Lack of Association between ctla-4 A49G Polymorphism and Vitiligo

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ABSTRACT

Background: Vitiligo is an acquired skin disorder that selectively destroys melanocytes in epidermis with an unknown etiology. **Objective:** To investigate the exon 1 A49G polymorphism of cytotoxic T lymphocyte antigen-4 (ctla-4) gene in vitiligo patients. **Methods:** The A49G polymorphism was detected by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method in 101 patients and 208 normal healthy age/ethnicity matched individuals. **Results:** The frequencies of heterozygote genotypes in patients and controls were found to be 42 (41.6%) of 101 and 85 (40.9%) of 208, respectively. The frequencies of homozygote A and G genotypes were 49 (48.5%) and 10 (9.9%) in 101 patients, whereas, these frequencies in 208 control individuals were 103 (49.5%) and 20 (9.6%), respectively. There was no significant difference between the genotype (P = 0.98) and allele (P = 0.86) frequencies of A49G polymorphism in patients and normal healthy individuals. **Conclusion:** Our results indicate that in contrast to several immune mediated disorders, there is no association between ctla-4 A49G gene polymorphism and vitiligo.

Keywords: Autoimmune, ctla-4, Melanocyte, PCR-RFLP, Polymorphism, Vitiligo

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INTRODUCTION

Vitiligo is an acquired melanin pigmentary disorder of the epidermis and hair follicles (1,2). The disease is associated with disappearance of functioning melanocytes and loss of melanin in the epidermis, which makes the lesional skin more sensitive to sun burns. Vitiligo affects 1-2% of the world's population (3,4), irrespective of gender and race. Age at onset is variable and the average age of onset is about 20 years (5). The etiology of the disease remains unknown and several hypotheses exist to explain the etiology. Autocytotoxic (self-destruct) hypothesis is based on the preferential destruction of melanocytes by toxic intermediates of melanin synthesis (6), while neural hypothesis suggests that the melanocytes could be damaged by neurochemical mediators released from the nerve endings supported by the association of vitiligo with neurological disorders or with peripheral nerve injury (7). The abnormal immune responses frequently found in vitiligo patients have led to the suggestion that, at least in some cases, the disease has an autoimmune origin (8). Additionally, genetic factors have also been proposed to play a role in the pathogenesis of vitiligo (9). If vitiligo is in part an autoimmune disorder, genetic factors that predispose an individual to autoimmunity may influence the development of the disease. One of the candidate genes which has a strong association with several autoimmune diseases is ctla-4 gene located in chromosome 2q33 region (10-14). Therefore, we investigated the possible association between ctla-4 gene polymorphism in exon 1 (A49G) and vitiligo in southern Iranian patients and compared the distribution of this polymorphism to matched control groups.

PATIENTS AND METHODS

Subjects. In this study 101 unrelated Iranian vitiligo patients (male: 42, female: 59) were referred to our laboratory by collaborating dermatologist. We included 208 (male: 198, female: 10) age/ethnicity matched unrelated healthy individuals, who were randomly selected from the blood donors in the same area as the control group. To additionally have a sex-matched control group, we analyzed 151 DNAs of healthy female individuals from previous studies (12,15). None of the healthy individuals had any evidence of vitiligo and autoimmune diseases or a positive family history of vitiligo and autoimmune diseases.
Information including: age, sex, occupation, age of onset, type of disease, history of major stress, history of autoimmune diseases, familial history of autoimmune diseases and familial history of vitiligo, were collected in the context of a questionnaire from patients. Emotional stress caused in the time of examination, the death of the first-degree relatives and migration from war areas were considered as major stresses.
Blood Samples and DNA Extraction. 10 ml of EDTA-anticoagulated venous blood samples was obtained from all the cases. Genomic DNA was extracted by salting out method as described by Miller et al. (16).
ctla-4 Gene Amplification. Polymerase chain reaction (PCR) was used to amplify the desired fragment of ctla-4 gene using the primers described by Donner et al. (11). The primer pairs including 5′-gCT CTA CTT CCT gAA gAC CT-3′ and 5′- AgT CTC ACT CAC CTT TgC Ag-3′ were used in the reaction and resulted in a fragment with 162 bp amplicon size (Fig. 1). A 25 µl PCR amplification mixture containing 12 ng/µl genomic DNA, 0.38 mM dNTPs, 1.65 mM $MgCl_2$, 0.08 U Taq DNA polymerase (Cinnagen, Iran), 0.5 pM of each primers (TIB Mol Biol, Germany) and 1X PCR buffer (20 mM Tris-HCl, pH=8.4 and 50 mM KCl) was used. The amplification was performed for 35 cycles under a touch down program: 3 min at 94˚C for initial denaturation, followed by 11 cycles of denaturation at 94˚C for 45s, annealing from 70˚C to 60˚C with 1˚C decreasing per each cycle for 45s, ex-

tension at 72˚C for 45s and 20 cycles of denaturation at 94˚C for 45s, annealing at 60˚C for 45s and extension at 72˚C for 45s, followed by 2 cycles of annealing at 59˚C for 45s and 2 cycles of annealing at 58˚C for 45s and extension at 72˚C for 45s in each cycle and final extension at 72˚C for 5 min.

Detection of Alleles. Restriction fragment length polymorphism was used for identification of various alleles in ctla-4 gene. 10 µl of PCR product was mixed with 1µl (2u/µl) of BbvI restriction enzyme solution (Fermentas, Lithuania) and was stored at 65˚C for 16 h. DNA fragments were resolved in 4% agarose gel at 80V. Ethidium bromide staining was used to reveal the fragments under UV light. The presence of a "G" allele at position 49 caused two 88/74 bp fragments but in the presence of an "A" allele no digestion of the 162bp PCR fragment occurred (Fig. 2).

Statistical Analysis. The data were analyzed using SPSS (Version 10; SPSS Inc., Chicago, IL., USA) and EPI Info 2000 softwares. Differences in the alleles or genotypes frequencies were examined by Chi-square or Fisher's exact test when appropriate. Consistency of genotype frequencies with the Hardy-Weinberg equilibrium was tested using a χ^2 test on a contingency table of observed vs. expected genotype frequencies in each group.

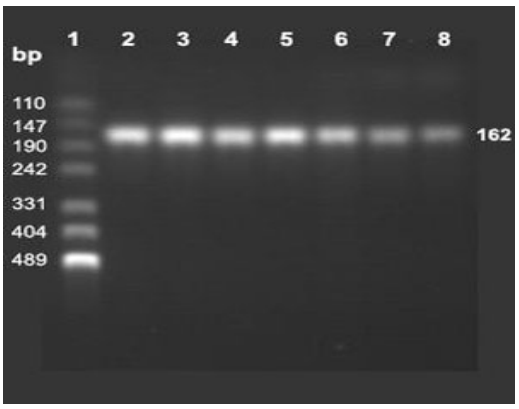

Figure 1. PCR amplification of the 162 bp fragment of exon 1 of ctla-4 gene. Lane 1: molecular weight marker (MassRuler DNA ladder, Fermentas, Lithuania), lanes 2-8: patients' DNA samples

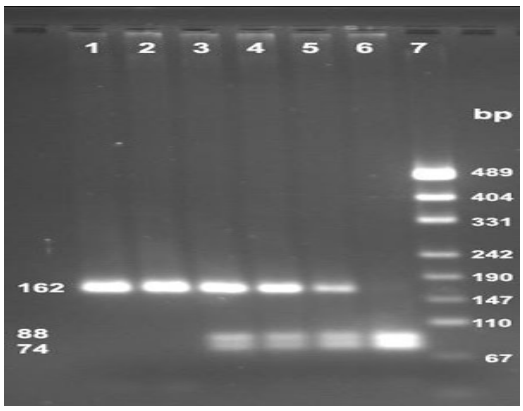

Figure 2. Results of RFLP analysis of the amplified fragment on a 4% agarose gel. Lanes 1 and 2 indicate AA genotype, lanes 3-5 indicate AG genotype and lane 6 indicates GG genotype. The molecular weight marker (MassRuler DNA ladder, Fermentas, Lithuania) is indicated in lane 7

RESULTS

Patients' ages ranged from 9 to 75 years (mean = 27.6 ± 14.4 years) and the age range of our control group was 17-68 years (mean = 37.1 ± 10.6 years). The disease age at onset ranged from 2-74 years (mean = 19.4 ± 13.8 years). 12 (13.3%) out of 90 patients had a positive family history of vitiligo, while only 2 (2.2%) of 92 patients had an associated autoimmune disease. Involvement of hair (leukotrichia) was not observed in the patients. The observed clinical subtypes of vitiligo among patients were: generalized (51%), acrofacial (39%), segmental (7%), focal (3%) and universal (0%).

Frequency and percentage of each genotype in patient and control groups are summarized in Table 1. The frequency of ctla-4 gene A49G polymorphism was compared in 101 vitiligo patients with 208 age/ethnicity and 359 sex/ethnicity matched controls. There were no significant differences between frequencies of ctla-4 genotypes in patients and age/ethnicity (P = 0.98) and sex/ethnicity (P = 0.85) matched controls. In addition, no deviation from Hardy-

Weinberg equilibrium was observed in the vitiligo patients (P = 0.99), age/ethnicity (P = 0.97) and sex/ethnicity (P = 0.99) matched controls. There were also no significant differences between allelic frequencies in patients and age/ethnicity (P = 0.86) and sex/ethnicity (P = 0.58) matched control groups (Table 2). A positive correlation between disease activity and gender in vitiligo patients was observed (P = 0.012), accordingly, a stable/regressive form of the disease was observed among females while the disease activity among male patients in most cases was progressive. A positive correlation between gender and familial history of vitiligo among patients was also observed (P = 0.008). Accordingly, male subjects were more likely to have a positive familial history of vitiligo than female subjects (25% *vs.* 5.5%). In addition, a trend of association between gender and history of major stress was observed suggesting more sufferers among females (P = 0.073).
No association was found between ctla-4 exon 1 (A49G) genotypes and clinical subtypes of vitiligo, disease activity, gender, familial history of vitiligo and history of stress.

Table 1. Genotypic frequencies of ctla-4 gene A49G polymorphism in patients and matched control groups

Genotype	Patients	Age/ethnicity* matched controls	Sex/ethnicity** matched controls
GG	10 (9.9%)	20 (9.6%)	39 (10.9%)
AG	42 (41.6%)	85 (40.9%)	157 (43.7%)
AA	49 (48.5%)	103 (49.5%)	163 (45.4%)
Total	101 (100%)	208 (100%)	359 (100%)

*$\chi^2 = 3.0$, P = 0.98
**$\chi^2 = 0.32$, P = 0.85

Table 2. Allelic frequencies of ctla-4 gene A49G polymorphism in patients and matched control groups

Allele	Patients	Age/ethnicity * matched controls	Sex/ethnicity** matched controls
A	140 (69.3%)	291 (70%)	483 (67.3%)
G	62 (30.7%)	125 (30%)	235 (32.7%)
Total	202 (100%)	416 (100%)	718 (100%)

*$\chi^2 = 3.0$, P = 0.86
** $\chi^2 = 0.3$, P = 0.58

DISCUSSION

Our results indicate that there is no association between ctla-4 A49G polymorphism and vitiligo in southern Iranian patients. In a previous study Kemp et al. (17) investigated the association of a microsatellite polymorphism lying in the 3′ UTR region of the ctla-4 gene with vitiligo. Their results indicated that in the absence of accompanying autoimmune diseases there was no association between the 106bp allele and vitiligo (17). Since in our study only 2.2% of the patients had a positive history of autoimmune diseases the comparison of the results with regard to this factor was not possible.
In our investigation no correlation was observed between genotypes of patients and disease type, disease activity and gender, which is in accordance with the results of Kemp et al. (17). However, our results are contrary to the frequently reported association of ctla-4 genotypes with several autoimmune diseases (11-13,18,19).

To date, several hypotheses have been proposed to define the etiology of vitiligo, some of which are based on the immunological pathogenesis evidences (8,20-22). However, most of these immunological pathogenic reactions can also be a consequence of the disease rather than being a cause (8,23). Therefore, it is possible that the autoreactivities observed in vitiligo arise in response to challenges to the immune system by non-immunological mechanisms (8). Accordingly, an "integrated theory" has been proposed for the disease in which, vitiligo is considered a primary melanocytorrhagic disorder (24). On the basis of this theory, autoimmune phenomena are secondary to the detachment of melanocytes (25,26), which can be a consequence of increased expression of tenascin (27) and/or local traumas to the skin (28).

Our results indicated that, at least in southern Iranian patients, male patients were more likely to have a familial history of vitiligo, and might suffer from a more progressive disease, while female patients were more likely to have a history of major stress and might suffer from a stable/regressive form of the disease. In this regard, it is logical to assume different etiologies in the occurrence of vitiligo in the studied male and female subjects. It is worth mentioning that neural hypothesis mostly describes segmental type of vitiligo, while autoimmune hypothesis is commonly related to the generalized vitiligo (21). Therefore, there might also be different etiologies for different types of the disease in male and female subjects.

In contrast to the studies in many other autoimmune diseases, we did not find any association between ctla-4 exon 1 polymorphism and vitiligo. Since the rediscovery of T reg cells, the immunoregulatory role of CTLA-4 protein in the immune responses and the importance of its deregulation in autoimmune diseases have been well established (29-31). A large body of information exists on the association of ctla-4 gene polymorphisms and various autoimmune diseases such as Graves' hyperthyroidism, Addison's disease, etc. (11-13,18,19,32-34). The lack of association between this polymorphism and vitiligo might therefore suggest the involvement of other (maybe unknown) immune regulatory genes and/or reflect a different nature or multiple etiologies for vitiligo.

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