Glutathione S-Transferases M1/T1 and P1 Polymorphisms in Patients with Alopecia Areata

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ABSTRACT

Introduction: Alopecia areata (AA) is a common, recurrent, chronic autoimmune and inflammatory disease. Many factors including genetic predisposition, emotional and environmental stress are thought to play important roles in its development. Since antioxidant/oxidant balance disturbance is a common feature in autoimmune, emotional and environmental stress, current evidences support the association between oxidative stress and AA. Glutathione S-transferases (GSTs) play a major role in the detoxification of various compounds. We aimed to determine the frequencies of GSTM1-GSTT1-GSTP1 polymorphisms in 176 patients with AA and 196 age- and sex-matched healthy controls.

Methods: GSTM1 and GSTT1 genotypes were determined by multiplex PCR whereas GSTP1 polymorphisms were analyzed by using PCR-RFLP technique.

Results: Genotype frequencies of the GSTM1, GSTT1 and GSTP1 polymorphisms showed significant differences between AA patients and healthy controls.

Conclusion: Our results suggested a significant association between the GSTP1, GSTT1 and/or GSTM1 null genotypes and AA. However, the potential role of GSTs as markers of susceptibility to AA needs further studies in larger patient groups.

Keywords: polymorphism; molecular genetics; GSTM1-GSTT1-GSTP1; alopecia areata, PCR-RFLP

INTRODUCTION

Alopecia areata (AA) is a common recurrent dermatological disease characterized by non-scarring hair loss of the scalp or body or both. Although the etiology of the disease remains incompletely understood, some evidences suggested the roles of immunological and genetic components in the generation of the disease [1, 2]. The life time risk of developing the disease in each individual is approximately 2% [3, 4].

AA may have many clinical presentations. In the most common forms, patches are seen in the scalp and beard regions. In a typical patch, the pathognomonic exclamation-mark hairs could be seen at the edges of the affected area. In severely affected individuals, AA may progress to include total loss of hairs on the scalp (alopecia totalis) or total loss of scalp and body hairs on the epidermis (alopecia universalis) [5].

Depending on their very different and important functions, such as to protect the skin from UV radiation and to suppress heat loss from body, the basic structure of the hair follicle is the most complicated and important annexes of skin. Normally, hair follicles have a cycle of growth with three phases: anagen, catagen and telogen. While anagen is the active phase that hair grows, hair growth is interrupted and keratinocyte apoptosis occurs during the catagen phase, it is also the short transition phase to the resting phase. The last, telogen phase is the resting phase of the hair follicle. Each follicle goes under changes independently during the cycle. In the patients with AA, hair loss may result from changes in these phases, such as premature transition from anagen to catagen or directly transition from anagen to telogen phases may happen. It is not known exactly what influences these abnormal alterations in phase transitions. However, there are evidences that immunological and genetic factors may play a role. Although most of AA cases are sporadic, the studies related with family-base linkage, monozygotic twin and heritability of the disease among first degree relatives showed that AA can be
characterized as a polygenic trait due to 50% concordance between the monozygotic twins [6, 7] and the incidence of a positive family history ranges between 10 and 42% among patients with AA [2].

Several research groups have reported that AA is an autoimmune disorder that results from infiltration of CD4+ and CD8+ T lymphocytes to the anagen hair follicles [8]. The inflammatory cell infiltration around the hair follicle in AA causes the disease to be associated with oxidative stress [9, 10].

The skin is chronically exposed to both endogenous and environmental pro-oxidant agents leading to the formation of ROS, which causes damaged cellular components such as nucleic acids, proteins and cell membrane lipids [11, 12]. As a result of permanent oxidative processes, the body has developed antioxidant defense mechanisms to prevent the attack of biological molecules.

The role of oxidative stress in AA and other skin diseases including vitiligo [13-15] has been studied by several research groups. Currently it is accepted that oxidative stress is a triggering event in the melanocytic destruction and is probably involved in the etiopathogenesis of vitiligo disease [16]. By the way, the strong association between the AA and vitiligo has been reported previously. It has been estimated that the risk increases two times in an AA case for development of vitiligo [17]. On the other hand, hair follicles are also exposed to excessive ROS that are generated through both endogeneous metabolic reactions and environmental pro-oxidant factors such as air pollutants, drugs, smoking inhalation. The ability of HF to maintain redox homeostasis requires effective antioxidant defense mechanisms to prevent the attack of biological molecules. Although the role of ROS in the pathogenesis of AA has been reported, the knowledge about the detoxification enzymes genotypes in AA susceptibility is unknown.

Glutathione-S-transferases (GSTs) refers to a group of detoxification enzymes that are pivotal components of the cellular defense against oxidative stress. The GSTs are a family of cytosolic enzymes (dimeric) composed of multifunctional subunits [18, 19] and this supergene family consists of eight different subclasses, including alpha, mu, theta, pi, zeta, sigma, kappa and omega, respectively [20] and act as a component of cellular defense against oxidative stress [21]. The GSTs gene family is associated with modulation of oxidative stress through the prostaglandin signaling pathways and regulation of inflammation as a result of regulation of normal cellular physiology [20, 22].

Although there are studies that support the association between oxidative stress and AA, and the expressions of GST isoforms have been shown in human skin, it is not, however, known if there is an association between the GST isoform genotypes and AA susceptibility. In the present study, we aimed to investigate the relationship between GSTM1, GSST1 and GSTP1 antioxidant genes polymorphisms and AA susceptibility in a Turkish cohort.

**Material - Methods**

**Selection of Subjects and Controls**

This study included 166 cases with AA and 196 healthy subjects as a control group. The patients diagnosed with AA by the dermatologists from Department of Dermatology of the Eskisehir Osmangazi University , have been included into the study. After explaining the aim of the study, written informed consent was obtained from each patient, and controls. The study protocol and procedure were approved by the Institutional Ethics Review Committee, and all the procedures pertaining to the study were conducted in accordance with the Declaration of Helsinki, local laws and regulations.

**DNA Isolation**

Genomic DNAs of the patients and control group subjects were was isolated from the peripheral blood samples by using Vivantis DNA Blood Extraction Kit (Vivantis technology, USA), according to the manufacture’s recommendations and stored at -20 °C. The average genomic DNA concentration was measured with NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA).

**Genotyping**

**Analysis of GSTM1 and GSTT1 deletion**

The contemporaneous GSTT1 and GSTM1 genotyping for gene deletion was carried out by the multiplex PCR method. The ubiquitous β-globin gene was used as an internal control. Briefly, multiplex PCR was carried out in a final volume of 25 μL containing 10 μL genomic DNA template, 5 μL of 10X PCR buffer A (Vivantis technology, USA), 10 nM of each primer, 2 μL of 50 mM MgCl$_2$ (Vivantis technology, USA), 0.5 μL of 10 μM dNTPs (Vivantis technology, USA), and 0.5 μL of 5 U Taq polymerase enzyme (Vivantis technology,
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USA). The DNA was initially denatured at 94 °C for 2 min prior to amplification. PCR amplification was accomplished by using 35 cycles, consisting of 30 sec denaturation at 94 °C, 30 sec of annealing at 55 °C and 1 min of extension at 72 °C and the final extension cycle at 72 °C for 7min.

The PCR fragments of GSTT1 and GSTM1 were separated by 3% agarose gel electrophoresis stained with ethidium bromide (250 ng/ml). PCR products of GSTT1 and GSTM1 were 480 base pairs (bp) and 215 base bp, respectively, whereas the presence or absence of these bands was determined in the presence of the control β-globin gene (268 bp). The null genotype was detected when no band appeared at specific region. The primer sequences and related product sizes are shown in Table 1.

### Table 1. Primer sequences for the polymerase chain reaction amplification of polymorphic sites genes and their related sizes for each polymorphism.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTT1</td>
<td>5′-tcct act ggt cct cac atc tc-3′ 5′-tcca cgg cat ggc cag ca-3′</td>
<td>215 bp</td>
</tr>
<tr>
<td>GSTM1</td>
<td>5′-gaa ctc cct gaa aag cta aag c-3′ 5′-gtt ggg ctc aaa tat aeg gtg g-3′</td>
<td>480 bp</td>
</tr>
<tr>
<td>β-globin</td>
<td>5′- caa ctt cat cca gct tca cc-3′ 5′-gaa gag cca agg aca ggt ac-3′</td>
<td>268 bp</td>
</tr>
</tbody>
</table>

**GSTP1 gene**

The GSTP1 gene, an A to G polymorphism at the nucleotide 313 in exon 5 leads to an amino acid substitution (A313G, Ile105Val) at 105 amino acid position. This residue lied in the substrate-binding site of the enzyme and the polymorphism has been shown to affect enzyme activity. The modified PCR-RFLP method by HpyCH4IV restriction endonuclease enzyme (Biolabs Inc NEB, New England) was used for the genotyping of GSTP1 gene in the patients and controls. The primer sequences for the GSTP1 forward and reverse primers were 5′- GGT GGA CAT GGT GAA TGA C-3′ and 5′- GAT GCT CAC ATA GTT GGT GGT AG-3′, respectively.

The digested PCR products were separated by 4% agarose gel electrophoresis stained with ethidium bromide and visualized on a UV transilluminator with 50 base pair DNA ladder. For the GSTP1 rs1695 polymorphism, the 313A (Ile/Ile) homozygous wild type allele was not digested (176 bp), while the 313G (Val/Val) homozygous mutant allele produced two fragments with the length of 91 bp and 66 bp and the heterozygous allele 313A/G (Ile/Val) demonstrated two fragments with the length of 91 bp and 176 bp.

**Statistical analysis**

Data collected from the patients were organized in a database and statistical calculations were performed by using IBM SPSS 21 package programme. Genotypic dependence between patients and controls was determined by the χ2 test, and odds ratio (OR) was calculated from 2x2 contingency tables. In addition, Mann Whitney U test and Spearman's rho test were employed to determine the correlation of age, gender, immune diseases and genotype for each study group. Yates’ correction was applied when the value of an expected frequency was <5. A p-value less than 0.05 was considered as statistically significant.

**RESULTS**

In this study, we examined deletion polymorphisms of the GSTM1/GSTT1 genes and p.Ile105Val polymorphism of the GSTP1 gene. The allelic and genotypic distributions of these three polymorphisms in AA patients and controls are given in Table 2.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LP (N=166) N(%)</th>
<th>Control (N=196) N(%)</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile/Ile</td>
<td>0 (0)</td>
<td>30 (15.3)</td>
<td>1</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Val/Val</td>
<td>68 (40.9)</td>
<td>0 (0)</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ile/Val</td>
<td>98 (59.1)</td>
<td>166(84.7)</td>
<td>1.590</td>
<td>(1.395-1.813)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GSTM1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>150 (90.9)</td>
<td>196(100)</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
For GSTT1 and GSTM1 polymorphisms, genotype was defined as ‘presence’ if at least one copy of the gene was present. Of the 166 cases, 28 (15.9%) showed null genotype whereas GSTT1 genotype was present in the remaining 138 (84.1%), but no gene deletion was revealed in the control group. A significant difference was observed between cases and controls in terms of GSTT1 genotypic distribution (P <0.001).

A statistically significant genotypic distribution difference was also seen between the cases and controls groups for the GSTM1 polymorphism (P <0.01).

A total of 150 (90.9%) of the cases and all of controls carried presence genotype for GSTM1. However, 16 (9.1%) cases with AA had null genotypes for this polymorphism (Table 2, Figure 1).

The GSTP1 genotyping and association studies was performed in 362 participants. The genotyping classification in both controls and patients was as follows: wild type AA (Ile/Ile), homozygous mutant GG (Val/Val) and heterozygous mutant AG (Ile/Val). The homozygous wild AA (Ile105Ile) genotype was not identified in the AA group while the frequency of the wild-type allele in the control group was 15.3%. The GSTP1 gene Val/Val homozygous mutant genotype could not be seen in the control group but 68 cases (40.9%) with AA. In other hand, 166 (84.7%) subjects from the control group and 98 (59.1%) cases with AA showed the heterozygous AG (Ile105Val) genotype. The genotype distributions and frequencies of GSTP1 gene polymorphisms in both the case and the control groups are given in table 3. Although no statistically significant difference was found between the patient and control groups for the wild-type allele, the differences between the groups were significant in both the heterozygous and the homozygous mutant genotypes.

### Table 3. Allel distributions in the patient and control groups for GSTP1 313 (Ile105Val) A>G gene deletion.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients (N=166) N (%)</th>
<th>Controls (N=196) N (%)</th>
<th>OR</th>
<th>P</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.001***</td>
<td></td>
</tr>
<tr>
<td>AA (reference genotype)</td>
<td>0 (0)</td>
<td>30 (15.3)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>68 (40.9)</td>
<td>0 (0)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>98 (59.1)</td>
<td>166 (84.7)</td>
<td>1.590</td>
<td>&lt;0.001</td>
<td>(1.395-1.813)</td>
</tr>
<tr>
<td>AG+GG</td>
<td>166 (50)</td>
<td>166 (50)</td>
<td>2.000</td>
<td>&lt;0.001</td>
<td>1.718-2.329</td>
</tr>
<tr>
<td>AA+AG</td>
<td>98 (33.3)</td>
<td>196 (66.7)</td>
<td>1.500</td>
<td>&lt;0.01</td>
<td>1.338-1.682</td>
</tr>
</tbody>
</table>

Figure 1. Agarose gel electrophoresis of multiplex PCR for GSTM1 and GSTT1 genotyping. PCR products were analyzed on 3% agarose gel. M, 100-bp DNA marker (GeneRulerTM). β-globin was used as an internal positive control (268 bp fragment). Lanes 1, 4: GSTM1 positive (215-bp fragment); lane 2: GSTT1 positive (480-bp fragment); lanes 3, 5: GSTT1 and GSTM1 positive (215-bp and 480-bp fragments); lane 4 represents null genotypes for both GSTM1 and GSTT1 genes.
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DISCUSSION

Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism and environmental factors, such as air pollutants, cigarette smoke and drugs. The production of ROS is not only a normal product of cellular metabolism, but also various environmental stresses, including air pollutants, cigarette smoking and drugs lead to over production of ROS. Since they are highly reactive molecules, they are involved in damaging cellular structures and altering their functions. Therefore, the protection of the balance between reducing and oxidizing states is crucial for cell viability, the continuity of cellular functions and also needs to be maintained to prevent damage to cellular macro molecules. It is known that oxidative damage of ROS in the tissues depends on the activities of nonenzymatic and enzymatic antioxidant defence mechanisms that are present in the tissues for scavenging excessive ROS. Since the skin is a major target of oxidative stress due to over production of ROS, the alterations in oxidative stress and antioxidant defense mechanisms have been implicated in the pathogenesis of various inflammatory skin diseases, including atopic dermatitis, psoriasis, vitiligo, acne rosacea, lichen planus and polymorphic light eruption disease.

Alopecia areata is an organ-specific autoimmune disorder that is associated with a genetic predisposition and environmental triggers, and is mediated by T lymphocytes that are directed to hair follicles in the anagen phase and produce ROS. Although continuous exposure to the ROS production during anagen is related with highly proliferation of keratinocytes and mitochondrial activities in the human outer root sheath and hair matrix [23] hair follicles are commonly believed to be comparatively ROS resistant if the ROS scavenging enzymes, such as GSTs are active. In the other hand, since production of ROS by the activated T cells causes the antioxidative glutathione response that needs to balance rising ROS and prevent cellular damage, Mak et al. (2017) has reported that glutathione is essential for T cell effector functions through its regulation of metabolic activity. The antioxidative glutathione pathway thus plays an unexpected role in metabolic integration and reprogramming during inflammatory T cell responses [24]. Many studies have reported the involvement of oxidative stress in AA, vitiligo, and accumulation of hydrogen peroxide in the epidermal layer of affected skin [25].

GSTs are pivotal components of the cellular defense against oxidative stress. Therefore, the lack of functional GST enzymes could increase ROS-mediated oxidative stress. Of the GSTs, GSTM1 and GSTP1 metabolize large hydrophobic electrophiles, whereas GSTT1 is involved in the metabolism of smaller compounds. The GSTM1 and GSTT1 null genotypes result in enzyme inactivation. However, coding for valine amino acid instead of isoleucine, due to A/G single nucleotide polymorphism (SNP) at at codon 105 (Ile105Val) reduces GSTP1 enzyme activity. Although GST polymorphisms have been evaluated by different research groups in the molecular pathogenesis of T cell-associated autoimmune diseases, especially in skin lesions, any studies concerning about the association of these three genes (GSTM1, GSTT1 and GSTP1) polymorphisms with AA have not been performed yet.

Figure 2. Agarose gel of the GSTP1 PCR products after digestion with HpyCH4IV for detection of GSTP1 polymorphisms. PCR products were separated by electrophoresis on a 4 % agarose gel. M, 50-bp DNA marker (Promega); lanes 1, 2 – AG, in the case of heterozygous individuals (Ile/Val), (91 and 176-bp fragments); lanes 3, 4 – GG, covering the mutant allele (Val), (66 bp and 91bp fragments).
In this study, the results showed that GG (Val/Val) genotype was associated with a higher risk of AA (p<0.001) for GSTP1 gene. The frequency of the genotype with G (AG + GG) was higher in the patient group than the control group (p<0.001). Because GSTP1 SNP in the gene (p.Ile105Val) leads to a partial or complete loss of enzyme activity, diminished enzyme activity may be associated with AA susceptibility. Similarly we detected significant differences in the deletions of GSTM1 and GSTT1 genes between the patient and control groups (P<0.01, P<0.001), respectively. Loss of activity for these enzymes may also lead to diminished antioxidant defense mechanism and increases the AA susceptibility.

The evidences indicate that the imbalance between antioxidation of skin and oxidative stress may be a pathogenic factor of skin diseases [26-28]. Yazici et al. have demonstrated a significant association between the GSTT1 and/or GSTM1 null genotypes and rosacea [29]. In the meta-analysis by Lu et al. [30], they have suggested a potential association between the GSTM1 null and GSTM1/T1 null/present polymorphisms and increased vitiligo risk, but no association between the GSTs polymorphisms and vitiligo susceptibility has been reported by [17]. In studies investigating the relationship between GST polymorphisms and psoriasis susceptibility, Srivastava et al. reported the null genotype of GSTT1 as a strong predisposing factor for psoriasis, while no relation was found by Solak et al. [31, 32].

The role of oxidative stress and shifts in oxidant/antioxidant balance in the pathogenesis of AA has been reported in the literature [33, 34], but the studies concerning about the association of development of AA with the gene polymorphisms of antioxidant enzymes that may change the amounts and the activities of the enzymes are very limited. In the only study by Kalkan et al., they examined whether there is a potential connection between the MnSOD Ala9Val and GPx1 Pro198 Leu polymorphisms and AA susceptibility but no association between AA patients and these two polymorphisms was detected [14].

The limitation of our study is the small sample size. The investigations performed in larger patient populations would be eligible for meaningful results, especially in the subgroups of patients with mutated GST genotypes. To the best of our knowledge, our study is the first study investigating the role of GSTs polymorphisms in AA. Therefore, we could not compare the results with those from other published studies, and from other ethnic groups.

In conclusion, genetic variation in other ROS defense genes and ROS-producing genes could be important modifiers in the relationship of GSTP1, GSTM1 and GSTT1 with AA. Our results indicated that there is a potential relationship between GST polymorphisms and AA disease, although many studies are needed for suggestion. Furthermore, to work other oxidants and antioxidant enzymes with GSTs polymorphisms that may be involved in AA disease could be contribute to explain the pathogenesis of the disease.

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