The relationship between VI
Tiligo and endothelial nitric oxide GLU298ASP and intron 4 VNTR polymorphism

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Abstract
Vitiligo is a chronic autoimmune depigmentation disease characterized by loss of melanocyte function in the epidermis. The pathogenesis of vitiligo remains unclear. In recent years, there is increasing evidence that some gene polymorphisms are risk factors for this disease. In this study, we designed to investigate whether there is a relationship between endothelial nitric oxide synthase (eNOS) gene Glu298Asp (G894T) and intron 4 VNTR polymorphisms and vitiligo. The study included 87 patients diagnosed with vitiligo and 96 healthy volunteers in the dermatology clinic of Bülent Ecevit University Medical Faculty. Polymerase chain reaction and enzyme restriction (PCR-RFLP) methods were used to detect Glu298Asp and intron 4 VNTR polymorphism. There was no significant difference between the groups in terms of GG, GT, and TT genotype distributions and allele frequencies in terms of eNOS Glu298Asp gene polymorphism (p=0.076). There was a significant difference between eNOS intron 4 VNTR polymorphisms in terms of genotype distribution and allele frequency (p=0.004). It was found that BB genotype increased the risk of disease 3.6 times compared to AB genotype. Although the pathogenesis is not known, genetic and immunological factors cooperate a role in the cause of vitiligo. Our study is thought to make an essential contribution to the literature in terms of being the first study on this subject.

Keywords: Vitiligo, nitric oxide, polymorphism

Introduction
Vitiligo is a skin disease that usually occurs in individuals aged 20 years and younger, with markedly different sizes and numbers of milky white stains. It is popularly known as ala, midwifery and white spot. Although it does not affect mortality and physical morbidity, it causes severe cosmetic concerns and psychological problems [1-5].

Although there are no definitive findings of the etiology and genetics of vitiligo, it is a disease that has been known for thousands of years because of its prominent appearance [3,4]. The first information about vitiligo BC In the 1550s, the Ebers Papyrus occurs, where two skin diseases are described, which cause discoloration of the skin. One of them is thought to be vitiligo, which is characterized by a lack of pigmentation. One of India’s Vedic writings, Atharva Veda, written in the same years, has been described as vitiligo “kilas”. Accordingly, “kilas” is based on the Sanskrit word “clay” which means white [6,7].

Recently, the term Vitiligo was first used by Roman physicist Celsus in De Medicina in the first century. The derivation of the remark vitiligo is thought to derive from the Latin word “vitium” which means stain or error. Depigmented areas in vitiligo are likened to white patches on mottled calves. In the late 19th century, dermatology books described vitiligo as “pigmentary dystrophy”. Moritz Kaposi (1837-1902) was the primary investigator to explain the histopathological findings of vitiligo. Studies on etiological factors show that vitiligo is affected by emotional stress or other traumatic factors, and its connection with the nervous system has also been shown as evidence. Today, vitiligo is outlined as the prototype of hypomelanotic disorders [1,7,8].

A variety of auto immunogenic and neurogenic pathogenic pathways have been proposed for the etiology of vitiligo. However, it remains unclear whether vitiligo is a disease in itself or a side effect of different processes [8].
Nitric oxide (NO) is a free radical molecule created by the nitric oxide synthase (NOS) gene in various tissues in the L-Citrulline reaction from L-Arginine. Nitric oxide has three isoenzymes; neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS). NO show a significant role in regulating peripheral and central nervous systems, cardiovascular system and immune system [9,10]. All isoforms were isolated in human skin [11].
eNOS has three most frequently studied genetic polymorphisms. One of them is the G894T polymorphism in exon 7 (rs1799983); this polymorphism is a dynamic polymorphism that leads to Glu-Asp exchange through protein synthesis.
Another T786C point mutation (rs2070744) significantly reduces the promoter activity of the enzyme eNOS. The other polymorphism of eNOS is intron 4 VNTR (27-bp), which leads to basal NO production [9,12].

Exposure to UVA and UVB can induce nitric oxide production, particularly in keratinocytes and melanocytes, during the creation of NOS, and may lead to an augment in tyrosinase activity and melanin synthesis [13-15].

Changes in NOS activity may affect melanocytes as follows: (a) decrease of melanogenesis and accretion of toxic intermediates of melanin synthesis; (b) increased reactive oxygen species; and (c) decreasing cells to compensate for oxidative stress.

As a potential result of atypical production of nitric oxide in light-induced tissue, there may be an increased inflammatory response, prolongation of the standard inflammatory response, facilitation of generation of autoimmunity, and consequently melanocyte death [11,14,15].

Several studies have shown a relationship between promoter polymorphisms in the iNOS gene and NO more production, thereby inducing melanocyte damage and death. Changes in NOS activity affect melanocytes by reducing the melanogenesis process, accumulating toxic intermediates of melanin synthesis, and growing production of reactive oxygen species [15,16].

Furthermore, in vitro experiments showed that endogenous nitric oxide showed anti-apoptotic effects in melanoma cells but not in normal human melanocytes [11]. However, high concentrations of exogenous NO are poisonous to normal human melanocytes [17,18], and the presence of NOS2 has been reported to be related to reduced survival in patients with melanoma metastasis [16].

Our study aimed to settle on whether there is a relationship between eNOS gene Glu298Asp (G894T) and intron 4 VNTR polymorphisms in patients with vitiligo.

Material and Methods

Patient and Control Group
Our study consisted of 87 patients diagnosed as vitiligo in Bülent Ecevit University Faculty of Medicine Dermatology Clinic and a control group composed of 96 healthy individuals considering the same age and sex characteristics. Following the approval of the ethics committee, information such as age, occupation, smoking and family history of vitiligo were recorded in the specially prepared information forms within the consent of the control and patient groups.

DNA isolation
For eNOS G894T and VNTR intron 4 polymorphisms, peripheral blood samples of patient and control groups were taken into sterile EDTA tubes, and genomic DNA was isolated using the QIAamp DNA Blood Mini Kit (Maryland, USA). The isolated DNA was stored at -20 °C until analysis.

Genotyping
Genotyping of eNOS gene polymorphisms was performed according to PCR and RFLP methods described by Zheng et al. For the eNOS G894T polymorphism, 5' - AAGGCCAGAGCACGTGGAGT-3' (forward) and 5' - CCCAGTCATCCCTTGTGTGCTCA-3' (reverse) primers were used. To amplify this region, the PCR reaction was performed with a mixture of 25 µl of 100 ng genomic DNA, 10 pmol forward and reverse primer, 200 pmol dNTP, 5U Taq polymerase, 2 mM MgCl2, 2µl 10X PCR buffer (Thermo Scientific, USA). PCR reaction GeneAmp PCR System thermal cycler (Thermo Fisher Scientific, USA) for 4 minutes at 94°C; 35 cycles were performed by applying 30 seconds at 94°C, 30 seconds at 65°C, 1 minute at 72°C and 5 minutes at 72°C for final elongation. The resulting 248 bp of PCR product was cut overnight at 37°C with the enzyme BanII (Thermo Scientific, USA). The products obtained were run on 2% agarose gel. When only 248 bp bands were observed, TT; 248-163-85 bp bands were seen as GT, and 163-85 bp bands were seen as GG, and genotyping was performed.

For the eNOS intron 4 VNTR polymorphism, 5' - AGGCCCTATGGTGCCCTT -3' (forward) and 5' - TCTCTTAGTGCTGTGGTCAC -3' (reverse) primers were used. To amplify this region, the PCR reaction was performed in the Applied Biosystems GeneAmp PCR System thermal cycler (Thermo Fisher Scientific, USA) by applying 35 cycles of 30 sec at 94°C, 30 sec at 63°C, 1 at 72°C and 5 minutes at 72°C for final elongation. The products obtained were run on 1.5% agarose gel. When only 248 bp bands were observed, TT; 248-163-85 bp bands were seen as GT, and 163-85 bp bands were seen as GG, and genotyping was performed.

Statistical Analysis
SPSS program (SPSS Inc, Chicago, IL., USA v18.0) was used for statistical analysis from the data obtained from the study and control groups. The comparison of patient and control group was evaluated by t-test, genotype distribution by χ2 test, and genotype distribution by One Way ANOVA test. A value of 0.05 was considered significant.

Results
The purpose of our work was to determine the correlation between the genotypic distribution of eNOS gene and blood samples taken from vitiligo patients and control subjects. The distributions of eNOS Glu298Asp and intron 4 VNTR polymorphisms were analyzed in the patient and control groups.
The distribution of GG, GT and TT genotypes in eNOS Glu298Asp gene polymorphism was 36 (41.4%), 38 (43.7%) and 13 (14.9%) in the patient group; in the control group 25 (26%), 56 (58.3%) and 14 (15.6%) were determined (Figure 1).

![PCR-RFLP gel electrophoresis image for the eNOS gene Glu298Asp polymorphism. GG homozygote, GT heterozygote, TT homozygote, bp: base pair](image1)

Figure 1.

Allele frequencies were 110 (63.21%) for the G allele and 64 (36.78%) for the T allele in the case group; 106 (55.20%) for the G allele and 86 (44.79%) for the T allele in the control group.

![PCR-RFLP gel electrophoresis image for intron 4 VNTR polymorphism of the eNOS gene. BB homozygous, AB heterozygous, AA homozygous, bp: base pair](image2)

Figure 2.

Table 1 shows the values and allele frequencies of the genotype distribution in the patient and control groups. In the analysis, no difference was found between the patient group and the control group in terms of genotype distribution and allele frequency for Glu298Asp polymorphism in the 7th exon gene (p = 0.076). Since GT frequency is higher in the control group, it can be argued that GT genotype has a protective effect compared to GG genotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control groups</th>
<th>Case groups</th>
<th>OR (% 95 Confidence Interval)</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>25</td>
<td>36</td>
<td>Ref.</td>
<td>G</td>
</tr>
<tr>
<td>% Distribution</td>
<td>26.0</td>
<td>41.4</td>
<td></td>
<td>% Distribution</td>
</tr>
<tr>
<td>GT</td>
<td>56</td>
<td>38</td>
<td>0.471 (0.245-0.908)</td>
<td>T</td>
</tr>
<tr>
<td>% Distribution</td>
<td>58.3</td>
<td>43.7</td>
<td>% Distribution</td>
<td>44.79</td>
</tr>
<tr>
<td>TT</td>
<td>15</td>
<td>13</td>
<td>0.602</td>
<td>Total</td>
</tr>
<tr>
<td>% Distribution</td>
<td>15.6</td>
<td>14.9</td>
<td>% Distribution</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1. Distribution of genotype and allele frequencies of eNOS Glu298Asp polymorphism in case and control groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control groups</th>
<th>Case groups</th>
<th>OR (% 95 Confidence Interval)</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>26</td>
<td>49</td>
<td>Ref.</td>
<td>B</td>
</tr>
<tr>
<td>% Distribution</td>
<td>27.1</td>
<td>56.3</td>
<td>% Distribution</td>
<td>63.02</td>
</tr>
<tr>
<td>GT</td>
<td>69</td>
<td>36</td>
<td>0.277 (0.148-0.516)</td>
<td>A</td>
</tr>
<tr>
<td>% Distribution</td>
<td>71.9</td>
<td>41.4</td>
<td>% Distribution</td>
<td>36.97</td>
</tr>
<tr>
<td>TT</td>
<td>1</td>
<td>2</td>
<td>1.061</td>
<td>Total</td>
</tr>
<tr>
<td>% Distribution</td>
<td>1</td>
<td>2.3</td>
<td>% Distribution</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Distribution of genotype and allele frequencies of eNOS intron 4 VNTR polymorphism in patient and control groups

Allele frequencies were 40 (22.98%) for the A allele and 134 (77%) for the B allele in the case group; the control group was 71 (36.97%) for the A allele and 121 (63.02%) for the B allele. Table 2 shows the values of allele frequencies and allele frequencies of the genotype distribution in the patient and control groups. In the analysis, a significant difference was found between the patient group and the control group in terms of genotype distribution and allele frequency for eNOS gene intron 4 VNTR polymorphism (p=0.004).

Vitiligo is a progressive pigment disorder that can occur at any age and may be hereditary or acquired with melanocyte loss and is very common all over the world. It has depigmented, sharp bounded and generally symmetrical macules of different size and localization. Although vitiligo is not a life-threatening disease, it may affect the social relationships and psychology of patients and may have adverse effects on quality of life [2,19,20].

Although the etiopathogenesis of vitiligo has not been fully
elucidated, various theories have been emphasized, and one or more of these are thought to be effective at the same time. These are otocytotoxic, autoimmune, neural and biochemical hypotheses. Otocytotoxic and autoimmune predictions are also commonly referred to as the combined hypothesis [7,8]. Also, family history should be considered [6,20,21].

The genetic susceptibility of vitiligo was first reported in 1933, and numerous studies were conducted on the underlying genetic causes of the disease, and the results demonstrated that genetic factors show a significant responsibility in the development of vitiligo. It was established that more than 30% of the patients with vitiligo were affected by one family member and more than 21% of the first-generation family members were affected [4,5]. Genes responsible for vitiligo have not been clearly defined yet. Today’s data complicate consensus on this issue and are rather remarkable for the multifactorial aspect of the disease [4,5,22-24].

There are several studies on the effect of various gene polymorphisms in vitiligo. Tarle et al. in family-based and case-control study performed in Brazil, establish a connection among the risk of developing vitiligo and CDH1 (rs10431924) polymorphism [25]. Onan et al. investigated the relationship between Interferon-induced helicase (IFIH1) gene and vitiligo. They found that each G allele for SNP1, T allele, and SNP2 was protective for the development of vitiligo. Thus, they confirmed that the IFIH1 gene locus has a role in GV sensitivity [26].

In a meta-analysis by Zhang et al., VDR revealed that Apal polymorphism increased the risk of vitiligo susceptibility and that there was a positive relationship between serum 25(OH)D deficiency and incidence of vitiligo [27]. Tuna et al. studied superoxide dismutase 1 and 2 genes polymorphism in Turkish vitiligo patients and found that the relative risk for the development of vitiligo was 2 times higher in TT genotype [28].

Conclusions

The causes and genetic predisposition of vitiligo, an autoimmune disease, is not yet known. Also, because of the necessity of a long and complicated treatment process and inadequate treatment methods, the search for new treatment is continuing. In our work we investigated if the eNOS G894T polymorphism in exon 7, and intron 4 VNTR polymorphism play any role in Turkish patients with vitiligo. To our information there is no published information on the association between this polymorphism and vitiligo. Also, we represent the first results on eNOS G894T polymorphism in exon 7, and intron 4 VNTR polymorphism and vitiligo in Turkish cohort. If this study is done by increasing the sample volume, it will give more accurate results. It is thought early diagnosis of vitiligo disease, prognosis, determination of heritability, whether vitiligo-nitric oxide relationship varies according to the populations, directing the treatment of vitiligo, contributing to the science of pharmacogenetics and genetic counseling will be necessary for vitiligo cases.

Conflict of interest

The authors declare that there are no conflicts of interest.

Financial Disclosure

All authors declare no financial support.

Ethical approval

Consent of ethics was approved by the local ethics committee.


