Original Article

Impacts of BMI & IL-4 Genetic Polymorphisms (rs2243250 C/T & rs2227284 A/C) on Iranian Breast Cancer Patients: a Pilot Study

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Abstract

Background & Objective: Breast cancer is a phenotypically complex and diverse genetic disease caused by changes in the structure and expression of specific genes. Immune system factors are also involved in the etiology of this neoplasm. This study aimed to evaluate the effect of genetic changes (rs2243250 & rs2227284) on the interleukin 4 gene and body mass index on breast cancer risk in Iranian women.

Material & Methods: From women referring to Shohada-Tajrish Hospital, 100 women with breast cancer and 100 healthy women were selected. After blood sampling and DNA extraction, the women's genotypes were determined using the RFLP-PCR technique. The results were evaluated by SPSS software version 21 and chi-square and logistic regression tests.

Results: Analysis of the results with different genetic models showed the effect of rs2243250 on breast cancer ($p<0.05$), but rs2227284 was not associated with breast cancer ($p>0.05$). People with the CC / TT genotype (polymorphism) were more likely to get breast cancer. Also, the increase in body mass index was significantly associated with both polymorphisms studied. Also, carriers of the TT genotype of rs2243250 polymorphism were more likely to develop breast cancer with aging.

Conclusion: Genetic alterations in the IL-4 gene and obesity probably contribute to breast cancer, and carriers of both genetic modifications (CC / TT) are more likely to develop breast cancer.

Keywords: Interleukin 4, rs2243250, rs2227284, Breast Cancer, Polymorphisms

Introduction

Cancer is the leading cause of death and a critical barrier to increasing life expectancy. Women's breast cancer has surpassed even the most common cancer, lung cancer. An estimate of 2.3 million new cancers shows that 11.7% is related to breast cancer, lung (11.4%), colon (10.0%), prostate (7.3%), and stomach (5.6%) are other cancers (1).

There has been a significant decrease in breast cancer deaths over the past 25 years, but its prevalence has increased by more than 30%. Factors such as gender, genetics, hormone therapy, lifestyle, poor diet, and age play roles in developing cancer (2). This systemic disease is characterized by long-term inflammation. Whether this

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inflammation is involved in initiating or supporting tumor formation depends on its tissue. Eventually, significant changes occur in the immune system as the tumor progresses (3). Interleukins and cytokines are the means of communication between innate and acquired immune cells. Interleukins play a pivotal role in the development, progression, and control of cancer and promote cancer by creating the right environment (4). IL-4 is a cytokine that is a potent regulator of the primary secretions of mast cells, Th2 cells, eosinophils, and basophils. It also plays a prominent role in leukocytes' survival, including Th2 cell-mediated immunity, IgE class switching in B cells, tissue repair, and homeostasis through macrophage activation under physiological and pathological conditions (5). This interleukin has the anti-tumor activity potent against varieties of tumors, including breast cancer, and causes apoptosis in cultured breast cancer cells. In addition, it plays a pivotal role in regulating estrogen synthesis enzymes such as 17 β-HSD and 3- β-HSD (6). IL-4 is a pleiotropic cytokine encoded by the IL-4 gene on chromosome 5q31.1. IL-4 is involved in some processes such as proliferation, differentiation, and apoptosis in various cells (7). This cytokine is a potent regulator of anti-tumor immune responses with both tumor-promoting and inhibitory properties and works by functions such as immune -suppressants and anti-angiogenesis (8). According to the research, the expression of IL-4 was up-regulated in many cancers, such as colon cancer, NSCLC, and cervical cancer (7). Because SNPs can influence disease risk, the effectiveness of drugs, and their side effects, it is necessary to study the genetic changes that affect diseases. A previous study on breast cancer and the interleukin gene had shown that the RP2/RP2 genotype of the *IL*-4 VNTR polymorphism could be a protective factor for breast cancer susceptibility (9). In this study, two polymorphisms rs2227284 and rs2243250 of IL-4 gene, which were not assayed for the risk of breast cancer in Iranian women, were studied.

Materials & Methods

Ethical and Legal Considerations

The study protocol designed in accordance with the guidelines of the Helsinki's Declaration was evaluated and approved by the Ethics Committee of Islamic Azad University of Medical Sciences. Considering the study setting, the institutional review board waived the requirement for informed consent. Informed consent was obtained from all participants and their information was received through a questionnaire.

Sample collection

This study was a case-control study. Patients with breast cancer were screened (mammography and clinical trials) by a specialist, and finally, 100 subjects who needed surgery, were selected as the case group. In addition, 100 ones in the same age range were selected as the control group, after ensuring the absence of diseases such as high blood pressure, diabetes, and cancer (especially breast cancer in first-degree relatives). The pathological report of malignancy, stage of cancer (I, II, III, IV) and type of cancer (Invasive Ductal Carcinoma, invasive carcinoma, carcinoma, and ductal carcinoma Carcinoma In Situ, Lobular Carcinoma In Situ) was extracted for cancer patients. The two groups were in a similar age range.

DNA extraction and genotyping

5-7 ml of blood was taken from all subjects. After DNA extraction by the salting-out method, the quantity and quality of DNA were evaluated using spectrophotometry and agarose gel (10). According to Table 1, Specific primers were designed for a gene fragment. Samples in a volume of 25 µL (including Master Mix Taq DNA Polymerase 2x Master Mix RED 10µL 10 (Danish amplicon), 1µL per primer (10 pmol/L), 12µL DNase/RNase free distilled water and 1µL from each DNA sample (100 ng) were amplified. Simultaneously with the amplification of the samples, a negative control sample (using water instead of DNA sample) was used.

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Then the samples were placed in a thermocycler with an initial denaturation program at 95°C for 5 minutes and denaturation at 95°C for 60 seconds. Primers binding was performed for rs2227284 polymorphism at 63°C and for rs2243250 at 60°C for 40 seconds. The template strand elongated at 72°C for 40 seconds (second to fourth steps repeated 35 times), and the final extension was performed for 7 minutes. To confirm the correctness of amplification, some of them were electrophoresed on 2% agarose gel. Then 10µl of PCR products were digested with restriction enzymes overnight at 37°C. After staining with DNA stain, the enzymatic cleavage products (rs2243250: Bmrl & rs rs2227284 : BspLl) were electrophoresed on 3% agarose gel at 100 volts for 40 minutes to observe the bands using a UV transducer.

Table 1. The sequence of primers and the length of the amplified fragment and the specific enzyme

Polymorphisms		Sequence 5'-3'	Restriction Enzyme	PCR Product	Digested Fragment
rs2243250 C/T	Forward	CTTGAGCCCAGGAATTTGAG	*Bmrl*	490bp	CC:381,109bp TT:490bp CT: 490,381,109bp
	Reverse	ACAGGTGGCATCTTGGAAAC			
rs2227284 A/C	Forward	TGACTCAGAAGGAGGCCTGT	*BspLl*	504bp	AA:293,211bp CC:504bp AC:504,490,381&109bp
	Reverse	CTTCGGGGGAACAAGAATTT			

Statistical analysis

The genotyping of samples was analyzed using SPSS software version 20. The genotype of the subjects and the frequency of alleles were examined using the Chi-square test and logistic regression. The odds ratio (OR) was calculated to estimate the association between the two IL-4 gene polymorphisms and the risk of breast cancer development. Odds ratios were determined with a 95% confidence interval (CI). Statistically, p-value <0.05 was considered significant.

Results

The results showed that the age range of the cancer patients was 43-76 years with a mean age of 60.76±0.89 years. The age range of the control group was 41-74 years with a mean age of 56.55±0.84 years. The mean Age (p=0.006), and free BMI (2.65×10^{-8}) were significant differences between the two groups. Information on age index, body mass, disease stage, and pathology report is summarized in Table 2.

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Table 2. Frequency distribution of demographic and biochemistry characteristics among cancer patients and controls

Variable		Patients	Control	P value
Age (year)	>50	15	28	0.006
	50 to 60	33	4	
	<60	52	31	
BMI (kg/m^2)	25≥	37	76	2.65×10^{-8}
	25<	63	24	
Stage of cancer	I	20		
	II	14		
	III	39		
	IV	27		
Type of cancer	Invasive Ductal Carcinoma (IDC)	40		
	Invasive Lobular Carcinoma (ILC)	34		
	Ductal Carcinoma in Situ (DCIS)	18		
	Lobular Carcinoma in Situ (LCIS)	8		

The results of counting genotypes for rs2243250 polymorphism showed that the homozygous CC genotype was 37% in the cancer group and 23% in the control group. Homozygous mutant TT genotype included 58% of the cancer group and 75% of the control group. Regarding CT genotype, 5% was observed in the cancer group and 2% in the control group. The frequency of the C allele was 0.39 in the cancer group and 0.24 in the control group. The mutant T allele was observed in the cancer group at 0.61 and the control group at 0.76.

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The counting of genotypes for rs2227284 polymorphism showed that the homozygous CC genotype was 44% in the cancer group and 52% in the control group. The mutant AA homozygous genotype was 19% in the cancer group, 13% in the control group, and the heterozygous AG/GA genotype was 37% in the cancer group and 35% in the control group. The frequency of the C allele was 0.39 in the cancer samples and 0.24 in the control samples. The frequency of the A allele was 0.61 in the cancer samples and 0.76 in the control samples. The frequency of genotypes of each polymorphism with different genetic models was examined in Table 3.

Five genetic models (dominant, recessive, log-additive, overdominant and codominant models) were used. By logistic regression test, the results indicated a significant relationship only in rs2243250 polymorphism. Genetic modeling of this polymorphism showed that the genetic model codominant (p=0.021), dominant (p=0.032) & recessive (p=0.012) had a meaningful relationship indicated in two groups. In addition, the frequency of alleles of each polymorphism with logistic regression confirmed the decreasing effect of the T allele of rs2243250 polymorphism.

Table 3. Shows the relationship among the frequency of genotypes and breast cancer odds ratio by logistic regression

SNP ID	Model	Genotype/ Allele	Control	Case	OR (CI95%)	P-value
2243250	Codominant	CC	37	23	1(Reference)	
		TT	58	75	0.481(0.258-0.896)	0.021
		CT	5	2	1.554(0.278-8.68)	0.615
	Dominant	CC	37	23	1(Reference)	
		TT-CT	63	77	0.509(0.274-0.943)	0.032
	Recessive	CC-CT	42	25	1(Reference)	
		TT	58	75	2.172(1.190-3.967)	0.012
	Log-Additive	CT	5	2	1(Reference)	
		CC-TT	95	98	0.388(0.073-2.047)	0.264
	Over-dominant	CC-TT	95	98	1(Reference)	
		CT	5	2	2.579(0.488-13.617)	0.264
	Allele	C	48	79	1(Reference)	
		T	152	121	0.484(0.314-0.744)	0.001

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2227284	Codominant	AA	19	13	1(Reference)	
		CC	44	52	0.579(0.257-1.304)	0.187
		AC	37	35	0.723(0.311-1.681)	0.452
	Dominant	AA	19	13	1(Reference)	
		CC-AC	81	87	0.637(0.296-1.373)	0.250
	Recessive	AA-AC	56	48	1(Reference)	
		CC	44	52	0.725(0.416-1.265)	0.258
	Log-Additive	AC	37	35	1(Reference)	
		AA-CC	63	65	0.917(0.515-1.634)	0.768
	Over-dominant	AA-CC	63	65	1(Reference)	
		AC	37	35	1.091(0.612-1.943)	0.768
	Allele	A	61	75	1(Reference)	
		C	139	125	1.36(0.902-2.071)	0.140

The relationship of combined genotypes between the patients and the control group was analyzed. The results showed a significant association between subjects carrying the CC/TT genotype and the risk of breast cancer (p=0.041, OR: 0.527, CI95%: 0.225-0.975). Carriers of the change in both polymorphisms were at a greater risk for breast cancer. The results of the studies are shown in Table 4.

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Table 4. The relationship among of the genotypes with the risk of breast cancer

rs 2243250 & rs 2227284	Case	Control	p-value	OR (CI95%)
AA/CC	6	1		1(Reference)
AA/TT	13	12	0.393	0.371(0.36-3.838)
AA/CT	-	-	-	-
CC/CC	2	0	-	-
CC/TT	24	39	0.041	0.527 (0.225-0.975)
CC/CT	18	13	0.258	1.567 (0.726-3.41)
AC/CC	13	9	0.304	1.605 (0.652-3.953)
AC/TT	21	24	0.755	0.899 (0.461-1.754)
AC/CT	3	2	0.612	1.599 (0.261-9.789)
Total	100	100		

There was no significant association between the frequency of polymorphism genotypes with disease stage and Gleason score. Concerning the body mass index, except for the CT genotype of rs2243250 polymorphism, other genotypes were associated with breast cancer development. The age index was also effective in carriers of TT genotype of rs2243250 polymorphism (p=0.040) and CC genotype of rs2227284 polymorphism (p=0.036) on breast cancer. The results are presented in Table 5.

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Table 5. Distribution of SNPs in Age, BMI , Stage of cancer and type of cancer among studied groups

Variable			rs2243250 polymorphism			rs2227284 polymorphism		
		Groups	CC	TT	CT	CC	AA	CA
Age (Range of years)	50<	Patient	4	11	0	7	4	4
		Control	3	25	0	15	4	9
	50 to 60	Patient	12	19	2	14	6	13
		Control	12	29	0	23	6	12
	60>	Patient	21	28	3	23	9	20
		Control	8	21	2	14	3	14
	P-value		0.239	0.040	0.290	0.036	0.378	0.227
BMI (Kg/m2)	≥25	Patient	12	21	4	16	3	18
		Control	16	59	1	39	9	28
	<25	Patient	25	37	1	28	16	19
		Control	7	16	1	13	4	7
	P-value		0.005	7.06×10^{-7}	0.427	1.37×10^{-4}	0.002	0.006

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Stage of cancer	I	8	10	2	8	6	6
	II	4	10	1	4	3	7
	III	16	22	0	21	3	15
	IV	9	16	2	11	7	9
	P-value	0.818	0.655	0.456	0.372	0.127	0.653
Type of cancer	IDC[1]	12	26	2	20	6	14
	ILC[2]	12	19	3	13	5	16
	DCIS[3]	8	10	0	8	7	3
	LCIS[4]	5	3	0	3	1	4
	P-value	0.315	0.514	0.491	0.757	0.129	0.150

Invasive Ductal Carcinoma
Invasive Lobular Carcinoma
Ductal Carcinoma in Situ

Discussion

Single nucleotide polymorphisms (SNPs), which often occur in the human genome, have been studied in cancer genetic studies. The findings indicate their pivotal role in the incidence and progression of cancer. Also, polymorphisms in cytokine genes concerning inflammation-related pathways are involved in susceptibility to various types of cancer (8).

Interleukin-4 (IL-4) in immune cells is responsible for functions such as lymphocyte proliferation and viability in addition to the polarization of macrophages to the pro-tumor M2 tumor-associated macrophage phenotype. Many signaling effects by regulating metabolism, including increased glycolysis in B lymphocytes and increased

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fatty acid oxidation in IL4 polarized macrophages, are exerted on immune cells (11).

The relationship between this anti-inflammatory cytokine and certain cancers' growth, including colon, breast, and lung cancer, has been recognized (12). It is identified that in many solid tumors such as triple-negative breast cancer (TNBC), increased levels of IL-4 receptor (IL-4R) help cancer cell proliferation, resistance to apoptosis, metastatic potential, and Th2 response in the Tumor environment (TME) (8). IL-4 functions have both positive and negative effects. It has an anti-tumor effect and increases tumor growth through its receptors on the surface of tumor tissue of the breast, kidney, prostate, colon, and lung (13). This cytokine is not only essential for Th2-type immune responses but is also the main factor in the growth of tumor cells in human breast cancer (6).

In this case-control study, the relationship of two polymorphisms rs2243250 & rs2227284 of the IL-4 gene with the risk of breast cancer development of Iranian women was assayed. The analysis results of the genetic model showed that rs2243250 under Co-dominant, Over-dominant and dominant models had a decreasing effect on breast cancer risk and the TT genotype of this polymorphism had a protective effect against cancer. Subjects who carried the CC mutant genotype of rs2227284 and the TT mutant genotype of rs2243250 were more likely to develop breast cancer. Recent studies have also indicated the potential association of these two polymorphisms with lung cancer (7) esophageal squamous cell carcinoma (14) and asthma and allergic rhinitis (15).

The results of the study by Joshi NN. et al. were in line with the results of our studies. They examined the effect of gene polymorphisms affecting the level of pro-inflammatory and anti-inflammatory cytokines such as rs2243250 on the risk of breast cancer. They concluded that the T allele in this polymorphism has a role in decreasing the risk of breast cancer development among western Indian women and anti-inflammatory genotypes were effective in modulating the risk of breast cancer incidence (16). Contrary to these results, Chin-Nan Ch and colleagues, despite examining three changes in the IL-4 gene promoter, such as rs2243250, found no association between these changes and breast cancer (13).

Examination of the effect of rs2243250 polymorphism on some diseases showed an association between it and the pathogenesis of prostate cancer. It also showed a correlation between rs2243250 genotypes with IL-4 gene transcription level and its plasma level. The T allele of this polymorphism reduces the activity of the IL-4 gene and is an effective factor in susceptibility to prostate cancer (17). But this polymorphism in the study of Maier LM. And colleagues showed no association with type I diabetes (18).

The present study indicated that rs2227284 polymorphism was not significantly associated with breast cancer. But Leung G et al. study of Lymphedema (LE) patients after breast cancer surgery showed this polymorphism was effective in this case. Their results showed genetic changes in the number of anti-inflammatory and pro-inflammatory genes such as rs2227284 caused Lymphedema (LE) after breast cancer treatment (19). However, this polymorphism was not significantly associated with other diseases including, rheumatoid arthritis (RA) (20).

In the present study, the results were different based on the ages and BMI of the control and patient groups. Subjects carrying the TT genotype of the rs2243250 polymorphism and the CC genotype of the rs2227284 polymorphism of the IL-4 gene had a higher chance of developing breast cancer with aging. Another pivotal factor was the increase in body mass index, which was significantly associated with the risk of breast cancer incidence in both polymorphisms. But these polymorphisms did not affect the stage of the disease and the degree of Gleason. In AL-Eitan LN et al. study, despite the lack of effect of variable number tandem repeat (VNTR) of IL-4 gene on breast cancer, body mass index and tumor differentiation were significantly associated with different genotypes of this polymorphism (21).

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Conclusion

Recent research has shown an association between pro-inflammatory cytokines and various cancers. By considering genetic factors as effectual factors on the serum level of these cytokines, they can play a role in the pathogenesis of cancers. In this study, mutant genotypes, all together, played a more effective role in breast cancer development, which indicates the importance of genetic changes in interleukin-4. Increased body mass index with genetic changes in IL-4 seems to be one of the factors involved in the development of breast cancer.

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Conflict of interest

The authors declare no conflict of interest.

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