DELTA-AMINOLEVULINIC ACID DEHYDRATASE (ALAD) GENETIC POLYMORPHISM IN TURKISH POPULATION

TÜRK POPULASYONUNDA DELTA-AMİNOLEVULİNİK ASİT DEHİDRATAZ (ALAD) GENETİK POLİMORFİZMİ

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ABSTRACT

Delta-aminolevulinic acid dehydratase (ALAD) is an enzyme involved in the heme biosynthetic pathway. ALAD is inhibited by lead. This enzyme is polymorphic with two alleles, ALAD\(^1\) and ALAD\(^2\). These alleles differ by a single base pair change, a guanine to cytosine transversion of coding nucleotide 177. This base substitution permits to apply a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay for genotyping the ALAD gene. In this study, polymorphism of the ALAD gene has been investigated in a Turkish population using PCR-RFLP method. The frequency of the ALAD\(^1\) was found to be 0.81. The comparison of this result with other population samples shows that significant difference exists among ethnic groups in ALAD gene polymorphism.

Key words: 5-aminolevulinic acid dehydratase, polymorphism, PCR-RFLP

ÖZET

Delta-aminolevulinik asit dehidrataz hem biosentetik yolağında yer alan bir enzimidir. ALAD kurşun tarafından inhibe olmaktadır. Bu enzim polimorfiktir ve ALAD\(^1\) ve ALAD\(^2\) diye 2 aleli vardır. Bu aleller 177. kodlama nükleotidi olan guaninin sitozone çapraz baz değişimi ile ayrılrırlar. Bu baz değişimi, polimeraz zincir reaksiyonu-kesilmiş parça uzunluk polimorfizmi (PCR-RFLP) yönteminin ALAD geninin genotiplemesi için uygulanmasına olanak sağlar. Bu çalışmada, ALAD genindeki polimorfizm PCR-RFLP metodu kullanılarak bir Türk populasyonunda incelendiştir. ALAD\(^1\) frekansi 0.81 olarak bulunmuştur. Bu sonucun diğer populasyon örnekleriyle karşılaştırılması, ALAD gen polimorfizminde etnik gruplar arasında önemli farklılığın olduğunu göstermektedir.

Anahtar kelimeler: 5-aminolevulinik asid dehidrataz, polimorfizm, PCR-RFLP
INTRODUCTION

δ-Aminolevulinate dehydratase (5-aminolevulinate hydrolyase, EC 4.2.1.24; ALAD) is second enzyme in the heme biosynthetic pathway (1). This enzyme catalyzes the self-condensation of two molecules of 5-aminolevulinic acid (ALA) to form the monopyrrole, phorhobilinogen (PBG), which is then utilized for the formation of porphyrins, cytochromes and other hemoproteins. The native human enzyme has a molecular weight of ~290 kDa and is composed of eight identical subunits of ~36kDa (2). ALAD is a zinc-containing enzyme, which is well-known to be inhibited by lead. The inhibition of ALAD activity is one of the most sensitive indicators of lead poisoning and decreased ALAD activity have been shown to correlate with lead exposure in humans (3).

Although the effects of lead have been investigated for centuries, lead levels in the workplace have been steadily decreased over the last few decades, and occupational lead exposure is still important problem worldwide. Non-occupational lead exposure is also significant problem from leaded-gasoline exhaust gases or fallout dust from the leaded paint of old houses (4,5).

The blood levels of patients from different countries have been varied because of the differences in environmental or occupational exposure and personal characteristics (6-9). In addition, genetic and ethnic differences should account for some of the variations. Because the human population is biologically diverse and genetically heterogeneous, it is not surprising that differences in susceptibility to disease among individuals with exposure to environmental or occupational chemicals should exist (10).

The use of molecular DNA techniques, one of the most important is the development of polymerase chain reaction-based methods, coupled with recent knowledge of some of the genes has aided in our ability to investigate the genetic basis for the interindividual differences observed in populations (11). The development of these techniques and the search for critical genes bring new insights in gene environment interactions in human populations. In these interactions, one of the important example is delta-aminolevulinic acid dehydratase (ALAD) gene (12). The genetic regulation of ALAD activity has been subject of interest in the study of gene environment interaction. The ALAD gene is located on chromosome 9q34 (13). This gene is a polymorphic enzyme coded by expression of two common alleles of the gene, which have
been designated ALAD$^1$ and ALAD$^2$, which are responsible for three phenotypes, ALAD 1-1, ALAD 1-2, and ALAD 2-2 (14).

The allele differ by a single base pair change, a guanine to cytosine transversion of coding nucleotide 177, which predicts the substitution of an asparagine for a lysine in the enzyme (15). The existence of this common polymorphism and the fact that ALAD is markedly inhibited by lead suggested a possible physiological relationship between the ALAD isozymes and lead poisoning. Although the activities of the erythrocytic ALAD1-1, 1-2, 2-2 isozymes are essentially same, it is hypothesized that individuals with ALAD$^2$ allele could be more susceptible to lead exposure (16). The ALAD2 isozyme is more electronegatively charged than the ALAD1 isozyme, and may bind lead more effectively (17). Thus, ALAD$^2$ heterozygotes and homozygotes might have higher blood lead concentrations, making them even more likely to express subclinical and clinical manifestations of exposure to lead either occupationally or environmentally.

In addition to human, the ALAD cDNAs for the mouse, rat, and *Escherichia coli* enzymes have been isolated and sequenced (18, 19, 20). The predicted amino acid sequences of the human and rodent enzymes both contain 330 amino acids and are 87% identical, whereas bacterial ALAD has only 37% identity and 53% similarity with human protein. In the mouse, rat, and human sequences, only three predicted amino acids differ in codons 55-87, one of which is codon 59, the site of the *Mspl* polymorphism. In fact, the asparagine in position 59 in the human ALAD$^2$ allele is also present in this position in the rat, mouse, and bacterial coding sequences. The conservation of this residue across species suggests that ALAD$^2$ was the ancestral allele. However, the fact that the ALAD 1-2 or ALAD 2-2 isozyme phenotype was not detected in an African population argues against this concept and implies that ALAD$^2$ allele may have become established in the Caucasian population by selection (21).

This polymorphic system has been studied in some populations, and the results suggested heterogeneity of gene distribution. The ALAD$^2$ allele is typically found in 11 to 20% of the white population, but it was not detected in an African population (21-23). The aim of this study was to examine the frequency of ALAD gene in a Turkish population. For this purpose polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (RFLP) method was modified and applied to the samples.
MATERIALS and METHODS

Subjects and DNA extraction

This study was sampled from 61 random samples of the Turkish population. Of the total participants, 65.6% of them were male; 57.4% were current smokers and 3.3% were ex-smokers. Blood samples were collected by venepuncture in heparin-containing tubes from unrelated healthy individuals.

Genomic DNA was isolated from aliquot of heparinized blood by modified high salt method (24). The concentration of each DNA sample was quantitated by UV spectroscopy. The extracted DNA purity was determined from the absorbance ratio at 260 and 280 nm.

Polymerase Chain Reaction (PCR)

A modified protocol based on the polymerase chain reaction was used for ALAD genotyping (25). For amplification of the 916 bp ALAD genomic sequence containing the $\text{ALAD}^1/\text{ALAD}^2$ polymorphic site, sense (5'-AGACAGACATTAGCTCAGTA-3’) and antisense (5’-GGCAAAGACCACGTCCATTC-3’) oligonucleotides which corresponded to ALAD intronic sequences were obtained from Genomed Biotechnologies, Inc. (USA).

PCR was performed with 5 μl (0.5 μg) of DNA solution, 30 pmol of each primer, 200 μM of dNTPs, 10 X PCR buffer (100 mM Tris-HCl, pH 8.8; 500 mM KCl; 1% Triton X-100), 3 mM MgCl₂, and 1 U Taq polymerase (Promega) in a final volume of 50 μl. Reactions were amplified on a Hybaid PCR Sprint Temperature Cycling System (UK) using the following thermocycling protocol:

a) one cycle of denaturation at 95°C for 2 minutes,

b) five cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, extension at 72°C for 1 minute,

c) thirty cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 30 seconds.

Negative control reactions with no added DNA were included in each amplification to ensure the reagents used contained no contaminating DNA.

It is important to optimize the amplification steps and reaction parameters for ALAD gene in PCR, because any factors those were capable of interfering with the amplification process. A
combination of suitable and systematic adjustments are needed to obtain maximum specificity, sensitivity and yield. Thus, optimization of PCR conditions were carried out by measuring the effect of different conditions on the final yield and specificity of ALAD gene product in this study.

Optimization of the annealing temperature is critical when using genomic DNA as a template for PCR amplification. Therefore, theoretical annealing temperature systematically varied by 1 °C increments over a 5-6 °C temperature range. Product yield was found to decrease with temperature. At annealing temperatures of 56 °C and below there were an increasing variety of misprimed non-target products generated. A high sensitivity of amplification was observed at 57 °C. Therefore 57 °C of annealing temperature was selected to obtain the best balance between purity and yield during the amplification process.

Annealing and extension times during PCR process were limited to only 30 seconds in order to decrease the cumulative error frequency of the polymerase during the PCR. The DNA templates were denatured at 94 °C for 30 seconds for complete denaturation.

PCR cycle number was adjusted to ensure that the selected primers produced the desired product. Experiments were performed for 25, 30, and 35 PCR cycles and optimal cycle number was found for 35 cycles. Taq polymerase titration from 0.5 U to 3.0 U was used to determine the optimal enzyme concentration for a PCR reaction. 1.0 U of enzyme was adjudged to give the best balance between product yield and amplification specificity.

The effect of primer concentration on PCR kinetics was monitored by performing PCR reactions using primer levels from 10 to 50 pmol per 50 μl reaction. From 10 to 30 pmol, the apparently limiting primer concentrations caused the yield of each product to increase in a linear manner. At concentrations of 40 and 50 pmol product yield approached a plateau. Misprimed non-target products were observed using 40 and 50 pmol concentrations. Thirty pmol primer concentration was found to be optimal achieving the best balance between product yield and amplification specificity for the ALAD gene product.

The concentration of each dNTP was titrated from 100 to 500 μM to examine if they contributed to plateau effect to ensure proper incorporation fidelity. Subsequent experiments were performed with a dNTP concentration of 200 μM to achieve an optimal balance between product yield and high fidelity reaction conditions.
The effect of magnesium ion concentration on PCR amplification was examined by varying the amount of total MgCl$_2$ from 1 to 5 mM. Three mM MgCl$_2$ concentration was selected to provide the best balance between product yield and high fidelity during the amplification process.

**Restriction Fragment Length Polymorphism (RFLP)**

After amplification 10 µl of the PCR products were subjected to *Mspl* endonuclease digestion in 20 µl reaction mixtures containing 1 X restriction endonuclease buffer at 37°C overnight.

Ten (10) µl of the digestion products were mixed with 2 µl of loading buffer (1% bromophenolblue, 0.1 M EDTA pH 8.0, 50% v/v glycerol, 1% w/v SDS) and analyzed by 1.5% agarose gel electrophoresis followed by staining with ethidium bromide.

The *Mspl* restriction sequence (CCGG) created by the G→C transversion in the ALAD$^2$ allele has permitted the using PCR-based method for ALAD genotyping as illustrated in Figure 1. Amplification and *Mspl* digestion of the 916 bp ALAD genomic fragment from the ALAD$^1$ or ALAD$^2$ alleles resulted in 582 and 511 bp fragments, respectively. To determine whether the nucleotide 177 G→C was present in other individuals the 916 bp ALAD PCR products were cleaved with *Mspl*. All ALAD 1-2 individuals had both the 582 bp and 511 bp fragments, consistent with heterozygosity for the ALAD$^1$ and ALAD$^2$ alleles and all ALAD 2-2 individuals had only the 511 bp fragment, consistent with homozygosity for the ALAD$^2$ allele.

**RESULTS AND DISCUSSION**

Molecular analyzes revealed that of the 61 healthy individuals tested for ALAD genotype, 40 (65.6%) were ALAD 1-1, 19 (31.1%) were ALAD 1-2 and 2 (3.3%) were ALAD 2-2 in this study. On the basis of these data, the allele frequency of ALAD$^1$ was 0.81 and the frequency for ALAD$^2$ was 0.19. The distribution of ALAD genotypes in the Turkish population samples is given in Table 1.
**Figure 1.** Molecular analysis of human ALAD\(^1\) and ALAD\(^2\) genotype. Amplification of 916 bp PCR product which permits analysis of Mspl genotype. Digestion with Mspl gives 582 bp (ALAD\(^1\)) or 511 bp (ALAD\(^2\)) fragments. The G→C transversion creates a Mspl site and predicts a lysine to asparagine substitution.
Table 1. The distribution of the ALAD gene polymorphism in Turkish population.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Numbers observed</th>
<th>Expected frequency</th>
<th>Numbers Expected</th>
<th>Gene Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAD</td>
<td>1-1</td>
<td>40</td>
<td>0.66</td>
<td>40</td>
<td>ALAD1: 0.81</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>19</td>
<td>0.31</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-2</td>
<td>2</td>
<td>0.03</td>
<td>2</td>
<td>ALAD2: 0.19</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>61</td>
<td>1.00</td>
<td>61</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Some populations have been examined for ALAD polymorphism, but the results suggested the existence of significant differences between them (21-23, 26, 27). No carriers of ALAD^2 were detected in a sample of negroid populations (Liberians) and this gene was found to be rarer among Japanese than among European populations by analysis of the erythrocyte isozyme phenotypes (21). In Caucasian populations, 80%, 18%, and 2% of individuals are reported to have ALAD 1-1, ALAD 1-2, and ALAD 2-2, respectively (14). The gene frequency of the ALAD^2 allele has been reported to be 0.10 in Italian, 0.11 in German, 0.058 in Japanese, 0.06 in Korean and absent in Liberian populations. Table 2 shows ALAD gene frequencies in different ethnic groups.

Table 2. The distribution of ALAD gene frequencies among different populations.

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Gene frequency</th>
<th>Frequency analysis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japanese</td>
<td>121</td>
<td>0.942 0.058</td>
<td>Phenotype</td>
<td>21</td>
</tr>
<tr>
<td>Taiwanese</td>
<td>630</td>
<td>0.980 0.020</td>
<td>Genotype</td>
<td>26</td>
</tr>
<tr>
<td>Korean</td>
<td>307</td>
<td>0.940 0.060</td>
<td>Genotype</td>
<td>27</td>
</tr>
<tr>
<td>Liberians</td>
<td>296</td>
<td>1.000</td>
<td>Phenotype</td>
<td>21</td>
</tr>
<tr>
<td>Germans</td>
<td>144</td>
<td>0.889 0.111</td>
<td>Phenotype</td>
<td>.21</td>
</tr>
<tr>
<td>Italian</td>
<td>762</td>
<td>0.900 0.100</td>
<td>Phenotype</td>
<td>22</td>
</tr>
<tr>
<td>Ashkenazi Jews</td>
<td>386</td>
<td>0.797 0.202</td>
<td>Phenotype</td>
<td>23</td>
</tr>
<tr>
<td>Turkish</td>
<td>61</td>
<td>0.810 0.190</td>
<td>Genotype</td>
<td>This work</td>
</tr>
</tbody>
</table>

In this study we have applied a PCR based method in order to examine the frequency of ALAD gene in a Turkish population. The ALAD^2 allele is higher in the Turkish people than Japanese, Taiwanese, and Korean. Our findings show that ALAD^2 carriers in the Turkish
population seem similar to European populations. Although our preliminary results have been obtained relatively insufficient sample number, this work is the first study on ALAD genetic polymorphism in the Turkish population.

Lead is common occupational and environmental toxin to which millions of people are exposed. The recent findings that individuals either heterozygous for the ALAD\(^2\) allele have higher blood lead levels when exposed to low or high levels of lead in the environment suggest that the lead binding and/or stability of the ALAD 2 subunit in the presence of lead is greater than those of the ALAD 1 subunit \((12, 28-30)\). These data suggest that in humans there is a genetic susceptibility to lead intoxication. In conclusion, the ability to rapidly and precisely determination of the ALAD genotype using the modified method has potential in the identification of individuals whom may be at increased risk for lead poisoning due to exposure in the workplace or the environment.

ACKNOWLEDGMENTS

This work was supported by grant \((98.03.001)\) from the Ankara University Research Council.

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Kabul Tarihi : 1.2.2001