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OX40 genetic variations in patients with breast cancer: a case-control study

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Breast cancer is the most frequently diagnosed life-threatening cancer in women [1]. To combat tumours, tumour-associated antigens must be presented by antigen-presenting cells (APCs) and recognized by cognate T cells. This interaction, however, is insufficient for full differentiation of naïve T cells; a secondary signal is also required to complete this process. This signal is commonly transferred through several co-stimulatory and co-inhibitory ligands and receptors. Depending on the balance between these co-stimulatory and co-inhibitory signals, the T cell-mediated response can be followed by lymphocyte anergy or activation [2].

Tumour necrosis factor receptor superfamily member 4 (TNFRSF4), also known as CD134, belongs to the tumour necrosis factor receptor superfamily with co-stimulatory properties. It is not constitutively expressed on resting naïve T cells, but is transiently expressed on CD4+ and CD8+ lymphocytes upon antigen stimulation. OX40–OX40L interaction regulates CD4+ lymphocyte differentiation and proliferation, primary clonal expansion, cytokine production, and memory development, as well as CD8+ cell activation [3]. Changes in OX40 protein expression have been shown to be associated with several pathological conditions [4–6]. In addition, it has been shown that OX40 plays an essential role in antitumour immunity [7], as it was reported that defects in the OX40 signalling pathway can be associated with progression in some tumours, e.g. in head and neck cancer [8]. Consistently, recent studies have highlighted that OX40 agonists can enhance antitumour immunity against a variety of tumours through several cellular and molecular mechanisms [9].

OX40 is located on chromosome 1p36. Several single nucleotide polymorphisms (SNPs) have been reported for this gene, which can positively and/or negatively affect normal functioning of *OX40* or its coded protein, which in turn impairs antitumoural immune responses and predisposes individuals to cancers. It has been consistently shown that some genetic variations in the molecules involved in the *OX40* signalling pathway are associated with a variety of malignancies. However, the association of *OX40* SNPs with breast cancer is unknown. We hypothesised roles for *OX40* SNPs in breast cancer by studying the distribution of rs17568A/G and rs2298211A/C polymorphisms in patients and healthy individuals.

A total of 203 patients with breast cancer were enrolled. Basic demographic and clinicopathological characteristics of the patients are shown in Table 1. The control group was 199 healthy individuals with no familial history of cancer or autoimmune disease. Informed consent was obtained from all participants. The study was approved by the ethics committee of Shiraz University of Medical Sciences.

Genomic DNA was extracted from EDTA-treated peripheral blood samples with a modified proteinase K salting-out method. Specific primers were designed with AlleleID®7 software packages (PREMIER Biosoft International, San Francisco, CA, USA) [10]. The forward and reverse primer sequences for rs17568 were 5′-GCAGCCGGCCAGCAATAGCT-3′ and 5′-AAGGGGTCTGCT GGGTGGGG-3′, respectively, and these sequences for rs2298211 were 5′- GGGCAGCCTCTGGTCCCTCC –3′ and 5′-TCACAGGGACCCTCCACCCG-3′. The genotypes inherited at both positions in *OX40* were determined with a polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) method. For each position, 0.25 µl (10 pmol/µl) of two specific forward and reverse primers, 0.45 µl of both dNTP (10 mM) and $MgCl_2$ (50 mM), 1.5 µl PCR buffer 10× and 1 unit of Taq DNA polymerase (all from Sinaclon, Tehran, Iran) were added to a tube containing 300 ng genomic DNA. The final volume was adjusted to 15 µl with distilled water. The samples were amplified in a thermocycler (Techne, Germany) with the following protocol: initial denaturation at 94 °C for 5 min, followed by 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 45 s; these three steps were repeated for 30 cycles and followed by a single final extension step at 72 °C for 10 min. The PCR products were then loaded on 1.5% agarose gels containing Gel Red (Biotium,

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Table 1. Demographic and clinicopathological characteristics of the patients with breast cancer.

Clinicopathological characteristic	Data: n (%)
Tumour type	
Infiltrating ductal carcinoma	174 (85.7)
Medullary carcinoma	12 (5.9)
Infiltrating lobular carcinoma	10 (4.93)
Others: metaplastic carcinoma and ductal carcinoma in situ	7 (3.45)
TNM stage	
0	2 (1.0)
I	42 (21.1)
II	109 (54.8)
III	46 (23.1)
Unreported	4
Lymph node status	
Free	97 (51.3)
Involved	92 (48.7)
Unreported	14
Lymphovascular invasion	
Negative	99 (49.7)
Positive	100 (50.2)
Unreported	4
Tumour size (cm)	
T1 (≤2)	72 (37.5)
T2 (2 < Size ≤ 5)	113 (58.8)
T3 (>5)	7 (3.6)
Unreported	11
Histological grade	
I (well differentiated)	55 (30.9)
II (moderately differentiated)	77 (43.3)
III (poorly differentiated)	46 (25.8)
Unreported	25
Distant metastasis at diagnosis	Negative: 100%
Tumour side	
Right	89 (45.6)
Left	106 (54.4)
Unreported	8

Fremont CA, USA) and were visualized under UV light. For RFLP reactions, PCR products were independently treated with 2.5 units of restriction enzymes *Alw*261 (Fermentas, Vilnius, Lithuania) or *Hpy*166II (NEB, Hitchin, Hertfordshire, UK) for rs17568A/G and rs2298211A/C, respectively, and incubated at 37 °C overnight. Then the digested products were mixed with loading dye, loaded on agarose gels containing Gel Red, and visualized under UV light.

Data were analysed by SPSS v15 and EPI info 2000. Chi-squared and Fisher exact tests were used to compare the genotype, allele and haplotype frequencies between cases and controls. P < 0.05 was considered statistically significant.

Genotype distributions at both rs17568A/G and rs2298211A/C positions were consistent with a Hardy–Weinberg equilibrium in both groups. There was no significant difference between cases and controls in the distribution of genotypes or alleles at either position. The frequencies of rs17568A/G genotypes and alleles were also compared among patients with different clinical and pathological characteristics. The AA genotype was significantly more frequent in patients with lower-stage tumours (18.2% in stage I vs. 5.8% in stages II/III; *P*= 0.03) and with smaller tumours (15.3% in <2 cm vs. 5.0% in ≥2; *P*= 0.03). No further associations were observed with other clinicopathological features.

The most common haplotype in both groups was GA (rs17568 G/rs2298211A; 64.7% of cases and 61% of controls) (Table 2). However, no significant differences in the distributions of predicted haplotypes were observed between cases and controls (*P*> 0.05).

A nonsynonymous SNP, rs17568A/G, is located in exon 5 of *OX40* and its functional consequence is the replacement of the glutamine codon with another codon for this amino acid [11]. Although this is a nonsynonymous mutation, several studies proposed that it might indirectly affect the pattern of *OX40* gene expression [4–6]. Our results did not show significant differences in the frequencies of genotypes and alleles of this SNP between cases and controls, although investigating the association of this SNP with clinicopathological features revealed a significant negative association between inheriting the rs17568 AA genotype and TNM stage as well as tumour size. Consistent with this observation, a recent study demonstrated that individuals with the GG genotype at the rs17568 locus have a > 2-fold higher risk of developing squamous cell carcinoma in the nose and paranasal sinuses [10]. In another study carriers of the G allele at this locus were more prone to developing acute coronary syndromes [12]. Although this group later reported that rs17568A/G SNP was not associated with this syndromes in the Han Chinese population, this SNP had an effect on serum high-density lipoprotein cholesterol (HDL-C) [4]. Ria *et al*. also showed that the G allele is associated with lower plasma levels of HDL in patients with the history of myocardial infarction [11]. Similar results were also reported by Wang X *et al*., who found that rs17568 polymorphism

Table 2. Genotype, allele and haplotype distribution of *OX40* rs17568 A/G and rs2298211 A/C polymorphisms in patients with breast cancer and controls.

		Patients (%) n = 203 2 n = 406	Controls (%) n = 199 2 n = 398	P value
rs17568 A/G				
Genotype	GG	101 (50.8)	9 9)49)	0.73
	GA	78 (39.2)	86 (42.6)	
	AA	20 (10.1)	17 (8.4)	
	Missing	0	1	
Allele	G	280 (70.3)	284 (70.3)	0.95
	A	118 (29.6)	120 (29.7)	
	Missing	0	2	
rs2298211 A/C				
Genotype	AA	171 (84.7)	165 (82.9)	0.89
	AC	28 (13.9)	31 (15.6)	
	CC	3 (1.5)	3 (1.5)	
	Missing	1	0	
Allele	A	370 (91.6)	361 (90.7)	0.75
	C	34 (8.4)	37 (9.3)	
	Missing	2	0	
rs17568 and rs2298211 haplotypes				
Haplotypes	GA	251 (64.7)	233 (61)	0.32
	AA	106 (27.3)	109 (28.5)	0.78
	AC	10 (2.9)	8 (2.1)	0.84
	GC	21 (5.4)	32 (8.4)	0.14

affects serum HDL levels [13]. Consistent with these findings, Liu *et al*. found that individuals homozygous for the A allele had higher levels of LDL and total cholesterol in comparison to G allele carriers [14]. The discrepancies in this data may arise from differences in the molecular pathology of the diseases investigated, as well as differences in minor allele frequencies in different populations.

The second SNP investigated, rs2298211, is an A-to-C variation in the *OX40* located at the 3′ end of intron 5. This SNP is in complete allelic relation with another *OX40* SNP, rs2298212. rs2298211 might modify a lariat intermediate branch sequence and consequently alter the third position of the consensus sequence, which could in turn lead to the production of another *OX40* transcript that might be upregulated, causing increased T cell recruitment and activation [11]. Our analysis of the associations in women with breast cancer found no differences between cases and controls in the genotype or allele distribution at this position, or in different clinicopathological characteristics. Patients with head and neck tumours who inherited the C allele at this position (AC and CC genotypes) had tumours with significantly higher histological grades [10]. Ria *et al*. found an association between rs2298212 (a SNP close to rs2298211) and myocardial infarction [11]. We found no significant differences in the distribution of rs17568 and rs2298211 haplotypes between patients with breast cancer and controls. Moreover, we found no associations between haplotype combinations and our patients' main clinicopathological characteristics, e.g. tumour type, TNM stage, lymph node status, tumour size or histological grade.

Our data represent an advance in biomedical sciences in that although rs17568 A/G and rs2298211 A/C SNPs in *OX40* may play no role in breast cancer, the rs17568 AA genotype appears to be associated with the stage and size of the tumour.

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Disclosure statement

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