Association of Toll-like Receptor 4 + 3725G/C Polymorphism in Egyptian Breast Cancer Patients

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A B S T R A C T

The activation of Toll-like receptor 4 (TLR4) may be an important event in the immune evasion of tumor cells. TLR4 polymorphisms in breast cancer patients have recently attracted great interest. However, there has been little research on the association between the TLR4 +3725G/C polymorphism and the risk of breast cancer. This study aimed to investigate the frequency of TLR4 +3725G/C gene polymorphism in Egyptian breast cancer patients in order to evaluate its prognostic role in this malignancy. This study was conducted on 75 newly diagnosed breast cancer female patients and 25 apparently healthy control females. Genotyping of TLR4 +3725G/C polymorphism was performed by PCR/RFLP. The present study revealed that the frequencies of GC and CC genotypes of TLR4 +3725G/C polymorphism (P = 0.018) and TLR4 +3725C allele frequency (P=0.003) were significantly higher in breast cancer cases than controls. Furthermore, there was a statistically significant relation between TLR4 +3725GC, CC genotypes and tumor size, LN infilteration, ER and PR status in BC patients (P<0.001). Our results suggested that TLR4 +3725G/C polymorphism might be associated with increased susceptibility to breast cancer in Egyptian women and could be used as a prognostic marker for this malignancy.

Introduction

Breast cancer (BC) has become the most common female malignancy around the world. While BC is a global issue, in Egypt, the figure for people suffering from BC is alarming. According to the data of National Cancer Registry Program of Egypt (NCRPE) between years 2008-2011, BC is estimated to be the most common cancer among females accounting for 32.04%. It is also the leading cause of cancer related mortality accounting for 29.1% [1-2].

The etiology of breast cancer is a complex combination of both environmental and genetic factors, so the determination of genetic polymorphisms provided a new way to investigate the risk of such complex genetic disease [3]. BC prognosis is determined by a multiple set of factors including traditional prognostic factors such as TNM classification and nuclear grading, as well as biological prognostic factors such as tumor ER, PR and HER-2 expression [4]. New promising biological prognostic factors for breast cancer

are continuously being introduced, but it is unclear which of them should be adapted to clinical use [5].

Toll- like receptors (TLRs) are type-I transmembrane proteins known as pattern recognition receptors (PRR) [6]. They play a vital role in innate immune responses, being involved in the regulation of inflammatory reactions and activation of the adaptive immune response to eliminate infectious pathogens and cancer debris [7].

The first discovered human TLR was TLR4, it is one of the most prominent members of TLRs which is expressed in both immune and non-immune cells [8]. The human TLR4 gene is located on chromosome 9 (9q32-33) and consists of four exons and three introns with an overall length of approximately 19 Kb[9].

TLR4 has been implicated in signal transduction events induced by lipopolysaccharide (LPS) of gram-negative bacteria initiating a cascade of signaling pathways [10]. This leads to the activation of transcription factor NF-κB, and induces the expression of inflammatory cytokines, chemokines, adhesion molecules, growth factors and interferons which help regulate the activity of the immune system [11]. Persistent activation of TLR4-induced inflammatory signaling in chronic inflammatory conditions can contribute to carcinogenesis [12].

TLR4 expression has been described in different human tumors [13]. One study has reported that 63% of breast cancer patients were reported to express TLR4 on tumor cells and the level of expression inversely correlated with the survival [14]. Dysregulation of TLR4 owing to single nucleotide polymorphisms (SNPs) can disrupt the normal cellular immune response and may alter ligand binding and the balance between

pro- and anti-inflammatory cytokines, thereby increasing the risk of chronic inflammation and cancer [15]. The association of TLR4 SNPs with cancer risk has been widely investigated, However, few studies has reported the correlation between TLR4 polymorphisms and breast cancer [16]. TLR4 +3725G/C polymorphism is a single nucleotide polymorphism, located in the 3'-untranslated region (3'UTR) of TLR4 gene at base pair position +3725 and it has been reported to be involved in inflammation and cancer [17].

In the present study, we conducted a case–control study to find out the relevance of TLR4 +3725G/C polymorphism in breast cancer patients. Furthermore we also investigated whether there is a relation between TLR4 +3725G/C polymorphism and the clinicopathological variables and immunohistochemical markers and hence evaluating its prognostic role in breast cancer.

Materials and Methods

Study subjects

From June 2015 to June 2017, a hospital-based case–control study was conducted on 75 histologically confirmed, recently diagnosed breast cancer female patients (mean age 52.12 ± 11.11 years), recruited from General Surgery Department of Tanta University, Egypt. In addition to 25 apparently healthy females (mean age 50.12 ± 11.88 years) serving as healthy controls matched by age, and geographic origin with the patients group.

Clinicopathological information on all BC patients was obtained from medical records and pathology reports. The cases who had not received any chemo or radiotherapy were chosen for this study. The patients were staged according to the most recent American

Joint Committee on Cancer, Tumor size, Lymph Nodes, and Metastases (TNM) system [18]. Patients with metastatic disease or prior history of any kind of malignancy were excluded from this study.

The control subjects were randomly selected from the outpatient clinic of Tanta University. At the time of the study, the healthy controls had no evidence of any malignancy or other critical chronic disease. The study was approved by the ethical committee of Tanta University, and a written informed consent was obtained from all participants.

Genotyping of TLR4 +3725G/C polymorphism

Genomic DNA was extracted from venous blood samples using the QIAamp DNA blood mini kit (Qiagen, Germany) and then stored at -20°C until use. The TLR4 +3725G/C SNP was detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. DNA extracts were amplified for TLR4 +3725G/C SNP to form undigested fragments of 361 bp (Fig. 1). The primers used were: forward (F: 5′ACAAGTGATGTTTGATGGAC-3') and reverse (R:5′ GCCATTCTACCTGGTATAAG-3').

Briefly PCR was carried out in a final volume of 25 µl containing 200 ng genomic DNA, 1.5 mM MgCl2, 0.5 µM primer, 2 µL 10X PCR buffer, 0.2 mM dNTP, and 1.2 U Taq polymerase. For PCR amplification, the standard program was used as follows: one initial denaturation step at 95◦C for 6min followed by 35 cycles of 94◦C for 1min, annealing at 55◦C for 1min, and extension at 72◦C for 2min. A final extension step was at 72◦C for 10 min.

The PCR products (10 µl) were digested overnight at 37◦C with 5U of restriction enzyme Ear I. Digestion products were separated by electrophoresis through 2.5% agarose gel. The wild-type genotype GG was characterized by 163 bp and 198 bp fragments on the gel; the heterozygous variant genotype GC was characterized by 163 bp, 198 bp and 361 bp fragments; and the homozygous variant genotype CC was characterized by a 361 bp fragment on the gel (Fig. 2).

Statistical analysis

Statistical presentation and analysis of the present study were conducted using chi-squared test by SPSS software (version 15.0; SPSS Inc., Chicago, Illinois, USA). A value of p <0.05 was considered statistically significant.

Results and Discussion

Characteristics of the study population

The distributions of selected characteristics in the 75breast cancer patients and 25 healthy controls are presented in Table 1. The patients and controls were shown to be adequately matched for age (P =0.321). Nulliparous status, positive history of OCP intake and positive family history of BC were significantly higher in BC patients than in controls (P = 0.048, 0.015 and 0.002 respectively). No significant difference in menopausal status was found between BC patients and healthy controls (P =0.336).

Genotype and allele frequencies of the TLR4 +3725G/C polymorphism among the patients and controls

Genotype and allele distributions of theTLR4 +3725G/C polymorphism in breast cancer patients and the healthy control group are summarized in Table 2. The observed genotype frequencies of the TLR4 +3725G/C polymorphism were in agreement with the

Hardy-Weinberg equilibrium in both breast cancer patients and control groups (both P > 0.05). Distributions of genotypes GG, GC, and CC were 52.0%, 33.3%, and 14.7% among BC patients and 84.0%, 12.0%, and 4.0 % among the controls. The frequencies of GC and CC genotypes were significantly increased in BC patients than in the controls (P = 0.018). Regarding the TLR4 +3725G/C allele frequency, the percentage of C allele was significantly higher in BC patients (47%) compared to control group (5%) (P=0.003).

Table.1 Characteristics of the breast cancer patients and healthy controls

Characteristic	BC patients (n=75) N (%)	Control subjects (n=25) N (%)	P value
Age (years) **≤40** **>40**	14 (18.7%) 61 (81.3%)	7 (28.0%) 18 (72.0%)	0.321
Parity **Nulliparous** **One or more**	28 (37.3%) 47 (62.7%)	4 (16.0%) 21 (84.0%)	0.048*
Menopausal status **Premenopausal** **Postmenopausal**	25 (33.3%) 50 (66.7%)	11 (44.0%) 14 (56.0%)	0.336
History of OCP intake **Negative** **Positive**	33 (44.0%) 42 (56.0%)	18 (72.0%) 7 (28.0%)	0.015*
Family history of BC **Negative** **Positive**	48 (64.0%) 27 (36.0%)	24 (96.0%) 1 (4.0%)	0.002*

Table.2 Genotype and allele frequencies of the TLR4+3725G/C polymorphism in breast cancer patients and healthy controls

Frequencies of genotype or allele	BC patients (n=75) N (%)	Control subjects (n=25) N (%)	P value
Genotype GG GC CC	39 (52.0%) 25 (33.3%) 11 (14.7%)	21 (84.0%) (12.0%)3 1 (4.0%)	**0.018***
Allele G C	103 (68.7%) 47 (31.3%)	45 (90.0%) 5 (10.0%)	**0.003***

Table.3 Relation between the TLR4+3725G/C polymorphism and clincopathological parameters in BC patients

Clinicopathological parameters	Genotypes			P value
	GG (n=39)	GC (n=25)	CC (n=11)	
Histological grade				
I+II	35 (89.7%)	19 (76.0%)	3 (27.3%)	<0.001*
III	4 (10.3%)	6 (24.0%)	8(72.7%)	
Tumor size				
T1+T2	35 (89.7%)	10 (40.0%)	3 (27.3%)	<0.001*
T3+T4	4 (10.3%)	15 (60.0%)	8 (72.7%)	
Lymph node infiltration				
N0	25 (64.1%)	2 (8.0%)	0 (0.0%)	<0.001*
N1+N2	14 (35.9%)	23(92.0%)	11(100.0%)	
Estrogen receptor				
Negative	11 (28.2%)	24 (96.0%)	9 (81.8%)	<0.001*
Positive	28 (71.8%)	1 (4.0%)	2 (18.2%)	
Progesterone receptor				
Negative	8 (20.5%)	23 (92.0%)	8 (72.7%)	<0.001*
Positive	31(79.5%)	2 (8.0%)	3 (27.3%)	
Her2/neu status				
Negative	22 (56.4%)	11 (44.0%)	6 (54.5%)	0.615
Positive	17 (43.6%)	14 (56.0%)	5 (45.5%)	

Figure.1 Representative agarose gel electrophoresis picture of TLR4 +3725G/C polymorphism (PCR bands of the amplified product at 361 bp as compared with DNA ladder)
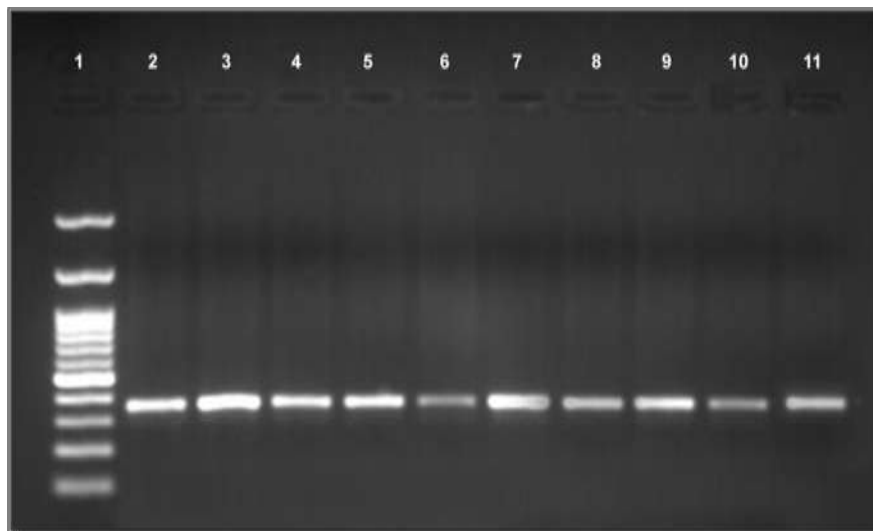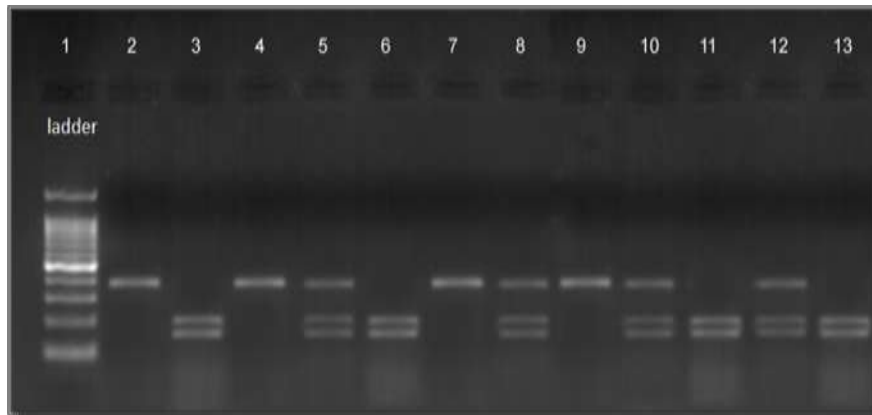
Figure.2 Representative agarose gel electrophoresis picture of TLR4 +3725G/C polymorphism
after treatment with Ear I restriction enzyme

Lanes 1: DNA ladder (100 bp marker)
Lanes 3, 6, 11 and 13: wild type (GG) [163 bp and 198 bp]
Lanes 5, 8, 10 and 12: heterozygous variant (GC) [163 bp, 198 bp and 361 bp]
Lanes 2, 4, 7 and 9: homozygous variant (CC) [361bp]

TLR4+3725G/C polymorphism and clincopathological parameters in BC patients

Table 3 shows the association of the TLR4+3725G/C polymorphism with various clinicopathological parameters in breast cancer patients.TLR4 +3725 CC genotype was significantly higher in BC patients with grade III (P<0.001). Also, GC and CC genotypes were significantly increased in BC patients with tumor size T3+T4, lymph node infiltration, ER -ve and PR −ve (P<0.001). However, there was no significant difference regarding Her2/neu status (P=0.615).

TLR4 can act as a double-edged sword in cancer because they can have both anti-tumorigenic and protumorigenic effect [19]. Cancer cells activated by TLR4 signals may release cytokines and chemokines that in turn may recruit immune cells and stimulate them to release further cytokines and chemokines. This process results in a cytokine profile that is associated with immune tolerance, cancer progression, and propagation of the tumor microenvironment [20].

TLR4 expression has been described in different human tumors. One study has shown that breast cancer cells have high expression levels of TLR4, indicating that the TLR4 may be critical in the development of breast cancer [21]. The response to TLR4 ligands may be impaired by SNPs that are present in TLR genes, resulting in a modified susceptibility to infectious or inflammatory diseases and cancer [22].

The TLR4 +3725G/C polymorphism is a single nucleotide polymorphism located in the 3'-untranslated region of TLR4 gene where it may have a direct effect on mRNA stability and thereby disrupt the innate immune response, inducing inflammation and subsequent carcinogenesis [23]. Although the biologic role of the present polymorphism remains yet to be clarified, the polymorphism might have some influence on transcription and/or translation of TLR4 [17].

This study aimed to investigate the association of TLR4 +3725G/C polymorphism with breast cancer risk in Egyptian women and to evaluate it prognostic role in BC.

In the current study, there was a significant difference in the distribution of the TLR4+3725 G/C genotypes between breast cancer patients and healthy controls. It was found that GC (heterozygous variant) and CC (homozygous variant) genotypes were significantly higher than GG genotype (wild type) in BC patients than the healthy controls. Also it was revealed that the frequency of C allele was significantly higher in breast cancer patients.

This finding was in agreement with Yang *et al.,* [24] who conducted the first study linking TLR4 +3725G/C polymorphism with breast cancer susceptibility and prognosis. Their study showed that TLR4 +3725GC genotype, CC genotype, and C allele were associated with an increased susceptibility to breast cancer in Chinese population.

Besides, Sato *et al.,* [23] demonstrated the biological significance of TLR4 +3725G/C polymorphism. They reported that monocytes from TLR4 +3725CC subjects expressed significantly higher levels of TLR4 on their surface than those from TLR4 +3725GG subjects. When peripheral blood mononuclear cells (PBMCs) were stimulated with LPS, a TLR4 ligand, the cells from the TLR4 +3725CC and GC subjects secreted significantly higher levels of the proinflammatory cytokine IL-8 compared to cells from the GG subjects.

The TLR4 +3725G/C SNP is associated with various diseases. For example, a study conducted by Zheng *et al.,* [25] revealed an association between TLR4 +3725G/C SNP and prostate cancer in Swedish patients.

Later, Fukusaki *et al.,* [26] found that +3725CC genotype was significantly higher in both moderate and severe periodontitis patients. In addition, Hishida *et al.,* [17] observed the GC or CC genotype is associated

with severe gastric atrophy in *Helicobacter pylori* seropositive Japanese subjects. These observations suggest that the TLR4 +3725G/C SNP may influence human inflammatory and/or malignant diseases [23].

Regarding TLR4 +3725G/C polymorphism and the tumor' clinicopathological characteristics in BC, the present study revealed a significant strong association between TLR4 +3725GC and CC genotypes with tumor size and LN infiltration in BC patients. Also +3725CC genotype was significantly higher in BC patients with advanced tumor grade III than those with tumor grade I+II.

This was in agreement with Yang *et al.,* [24] who reported that tumor characteristics such as nodal involvement, poor histological grade, and advanced tumor stage were all found to be significantly associated with TLR4 +3725G/C polymorphism (GC, CC genotypes) and poor outcome in breast cancer. Additionally, they reported that results of Cox multivariate regression survival analysis showed that breast cancer patients carrying the mutant C allele presented a significantly lower survival rate than those with wild-type G allele. Thus, individuals with C allele indicated a worse prognosis of breast cancer.

To our knowledge this is first study to evaluate the relation of TLR4 +3725G/C polymorphism with the immunohistochemical markers status (ER, PR and HER2/neu) in breast cancer patients.

The current study revealed that TLR4 +3725GC and CC genotypes were significantly higher in estrogen receptor/progesterone receptor-negative breast cancer; however there was no significant association with HER2/neu.

This was in agreement with other studies that evaluated the association of TLR4 expression or other TLR4 polymorphisms with the hormonal receptor status in BC. Semlali *et al.,* [27] reported that The TLR4 polymorphism rs4986790 was strongly associated with BC in the ER−ve patient groups. Mehmeti *et al.,* [28] revealed that all ER+ cell lines were negative for TLR4, also TLR4 expression correlated significantly with the ER/PR-negative patient group, however it did not correlate to Her2/neu expression.

In conclusion, this study suggested that TLR4 +3725G/C polymorphism might be associated with increased risk of breast cancer in Egyptian women. In addition, it was also shown to be strongly associated with poor prognosis in BC patients, providing a better understanding of the implication of TLR-4 +3725G/C polymorphism in breast tumorigenesis and for its eventual use as a cancer prognostic marker. However, still more comparative studies on large sample size are needed to evaluate the associations between TLR4 +3725G/C polymorphism and breast cancer risk.

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