Role of ATG16LI (rs2241880) and Interleukin 10 (rs1800872) Polymorphisms in Breast Cancer Among Egyptian Patients

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This study was performed to determine the role of autophagy-related 16-like 1 (ATG16L1, rs2241880) and IL10 (rs1800872) polymorphisms in the susceptibility to and early prediction of breast cancer in Egyptians. The study included 50 breast cancer patients and 50 apparently healthy controls. The PCR-RFLP technique was used to detect ATG16L1 (rs2241880) and IL10 (rs1800872) genotypes. IL10 level was determined in serum by ELISA. The mean age of the patients was 54.2 years. Among the patients, 80% had no family history for breast cancer, 70% were postmenopausal, and 72% exhibited grade II tumors. Metastasis was detected in 18% of the patients, and 6% of the cases exhibited triple-negative receptor (TNR) status. In the ATG16LI (rs2241880) gene, the GG genotype frequency was significantly higher in patients than in controls (14% in patients versus 2% in controls, *P* =0.02), and no metastasis was observed in patients with the AA genotype (*P*=0.03). In the IL10 (rs1800872) gene, the A allele was observed in 30% of patients and 23% of controls, but the difference was insignificant (*P*=0.26). Also, the prevalence of the AA genotype was 8% in patients and 4% in controls (*P*=0.54). Serum IL10 levels were higher in patients than in controls (*P*<0.001). Within the patient group, individuals with the IL10 (rs1800872) AA genotype showed significantly higher serum IL10 levels than those with the CC and CA+CC genotypes (*P* =0.03 and 0.04, respectively). In conclusion, in Egyptian breast cancer patients, the GG genotype of ATG16LI (rs2241880) may be associated with increased disease risk, and the AA genotype could be protective against metastasis.

Breast cancer is the most common type of cancer and the top cause of cancer deaths among females all around the world and the second leading cause after lung cancer in both sexes [1,2]. Several genetic and environmental factors contribute to breast cancer etiology. Obesity, physical inactivity, and delayed childbearing are considered the most important environmental factors [3]. Patients at high risk can be determined by fully understanding the immunological mechanisms and genetic factors that play an important role in the disease pathogenesis [4]. Early detection of breast cancer reduces death rates and improve the therapeutic response [5].

Autophagy (type II programmed cell death) is a catabolic pathway that plays an essential role in cellular homeostasis [6]. Through autophagy, endogenous long-lived proteins and invading pathogens are degraded by lysosomes via three different pathways: micro-autophagy, macro-autophagy, and Chaperone-mediated autophagy [7,8].

Various autophagy-associated genes and the secreted proteins they encode are responsible for the formation of autophagosomes. An autophagosome is a double-walled membranous structure

containing a specific cargo or part of the cytoplasm that fuses with a lysosome to form an autolysosome for the degradation and recycling of its contents of unwanted cellular components [9]. Several studies support the role of autophagy defects in oncogenesis and the response of breast cancer patients to anti-cancer drugs [10].

The autophagy-related 16-like 1 (ATG16LI) gene of mammals is homologous to Atg16 in yeast. Atg16 is conjugated non-covalently to the Atg12-Atg5 complex for the formation of Atg12-Atg5/Atg16 oligomers, which play an important role in autophagosome formation [11]. Several studies have demonstrated a strong association of ATG16LI polymorphisms (rs2241880 A/G) with Crohn's disease [12,13]. Another study concluded that Atg16L2 (rs1000620) was associated with breast cancer [14].

Interleukin 10 (IL10) plays an essential role in autoimmune and malignant disease pathogenesis [15]. Two controversial roles of IL 10 have been observed; the first is its immunosuppressive function when secreted from T-helper-2 (TH2) and regulatory T cells (T-regs), which it down regulates the immune system [16]. On the other hand, it is considered to act as an immune-stimulatory cytokine when used in cancer therapy by increasing the cytotoxic activity of CD^{+8} T cells [17]. Single nucleotide polymorphisms (SNP) in IL 10 genes such as IL10 rs1800871, rs1800872, and rs1800896 were recently studied to determine their role in development and prognosis of different malignant diseases [18].

In the current study, we aimed to determine whether polymorphisms in the autophagy-associated genes ATG16L1 (rs2241880) and IL10 (rs1800872) affect the susceptibility to and prognosis of breast cancer in Egyptian patients and determine the serum level of IL10 in patients and controls.

Materials and Methods

Fifty unrelated female breast cancer patients and 50 unrelated apparently healthy controls without a family history of breast cancer or any other type of cancer were enrolled in this case-control study. All the patients were over 18 years of age and showed no distant metastasis at the time of diagnosis. Patient samples were obtained from the Oncology Department of Qena University Hospitals, South Valley University. The study was conducted from June 2018 to July 2019.

Patient histories were taken, including their age, family history, physical inactivity, menstrual state, disease recurrence, and received methods of treatment. Clinical and laboratory examinations are routinely performed at the Oncology Department, including the evaluation of the body mass index, mass size, lymph node involvement, cancer grade, histopathological type, hormonal receptors, and signs of metastasis. For the diagnosis of metastasis, abdominal ultrasonography and CT scans of the brain were performed for all patients to detect any focal lesions in the liver or brain. If clinical manifestations indicated suspected bone metastasis, a bone scan and MRI were performed. Informed written consent was obtained from all patients. The study was reviewed and approved by the Ethics Committee of the Qena Faculty of Medicine, South Valley University.

DNA extraction: Five milliliters of peripheral venous blood were obtained from the patients and controls. Four milliliters of EDTA venous blood was prepared for DNA extraction by the salting-out method as previously described by Miller et al. (1988) [19]. Briefly, a double volume of lysis buffer (containing 320 mM sucrose, 1% Triton X-100, 5 mM $MgCl_2$, and 10 mM Tris-HCl, with the pH adjusted to 7.6) was added to 4 ml of EDTA venous blood in a 15 ml polypropylene tube. This was, followed by centrifugation at 1200 x g for 10 min in a cooling centrifuge. A small white pellet formed at the bottom of the tube, consisting of white blood cells (WBCs). After removal of the supernatant, 2 ml of WBC lysis buffer (25 mM EDTA, 75 mM NaCl, with the pH adjusted to 8.0) was added to the pellet, followed by mixing well to dissolve the pellet. Next, 15 µl of 10 mg/ml proteinase K, and 35 µl of 20% SDS were added to the mixture, followed by incubation at 37°C overnight. Five moles of saturated

NaCl was added to each tube, followed by centrifugation at 1200 x g for 20 min. The supernatant was transferred to a new 15 ml polypropylene tube, then 8 ml of absolute cold ethanol was added to the tube. DNA molecules were precipitated to the bottom of the tube. The supernatant was removed after centrifugation, and the DNA was left to dry for 2 hours. Thereafter, 200 µl of TE buffer (10 mM Tris, 1-mM EDTA, pH adjusted to 7.5) was added to dissolve the DNA. The DNA was stored at -20°C until used.

Gene amplification by PCR: For gene amplification, a forward and reverse primers were used. For the ATG16L1 gene (rs2241880), the sequence of the forward primer was 5′-CTCTGTCACCATATCAAGCGTGG -3′, and that of the reverse primer was 5′-TCTAGAAGGACAGGCTATCAACAGATG-3′. For the amplification of the IL 10 gene (rs1800872), the sequence of the forward primer was 5′-GGTGAGCACTACCTGACTAGC-3′, and that of the reverse primer was 5′ CCTAGGTCACAGTGACGTGG-3′, as previously described by Diler et al. (2018) and Mohammadi *et al*. (2019) [20,21]. The PCR volume was 25 µl for both genes, containing the following components: 12.5 µl master mix (Bioline, Catalog no. BIO-25043, UK), 1 µl forward primer, 1 µl reverse primer, 2 µl extracted DNA, and 8.5 µl H2O.

The PCR was performed using a thermal cycler (Peqstar, VWR International, UK). The reaction conditions for the amplification of the ATG16L1 and IL10 genes included an initial denaturation step of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing for 40 seconds at 55°C (for ATG16L1 rs2241880) or at 63°C (for IL10 rs1800872), and an extension at 72°C for 40 seconds, with a final extension step at 72°C for 5 min.

Two aliquots of PCR products were separated by gel electrophoresis (Multisub Horizontal Gel System, Cleaver Scientific, UK) in a 2% agarose gel containing 5% ethidium bromide. The first to confirm the presence of the PCR product and the second to determine the genotypes after restriction enzyme digestion for restriction fragment length polymorphism (RFLP) analysis.

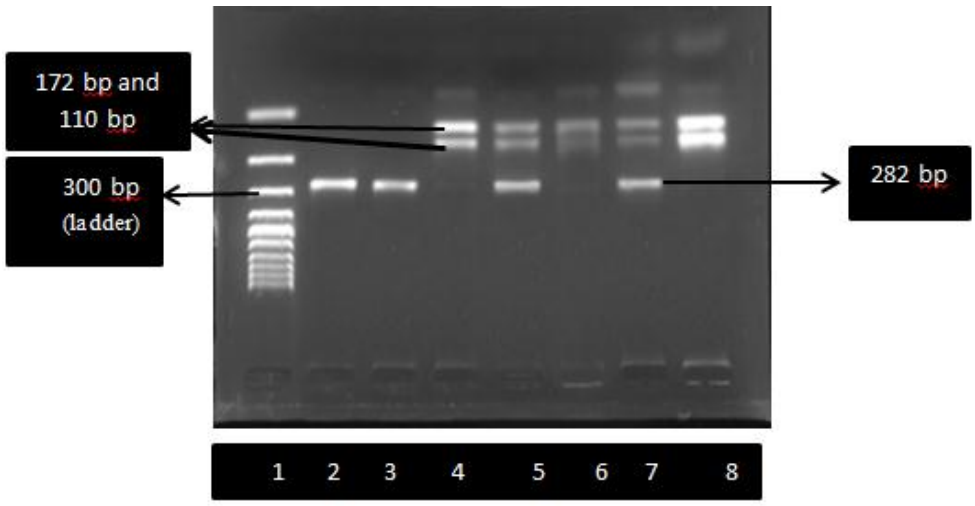
Determination of genotypes: RFLP analysis for the detection of the ATG16L1 genotypes (rs2241880) was performed using a commercially available kit SfaNI (New England BioLabs, USA), according to the manufacturer's instructions. The total reaction volume was 25 µl containing 8 µl of the PCR product, 2.5 µl NE Buffer, 2 µl restriction enzyme, and 12.5 µl H2O, and incubated at 37°C for 60 min. As shown in Figure 1a, the genotypes were classified as follows: AA genotype (282 base pairs (bp)), AG genotype (282 bp, 172 bp, and 110 bp), and GG genotype (172 bp, and 110 bp).

RFLP analysis for the detection of the IL10 genotype (rs1800872) was performed using a commercially available kit RsaI (New England BioLabs, USA), according to the manufacturer's instructions. The total reaction volume was 25 µl containing 8 µl of the PCR product, 2.5 µl NE Buffer, 1 µl restriction enzyme, and 13.5 µl H2O, and incubated at 37°C for 15 min. As shown in Figure 1b, the genotypes were classified as follows: CC genotype (412 bp), AC genotype (412 bp, 236 bp, and 176 bp), and AA genotype (236 bp, and 176 bp).

Serum obtained from the remaining blood sample was used to determine the level of IL10 in patients and controls. Assessment of IL10 level in sera of breast cancer patients and controls was performed using a human IL-10 ELISA kit (Biospes, China), according to the manufacturer's instructions. ELISA optical densities readings, measured at 450 nm, were obtained using a microtiter plate reader (Infinite F50, Tecan, Austria). The results were expressed in picograms/ml.

Statistical Analysis

Analysis was performed using SPSS software version 22. Quantitative variables are presented as the mean± standard deviation. Qualitative variables are presented as percentages. The Chi-squared test, Fisher's exact test, and ANOVA were performed when appropriate. The odds ratio was calculated. A *P* value of 0.05 or less was considered significant.



Figure(1a)

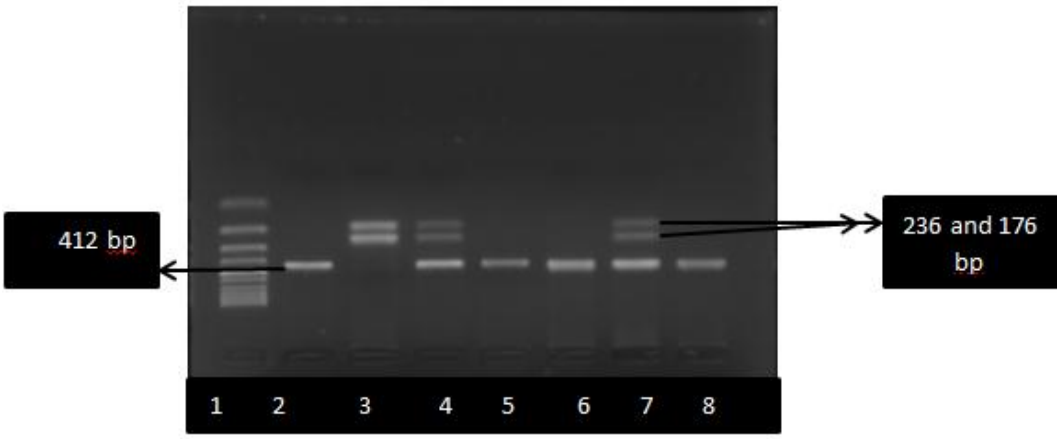


Figure (1b)

Figure 1. Restriction fragment length polymorphism (RFLP) analysis of ATG16L1 (rs2241880, A/G) (Figure 1a) and IL 10 (rs1800872, C/A) (Figure 1b). Lane 1 shows a 100 bp DNA ladder of 100-1000 bp. In Figure 1a, lanes 2 and 3 show the AA genotype; lanes 4, 6, and 8 show the GG genotype; and lanes 5 and 7 show the AG genotype. In Figure 1b, lanes 2, 5, 6, and 8 show the CC genotype; lane 3 shows the AA genotype; and lanes 4 and 7 show the AC genotype.

Results

As shown in Table 1, the age of the breast cancer patients ranged from 32 years to 77 years, with a mean of 54.2±10.6 years. Of the studied patients, 80% had no family history for breast cancer. Among the cases, 18% were diagnosed as metastatic breast cancer (MBC). Bone was the most common site of metastasis (observed in 12% of patients). Ninety percent of the patients expressed the estrogen receptor (ER) or

progesterone receptor (PR). Double ER and PR positivity was detected in 82% of breast cancer patients, while 8% of the patients were positive for human epidermal growth factor receptor 2 (HER2), and 6% showed a triple-negative receptor (TNR) status.

Table 1 Clinical and histopathological characteristics of Egyptian breast cancer patients

	Number of cases = 50 (%)
Age	54.2 ± 10.6
Menopausal state	
Premenopausal	15 (30%)
Postmenopausal	35 (70%)
Family history	
Positive	10 (20%)
Negative	40 (80%)
Grade I	5 (10%)
Grade II	36 (72%)
Grade III	9 (18%)
Lymph node involvement	
Yes	36 (72%)
No	14 (28%)
Mass size	
Less than 5 cm	21 (42%)
Equal to or more than 5 cm	29 (58%)
Distal metastasis	9 (18%)
Bone	6 (12%)
Liver	2 (4%)
Brain	1 (2%)
ER +ve	45 (90%)
PR +ve	45 (90%)
HER2 +ve	4 (8%)
Triple negative	3 (6%)
Single positive	3 (6%)
Triple positive	3 (6%)
Double positive (ER+, PR+)	41 (82%)

Table 2 shows comparison of ATG16L1 (rs2241880, A/G) genotypes between the patients and controls. These was no difference in presence of AA genotype in patients (28%) versus controls (24%) (*P* =0.64). The same was observed for AG genotype (*P* =0.09). However, GG genotype was significantly higher in patients (14%) than in controls (2%) (*P* =0.02). There was no significant difference in the frequency of the G allele of ATG16L1 (rs2241880, A/G) between patients and control subjects.

Table 2. The genotype and allele frequencies of ATG16L1 (rs2241880, A/G) in breast cancer patients and controls.

Genotype and allele frequencies of ATG16L1 (rs2241880, A/G)	BC patients N=50 (%)	Healthy Controls N=50 (%)	OR (95% CI)	*P* value
Alleles				
A	57 (57)	61 (61)		NS
G	43 (43)	39 (39)	0.84 (0.48-1.49)	
Genotypes (Co-dominant model)				
AA	14 (28)	12 (24)	References	0.03*
AG	29 (58)	37 (74)	0.13 (0.01-1.14)	
GG	7 (14)	1 (2)	8.93 (1.03-76.74)	
Dominant model				
AA	14 (28)	12 (24)	Reference	NS
AG+GG	36 (72)	38 (76)	1.23 (0.50-3.01)	
Recessive model				
AA+AG	43 (86)	49 (98)	Reference	0.02*
GG	7 (14)	1 (2)	7.97 (0.94-67.45)	

* Significant *P* value ≤0.05; 95% CI, confidence intervals; OR, odds ratio

Regarding IL10 (rs1800872, C/A) region polymorphisms, the frequency of the C allele was lower in patients than in controls (70% versus 77%, respectively), and the A allele was detected in 30% of patients versus 23% of controls; these differences did not reach statistical significance (Table 3). Additionally, the frequencies of the AA and AC genotypes were higher in patients than in the control group (the AA genotype frequency was 8% in patients versus 4% in controls, while the AC genotype frequency was 44% in patients versus 38% in controls), but the difference was not significant (Table 3). Neither the presence of the dominant model nor that of the recessive model showed a significant difference between the cancer patients and controls (Table 3).

Table 3. Genotype and allele frequencies of IL 10 (rs1800872, C/A) in breast cancer patients and controls.

Genotype and alleles frequencies of IL 10 (rs1800872, C/A)	BC patients N=50 (%)	Healthy Controls N=50 (%)	OR (95% CI)	*P* value
Alleles				
C	70 (70)	77 (77)		NS
A	30 (30)	23 (23)	1.43 (0.76-2.70)	
Genotypes (Co-dominant model)				
CC	24 (48)	29 (58)	Reference	NS
AC	22(44)	19 (38)	0.57 (0.09-3.52)	
AA	4 (8)	2 (4)	1.72 (0.28-10.5)	
Dominant model				
CC	24 (48)	29 (58)	Reference	
AC+AA	26 (52)	21(42)	0.66 (0.30-1.47)	NS
Recessive model				
CC+AC	46 (92)	48 (96)	Reference	NS
AA	4 (8)	2 (4)	2.08 (0.36-11.94)	

95% CI, confidence intervals; OR, odds ratio

* Significant *P* value ≥0.05 is not significant (NS)

Table 4 shows effects of the ATG16L1 (rs2241880, A/G) and IL10 (rs1800872, C/A) genotypes on disease progression. Regarding to the ATG16L1 (rs2241880) genotype, there was a significant association between patients with an AA genotype and lack of metastasis (*P* =0.03).

The level of IL10 in the serum was assessed and compared in different genotypes of the IL10 (rs1800872, C/A) region polymorphism. A significantly higher level of serum IL10 was observed in breast cancer patients than in the control group (69 pg/ml ±15.07 in patients versus 12.4 pg/ml ±2.45 in controls, *P* <0.001). As shown in Figure 2, the highest level of IL10 between patients was found among those with an AA genotype (93 pg/ml ±2.16), while the lowest level was found in those with a CC genotype (63.75 pg/ml ± 10.7) (*P*=0.03). Also, the serum level of IL10 in AA genotype was significantly higher than in AC+CC genotypes (*P* =0.04).

Table 4. The effect of ATG16L1 (rs2241880) and IL10 (rs1800872) on disease progression (lymph node enlargement, metastasis, and mass size).

	Lymph node		*P* value	Metastasis		*P* value	Mass size		*P* value
	Involved N=36	Not involved N=14		Yes N=9	No N=41		Less 5 cm N=21	More than 5 cm N=29	
ATG16L1 (rs2241880, A/G)									
AA	11	3	NS	0	14	0.03*	8	6	NS
AG	19	10	NS	7	22	NS	10	19	NS
GG	6	1	NS	2	5	NS	3	4	NS
IL 10 (rs1800872, C/A)									
CC	20	4	NS	4	20	NS	10	14	NS
AC	14	8	NS	4	18	NS	9	13	NS
AA	2	2	NS	1	3	NS	2	2	NS

* Significant *P* value ≥0.05 is not significant (NS)

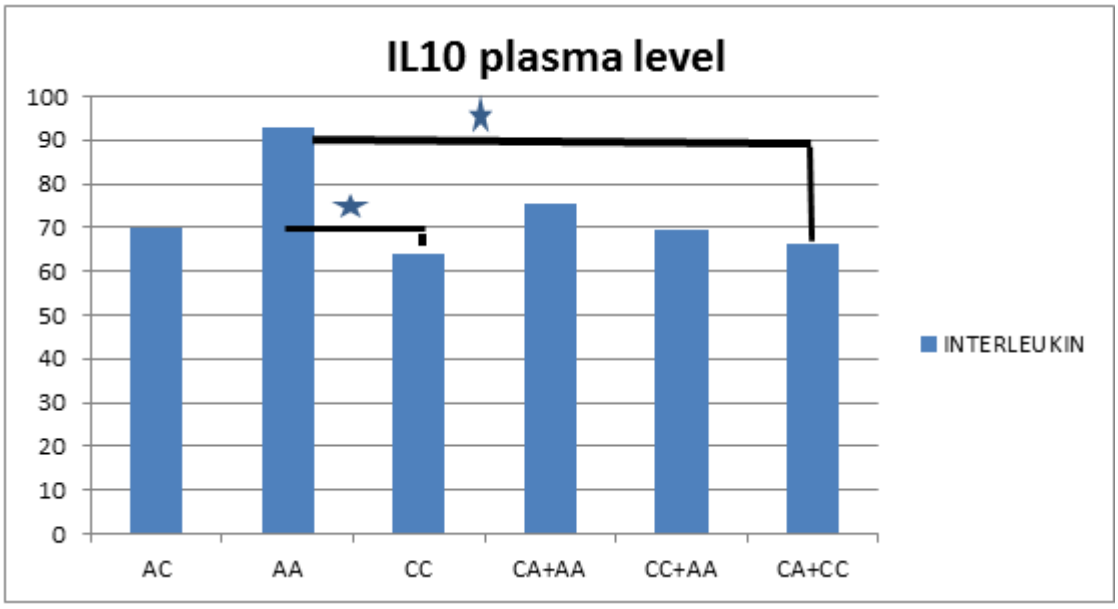


Figure 2. The correlations between different patients genotypes of IL 10 (rs1800872, C/A) and Interleukin 10 serum levels. *significant correlation between AA and CC genotypes. *significant correlation between AA and CA+CC genotypes

Discussion

The critical role of Atg16L1 conjugated with the Atg12-Atg5 complex to form a larger oligomer, required for the lipidation of microtubule-associated protein 1 light chain 3 (LC3) on the autophagosomal membrane has been documented [22]. However, to the best our knowledge, there is no formal studies which investigated the association of between ATG16L1 (rs2241880) polymorphism and breast cancer.

In our study, the GG genotype was more commonly found in patients than in the control group (14% of patients versus 2% of the control group) and was significantly associated with increased risk of breast cancer. Diler *et al.* (2018) found that ATG16L1 (rs2241880) polymorphism did not confer any risk of susceptibility for two different types of cancer (prostate and bladder cancer) in a Turkish population [20]. However, Tan *et al.* (2018) found that ATG16L2 polymorphism was significantly associated with breast cancer risk [14]. ATG16L1 (rs2241880) polymorphism has been analyzed in diseases other than cancer, such as Crohn's disease, and a significant association was found between the GG genotype and Crohn's disease risk in Hungarian patients [13]. The genetic variation between different populations explains the differences between previous results.

According to our results, a protective effect of the AA genotype of rs2241880 against breast cancer metastasis was observed. While neither of two other SNPs (rs2241880 or rs1800872) showed significant relationship with lymph node involvement or mass size.

In our study, the frequency of the mutant allele (A) of the IL10 region polymorphism (rs1800872, C/A) was higher in the patients than in the control group (30% versus 23%, respectively), however, the difference was not significant. Also, the difference observed between the dominant and recessive models was insignificant (P =0.32 and 0.4, respectively). This result is similar to those of other studies [18,23,24] showing an insignificant association between rs1800872 (C/A) and breast cancer. Although most studies have found an insignificant association of IL10 region polymorphism (rs1800872, C/A) with breast cancer, de Oliveira *et al.* (2015) and Zidi *et al.* (2015) found that a high risk was conferred by IL10- rs1800872 (C/A) for gastric and cervical cancer, respectively [25,26].

According to our results, IL10 levels in patient sera were significantly higher than those in the controls. Several studies have obtained similar results to our findings [27,28]. Such observation indicates that a high level of IL10 is associated with a poor prognosis due to the immunosuppressive role of IL10 in the down regulation of the immune system [14]. Although the IL10 serum level was significantly higher in patients with the AA genotype (IL10 region polymorphism rs1800872) than in those with the CC and CA+CC genotypes, there was no association observed between disease prognosis (lymph node enlargement, metastasis, and mass size) and AA genotype in the current study. IL10 is considered to represent a two-edged sword: in addition to its immunosuppressive function, it shows synergistic action with IL2 in increasing the activity of cytotoxic T cells [28].

In our study, breast cancer was mostly diagnosed in the fifth decade of life at a mean age of 54.2 years, which is similar to the results of another study from Egypt

showing that the mean age of patients was 52 years [29]. Lakkis *et al.* (2017) found two peaks of breast cancer in two different age groups (45-49 years and above 75 years) of Lebanese patients [30]. While a study from the USA involving 39 states found that the mean age of breast cancer patients was 61.8 years [31]. The age at the diagnosis of breast cancer varies between different countries, as several factors are implicated in breast cancer pathogenesis in addition to genetic factors, such as physical inactivity and obesity [3].

Only 6% of our patients showed TNR status. Scott *et al.* (2019) found that 8.4% of breast cancer cases were categorized as TNR, which is similar to our results [31]. The range of TNR status in different studies is 10-20% among all cases of breast cancer [32]. A total of 18% of our patients suffered from distal metastasis, and the most common site of metastasis was bone. Whereas DeSantis *et al*. (2016) found that the percentage of metastasis in breast cancer in different ethnic groups in the USA ranged from 5-9% [33]. The rate of a good prognosis of breast cancer cases in high-income countries is relatively high due to early diagnosis, while late diagnosis in low- and middle-income countries is associated with a high percentage of metastasis [34]. In our study, 20% of the breast cancer patients had a positive family history, while Shiovitz & Korde (2015) found that 10% of patients had positive family history [35]. Different variables are responsible for the differences in the percentage of family history between different breast cancer patients, such as ethnicity, environmental factors, and the size of their family [36].

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