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Tumor necrosis factor-α and -β genetic polymorphisms as a risk factor in Saudi patients with vitiligo

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ABSTRACT. Vitiligo is an acquired depigmentary disorder of the skin, characterized by multiple susceptibility loci and genetic heterogeneity. The etiology of vitiligo is unknown but several hypotheses, including an autoimmune origin, have been proposed. Tumor necrosis factor (TNF)-α, a pleiotropic proinflammatory cytokine, has been shown to play a critical role in several autoimmune diseases including vitiligo. The aim of present study was to determine the association of TNF-α and -β gene polymorphisms with vitiligo in Saudi patients. TNF-α and -β genes were amplified in 123 Saudi patients and 200 matched controls using polymerase chain reaction to search for polymorphisms involved at positions -308, and intron 1 +252. The frequency of the TNF-α (-308) GA genotype was higher and the frequencies of the GG and AA genotypes were significantly lower in vitiligo patients compared to controls. These findings suggested that genotype GA-positive individuals at position -308 of TNF-α are susceptible to vitiligo, whereas the GG and AA genotypes might exert a protective effect. The frequency of allele A (TNF-α 2-allele) was significantly higher and that of allele G (TNF-α 1-allele) was lower in vitiligo patients compared to controls, indicating an association of allele A with susceptibility to

vitiligo in Saudi patients. The results of our examination of TNF-β (intron 1 +252) polymorphisms showed a significant increase in the frequency of the GG genotype and allele G (TNF-β 1-allele) in vitiligo patients, suggesting a susceptibility of the GG genotype and allele G for vitiligo. By contrast, the high frequency of the GA genotype in controls might indicate a protective effect. The results of the present study strongly support a link between TNF-α (-308) and -β (intron 1 +252) polymorphisms and vitiligo in Saudi patients.

Key words: Vitiligo; Tumor necrosis factor; Polymorphism; Saudi

INTRODUCTION

Vitiligo is a depigmenting disorder of the skin that has a spontaneous onset. It is an acquired progressive disorder in which some or all of the melanocytes in the interfollicular epidermis and occasionally those in the hair follicles, are selectively destroyed. Patients with vitiligo present one to several milky white amelanotic macules. The macules are round or oval, often with scalloped margins (Sehgal and Srivastava, 2007). The disease can affect children as well as adults. It predominantly involves the hands, feet, wrists, axilla, and periorbital, perioral, and anogenital skin. Although vitiligo does not restrict the capacity to work or reduce life expectancy, it causes cosmetic disfigurement, leading to psychological trauma to the patients. Vitiligo is accompanied by stigma and affected individuals, especially girls, are socially ostracized for marital purpose.

Vitiligo occurs worldwide with an overall prevalence of 1%. The highest incidences of the condition have been recorded in Indians from the Indian subcontinent, followed by Mexicans and Japanese (Sehgal and Srivastava, 2007). The etiology of vitiligo is far from clear, although the loss of melanocytes has been attributed to autoimmune and autocytotoxic/metabolic mechanisms, neural dysfunction, and genetic factors (Moretti et al., 2002; Ongenae et al., 2003; Zhang et al., 2005). Recent research on vitiligo has suggested that various local triggers alert the innate immune system of the skin, which may precede adaptive immune responses targeting melanocytes. This phenomenon is akin to that of other common skin inflammatory disorders such as psoriasis and atopic dermatitis (Taieb, 2012). Moreover, ample evidence suggests that cytokines, particularly tumor necrosis factors (TNFs) play a role in the depigmentation process of vitiligo (Moretti et al., 2002; Namian et al., 2009; Kim et al., 2011).

TNF-α and -β, also known as lymphotoxin-α, give rise to a similar proinflammatory response that plays a critical role in the pathogenesis of several dermatological disorders including vitiligo (Taieb, 2012). The genes for TNF-α (OMIM 191160) and -β (MIM 153440), located within the major histocompatibility complex III region of chromosome 6, show close linkage to the genes for human leukocyte antigen (HLA) classes I (HLA-B) and II (HLA-DR). Studies using *ex vivo* endotoxin stimulated whole blood samples from monozygotic twins and their 1st degree relatives have provided evidence that 60% of the variation in the production capacity of TNF-α is genetically determined (Westendorp et al., 1997). Several polymorphisms within the promoter region of TNF-α and the intron 1 of TNF-β in particular have been associated with altered levels of circulating TNF-α (Sharma et al., 2008). One of the best

described single-nucleotide polymorphisms is located at nucleotide position -308 within the TNF-α promoter region (rs1800629), which affects a consensus sequence for a binding site of the transcription factor activator protein 2 (Abraham and Kroeger, 1999). TNF-α promoter polymorphism leads to a less common allele A (2-allele), which has been associated with increased TNF production *in vitro* (Braun et al., 1996) and a higher rate of TNF transcription than that associated with the wild-type GG genotype (Wilson et al., 1997). It has also been linked to increased susceptibility to several chronic metabolic degenerative, inflammatory, and autoimmune diseases (Cuenca et al., 2001; Qidwai and Khan, 2011). Conversely, in the TNF-β gene, a polymorphism at nucleotide position +252 within the first intron (A252G) (rs909253) affects a phorbol ester-responsive element. The presence of G at this position defines the mutant allele known as TNF-β * 1 (1-allele), which is associated with higher TNF-α and -β production (Messer et al., 1991; Abraham et al., 1993).

Several studies have attempted to show links between susceptibility to diseases and TNF gene polymorphisms (Qidwai and Khan, 2011). The concomitant analysis of polymorphisms at TNF-α and -β given that both are involved in the expression of TNF-α in addition to their critical role in the pathogenesis of autoimmune diseases, might help in the development of better strategies for the prevention and treatment of vitiligo. In this study, the genotype and allele frequencies of TNF-α (-308) and -β (+252) polymorphisms in Saudi patients with vitiligo and control subjects were investigated.

MATERIAL AND METHODS

Patients and controls

A total of 323 subjects visiting Riyadh Military Hospital (Saudi Arabia) were recruited for this study. One hundred and twenty-three unrelated Saudi vitiligo patients (62 males and 61 females) ranging from 6 to 79 years (mean age: 27.85 ± 12.43 years) and 200 unrelated healthy matched voluntary blood donors (143 males and 57 females) ranging from 20 to 65 years from the same population were studied. Written informed consent was obtained from each subject before recruitment.

Polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from the blood of vitiligo patients and controls using the QIA amp^R DNA mini kit (Qiagen, CA, USA). TNF-α and -β genes were amplified using an amplification refractory mutation system PCR methodology described elsewhere (Al-Rayes et al., 2011) to detect any polymorphism at position -308 and intron 1 +252 of TNF-α and -β genes, respectively. The primers used to amplify the various polymorphisms are summarized in Table 1. PCR amplification was carried out in PuReTaq Ready-to-Go PCR Beads (GE Healthcare, Buckinghamshire, UK). The reaction conditions consisted of 10 cycles of denaturation for 15 s at 94°C, annealing for 50 s at 65°C and extension for 40 s at 72°C. Then, 25 cycles of denaturation for 20 s at 94°C, annealing for 50 s at 59°C, and extension for 50 s at 72°C were performed. A final extension was performed at 72°C for 7 min. A positive control was included in the PCR assay via amplification of the human growth hormone gene.

Table 1. Sets of sense and antisense primers used to amplify TNF-α and -β (LT-α) to detect polymorphism.

Locus	Generic (antisense) primers	Sense primers
TNF-α (G-308A)	5'-TCT CGG TTT CTT CTC CAT CG-3'	5'-ATA GGT TTT GAG GGG CAT GG-3' 5'-AAT AGG TTT TGA GGG GCA TGA-3'
TNF-β (LT-α) intron 1 +252	5'-AGA TCG ACA GAG AAG GGG ACA-3'	5'-CAT TCT CTG TTT CTG CCA TGG-3' 5'-CAT TCT CTG TTT CTG CCA TGA-3'

LT-α = lymphotoxin-α.

Statistical analysis

The differences in genotype and allele frequencies between patients and controls were analyzed by the Fisher exact test using the CalcFisher software (http://www.jstatsoft.org/v08/i21/paper).

P values ≤0.05 were considered to be significant. The strength of the association of disease with respect to a particular genotype/allele was expressed with odds ratio interpreted as relative risk (RR) following the method of Woolf as described by Schallreuter et al. (1993). RR indicates how many times more frequent a disease is in the positive subjects compared with allele-/genotype-negative subjects. It is calculated for a genotype/allele that is increased or decreased in vitiligo patients compared to the frequency in normal Saudi subjects. RR was calculated for all the subjects using the following formula: RR = (a) x (d) / (b) x (c), where *a* = number of patients expressing the allele or genotype; *b* = number of patients without allele or genotype expression; *c* = number of controls expressing the allele or genotype; *d* = number of controls without allele or genotype expression.

The etiological fraction (EF) indicates the hypothetical genetic component of the disease. EF values >0.00-0.99 are significant. It is calculated for positive associations (RR > 1) using the following formula proposed by Svejgaard et al. (1983): EF = (RR - 1)f / RR, where $f = a / a + c$.

Preventive fraction (PF) indicates the hypothetical protective effect of one allele/genotype for a disease. It is calculated for negative associations (RR < 1) using the following formula (Svejgaard et al., 1983). PF = (1 - RR)f / RR (1 - f) + f, where $f = a / a + c$. Values <1.0 indicate the protective effect of an allele/genotype against the manifestation of disease.

RESULTS

The demographic/clinical features of vitiligo subjects are summarized in Table 2. All vitiligo patients were diagnosed as having the nonsegmental (generalized or localized) type of the disease. The distribution of frequencies of the TNF-α -308 promoter polymorphism was significantly different in vitiligo patients compared to controls (Table 3). The frequency of the GA genotype in vitiligo patients was significantly higher than in controls (P = 0.0001, RR = 8.402, EF = 0.594). Conversely, the frequencies of homozygous GG and AA genotypes in vitiligo patients were significantly lower than in controls (P = 0.0001, RR = 0.160, PF = 0.514 and P = 0.001, RR = 0.183, PF = 0.444, respectively). The frequency of allele A in patients was also higher than in control subjects (P = 0.0007), whereas the frequency of allele G in the patient group was lower than in controls (P = 0.0007).

Table 2. Demographic/clinical features of vitiligo patients.

Age of patients [mean ± SD (range) years]	27.85 ± 12.43 (6-79)
Gender (male:female)	61:62
Age of onset [mean ± SD (range) years]	22.57 ± 15.42 (2-60)
Duration of disease [mean (range) years]	6.2 (1-19)
Type of vitiligo (nonsegmental)	
Generalized	34%
Focal	34%
Acrofacial	19%
Lip-tip	12%
Universalis	1%

Table 3. Genotype and allele frequencies of the (G-308A) TNF-α polymorphism in vitiligo patients and matched controls.

Genotype/allele	Vitiligo (N = 123)		Control (N = 200)		P value	RR	EF[a]/PF
	N	%	N	%			
GG	17	13.82	100	50	0.0001*	0.160	0.514
GA	103	83.74	76	38	0.0001*	8.402	0.594[a]
AA	03	2.44	24	12	0.001*	0.183	0.444
G allele (TNF-α 1-allele)	137	55.69	276	69	0.0007*	0.564	0.238
A allele (TNF-α 2-allele)	109	44.31	124	31	0.0007*	1.770	0.240[a]

N = number of subjects; *statistically significant; RR = relative risk; EF = etiological fraction; PF = preventive fraction.

The distribution of frequencies of the TNF-β (intron 1 +252) promoter polymorphism also varied significantly between Saudi vitiligo patients and control subjects (Table 4). The frequency of homozygous GG genotypes in patients was significantly higher than that in controls (P = 0.0004, RR = 3.071, EF = 0.459), whereas heterozygous genotype GA in patients was significantly lower than that in controls (P = 0.002, RR = 0.464, PF = 0.277). The difference in frequency of AA genotypes of vitiligo patients and control subjects was statistically insignificant (P = 0.588). The frequency of the G allele (1-allele) of TNF-β was significantly higher in vitiligo patients compared with that in controls (P = 0.009, RR = 1.200, EF = 0.179), whereas the frequency of the A allele (2-allele) was significantly lower in vitiligo patients compared with that in controls (P = 0.009, RR = 0.832, PF = 0.179).

Table 4. Genotype and allele frequencies of the TNF-β (intron 1 +252) polymorphism in vitiligo patients and matched controls.

Genotype/allele	Vitiligo (N = 123)		Control (N = 200)		P value	RR	EF[a]/PF
	N	%	N	%			
GG	41	33.33	28	14	0.0004*	3.071	0.459[a]
GA	70	56.91	148	74	0.002*	0.464	0.277
AA	12	9.76	24	12	0.588	0.792	0.052
G allele (TNF-β 1-allele)	152	61.79	264	51	0.009*	1.200	0.179[a]
A allele (TNF-β 2-allele)	94	38.21	196	49	0.009*	0.832	0.179

N = number of subjects; *statistically significant; RR = relative risk; EF = etiological fraction; PF = preventive fraction.

DISCUSSION

Our study on TNF promoter polymorphism in Saudi vitiligo patients suggested that

genotype GA-positive individuals at position -308 of TNF-α are at higher risk for vitiligo, whereas GG and AA genotypes might confer resistance against the disease. Conversely, the GG genotype at the intron 1 +252 position of TNF-β was positively associated with vitiligo, whereas genotype GA might be refractory.

The results of this study further showed that allele A (TNF-α 2-allele) is susceptible, whereas allele G (TNF-α 1-allele) is resistant to vitiligo. Allele A (TNF-α 2-allele) lies on the extended haplotype HLA-A1-B8-DR3-DQ2, which is associated with autoimmunity and high TNF-α production. The TNF-α 2-allele (allele A) has been demonstrated to be a much stronger transcriptional activator than the common TNF-α 1-allele (allele G) is in a human B-cell line (Chen et al., 1996). Moreover, polymorphism at -308 in the TNF-α promoter has a direct effect on TNF-α gene regulation and may be responsible for the association of allele A with the high TNF-α phenotype and more severe disease (Wilson et al., 1997). Further populations bearing a higher proportion of the TNF-α 2-allele (A allele) are reportedly predisposed to several metabolic, degenerative, inflammatory, and autoimmune diseases (Cuenca et al., 2001; Aguillón et al., 2002).

This report clearly showed a direct association between TNF-α promoter polymorphism and vitiligo in Saudi patients. A similar association between the TNF-α -308 (G/A) polymorphism and vitiligo was recently reported in Iranian patients (Namian et al., 2009). Contrary to these reports, Yazici et al. (2006) found no association between TNF-α -308 promoter polymorphism and vitiligo in Turkish patients. This difference in the distribution of TNF-α promoter polymorphism may be attributed to ethnicity. Significant ethnic variations in TNF-α genotypes have been reported in healthy individuals as well as among patients with other diseases (Cuenca et al., 2001; Nemec et al., 2008; Al-Rayes et al., 2011). Linkage and association studies have also provided strong evidence for the presence of multiple vitiligo susceptibility genes on different chromosomes. Different candidate genes for vitiligo have been identified among different populations (Gavalas et al., 2006; Shajil et al., 2006; Namian et al., 2009; Alkhateeb and Qarqaz, 2010; Spritz, 2012; Karaca et al., 2013). Our earlier study showed a significant association between HLA-DRB4* 010101 and HLA-B7, -B15, -Bw6, -Cw6, and -Cw7 and vitiligo in Saudi patients (Abanmi et al., 2006), suggesting that various genetic factors operate simultaneously in the pathogenesis of the disease.

This report is the first to show an association between the TNF-β (intron 1 +252) polymorphism and vitiligo in Saudi patients. The 252 A/G polymorphism in intron 1 of TNF-β is in almost complete linkage disequilibrium with a missense variant (Thr 26 Asn) in exon 3. It is closely linked to TNF-α and found in linkage disequilibrium with HLA-A, -B8, and -DR3 (Messer et al., 1991; Wilson et al., 1993) and has also been reported to define a high TNF-α expressing haplotype in addition to modifying expression of TNF-β itself (Messer et al., 1991). The TNF-β (intron 1 +252) polymorphism has also been associated with numerous autoimmune diseases (Lu et al., 2005; Watanabe et al., 2010; Al-Rayes et al., 2011).

In addition to highlighting the similarity in TNF-α and -β functions, some reports have suggested that polymorphisms in their genes act in tandem to influence TNF-α production (Messer et al., 1991; Abraham et al., 1993). This effect is evident in the fact that peripheral blood mononuclear cells from individuals carrying the TNF-β 1-allele (allele G) secreted more TNF-α than TNF-β2 (allele A). Our results suggested that the high frequency of allele A and GA genotype of TNF-α together with allele G and GG genotype of TNF-β in Saudi vitiligo patients caused the increased production of TNF-α. Several recent studies have shown that

vitiligo patients have increased tissue and serum levels of proinflammatory soluble mediators, interleukin (IL)-1, IL-6, and TNF-α (Moretti et al., 2002). TNF-α has been shown to inhibit melanogenesis and promote melanocyte apoptosis (Swope et al., 1991; Kim et al., 2007). Cases of vitiligo treated with TNF-α inhibitors, although achieving mixed results, have been reported as well (Rigopoulos et al., 2007; Campanati et al., 2010). A recent study has shown that the anti-TNF-α agent etanercept can stop vitiligo progression and induce repigmentation in some cases (Kim et al., 2011). *In vitro* direct analyses of skin T cells from the margins of vitiliginous skin have shown that polarized type-1 T cells (CD4+ and CD8+), which secrete predominantly TNF-α and interferon-γ, are associated with the destruction of melanocytes during active vitiligo (Huang et al., 2002; Wankowicz-Kalinska et al., 2003). In addition to causing apoptosis of melanocytes, TNF-α also inhibits melanocytogenesis through an inhibitory effect on tyrosinase and tyrosinase-related protein (Martínez-Esparza et al., 1998).

By binding 2 distinct cell-surface receptors, TNF-R1 and TNF-R2, TNF-α and -β have been shown to act in tandem to produce apoptosis in many cell types. Several mechanisms have been proposed through which these cytokines induce expression of proapoptotic genes. The messenger RNA expression for TNF-α and IL-6 is reportedly increased in the epidermis of vitiligo patients, whereas their expression was practically undetectable in the skin of control subjects. Apoptotic keratinocytes in lesional skin were also more abundant than those in prelesional skin in vitiligo patients and were absent in the epidermis of control subjects (Moretti et al., 2009). Melanocytes and keratinocytes seem to exhibit a functionally and structurally close relationship to a great extent that is mediated by keratinocyte-derived cytokines. Gauthier et al. (2003) described the presence of a large extracellular space between the melanocytes, vacuoles in keratinocytes containing tenascin, which can promote the extracellular detachment of melanocytes. Moreover, keratinocytes produce and release high amounts of proinflammatory cytokines including TNF-α, which promotes the expression of adhesion molecules on the melanocyte membrane (intercellular adhesion molecule 1), leading to further lymphocyte recruitment. By contrast, TNF-α directly interferes with some mitochondrial activities through the production of peroxides, leading to a mitochondrial-dependent cell death as well as activation of inflammatory genes through the nuclear translocation of nuclear factor κB (Moretti et al., 2002; Wankowicz-Kalinska et al., 2003).

The present study clearly demonstrated an association between TNF-α (-308) and -β (intron 1 +252) polymorphisms and vitiligo. However, further studies are warranted to determine the role of various cytokine gene polymorphisms in the pathogenesis of vitiligo and to identify possible gene-environment interactions in various populations.

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