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Evaluation of the circulating levels of IL-12 and IL-33 in patients with breast cancer: influences of the tumor stages and cytokine gene polymorphisms

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ABSTRACT

Objective(s): IL-12 as an anti-tumor cytokine and IL-33 a novel identified cytokine with both pro- or anti-tumor activities, play important roles in response against tumor cells. Our aim was to evaluate the IL-12 and IL-33 levels and single nucleotide polymorphisms (SNP) in their genes in patients with breast cancer.

Materials and Methods: Blood samples were collected from 100 patients with breast cancer, and 100 healthy women were controls. The serum IL-12 and IL-33 levels were measured by ELISA. The SNP rs3212227 (in IL-12 gene) and rs1929992 (in IL-33 gene) were determined using PCR-RFLP.

Results: The IL-12 levels similarly expressed in patients and controls. IL-12 levels in patients at stage I were significantly lower than in the healthy group (P<0.05). IL-33 levels and the IL-33/IL-12 ratio were significantly higher in patients than the control group (P<0.001). The IL-33 levels and IL-33/IL-12 ratio in stage IV patients were significantly higher than other stages and controls (P<0.0001 and P<0.001, respectively). There were no significant differences in the frequencies of genotypes in rs3212227 and rs1929992 between patients and the control group. No significant differences were observed between subjects with various genotypes at rs3212227 and rs1929992 with respect to related cytokine levels.

Conclusion: These results indicate that the diminished IL-12 production may contribute to the tumor establishment. The higher IL-33 levels and IL-33/IL-12 ratio in patients also indicate an imbalance in Th1/Th2 responses that may contribute to tumor development. Thus, correcting the imbalance of Th1/Th2 could be an important strategy for cancer immunotherapy.

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Introduction

Breast cancer is the most frequent cancer among women, comprising approximately one-fourth of female cancers worldwide (1, 2). Despite the development of the novel treatment over the past years and relatively good overall survival rate at present, the breast cancer causes over 410,000 deaths worldwide per year (2). The function of the immune system is defending against infections and tumors. It has been demonstrated that there is immune dysfunction in breast cancer patients (3, 4). However, the exact immunopathological mechanisms in breast cancer development are not clearly understood. The results of many studies suggest that the modulation of the innate and adaptive immune parameters such as B cells, T cells, macrophages, dendritic cells (DC), and natural killer (NK) cells is important for initiation and progression of breast cancer (5, 6). The role of inflammation in the development of breast cancer has also been reported (7, 8). Several cytokines including interferon (IFN)-α, β, and γ, IL-2, IL-4, IL-6, IL-8, IL-10, and IL-19, and tumor necrosis factor-α (TNF-α) are known to play An important role in a coordinated manner in breast

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cancer development (9-12).

The helper T (Th) cell responses also exert an important role in the immune responses to tumor antigens. The Th cells are divided into several subsets including Th1, Th2 and regulatory T (Treg) cells, which release distinct cytokines (13). The Th1 cytokines such as INF-γ and TNF-α increase immune responses to tumor antigens, which leads to tumor regression, whereas the Th2-related cytokines (such as IL-4 and IL-10) and Treg-related cytokines (TGF-β and IL-10) reduce the responses to tumor antigens (14, 15). IL-12 and IFN-γ cause the differentiation of the naive $CD4^+$T cells to Th1 cells. Binding of IL-12 to its receptor, IL-12R, activates signal transducer and activator of transcription protein 4 (STAT4), which up-regulates IFN-γ, which in turn, induces STAT1 and Th1-differentiation specific transcription factor, T-bet (16). On the other hand, IL-4 induces differentiation of the activated CD4+T cells into Th2 cells. Binding of IL-4 to its receptor, IL-4R, activates STAT6, which in turn, induces Th2-specific transcription factor, GATA-3 (16). Moreover, TGF-β induces the naïve CD4+T cells to up-regulate FOXP3 expression and differentiate into Treg cells (17). In our previous study higher levels of a Th2/Treg-related chemokine (CCL22) were observed in patients with breast cancer, levels of which were correlated with tumor stages (18).

Structurally, IL-12 consists of two covalently linked P35 and P40 subunits (encoded by the IL-12A and IL-12B genes, respectively), which perform an interconnection between the innate and adaptive immunity. The main producer cells of IL-12 are activated antigen presenting cells including dendritic cells and macrophages (19). IL-12 receptor is composed of two polypeptide chains, IL-12Rβ1 and IL-12Rβ2. IL-12 receptor is expressed on a number of leukocytes such as NK cells, T and B lymphocytes (19, 20). The main actions of IL-12 are as follows: induction of Th1 immune response, increasing production of IFN-γ from NK and $CD4^+$ T cells, stimulation of proliferation and cytotoxicity of NK cells and $CD8^+$ T cells, enhancement of antibody-dependent cellular cytotoxicity (ADCC) against tumor cells and the induction of IgG, and suppression of IgE production from B cells (19, 21). It has been demonstrated that IL-12 is able to suppress tumorigenesis and induce regression of established tumors by promoting Th1-related adaptive immunity together with cytotoxic response (22). Other antitumor activities of IL-12 have been attributed to its antiangiogenic effects via induction of antiangiogenic cytokine and chemokine secretion, influencing the tumor microenvironment, suppressing the myeloid-derived suppressor cells and increasing expression of MHC class I molecules (19).

IL-33 is a new member of the IL-1 cytokine family that signals via a heteromeric receptor that consists of ST2L (or ST2) and IL-1R accessory protein (IL-1RAcP). ST2 is expressed on many cells especially on mast cells and activated Th2 cells (23). Binding of IL-33 to its receptor induces the production of pro-inflammatory cytokines in mast cells and Th2 lymphocytes (23), induces the chemotaxis of Th2 cells (24), promotes the activation of the eosinophil, basophil and natural killer cells (25, 26), potentiates both Th1- and Th2-related responses (25), induces IFN-γ production in iNKT and NK cells, and increases the numbers of splenic iNKT cells (25, 27). It has been also demonstrated that IL-33 strongly induces the expression of Th2-associated cytokines such as IL-4, IL-5 and IL-13, and IL-33-treated mice have higher serum levels of IgE (28). These findings signify that IL-33 may have both pro and/or anti-tumor activities.

The gene encoding IL-12B is located on chromosome 5q31–33 in humans and the polymorphism 1188 A/C polymorphism (rs3212227) is located in the 3-untranslated region of the cytokine gene. Based on HGMD, 14 important polymorphisms have been found in the IL-12B gene to date (www.portal.-biobaseinternational.com), and many investigations have been focused on rs3212227 (22, 29). The rs3212227 was reported as a functionally important SNP that alters IL-12 production, and it has been reported that rs3212227 was associated with a significantly higher overall risk of cancer (22, 29). Conversely, it has been suggested that rs3212227 could play a protective role against cancers (30). However, it has been reported that SNP rs3212227 may increase the risk of cancer among Asians (29), but there is no investigation regarding the association of this SNP and breast cancer in the Iranian population.

Human IL-33 gene is mapped on chromosome 9p24 and several SNPs have been reported in the cytokine gene, of those the 9894 A/G polymorphism (rs1929992) has been located in intron 3 region (31, 32). The association of the SNP rs1929992 with several non-malignant diseases such as cedar pollinosis (31), ankylosing spondylitis (33), ischemic stroke (34), and Behcet's disease (35) have been investigated in studies from different countries. Although, a large number of studies use polymorphic markers to discover the involvement of genetic components in the pathogenesis of breast cancer (36), however, no study was found regarding the association of the genetic variation in IL-33 gene and cancers such as breast cancer.

As regards, there are differences in genotype, and allele frequencies of cytokine genes depend on ethnicity and race, this study was conducted for the first time to evaluate the serum IL-12 and IL-33 levels in Iranian patients with breast cancer and also

to investigate their associations with tumor stages and SNP rs3212227 and rs1929992 to make clear any relationship.

Materials and Methods

Subjects

In total, 100 women (age 45.73 ± 10. 52 years) with breast cancer were selected from patients who referred for operation to hospitals affiliated to Shiraz Medical School between April 2012 and May 2013. None of the patients had been treated with chemotherapy or radiotherapy before sample collection. Breast cancer was confirmed by oncologists according to the surgical and pathological reports. The distribution of patients according to tumor stages was as follows: stage I: n= 14; stage II: n= 29; stage III: n= 39 and stage IV: n= 18. Furthermore, 17 patients had a family history of breast cancer and 31 patients were in menopause age. Moreover, 100 healthy women (44.23±10.50 years) were enrolled into the study as a control group. The healthy subjects were recruited among blood donors and interviewed with regard to malignancy; none of them had any history of malignant disease. All control subjects were basically healthy, with no acute or chronic illnesses. Indeed sick individuals (with a history of recurrent infections, asthma, allergy and atopic diseases, or any suspected immunological disorders), cigarette smokers and drug users were excluded from the study. The other exclusion criteria were surgery and major trauma within 6 months prior to blood collection. This study was evaluated and approved by the Ethical Committee of Kerman University of Medical Sciences, Kerman, Iran. Moreover, patients were recruited if they agreed to blood sampling. A peripheral blood sample (3–5 ml) was obtained from all participants and the sera were separated and stored at −70 ˚C until analysis.

DNA Extraction

Peripheral blood was collected in EDTA pre-coated tubes and then genomic DNA was extracted by a commercial kit (Bioneer, South Korea). Extracted DNA samples were stored at −20 ˚C for further use.

Polymorphism Genotyping

The polymerase chain reaction–fragment restriction length polymorphism (PCR–RFLP) method was performed to determine the IL-12 and IL-33 gene polymorphisms at rs3212227 and rs1929992, respectively. The PCR performed in a 25 µl reaction mixture containing 12.5 µl of PCR master mix (Cinagen, Iran), 1 µl of prepared DNA, 2 µl of each primer and 7.5 µl of sterile double-distilled water. Primers sequences of SNP rs3212227 were (F:5-TTTGGAGGAAAAGTGGAAGA-3,R:5-AACA-TTCCAT ACATCCTGGC-3), and for SNP rs1929992 they were (F:5-GAAGTCATCATCAACTTGGAACC-3,R:5GGATTGGAATCCCATGGTC-3). The PCR reactions were performed under standard conditions according to the following program: one initial phase of 95 ˚C for 10 min, followed by 35 cycles including denaturation phase in 95 ˚C for 30 sec, annealing phase in 61 ˚C for 30 sec and elongation phase in 72 ˚C for 30 sec.

The lengths of PCR products were 300 bp for rs3212227 and 300 bp for rs1929992. The PCR products were digested with restriction enzymes according to the manufacturer's instruction. Electrophoresis of the digested PCR product was performed on a 2.5% agarose gel, then stained with ethidium bromide and finally analyzed by a Chemi-Doc model XRS (Bio-Rad, USA). The amplified PCR product of SNP rs3212227 was digested with TaqI restriction enzyme (New England Biolabs, England). In the homozygotic situation (AA), a 300 bp fragment (without any digestion); in the heterozygotic form (CA), three different fragments with 300, 166 and 134 bp; and in the homozygotic situation (CC) two different fragments with 166 and 134 bp were observed.

The amplified PCR product of SNP rs1929992 was digested with SspI restriction enzyme (New England Biolabs, England). In the homozygotic situation (GG), a 217 bp fragment (without any digestion); in the heterozygotic form (AG), three different fragments with 217, 134 and 83 bp; and in the homozygotic situation (AA), two different fragments with 134 and 83 bp were observed.

Cytokine assay

The serum IL-12 and IL-33 levels measured by commercial ELISA kits (R & D systems, UK) according to the manufacturers guidelines. The sensitivity of the assay was 2 pg/ml.

Statistical Analysis

Differences in variables were analyzed using Student's t, ANOVA and $\chi 2$ tests as appropriate, and *P*-values of less than 0.05 were considered significant. The statistical analyses were performed using SPSS version 15 (Chicago, IL, USA).

Results

The levels of IL-12 in breast cancer and control groups

The mean serum levels of IL-12 in patients with breast cancer and the healthy control group have been demonstrated in Figure 1. The mean serum levels of IL-12 similarly expressed in patients with breast cancer and healthy group (56.57±12.70 pg/ml and 54.54±10.16 pg/ml, respectively). The mean serum level of IL-12 in patients with breast cancer according to tumor stages have been demonstrated

Table 1. The frequency of IL-12 gene polymorphism in breast cancer (BC) patients and controls

Genotypes	BC patients No. (%)	Healthy subjects No. (%)	*P*-value
CC	10 (10%)	8 (8%)	0.62
CA	32 (32%)	33 (33%)	0.88
AA	58 (58%)	59 (59%)	0.88
C	52 (26%)	49 (24.5%)	0.862
A	148 (74%)	151 (75.5)	0.848

in Figure 2. The serum levels of IL-12 were 10.50±2.12 pg/ml, in patients with tumor stage I, 53.92±12.82 pg/ml in patients with tumor stage II, 54.64±27.22 pg/ml in patients with tumor stage III, and 99.35±42.71pg/ml in patients with tumor stage IV. The mean serum level of IL-12 in patients with tumor stage I was significantly lower than that observed in the healthy control group (P<0.05). No significant differences were observed between patients with tumor stages II or III and the control group regarding the mean serum levels of IL-12. Although, the mean serum level of IL-12 in patients with tumor stage IV was higher than that in the control group, but the difference was not significant (P<0.12).

The levels of IL-33 in breast cancer and control groups

The mean serum levels of IL-33 in patients with breast cancer and the healthy control group has been demonstrated in Figure 3. The mean serum level of IL-33 in patients with breast cancer (1043.02± 200.98 pg/ml) was significantly higher than that in the control group (296.29±70.21 pg/ml, P<0.001). The mean serum levels of IL-33 in patients with breast cancer according to tumor stages have been demonstrated in Figure 4. The serum levels of IL-33

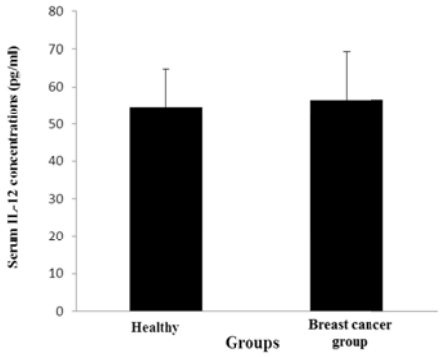

Figure 1. Comparison of the serum IL-12 levels between patients with breast cancer and the healthy group
The mean serum levels of IL-12 similarly expressed in patients with breast cancer and healthy group

was 338.13±235.53 pg/ml in patients with tumor stage I, 359.44±190.18 pg/ml in patients with tumor stage II, 646.44±280.80 pg/ml in patients with tumor stage III and 3437.90±562.74 pg/ml in patients with tumor stage IV. The serum levels of IL-33 increased with advanced stages. The mean serum levels of IL-33 in patients with stages I, II and III were also higher than the healthy control groups, but the differences were not statistically significant. The mean serum level of IL-33 in patients with tumor stage IV was significantly higher than controls (P<0.0001). Moreover, the mean serum level of IL-33 in patients with tumor stage IV was significantly higher than other stages (P<0.0001).

The IL-33/IL-12 ratio in breast cancer and control groups

The mean IL-33/IL-12 ratios in patients with breast cancer and the healthy control group have been

Table 2. Serum levels of IL-12 in breast cancer (BC) and healthy groups according to chemokine gene polymorphisms at rs3212227

Groups	Genotypes	No. (%)	IL12 levels* (Mean± SEM)	*P*-value
BC	CC	10 (10%)	118.82 ± 85.85	0.23
	CA	32 (32%)	71.28 ± 27.71	
	AA	58 (58%)	41.75 ± 11.51	
	C	52 (26%)	79.67 ± 26.91	0.28
	A	148 (74%)	51.96 ± 12.18	
Healthy	CC	8 (8%)	58.41± 28.26	0.21
	CA	33 (33%)	28.05± 8.84	
	AA	59 (59%)	67.48±15.51	
	C	49 (24.5%)	34.99 ±9.40	0.33
	A	151 (75.5)	54.17±10.86	
Total	CC	14 (8%)	84.30 ± 39.10	0.56
	CA	55 (31.42%)	50.03 ± 14.92	
	AA	106 (60.57%)	54.61 ± 9.69	
	C	83 (23.71%)	57.00 ± 14.25	0.81
	A	267 (76.28%)	53.05 ± 8.14	

* The serum levels of cytokine expressed as pg/ml

Table 3. The frequency of IL-33 gene polymorphism in patients with breast cancer (BC) and the healthy control group

Genotype	BC patients No. (%)	Healthy subjects No. (%)	*P*-value
GG	15 (15.00%)	13 (13.00%)	0.68
GA	46(46.00%)	38 (38.00%)	0.25
AA	39(39.00%)	49 (49.00%)	0.15
G	76 (38.00%)	64 (32.00%)	0.459
A	124 (62.00%)	136 (68.00%)	0.310

demonstrated in Figure 5. The mean IL-33/IL-12 ratio in patients with breast cancer (72.71±18.87) was significantly higher than that in the. control group (10.26±1.94, *P*<0.001). The IL-33/IL-12 ratios in patients with breast cancer according to tumor stages have been demonstrated in Figure 6The IL-33/IL-12 ratio was 22.47±13.56 in patients with tumor stage I, 13.11±2.92 in patients with tumor stage II, 62.75±34.70 in patients with tumor stage III, and 198.78±55.15 in patients with tumor stage IV. The IL-33/IL-12 ratio in patients with stage I and II was higher than the healthy control groups, but the differences were not statistically significant. The IL-33/IL-12 ratio in patients with tumor stages III and IV was significantly higher than controls (*P*<0.01 and *P*<0.001, respectively). Moreover, the IL-33/IL-12 ratio in patients with tumor stage IV was significantly higher than those in stages I, II and III (*P*<0.01, *P*<0.001, and *P*<0.05, respectively).

The IL-12 gene polymorphism in breast cancer and control groups

Table 1 summarizes the frequencies of genotypes and alleles at SNP rs3212227 in IL-12 gene in patients with breast cancer and the healthy control group. Statistical analysis showed no deviation of genotype frequencies from the Hardy-Weinberg equilibrium, either in the patients or in the

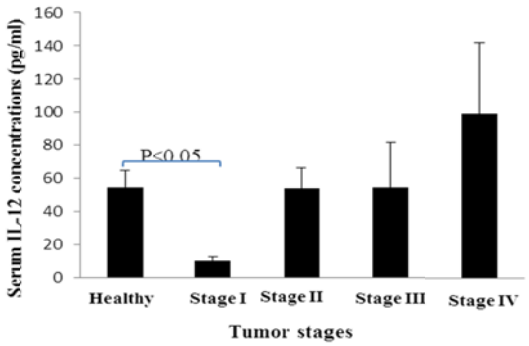

Figure 2. Comparison of the serum levels of IL-12 in the healthy group and patients with different stages of breast cancer
The mean serum level of IL-12 in patients with stage I was significantly lower than the control group (*P*<0.05). No significant differences were observed between patients with stages II or III tumors and the control group regarding the serum levels of IL-12. Although, the serum IL-12 levels in patients with stage IV tumors were higher than the control group, but the difference was not significant (*P*<0.12)

controlsStatistical analysis also revealed no significant differences in the frequencies of genotypes (CC, CA and AA genotypes) and alleles (C and A alleles) at SNP rs3212227 in IL-12 gene between breast cancer patients and controls.

The levels of IL-12 according to cytokine gene polymorphisms

The levels of IL-12 according to cytokine gene polymorphism at rs3212227 in breast cancer and the control groups have been demonstrated in Table 2. No significant differences were observed between subjects with CC, CA or AA genotypes (or between subjects with C and A alleles) at SNP rs3212227 regarding the mean serum levels of IL-12 either in breast cancer patients or in the healthy control group (Table 2).

Table 4. Serum levels of IL-33 in breast cancer (BC) and healthy groups according to the cytokine gene polymorphisms at rs1929992

Groups	Genotypes	No. (%)	IL33 levels* (mean± SEM)	*P*-value
BC	GG	15 (15.00%)	362.20± 278.23	0.44
	GA	46 (46.00%)	1152.16± 294.16	
	AA	39 (39.00%)	1128.5± 351.60	
	G	76 (38.00%)	988.49 ± 243.27	0.65
	A	124 (62.00%)	1142.56 ± 224.73	
Healthy	GG	13 (13.00%)	160.35 ± 95.45	0.71
	GA	38 (38.00%)	353.41 ± 145.61	
	AA	49 (49.00%)	283.3±82.33	
	G	64 (32.00%)	309.54±114.71	0.93
	A	136 (68.00%)	313.71±78.18	
Total	GG	28 (12.00%)	266.08± 150.78	0.33
	GA	84 (43.42%)	794.83 ± 180.12	
	AA	88 (44.57%)	651.56 ± 165.87	
	G	140 (35.00%)	680.36 ± 146.18	0.73
	A	260 (65.00%)	722.71 ± 122.03	

* The serum levels of cytokine expressed as pg/ml

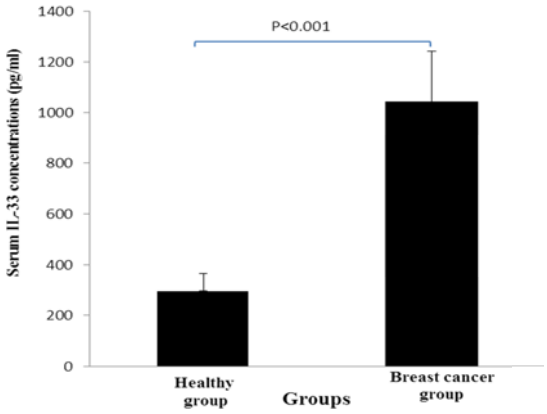

Figure 3. Comparison of the serum IL-33 levels between patients with breast cancer and the healthy group
The mean serum level of IL-33 in patients with breast cancer was significantly higher than that in the healthy group (*P* <0.001)

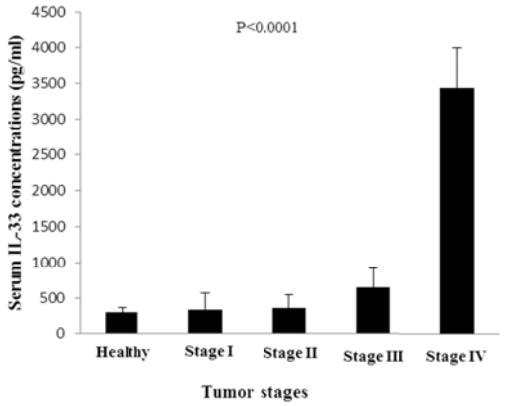

Figure 4. Comparison of the serum levels of IL-33 in the healthy group and patients with different stages of breast cancer
The serum level of IL-33 increased with advanced stages. The mean serum level of IL-33 in patients with stage IV tumors was significantly higher than the controls and other stages (*P*<0.0001)

The IL-33 gene polymorphism in breast cancer and the control groups

Table 3 summarizes the frequencies of genotypes and alleles at position rs1929992 in the IL-33 gene in patients with breast cancer and the healthy control group. Statistical analysis showed no deviation of genotype frequencies from the Hardy-Weinberg equilibrium, either in the patients or in the controls. Statistical analysis revealed no significant differences in the frequencies of genotypes (CC, CT and TT genotypes) and alleles (C and T alleles) at SNP rs1929992 in IL-33 gene between breast cancer patients and the controls, either.

The levels of IL-33 according to cytokine gene polymorphisms

The levels of IL-33 according to cytokine gene polymorphism in breast cancer and the control groups have been demonstrated in Table 4. The mean serum level of IL-33 in patients with AA or GA genotype was higher than that in patients with GG genotype, but the differences were not statistically significant. Moreover, no significant differences were observed between healthy subjects with AA, GA or AA genotypes regarding the mean serum levels of IL-33. In both breast cancer and healthy groups, no significant differences were observed between subjects with various alleles at rs1929992 with respect to the levels of IL-33 (Table 4).

Discussion

The results of the present study showed that the IL-12 levels similarly expressed in patients with breast cancer and the healthy group. The anti-tumoral activities of IL-12 may be performed

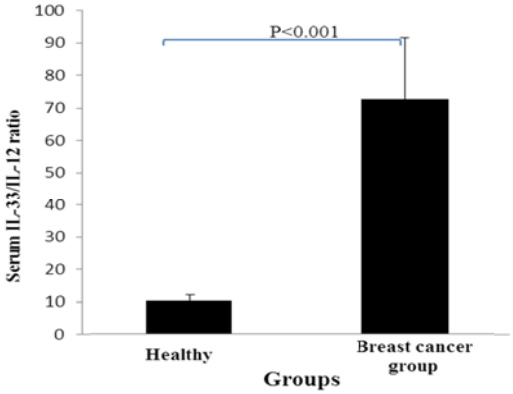

Figure 5. Comparison of the serum IL-33/IL-12 ratio between patients with breast cancer and the healthy group
The serum IL-33/IL-12 ratio in patients with breast cancer was significantly higher than that in the healthy group (*P*<0.001)

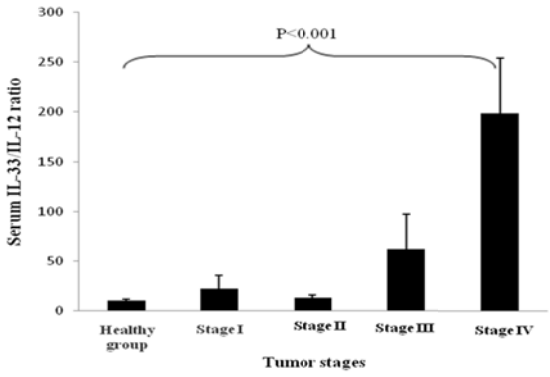

Figure 6. Comparison of the serum IL-33/IL-12 ratio in the healthy group and patients with different stages of breast cancer
The IL-33/IL-12 ratio in patients with stage IV tumors was significantly higher than those in stages I, II and III (*P*<0.01, *P*<0.001 and *P*<0.05, respectively)

through the induction of Th1 immune response, increasing production of IFN-γ from NK and $CD4^+$ T cells, stimulating the proliferation and cytotoxicity of NK cells and $CD8^+$ T cells, enhancement of ADCC against tumor cells, induction of antiangiogenic cytokine and chemokine production, changing of the tumor microenvironment, suppressing the myeloid-derived suppressor cells, and increasing expression of MHC class I molecules (19, 21, 22). Although, the results of the present study showed that the IL-12 levels similarly expressed in patients with breast cancer and the healthy group, however, our results showed that the IL-12 levels in patients with stage I were significantly lower than those observed in the healthy group. There were no significant differences between patients with tumor stages II, III or IV and the control group regarding the mean serum level of IL-12. This finding represents that diminished IL-12 production may contribute in the initial phase of tumor establishment and development. It has also been reported that the individuals with low serum concentrations of IL-12 have a high risk of gastric and colorectal cancer development (37, 38), which is consistent with the results of this study. IL-12 has been also reported as a perfect candidate for tumor immunotherapy, due to its ability to activate both innate and adaptive immune responses against tumor cells (19). Furthermore, IL-12-based immunotherapy may be more effective in cancer patients with defects in IL-12 production (19, 39).

The results of the present study also showed that the IL-33 levels were significantly higher in patients with breast cancer than in the control group. The IL-33 levels increased with advanced stages so that its levels were significantly higher in patients with tumor stage IV in comparison to the healthy controls. The precise mechanisms that are responsible for the higher expression of IL-33 in breast cancer remain to be clarified. However, the results of the present study signify that IL-33 may have an important role in the development of breast cancer. In agreement with our findings, Liu *et al* also indicated higher serum levels of IL-33 in breast cancer patients in comparison to patients with benign breast disease, and higher expression of IL-33 in carcinomatous tissues as compared with normal breast tissues from the same patients (40). Moreover, they found no significant differences between tumor stages I, II and III with respect to the serum levels of IL-33, which is in accordance with our results.

There are a few studies about the role of IL-33 and its receptor, ST2, in anti-tumor immunity or tumor growth with controversial results. The results of some investigations suggest that IL-33 may have pro-tumor activities, whereas the others support anti-tumor properties of IL-33 (41, 42). It has been demonstrated that the exogenous administration of IL-33 enhances tumor growth and inhibits innate anti-tumor immunity (43). Moreover, it has been reported that IL-33 acts as an important inducer of alternatively activated macrophages (M2) and also significantly reduces NK cell activity (44). The M2 macrophages produce IL-10 and suppress innate and adaptive anti-tumor immune responses (12). Furthermore, it has been also demonstrated that deletion of ST2 signaling may enhance anti-tumor immune response in a murine model of breast carcinoma (45). Moreover, ST2 deletion has been associated with higher numbers of $CD4^+$ and $CD8^+$ T cells in lymphoid organs, higher cytotoxicity of NK and $CD8^+$ T cells, higher numbers of IFN-γ expressing NK cells, higher serum levels of IFN-γ and TNF-α, decreased IL-4 levels, and decreased frequencies of M2 macrophages in spleen after tumor challenge (45). The increased anti-tumor immune response in ST2-deficient mice has been also associated with Th1/Th17 cell polarization and enhanced NK cell activity (45). In ST2-deficient mice, IL-12 produced by classically activated M1 macrophages promote maturation of DCs and consequently potentiate the Th1/Th17 response that activate NK, NKT cells and $CD8^+$ T lymphocytes (45). Accordingly, in overexpression of IL-33, it binds to ST2 and activates Th2 cells and promotes the generation of relatively immature dendritic cells that do not produce IL-12. Immature DC promotes generation of Treg cells and, therefore, facilitate tumor progression and metastasis (46). In addition, ST2 signaling may induce the production of thymic stromal lymphopoietin (TSLP) by tumor cells. TSLP induces the production of IL-4 and subsequently IL-10 and IL-13 producing Th2-cells that promote cancer escape (47). It has been also demonstrated that the administration of IL-33 increases tumor growth and promotes the lung and liver metastases, which was associated with increased intratumoral accumulation of myeloid-derived suppressor cells (MDSCs) and Treg cells (48). The mentioned observations are in accordance with our findings.

Although, we have observed unchanged serum levels of IL-12 in patients with breast cancer, the ratio of IL-33/IL-12 levels was significantly higher in patients with breast cancer as compared to healthy subjects. These observations represent an imbalance in the Th1/Th2 -associated immune responses with a tendency toward the Th2 responses that may contribute in the tumor development and progression. The results of many studies have demonstrated the Th1/Th2 immune imbalance in patients with cancer. The Th1/Th2 immune imbalance has been reported in cancers such as liver cancer (49), breast cancer (50), colon cancer (51), laryngo-pharyngeal cancer (52), gastric cancer (53), and gastro-intestinal cancer (54). We have observed that IL-33/IL-12 ratio was higher in patients with tumor stages III and IV in comparison with those in

tumor stages I and II. Our observations and those mentioned above indicate that Th2 tendency is one of the common mechanisms for immune escape of cancer cells and is closely related to initiation and development of cancers. Thus, correcting the imbalance of Th1/Th2 could be an important strategy for cancer immunotherapy.

The results of the present study also demonstrated that there were no significant differences in the frequencies of genotypes and alleles rs3212227 in IL-12B gene between breast cancer patients and controls. There are some investigations supporting that SNP rs3212227 is probably associated with higher cancer risk. Recently, the results of a study on the Iranian population showed that the frequency of CA genotype at SNP rs3212227 was significantly higher in patients with bladder cancer as compared to the control group (55). Tamandani *et al* also reported that the presence of AC/CC genotypes at SNP rs3212227 increases the risk of cervical cancer in Iranian women (56). Moreover, the results of a meta-analysis showed a significant association between the presence of the AC or CC genotype at SNP rs3212227 and susceptibility to cervical and nasopharyngeal cancers (22, 57). The results of a study on women from Croatia also indicated a significant association between SNP rs3212227 and breast cancer (58). However, no significant association has been reported between SNP rs3212227 and colorectal cancer (22). Conversely, the results of another meta-analysis showed that SNP rs3212227 may play a protective role against cancers including nasopharyngeal cancer and hepatocellular carcinoma (29). Accordingly, SNP rs3212227 might play a distinctive role in the pathogenesis of different cancers. Moreover, this discrepancy may be attributed to the differences in parameters such as genetic and ethnic backgrounds of patients, sample size or matching criteria. In the majority of circumstances, however, it is not clear whether such polymorphisms perform the functional effects in a direct manner or in linkage disequilibrium with another functional SNP in their proximity.

Our results also indicated no significant differences in the frequencies of genotypes and alleles at SNP rs1929992 in IL-33 gene between breast cancer patients and controls. Accordingly, we could not find any association between SNP rs1929992 and breast cancer. The association of the IL-33 gene polymorphism with some non-malignancy diseases has been investigated in a number of studies. The results of a study on a Japanese population showed a correlation between the SNP rs1929992 and susceptibility to Japanese cedar pollinosis (31). The results of a study on a population from China also demonstrated a significant association between the SNP rs1929992 with ischemic stroke (34). Moreover, Fan *et al* also reported a significant association between SNP rs1929992 and ankylosing spondylitis in Chinese population (33). However, another study found no association between SNP rs1929992 and Behcet's disease in the Turkish population, either (35).

The association of SNPs rs1929992 and rs3212227 with related cytokine levels has also been evaluated in the present investigation. No significant differences were observed between subjects with various genotypes at SNP rs3212227 regarding the IL-12 levels, either in breast cancer patients or in the healthy control group. Our results also indicated that in both breast cancer and the healthy control group, no significant differences were observed between subjects with various genotypes at SNP rs1929992 with respect to the mean serum level of IL-33. These data signify that the IL-12 and IL-33 levels were not influenced by genetic variation at SNPs rs3212227 and rs1929992, respectively. However, more studies with bigger sample size were suggested in this field.

Conclusion

The results of the present study showed that the IL-12 levels were significantly lower in patients with stage I than in the healthy group. These findings indicate that diminished IL-12 production may contribute in the initial phase of tumor establishment and development. Moreover, the IL-33 levels in patients with breast cancer were significantly higher than that in the control group. The IL-33 levels increased with advanced stages so that they were significantly higher in patients with tumor stage IV in comparison to healthy controls. These results represent that the IL-33 may have an important role in the development of breast cancer. Furthermore, the ratio of IL-33/IL-12 levels was significantly higher in patients with breast cancer as compared to the healthy subjects. These observations indicate an imbalance in the Th1/Th2 - associated immune responses with a tendency toward the Th2 responses that may contribute to the tumor development and progression. We have observed that IL-33/IL-12 ratio was higher in patients with tumor stages III and IV, which indicates that Th2 tendency may be a mechanism for immune escape of cancer cells and is related to initiation and development of cancers. Thus, correcting the imbalance of Th1/Th2 could be an important strategy for cancer immunotherapy. The results of the present study also demonstrated that there were no significant differences in the frequencies of genotypes and alleles at rs3212227 and rs1929992 between breast cancer patients and the controls. Accordingly, these polymorphisms may have no influence on susceptibility to breast cancer. These data showed that the IL-12 and IL-33 levels were not

influenced by genetic variation at SNPs rs3212227 and rs1929992, respectively.

Conflict of interest

The authors report no conflicts of interest.

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