using data from Egyptian women with histologically confirmed BC who were recruited from the general surgery department at Kasr Al-Einy Hospital between 2012 and 2016. Written informed consent was obtained from all subjects. The study protocol was approved by the ethics committee of the Faculty of Pharmacy, Cairo University, and conformed to 1975 Declaration of Helsinki.

A total of 276 eligible data points from 146 Egyptian women with histologically confirmed BC and 130 cancer-free controls were available for the study. BC patients' age ranged from 29 to 70 years, with a median age of 52 years. A total of 8.7% of patients with BC were aged < 40 years. About 50% of the women had a family history of BC. In order to create a more representative sample of patients without enrichment for genetic risk factors such as family history, we ensured that the other 50% of data were from patients who had no family history of BC. Control data were from participants who were proven to be cancer-free during a routine checkup, with no previous cancer history, and controls were age-matched to members of the BC group. In total 115 BC cases and 120 controls whose data included all the 6 investigated SNPs were selected for use in this study.

Selection of SNPs and Genotyping Method

We analyzed the effect of combination and SNP-SNP interactions between 6 SNPs with reported relation to BC risk and of 4 genes acting on common cancer pathways. Three of the selected SNPs had not been previously described in the literature in Egyptian women with BC (CHI3L1-rs4950928, VDR FokI-rs2228570, and VDR BsmI-rs1544410); however, the single-locus effects of the other 3 SNPs (RANKL-rs9533156, OPG-rs2073618, and OPG-rs2073617) on BC susceptibility in Egyptian women were published.[23] All selected SNPs were functional and were observed/predicted to affect transcription, translation, or structure of their corresponding proteins.[30-36] SNPs chromosome and genomic position, alteration site, function, and biological process are listed in Table 1.

Genotyping of *RANKL*, *OPG*, and *VDR* SNPs was determined using a PCR restriction fragment length polymorphism assay after DNA extraction from whole blood as previously described.[23,26] CHI3L1-rs4950928 was genotyped in a Qiaplex real-time PCR system using the TaqMan SNP genotyping assay (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA).

Table 1 SNP Position, Function, Biologic Processes, and Effect on Breast Cancer Susceptibility

Gene	dbSNP, Major/Minor Alleles[a]	Chromosome and Genomic Position	Alteration	Function	Breast Cancer Association	Selected Biologic Process
TNFSF11 (RANKL)	rs9533156 (T > C)	13:42573535	5′ UTR variant 2 kb upstream	Promoter SNP that is predicted to alter transcription factor binding sites or RNA secondary structure thus could affect RANKL gene expression.	Yes[23]; no[24]	Positive regulation of PKB, TNF-mediated signaling pathway, cytokine activity, cytokine–cytokine receptor interaction, osteoclast differentiation, immune response, positive regulation of MAPK signaling
TNFRSF11B (OPG)	rs2073618 (G > C)	8:118951813	Missense	Lies in OPG first exon and changes third amino acid from lysine (basic) to asparagine (uncharged polar) thus influences OPG secretion from cells. CC genotype is associated with lower OPG level.[30]	Yes[23]; no[24]	TNF-mediated signaling pathway, cytokine activity, cytokine–cytokine receptor interaction, osteoclast differentiation, immune response, inflammatory response, apoptosis, positive regulation of MAPK signaling
	rs2073617 (T > C)	8:118952044	UTR variant 5′ 2 kb upstream	Promoter SNP which may affect transcription and translation of OPG by altering secondary structure.[31]	Yes[23]	
CHI3L1	rs4950928 (C > G)	1:203186754	UTR variant 5′ 2 kb upstream	Promoter SNP located within binding site for MYC and MAX transcription factors; minor G allele disrupts binding of MAX and MYC, and is associated with reduced transcription and reduced circulating YKL-40 protein level.[32] Conversely, C allele was associated with increased serum YKL-40 levels.[33]	No[25]	Hydrolyzing *O*-glycosyl compounds, positive regulation of PKB, inflammatory response, apoptosis, positive regulation of MAPK signaling, response to TNF
VDR	rs2228570 (FokI) (T > C)	12:47879112	Missense	Formation of second methionine start site that leads to production of shorter protein receptor, with higher transcriptional activity than longer-type receptor.[34]	Yes[26,27]; no[28,29]	C4 zinc finger nuclear receptor, RNA polymerase II transcription factor binding, transcription factor activity, signal transducer activity, growth factor signaling, positive regulation of tissue remodeling, response to external stimulus
	rs1544410 (BsmI) (G > A)	12:47846052	Intron variant	Associated with different length poly-adenylate sequence within 3′ UTR of *VDR* gene,[35] with reduced VDR level in BsmI (B) allele versus GG (bb) genotype.[36]	Yes[28,29]; no[26]	

Abbreviations: dbSNP = Single Nucleotide Polymorphism Database; MAPK = mitogen-activated protein kinase; OPG = osteoprotegerin; PKB = protein kinase B; RANKL = receptor activator of nuclear factor κB ligand; TNF = tumor necrosis factor; UTR = untranslated region; VDR = vitamin D receptor.
[a]Major and minor alleles according to their frequencies in our control population.

Bioinformatics Analysis

We performed functional annotation analysis by conducting Go-enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (https://www.genome.jp/kegg/) as well as protein interactions regarding our selected genes using David Bioinformatics Resources 6.8 (National Institute of Allergy and Infectious Diseases/National Institutes of Health) via the Gene ID Conversion Tool[37] (https://david.ncifcrf.gov/conversion.jsp?VFROM=NA).

Statistical Analysis

We sought evidence of association of each of the 6 SNPs, and their interactions and combinations with BC risk in a multistep process. At the first stage, we calculated crude allele and genotype frequencies for each individual polymorphism and evaluated it via Hardy-Weinberg equilibrium (HWE) using a chi-square test with 1 degree of freedom among controls and cases. For testing the association of each SNP with BC risk, a multiple logistic regression model was used for calculating odds ratios (ORs), 95% confidence intervals (CIs), and corresponding *P* value of codominant, dominant, recessive, and log-additive models, controlling for age and family history of BC as covariates. In all analyses, the major allele or the common homozygote genotype in the control population were defined as the reference category. Akaike information criterion was also used to select the best genetic effect for each SNP.

At the second stage, we used a statistically oriented approach to examine the epistatic effects between selected SNPs, where pairwise SNP-SNP interactions were investigated using multivariate logistic models with adjustment for age and family history of BC. Because there is large number of interactions analyzed that could lead to false-positive results, Bonferroni correction of *P* value was conducted. Allelic combination (haplotype) and linkage disequilibrium (LD) analyses were also performed. Haplotypes with total frequency (pooled frequency of controls and BC cases) of > 0.05 were only considered, while rare haplotypes (total frequency < 0.05) were omitted. The overall difference in the frequency distribution of haplotypes between controls and BC cases (global test), and each haplotype association test was estimated, controlling for age and

family history of BC as covariates. Interaction of haplotypes with pathologic data was also examined.

Concerning SNPs, all tests were carried out by SNPStats online software (Institut Català d'Oncologia, Barcelona, Spain; https://www.snpstats.net/start.htm), which uses the maximum likelihood method.[38] For demographic and clinical data of cases and controls, *t* test and the Fisher exact test were conducted for analyzing quantitative and categorical variables, respectively, using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). $P < .05$ was considered significant.

Results

The selected background characteristics of the study subjects are listed in Table 2. There were no significant differences between the BC and control groups with respect to menstrual history ($P = .15$). The average age of patients in the BC group was slightly higher than in the control group, but the difference was not significant ($P = .057$). The number of BC patients with a family history of BC was significantly higher in the BC group than in the control group ($P < .0001$).

Regarding the pathologic data of BC cases (Table 2), 88.7% of tumors were invasive ductal tumors. Seventy-three percent of cases were tumor, node, and metastases stages II and III, and 74.8% were small tumors (< 5 cm). Eighteen percent of cases were positive for estrogen receptor (ER)/progesterone receptor (PR) hormones. Bone metastasis was present in 28% of BC cases; all had small tumor size, and 29% of them had positive ER/PR status.

Single Locus Effects of Studied SNPs

The minor allele frequencies (MAF) of all SNPs were > 0.1. MAFs of some SNPs in controls were similar to the global MAF reported in the National Center for Biotechnology Information Single Nucleotide Polymorphism Database (dbSNP) or TransOmics for Precision Medicine (TOPMed), or were close to the highest population MAF reported in Ensembl GRCh37 release 89 (2017) for others (Supplemental Table 1 in the online version). The genotype distribution of OPG-rs2073618, OPG-rs2073617, and VDR FokI-rs2228570 in controls and cases; CHI3L1-rs4950928 in controls; and VDR BsmI-rs1544410 in cases followed HWE ($P > .05$). Cases of CHI3L1-rs4950928; controls of VDR BsmI-rs1544410; and both cases and controls of RANKL-rs9533156 showed deviation from HWE ($P < .05$) (Supplemental Table 2 in the online version).

Regarding allele frequencies, the frequencies of the minor alleles OPG-rs2073618 C and VDR BsmI-rs1544410 A were significantly higher in the BC group (0.53 and 0.57, respectively) than in the control subjects (0.27 and 0.43, respectively) (OR = 1.99; 95% CI, 1.38-2.88; $P = .0002$; OR = 1.72; 95% CI, 1.2-2.5; $P = .004$, respectively), while the frequencies of the major alleles RANKL-rs9533156 T, OPG-rs2073617 T, and VDR FokI-rs2228570 T were significantly higher in the BC group (0.71, 0.76, 0.78, respectively) than in the control group (0.53, 0.51, 0.6, respectively) [OR = 2.22, 95% CI, 1.52-3.22; OR = 2.94, 95% CI, 2-4.35; OR = 2.32, 95% CI, 1.53-3.57, respectively; $P < .0001$ for each]. BC risk reduction was observed with RANKL-rs9533156 C, OPG-rs2073617 C, and VDR FokI-rs2228570 C minor alleles. Conversely, the allele frequencies of CHI3L1-rs4950928 were not significantly different between the two groups ($P = .73$) (Table 3). When a more stringent *P* value ($P < .002$) was applied, only the rs9533156, rs2073618, rs2073617, and rs2228570 alleles were associated with BC risk in our patients.

Table 2 Demographic, Clinical, and Pathologic Characteristics of Studied Population

Characteristic	Breast Cancer (N = 115)	Control (N = 120)	*P*
Age (years)	51.33 ± 8.685	48.96 ± 10.17	.057
Menstrual History			
Premenstrual	56 (48.7)	70 (58.3)	
Postmenstrual	59 (51.3)	50 (41.6)	.15
Family History			< .0001
Yes	64 (55.7)	11 (9)	
No	51 (44.3)	109 (91)	
Tumor Type			
Invasive duct	102 (88.7)	—	—
Invasive lobular	13 (11.3)	—	—
TNM Stage			
II	18 (15.7)	—	—
III	66 (57.3)	—	—
IV	31 (27)	—	—
Tumor Size			
5 cm	86 (74.8)	—	—
> 5 cm	29 (25.2)	—	—
Metastasis			
No	83 (72)	—	—
Yes	32 (28)	—	—
ER/PR			
Positive	21 (18.3)	—	—
Negative	79 (68.7)	—	—
Unknown	15 (13)	—	—

Data are presented as n (%) unless otherwise indicated.
Abbreviations: ER = estrogen receptor; PR = progesterone receptor; TNM = tumor, node, metastasis classification system.

Regarding genotype frequencies, all studied SNPs were associated with BC risk at least in one model compared to controls with adjustment for age and family history (Supplemental Table 3 in the online version). Although the allele frequencies of CHI3L1-rs4950928 were not significantly associated with BC risk, the GG genotype was associated with increased BC risk (adjusted OR = 8.92; 95% CI, 1.7-46.84; $P < .0001$), while the CG genotype was associated with decreased BC risk (adjusted OR = 0.31; 95% CI, 0.14-0.69; $P < .0001$) in the codominant model. With the application of a more stringent *P* value ($P < .002$), 5 out of the 6 SNPs were still associated with BC risk in at least one model, while VDR BsmI-rs1544410 lost statistical significance (overdominant AG vs. GG+AA, adjusted OR = 2.21; 95% CI, 1.13-4.31; $P = .018$).

SNP-SNP Interactions and BC Risk

We analyzed gene–gene interaction using multiple logistic regression models (Table 4). An interaction of RANKL-rs9533156 carrying at least one T allele (TC genotype) with other SNPs was

Table 3 Association of Studied SNPs With Breast Cancer Risk in Study Population

Allele	Control (N = 120)		Breast Cancer (N = 115)		OR (95% CI)	*P*
	N	%	N	%		
RANKL-rs9533156 (T > C)						
T	127	0.53	164	0.71	1.00 (reference)	
C	113	0.47	66	0.29	0.45 (0.31-0.66)	< .0001*
OPG-rs2073618 (G > C)						
G	176	0.73	108	0.47	1.00 (reference)	
C	64	0.27	122	0.53	1.99 (1.38-2.88)	.0002*
OPG-rs2073617 (T > C)						
T	123	0.51	174	0.76	1.00 (reference)	
C	117	0.49	56	0.24	0.34 (0.23-0.50)	< .0001*
CHI3L1-rs4950928 (C > G)						
C	191	0.8	186	0.81	1.00 (reference)	
G	49	0.2	44	0.19	0.92 (0.58-1.45)	.73
VDR FokI-rs2228570 (C/T = F/f)						
T	145	0.6	179	0.78	1.00 (reference)	
C	95	0.4	51	0.22	0.43 (0.28-0.65)	< .0001*
VDR BsmI-rs1544410 (A/G = B/b)						
G	136	0.57	99	0.43	1.00 (reference)	
A	104	0.43	131	0.57	1.72 (1.2-2.5)	0.004*

In VDR FokI and BsmI, underlining denotes minor allele in control group.
Abbreviations: CHI3L1 = chitinase-3–like protein 1; CI = confidence interval; OPG = osteoprotegerin; OR = odds ratio; RANKL = receptor activator of nuclear factor κB ligand; SNP = single nucleotide polymorphism; VDR = vitamin D receptor.
*Statistically significant.

observed. The presence of RANKL-rs9533156 with OPG-rs2073618 GC or CC alleles were associated with increased BC risk; the strongest interaction was rs9533156 TT genotype with rs2073618 GC (adjusted OR = 11.5; 95% CI, 2.47-5.52, $P_{Bonferroni}$ = 0.001). Conversely, the combination of RANKL-rs9533156 TC genotype with OPG-rs2073617 CC (adjusted OR = 0.02; 95% CI, 0.00-0.1, $P_{Bonferroni}$ = 0.001), CHI3L1-rs4950928 CG (adjusted OR = 0.11; 95% CI, 0.03-0.35, $P_{Bonferroni}$ = 0.001), and VDR FokI-rs2228570 CC (adjusted OR = 0.12; 95% CI, 0.03-0.56, $P_{Bonferroni}$ = 0.003) was strongly associated with decreased BC risk.

Furthermore, the presence of OPG-rs2073618 GG and OPG-rs2073617 CC was protective (adjusted OR = 0.08; 95% CI, 0.01-0.48, $P_{Bonferroni}$ = 0.03). In contrast, combinations of OPG-rs2073618 GC with CHI3L1-rs4950928 GG (adjusted OR = 33.33; 95% CI, 2.98-377, $P_{Bonferroni}$ = 0.001) or VDR BsmI-rs1544410 AG (adjusted OR = 12.79; 95% CI, 3.41-47.95, $P_{Bonferroni}$ = 0.001) and OPG-rs2073618 CC with CHI3L1-rs4950928 CC (adjusted OR = 15.13; 95% CI, 3.82-61.35, $P_{Bonferroni}$ = 0.001), VDR FokI-rs2228570 TT (adjusted OR = 46.08; 95% CI, 4.68-453.8, $P_{Bonferroni}$ = 0.001), and VDR BsmI-rs1544410 AG (adjusted OR = 26.89; 95% CI, 3.57-202.4, $P_{Bonferroni}$ = 0.001) or AA (adjusted OR = 19.7; 95% CI, 2.19-166.08, $P_{Bonferroni}$ = 0.001) were strongly associated with BC risk.

There was a strong association of OPG-rs2073617 TT and VDR BsmI-rs1544410 AG (adjusted OR = 5.29; 95% CI, 1.41-24.77, $P_{Bonferroni}$ = 0.03) with increased BC risk. On the other hand, the presence of OPG-rs2073617 TC with CHI3L1-rs4950928 CG (adjusted OR = 0.09; 95% CI, 0.03-0.28, $P_{Bonferroni}$ = 0.001) and VDR FokI-rs2228570 CT (adjusted OR = 0.07; 95% CI, 0.02-0.22, $P_{Bonferroni}$ = 0.003) or OPG-rs2073617 CC with CHI3L1-rs4950928 CC (adjusted OR = 0.1; 95% CI, 0.03-0.37, $P_{Bonferroni}$ = 0.001) and VDR FokI-rs2228570 CT (adjusted OR = 0.03; 95% CI, 0.01-0.13, $P_{Bonferroni}$ = 0.001) was strongly protective against BC risk. Moreover, the interaction of CHI3L1-rs4950928 CC with VDR FokI-rs2228570 CT (adjusted OR = 0.11; 95% CI, 0.04-0.29, $P_{Bonferroni}$ = 0.001) and CC (adjusted OR = 0.07; 95% CI, 0.01-0.34, $P_{Bonferroni}$ = 0.001) genotypes, CHI3L1-rs4950928 CG with VDR FokI-rs2228570 TT (adjusted OR = 0.12; 95% CI, 0.03-0.41, $P_{Bonferroni}$ = 0.006) and CT (adjusted OR = 0.06; 95% CI, 0.02-0.22, $P_{Bonferroni}$ = 0.001) genotypes, and VDR BsmI-rs1544410 GG (adjusted OR = 0.09; 95% CI, 0.02-0.45, $P_{Bonferroni}$ = 0.045) was a decreased risk factor of BC susceptibility. The interaction of VDR FokI-rs2228570 TT with VDR BsmI-rs1544410 AG (adjusted OR = 4.79; 95% CI, 1.43-16.03, $P_{Bonferroni}$ = 0.03) was a candidate risk factor of BC risk.

Results of LD Analysis

We carried out pairwise LD analysis for all studied SNPs (Supplemental Figure 1 in the online version). LD is the nonrandom association of haplotypes of 2 loci at the same chromosome or even at different chromosomes.[39] No SNPs were in strong LD ($D' > 0.7$, $r^2 > 0.25$) in our patients; however, the most significant weak LDs existed between RANKL-rs9533156 and VDR FokI-rs2228570 (D' = 0.22, r^2 = 0.036; P = .00006), and

Table 4 Gene–Gene Interactions Between Studied SNPs in BC

SNP-SNP Interaction		Combination		Frequency, N (%)		Adjusted OR (95% CI)	P^a	P^b
		Genotype	Genotype	Control	BC			
RANKL	OPG	TT	GG	16 (13.33)	7 (6.08)	1.00 (reference)		
Rs9533156	rs2073618		GC	5 (4.16)	28 (24.34)	11.5 (2.47-53.52)	< 0.0001	0.001*
			CC	3 (2.5)	14 (12.17)	8.56 (1.35-54.48)	0.001	0.015*
		TC	GG	43 (35.8)	16 (13.91)	0.79 (0.21-2.95)	0.79	
			GC	32 (26.6)	34 (29.56)	2.7 (0.75-9.74)	0.14	
			CC	4 (3.33)	16 (13.91)	10.21 (1.87-51.71)	0.002	0.03*
	OPG	TT	TT	4 (3.33)	22 (19.13)	1.00 (reference)		
	rs2073617		TC	20 (16.67)	24 (20.87)	0.12 (0.03-0.5)	0.017	0.255
		TC	TT	25 (20.83)	43 (37.4)	0.25 (0.07-0.96)	0.049	0.739
			TC	24 (20)	20 (17.4)	0.11 (0.03-0.43)	0.002	0.03*
			CC	30 (25)	3 (2.6)	0.02 (0.00-0.1)	< 0.0001	0.001*
	CHI3L1	TT	CC	13 (10.83)	37 (32.17)	1.00 (reference)		
	rs4950928		CG	11 (9.17)	9 (7.83)	0.3 (0.07-1.21)	0.016	0.24
		TC	CC	49 (40.8)	46 (40)	0.35 (0.15-0.85)	0.002	0.03*
			CG	28 (28.33)	11 (9.57)	0.11 (0.03-0.35)	< 0.0001	0.001*
			GG	2 (1.67)	9 (7.83)	3.38 (0.57-20.06)	0.71	
	VDR FokI	TT	TT	13 (10.83)	33 (28.7)	1.00 (reference)		
	rs2228570		CT	10 (8.33)	11 (9.56)	0.37 (0.1-1.34)	0.16	
			CC	1 (0.83)	5 (4.34)	1.06 (0.08-14.63)	1	
		TC	TT	26 (21.67)	40 (34.78)	0.67 (0.25-1.76)	0.22	
			CT	38 (31.67)	22 (19.13)	0.19 (0.07-0.51)	0.0004	0.006*
			CC	15 (12.5)	4 (3.48)	0.12 (0.03-0.56)	0.0002	0.003*
	VDRBsmI	TT	GG	12 (10)	13 (11.3)	1.00 (reference)		
	rs1544410		AG	7 (5.83)	17 (14.78)	1.66 (0.43-6.38)	0.24	
			AA	5 (4.16)	19 (16.5)	1.31 (0.28-6.08)	0.07	
		TC	GG	32 (26.67)	13 (11.3)	0.29 (0.09-0.93)	0.045	0.675
			AG	24 (20)	30 (25.22)	0.99 (0.33-2.9)	1	
			AA	23 (19.16)	23 (20)	0.49 (0.16-1.57)	1	
OPG	OPG	GG	TT	15 (12.5)	14 (12.17)	1.00 (reference)	-	
rs2073618	rs2073617		TC	29 (24.16)	7 (6.08)	0.15 (0.04-0.6)	0.01	0.15
			CC	22 (18.33)	2 (1.73)	0.08 (0.01-0.48)	0.002	0.03*
		GC	TT	18 (15)	39 (33.91)	2.32 (0.71-7.54)	0.09	
			TC	20 (16.67)	21 (18.26)	0.87 (0.27-2.84)	1	
			CC	6 (5)	2 (1.73)	0.95 (0.09-4.85)	0.38	
		CC	TC	8 (6.67)	16 (13.91)	1.51 (0.35-6.54)	0.26	
			CC	2 (1.67)	2 (1.73)	1.37 (0.11-16.66)	1	
	CHI3L1	GG	CC	42 (35)	17 (14.78)	1.00 (reference)		
	rs4950928		CG	24 (20)	2 (1.73)	0.17 (0.03-1.01)	0.052	
		GC	CC	27 (22.5)	44 (38.26)	5.52 (2.11-14.42)	0.001	0.015*
			CG	16 (13.33)	13 (11.3)	1.88 (0.57-6.17)		
			GG	1 (0.83)	5 (4.35)	33.35 (2.98-377)	< 0.0001	0.001*
		CC	CC	4 (3.33)	22 (19.13)	15.13 (3.82-61.35)	< 0.0001	0.001*
			CG	5 (4.16)	5 (4.35)	3.95 (0.52-29.96)		
			GG	1 (0.83)	3 (2.6)	2.82 (0.07-117.89)	0.085	
	VDR FokI	GG	TT	24 (19.16)	11 (9.56)	1.00 (reference)		
	rs2228570		CT	29 (24.16)	11 (9.56)	0.65 (0.19-2.22)	0.73	
			CC	13 (10.8)	1 (0.87)	0.18 (0.02-2.07)	0.08	
		GC	TT	17 (14.16)	39 (33.91)	6.22 (1.98-19.5)	0.0009	0.013*
			CT	23 (19.16)	16 (13.91)	1.34 (0.4-4.48)	0.47	
			CC	4 (3.33)	7 (6.08)	3.47 (0.53-22.93)	0.08	
		CC	TT	1 (0.83)	23 (20)	46.08 (4.68-453.8)	< 0.0001	0.001*

Table 4 Continued

SNP-SNP Interaction		Combination		Frequency, N (%)		Adjusted OR (95% CI)	P^a	P^b
		Genotype	Genotype	Control	BC			
			CT	9 (7.5)	6 (5.21)	1.53 (0.31-7.49)	0.72	
	VDRBsmI	GG	GG	25 (20.83)	7 (6.08)	1.00 (reference)		
	rs1544410		AG	23 (19.16)	9 (7.82)	1.38 (0.36-5.27)	0.77	
			AA	18 (15)	7 (6.08)	0.77 (0.17-3.54)	0.75	
		GC	GG	19 (15.83)	13 (11.3)	2.82 (0.76-10.4)	0.16	
			AG	11 (9.16)	28 (24.35)	12.79 (3.41-47.95)	< 0.0001	0.001*
			AA	14 (11.66)	21 (18.26)	3.95 (1.03-15.18)	0.048	0.72
		CC	GG	6 (5)	6 (5.21)	4.7 (0.87-25.43)	0.13	
			AG	2 (1.66)	10 (8.7)	26.89 (3.57-202.4)	< 0.0001	0.001*
			AA	2 (1.66)	14 (12.17)	19.7 (2.19-166.08)	< 0.0001	0.001*
OPG	CHI3L1	TT	CC	22 (18.33)	46 (40)	1.00 (reference)		
rs2073617	rs4950928		CG	10 (8.33)	12 (10.45)	0.45 (0.12-1.67)	0.3	
			GG	1 (0.83)	7 (6.08)	4.68 (0.46-48.01)	0.45	
		TC	CC	31 (25.8)	32 (27.83)	0.35 (0.14-0.85)	0.045	0.675
			CG	25 (20.83)	8 (6.97)	0.09 (0.03-0.28)	< 0.0001	0.001*
			GG	1 (0.83)	4 (3.48)	2.52 (0.2-31.16)	1	
		CC	CC	20 (16.66)	5 (4.35)	0.1 (0.03-0.37)	< 0.0001	0.001*
	VDR FokI	TT	TT	11 (9.17)	41 (35.65)	1.00 (reference)		
	rs2228570		CT	14 (11.66)	19 (16.52)	0.22 (0.06-0.76)	0.007	0.105
			CC	8 (6.66)	5 (4.35)	0.11 (0.02-0.58)	0.014	0.21
		TC	TT	27 (22.5)	29 (25.22)	0.17 (0.06-0.47)	0.005	0.075
			CT	26 (21.66)	11 (9.57)	0.07 (0.02-0.22)	< 0.0001	0.001*
			CC	4 (3.33)	4 (3.48)	0.11 (0.01-0.79)	0.01	0.15
		CC	TT	4 (3.33)	3 (2.6)	0.26 (0.04-1.9)	0.065	
			CT	21 (17.5)	3 (2.6)	0.03 (0.01-0.13)	< 0.0001	0.001*
	VDRBsmI	TT	GG	14 (11.67)	13 (11.3)	1.00 (reference)		
	rs1544410		AG	5 (4.16)	29 (25.22)	5.29 (1.41-24.77)	0.002	0.03*
			AA	14 (11.66)	23 (20)	0.73 (0.2-2.68)	0.3	
		TC	GG	25 (20.83)	11 (9.57)	0.28 (0.08-0.99)	0.048	0.72
			AG	18 (15)	16 (13.91)	0.62 (0.18-2.12)	0.98	
			AA	14 (11.67)	17 (14.78)	0.39 (0.1-1.49)	0.75	
		CC	GG	11 (9.16)	2 (1.73)	0.15 (0.02-0.97)	0.045	0.72
			AG	13 (10.83)	2 (1.73)	0.12 (0.02-0.86)	0.042	0.63
			AA	6 (5)	2 (1.73)	0.3 (0.04-2.34)	0.39	
CHI3L1	VDR FokI	CC	TT	21 (17.5)	58 (50.43)	1.00 (reference)		
rs4950928	rs2228570		CT	40 (33.33)	20 (17.39)	0.11 (0.04-0.29)	< 0.0001	0.001*
			CC	12 (10)	5 (4.35)	0.07 (0.01-0.34)	< 0.0001	0.001*
		CG	TT	20 (16.66)	11 (9.57)	0.12 (0.03-0.41)	0.0004	0.006*
			CT	20 (16.66)	7 (6.08)	0.06 (0.02-0.22)	< 0.0001	0.001*
			CC	5 (4.16)	2 (1.73)	0.16 (0.02-1.51)	0.057	
		GG	TT	1 (0.83)	4 (3.48)	1.24 (0.09-17.29)	1	
			CT	1 (0.83)	6 (5.22)	3.67 (0.37-36.68)	0.67	
	VDRBsmI	CC	GG	26 (21.66)	20 (17.39)	1.00 (reference)		
	rs1544410		AG	24 (20)	33 (28.7)	1.38 (0.54-3.54)	0.16	
			AA	23 (19.16)	30 (26.08)	0.72 (0.26-1.97)	0.2	
		CG	GG	22 (18.33)	3 (2.6)	0.09 (0.02-0.45)	0.003	0.045*
			AG	12 (10)	10 (8.7)	0.84 (0.24-2.96)	1	
			AA	11 (9.16)	7 (6.08)	0.31 (0.06-1.51)	0.78	
		GG	GG	2 (1.67)	3 (2.6)	2.17 (0.23-20.23)	0.64	
VDR FokI	VDRBsmI	TT	GG	22 (18.33)	18 (15.65)	1.00 (reference)		

Table 4 Continued

SNP-SNP Interaction		Combination		Frequency, N (%)		Adjusted OR (95% CI)	P^a	P^b
		Genotype	Genotype	Control	BC			
rs2228570	rs1544410		AG	9 (7.5)	25 (21.74)	4.79 (1.43-16.03)	0.002	0.03*
			AA	11 (9.16)	30 (26.08)	2.63 (0.79-6.77)	0.05	
		CT	GG	21 (17.5)	7 (6.08)	0.33 (0.09-1.24)	0.12	
			AG	25 (20.83)	22 (19.13)	0.91 (0.33-2.53)	0.9	
			AA	15 (12.5)	4 (3.48)	0.1 (0.02-0.55)	0.03	0.45
		CC	GG	7 (5.83)	1 (0.87)	0.39 (0.04-3.85)	0.12	
			AA	8 (6.66)	8 (6.95)	0.58 (0.13-2.58)	0.75	

Combinations with zero frequencies in either group were omitted.
Abbreviations: BC = breast cancer; CI = confidence interval; CHI3L1 = chitinase-3—like protein 1; OPG = osteoprotegerin; OR = odds ratio; RANKL = receptor activator of nuclear factor κB ligand; SNP = single nucleotide polymorphism; VDR = vitamin D receptor.
[a]Adjusted for age and family history in logistic regression model.
[b]Bonferroni-corrected *P*.
*Statistically significant ($P < .05$).

OPG-rs2073618 and VDR FokI-rs2228570 ($D' = 0.3$, $r^2 = 0.026$; $P = .0004$). However, they were still significant after application of $P_{Bonferroni}$.

Haplotype Analysis and BC Risk

Haplotype analysis of all the 6 polymorphisms revealed 45 different combinations, of which only 20 haplotype combinations were present in both controls and cases. Of these, only 4 haplotypes were of pooled frequencies of cases and controls > 0.05 (Table 5). Testing the association of these 4 haplotypes with BC risk revealed that the TCTCTG-rs9533156-rs2073618-rs2073617-rs4950928-rs2228570-rs1544410 haplotype was associated with increased BC risk (adjusted OR = 8.33; 95% CI, 1.32-52.46; $P = .025$) compared to controls in the recessive model with adjustment for age and family history.

Interaction of Haplotypes With BC Pathologic Data

We stratified BC patients into different disease subtypes (positive vs. negative ER/PR status, bone metastatic vs. nonmetastatic, and large vs. small tumor size); their causes and risk factors are therefore different. A separate analysis of the association of haplotypes with these pathologic data was performed using multiple regression models controlling for age and family history (Table 6). Analysis revealed that the TCCCTG haplotype was inversely correlated with positive ER/PR (adjusted OR = 0.05; 95% CI, 0.01-0.71; $P = .029$) in the recessive model. TCTCTA was positively associated with bone metastasis (adjusted OR = 7.37; 95% CI, 2.9-18.6; $P = .01$) in the dominant model. Conversely, TCTCTG (adjusted OR = 0.03; 95% CI, 0.004-0.24; $P < .0001$) and TGTCTG (adjusted OR = 0.05; 95% CI, 0.007-0.48; $P < .0001$) were inversely correlated with metastasis in the recessive model. No haplotype was significantly correlated with tumor size ($P > .05$).

While the TCTCTA was a risk factor for metastasis, TCTCTG was unlikely. The difference is in the presence of G/A alleles of VDR BsmI-rs1544410. We further analyzed the influence of this SNP on metastasis and found that the protective VDR BsmI-rs1544410 G was more prevalent in the nonmetastatic group (53% vs. 17%; $P < .0001$), with GG+AG versus AA (adjusted OR = 0.14; 95% CI, 0.05-0.4; $P < .0001$) negatively associated with metastasis. Interestingly, the CG CHI3L1-rs4950928—VDR BsmI-rs1544410 diplotype was associated with decreased risk of metastasis in BC patients (CG vs. CA diplotype, adjusted OR = 0.148; 95% CI, 0.05-0.42; $P < .0001$).

Results of Functional Annotation Analysis

Selected common biological processes and pathways for the studied genes are presented in Table 1. Investigating protein interaction with the selected gens revealed 13 proteins commonly interacting with our 4-gene list, of which signal transducer and activator of transcription (STAT) 5B, STAT, STAT1, SRY, BACH2, and IK3 revealed statistically significant interactions ($P < .05$) (Table 7).

Discussion

Recent reports have focused on SNPs and their epistatic interactions to explore the unexplained genetic susceptibility of BC risk.[10,11] To our knowledge, this is the first study investigating the association of SNP-SNP interactions with BC predisposition in Egyptian women. In this explanatory study, we identified multiple SNP-SNP interactions between *RANKL, OPG, CHI3L1,* and *VDR* genes that were strongly associated with BC susceptibility and contributed to an overall higher risk than individual SNPs, suggesting the role of SNP-SNP interactions between these genes in BC development. Analysis of the combined genotypes of studied genes revealed a significant increase in BC risk, with increasing numbers of high-risk alleles. We demonstrated that the TCTCTG haplotype, which contains 4 high-risk alleles of RANKL-rs9533156, OPG-rs2073618, OPG-rs2073617, and VDR FokI-rs2228570 was significantly associated with an 8-fold higher risk of BC compared to controls. It is noteworthy that all studied SNPs had weak single-locus effects on BC susceptibility in our study and were candidates for SNP-SNP interaction analysis. In addition, all studied SNPs were functional and were observed or predicted to affect gene expression level or structure, and thus secretion or function, of their corresponding proteins.[30-36]

We further carried out functional annotation analysis to explore possible biological insights into the observed SNP-SNP interactions. We observed that the SNP-SNP interactions in the studied genes

Table 5 Association of Haplotypes With Breast Cancer Risk

Haplotype						Total Frequency	Frequency in Control Subjects	Frequency in Breast Cancer Patients	Adjusted OR (95% CI)	*P*
SNP1	SNP2	SNP3	SNP4	SNP5	SNP6					
T[a]	G	T[a]	C	T[a]	A[a]	0.0849	0.0169	0.0693	1.00	—
T[a]	G	T[a]	C	T[a]	G	0.0721	0.0505	0.07	1.23 (0.25-6.16)	.8
T[a]	C[a]	T[a]	C	T[a]	G	0.067	0.0343	0.1039	8.33 (1.32-52.46)	.025*
C	C[a]	T[a]	C	T[a]	A[a]	0.0638	0.0058	0.0732	3.58 (0.60-21.36)	.16

Adjusted by age and family history in multiple logistic regression by SNPStats online software using recessive model.
Abbreviations: CHI3L1 = chitinase-3–like protein 1; CI = confidence interval; OR = odds ratio; SNP = single nucleotide polymorphism; SNP1 = RANKL-rs9533156 (T/C); SNP2 = OPG-rs2073618 (G/C); SNP3 = OPG-rs2073617 (T/C); SNP4 = CHI3L1-rs4950928 (C/G); SNP5 = VDR FokI-rs2228570 (C/T); SNP6 = VDR BsmI-rs1544410 (A/G).
Global haplotype association; $P < .0001$.
[a]Risk alleles.
*Statistically significant ($P < .05$).

were complemented by common involvement in biological processes, including immune response, regulation of mitogen-activated protein kinase (MAPK), protein kinase B signaling, tumor necrosis factor (TNF) signaling, cytokine–cytokine receptor interaction, angiogenesis, and apoptosis (Table 1); observed/predicted direct or indirect interactions among proteins encoded by participating genes or with other proteins linked to carcinogenesis; and protein/transcription factor–gene interactions. Our bioinformatic analysis of protein interactions revealed 6 significant predicted proteins/transcription factors (STAT5B, STAT, STAT1, SRY, BACH2, and IK3) that were in common interaction with *RANKL, OPG, CHI3L1,* and *VDR* genes; the most significant was STAT5B. STAT5 signaling mediated mesenchymal phenotypes of basal-type BC cells.[40] PR and STAT5 signaling cross-talk through RANKL in mammary epithelial cells.[41] OPG interacts with and inhibits TRAIL; TRAIL induces RANKL expression through a STAT6-dependent transcriptional regulatory mechanism in bone marrow stromal/preosteoblast cells.[42] The robust cytokine-driven expression of YKL-40 requires both STAT3 and nuclear factor kappa–light-chain enhancer of activated B cells (NF-κB; RelB/p50 complex) binding elements of the *CHI3L1* promoter.[43] RANKL/RANK binding activates NF-κB, and downstream pathways and can activate a noncanonical NF-κB pathway with translocation of RelB/52 heterodimers to the nucleus[44] and thus could induce YKL-40 expression. The $1,25(OH)_2D_3$/VDR signaling regulates the expression of RANKL and OPG.[45] The $1,25(OH)_2D_3$ downregulated RANKL expression and inhibited osteoclastogenesis through inhibiting JAK2/STAT3 and p38 MAPK/NF-κB signalling.[46] The $1,25(OH)_2D_3$ through VDR also suppressed RANKL expression in Th17 cells via suppressing NF-κBp65 nuclear translocation.[47] Vitamin D/VDR signaling may reprogram T cells to decrease essential STAT activation and proinflammatory cytokine output.[48] Together, these findings explore that the impact of the observed SNP-SNP interactions on BC susceptibility could be through direct or indirect cross-talk between their encoded proteins through a complex interaction network that could affect the tumor microenvironment.

More specifically, our study revealed that the epistatic interaction between RANKL-rs9533156 and OPG-rs2073618 carrying one or two C risk alleles was a candidate risk factor for BC. These results may in part be attributed to a functionally altered RANKL/OPG ratio by the combined genotypes, with a high RANKL/OPG ratio supposedly predisposing to BC risk as well as BC-associated bone metastasis.[49-51] Carriers of the OPG-rs2073618 CC genotype were more likely to have a lower OPG level and a higher RANKL/OPG ratio than carriers of the GG genotype.[30,49] Similarly, a trend of elevation of serum OPG level in OPG-rs2073618 GG genotype than GC or CC genotypes, and a slightly higher serum RANKL in RANKL-rs9533156 TT than TC genotype carriers was observed in Egyptian women with BC; however, results were statistically nonsignificant.[23] Although the exact mechanism of RANKL-rs9533156 is not known, carriers of a nearby RANKL-rs7984870 promoter SNP was associated with higher RANKL level as well as higher RANKL/OPG ratio.[49]

In contrast to OPG-rs2073618, the presence of RANKL-rs9533156 heterozygosity with OPG-rs2073617 carrying one or two nonrisk C alleles was protective against BC risk in our study.

Table 6 Correlation of Haplotypes With ER/PR Status, Bone Metastasis, and Tumor Size in Breast Cancer Group

Haplotype						Total Frequency	Frequency		Adjusted OR (95% CI)	P[a]	Global Association P[a]
SNP1	SNP2	SNP3	SNP4	SNP5	SNP6		Negative ER/PR (N = 79)	Positive ER/PR (N = 21)			
T	C	T	C	T	A	0.1045	0.046	0.0955	1.00	—	.066
T	C	T	C	T	G	0.1039	0.0639	0.1549	2 (0.39-10)	.4	
C	C	T	C	T	A	0.0732	0.0774	0.1105	1.66 (0.26-10.6)	.59	
T	G	T	C	T	G	0.07	0.0756	0.0896	0.6 (0.11-3.28)	.55	
T	G	T	C	T	A	0.0693	0.1082	0.1243	0.78 (0.08-7.38)	.83	
C	G	T	C	T	A	0.0576	0.0268	0.0508	1.3 (0.39-7.3)	.7	
T	C	C	C	T	G	0.052	0.065	0.0080	0.05 (0.01-0.71)	.029	
							Without Metastasis (N = 83)	**With Metastasis (N = 32)**			
T	C	T	C	T	A	0.1045	0.0625	0.3211	1.00	—	< .0001*
T	C	T	C	T	G	0.1039	0.13680	0.0052	0.03 (0.004-0.24)	< .0001	
C	C	T	C	T	A	0.0732	0.0307	0.0688	4.35 (0.29-10)	.29	
T	G	T	C	T	G	0.07	0.0731	0.005	0.05 (0.007-0.48)	< .0001	
T	G	T	C	T	A	0.0693	0.1057	0.0205	0.1 (0.01-7.69)	.29	
C	G	T	C	T	A	0.0576	0.0058	0.1223	6.66 (0.6-100)	.13	
							Tumor Size < 5 cm (N = 86)	**Tumor Size > 5 cm (N = 29)**			
T	C	T	C	T	A	0.1045	0.0655	0.1776	1.00	—	.15
T	C	T	C	T	G	0.1039	0.1386	0	—	—	
C	C	T	C	T	A	0.0732	0.0895	0.0211	0.57 (0.03-9.86)	.7	
T	G	T	C	T	G	0.07	0.0592	0.1066	3.24 (0.26-39.7)	.36	
T	G	T	C	T	A	0.0693	0.0968	0.0219	0.41 (0.03-5.62)	.51	
C	G	T	C	T	A	0.0576	0.0812	0.0323	0.74 (0.09-5.99)	.78	
T	C	C	C	T	G	0.052	0.0654	0.0518	0.87 (0.07-10.5)	.92	

Abbreviations: CI = confidence interval; CHI3L1 = chitinase-3–like protein 1; ER = estrogen receptor; OR = odds ratio; PR = progesterone receptor; SNP1 = RANKL-rs9533156 (T/C); SNP2 = OPG-rs2073618 (G/C); SNP3 = OPG-rs2073617 (T/C); SNP4 = CHI3L1-rs4950928 (C/G); SNP5 = VDR FokI-rs2228570 (C/T); SNP6 = VDR BsmI-rs1544410 (A/G).

[a]Adjusted by age and family history in logistic regression model in SNPStats online software using recessive model.

*Statistically significant ($P < .05$).

SNP-SNP Interactions

Table 7 Protein Interaction With 4 Studied Genes (*RANKL, OPG, VDR,* and *CHI3L1*)

Category	Protein	Interaction With Genes				
		Genes	Count	%	*P*-Value	Benjamini
UCSC_TFBS	STAT5B	[redacted]	4	100.0	1.9E-2	9.6E-1
UCSC_TFBS	STAT	[redacted]	4	100.0	2.3E-2	8.5E-1
UCSC_TFBS	STAT1	[redacted]	4	100.0	3.5E-2	8.5E-1
UCSC_TFBS	SRY	[redacted]	4	100.0	4.0E-2	8.0E-1
UCSC_TFBS	BACH2	[redacted]	4	100.0	4.4E-2	7.6E-1
UCSC_TFBS	IK3	[redacted]	4	100.0	4.8E-2	7.3E-1
UCSC_TFBS	ROAZ	[redacted]	4	100.0	5.4E-2	7.1E-1
UCSC_TFBS	HAND1E47	[redacted]	4	100.0	5.6E-2	6.8E-1
UCSC_TFBS	RSRFC4	[redacted]	4	100.0	5.9E-2	6.6E-1
UCSC_TFBS	GRE	[redacted]	4	100.0	6.0E-2	6.3E-1
UCSC_TFBS	ER	[redacted]	4	100.0	7.6E-2	6.8E-1
UCSC_TFBS	RP58	[redacted]	4	100.0	8.0E-2	6.7E-1
UCSC_TFBS	TATA	[redacted]	4	100.0	9.4E-2	7.0E-1

Analysis was performed by David Bioinformatics Resources 6.8, National Institute of Allergy and Infectious Diseases/National Institutes of Health via Gene ID Conversion Tool. List of 4 studied genes was introduced, and protein interaction option by UCSC_TFBS genome browser was chosen.

Notably, the C allele of OPG-rs2073617 was associated with decreased risk of advanced prostate cancer.[52] A possible explanation is that rs2073617 SNP is associated with an altered secondary structure, which may cause differences in OPG messenger RNA expression.[31] However, the OPG-rs2073617 T/C alleles had no effect on serum OPG in prostate cancer.[52] Thus, the mechanism of the protective effect of OPG-rs2073617 should be further investigated.

Reports about the association of *CHI3L1* polymorphisms with BC were few. We found significant association between CHI3L1-rs4950928 and BC, where the minor homozygote GG genotype was associated with increased BC risk, whereas the CG heterozygote was protective. In contrast, CHI3L1-rs4950928 was not a significant predisposing factor of BC in a large case–control study.[25] Furthermore, no association was found for CHI3L1-rs4950928 with glioblastoma,[53] uterine cancer,[54] or hepatocellular carcinoma.[55] On the other hand, the C allele of CHI3L1-rs4950928 was significantly higher in patients with rectal cancer than those with colon cancer in an Egyptian population.[33] These discordant results may be attributable to different tumors, sample sizes, and populations studied.

We observed significant interactions between RANKL-rs9533156 and *OPG* SNPs with CHI3L1-rs4950928 in relation to BC risk. These interactions are consistent with the link between these genes and diseases characterized by inflammation and tissue remodeling. High serum YKL-40 level is a nonspecific inflammatory marker and was associated with poor prognosis in BC.[56,57] Genetic variants of *CHI3L1* and *RANK/RANKL* genes were associated with inflammatory diseases such as rheumatoid arthritis.[58,59] Genetic variants in the OPG locus have been implicated in ankylosing spondylitis.[60] RANKL might induce CHI3L1 indirectly through NF-κB signaling.[43,44] A cross-talk between OPG and YKL-40 could be through enhancing angiogenesis; both stimulate endothelial cell survival and tube formation by inducing ERK1/2 and Akt phosphorylation.[17,61]

We demonstrated association of VDR FokI and BsmI polymorphisms with BC predisposition, with the minor BsmI A (B) allele and AG genotype associated with increased BC risk whereas the minor FokI C (F) allele and the presence of CT+CC genotypes were protective in our studied population. Other genetic variants in VDR, ApaI and TaqI, also conferred high BC susceptibility, particularly in Egyptian women.[62] Together, these results support the notion that breast carcinogenesis may be affected by vitamin D/VDR signaling. Previous reports demonstrated inconsistent results about association of FokI and BsmI with BC risk among different populations.[22,26-29] This variability among different studies was suggested to be due to different proportions of patients with a family history of BC.[26,29]

The observed interaction of *VDR* polymorphisms and *RANKL/OPG* SNPs could be explained by the idea that vitamin D through VDR regulates *RANKL* and *OPG* gene expression.[45-47] A direct relationship between *VDR* and *RANKL* gene expression has been reported. The $1,25(OH)_2D_3$ activated human RANKL promoter through vitamin D–responsive elements located at distal regions −1584/−1570 by binding VDR and RXR-α heterodimers in a ligand-dependent manner.[63] Furthermore, an interplay between low plasma RANKL and VDR-FokI polymorphism in lumbar disc herniation has also been demonstrated.[64] Higher RANKL and lower plasma OPG levels were observed with the VDR-FokI F allele compared to the f allele and were linked to T-cell activation in multiple sclerosis patients.[65] As VDR and RANKL/RANK/OPG

have identified roles in immune system modulation, interactions between several immune-related genes were demonstrated to be associated with susceptibility to breast invasive ductal carcinoma.[66] Notably, 88.7% of our patients had invasive duct tumors. Together, these results implicate that interaction of certain genotypes of specific functional SNPs could predispose cancer through cross-talk of their proteins.

We carried out a haplotype analysis in BC subgroups to identify haplotypes associated with BC prognosis. Our results revealed that TCCCTG haplotype stratified patients according ER/PR status. TCTCTG and TGTCTG haplotypes were inversely correlated, whereas TCTCTA was positively associated with bone metastasis. Our results indicate that some combinations of these selected SNPs could contribute to BC progression and poor prognosis. In line with our results, the VDR-BsmI polymorphism was linked to BC metastasis, with women carried the minor homozygote had almost a 4 times higher risk of developing metastases than women carrying the major homozygote genotype.[67] CHI3L1-rs4950928 may be a potential candidate for predicting poor hepatocellular carcinoma prognosis and clinical status, where CG+GG genotype carriers among hepatocellular carcinoma patients indicated a greater risk of vascular invasion.[55] The OPG-rs2073618 stratified BC patients into invasive or noninvasive tumors.[24] The OPG-rs2073617 C allele was negatively correlated with metastatic prostate cancer.[52] Although the genotype distributions of the studied RANKL and OPG SNPs were not significantly different between bone metastatic and nonmetastatic BC in an Egyptian study,[23] elevated serum RANKL, OPG, and vitamin D, but not YKL-40, were significantly associated with bone metastasis in BC in Egyptian patients.[68]

Our study improves on previous studies in that we examined the interaction between a number of biologically plausible polymorphisms in different genes that are involved in a wide range of cancer-related processes, not just SNPs in a single gene or in genes acting on a specific pathway. The observed interactions have not been previously reported. Our results also confirm the notion that SNP-SNP interactions could facilitate the understanding of the additional missing heritable components of BC risk, possibly through interplay in several pathways contributing to BC etiology. We think that our interaction results are relevant in BC screening because genotyping a small number of SNPs (using the widely available PCR techniques) and testing for interaction is less cumbersome and easier to interpret than genome-scale methods that disregard such interactions.

We acknowledge the following limitations of our study. First, selection bias was inevitable; 56% of our patients had a family history of BC, which might have enriched the study with a genetic component. This result is higher than the 17% to 22% frequency reported in large case–control studies,[44,69] but it is consistent with the 58% frequency in another Egyptian study.[70] However, we controlled for this by adjusting all results with family history. Also, the majority of our patients had nonmetastatic disease (72%), so association of haplotypes with bone metastasis could be biased. Second, although studied SNPs demonstrated weak single-locus effects on BC susceptibility in our study, results were inconsistent among different populations[23-29] and thus might not be reproducible. Third, we used a statistically oriented approach for epistasis detection; with adjustments for multiple comparisons, the BsmI association did not meet the conservative Bonferroni *P* value and thus might be due to chance. Fourth, we limited our analyses to Egyptian women, which may limit the generalizability of our findings; however, population homogeneity is a study strength by reducing genetic variability. Fifth, our findings are based on a small number of case subjects, and their replication in a larger study is unclear and so should be interpreted with caution. Future large independent studies are warranted to further validate our results. SNP–environment interactions should be also evaluated to comprehensively account for BC risk. Nevertheless, we believe that our findings reflect important, sound biology to clinical medicine rather than simply statistical findings.

In summary, our results suggested that the stronger combined effect of SNPs in the *RANKL, OPG, CHI3L1,* and *VDR* genes via gene–gene interaction may help to predict BC risk and its prognosis. Our data have potential implications in genetic counseling, BC screening, and BC prognosis.

Clinical Practice Points

- Most of the heritability of BC is still unexplained. Gene–gene interactions (epistasis) are assumed to contribute to overall BC risk. Genetic variants of *RANKL, OPG, CHI3L1,* and *VDR* play an overall role in breast carcinogenesis; however, their epistatic effects on BC susceptibility remain unknown.
- We provided the first evidence of an association of multiple SNP-SNP interactions and TCTCTG haplotype of 6 SNPs in *RANKL, OPG, CHI3L1,* and *VDR* with a genetic predisposition of BC. We also demonstrated the association of certain haplotypes with BC prognosis.
- The TCCCTG haplotype was inversely correlated with positive ER/PR. The TCTCTA, TCTCTG, and TGTCTG haplotypes were correlated with bone metastasis.
- Bioinformatic analysis of protein interactions revealed that the 4 genes interact most significantly with STAT5B. Our results suggested that a stronger combined effect of SNPs in the *RANKL, OPG, CHI3L1,* and *VDR* genes via gene–gene interaction may help predict BC risk and prognosis.
- Our work explored the complex biology of BC via gene–gene interaction as well as protein–gene interaction that may shed more light into unexplained heritability of BC. Our work also identified new genetic markers for BC screening and prognosis.
- Our findings reflect important, sound biology relevant to clinical medicine, rather than simply statistical findings, and have potential implications in genetic counseling, BC screening, and BC prognosis.

Disclosure

The authors have stated that they have no conflict of interest.

Supplemental Data

A supplemental figure and tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.clbc.2018.09.004.

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Supplemental Data

Supplemental Table 1 Minor Allele Frequencies of Studied SNPs

Gene	dbSNP	dbSNP Alleles	MAF[a]	Global MAF	Highest Population MAF[b]
TNFSF11 (RANKL)	rs9533156	C/T	C = 0.47	0.458[c] 0.463[d]	0.5
TNFRSF11B (OPG)	rs2073618	C/G	C = 0.27	0.333[c] 0.394[d]	0.5
	rs2073617	C/T	C = 0.49	0.378[c] 0.426[d]	0.5
CHI3L1	rs4950928	A/C/G/T	G = 0.2	0.2145[c] 0.229[d]	0.44
VDR	rs2228570 (FokI)	A/C/G/T	C = 0.4	0.328[c] 0.34[d]	0.48
	rs1544410 (BsmI)	A/G	A = 0.43	0.295[c] 0.334[d]	0.48

Abbreviations: dbSNP = Single Nucleotide Polymorphism Database; CHI3L1 = chitinase-3–like protein 1; MAF = minor allele frequency; OPG = osteoprotegerin; RANKL = receptor activator of nuclear factor κB ligand; SNP = single nucleotide polymorphism.
[a]MAF calculated from control samples.
[b]According to Ensembl GRCh37 release 89, May 2017.
[c]According to 1000 genomes (dbSNP).
[d]Trans-Omics for Precision Medicine (TOPMed).

Supplemental Table 2 Hardy-Weinberg Equilibrium for 6 SNPs in Patients With BC and in Control Subjects

SNP No.	Observed Frequencies			Expected Frequencies			*P*
RANKL-rs9533156 (T > C)	TT	TC	CC	TT	TC	CC	
Controls (n = 120)	20	65.8	14.2	28	50	22	.001*
BC (n = 115)	42.6	57.4	0	50.4	41.2	8.4	< .0001*
OPG-rs2073618 (G > C)	GG	GC	CC	GG	GC	CC	
Controls (n = 120)	55	36.7	8.3	53.3	39.4	7.3	.56
BC (n = 115)	20	54	26	22	55	28	.556
OPG-rs2073617 (T > C)	TT	TC	CC	TT	TC	CC	
Controls (n = 120)	27.5	47.5	25	26	50	24	.61
BC (n = 115)	56.5	38.3	5.2	57.8	36.4	5.8	.66
CHI3L1-rs4950928 (C > G)	CC	CG	GG	CC	CG	GG	
Controls (n = 120)	60.8	37.5	1.7	64	32	4	.11
BC (n = 115)	71.6	17.4	10.4	65.6	30.8	3.6	< .0001*
VDR FokI-rs2228570 (C/T = F/f)	TT	CT	CC	TT	CT	CC	
Controls (n = 120)	35	50.8	14.2	36	48	16	.53
BC (n = 115)	63.5	28.7	7.8	60.8	34.4	4.8	.086
VDR BsmI-rs1544410 (A/G = B/b)	GG	AG	AA	GG	AG	AA	
Controls (n = 120)	41.7	30	38.3	32.5	49	18.5	.00*
BC (n = 115)	22.6	41.9	36.5	18.5	49	32.5	.119

Abbreviations: BC = breast cancer; CHI3L1 = chitinase-3–like protein 1; OPG = osteoprotegerin; RANKL = receptor activator of nuclear factor κB ligand; VDR = vitamin D receptor.

Supplemental Table 3 Genotype Association of Studied SNPs With Breast Cancer Risk

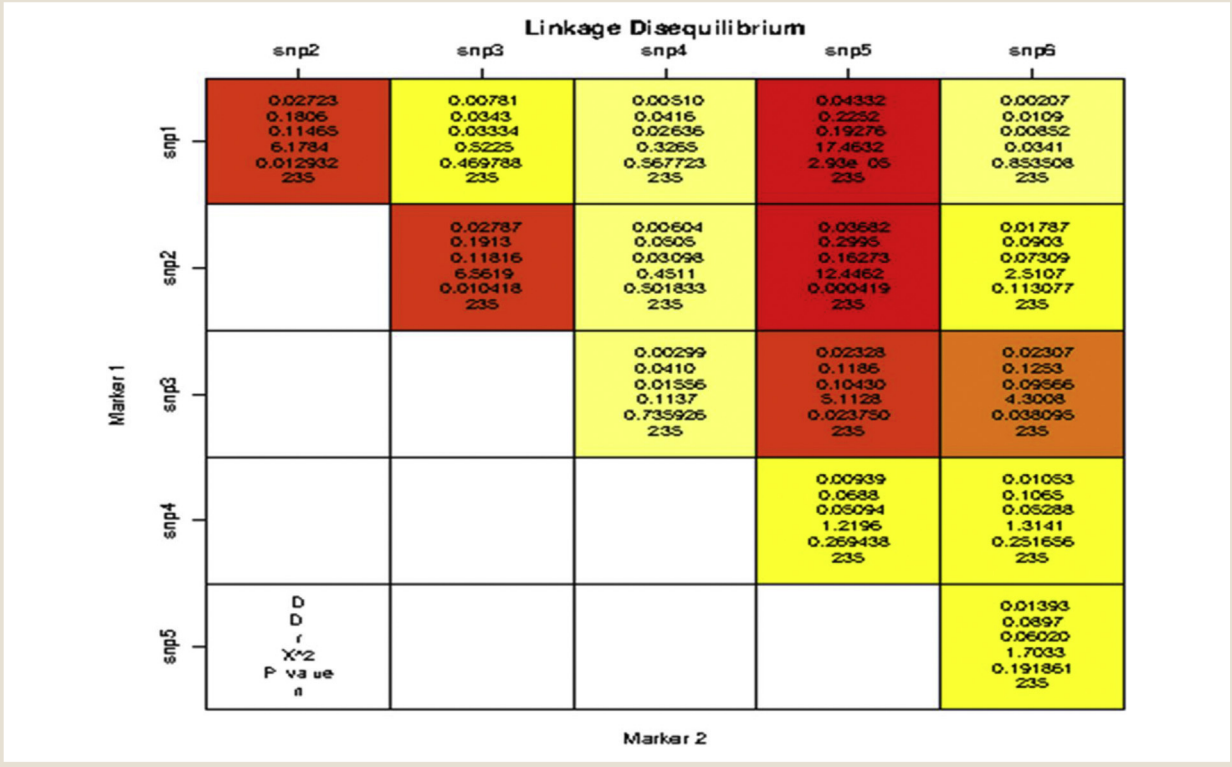
SNP Model	Genotype	Control (N = 120)	BC (N = 115)	Adjusted OR (95% CI)	P[a]	AIC	BIC
RANKL-rs9533156 (T > C)							
Codominant	T/T	24 (20%)	49 (42.6%)	1.00	<.0001*	227.2	244.5
	T/C	79 (65.8%)	66 (57.4%)	0.44 (0.22-0.89)			
	C/C	17 (14.2%)	0 (0%)	—			
Dominant	T/T	24 (20%)	49 (42.6%)	1.00	.0048*	238	251.8
	T/C-C/C	96 (80%)	66 (57.4%)	0.37 (0.18-0.74)			
Recessive	T/T-T/C	103 (85.8%)	115 (100%)	1.00	1e-04*	230.5	244.3
	C/C	17 (14.2%)	0 (0%)	—			
Overdominant	T/T-C/C	41 (34.2%)	49 (42.6%)	1.00	.38	245.2	259
	T/C	79 (65.8%)	66 (57.4%)	0.75 (0.39-1.43)			
Log additive	—	—	—	0.31 (0.17-0.58)	1e-04*	231	244.8
OPG-rs2073618 (G > C)							
Codominant	G/G	66 (55%)	23 (20%)	1.00	<.0001*	221.8	239.1
	G/C	44 (36.7%)	62 (54%)	4.91 (2.28-10.57)			
	C/C	10 (8.3%)	30 (26%)	9.88 (3.35-29.12)			
Dominant	G/G	66 (55%)	23 (20%)	1.00	<.0001*	221.8	235.6
	G/C-C/C	54 (45%)	92 (80%)	5.73 (2.74-11.98)			
Recessive	G/G-G/C	110 (91.7%)	85 (73.9%)	1.00	.0046*	237.9	251.7
	C/C	10 (8.3%)	30 (26.1%)	3.73 (1.46-9.55)			
Overdominant	G/G-C/C	76 (63.3%)	53 (46.1%)	1.00	.0087*	239.1	252.9
	G/C	44 (36.7%)	62 (53.9%)	2.35 (1.23-4.47)			
Log additive	—	—	—	3.48 (2.05-5.89)	<.0001*	221.3	235.2
OPG-rs2073617 (T > C)							
Codominant	T/T	33 (27.5%)	65 (56.5%)	1.00	<.0001*	225.1	242.4
	T/C	57 (47.5%)	44 (38.3%)	0.27 (0.13-0.57)			
	C/C	30 (25%)	6 (5.2%)	0.11 (0.04-0.33)			
Dominant	T/T	33 (27.5%)	65 (56.5%)	1.00	<.0001*	226	239.9
	T/C-C/C	87 (72.5%)	50 (43.5%)	0.22 (0.11-0.44)			
Recessive	T/T-T/C	90 (75%)	109 (94.8%)	1.00	.0017*	236.1	250
	C/C	30 (25%)	6 (5.2%)	0.22 (0.08-0.62)			
Overdominant	T/T-C/C	63 (52.5%)	71 (61.7%)	1.00	.038*	241.6	255.5
	T/C	57 (47.5%)	44 (38.3%)	0.51 (0.27-0.97)			
Log additive	—	—	—	0.31 (0.18-0.52)	<.0001*	223.3	237.2
CHI3L1-rs4950928 (C > G)							
Codominant	C/C	73 (60.8%)	83 (72.2%)	1.00	<.0001*	228	245.3
	C/G	45 (37.5%)	20 (17.4%)	0.31 (0.14-0.69)			
	G/G	2 (1.7%)	12 (10.4%)	8.92 (1.70-46.84)			
Dominant	C/C	73 (60.8%)	83 (72.2%)	1.00	.12	243.5	257.3
	C/G-G/G	47 (39.2%)	32 (27.8%)	0.58 (0.30-1.15)			
Recessive	C/C-C/G	118 (98.3%)	103 (89.6%)	1.00	9e-04*	234.9	248.7
	G/G	2 (1.7%)	12 (10.4%)	11.75 (2.26-60.94)			
Overdominant	C/C-G/G	75 (62.5%)	95 (82.6%)	1.00	7e-04*	234.4	248.2
	C/G	45 (37.5%)	20 (17.4%)	0.28 (0.13-0.60)			
Log additive	—	—	—	1.01 (0.59-1.73)	.97	245.9	259.8
VDR FokI-rs2228570 (C/T = F/f)							
Codominant	T/T	42 (35%)	73 (63.5%)	1.00	1e-04*	229.3	246.6
	C/T	61 (50.8%)	33 (28.7%)	0.24 (0.12-0.49)			
	C/C	17 (14.2%)	9 (7.8%)	0.25 (0.08-0.77)			

Supplemental Table 3 Continued

SNP Model	Genotype	Control (N = 120)	BC (N = 115)	Adjusted OR (95% CI)	P[a]	AIC	BIC
Dominant	T/T	42 (35)	73 (63.5)	1.00	<.0001*	227.3	241.2
	C/T-C/C	78 (65%)	42 (36.5%)	0.24 (0.12-0.47)			
Recessive	T/T-C/T	103 (85.8%)	106 (92.2%)	1.00	.17	244.1	257.9
	C/C	17 (14.2%)	9 (7.8%)	0.48 (0.17-1.40)			
Overdominant	T/T-C/C	59 (49.2%)	82 (71.3%)	1.00	5e-04*	233.7	247.6
	C/T	61 (50.8%)	33 (28.7%)	0.31 (0.16-0.61)			
Log additive	—	—	—	0.37 (0.22-0.63)	1e-04*	230.8	244.7
VDR BsmI-rs1544410 (A/G = B/b)							
Codominant	A/A	34 (28.3%)	42 (36.5%)	1.00	.054	242.1	259.4
	A/G	36 (30%)	47 (40.9%)	1.95 (0.87-4.35)			
	G/G	50 (41.7%)	26 (22.6%)	0.80 (0.35-1.81)			
Dominant	A/A	34 (28.3%)	42 (36.5%)	1.00	.51	245.5	259.3
	A/G-G/G	86 (71.7%)	73 (63.5%)	1.27 (0.62-2.60)			
Recessive	A/A-A/G	70 (58.3%)	89 (77.4%)	1.00	.076	242.8	256.6
	G/G	50 (41.7%)	26 (22.6%)	0.54 (0.28-1.07)			
Overdominant	A/A-G/G	84 (70%)	68 (59.1%)	1.00	.018*	240.4	254.2
	A/G	36 (30%)	47 (40.9%)	2.21 (1.13-4.31)			
Log additive	—	—	—	0.87 (0.58-1.30)	.49	245.5	259.3

Abbreviations: AIC = Akaike-information-criterion; BIC = Bayesian-information-criterion; CHI3L1 = chitinase-3—like protein 1; CI = confidence interval; OPG = osteoprotegerin; OR = odds ratio; RANKL = receptor activator of nuclear factor κB ligand; SNP = single nucleotide polymorphism; VDR = vitamin D receptor.
[a]Adjusted for age and family history in logistic regression model.
*Statistically significant.

Supplemental Figure 1 **Linkage Disequilibrium Between Studied SNPs. Red-To-Blue Scale is Presented. LD was Performed in Studied Patients, 120 Controls and 115 Breast Cancer Patients. SNP1 Indicates RANKL-rs9533156 (T/C); SNP2, OPG-rs2073618 (G/C); SNP3, OPG-rs2073617 (T/C); SNP4, YKL40-rs4950928 (C/G); SNP5, VDR FokI-rs2228570 (C/T = F/f); and SNP6, VDR BsmI-rs1544410 (A/G = B/b). SNPStats Online Software was Used**



Abbreviations: LD = linkage disequilibrium; OPG = osteoprotegerin; RANKL = receptor activator of nuclear factor κB ligand; SNP = single nucleotide polymorphism; VDR = vitamin D receptor.