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Original Article

Relationship between manganese superoxide dismutase (MnSODAla-9Val) and glutathione peroxidase (GPx1 Pro 197 Leu) gene polymorphisms and alopecia areata

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Abstract: Objective: The role of the oxidative stress in alopecia areata (AA) has been studied by several researchers in a few studies with conflicting results. These results suggested that lipid peroxidation and alterations in the oxidant-antioxidant enzymatic system may play a role in the pathogenesis of AA. Therefore, we aimed to examine the possible associations between the MnSOD Ala-9Val and GPx1 Pro 198 Leu polymorphisms and AA susceptibility and disease progression in Turkish population. Methods: The study group consisted of 119 unrelated patients with AA and 104 unrelated healthy controls with no scalp lesions in their personal history or on clinical examination. Genotyping was performed to identify MnSOD Ala-9Val and GPx1 Pro 198 Leu polymorphisms by a method based on PCR amplification and detection of polymorphisms with hybridization probes labeled with fluorescent dyes. Genotype and allele frequencies were compared between patients with AA and healthy control subjects. Results: There was no significant difference between the MnSOD Ala-9Val SNP genotype distributions and allele frequencies of the AA patients and the control group (P=0.168 and P=0.820, respectively). There was not any association between clinical and demographical features of the study patients with AA and MnSOD Ala-9Val and GPx1 Pro 198 Leu polymorphism genotypes except gender. Conclusions: This study is unique since an investigation to reveal the possible associations between the MnSOD Ala-9Val and GPx1 Pro 198 Leu polymorphisms and AA susceptibility and in Turkish population.

Keywords: Alopecia areata, MnSOD Ala-9Val, GPx1 Pro 198 Leu, polymorphism

Introduction

Alopecia areata (AA) is a chronic inflammatory disorder that is assumed to be a tissue-specific, T-cell mediated autoimmune disease of the hair follicle, clinically characterized by well-defined patches of non-scarring hair loss, which can promote to complete loss of all body hair in severe cases [1, 2]. AA has a multifactorial background that many factors including genetic predisposition, autoimmunity, environmental factors, infections agents, drugs and emotional stress have been implicated. Even if there have been significant progresses for illuminating the pathogenesis of AA, the exact aetiopathogenesis of the disease can not been completely understood yet [1-4]. To be able to contribute this complicated and unclear pathogenesis of AA, the role of the oxidative stress in AA has also been studied by several researchers in a few studies with conflicting results. These results suggested that oxidant/antioxidant imbalance may play a role in the etiopathogenesis of AA [5-10].

Inadequate antioxidant protection or excess reactive oxygen species (ROS) production creates a condition known as an oxidative stress, contributing to the development of cutaneous disease and disorders like atopic dermatitis,

psoriasis, vitiligo, acne vulgaris, pemphigus vulgaris and alopecia areata [10-14]. The intention of ROS against the skin can be explained by damaging effect on the cell compounds such as protein, lipid and DNA. ROS production in cells is controlled by the antioxidant enzymes, such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) [15, 16]. The manganese SOD (MnSOD), the most significant type, is an intramitochondrial enzyme that scavenges the superoxide anions produced by mitochondrial energy metabolism, and which converts O_2 - into hydrogen peroxide (H_2O_2) [17, 18]. GPx acts against ROS by reducing hydrogen peroxide (H_2O_2) to H_2O. GPx1 is most abundant izoenzyme of identified four different GPx izoenzymes which is selenium dependent and expressed in epithelial tissues of human breast and other organs [19, 20]. The ultimate levels of mitochondrial ROS likely depend on the activities of both MnSOD and GPx1 [15-17, 19].

The MnSOD enzyme is encoded by a gene located in the long arm (6q25) of chromosome 6. The Ala-9Val polymorphism in exon 2 of the MnSOD gene is the most extensively studied single nucleotide polymorphism (SNP) at codon 16 (rs4880), with the Ala-to-Val substitution possibly resulting in the alteration of MnSOD activity in human mitochondria. A high activity of the MnSOD Ala allele could increase production of H_2O_2 and contribute to the accumulation of ROS [21, 22].

GPx1 enzyme is encoded by GPx1 gene, one of the major factors regulating GPx1 activity, which is located at chromosome 3p21 and contains two exons. A transition of C to T at nucleotide 594 in exon 2 of the GPx1 gene has been detected, corresponding to an amino acid change from proline (Pro) to leucine (Leu) at codon198 (rs1050450). This polymorphism is functional in humans and impacts the response of GPx1 activity [23].

Therefore, genetic differences in the antioxidant genes coding for the GPxs, catalase CAT, and SOD enzymes may modify ROS detoxification and may alter disease risk [18]. The role of oxidative stress and oxidant/antioxidant imbalance in the pathogenesis of AA, has been established in some studies, however any studies concerning about the association of these two polymorphisms with AA have not been performed yet. As a result, we performed this study to be able to establish a potential connection between the MnSOD Ala-9Val and GPx1 Pro 198 Leu polymorphisms and AA susceptibility in Turkish population.

Materials and methods

Subjects

The study group consisted of 119 unrelated patients with AA (68 male and 51 female; mean age: 32.55±9.606 standard deviation [SD] years), and 104 (54 male and 50 female; mean age: 32.28±11.908 SD years) unrelated healthy controls with no scalp lesions in their personal history or on clinical examination. AA patients were gathered from Department of Dermatology of Gaziosmanpasa University, Tokat, Turkey. Clinical and demographical data were obtained from all patients, including gender, age, disease duration, number of attacks, family history, associated psychiatric disorders, focal infection, nail dystrophy, the severity of AA; more than 50% involvement as diffuse and less than 50% involvement as localized and the localization of the AA lesions; scalp, beard/mustache, eyebrow, eyelash. The inclusion criterion was a diagnosis of AA according to standard criteria [24]. AA clinical subtype was determined according to the AA investigational assessment guidelines. Patients were categorized as having patchy AA, alopecia totalis (AT), alopecia totalis/universalis (AT/AU) or alopecia universalis [24]. All participants, patients and healthy controls, were of Turkish origin, from inner Central Black Sea region of Turkey. The healthy controls matched for age and gender with AA patients. The protocol of this study was approved by the Institutional Ethics Committee, and all participants gave written informed consent before entering the study.

Genotyping

DNA isolation: Blood specimens were drawn into EDTA containing tubes and genomic DNA samples were extracted from the peripheral leukocytes of the collected venous blood by the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the instructions of the manufacturer.

MnSOD Ala-9Val and GPx Pro 198 Leu genotyping: To identify MnSOD Ala-9Val and GPx1 Pro 198 Leu SNPs, genotyping was performed using PCR amplification and polymorphisms

Table 1. Baseline clinical and demographical features of the study patients with AA stratified according to manganese superoxide dismutase (MnSODAla-9Val) and glutathione peroxidase (GPx1 Pro 197 Leu) gene polymorphisms

Characteristic	Total n=119	MnSOD				GPx1			
		Ala/Ala n=21	Ala/Val n=63	Val/Val n=35	p	Pro/Pro n=56	Pro/Leu n=54	Leu/Leu n=9	p
Gender, male/female, n (%)	68/51 (57.1/42.9)	18/3 (85.7/14.3)	32/31 (50.8/49.2)	18/17 (51.4/48.6)	**0.014**	37/19 (66.1/33.9)	24/30 (44.4/55.6)	7/2 (77.8/22.2)	**0.031**
Age (years)	32.55±9.606	31.67±9.593	33.98±9.925	30.51±8.826	0.208	32.63±9.766	32.19±9.443	34.33±10.488	0.825
Disease duration (months)	22.51±54.066	13.90±43.916	24.16±63.467	24.70±40.122	0.727	13.90±34.908	32.31±70.732	17.22±19.867	0.195
Number of attacks	1.21±3.220	0.57±0.746	1.27±4.096	1.49±2.174	0.580	0.61±1.317	1.87±4.476	1.00±1.658	0.118
Family history, n (%)					0.927				0.514
Positive	4 (3.4)	1 (4.8)	2 (3.2)	1 (2.9)		3 (5.4)	1 (1.9)	0	
Negative	114 (96.6)	20 (95.2)	61 (96.8)	33 (97.1)		53 (94.6)	53 (98.1)	8 (100)	
Stress, n (%)					0.417				0.978
Positive	70 (58.8)	15 (71.4)	36 (57.1)	19 (54.3)		33 (58.9)	32 (59.3)	5 (55.6)	
Negative	49 (41.29	6 (28.6)	27 (42.9)	16 (45.7)		23 (41.1)	22 (40.7)	4 (44.4)	
Focal infection, n (%)					0.417				0.385
Positive	13 (10.9)	1 (4.8)	9 (14.3)	3 (8.6)		8 (14.3)	5 (9.3)	0	
Negative	106 (89.1)	20 (95.2)	54 (85.7)	32 (91.4)		48 (85.7)	49 (90.7)	9 (100)	
Nail dystrophy, n (%)					0.759				0.746
Positive	40 (33.6)	6 (28.6)	23 (36.5)	11 (31.4)		19 (33.9)	19 (35.2)	2 (22.2)	
Negative	79 (66.4)	15 (71.4)	40 (63.5)	24 (68.6)		37 (66.1)	35 (64.8)	7 (77.8)	
Alopecia severity					0.513				0.359
<50%	115 (96.6)	20 (95.2)	62 (98.4)	33 (94.3)		54 (96.4)	53 (98.1)	8 (88.9)	
>50%	4 (3.4)	1 (4.8)	1 (1.6)	2 (5.7)		2 (3.6)	1 (1.9)	1 (11.1)	
Alopecia localization					0.517				0.898
Scalp	85 (71.4)	12 (57.1)	47 (74.6)	26 (74.3)		42 (75.0)	36 (66.7)	7 (77.8)	
Beard/mustache	28 (23.5)	8 (38.1)	14 (22.2)	6 (17.1)		12 (21.4)	14 (25.9)	2 (22.2)	
Eyebrow	4 (3.4)	1 (4.8)	1 (1.6)	2 (5.7)		1 (1.8)	3 (5.6)	0	
Eyelash	2 (1.7)	0	1 (1.6)	1 (2.9)		1 (1.8)	1 (1.9)	0	

Data were analyzed by analysis of variance and χ^2 test. Mean plus standard deviation values are presented for age, disease duration and number of attacks. AA: Alopecia areata; MnSOD: Manganese superoxide dismutase; GPX: Glutathione peroxidase. The results that are statistically significant are shown in boldface.

were detected with hybridization probes labeled with fluorescent dyes (LightCycler 480 II Real-Time PCR System, Roche Diagnostics, Mannheim, Germany). Target fragments of the human MnSOD and GPX1 genes were amplified with specific primers. To detect the MnSOD Ala-9Val polymorphism, we applied 10 pmol of the forward primer 5'-CAGCCTGCGTAGACGGTCCC-3' and the reverse primer 5'-CGTGGTGCTTG-CTGTGGTGC-3', and 3 pmol of the sensor probe 5'-CTCCGGCTTTGGGGTATCTG-fluorescein-3' and the anchor probe 5'-LC Red 640-GCTCC-AGGCAGAAGCACAGCCTCCp-3'. To detect the GPX1 Pro 198 Leu polymorphism, we also used 10 pmol of the forward primer 5'-ACTTTGAGA-AGTTCCTGGTG-3' and the reverse primer 5'-TT-CCTCCCTCGTAGGTTTAG-3', and 3 pmol of the sensor probe 5'-CAGACCATTGACATCGAGCCTG-ACATCGAA-fluorescein-30 and the anchor probe 50-LC Red 640-TGCTGTCTCAAGGGCC-CAG-p-3'. The LC FastStart Master Hybridization Probes buffer (Roche Diagnostics Inc.) was used as a reaction buffer. All primers and hybridization probes were designed and synthesized by TIB MOLBIOL (Berlin, Germany). The genotypes were identified by running a melting curve with specific Tm. Wild type MnSOD Ala exhibits a Tm of 65±0.5°C, while wild type GPX1 Pro yields a Tm of 66±0.5°C. The allele variant MnSOD Val exhibits a Tm of 56±0.5°C, and the allele variant GPX1 Leu exhibits a Tm of 57±0.5°C. The PCR reaction was as follows: Initial denaturation at 95°C for 10 min, followed by 20 cycles at 95°C for 10 s, annealing at 60°C (MnSOD) and 50°C (GPx1) for 20 s, extension at 72°C for 20 s. And then a melting curve was recorded by an initial increase in temperature to 95°C, cooling the reaction mixture to 40°C at 20°C/s holding for 30 s and then slowly heating it to 85°C at 0.1°C/s with continuous acquisition. Finally, the fluorescence signal was plotted against temperature in real time to produce melting curves for each sample.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 20.0) and the OpenEpi Info software package version 3.01 (www.openepi.com). Results were given as mean ± standard deviation (S.D.). The relationships between MnSOD Ala-9Val and GPx1 Pro 198 Leu gene polymorphisms and the clinical and demographics features were analyzed by using χ^2 test or analysis of variance (ANOVA) statistics. χ^2 test and Fisher's exact test were used to compare categorical variables appropriately, and odds ratio (OR) and 95% confidence interval (CI) were used for the assessment of risk factors. All *P* values were 2-tailed, and *p* values less than 0.05 were considered as significant.

Results

Baseline clinical and demographical features of the study patients with AA were shown in **Table 1**. Gender, age, disease duration, number of attacks, family history, stress, focal infection, nail dystrophy, alopecia severity and alopecia localization of AA patients were analyzed. Age and gender were not different between patient and control groups (P=0.85 and 0.50, respectively). There was not any association between clinical and demographical features of the study patients with AA and MnSOD Ala-9Val andGPx1 Pro 198 Leu polymorphism genotypes except gender (**Table 1**). The significant association between gender MnSOD Ala-9Val and GPx1 Pro 198 Leu polymorphism was found as P=0.014 and 0.031, respectively).

The genotype distributions and allele frequencies of the MnSOD Ala-9Val and GPx1 Pro 198 Leu SNPs in patients and controls are shown in **Table 2**. The frequencies of Pro/Pro, Pro/Leu and Leu/Leu genotypes of GPx1 Pro 198 Leu polymorphism in the patients were 47.1%, 45.4%, and 7.6%; in the controls were 41.3%, 41%, and 14% (**Table 2**). Pro and Leu allele frequencies were 69.7% and 30.3% in patient group and 65.9 % and 34.1% in control group (**Table 2**). There was no significant difference between the MnSOD Ala-9Val SNP genotype distributions and allele frequencies of the AA patients and the control group (P=0.168 and P=0.820, respectively). The frequencies of Ala/Ala, Ala/Val and Val/Val genotypes of MnSOD Ala-9Val polymorphism in the patients were 17.6%, 52.9%, and 29.4%; in the controls were 12.5%, 65.4%, and 22.1% (**Table 2**). Ala and Val allele frequencies were 44.1% and 55.9% in patient group and 45.2% and 54.8% in control group (**Table 2**). There was also no significant difference between distributions of the genotype or allele frequencies of the GPx1 Pro 198 Leu SNP of the patient groups and control subjects (P=0.657 and P=0.383, respectively).

Table 2. Genotype and allele frequencies of MnSOD and GPx1 gene polymorphisms in patient and control groups

Gene	AA patients n=119 (%)	Healthy controls n=104 (%)	P	OR (CI 95%)
MnSOD (Ala-9Val)				
Genotypes				
Ala/Ala	21 (17.6)	13 (12.5)	0.168	
Ala/Val	63 (52.9)	68 (65.4)		
Val/Val	35 (29.4)	23 (22.1)		
Alleles				
Ala	105 (44.1)	94 (45.2)	0.820	1.04 (0.72-1.52)
Val	133 (55.9)	114 (54.8)		
GPx1 (Pro 197 Leu)				
Genotypes				
Pro/Pro	56 (47.1)	43 (41.3)	0.657	
Pro/Leu	54 (45.4)	51 (49.0)		
Leu/Leu	9 (7.6)	10 (9.6)		
Alleles				
Pro	166 (69.7)	137 (65.9)	0.383	0.84 (0.56-1.25)
Leu	72 (30.3)	71 (34.1)		

AA: Alopecia areata; mnSOD: Manganese superoxide dismutase; GPx1: Glutathione peroxidase.

Discussion

In this study, we examined the associations of MnSOD Ala-9Val and GPx1 Pro 198 Leu gene polymorphisms in a group of Turkish patients with AA to expose whether it is a risk factor or not for the development of AA. However, we could not detect statistically significant association between AA patients and controls according to genotype and allele distribution of MnSOD Ala-9Val and GPx1 Pro 198 Leu gene polymorphisms. To the best of our knowledge, this is the first study investigating the association between AA and MnSOD Ala-9Val and GPx1 Pro 198 Leu gene polymorphisms.

The role of oxidant/antioxidant status has been proposed as one of the striking probable new pathogenetic mechanisms because of apoptotic and autoimmune features of AA. It has been proposed that ROS forms covalent bond with endogen proteins and causes the formation of hair follicle antigen by producing structural changes in the proteins. Autoimmun mechanisms are triggered by formation of new antigens [25]. These autoimmune process results hair follicle cell injury and, ultimately, cell death [15]. Therefore it can be also concluded that oxidative stress triggers apoptosis and contributes to development of AA [26]. Although these two theories support the initiating role of oxidative stress in the aetipathogenesis of AA, it is still not known if oxidative stress is initiator for AA or if it develops as the result of inflammation.

The previous studies reporting the oxidant/antioxidant status of AA is limited and conflicting [5-10]. In the study Naziroglu & Kokcam performed, increased oxidative stress and decreased GSH-Px activity were detected [7]. Koca et al. 10,4 also observed increased lipid peroxidation and decreased SOD activity in the serum of subjects with AA compared with controls [8]. Abdel Fattah et al. found significantly higher MDA levels and lower SOD activity in patients with AA compared with healthy controls in both blood and the scalp tissues of subjects with AA [4]. Bilgili et al. found serum total antioxidant capacity levels and paraoxonase-1 activity were significantly lower in the subjects with AA than controls whereas total oxidant status levels were significantly higher in the subjects with AA [27].

Different from these studies, Akar et al. found the levels of oxidative stress, SOD and GSH-Px in scalp of patients with AA significantly higher than those of controls [4]. Similarly, Gungor et al. reported decreased erythrocyte SOD and GSH-Px levels and increased serum lipid peroxidation in the patients with AA when compared with the control group [10]. These conflicting results may be due to the differences between the duration of the disease, pattern or severity of hair loss in the AA patients of the studies.

A polymorphism in the sequence of an important antioxidant enzyme, Mn-SOD, may influence the biological behavior of this enzyme and therefore the clinical diversity of AA. A change in enzyme concentration through the action of Ala-9Val, may result in ROS accumulation and

hence cell injury [17, 21, 22]. In various studies; Ala allele found to be associated with higher activity, which results in more efficient transport of MnSOD into the mitochondria [21, 28] while in a study Val allele is found to be associated with a lower MnSOD content [29].

Number of studies has identified the association of MnSOD polymorphism with various diseases including diabetes mellitus, hypertension, Parkinson's disease, schizophrenia, asthma, nonfamilial idiopathic cardiomyopathy [21, 28-32]. Because MnSOD plays a critical role in the defense against oxidant-induced injury and apoptosis of rapidly growing cancer cells, it is also considered a unique tumor suppressor protein [33]. Therefore most of the studies have performed to be able to establish the association between MnSOD polymorphism and various cancer types like, breast cancer, ovarian carcinoma, lung cancer, prostate cancer with variable results [18, 22, 34-36].

Previous studies have shown that erythrocyte *GPx-1* activity was modulated by the *GPx-1* Pro 198 Leu polymorphism. This variant influences *GPx-1* enzyme activity; with lower activity being associated with the Leu allele. These indicate that the amino acid substitution has a biological phenotype [37, 38]. However, the results of association of GPx1 Pro 198 Leu polymorphism and GPx activity are inconsistent. Erdem et al. reported there were no relationships between GPx1 polymorphism and erythrocyte GPx activity [33]. GPx1 polymorphisms have been investigated in several types of malignancies, in diverse populations, such as prostate cancer, breast cancer, lung cancer and bladder cancer [20, 23, 37-40]. The results supported the hypothesis that the *GPx-1* Pro 198 Leu polymorphism serves as a potential susceptibility tumor marker. Previous investigations also found that the frequency distribution of Leu allele significantly varied in different ethnicities (36% in Caucasians, 33% in Africans and 5% in Japanese) [39, 40]. This study showed that the mean frequency of the GPX1 variant Leu allele was 30.3%.

The *FAS/FASLG* polymorphisms that have an important role in apoptosis and in autoimmune diseases and have been studied in AA before and GG genotype of *FAS*-670A/G polymorphism was found to be associated with a lower risk for susceptibility to alopecia areata [41]. However, even if MnSOD Ala-9Val and GPx1 Pro 198 Leu gene polymorphisms share the common pathogenetic mechanisms, we could not find any significant association. In this study, most of the patients were composed of localized AA patients with less than 50% involvement mostly on the scalp. Even if there has been an association reported between oxidative stress and the disease duration, pattern or severity of hair loss in several studies previously mentioned, we could not find a statistically significant association between clinical and demographical features of the AA patients and MnSOD Ala-9Val and GPx1 Pro 198 Leu polymorphism genotypes except gender. A male predominancy has been observed in Ala/Ala genotype and Leu/Leu genotypes. However, we observed that the Ala/Ala genotype and Leu/Leu genotype were less frequent than the other genotypes. The variability of the clinical characteristics of the disease and other gene-environment interaction, the racial and ethnic differences may also effect the association between these polymorphisms.

Genetic variation in other ROS defense genes and ROS-producing genes could be important modifiers in the relationship MnSOD and GPx1 with AA. Other antioxidant enzyme gene polymorphisms as well as oxidant enzymes should thus be taken into account in future studies. To be able to evaluate the functional consequence of the polymorphisms, it would be better to measure the plasma levels of anti-oxidants. Therefore, further well-designed studies with wider spectrum of subjects on different populations and ethnicities will be required to comprehensively investigate MnSOD Ala-9Val and GPx1 Pro 198 Leu gene polymorphisms together with the activity and levels of the anti-oxidant enzymes, and will be able to comprehensively interpretate the association between these polymorphisms and AA, elucidate the complex immunopathogenesis and contribute the clinical management AA. The study we presented here generated a skeleton for the next studies about the role of MnSOD Ala-9Val and GPx1 Pro 198 Leu gene polymorphisms in the susceptibility of complex diseases caused by oxidative stress exposure.

Disclosure of conflict of interest

None.

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