ORIGINAL RESEARCH

The influence of an *IL-4* variable number tandem repeat (VNTR) polymorphism on breast cancer susceptibility

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Backgrounds: Breast cancer (BC) is one of the most widespread cancers globally. Understanding the etiology of BC may help in determining the various risk factors involved in its malignancy. Certain genetic mutations are considered to play a key role in increasing the risk of BC.

Objectives: In this study, we explored the correlation between a variable number tandem repeat (VNTR) polymorphism in the *IL-4* gene and BC.

Methods: PCR and subsequent gel electrophoresis were used to genotype this variant in 360 Jordanian women (180 BC patients and 180 controls). In addition, phenotype–genotype analysis was carried out.

Results: Our findings illustrate that there is no significant relationship between the variant genotypes in the *IL-4* gene and BC among Jordanian females. Other than body mass index and tumor differentiation ($p < 0.05$), none of the clinical and pathological parameters of BC patients exhibited any association with the variant genotypes.

Conclusions: From this study, we propose that the *IL-4* genetic variant does not impact BC development and progression but that it could influence the disease prognosis.

Keywords: breast, cancer, IL-4, genetic variation, prognosis

Introduction

According to the World Health Organization, breast cancer (BC) constitutes a major burden on worldwide female health and is the leading cause of death among women.[1] However, survival rates among BC patients in the developed world are much higher than those in less developed countries.[2] In 2012, the estimated number of women with BC was 651,000, accounting for more than a third of worldwide cancer cases.[3,4] In Jordan, the mean age of BC diagnosis is 51 years, and it is the most common fatal cancer among Jordanian females.[5]

BC is a complex disease which is influenced by a range of factors, including the environment, sociobiology, family history, hormones, obesity, and the immune system of the individual.[6,7] Despite the fact that only 5–6% of BC cases are inherited, genetic mutations are one of the most predictable risk factors that contribute to BC development, with many studies reporting different genetic variants in critical genes that may induce BC onset.[8,9]

One of the most frequently investigated genetic variants is the variable number tandem repeat (VNTR), a region in the genome with interindividual differences in length consisting of repeated nucleotides that lie adjacent to one another.[10]

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Different studies have screened the VNTR in the *IL-4* gene in order to detect if it is related to the progression of different cancers, including BC.[11] *IL-4* is a cytokine produced by T lymphocytes and is known for its antitumor ability, the latter of which is hypothesized to occur by inducing apoptosis in BC cells and regulating estrogen synthesis.[12–14]

Together with the published data on the subject, the aforementioned functions of *IL-4* make it ideal for candidate gene association analysis.[15–17] The *IL-4* gene is located on chromosome 5 and contains a 70-bp VNTR polymorphism within intron 3 (Figure 1) that includes two common alleles: allele 1 (one repeat) and allele 2 (two repeats). These alleles are more frequent among the population than allele 3 (3 repeats), which is considered a rare variant.[18,19]

In this case–control study, we aim to explore the association between the VNTR polymorphism in the *IL-4* gene and the development of BC among Jordanian women. This study also investigates the relationship between clinicopathological parameters and disease progression among BC patients.

Materials and methods
Study design
This study involved 180 healthy Jordanian women with no family history of BC and 180 Jordanian BC patients, all of whom were randomly chosen and matched for gender and age. Written informed consent was obtained from the participants in this study. Ethical approval was obtained from the Institutional Review Board (IRB) at Jordan University of Science and Technology (ethical approval code number 32/104/2017). This study was also conducted in accordance with the Declaration of Helsinki. Furthermore, clinical and demographic information was obtained from BC patients. Blood samples were collected in cooperation with Jordanian Royal Medical Services (JRMS).

Molecular analysis
Each participant gave 5 mL of peripheral blood, and DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). The quantity and purity of the extracted DNA were verified using the NanoDrop (Thermo Fisher Scientific Inc., DE, USA). PCR was used to genotype the *IL-4* VNTR using a specific forward primer (5′AGGCTGAAAGGGGGAAAGC3′) and reverse primer (5′CTGTTCACCTCAACTGCTCC3′).[20] The researchers added 50 ng of DNA to a 25 µL PCR tube containing 12.5 µL Master Mix, 10 µL deionized distilled water, and 2 µL of each of the forward and reverse primers. The PCR program consisted of an initial denaturation phase at 94°C for 5 mins followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 61°C for 30 s, and extension at 72°C for 45 s. PCR was concluded by a final extension step at 72°C for 5 mins.[21] The PCR product was separated and visualized using 2% agarose, and a 100 bp ladder was used to identify their sizes.

Statistical analysis
Frequency distribution for genotypes and alleles was statistically calculated. Additionally, Pearson's Chi-squared test
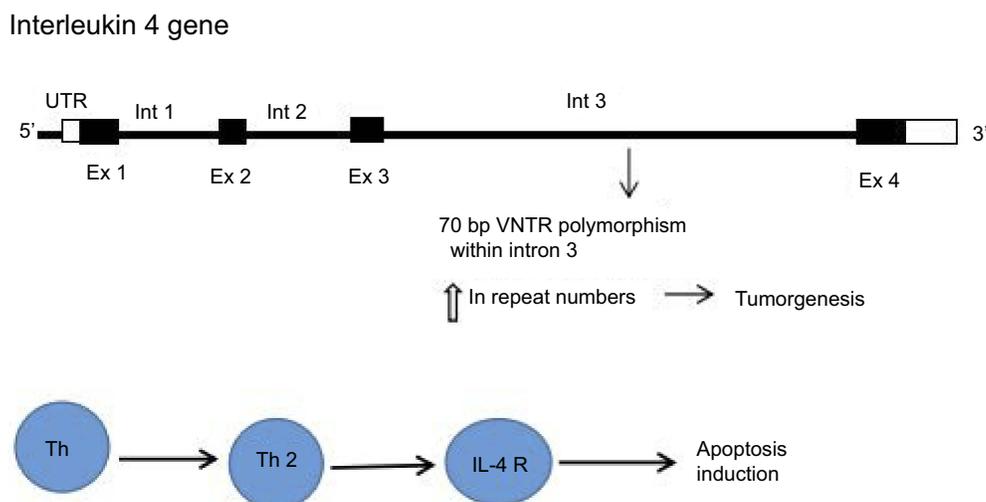
Figure 1 VNTR polymorphism locus within the *IL-4* gene.
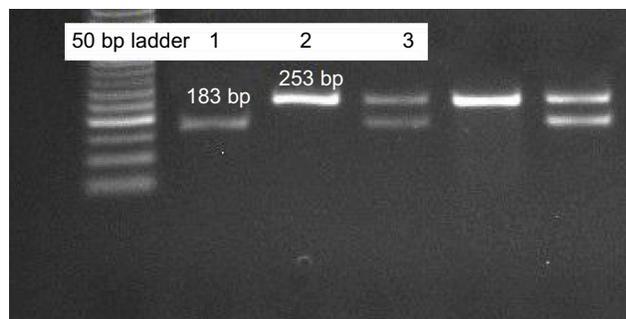**Abbreviation:** VNTR, variable number tandem repeat.

was used to calculate the differences between cases and controls.[22] The genetic association and phenotype-genotype analyses were conducted using the Statistical Package for the Social Sciences (SPSS), version 25.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

Results

VNTR of the IL-4 gene

The genotypes of the VNTR within the *IL-4* gene were analyzed using PCR and subsequent gel electrophoresis. Figure 2 shows the visualization of the resulting genotypes on the agarose gel. Three genotypes were detected in terms of their band sizes: allele 1 (1R) was present at a size of 183 bp and allele 2 (2R) at 253 bp, while the presence of the heterozygous alleles (2R\1R) was indicated by the appearance of both bands.

However, the genotypic and allelic frequency distribution of both the control and patient groups was analyzed and tested using Pearson's Chi-squared test (Table 1). As Table 1 illustrates, 79.4% of patients had 2R on both copies of the *IL-4* gene, which was not markedly different from the control group. Our results did not estimate the homozygous 1R genotype among patients, while only two healthy individuals carried the (1R\1R) genotype. The frequency of the heterozygous genotype (2R\1R) among patients was 26.7% compared to 19.5% in the control group; however, this was found to be nonsignificant ($p = 0.303$). Moreover, the allelic distribution of 1R and 2R genotypes was not significantly different between controls and patients (Table 1). Moreover, genetic association analysis using genetic models was performed. 2R/1R vs 2R/2R was the only applicable model and revealed no

Table 1 Genotypic and allelic frequency distribution of the VNTR in the *IL-4* gene for cases and controls

Genotypes and alleles	Case group	Control group	*p*-value*
1R	39 (10.8%)	48 (13.3%)	0.107
2R	321 (89.2%)	312 (86.7%)	
1R\1R	2 (1.1%)	0 (0%)	0.303
2R\2R	143 (79.4%)	132 (73.3%)	
1R\2R	35 (19.5%)	48 (26.7%)	

Note: *$P < 0.05$ is considered significant using Pearson Chi–squared test.

association between IL-4 VNTR and BC (OR: 0.67, 95% CI: 0.41–1.11, χ2: 2.47).

In addition to genetic association analysis, in this study, the correlation between the clinicopathological parameters and the VNTR of *IL-4* was investigated. Table 2 demonstrates the

Table 2 Association of *IL-4* polymorphism with clinical characteristics of breast cancer patients

Clinical characteristics		Genotype frequency (1R\2R) (%)	Genotype frequency (2R\2R) (%)	*p*-value
BMI**	≤25	39.10	21	0.035
	>25	60.90	79	
First pregnancy (age)*	<20	24.30	33.60	0.684
	≥20	75.70	66.4	
Age at breast cancer diagnosis*	<45	32.60	33.80	0.847
	≥45	67.40	66.20	
Age at first menstruation**	<13	28.90	30.90	0.190
	≥13	71.10	69.10	
Breastfeeding status*	Yes	65.20	66.20	0.702
	No	34.80	33.80	
Age at menopause**	<50	45.80	45.80	0.679
	≥50	54.20	54.2	
Family history*	Yes	26.1	34	0.863
	No	73.9	66	
Allergy*	Yes	21.70	29.80	0.973
	No	78.30	70.20	
Smoking*	Yes	29.50	29	0.582
	No	70.50	71	
Comorbidity*	Yes	45.70	34.30	0.820
	No	54.30	65.70	

Notes: *Genotype–phenotype association *p*-value using Pearson Chi-squared test. **Genotype–phenotype association *p*-value using ANOVA test. $p < 0.05$ is considered significant.

Figure 2 Agarose gel electrophoresis showing different VNTR genotypes in the *IL-4* gene. The size of the bands was determined through comparison to a 50 bp ladder. Lane 1 represents the 1R\1R genotype, with one band at 183 bp; lane 2 contains the 2R\2R genotype, as indicated by one band at 253 bp. On the other hand, the heterozygous genotype 1R\2R is represented by lane 3 (two bands with sizes of 183 bp and 253 bp, respectively).

relationship between a number of clinical features and the VNTR genotypes of BC patients. We found that 66% of BC patients were older than 45 years and had the 2R\2R genotype, while only 33% of the cases were under the age of 45 and had the 2R\2R genotype. However, this difference between the two groups was not statistically significant (p= 0.8).

Other clinical features, such as age at first pregnancy and age at menarche, were also explored in terms of the relationship between exposure to endogenous estrogen and different VNTR genotypes among BC patients. In the present study, we found that the only critical clinical parameter in the context of the VNTR genotypes was body mass index (BMI). Most patients with a BMI of more than 25 and double 2R genotypes (79%) were far more frequent than patients who had only one 2R and one 1R (p= 0.035). Additionally, the relationship between the pathological characteristics exhibited by BC patients and VNTR genotypes was studied (Table 3). In this study, we found none of the features to be critical for BC risk among the different VNTR genotypes, except for tumor differentiation (p= 0.028).

Discussion

BC is a serious problem that affects both genders, but it is particularly common among females.[1,4] While the cause of

BC has yet to be definitively determined, it is believed to be triggered by a combination of many risk factors, including environment, physiology, and individual genetics.[2,23] With regard to the genetic component of the disease, several studies have investigated the relationship between different genetic polymorphisms and the increased risk of developing BC in different populations.[24–27] For example, many studies have been conducted on the Jordanian population to investigate the genetic predisposition to BC. A range of genetic variants within significant genes have been explored among Jordanian BC patients, such as the IL-10 haplotype,[28] ERα PvuII and XbaI polymorphisms,[29] BRCA1/2[30,31] and RAD51.[32]

In the present study, we screened 180 healthy Jordanian women and 180 Jordanian BC patients to determine the relationship between the VNTR of a 70-bp sequence located in intron 3[22,23] within the *IL-4* gene and BC. Two different alleles were detected within the VNTR region of the *IL-4* gene: 2 repeats and 3 repeats. *IL-4* is a pleiotropic cytokine secreted by a wide range of epithelial cells, including BC cells. This cytokine has been implicated in the progression of BC and resistance to common therapeutic regimens driven by tumor-initiating cells (TICs).[33] The distribution frequency of the *IL-4*

Table 3 Association of *IL-4* polymorphism with pathological characteristics of breast cancer patients

Pathological characteristics		Genotype frequency (1R\2R) (%)	Genotype frequency (2R\2R) (%)	*p*-value
Progesterone receptor*	Positive	44.40	54.70	0.247
	Negative	55.60	45.30	
Estrogen receptor*	Positive	75.60	79.50	0.189
	Negative	24.40	20.50	
Tumor differentiation*	Low differentiation	29.50	33.60	0.028
	Mid & high differentiation	70.50	66.40	
Axillary lymph nodes*	Free of tumor	47.90	50	0.771
	Show metastatic Carcinoma	52.10	50	
Tumor stage*	Stage 1&2	95.10	89.50	0.246
	Stage 3&4	4.90	10.50	
Histology classification*	In situ carcinoma	13.30	20.50	0.093
	Invasive carcinoma	86.70	79.50	
Tumor size**	≤2CM	23.40	23.70	0.592
	2<x≤5	29.80	37.40	
	>5	46.80	38.90	
Lymph node involvement*	Yes	84.80	82.40	0.340

Notes: *Genotype–phenotype association *p*-value using Pearson Chi-squared test. **Genotype–phenotype association *p*-value using ANOVA test. *p*<0.05 is considered significant.

VNTR alleles varies among different populations, for example, the estimated frequencies for (1R, 2R) in China,[34] Malaysia,[11] North India[35], and Taiwan[36] were: (76.9%, 23.1%), (62.81%, 37.19%), (21.7%, 78.8%), respectively. In the current study, we found that 1R frequency among healthy Jordanian was 13.3%, while the 2R frequency was 86.7%.

The data related to *IL-4* VNTR and BC are very scarce. However, the 1R allele of the *IL-4* VNTR has been assigned as a risk factor for malignancy development, and in Taiwan 1R allele of *IL-4* VNTR was associated with bladder[36] and oral cancers in China.[37] In addition, the 1R/2R genotype of *IL-4*VNTR increased the risk of developing cervical cancer among North Indian women.[38]

In a recent study, Ibrahimi et al suggested that RP2\RP2 genotypes might be a potent protective factor against BC.[39] In addition, Jia et al[40] examined in a comprehensive meta-analysis the correlation between different types of genetic variants within the *IL-4* gene, including the VNTR, and cancer risk. In their analysis, only one study among Indian women reported a significant correlation between *IL-4* VNTR polymorphism and BC risk. That study stands in contrast to our results, which suggest that the *IL-4* VNTR is not associated with BC risk among Jordanian women.

In the present study, we described a group of the clinical and pathological features that might be associated with increased BC risk among Jordanian women. Moreover, we speculated regarding the correlation between these parameters and the VNTR of *IL-4*. Our results reveal that 33.8% of patients under the age of 45 had the homozygous 2R genotype, which is comparable to the proportion of patients with the heterozygous 2R\1R genotype at the same age (32.6%). Furthermore, age at first pregnancy (<20) and age at first menstruation (<13) were analyzed to detect the effect of endogenous estrogen. Though patients with the 2R\2R genotype were evidently more numerous than patients with the 1R\2R genotype at the critical age, there was no statistically significant association between each of the age at first pregnancy and the age at first menstruation on the one hand, and BC on the other. Moreover, 34% of patients with a family history of BC possessed the 2R\2R genotype, while 26.1% of such patients had the 1R\2R genotype. Despite this, we suggest that family history did not interfere with *IL-4* polymorphism in this study.

On the other hand, the relationship between allergy and BC has not been fully elucidated. Hedderson et al[41] declared that allergy history may be linked with reduced risk of BC for women who were diagnosed early. Although we did not find any significant relationship between BC and allergy, the estimated frequency of patients with a history of allergy and who possessed the 2R\2R genotype (29.8%) was lower than patients with the same genotype but without any history of allergy (70.2%). Otherwise, we could not assess any correlation between BC and the remaining clinical characteristics of menstrual condition, breastfeeding status, smoking, and comorbidity. Remarkably, the only clinical characteristic to show any significant association with BC was BMI. Of patients with the 2R\2R genotype, 79% had a BMI >25, while 60.9% of patients with a BMI >25 had the 2R\1R genotype. Correspondingly, we can mark BMI as a factor that may influence BC prognosis among patients who carry or are susceptible to the *IL-4* variant.

Similarly, we analyzed the pathological factors that may influence BC risk. Molecular receptors such as progesterone receptor (PR) and estrogen receptor (ER) status were examined. (ER) is one of the crucial predictive markers in BC prognosis and in anticipating response to hormone therapies.[42] We found that, of the patients carrying the 2R\2R genotype, 54.7% and 79.50% were screened with positive progesterone and ERs, respectively, which is slightly higher than among patients carrying the 1R\2R genotype.

Moreover, patients were placed into two different groups (low differentiation and medium to high differentiation) based on the rate of cancer progression. The variation between patients carrying the 2R\2R and 1R\2R genotypes was striking, as 70.5% of patients with medium and high differentiation had the 2R\1R genotype compared to 66.4% of those with the 2R\2R genotype. Correspondingly, our result revealed an association between tumor differentiation of BC and *IL-4* VNTR, suggesting that the 2R allele reduces the risk of BC. Other parameters, such as tumor size, lymph node involvement, and histology classification, did not show any significant differences between the different VNTR genotypes among BC patients. However, in this study, tumor differentiation was the only pathological factor to show a significant relationship with *IL-4* VNTR.

In conclusion, there is no significant relationship between the VNTR polymorphism within the *IL-4* gene and BC among Jordanian women. Furthermore, BMI and the tumor differentiation parameters of BC were associated with the variant genotypes. To the best of our knowledge,

this study is one of only a few in the Arab world to tackle the genetic component of BC in an Arab, and particularly the Jordanian, population. Due to the rapid rise of BC incidence in the developing world, more studies must be carried out in order to enhance survival rates by tailoring treatment regimens to the individual. Together with the established prognostic factors, the selection of BC genetic markers can help facilitate the rise of personalized medicine in the Arab world.

Data availability

The datasets generated and/or analyzed over the course of the study are not publicly available but are available from the corresponding author on reasonable request.

Ethical approval and informed consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the IRB at Jordan University of Science and Technology with ethical code number 32/104/2017. Informed consent was obtained from all individual participants included in the study.

Acknowledgment

Disclosure

The authors report no conflicts of interest in this work.

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