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Association of FAS Gene Promoter Functional Polymorphisms and the Risk of Vitiligo in Turkish Populations

Ümit Türsen,[1]* MD, Tamer İrfan Kaya,[1] MD, Bilal Bulut,[1] MD, Mehmet Emin Erdal,[2] MD, Özlem İzci Ay,[2] MD, Belma Türsen,[3] MD

Address: [1]Department of Dermatology and [2] Department of Genetics, School of Medicine, Mersin University, [3]Department of Dermatology, Mersin State Hospital, Mersin, Turkey

E-mail: utursen@mersin.edu.tr

** Corresponding Author:* Dr. Ümit Türsen, MD, Mersin University, School of Medicine, Department of Dermatology, Mersin, Turkey.

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Abstract

Background: The FAS/FASLG system shows a key role in regulating apoptosis.

Objectives: Previous findings have indicated that a significantly increased risk of vitiligo was associated with FAS-1377 AA genotype and the FAS-1377 AG genotype when compared with FAS-1377 GG genotype in Chinese population.

Material and Methods: We investigated the FAS-670, FAS-1377, FAS-844 and FAS-124 polymorphisms in 113 vitiligo patients and also compared 196 controls.

Results: We found that a significantly increased vitiligo was associated with the FAS-670 GA genotype [adjusted odds ratio (OR), 2.87; 95% confidence interval (CI), 1.37-6.03] when compared with the controls. However, there were no evident risk associated with FAS-1377, 844 and 124 genotypes.

Conclusion: This is the first report of an association between FAS-670, FAS-1377, FAS-844 and FAS-124 gene polymorphism and vitiligo in a Turkish population. The results of this study suggest that the presence of FAS-670 GA genotype can be regarded as risk factor for vitiligo, and especially for vitiligo in Turkish population.

Introduction

Vitiligo is an acquired pigmentation disorder of skin and hair, characterized by symmetrically arranged, well defined white patches, as a result of the selective destruction and disappearance of functioning melanocytes from the epidermis and follicular reservoir [**1**]. It is an enigmatic disease with variable clinical presentations and an unpredictable prognosis. The pathogenesis of this cosmetically disfiguring disease has still not been completely clarified. The disappearance of melanocytes from the achromic patches appears to be multifactorial and has been so far explained as either caused by hereditary factors, autoimmunization, neurological disorders and autodestruction. Vitiligo occurs in 1-4% of the population, mostly between the ages of 10 and 30 years [**2, 3**]. But it is much more frequent among probands' relatives: 7.0% among all first degree relatives, 6.1% among siblings, and 23% among monozygot twins.

The genetics of vitiligo cannot be explained by simple Mendelian genetics, it is characterized by incomplete penetrance. Family clustering of cases and segregation analyses have suggested multifactorial, polygenic inheritance [**4, 5**].

Despite the lack of conclusive evidence, the finding of melanophagia and the absence of a prominent inflamation in vitiligo lesions favor the theory that apoptosis is responsible for the melanocyte death in vitiligo. In a recent study, Ruiz-Arguelles et al. demonstrated that residual melanocytes in biopsies from vitiligo patients expressed anti-apoptotic markers, which strongly supports that those cells where the expression of proapoptotic genes were predominant incurred rapidly in apoptosis and they are no longer found as residual melanocytes. They also showed the presence of serum antibodies to melanocytes in vitiligo patients, which penetrate into cultured melanocytes in vitro and trigger them to undergo into apoptosis [**6, 7**]. Apoptosis has also recently been proposed as a mechanism for intracellular redistribution and exposure and unusual cleavage fragments of autoantigens. The exposure of previous cyriptic determinants on apoptotic cells could consequently cause an autoimmune response which may induce autoimmunity in vitiligo [**8, 9**].

Autoimmune mechanism stems from the fact that many vitiligo patients also exhibit other autoimmune disorders and some patients respond to immunosuppressive treatments. Melanocytes are particularly immunogenic by the contents of their melanosomes, generating melanogenic enzymes and structural components, including tyrosinase, MART-1, gp100, TRP-1 and TRP-2. These molecules are also prime targets of the immune response in both vitiligo and melanoma. An autoimmune response targeting epidermal melanocytes has been demonstrated in vitiligo patients which tends to be subclinical. Studies demonstared circulating antibodies and auto reactive T cell, particularly cytotoxic T cell, infiltrations at the margins of lesions against these melanosomal antigens in affected subjects during disease acceleration phases [**8, 9, 10, 11**]. CD8+ T cell mediated targeted cell death involves two principal mechanisms: perforin and Fas-mediated signaling. CD4+ T cell mediated killing occurs via Fas-FasL interactions in MHC class II-expressing cells such as melanocytes. In a mouse vitiligo model, Lambe T et al showed that perforin knockout mice continued to develop severe vitiligo, whereas, the incidence and severity of vitiligo in mice carrying a mutation in FasL (gld/gld) was considerably reduced. These results demonstrate the destruction of melanocytes and the severity of disease are both partially dependent on Fas. They also suggest that CD4+ T cells are sufficient to initiate the destruction of melanocytes, in addition to being adept at providing help to other effector lineages [**12**].

Recently *Li* et al, found that a significantly increased risk of vitiligo was associated with the FAS-1377 AA genotype and the FAS-1377 AG genotype when compared with the FAS-1377 GG genotype in Chinese population [**13**], however these polymorphisms have not been investigated in different ethnic groups. To determine the generality of this association, we studied Fas gene polymorphisms in a Turkish population. In addition to these polymorphisms, our study was the first to investigate the relationship between FAS-124 polymorphism and vitiligo.

Materials and Methods

Subjects: The study was designed as a case controlled study. We examined 112 patients with vitiligo (56 men, 55 women) and 196 healthy control subjects (99 men, 97 women), matched according to age and sex. The mean age of the control subjects was 30.08±13.34 years and of the patients was 35.27±16.71 years. The diagnosis of vitiligo was based on a thorough physical examination, examination with Wood's light (UVA light with a wavelength of 365 nm), and evaluation of the patient's medical history. Also, the clinical types of disease (vitiligo vulgaris or acrofacial vitiligo) were documented. Of the 112 patients, 73 (66%) suffered from vitiligo vulgaris, 39 (34%) acrofacial vitiligo. The control group were chosen from the healthy individuals without any systemic and dermatologic disease. The study was approved by the local ethic comittee, and informed consent was obtained from all patients.

Molecular analysis

DNA extraction and analysis: With written informed consent, a blood sample was drawn from each individual. Venous blood samples were collected in ethylenediaminetetra acetic acid (EDTA) contai-

ning tubes. DNA was extracted from whole blood by salting out procedure [**14**].

Genotypic Analysis of the FAS (CD95, FAS/APO1) -670 G/A (rs 1800682) Polymorphism: W.Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay were used to determine FAS (CD95, FAS/APO1) -670 G/A (rs 1800682) polymorphism. The oligonucleotide primers used to determine the -670 G/A polymorphism in promoter region within the FAS gene were described previously [**15, 16**]. The primers (GenBank accession no: AY450925), forward 5'-CTACCTAAGAGCTATCTACCGTTC-3'; reverse 5'-GGCTGTCCATGTTGTGGCTGC-3' were used to amplify the 332bp PCR fragment. Polymerase Chain Reaction was performed in a 25 µl volume with 100 ng DNA, 100 µm dNTPs, 20 pmol of each primer, 1.5 mM MgCl2, 1x PCR buffer with (NH4)2SO4 (Fermentas, Vilnius, Lithuania) and 2U Taq DNA polymerase (*Fermentas, Vilnius, Lithuania*). Amplification was performed on an automated Thermal Cycler (*Techne Flexigene, Cambridge*, UK). Polymerase Chain Reaction conditions were 2 min for initial denaturation at 95°C; 35 cycles at 95°C for 45 s for denaturation, 1 min at 62°C for annealing and 90 s at 72°C for extension, followed by 7 min at 72°C for final extension. The PCR products were digested with 10 U MvaI (BstNI *Fermentas, Vilnius, Lithuania*) at 37°C for 14 hr, the genotyping of the FAS gene was determined by fragment separation at 120 V for 40-50 min on a 3 % Agarose gel containing 0.5µg/ml ethidium bromide. A 100 bp marker (100 bp DNA Ladder, *Fermentas*) was used as a size standard for each gel lane. The gel was visualized under UV light using a gel electrophoresis visualizing system (*Vilber Lourmat*). The MvaI restricted products of FAS -670; GG, GA and AA genotypes had band sizes of 184bp/101bp/47bp, 231bp/184bp/101bp/47bp and 231bp/101bp, respectively.

Genotypic Analysis of the FAS (CD95, FAS/ APO1) -1377 G/A (rs2234767) Polymorphism: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay were used to determine FAS -1377 G/A (rs2234767) polymorphism. The oligonucleotide primers used to determine the -1377 G/A polymorphism within the FAS gene were described previously [**17, 18**]. The primers (GenBank accession no: AY450925), forward 5'-TGTGTGCACAAGGCTGGCGC-3' (mismatch base is underlined); reverse 5'-TGCATCTGTCACTGCACTTACCACCA-3'were used to amplify the 122bp PCR fragment. PCR was performed in a 25 µl volume with 100 ng DNA, 100 µm dNTPs, 20 pmol of each primer, 1.5 mM MgCl2, 1x PCR buffer with (NH4)2SO4 (Fermentas, Vilnius, Lithuania) and 2U Taq DNA polymerase (*Fermentas, Vilnius, Lithuania*). Amplification was performed on an automated Thermal Cycler (*Techne Flexigene, Cambridg*e, UK). The Bsh1236I restricted products of FAS -1377; GG, GA and AA genotypes had band sizes of 104bp/18bp, 122bp/104bp/18bp and 122bp, respectively.

Genotypic Analysis of the FASL -844 T/C (rs763110) Polymorphism: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay were used to determine FASL -844 T/C (rs763110) polymorphism. The oligonucleotide primers used to determine the -844 T/C polymorphism within the FASL gene were described previously [**19, 20**]. The primers (GenBank accession no: AF027385, Z96050), forward 5'-CAGCTACTCGGAGGCCAAG-3'; reverse 5'-GCTCTGAGGGGAGAGACCAT-3'were used to amplify the 401bp PCR fragment. PCR was performed in a 25 µl volume with 100 ng DNA, 100 µm dNTPs, 20 pmol of each primer, 1.5 mM MgCl2, 1x PCR buffer with (NH4)2SO4 (Fermentas, Vilnius, Lithuania) and 2U Taq DNA polymerase (*Fermentas, Vilnius, Lithuania*). Amplification was performed on an automated Thermal Cycler (*Techne Flexigene, Cambridge*, UK). The BseMI restricted products of FASL -844; TT, TC and CC genotypes had band sizes of 401bp, 401bp/233bp/168bp and 233bp/168bp, respectively.

Genotypic Analysis of the FASL IVS2nt -124 A/G (rs5030772) Polymorphism: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay were used to determine FASL IVS2nt -124 A/G (rs5030772) polymorphism. The oligonucleotide primers used to determine the IVS2nt -124 A/G polymorphism within the FASL gene were described previously [**15, 18**]. The primers (GenBank accession no: AF027385, Z96050), forward 5'-GCAGTTCAGACCTACATGATTAGGAT-3' (mismatch base is underlined); reverse 5'-CCAGATACAGACCTGTTAAATGGGC-3' were used to amplify the 219bp PCR fragment. PCR was performed in a 25 µl volume with 100 ng DNA, 100 µm dNTPs, 20 pmol of each primer, 1.5 mM MgCl2, 1x PCR buffer with $(NH4)_2SO_4$ (*Fermentas, Vilnius, Lithuania*) and 2U Taq DNA polymerase (*Fermentas, Vilnius, Lithuania*). Amplification was performed on an automated Thermal Cycler (*Techne Flexigene, Cambridge*, UK). The BseGI restricted products of FASL IVS2nt -124 A/G; AA, AG and GG genotypes had band sizes of 219bp, 219bp/190bp/29bp and 190bp/29bp, respectively. All procedures were conducted in a manner blind to the case status and other characteristics of the participants. Scoring of gels and data entry were conducted independently by two persons. We performed the PCRs and evaluated the results without knowing the groups of the

subjects. At least 10% of the samples were retested, and the results were 100% concordant.

Statistical Tests

All statistical tests were carried out using MedCalc Software Verrsion 12.3.0 (1993-2012 Broekstraat 52, 9030 Mariakerke, Belgium). All values were presented as mean ± standard deviation (SD). The strength of association was estimated by calculating the odds ratios (ORs) with 95% confidence intervals (95% CIs), which were generated by logistic regression analysis. To calculate the alignment of distribution of alleles and genotypes (*Hardy-Weinberg* equilibrium), chi-square test was applied. Potential risks of genotypes and alleles were determined by calculating the Odds ratio. The relationship between groups and gender was analyzed using chi-square test. P values <0.05 were considered statistically significant.

Results

As a result of the *Hardy-Weinberg* equilibrium control, all polymorphisms were stable in the control groups (p>0,05). Except for FAS 670 patients group (p<0,05), the other polymophisms were in the *Hardy Weinberg* equilibrium (p>0,05). When calculations of FAS 670 and FAS 1377 polymorphisms were performed, GG genotype was taken as a reference. For FASL-844, TT genotype and for FASL, 124 AA genotype was taken as reference. As shown in **Table 1**, the frequencies of FAS-670 genotypes was statistically significant between patients and controls (p=0,0035). This difference come from GG (p=0,019) and GA (p=0,0172) groups. In order to interpret the odss ratio, the results must be statistical significant. For FAS-670, OR was statistically significant between GG and GA (p=0,0052). GA genotype was 2.874 times more frequent in patients than in controls (OR=2,874 ; 95%CI, 1,37-6,03, p=0,0052). The other ORs couldn't interpret, since there weren't statistically significant results (p>0,05). According to allele frequencies, patient and control groups were similar (p=0,4159). As shown in **Table 2**, the frequencies of FAS-1377 genotypes were not statistically significant between patients and controls (p=0,5812). The ORs results could not interpret, since there weren't statistically significant (p>0,05). As shown in **Table 3**, the frequencies of FAS-844 genotypes were not statistically significant between

Table 1. Genotypic Frequencies of FAS-670 Polymorphisms in Patients and Controls and Their Associations with Risk of Vitiligo

FAS 670	Controls (n=196)		Patients (n=113)		Chi-square		OR
Genotype	n	%	n	%	p	OR(%95 CI)	p
GG	41	20,9	11	9,8	0,0035	1,00	Reference
GA	83	42,4	64	57,2		2,874 (1,37-6,03)	0,0052
AA	72	36,7	38	33,0		1,915 (0,88-4,16)	0,1001
HW (p)	0,066		0,028				
Allel Frequency	n	%	n	%			
G	165	42,1	86	38,4	0,4159	----	
A	227	57,9	140	61,6		1,166 (0,83-1,63)	0,3689

Table 2. Genotypic Frequencies of FAS-1377 Polymorphisms in Patients and Controls and Their Associations with Risk of Vitiligo

FAS 1377	Controls (n=196)		Patients (n=113)		Chi-square		OR
Genotype	n	%	n	%	p	OR(%95 CI)	p
GG	138	70,4	78	69,0	0,5812	1,00	Reference
GA	51	26,0	33	29,2		1,145 (0,68-1,92)	0,6092
AA	7	3,6	2	1,8		0,505 (0,10-2,49)	0,4021
HW (p)	0,405		0,480				
Allel Frequency	n	%	n	%			
G	325	83,6	199	83,4	0,9643	----	
A	68	16,4	37	16,6		1,015 (0,65-1,58)	0,9460

Table 3. Genotypic Frequencies of FAS-844 Polymorphisms in Patients and Controls and Their Associations with Risk of Vitiligo

FASL 844	Controls (n=196)		Patients (n=113)		Chi-square		OR
Genotype	n	%	n	%	p	OR(%95 CI)	p
TT	46	23,5	31	27,4	0,6956	1,00	Reference
TC	99	50,5	56	49,6		0,839 (0,48-1,47)	0,5407
CC	51	26,0	26	23,0		0,756 (0,39-1,46)	0,4045
HW (p)	0,8791		0,9415				
Allel Frequency	n	%	n	%			
T	191	48,7	118	52,2	0,4522	----	
C	201	51,3	108	47,8		0,869 (0,63-1,21)	0,4037

Table 4. Genotypic Frequencies of FAS-124 Polymorphisms in Patients and Controls and Their Associations with Risk of Vitiligo

FAS 124	Controls (n=196)		Patients (n=113)		Chi-square		OR
Genotype	n	%	n	%	p	OR(%95 CI)	p
AA	134	68,4	78	69,0	0,6233	1,00	Reference
GA	57	29,1	30	26,5		0,90 (0,54-1,52)	0,7057
GG	5	2,5	5	4,5		1,72 (0,48-6,12)	0,4039
HW (p)	0,7145		0,3456				
Allel Frequency	n	%	n	%			
A	325	82,9	186	82,3	0,9348	----	
G	67	17,1	40	17,7		1,04 (0,68-1,60)	0,8476

patients and controls (p=0,6956). The ORs results couldn't interpret, since there weren't statistically significant (p>0,05). As shown in **Table 4**, the frequencies of FAS-124 genotypes were not statistically significant between patients and controls (p=0,6233). The ORs results couldn't be interpreted, since they were not statistically significant (p>0,05).
Correlations between clinical types of vitiligo (vitiligo vulgaris and acrofacial vitiligo) and different polymorphisms have been done. No differences in FAS-124, FAS-670, FAS-844 ve FAS 1377 polymorphisms were detected between vitiligo vulgaris and acrofacial vitiligo.

Discussion

The highest risk of the vitiligo has been recorded in patients with autoimmune diseases and also positive family history of vitiligo [**11**]. In this study, we explored the association of the FAS 670, FAS 1377, FASLG 844 and FASL 124 polymorphisms with the risk of vitiligo in a Turkish population. The FAS 1377, FASLG 844 and FASL 124 polymorphisms did not affect the risk of vitiligo. However, we found that a increased risk of vitiligo risk appeared to be associated with FAS 670GA. To the best of our knowledge, this is the first study to investigate whether or not FAS and FASLG polymorphisms are associated with a risk of vitiligo in Turkish population. Published association studies of FAS polymorphisms and autoimmune disease risk include *Sjögren*'s syndrome, systemic lupus erythematosus, autoimmune hepatitis, alopecia areata and *Guillain–Barré* syndrome [**21, 22, 23, 24 ,25**]. Fas-FasL signaling has also been implicated in other forms of cell-specific autoimmunity including experimental autoimmune encephalomyelitis and autoimmune diabetes [**26, 27**].

Apoptosis, or programmed cell death, is an important mechanism used to delete autoreactive lymphocytes and may be a key factor in the development of autoimmunity. In addition, increased expression of Fas, Fas ligand (FasL), and increased apoptosis may play a part in the observed cutaneous melanocyte destruction although this remains controversial [**28**]. Redistribution of the normally intracellular Ro and La autoantigens to surface membrane blebs on apoptotic cells has recently been demonstrated, a process by

which these autoantigens may be accessible to the immune system. It is therefore conceivable that apoptosis may underlie the induction and facilitation of Ro/La autoantibody responses in primary Sjögren's syndrome. After apoptosis, cell corpses are rapidly recognized and phagocytosed by professional phagocytes, such as macrophages and dendritic cells. The rapid removal of cell corpses by phagocytes prevents the release of potentially toxic or immunogenic materials from dying cells [**29**]. Vitiligo is an acquired melanocyte loss disorder resulting from an immunologically mediated attack on melanocytes, and autoimmunity may play a part in its pathogenesis. It is suspected to be a T cell-mediated autoimmune disease of the melanocytes, where Fas is expressed on melanocytes and FasL on perilesional infiltrates. Vitiligo is an autoimmune disease however the mechanism behind tolerance breakdown of the self-peptides remains unclear [**30, 31**]. Similarly, it is therefore conceivable that apoptosis may underlie the induction and facilitation of autoimmune responses in vitiligo.

Li et al studied functional polymorphisms of the FAS and FASLG genes in 750 vitiligo patients and 756 controls, all of Han Chinese descent, with respect to the FAS-1377 G>A, FAS-670 A>G, and FASLG-844 T>C polymorphisms. Their ORs (95% CI) for vitiligo of FAS-1377 AA and AG genotypes were 1.49 (1.07-2.08) and 1.31 (1.05-1.63). They observed a significant increase in the frequency of the AA and AG allele in vitiligo patients as compared with that of the FAS-1377 GG groups. They found that a significantly increased risk of vitiligo was associated with the FAS-1377 AA genotype (adjusted OR, 1.49; 95% CI, 1.07–2.08) and the FAS-1377 AG genotype (adjusted OR, 1.31;95% CI, 1.05–1.63) when compared with the FAS-1377 GG genotype. They suggested that allelic variance in the FAS gene itself or by other genes in linkage disequilibrium with this gene could predispose to the development of vitiligo [**13**]. It can be hypothesized that apoptosis can cause the presentation to the immune system. In vitiligo, however, T cell tuning allows T cells with higher affinity for melanocyte differentiation antigens to enter the circulation after escaping clonal deletion in primary lymphoid organs. The resulting efficacious and progressive autoimmune response to melanocytes provides a roadmap for melanoma therapy. This is the first report of an association of Fas gene polymorphism and vitiligo in a Caucasian population. This is the also first study of an association between FAS-124 gene polymorphism and vitiligo. The results of this study suggest that the presence of FAS-670 GA genotype can be regarded as risk factor for vitiligo, and especially for vitiligo in Turkish population. The influence of a functional single-nucleotide polymorphism at position -670 in the promoter of the apoptosis gene FAS on susceptibility to autoimmune diseases needs further investigations. Although it has not yet been possible to assign any particular allele or genotype to the control of FAS expression, this polymorphism has been described to be associated with several autoimmune diseases including lupus erythematosus [**22**]. Our finding suggests that apoptosis may contribute to development of autoimmune reactions and that FAS function might be relevant for vitiligo.

In view of these data the involvement of other genetic and/or enviromental factors seems to be required and to be more important in vitiligo. Further analysis of other FAS and cytokine polymorhisms and extended samples may clarify the current findings, eventually resulting in a better understanding of the genetic and immunologic components of vitiligo.

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