Novel plasminogen gene mutations in Turkish patients with type I plasminogen deficiency

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The plasminogen (Plg) protein is the inactive proenzyme form of plasin that dissolves fibrin thrombi by a process called fibrinolysis. It has been shown that homozygous or compound-heterozygous deficiency of this protein is a major cause of a rare inflammatory disease affecting mainly mucous membranes found in different body sites. In this study, five individual Turkish patients and nine Turkish families with type 1 Plg deficiency were investigated for PLG gene mutations. All of the coding regions of the PLG gene mutations were screened for mutations using denaturing high-pressure liquid chromatography (DHPLC). Samples showing a different DHPLC profile were subjected to DNA sequencing analysis. Here, we described five novel mutations namely, Cys49Ter, +1 IVS6 G>A, Gly218Val, Tyr283Cys, and Gly703Asp. Previously identified five nonsynonymous (Lys38Gluf, Gly180Lys, Gly420Asp, Asp453Asn, Pro763Ser), five synonymous (330 C>T, 582 C>T, 1083 A>G, 2286 T>G), and a 3’ untranslated region (3’ UTR) mutation (c.+45 A>G) were also reported in this present study. In this study, we have identified a total of eight mutations, five of which are novel. The mutations/polymorphisms identified in eight of the patients do not explain the disease phenotype. These cases probably carry other pathological mutations (homozygous or compound heterozygous) that cannot be detected by DHPLC. Blood Coagul Fibrinolysis 26:000–000 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

Keywords: fibrinolysis, plasminogen, plasminogen deficiency, plasminogen mutations

Introduction

The physiological or pharmacological dissolution of thrombi is ultimately accomplished by the serine protease plasin. Plasin is derived from its precursor plasminogen (Plg) in a reaction catalyzed by Plg activators. Plasin proteolytically cleaves the fibrin network supporting blood clots and, as a result, restores blood flow to the affected tissue [1].

Plg is primarily synthesized in liver tissue [2], but in humans, the cornea has been described as an extrahepatic site of Plg synthesis [3]. The PLG gene maps to chromosome 6q26 [4]. According to the last data in genome browsers, the longest PLG transcript has 810 amino acid residue, with 19 exons and 2741 base pairs of DNA [5]. It was shown that PLG gene shows strong structural homology with apolipoprotein(a) (apo(a)) gene and both genes were shown to be located on 6q26 [6].

Inherited Plg deficiency in humans can be divided into two types: type I Plg deficiency (hypoplasminogenemia) that represents a quantitative deficiency and type II Plg deficiency (dysplasminogenemia) that represents a qualitative deficiency. Dysplasminogenemia does not lead to a specific clinical manifestation and probably represents only a polymorphic variation in the general population, mainly in Asian countries. Severe hypoplasminogenemia named as ligneous conjunctivitis, is associated with compromised extracellular fibrin clearance during wound healing, leading to pseudomembranous (ligneous) lesions on affected mucous membranes mainly in the eyes [7].

Increased number of cases and disease-related PLG gene mutations identified and reported from Turkey or in patients of Turkish descent were stressed and this situation is suggested to be related to founder effects or intermarriage within some communities [8].

The present study was conducted in five individual patients and nine patients with type I Plg deficiency with their selected family members. We identified a total of 16 mutations in PLG gene, five of which are novel. We examined family members to show the genetic transition of the mutations and to compare the patient and healthy family members to elucidate genotype–phenotype correlation.
Methods

Patients

In this study, five individual patients and nine patients with their selected family members were studied. They all had ligneous conjunctivitis; in addition few of them had additional pseudomembranous lesions or hydrocephalus. The protocol of the study was approved by the local ethics committee of the Ankara University and abides by the tenets of the Helsinki protocol. Informed consent was taken from all of the patients and family members. The Plg activity of the patients was tested in the clinics they were diagnosed.

DNA isolation

Genomic DNA was prepared from peripheral blood samples of all patients with type I Plg deficiency as well as from healthy family members. DNA was extracted using conventional phenol-chloroform method. After isolation, purity and amount of DNA were assessed spectrophotometrically and by running on 1% agarose gel electrophoresis.

PCR

DNA samples were amplified by PCR using a set of primer pairs flanking all 19 exons including intron boundaries of the human PLG gene. For this purpose, firstly PLG regions that homologous to apo(a) gene were identified. For several exons that have high homology with apo(a), two pairs of primers (nested PCR) were used for specific amplification. A total of 27 pairs of primers were used to amplify all 19 exons of PLG. PCR fragments were evaluated on 2% agarose gel electrophoresis.

PCR was performed in 25 μl reaction mixture for denaturing high-pressure liquid chromatography (DHPLC) analysis and 50 μl for DNA sequence analysis. NH4PCR buffer, dNTP mix, Taq polymerase (Fermentas, Lithuania); DNA primer (Metabion, Germany), and 100 ng template DNA or diluted post-PCR mixture (for nested PCR) were used for DNA sequencing. NH4PCR buffer, dNTP mix, Taq polymerase (Fermentas, Lithuania); DNA primer (Metabion, Germany), and 100 ng template DNA or diluted post-PCR mixture (for nested PCR) were used for DNA sequencing.

Denaturing high-pressure liquid chromatography analysis

PCR samples were evaluated by DHPLC analysis using Transgenomic WAVE MD System 4000 Plus (Transgenomic, Omaha, Nebraska, USA). After heteroduplex formation, PCR samples were analyzed by DHPLC system in partially denaturing conditions (two to four different temperatures for each exon) and classified according to differences in DHPLC profile.

PCR purification

PCR fragments that were prepared in 50 μl total volume for DNA sequencing were purified with Promega Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, Wisconsin, USA). Purified PCR products then evaluated on 2% agarose gel electrophoresis and were used for DNA sequencing.

DNA sequence analysis

DNA fragments with different DHPLC profile pattern were directly sequenced on Beckman Coulter CEQ8000XL (Beckman Coulter, Brea, California, USA). Purified PCR products were used as DNA template for DNA sequencing reaction.

Results

In five individual patients and nine patients with type I Plg deficiency with their selected family members, we identified a nonsense mutation, eight nonsynonymous mutations, five synonymous mutations, a splice site mutation and a substitution at 3′ untranslated region (3′ UTR) of PLG gene. To the best of our knowledge, the following PLG gene mutations are novel: Cys49Ter, Gly218Val, Tyr283Cys, Gly703Asp, and +1 IVS6 G>A (Table 1).

We have identified two novel nonsynonymous mutations in two patients (PLG 44 and PLG 196). These two patients are homozygous for Tyr283Cys and Gly703Asp mutations (Fig. 1a and c).

The correlation of nonsynonymous mutations to genetic disease is not always straightforward as they can also be seen as polymorphisms. Here, we report two previously identified nonsynonymous mutations, namely Lys38Glu and Pro763Ser, in homozygous state in two patients (PLG 53 and PLG 187, respectively) (Fig. 1b and d). Family members of these two patients who are heterozygous for these mutations are healthy.

Two of our patients (PLG 178 and PLG 195) were found to carry a novel homozygous splice site mutation, IVS6 +1G>A (Fig. 2a and c). Because of the unavailability of RNA samples of these patients, we could not demonstrate the probable splicing defect of this mutation at transcript level. However, as the mutation is located at transcript level. However, as the mutation is located at

Table 1 PLG gene mutations identified in the study

<table>
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<tr>
<th>Type of alteration</th>
<th>Nucleotide or amino acid change</th>
<th>Novel reported</th>
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<td>Mutations</td>
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<tr>
<td>Nonsense</td>
<td>Cys49Ter</td>
<td>Novel</td>
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<td>Splice site</td>
<td>+1 IVS6 G&gt;A</td>
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<td>Nonsynonymous</td>
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<td>Synonymous</td>
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<td></td>
<td>930 C&gt;T</td>
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<td>582 C&gt;T</td>
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<td>771 T&gt;C</td>
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<td>1083 A&gt;G</td>
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<td></td>
<td>2286 T&gt;G</td>
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<td>3′UTR Region</td>
<td>c.*45 A&gt;G</td>
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the 100% conserved first base of the splicing consensus site, it is highly probable that this mutation is the cause of Plg deficiency in these patients.

In another patient (PLG 183), we have identified a novel nonsense mutation (Cys49Ter) in his paternal allele and a previously reported nonsynonymous mutation in his maternal allele (Fig. 2b).

In our study group, there are cases in which the identified PLG gene mutation cannot be correlated with the disease phenotype. One of these patients, who was heterozygous
for Lys38Glu mutation (PLG 181), is Plg deficient (Fig. 3d). On the contrary, we have identified patients with only one or more synonymous mutations (Fig. 3a–c) who are Plg deficient.

Moreover, in two of the patients (PLG 193 and PLG194), we have identified a novel nonsynonymous mutation Gly218Val in heterozygous state (Fig. 3e), although these two patients are compound heterozygotes for Gly218Val and Asp472Asn. We cannot clearly identify the effect of Asp472Asn, as PLG182, the mother of PLG183, depicted in Fig. 2b is homozygous for this mutation and is healthy.

**Discussion**

Type I Plg deficiency, also known as hypoplasminogenemia, represents a quantitative deficiency. In this situation, both immunoreactive Plg level and functional activity are reduced [7]. The most common symptom for severe hypoplasminogenemia is ligneous conjunctivitis and is associated with compromised extracellular fibrin clearance during wound healing, leading to pseudomembranous (ligneous) lesions on affected mucous membranes mainly in the eyes [8].

The first study of ligneous conjunctivitis in a 46-year-old man with bilateral pseudomembranous conjunctivitis was published as early as 1847 [9]. Worldwide, more than 150 patients with this disease have been reported since the date of its first description [7]. As the pseudomembranes have a wood-like consistency, the disease was later termed ‘ligneous’ conjunctivitis [10]. In 1997, severe type I Plg deficiency has for the first time been shown to be present in three unrelated girls who all suffered from ligneous conjunctivitis [11]. It has been shown by several
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Fig. 3

Mutations which phenotypic effects are unclear. Dark arrows, homozygous mutations; light arrows, heterozygous mutations.
Authors since 1995 that homozygous or compound-heterozygous mutations in \textit{PLG} gene that cause Plg deficiency is a major cause of a rare inflammatory disease affecting mainly mucous membranes in different body sites \cite{12-20}.

Although Plg deficiency was initially believed to be related with the development of venous thromboembolic disease, more recent data suggest that decreased Plg levels may not increase the risk of thrombosis \cite{8}. There are no studies in the literature that prove patients with ligneous conjunctivitis ever suffered from venous thrombosis, the reason for why even homozygous type I Plg deficiency does not predispose to thrombosis is not yet clear \cite{13}. The presence of an alternative system for the dissolution of intravascular fibrin, which apparently is not active in the extravascular compartments, is one of the speculations \cite{14}.

\textit{PLG} gene mutations were previously described in Turkish patients \cite{13-17,19-21}. In 1987, ligneous conjunctivitis reported in a Turkish sibling. The sister developed conjunctivitis at 3 weeks of age, which was followed by pseudomembranes in various parts of the body including but not limited to the laryngotracheobronchial tree and renal collecting system. The brother developed conjunctivitis at 9 months of age, he also had membrane formation in the pharynx and in the renal collecting system and some other parts of the body \cite{22}. These two patients were examined for genetic mutations and were found to be compound-heterozygous for a deletion (del Lys212) and splice site mutation (Ex17+1del-g) \cite{16}.

In 1997, two unrelated Turkish girls who had severe Plg deficiency were examined. They were both suffering from ligneous conjunctivitis and occlusive hydrocephalus. It was found that, one of the girls had homozygous Arg216His mutation, whereas her healthy family members carried this mutation in the heterozygous state. The other girl had Trp597Ter mutation in the homozygous manner, whereas her healthy family members had this mutation in heterozygous state \cite{13}.

In 1998, a case study of a Turkish child with homozygous Plg deficiency and ligneous conjunctivitis was published. Other symptoms included hyperviscosity of tracheobronchial and nasopharyngeal secretions, impaired wound healing, and internal hydrocephalus. In this patient, homozygous Glu460Ter mutation that abolishes the catalytic domain of plasmin was identified. The healthy members of the family had the same mutation in the heterozygous state \cite{14}.

In 2001, Özçelik et al. \cite{17} reported a 2.5-year-old girl with ligneous conjunctivitis who had tracheoalveolar involvement and homozygous type I Plg deficiency. There was no history of venous thrombosis in the family. The patient was shown to be homozygous for frameshift mutation in the Plg exon 14 (Gly565ins-G) that resulted in a premature stop codon \cite{17}.

In 2003, Çifçi et al. \cite{19} reported a 1-month-old Turkish boy who had pseudomembranous conjunctivitis, occlusive hydrocephalus, and hydrocele. After screening for mutations in the \textit{PLG} gene, a homozygous L650fsX652 mutation (deletion of 2081C) was detected. The authors stressed that ligneous conjunctivitis related to type I Plg deficiency was relatively common in the Turkish population; however, mutations were heterogeneous and a common founder is unlikely \cite{19}.

In 2006, Schuster et al. \cite{7} examined a series of 50 patients with Type I Plg deficiency. In nine of 12 patients with Turkish origin, a homozygous combination of three
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common PLG gene polymorphisms, 406T>C, 1490A>G, and 2362G>T, was identified, suggesting a founder effect [7].

PLG gene mutations were previously described in Turkish patients who had been investigated in several studies [13–17,19–21]. In these patient groups, Turkish patients had been analyzed individually and mutations were identified [7,13,14,17,19]. PLG and type I Plg deficiency-associated mutations are found to be common in Turkish patients. The present study was conducted in five individual patients and nine patients with type I Plg deficiency with their selected family members. We identified a total of 16 mutations in PLG gene, five of which are novel (one nonsense, one splice site, three nonsynonymous). We examined family members to show the genetic transition of the mutations and to compare the patient and healthy family members to elucidate genotype-phenotype correlation. The mutations/polymorphisms identified in eight of the patients do not explain the disease phenotype (Fig. 3a–e). These cases probably carry other pathological mutations (homozygous or compound heterozygous) that cannot be detected by DHPLC.

Tyr283Cys in exon eight was identified in the homozygous state in a boy with ligneous conjunctivitis (PLG44) (Fig. 1a) and homozygous Gly703Asp mutation in exon 17 was identified in a girl with ligneous conjunctivitis (PLG196) (Fig. 1c) as nonsynonymous novel mutations (Fig. 1). Tyrosine at residue 283 and glycine at residue 703 were found to be conserved between different species (human, chimpanzee, pig, and mouse) throughout evolution. As the patients who have Tyr283Cys and Gly703Asp mutations have not got any other significant mutations except common variations, it seems that these mutations are significant on the onset of the disease. Moreover, it should be noted that the Plg activity of PLG44 and PLG196 is 16 and 13%, respectively.

Lys38Glu mutation was previously named as K19E [23] and shown to be a common cause of hypoplasminogenemia [7]. Lys38Glu mutation was identified in one patient with 47% Plg activity in homozygous state (PLG 53) (Fig. 1b). In a study of 28 unrelated participants with familial Plg deficiency were investigated for detecting mutations. In this study, Lys38Glu mutation in the PLG gene was found in 13 of the 15 propositi with hypoplasminogenemia, in one of these in a homozygous manner. The individual who carried this mutation in the homozygous state showed Plg activity of only 15% and no evidence of venous thrombosis or ligneous conjunctivitis. As a conclusion in this study, it was written that Lys38Glu mutation was a common genetic defect in participants with hypoplasminogenemia and in a homozygous manner, this mutation was often, but not always, associated with ligneous conjunctivitis [7]. In our patient group, the patient who has homozygous Lys38Glu mutation has ligneous conjunctivitis. Contrary to what one would expect from an autosomal recessive disorder, patient (PLG181) with a heterozygous Lys38Glu mutation showed disease phenotype as well (Fig. 3d). Unfortunately, neither the patient’s nor her relatives’ Plg activity results were available to be able to comment more on the situation.

Pro763Ser mutation was identified in one patient in homozygous state (PLG187) (Fig. 1d). This patient’s Plg activity was found to be 2%. This mutation has been reported before [7]. This mutation is identified in the heterozygous state in the patient’s healthy sisters.

+1 IVS6 G>A splice site mutation was identified in the first base of the intron that follows the sixth exon of the PLG gene as a novel mutation. +1 IVS6 G>A mutation was identified to be homozygous in two patients (PLG178, PLG195) (Fig. 2a and c). One of the homozygous patients whose parents were heterozygous (PLG178) had hydrocephalus in addition to ligneous conjunctivitis. This patient’s Plg activity was measured as 21%, whereas the Plg activity of the other patient was not available. Because the mutation exists in conserved G base of GT sequence, which is a signal for splicing, it is supposed to affect transcript structure and thus function of the protein. It seems that carrying this mutation in the homozygous state is significant on the onset of the disease, because parents of the patient who carry this mutation heterozygously are healthy. It is compatible again with the fact that for recessive disorders having the mutation in homozygous form is significant for existence of the disease.

Cys49Ter nonsense mutation in exon two of PLG gene was found to be heterozygous in a girl who has type I Plg deficiency with ligneous conjunctivitis (PLG183) as a novel mutation (Fig. 2b). Her clinically healthy father was screened for this mutation too and determined not to have this nonsense mutation. In addition, this patient with Plg activity of 50% was also found to have a novel heterozygous Gly180Lys mutation in exon five (Fig. 2b). Glutamic acid at residue 180 was found to be conserved between different species (human, chimpanzee, pig, and mouse) among evolution suggesting that Glu180 might be critical for Plg function. The fact that the phenotypically healthy father is positive for heterozygous Gly180Lys mutation while the patient is positive for both heterozygous Gly180Lys and heterozygous Cys49Ter mutations is consistent with the literature proposing the underlying effect of PLG compound heterozygous mutations on the onset of the disease.

It is interesting that, a reported nonsynonymous Gly420Asp mutation in PLG gene was found in a patient’s deaf mother but not any of the patients (Fig. 3b). Gly218-Val nonsynonymous mutation in exon six was identified heterozygously in a boy (PLG194) and a girl with ligneous conjunctivitis (PLG193) as a novel mutation.
(Fig. 3e). The girl had 10% Plg activity and has fibrin deposits in the genitourinary system in addition to ligneous conjunctivitis. Glycine at residue 218 was found to be conserved between different species (human, chimpanzee, pig, and mouse) among evolution suggesting that Gly218 might be critical for Plg function. These patients also have Asp472Asn nonsynonymous mutation (Fig. 3e). This alteration was reported by Petersen et al. [24] and was thought to be a polymorphism as it was also present in normal human population. Concordant with the data of Petersen et al. [24], we found this alteration in healthy family members as well as the patients in both heterozygous and homozygous states. So in the light of the fact that these two patients are compound heterozygous for Gly218Val and Asp472Asn, it might be speculated that either there is another mutation in these two patients which is responsible for evoking the disease state but was perhaps missed by DHPLC or that Asp472Asn creates the disease phenotype only when in compound heterozygosity with Gly218Val.

In this study, we have identified a total of eight mutations, five of which are novel (one nonsense, one splice site, three nonsynonymous). The mutations/polymorphisms identified in eight of the patients do not explain the disease phenotype (Fig. 3a–c). These patients probably carry other pathological mutations (homozygous or compound heterozygous) that cannot be detected by DHPLC.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References