Background and Aim: The immune system plays an important role in the breast cancer development. OX40L (also known as TNFSF4), a membrane protein, which is a member of the tumor necrosis factor super family binds to its receptor OX40 and this co-stimulation has a crucial role in T cell proliferation, survival and cytokine release. Previous studies have shown that OX40L is associated with cancer. Due to the importance of the T cells in antitumor activities of OX40L we studied the association of rs3850641 (A>G) polymorphism of OX40L gene with the breast cancer. Materials and Methods: The study included 123 women with breast cancer and 126 healthy volunteers with no signs of cancer. Genomic DNA was extracted from blood leukocytes. Genotype and allele frequencies were determined in patients and control cases with the method of polymerase chain reaction-restriction fragment length polymorphism and the statistical analysis was performed by Med Calc. Results: The prevalence of genotype frequencies of AA/AG/GG were 60.9%, 30.08% and 8.9% in patients with breast cancer cases and 74.6%, 18.25% and 7.14% in healthy volunteers while the A and G allelic frequency was 76.01 and 23.98% in patients and 83.73 and 16.26% in healthy controls, respectively. Statistical analysis has shown significant difference from the comparison of genotype ($p = 0.03$). Conclusion: It is concluded that the rs3850641 SNP is significantly associated with the breast cancer susceptibility in Iranian population. However, further studies in larger populations including other genetic and environmental factors are required to achieve conclusion.

Key Words: OX40L, gene polymorphism, breast cancer, PCR-RFLP.

Breast cancer is the most common invasive malignancy among women worldwide with an incidence reaching more than one million new cases [1]. Studies have indicated that the mean age of patients with breast cancer in Iran is approximately 10 years younger than in western countries [2]. Genetics and environmental factors have been shown to be important in breast cancer initiation and promotion. Many genes have been shown to have important roles in breast cancer onset, invasion, metastasis and prognosis. OX40L which is also known as TNFSF4 and gp34 is a member of the tumor necrosis factor (TNF) super family and was first identified as gp34 on HTLV-I transformed cells [3]. OX40L is a type II glycoprotein which is located on the human chromosome 1 [4]. OX40L is expressed on activated antigen presenting cells like B cells [5], dendritic cells [6] and macrophages [7] as well as natural killer cells (NK cells) [8], Langerhans cells [9], airway smooth muscle cells [10] and vascular endothelial cells [11]. OX40L is also expressed on the surface of activated T cells which enhances further T cell responses [12]. Its receptor OX40 is expressed on activated T cells and its ligation to OX40L promotes pathways which regulate the T cell proliferation, effective activation, survival and cytokine release from the T cells [13]. Unlike OX40 expressing T cells, OX40L expressing cells are only found in inflammatory sites and only those T cells which migrate to inflammatory sites can receive the co-stimulatory effect of OX40L [7]. This prevents the widespread activation of OX40 on activated T cells [14]. OX40 stimulation results in IL-2R expression and IL-2 production which affects the T cell survival and effector differentiation through downstream molecules like nuclear factor kappa-B (NF-kB) and survivin [15]. The ligation of OX40 leads to the activation of the NF-kB by TRAF-2 induction which promotes the transcription of the target genes [16], and the activation of PI-3K and protein kinase B (also known as Akt activation) [17]. The activation of Akt results in the expressing anti-apoptotic molecules like Bcl-2 and Bcl-xl [18].

The anti-apoptotic protein survivin which is activated by an AKT/PI3K-dependent activation increases the survival of the activated T cell, which leads to the increase of T cell clonal expansion [19]. OX40 stimulation leads to enhanced CD8 T cell differentiation against soluble or tumor associated antigen, which results in the increased expression of CD25 and granzyme B expression and enhanced cytolytic activity [20] and also the accumulation of CD8 at the tumor site [21].

It has been shown that OX40L polymorphisms are associated with diseases like systemic lupus erythematosus (SLE) [22] and systemic sclerosis [23]. Some studies have shown that OX40L fusion protein inhibited the growth of mouse 4T1 breast tumor model [24]. The OX40L rs3850641SNP is located at intron 1 of the OX40L. Variants in the intron of a gene may influence its expression and regulate its function. In this study we aimed to determine the association of the OX40L polymorphism with breast cancer in Iran.

**MATERIALS AND METHODS**

Blood samples were collected from a total of 123 patients with sporadic breast cancer and 126 healthy blood donors as control cases with no signs of any cancer. The study has been approved...
by the appropriate ethics committee and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study.

We extracted the genomic DNA from blood cells by using Gpp Solution DNA extraction kit (Genpajoohan, Iran), Chloroform, 90% and 70% ethanol and NaCl. The target genes OX40L, with a 769 base pair length, was amplified by using forward and reverse primers (Table 1) (Generay, China) by polymerase chain reaction (PCR). The PCR mixture contained 10 μl Master mix, 1 μl of each primer, 5 μl template DNA and 3 μl deionized water. Amplification was performed as followed: denaturation for 5 min at 95 °C and 35 cycles with the program 95 °C in 45 s, annealing temperature 55 °C for 45 s, 72 °C for 45 s and finally 72 °C for 5 min to ensure the product extension.

The PCR products were electrophoresed on 2% agarose gel and visualized by UV light. After being sure of having PCR products the products were digested by HpyCH4III which cutes the DNA in the ACN’GT restriction site and the result is DNA fragments with the sizes of 769, 521, and 248 base pairs. The products were electrophoresed on 2% agarose gel (Figure).

![Figure](image)

**Figure.** Agarose gel electrophoresis after the digestion of the DNA of the patient group by HpyCH4III. A 769bp segment represents A/A homozygosis (A) while 769, 521, and 248 base pairs represent A/G Heterozygosis (B). Segments sized 521 and 248 represent G/G mutant homozygosis.

###  Statistical analysis

The statistical analysis was performed by MedCalc chi-square test (version 14.8.1) to make a comparison between genotypes and alleles. We used Hardy — Weinberg equilibrium (HWE) to evaluate deviation between observed and expected frequencies. A value of \( p < 0.05 \) was considered statistically significant.

### RESULTS

The study included 123 controls and 126 patients with breast cancer. The patients ranged in the age group from 20 to 80 years old, and the healthy controls aged from 20 to 70 years old. The PCR product had a 769 base pair length and the details of enzyme digestion are shown in Table 2. The Hardy — Weinberg analysis is shown in Table 3. Distribution of OX40L genotypes was incompatible with HWE for control group but not for breast cancer patients (\( p = 0.00082 \) and \( p = 0.07 \), respectively). Deviation from HWE can be caused by some reasons such as small number of subjects or random genetic drift. The allele and genotypes frequencies of the studied genetic variants in breast cancer cases and healthy subjects are shown in Table 4. The genotype frequencies of AA/AG/GG were 60.9%, 30.08% and 8.9% in breast cancer cases and 74.6%, 18.25% and 7.14% in healthy volunteers.

### Table 1. Forward and reverse primer designation used in amplification

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Primer sequences</th>
<th>Size of DNA fragments</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5'-TCTATGCAAAAAAAGTTGAGT-3'</td>
<td>769 bp</td>
<td>5'-ACNGT...3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TAGCTCAACAAAAAGGGAACAGA-3'</td>
<td>21 bp</td>
<td>3'-TCNT...5'</td>
</tr>
</tbody>
</table>

**The PCR**

The PCR products were electrophoresed on 2% agarose gel and visualized by UV light. After being sure of having PCR products the products were digested by HpyCH4III which cutes the DNA in the ACN’GT restriction site and the result is DNA fragments with the sizes of 769, 521, and 248 base pairs. The products were electrophoresed on 2% agarose gel (Figure).

### Table 2.restriction length polymorphism data of the restriction site of HpyCH4III and the sizes of DNA fragments after digestion by this enzyme

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>HpyCH4III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of DNA segments</td>
<td>Restriction site</td>
</tr>
<tr>
<td>T/C genotype</td>
<td>T/C genotype</td>
</tr>
<tr>
<td>Size of DNA fragments</td>
<td>769 bp</td>
</tr>
<tr>
<td>5´-TCTTATGCCAAAAGTGTTGAGT-3´</td>
<td>5´-TCTTATGCCAAAAGTGTTGAGT-3´</td>
</tr>
</tbody>
</table>

**The AG and GG genotypes of the polymorphism were seen in 48 patients with breast cancer (39.0%) and 32 controls (25.4%) (dominant model: OR = 1.88; 95% CI 1.09–3.23; \( p = 0.021 \). The association was also significant when analyzing the AG genotype in overdominant model (OR = 1.93; 95% CI 1.06–3.49; \( p = 0.029 \). The A allele frequency was 76.01% in patients and 83.73% in control groups. The prevalence of G allele was 23.98% in patients and 16.26% in the control group. The results show that there is a significant difference in the genotype and allele frequencies between two groups (\( p = 0.04 \) and \( p = 0.03 \), respectively) (see Table 4).

### Table 3. Hardy — Weinberg analysis

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Controls, n (%)</th>
<th>Patients, n (%)</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>94 (75)</td>
<td>23 (18)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>23 (18.2)</td>
<td>37 (30.1)</td>
<td>2.02 (1.10–3.68)</td>
<td>0.061</td>
</tr>
<tr>
<td>GG</td>
<td>9 (7.1)</td>
<td>32 (25.4)</td>
<td>1.53 (0.60–3.89)</td>
<td>0.012</td>
</tr>
<tr>
<td>A</td>
<td>103 (81.8)</td>
<td>103 (81.8)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>32 (25.4)</td>
<td>48 (39.0)</td>
<td>1.88 (1.09–3.23)</td>
<td>0.021</td>
</tr>
<tr>
<td>A</td>
<td>117 (92.9)</td>
<td>117 (92.9)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>9 (7.1)</td>
<td>9 (7.1)</td>
<td>1.28 (0.51–3.20)</td>
<td>0.6</td>
</tr>
<tr>
<td>AA</td>
<td>103 (81.8)</td>
<td>103 (81.8)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>23 (18.2)</td>
<td>23 (18.2)</td>
<td>1.93 (0.60–3.49)</td>
<td>0.029</td>
</tr>
<tr>
<td>A</td>
<td>21 (16.2)</td>
<td>21 (16.2)</td>
<td>1.48 (0.99–2.21)</td>
<td>0.54</td>
</tr>
<tr>
<td>G</td>
<td>41 (32.4)</td>
<td>41 (32.4)</td>
<td>1.62 (1.04–2.53)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**The AG and GG genotypes of the polymorphism were seen in 48 patients with breast cancer (39.0%) and 32 controls (25.4%) (dominant model: OR = 1.88; 95% CI 1.09–3.23; \( p = 0.021 \). The association was also significant when analyzing the AG genotype in overdominant model (OR = 1.93; 95% CI 1.06–3.49; \( p = 0.029 \). The A allele frequency was 76.01% in patients and 83.73% in control groups. The prevalence of G allele was 23.98% in patients and 16.26% in the control group. The results show that there is a significant difference in the genotype and allele frequencies between two groups (\( p = 0.04 \) and \( p = 0.03 \), respectively) (see Table 4).

**DISCUSSION**

The objective of this study was to examine the relationship between OX40L rs3850641 polymorphism and breast cancer susceptibility in Iran. Result reveal that rs3850641 G allele is associated with a significantly higher risk of breast cancer.

Breast cancer is the most common invasive malignancy type worldwide. It is estimated that more than one million women are diagnosed with breast cancer and 450,000 cases die annually by this disease [1]. OX40L, a type 2 membrane protein, is a member of the
TNF superfamily [5]. OX40L is expressed by activated B cells, vessel endothelial cells, macrophages, dendritic cells and certain activated T cells. This generates co-stimulatory signals by interacting with TNFRSF4 on activated T lymphocytes and enhances the proliferation and differentiation of T lymphocytes and the development and survival of memory T cells [25]. A study by Zaini et al. [26] has shown that TNF-α-stimulated dendritic cells maturation in mice was accompanied by increased expression of OX40 ligand (OX40L), the lack of which resulted in an inability of mature dendritic cells to generate cellular antitumor immunity. Furthermore, intratumoral administration of dendritic cells modified to express OX40L suppressed tumor growth through the generation of tumor-specific cytolytic T cell responses, which were mediated by CD4 T cells and NK T cells. In the tumors treated with OX40L-expressing dendritic cells, the NK T cell population significantly increased and exhibited a substantial level of IFN-γ production essential for antitumor immunity. These results indicate that the OX40L expression on dendritic cells is crucial in the development of antitumor immunity, possibly by coupling innate to adaptive immunity [26].

The proliferation of naive CD8 T cells or previously activated CD8+ T cells were suppressed by regulatory T cells (Tregs), though not to the same extent as CD4+ T cells. Tregs suppressed the proliferation of naive CD4+, naive CD8+ T and CD25+ T cells after TCR triggering, in contrast, OX40 co-stimulated Tregs that reduced Foxp3 expression reversed the suppressive function [27].

Studies have shown that OX40L polymorphisms are associated with SLE [22]. Another study has shown the association of the OX40L polymorphism in systemic sclerosis [23]. Polymorphisms of OX40L are also associated with artherosclerosis [28]. Other studies suggested that OX40L polymorphism was associated with myocardial infarction [29]. Fu et al. [30] conducted a meta-analysis with 9 articles to assess the relationship between rs3850641 and breast cancer risk under additive and dominant models (OR = 0.01042 and p = 0.01942, respectively). Moreover, rs3850641G allele could increase the susceptibility to breast cancer (p = 0.009662).

It is found that in a mouse model of allergic asthma basophiles highly expressed OX40 ligand (OX40L) after activation. The results reveal a critical role of OX40L presented by the activated basophiles to initiate Th2 responses in an allergic asthma model, implicating OX40-OX40L signaling as a potential therapeutic target in the treatment of allergic airway inflammation [12]. Blocking OX40-OX40L interaction has been considered an approach to the treatment of various diseases in which T cells play a critical role. In animal models, OX40L blockade in vivo has been shown to weaken harmful diseases in many inflammatory and autoimmune models like allergic asthma, colitis, diabetes, arthritis, atherosclerosis and allograft rejection. Evidences showed that inflammatory responses play a crucial role at different stages of tumor development and contributes to the initiation and progression of cancer [33].

It is concluded that the rs3850641 polymorphism is associated with the susceptibility of breast cancer in Iran. It is necessary to confirm this result with larger sample size in multiethnic groups in the future.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

REFERENCES


