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TNF alpha promoter polymorphisms analysis in benign and malignant breast lesions

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ABSTRACT

Polymorphisms in genes involved in the complex mechanisms of carcinogenesis may affect the susceptibility to cancer. The multifunctional cytokine, tumor necrosis factor alpha (TNF alpha) has an important role in the pathogenesis of inflammatory, autoimmune and malignant diseases. It has a large spectrum of activities, including both antitumorigenic and protumorigenic. In recent years, several TNF alpha promoter polymorphisms have been identified and related to the expression level of cytokine and to the susceptibility to solid tumors. The aim of our study was to investigate the frequency of three TNF alpha promoter polymorphisms (-1031, -308 and -238) in benign (fibrocystic changes) and malignant (invasive carcinoma) breast lesions. Using "real-time" PCR SNP analysis these polymorphisms were determined in 76 patients with benign and 158 patients with malignant breast lesions. The high expression genotypes at any of the three SNP polymorphisms were more frequent in invasive breast carcinoma (in 81 of 158 examined, 51,3%) than in fibrocystic changes (in 33 of 76 examined, 43,4%). The combined frequency of high production genotypes (-1031 T/C and C/C, -308 G/A and A/A and -238 G/A and A/A) was higher in patients with invasive breast carcinoma than in those with fibrocystic changes. However, these results were not statistically significant. Further studies on a larger group of patients are needed to evaluate the significance of potential differences in TNF alpha genotypes in different breast lesions.

Keywords: breast lesions, TNF alpha promoter polymorphisms

INTRODUCTION

The etiology of breast cancer is extremely complex and while not yet elucidated, appears to involve numerous genetic, endocrine, and external environmental factors. Breast cancer is the most common non-cutaneous malignancy in women and is second only to lung cancer in mortality rates (Tsongalis and Ricci, 2003). The increased incidence of breast cancer in the last 20 years is coincident with a large improvement in early detection due to the implementation of mammography as the standard for surveillance (Tabar et al., 2001). Despite the mass screening efforts and some advances in adjuvant therapy, the overall mortality rate for breast cancer has not yet changed significantly to reflect the rather dramatic shift to earlier stage detection (Olsen and Gotzsche, 2001). A better understanding of tumor cell biology and development of novel therapeutic drugs are expected to make major advances in improving breast cancer outcomes. The role of genetic factors in epidemiology and pathogenesis of both sporadic breast cancer and familial breast cancer are now well established. Only a small minority (~5%) of patients with breast cancer develop the disease as a result of inheritance of germline mutations in dominant, highly penetrant susceptibility genes such as BRCA1 and BRCA2. However, polymorphisms in genes involved in the complex mechanisms of carcinogenesis may confer low penetrant susceptibility to breast cancer in a significant proportion of the remainder of the patients (Greene, 1997; Coughlin and Piper, 1999). The multifunctional cytokine, tumor necrosis factor alpha (TNF alpha), is involved in the promotion of inflammatory responses and plays a critical role in the pathogenesis of inflammatory, autoimmune and malignant diseases (Bazzoni and Beutler, 1996). Initially proposed to have anti-carcinogenic effects (Jaattela, 1991), TNF alpha was later shown to be tumorigenic in both *in vitro* (Komori et al., 1993) and *in vivo* studies (Fujiki and Suganuma, 1994). High plasma TNF alpha levels in cancer patients are associated with a poor disease outcome (Warzocha et al., 1997; and Nakashima et al., 1998). TNF alpha is also a key angiogenic molecule that may promote angiogenesis directly by stimulating endothelial cell proliferation and indirectly by modulating expression of other proangiogenetic factors (Leek et al., 1998). Several TNF alpha promoter polymorphisms have been identified and have been implicated in the regulation of TNF alpha transcription (Kroeger et al., 1997; Wilson et al., 1997). Single nucleotide polymorphisms at -308 and -238 of the promoter region of the TNF alpha gene have been commonly studied. The -308 polymorphism is a G → A substitution and reportedly affects gene expression, the rare A allele resulting in higher TNF alpha production *in vitro* (Bouma et al., 1996). For the -238 polymorphisms, the rare A allele has been shown to be associated with high

TNF alpha production (Grove et al., 1997). The -1031 polymorphism is a T → C substitution and the rare C allele has been shown to be associated with high TNF alpha production (Higuchi et al., 1998).

The aim of our study was to investigate the frequency of these three polymorphisms in breast lesions, fibrocystic changes and invasive breast carcinoma.

MATERIAL AND METHODS

Patients and tissue specimens

This retrospective study was carried out using specimens of 76 benign (fibrocystic changes without proliferative breast disease) and 158 malignant (invasive carcinoma) breast lesions. All specimens were obtained from the Croatian Human Tumor Bank (Spaventi et al., 1993). All specimens were obtained through routine surgery; the diagnoses were established by standard diagnostic procedures and confirmed histopathologically. The study included 76 women with benign breast lesions with age range between 19 and 75 years (mean age 45.1 years) and 158 women with malignant breast lesions with age range between 23 and 82 years (mean age 59.2 years).

Fresh samples of resected tissue were snap-frozen in liquid nitrogen and stored in the Human Tumor Bank at -80°C until further use. Before inclusion in the study, each specimen was verified by a histopathologist (S.K.).

DNA isolation

DNA isolation from frozen-tissue sections was performed using digestion buffer (50mM Tris, pH 8.5, 1 mM EDTA, 0.5% Tween 20) and proteinase K (2 mg/ml, Sigma).

"Real-time" PCR analysis of TNF alpha SNP promoter polymorphisms

"Real-time" PCR analysis for three TNFα SNP promoter polymorphisms was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA) and predeveloped TaqMan assay reagents, C_7514871_10 (rs1799964) for TNF alpha -1031 C/T SNP, C_7514879_10 (rs1800629) for TNF alpha -308 A/G SNP and C_2215707_10 (rs361525) for TNF alpha -238 A/G SNP. The PCR reaction was carried out according to the manufacturer's protocol.

Statistical analysis

The data were analyzed using $\chi 2$ statistics. Difference was considered significant when P value was less than 0.05.

RESULTS

Genetic polymorphisms of TNFα were determined in 76 patients with fibrocystic changes and 158 with invasive breast carcinoma. Using "real-time" PCR SNP analysis we detected TNF alpha -1031, -308 and -238 genotypes. The genotype frequencies of the three polymorphisms are presented in Table 1. Genotypes frequencies were distributed in accordance with Hardy-Weinberg equilibrium.

The high expression genotypes at any of the three SNP polymorphisms were more frequent in invasive breast carcinoma (in 81 of 158 examined, 51,3%) than in fibrocystic changes (in 33 of 76 examined, 43,4%), but not statistically significant (Figure 1).

The combined frequency of high production genotypes (-1031 T/C and C/C, -308 G/A and A/A and -238 G/A and A/A) was higher in patients with invasive breast carcinoma than in those with fibrocystic changes but not statistically significant (Table 1, Figure 2).

The haplotype analysis revealed no statistically significant difference in haplotype distribution between two groups of breast lesions (Table 2).

DISCUSSION

TNF alpha has a large spectrum of activity including both protumorigenic and antitumorigenic activity (Naylor et al., 1990; Balkwill, 2002). It has been shown that dysregulation and overproduction of TNF alpha could be involved in cancer development and progression (Mocellin et al., 2005). Blood levels of TNF alpha are significantly higher in patients with solid tumors including breast cancer (Ardizzoia et al., 1992; Anderson, 2004). Several mechanisms of pro-tumor activities of TNF alpha in breast carcinoma have been suggested: induction of promalignant chemokines, matrix metalloproteinases, endothelial adhesion molecules, angiogenic mediators and reactive oxygen intermediates (Ben-Baruch, 2003).

In recent years, several TNF alpha promoter polymorphisms have been identified and their association with susceptibility to solid tumors as well as with high TNF alpha expression has been shown. Single nucleotide polymorphisms at -308 and -238 of the promoter region of the TNF alpha gene have been commonly studied. Many studies have shown that inheritance of the TNF alpha-308 A allele is associated with increased production of TNF

alpha (Baseggio et al., 2001; Sallakci et al., 2005; Jeong et al., 2004). Several authors have directly demonstrated markedly higher transcription of TNF alpha *in vitro* in association with -308A allele (Abraham et al., 1999; Kroeger et al., 1997; Kroeger et al., 2000, Wilson et al., 1997), but some other studies didn't support these findings (Brinkman et al., 1996, Stuber et al., 1996, Uglialoro et al., 1998). These differences might be related to cell-specific factors and the differences in the stimuli that have been used (Suriano et al., 2005). For the -238 polymorphism, the rare A allele has been shown to be associated with high TNF alpha production (Grove et al., 1997). But data on the association between the -238 polymorphism and the TNF alpha production are not equivocal. Some authors have found no association between -238 polymorphism and TNF alpha production (Paciot et al.,1995; Kaijzel et al., 1998). The -1031 polymorphism is a T → C substitution and the rare C allele has been shown to be associated with high TNF alpha production (Higuchi et al., 1998). The aim of our study was to investigate the frequency of -1031, -308 and -238 TNF alpha promoter polymorphisms in breast lesions: fibrocystic changes and invasive breast carcinoma.

It was shown that TNF alpha -308 A/A genotype may play an important role in the tumorigenesis of breast carcinoma (Mestiri et al., 2001). Azmy and coworkers (2004) demonstrated no association between the -308 and -238 TNF alpha polymorphism and susceptibility to breast cancer in a North European population. However, the -308 polymorphism was found to be associated with the presence of vascular invasion in breast tumors. Park and coworkers (2002) did not find the association between -1031, -863, -857 and -308 SNPs in TNF alpha promoter and susceptibility to breast cancer. Smith and coworkers (2004) found a non-significant trend for association between the TNF alpha -308 GG genotype and breast cancer compared to controls. To our knowledge, our study is one of the first reports showing the frequency of TNF alpha promoter polymorphisms in fibrocystic breast changes compared with their frequencies in invasive breast carcinoma.

In this study, using "real-time" PCR SNP analysis, genetic polymorphisms of TNF alpha were determined in 51 patients with fibrocystic changes and 82 with invasive breast carcinoma. The high expression genotypes at any of the three SNP polymorphisms were more frequent in invasive breast carcinoma (in 35 of 82 examined, 42.9%) than in fibrocystic changes (in 17 of 51 examined, 33.3%), but not statistically significant. Samples with two high expression genotypes were also more frequent in invasive breast carcinoma than in samples with fibrocystic changes but not statistically significant. The combined frequency of high production genotypes (-1031 T/C and C/C, -308

G/A and A/A and -238 G/A and A/A) was higher in patients with invasive breast carcinoma than in those with fibrocystic changes but not statistically significant.

Previous breast biopsy showing benign conditions is considered as a possible risk factor for breast cancer. However, only a few types of benign breast lesions have significant premalignant potential (Allred and Mohsin., 2000). The magnitude of the risk depends a great deal on the histological category of the benign breast lesion, degree of proliferative changes and the atypia presence in the biopsy material being the most important factors (Schnitt, 2003; Bilous et al., 2005). Women with proliferative lesions without atypia have 1.5 to 2 fold increase in risk (Page and Dupont., 1990), while women with atypical hyperplasia have fourfold to fivefold increase in breast cancer risk (Fitzgibbons et al., 1998). Several clinical factors modify the risk associated with these lesions such as time elapsed since biopsy, menopausal status, and family history of breast cancer (Schnitt, 2003). Benign breast lesions included in this study were of the non-proliferative type. The consensus view is that women with nonprolifetarive fibrocystic changes are not at significantly increased risk of developing breast cancer (Richie and Swanson, 2003; Dupont and Page, 1985; Fitzgibbons et al., 1998).

Our results show a difference between TNF alpha genotypes associated with fibrocystic changes and those found in invasive breast carcinoma but this diference was not statistically significant. Further studies on a larger group of patients are needed to evaluate the significance of potential differences in TNF alpha genotypes in different breast lesions.

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FIGURE LEGENDS

Figure 1.Frequency of common and high expression genotypes in fibrocystic changes and invasive breast cancer.

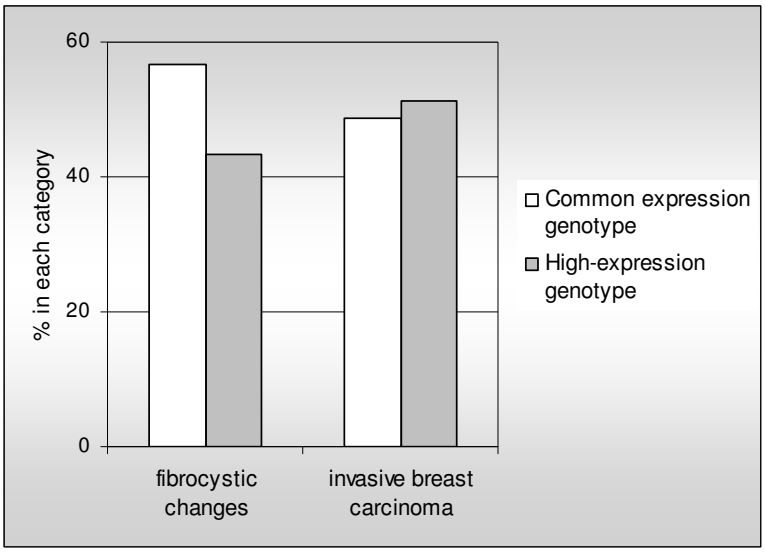
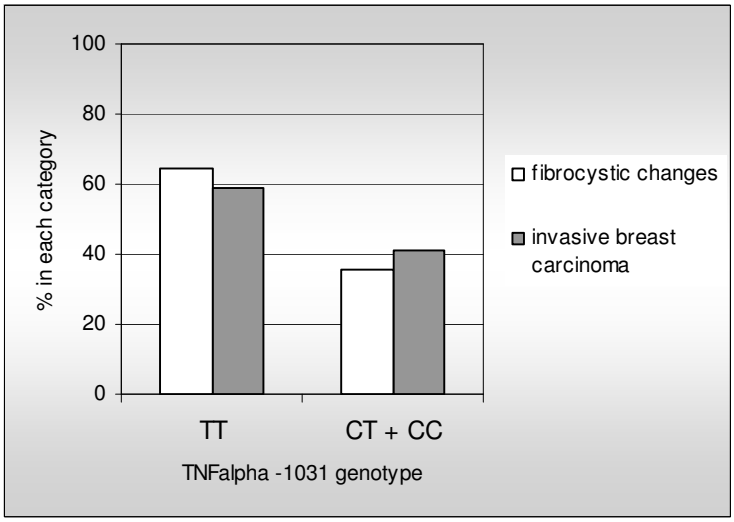
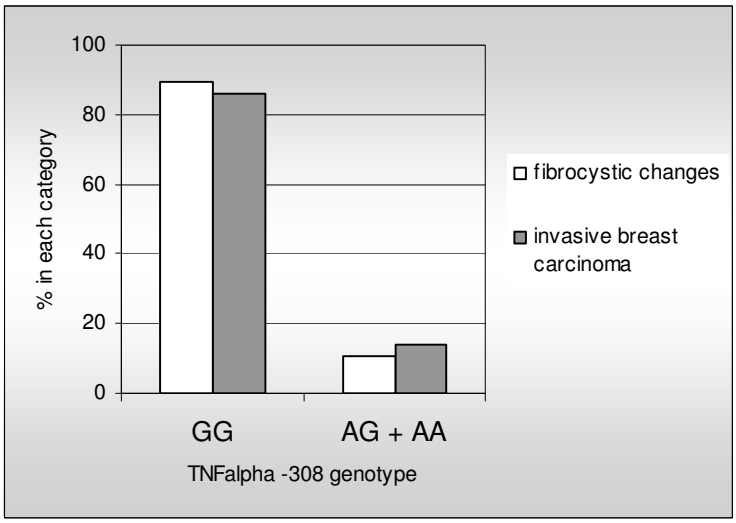


Figure 2. Frequency of TNFalpha promoter polymorphisms in fibrocystic changes and invasive breast cancer. A. -1031 polymorphism; B. -308 polymorphism; C. -238 polymorphism.



B


100
80
60
40
20
0
% in each category
fibrocystic changes
invasive breast carcinoma
GG
AG + AA
TNFalpha -308 genotype

C

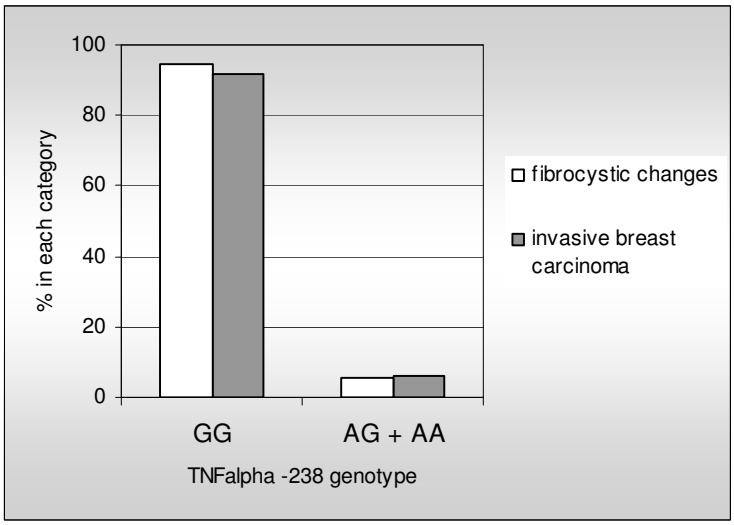

100
80
60
40
20
0
% in each category
fibrocystic changes
invasive breast carcinoma
GG
AG + AA
TNFalpha -238 genotype

Table 1. Genotype and allele frequencies for each TNFα SNP promoter polymorphism in patients with benign breast lesions (fibrocystic changes) and invasive breast cancer

		% Patient (n) Fibrocystic changes	% Patient (n) Breast cancer	P
TNFα-1031	T/T	64.5 (49)	58.9 (93)	
	T/C	31.6 (24)	35.4 (56)	
	C/C	3.9 (3)	5.7 (9)	0.671
	T/C + C/C	35.5 (27)	41.1 (65)	0.410
	T allele	80.3 (122)	76.6 (242)	
	C allele	19.7 (30)	23.4 (74)	0.370
TNFα-308	G/G	89.5 (68)	86.1 (136)	
	G/A	10.5 (8)	13.9 (22)	
	A/A	0 (0)	0 (0)	0.467
	G/A + A/A	10.5 (8)	13.9 (22)	0.467
	G allele	94.7 (144)	93.0 (294)	
	A allele	5.3 (8)	7.0 (22)	0.482
TNFα-238	G/G	94.7 (72)	93.7 (148)	
	G/A	5.3 (4)	5.7 (9)	
	A/A	0 (0)	0.6 (1)	0.777
	G/A + A/A	5.3 (4)	6.3 (10)	0.747
	G allele	97.4 (148)	96.5 (305)	
	A allele	2.6 (4)	3.5 (11)	0.625

Table 2. Haplotype frequencies for three TNFα SNP promoter polymorphism in patients with benign breast lesions (fibrocystic changes) and invasive breast cancer

TNFα haplotype -1031/-308/-238	% Patient (n) Fibrocystic changes	% Patient (n) Breast cancer
TT GG GG	56.7 (43)	48,7 (77)
CT GG GG	25.0 (19)	27.2 (43)
TT AG GG	7.9 (6)	10.1 (16)
CT GG AG	3.9 (3)	5.1 (8)
CC GG GG	2.6 (2)	3.8 (6)
CT AG GG	2.6 (2)	3.2 (5)
CC GG AG	1.3 (1)	0.63 (1)
CC GG AA		0.63 (1)
CC AG GG		0.63 (1)