Role of the 4G/5G Polymorphism of Plasminogen Activator Inhibitor-1 Gene in Idiopathic Sudden Sensorineural Hearing Loss, A Case Control Study

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Short running head: 4G/5G PAI-1 polymorphism in idiopathic sudden sensorineural hearing loss

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ABSTRACT

Background: Lower plasminogen activator inhibitor-1 (PAI-1) level can provide protective effects on inflammation, local microcirculatory disturbance, and fibrotic changes, which are likely associated with developing ISSHL. The role of the 4G/5G PAI-1 polymorphism in the development and clinical outcome of idiopathic sudden sensorineural hearing loss (ISSHL) is evaluated.

Methods: 103 patients with ISSHL and 113 age and sex-matched controls were enrolled at University of Ferrara, Italy and hearing loss outcome was measured at least 3 months after the onset of hearing loss. Genomic DNA was isolated from peripheral blood using the QiAMP kit and the 4G/5G polymorphism in the -675 promoter region was genotyped with an allele-specific PCR. Genotype distribution was tested in patients and compared to controls by chi-square and odd-ratio analysis.

Results: In this population, 5G/5G genotype had a two-time lower frequency in ISSHL patients compared to healthy controls (15.5% vs 30.1%) and was associated with decreased odds compared to 4G/5G genotype (OR 0.37, 95% CI 0.19-0.75, p = 0.005). In addition, the patients with 5G/5G genotype showed a trend of 2-3 times higher ratio of hearing recovery (> 20 dB) after treatment compared to other genotypes, suggesting a better prognosis as a clinical outcome.

Conclusion: The 5G/5G genotype of PAI-1 may have a protective effect against developing ISSHL and function as a prognostic factor in recovery from ISSHL.

Key Words: Sudden hearing loss, Plasminogen activator inhibitor-1, 4G/5G polymorphism
BACKGROUND

Idiopathic sudden sensorineural hearing loss (ISSHL) is a syndrome characterized by rapid progression of hearing loss (> 30 dB) over seconds to days [1]. The majority of patients with this disease have no identifiable causes and are thus classified as “idiopathic” [2]. ISSHL affects 5 to 20 persons for each 100,000 individuals annually and can be devastating because they can lose their hearing permanently. The etiology of ISSHL is still unclear although the most recent studies suggest infection, vascular impairment, microcirculation disturbance, and autoimmune process as possible causes [1, 3].

Plasminogen activator inhibitor-1 (PAI-1) is the principal inhibitor of tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), which actively facilitate plasminogen and hence fibrinolysis. PAI-1 is a key molecule for thrombus formation and inflammation [4]. Elevation in plasma levels of PAI-1 has been reported to be associated with many diseases, such as cardiovascular diseases [4], stroke [5] and asthma [6-8]. Marcucci et al [9] also report that plasma levels of PAI-1 were significantly higher in patients with ISSHL compared to control subjects. One of most probable causes of ISSHL appears to be impaired cochlear blood circulation involving the pathogenic micro-thrombotic mechanism [10, 11]. Our previous study demonstrated the effectiveness of fibrinolytic therapy for sudden hearing loss with an improvement of 50 dB using a recombinant tPA which is appeared to improve the microcirculation of the inner ear [11]. These findings suggest PAI-1 as one of the possible risk factors and potential therapeutic targets for ISSHL.

The PAI-1 gene has variation in the promoter region on the basis of a single guanosine insertion-deletion (5G or 4G), and the 4G allele is correlated with increased plasma levels of
PAI-1 [6, 12]. Studies show that the 4G/5G polymorphism of PAI-1 gene is associated with cardiovascular and thromboembolic diseases [6, 13].

In this study we investigated the 4G/5G polymorphism of PAI-1 gene in ISSHL patients to see if this polymorphism is a risk factor in developing ISSHL and can be a prognostic indicator for clinical outcome in patients with this disease. We demonstrated a significant contribution of 5G/5G polymorphism to lowering the risk of developing ISSHL and a trend of improved hearing recovery in follow up evaluation after treatment of ISSHL.
METHODS

Patients and controls

One hundred and three patients (mean age: 56.5 (23-83), sex ratio: M/F 0.44/0.56) with a diagnosis of ISSHL who referred to the Department of Audiology at the University of Ferrara and the University of Modena, Italy were enrolled. All patients received complete history taking, general physical examination, and complete audiological examination. The diagnosis of ISSHL was made by experienced otolaryngologists by excluding other causes of sudden deafness such as congenital, viral, inflammatory, degenerative or traumatic. The age and sex-matched control population (n = 113) was selected. Exclusion criteria for patients and controls were any history of arterial or venous thrombotic disease or other chronic diseases. All patients were treated with intravenous steroids within 3 days after onset of ISSHL and hearing loss outcome was measured by pure tone audiometry at least 3 months after the onset of hearing loss. All subjects provided their informed consent for the study, which was approved by the Institutional Review Board of the University of Ferrara and the University of Modena.

DNA extraction

Blood was taken from each patient or control for genomic DNA extraction, which was performed using the QIamp kit (Qiagen Inc., Valencia, CA) as directed to obtain 3 to 12 µg of DNA from 200 µl whole blood.

PAI-1 genotyping

The PAI-1 4G/5G genotype was analyzed with an allele-specific PCR modified from that of Falk et al. [12], using an alternative forward primer (GTCTGGACACGTGGGGG for the 5G allele or
GTCTGGACACGTGGGGA for the 4G allele) with a common reverse primer (TGCAGCCAGCCACGTGATTGTCTAG, designed to minimize primer-dimer formation) and a control reverse upstream primer (AAGCTTTTACC ATGGTAACCCCTGGT). The PCR procedure included a hot-start initial step to avoid primer-dimer artifacts. The PCR mixture was subjected to 30-step cycles of 94°C (1 minute), 60°C (1 minute) and 72°C (1 minute). The PCR reaction was performed in a total volume of 25 µl with 0.5 µg of genomic DNA by using C1000™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). The reaction mixture contained 10 mmol/L TRIS-HCl (pH 8.0), 2.5 mmol/L MgCl₂, 200 µmol/L deoxyribonucleoside triphosphates, and 25 pmol of each primer. For each PCR, 2.5 U of Taq polymerase (Promega, Madison, MI) was used. Electrophoresis was performed in 2% agarose with 1 x TAE buffer. The gels were photographed after ethidium bromide staining. As a control of this PCR technique, PCR analysis was performed on DNA samples of known genotypes.

**Statistical analysis**

Genotype distribution and allele frequencies were tested in patients and compared to controls by \( \chi^2 \) and odd-ratio analysis using GraphPad Prism for Windows version 4.03 (GraphPad Software Inc., San Diego, CA). A \( p \) value of less than 0.05 was considered to be statistically significant. All odds ratios (OR) are given with their 95% confidence interval.
RESULTS

A total of 103 ISSHL patients and 113 healthy controls were analyzed for 4G and 5G PAI-1 gene alleles. The frequencies of allele 4G were 48.7% in ISSHL and 47.1% in controls, comparable to those for allele 5G of 51.3% (ISSHL) and 52.9% (controls). The frequencies of genotypes 4G/5G, 4G/4G, and 5G/5G were 61.2%, 23.3%, and 15.5%, respectively, in ISSHL, and 44.2%, 25.7%, and 30.1% in controls (Table 1). The prevalence of 5G/5G genotype in the ISSHL patients (15.5%) was two times lower than that in control (30.1%). The 5G/5G genotype appeared to have the risk effect three times lower than that of the 4G/5G genotype (OR 0.37, 95% CI 0.19-0.75, \( p < 0.005 \)), which is the most prevalent genotype. When we compared the 5G/5G genotype with combined non-5G/5G genotypes (4G/5G+4G/4G), the 5G/5G genotype had two times higher risk of developing ISSHL than non-5G/5G genotype. However, the 4G/4G genotype did not have significantly increased risk compared to the 4G/5G genotype.

We further examined to see if the polymorphism of PAI-1 gene is associated with clinical outcome in patients with ISSHL (Table 2). We were able to obtain follow-up pure tone audiometry from only 34 patients. Among these ISSHL patients, patients with 5G/5G genotype showed a tendency to have better outcome with 60% of patients having > 20 dB recovery after treatment, while patients with 4G/5G and 4G/4G genotypes had only 39.1% and 33.3% recovery rates (> 20 dB improvement), respectively. The genotype 5G/5G appeared to have a recovery effect 2.3 times higher than that of 4G/5G (OR 2.33; 95% CI 0.32 - 16.83). However, this finding of better clinical outcome in patients with the 5G/5G genotype was not statistically significant due to relatively small number of patients who had received the available follow-up pure tone audiometry.
DISCUSSION

One of most probable mechanisms of ISSHL appears to be impaired cochlear blood circulation involving the pathogenic micro-thrombotic mechanism [11]. There are precedent polymorphism studies on the roles of various prothrombotic risk factors in ISSHL, including GPIa C807T and FV 1691 G-A [14], MTHFR 677 C-T [10], and G20210A [15]. It has been known that elevated plasma levels of PAI-1 are associated with ISSHL [9], but the role of the PAI-1 is controversial [9, 14]. In this study, we found that the 5G/5G genotype of PAI-1 gene was associated with reduced risk of developing ISSHL.

To investigate the potential contribution of polymorphism within the PAI-1 gene to the development of ISSHL, we recruited ISSHL patients and control subjects from Ferrara and Modena, Italy, from a white, homogeneous population. In this population, we found the frequencies of 5G allele (51.3%, 52.9%) had no significant difference from those of 4G allele (48.7%, 47.1%) either in patients or in controls. However, the frequency of the 5G/5G genotype was two times lower in the ISSHL group (15.5%) compared to that in the control group (30.2%). This 5G/5G genotype showed 2-3 times lower risk effect than 4G/4G and 4G/5G. In this study, we found the 4G/4G genotype had no significant risk ratio in developing ISSHL. However, the 5G/5G genotype appeared to have the protective effect against developing ISSHL. Our findings are supported by several previous reports that the patients with 5G/5G genotype are known to have lower plasma levels of PAI-1 compared to those with 4G/4G [6, 16] and lower PAI-1 level can provide protective effects on inflammation, local microcirculatory disturbance, and fibrotic changes, which are likely associated with developing ISSHL [3, 9, 17]. Our data showing the significance of the 5G/5G genotype are different from a previous study on German patients with ISSHL [14], where the 5G/5G genotype had no significant difference in frequency compared to
the controls. Notably, their study elected only severe ISSHL patients with a loss of 60 dB or more. In contrast, our study recruited patients with a hearing loss of 30 dB or more. It is not clear whether the discrepancy between these two studies is due to different population (Italian vs German) or different severity of the disease.

It has been known that there is no effective treatment of ISSHL other than systemic corticosteroids which is generally used but has limitation to some patients [2]. Our study suggests that lowering plasma levels of PAI-1 may be a strategy to prevent ISSHL, especially in people who are likely to have high plasma levels of PAI-1 such as subjects with obesity, diabetes, and smokers [6, 8]. Our study also showed that the patients with the 5G/5G genotype had a tendency of 2-3 times higher ratio of hearing recovery (> 20 dB) compared to those with the 4G/4G and 4G/5G genotypes although it was not statistically significant. We previously reported a case which showed significant improvement of hearing (> 50 dB) with recombinant tPA treatment two years after the development of ISSHL [11]. The findings we report here further suggest that lowering plasma levels of PAI-1 may be an alternative way to improve clinical outcome in patients with ISSHL.

This study suggests that the individuals with the 5G/5G genotype of PAI-1 have less risk of developing ISSHL, and the 5G/5G genotype may function as a prognostic factor in recovery from ISSHL. This result may be of clinical significance in diagnosis, treatment, and prognosis for ISSHL patients and may provide a new therapeutic strategy for ISSHL.
LIST OF ABBREVIATIONS USED

ISSHL, Idiopathic sudden sensorineural hearing loss; PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator.

COMPETING INTERESTS

Authors declare that there is no financial or non-financial competing interests in relation to this manuscript.

AUTHORS’ CONTRIBUTIONS

SC, SP, MB, AM and TY conceived of the study, and participated in its design and coordination and helped to draft the manuscript. HC, CY and JK participated in the design of the study and performed the statistical analysis. IK and CC carried out the molecular genetic studies, participated in drafted the manuscript. All authors read and approved the final manuscript.
REFERENCES


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**Tables**

**Table 1. Genotype distribution of PAI-1 polymorphism in ISSHL and controls**

<table>
<thead>
<tr>
<th>Model</th>
<th>Genotype</th>
<th>Controls, n = 113 (Frequency)</th>
<th>ISSHL, n = 103 (Frequency)</th>
<th>OR</th>
<th>95% CI</th>
<th>( \chi^2 )</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4G/5G</td>
<td>50 (44.2%)</td>
<td>63 (61.2%)</td>
<td>1</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>4G/4G</td>
<td>29 (25.7%)</td>
<td>24 (23.3%)</td>
<td>0.66</td>
<td>0.34 – 1.27</td>
<td>1.59</td>
<td>0.210</td>
</tr>
<tr>
<td></td>
<td>5G/5G</td>
<td>34 (30.1%)</td>
<td>16 (15.5%)</td>
<td>0.37</td>
<td>0.19 - 0.75</td>
<td>7.83</td>
<td><strong>0.005</strong></td>
</tr>
<tr>
<td></td>
<td>4G/4G-4G/5G</td>
<td>79 (69.9%)</td>
<td>87 (84.5%)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5G/5G</td>
<td>34 (30.1%)</td>
<td>16 (15.5%)</td>
<td>0.43</td>
<td>0.22 - 0.83</td>
<td>6.42</td>
<td><strong>0.011</strong></td>
</tr>
</tbody>
</table>

ISSHL, idiopathic sudden sensorineural hearing loss; OR, odd ratio; CI, confidence interval
Table 2. Significant hearing improvement (> 20 dB) in PAI-1 polymorphism

<table>
<thead>
<tr>
<th>Genotype</th>
<th>4G/5G, n = 23 (Frequency)</th>
<th>5G/5G, n = 5 (Frequency)</th>
<th>4G/4G, n = 6 (Frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No improvement</td>
<td>14 (60.9%)</td>
<td>2 (40.0%)</td>
<td>4 (66.7%)</td>
</tr>
<tr>
<td>Improvement</td>
<td>9 (39.1%)</td>
<td>3 (60.0%)</td>
<td>2 (33.3%)</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1</td>
<td>2.33 (0.32 - 16.83)</td>
<td>0.78 (0.12 - 5.17)</td>
</tr>
<tr>
<td>$\chi^2; p$</td>
<td></td>
<td>0.73; 0.39</td>
<td>0.068; 0.79</td>
</tr>
</tbody>
</table>

OR, odd ratio; CI, confidence interval