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ARTICLE

Association of TNF-α and TNF-β Gene Polymorphism with Steroid Receptor Expression in Breast Cancer Patients

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The presence of estrogen and progesterone receptors is correlated with good prognosis in breast cancer. The effect of TNF-α on down-regulation of estrogen receptor and blocking the proliferative response of breast cancer cells to estradiol have been demonstrated. However, the effect of TNFA and TNFB gene polymorphisms on the expression of steroid receptors in breast cancer cells is not well documented. Therefore, 160 breast cancer patients were recruited to investigate the association of TNFA and TNFB gene polymorphism with the level of steroid receptor expression. This association was not found to be significant for TNFA polymorphism and estrogen receptor expression (p=0.07). However, when combined genotypes of TNFA and TNFB polymorphism was considered, homozygous patients for lower TNF-α producer genotypes (TNFA1/A1 and TNFB1/B1) showed significantly higher progesterone receptor expression (p=0.041). Our findings indicate that TNFA and TNFB polymorphisms may be associated with the levels of steroid receptor expression in breast cancer patients. Further studies on a larger group of breast cancer patients are recommended. (Pathology Oncology Research Vol 11, No 2, 99–102)

Key words: breast cancer, steroid receptors, TNF, polymorphism

Introduction

Steroid hormones bind with high specificity and affinity to intracellular receptors. These steroid receptors belong to a "superfamily" of proteins whose function is to control the transcription of a repertoire of other cellular genes.[1] Steroid receptors such as estrogen receptor (ER) and progesterone receptor (PR) are responsible for the regulation of some genes which are involved in controlling cell growth. In fact, the majority of breast tumors are initially dependent upon estrogen to support their growth, with the highest incidence of breast cancers occurring in postmenopausal women at time when ovarian production of estrogens has ceased. In this group of women estrogens continue to be produced at extraglandular sites, such as adipose tissues.[2] There is now convincing evidence implicating TNF-α in regulating the activities of the enzymes that are involved in estrogen synthesis,[3-5] and also in blocking the proliferative response of breast cancer cells to estradiol through down-regulation of ER.[6] TNF-β (lymphotoxin) is another cytokine which has close structural homology and about 30% amino acid sequence identity to TNF-á, and is recognized by the same widely distributed cellular TNF receptors.[7] As a consequence, many of their numerous effects are similar. Therefore, it is possible that individuals who genetically produce high level of these cytokines might show reduced expression levels of ER in breast cancer tissues.

Of interest, the secretion of TNF-α and TNF-β is believed to be influenced by genetic polymorphisms within their genes. A polymorphism at position -308 in the promoter region of the TNFA gene, consisting of a G in the common allele (TNFA1) and an A in the uncommon allele (TNFA2), modifies gene expression.[8] In vitro studies show that TNFA -308 A allele displays increased gene transcription as compared with the common allele.[8] A polymorphism also exists at position 252 within the first intron of the TNFB gene, consisting a G (TNFB +252 G)

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on one allele and an A (TNFB +252 A) on the alternate allele.[9] Individuals homozygous for TNFB +252 A (known as TNFB2 in most studies) have been shown to secrete increased levels of TNF-α in response to sepsis.[10] Therefore, in the present study we asked whether the mentioned di-allelic polymorphisms within the genes of these cytokines might play a role in steroid receptor expression in breast cancer.

Table 1. **Primer sequences and methods for determination of TNFA (-308) and TNFB (+252) gene polymorphisms**

Locus	*Primers*	*Methods*
TNF-α (-308)	common primer, 5′-tct cgg ttt ctt ctc cat cg-3′ G allele primer, 5′-ata ggt ttt gag ggg cat gg-3′ A allele primer, 5′-ata ggt ttt gag ggg cat ga-3′	ASO-PCR[1]
TNF-β (+252)	5′-ccg tgc ttc gtg gtt tgg act-3′ 5′-aga ggg gtg gat gct tgg gtt c-3′	*Nco*I based PCR-RFLP[2]

[1]Allele-specific oligonucleotide polymerase chain reaction
[2]PCR-restriction fragment length polymorphism

Materials and methods

Patients

The study included 160 consecutive patients from Fars province, Iran, presenting with operable primary breast cancer. Patients with infiltrative ductal carcinoma were included in the present study. None of the patients was under tamoxifen or hormone replacement therapy. The median age of patients was 49.6 years (ranging 27-85 years).

The level of ER and PR expression

Tumor site was determined by pathologists on fresh tissue. For immunohistochemical studies, the most representative paraffin blocks were selected for the study of molecular markers. To determine the level of ER and PR expression, an antigen retrieval protocol based on oven heating was applied to tissue sections, and immunohistochemistry was performed according to the instructions of the manufacturer (LSAB2 kit, DAKO Corporation, Denmark). Scores of 0, 1+, 2+, and 3+ were given according to the summation of intensity and proportion of positive cells in immunohistochemically stained sections. Scores =2 were considered as positive.

Determination of cytokine genotype

Genomic DNA was extracted from peripheral blood leukocytes by a salting out procedure. An allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) was used to detect the polymorphism at position -308 of TNFA gene.[11] As an internal control, the b-globin-specific primers were included in the ASO-PCR (5' primer: 5'-ACACAACTGTGTTCACTAGC-3', and 3' primer: 5'-CAACTTCATCCACGTTCACC-3'). The genotypes of TNFB were determined by PCR-RFLP,[10] using primer sequences given in *Table 1*. For TNFA genotyping, 50 µl of PCR reaction mixture consisting of 250 ng of genomic DNA, 200 mmol/L dNTPs, 2 mM $MgCl_2$, 1x Taq DNA polymerase buffer, 2 units of Taq DNA polymerase (Boehringer Mannheim, Germany), 10 pmol of each test primer and 5 pmol of internal control primers were employed. Cycling conditions included: 95°C for 5 minutes; 31 cycles at 94°C for 30 seconds, 61°C for 150 seconds, and 72°C for 30 seconds; 72°C for 10 minutes. Cycling conditions of PCR for TNFB genotyping were those for TNFA. Subsequent to the amplification, the PCR product (10 ìL) was digested with 5 U *NcoI* at 37°C for >16 hours. The TNFB1 allele gave 2 fragments of 586 and 196 bp, and the TNFB2 allele gave a single 782-bp fragment. Reaction products of TNFA and TNFB were separated on a 2-2.5% agarose gel and stained with ethidium bromide (*Figure 1*).

Statistical methods

Data were analyzed using chi-square test and Fisher's exact test when appropriate. All tests were performed two tailed with a confidence interval (CI) of 95%. Statistical calculations were carried out using the Epi Info 2000 software.

Results

Immunohistochemical analysis of 160 formalin-fixed and paraffin-embedded breast cancer tissues revealed that 27.0% and 33.1% of cases were positive for ER and PR, respectively. Frequency of TNFA and TNFB genotypes is showed in *Table 2*. When the patients were stratified according to TNFA genotypes, ER expression of patients carrying the TNFA1/A1 genotype showed a trend to be higher than that of patients carrying the TNFA1/A2 genotype (29.9% vs. 9.1%, respectively; p=0.07). Accordingly, TNFA2 allele showed a near significant association with the absence of ER by Yates-corrected chi-square analysis (p=0.086; OR=3.96, CI=0.87-25.1). No significant association was observed between PR expression and TNFA genotypes (p=0.5; R=1.35, 95% CI=0.64-2.93) or allele frequencies (p=0.7; OR=1.35, 95% CI=0.48-3.99). Also, stratification of steroid receptor data according to TNFB genotypes showed no significant association between ER and PR expression and TNFB genotypes (p<0.53 and

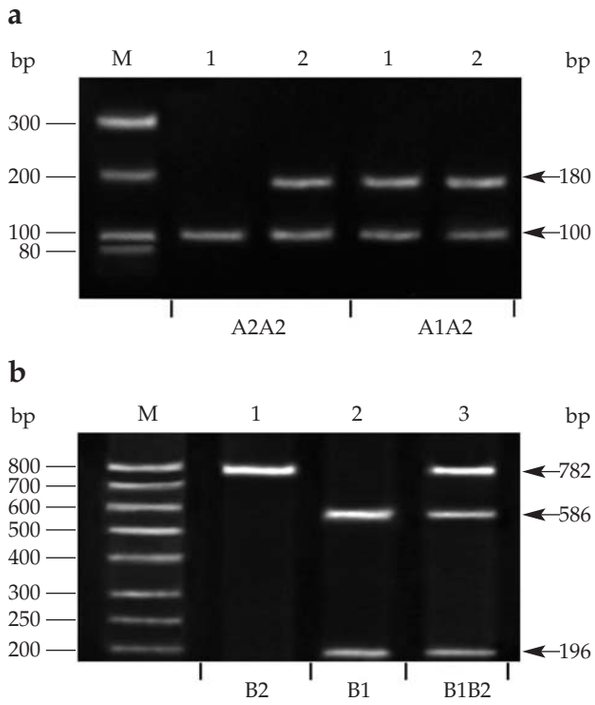

Figure 1. *Band patterns of the PCR products for TNF-α and TNF-β polymorphisms. M stands for molecular size marker.* ***(a)*** *Determination of TNF-α genotypes by ASO-PCR: 100 bp internal control. Band of 184 bp allele present or absent; lane 1: TNFA1 allele, lane 2: TNFA2 allele. Genotypes of each individual are shown beneath the figure.* ***(b)*** *Determination of inherited TNF-β alleles in three different individuals by PCR-RFLP: 782 bp B2 allele; 586, 196 bp B1 allele. Alleles of each individual are shown beneath the figure.*

p<0.2, respectively). In the same manner, allelic distribution of the TNFB gene also showed no significant association with ER or PR expression (p=0.54 and p=0.81, respectively). However, when the combined genotypes of TNFA and TNFB genes were taken into consideration, low producer genotypes of TNF-α (patients who are homozygous for TNFA1 and TNFB1 genotype) showed significantly higher percentage of PR positive tumors compared to those with high producer genotypes of TNF-α (p=0.041). TNFA and TNFB genotype distributions in breast cancer patients with steroid receptor positive and receptor negative status are shown in *Table 2*.

Discussion

Human breast cancer is a neoplasia characterized by considerable heterogeneity in its behavior.[12] At the present time, steroid hormone receptor expression is one of the best prognostic factors frequently used to identify patients who can benefit from hormone therapy. Furthermore, the presence of steroid receptors in tumor tissue is an important prognostic factor that correlates with higher survival rates and lower risk of relapse.[13] Therefore, identification of new factors that affect steroid receptor expression in breast cancer would yield further insight into breast tumorigenesis. There is now increasing evidence showing the importance of TNF-α in extraovarian synthesis of estradiol,[3-5] and also in the control of ER expression.[6] Furthermore, a clear inhibition of progesterone-induced transcriptional activity was reported when TNF-α was added to the culture of HeLa and T47D cells.[14] Therefore, in the present study the possibility that di-allelic functional polymorphisms within the genes for TNF-α and TNF-β might determine the expression levels of ER and PR in breast cancer was investigated. Our results showed a marginally significant association between TNFA -308 A/G polymorphism and the expression levels of ER in our set of breast cancer patients (p=0.07). In fact, as shown in *Table 2*, 90.9% of patients who carry the TNFA2 allele (high producer allele) were negative for ER. Our finding is supported by the report of Danforth et al. showing that TNF-α down-regulated ER expression.[6] This process is enhanced in the presence of estradiol,[6] and TNF-α could synergize its effect on the down-regulation of ER by the conversion of estrone to estradiol.[4]

In addition to the similarity in TNF-α and TNF-β function, there are reports which suggest that polymorphisms in their genes mutually influence TNF-α production. In fact, peripheral blood mononuclear cells (PBMC) from individuals carrying the TNFB2 allele secreted more TNF-α than those from TNFB1/B1homozygous ones.[10,15] Yet again, PBMC from individuals carrying a TNFA2 allele produced more of both TNF-α and TNF-β under the stimulation of T lymphocytes than did PBMC from individuals homozygous for TNFA1.[16] Because of these evidences that different combinations of alleles at the TNFA and TNFB loci can affect cytokine secretion, we considered it important to include both loci in our investigation of relationship between TNF polymorphisms and steroid receptor expression in breast cancer. It is surprising that, contrary to the above mentioned marginally significant association between TNFA –308 A/G polymorphism and the expression levels of ER (p=0.07), investigation of the simultaneous effect of TNFA and TNFB genotype on the level of ER expression showed no significant association between high TNF-α producing genotypes and lower percentage of ER expression (p=0.7). The possible biological explanation for this observation is not clear. However, our results showed that patients who carry the TNFA2 and/or TNFB2 allele had lower percentage of PR positive tumors compared to patients with TNFA1/A1 and TNFB1/B1 homozygous genotypes (p=0.041). Therefore, carrying the high producer TNFA genotypes (TNFA1/A1 or TNFA1/A2+ TNFB1/B2 or TNFB2/2 and TNFA1/A2+TNFB1/B1)

Table 2. **TNFA and TNFB genotype frequencies in breast cancer patients stratified according their steroid receptor status.**

	TNFA		*TNFB*		
	A1/A1 number(%)	*A1/A2 number(%)*	*B1/B1 number(%)*	*B1/B2 number(%)*	*B2/B2 number(%)*
ER					
+	41(25.8)	2(1.2)	2(1.3)	18(11.6)	19(12.2)
–	96(60.4)	20(12.6)	13(8.4)	48(31.0)	55(35.5)
PR					
+	47(29.4)	6(3.7)	8(5.1)	22(14.1)	22(14.1)
–	91(56.9)	16(10.0)	7(4.5)	44(28.2)	53(34.0)

would determine the higher percentage of PR^- tumors (69.3%; 97/140) compared to low producer TNF-α genotypes (36.4%; 4/11) in breast cancer. Of interest, TNF-α was found in high concentrations in patients with cancer,[17] and endogenous TNF-α production was linked to protection against cytotoxicity of exogenous TNF-α.[18] These reports suggest that TNF-α may act as an endogenous tumor promoter in vivo. There are also many reports indicating that the presence of steroid receptors is correlated with good prognosis in breast cancer.[19] By considering the above mentioned reports and results of the present study, we hypothesized that genetic ability to produce high levels of endogenous TNF-α could promote breast cancer progression by down-regulation of PR.

In conclusion, the present study defines an association between TNFA and TNFB gene polymorphisms with the expression of steroid receptors in breast cancer. These findings indicate an important interaction between the immune and endocrine systems. In fact, genetic ability to produce higher levels of TNF-α by immune cells and adipocytes could down-regulate PR expression at the tumor site, and thereby affect breast cancer progression.

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