Molecular characterization of an Italian patient with plasminogen deficiency and ligneous conjunctivitis

Simona M. Siboni, Marta Spreafico, Marzia Menegatti, Ida Martinelli and Flora Peyvandi

Plasminogen deficiency is a rare disease characterized by ligneous conjunctivitis and infections. We observed a 3-year-old Italian boy presenting ligneous conjunctivitis and low plasma levels of plasminogen. Twenty-three different mutations on the *PLG* gene have been reported to date, but mutation analysis had been troublesome for the presence of highly homologous genes. The aim of the study was to identify the underlying mutation avoiding coamplification of unwanted genetic materials using a long polymerase chain reaction strategy, instead of the previously reported subcloning methods. By this simple strategy the complete sequence analysis of *PLG* gene was performed, and a previously reported missense homozygous mutation (K19E) was identified. *Blood Coagul Fibrinolysis* 18:81–84

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Angelo Bianchi Bonomi Haemophilia and Thrombosis Centre, University of Milan and Department of Medicine and Medical Specialities, IRCCS, Maggiore Hospital, Mangiagalli and Regina Elena Foundation, Milan, Italy

Correspondence and requests for reprints to Dr Simona Maria Siboni, Angelo Bianchi Bonomi Haemophilia and Thrombosis Centre, University of Milan and Department of Medicine and Medical Specialties, IRCCS, Maggiore Hospital, Mangiagalli and Regina Elena Foundation, Via Pace 9, 20122 Milan, Italy
Tel: +39 2 55035414; fax: +39 2 54100125;
e-mail: simona.siboni@unimi.it

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Introduction

Type I plasminogen deficiency is a rare condition that can be associated with an uncommon and severe form of ligneous conjunctivitis. This inherited disease mainly affects children and is frequently associated with infections of the upper respiratory trait, otitis media and vulvovaginitis, and it is characterized by conjunctival wood-like consistency membranes due to fibrin deposition. The severity of the disease is related to the degree of plasminogen deficiency, which is caused by homozygous or double heterozygous gene mutations transmitted as an autosomal recessive trait [1]. The *PLG* gene, consisting of 19 exons separated by 18 introns, spans about 52.5 kb on chromosome 6q26-27 [2]. The primary structure of *PLG* (791 amino acids) consists of a preactivation peptide, five tandemly repeated 'kringle' domains and a catalytic domain [2]. Plasminogen is activated by tissue plasminogen activator or urokinase, resulting in the formation of plasmin. Plasmin itself may cleave the carboxy-terminal end of plasminogen or plasmin at Lys77–Lys78 residues, enhancing the affinity of the Lys-plasminogen or plasmin for fibrin [3]. An extraordinary sequence homology between *PLG* and apolipoprotein(a) (*LPA*) genes was reported, suggesting the possibility of a common origin resulted from a gene duplication [4]. Furthermore, a 95% homology between the *PLG* gene sequence and exons 1–5, 17, 18 and 19 of plasminogen-related genes (*PRGs* genes) was reported [5]. Twenty-three mutations on *PLG* gene have been previously described [1,5–11], some of them reported in more than one patient (A600T and K19E). In this report we describe the molecular characterization of a young patient affected by plasminogen deficiency with ligneous conjunctivitis and recurrent tracheo-bronchitis.

Materials and methods

Case report

A 3-year-old boy, the only child of healthy nonconsanguineous Italian parents, was referred to our centre for plasminogen deficiency after two episodes of ligneous conjunctivitis. His family history was negative. The patient had suffered from recurrent tracheo-bronchitis since the first year of life, with a frequency of four to six episodes per year. His first ligneous conjunctivitis was treated with aspecific eye-drop therapy, whereas the second episode was treated with corticosteroid eye drops and required surgical excision twice. Both episodes involved the right eye. In September 2004, after the diagnosis of plasminogen deficiency was completed, he had a third episode of ligneous conjunctivitis in the same eye. On this occasion the patient had early heparin eye-drop treatment, and in approximately 1 week had complete healing. Informed consent was obtained from the patient's family.

Plasminogen functional assay

A plasminogen functional activity in citrated plasma, after activation with streptokinase and fibrinogen, was performed using a chromogenic assay with *p*-nitroaniline as the substrate. Plasminogen levels were revealed automatically by the ACL system (Instrumentation Laboratory, Milan, Italy).

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Molecular analysis

DNA extraction

Ten millilitres of peripheral blood were collected in sodium citrate 0.105 mol/l from the patient and his parents. Plasma and cells were separated after centrifugation at $4000 \times g$ for 30 min. DNA was isolated from blood cells according to the salting out method [12].

Amplification strategies

All coding regions and intron/exon boundaries of the *PLG* gene were analysed by direct sequencing after polymerase chain reaction (PCR) amplification, using sets of intronic primers designed on the basis of previously reported primer sequences [9]. Considering the extraordinary sequence homology between *PLG* [National Centre for Biotechnology Information (NCBI) accession number NT_007422], *LPA* (NCBI accession number NT_007422) and *PRGs* genes (NCBI accession number NT_022184) [2,4], their sequences were aligned. This allowed one to identify the most homologous regions and to draw adequate sets of primers in order to avoid coamplification during the PCR. Specific sets of primers were found to singly amplify all exons (see Table 1) except for exons 1–5, 11, 12, 16 and 17. Exons 1–5 were therefore amplified by a different strategy based on a long PCR, using the forward primer of exon 1 and the reverse primer of exon 5. The amplification was performed using the Expand Long Template PCR System (Roche, Penzberg, Germany), according to the manufacturer's instruction with an annealing temperature of 52°C. The obtained fragment, 11 099 bp long, was resolved on 1.5% SeaKem gel and then gel-extracted by the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Then 1 μl of the obtained solution was used as a template for further nested PCRs using different sets of primers for each exon (see Table 1). The fragments containing, respectively, exons 11–12 and 16–17 were amplified using the forward primer of exons 11 or 16 and the reverse primer of exons 12 or 17 (Table 1) by two separate PCR amplifications. The obtained PCR fragments were sequenced using both forward and reverse primers in order to analyse the two comprised exons of each couple.

PCR and sequencing conditions

DNA samples were amplified by PCR in a final volume of 30 μl with 0.33 mmol/l each primer, 0.2 mmol/l each dNTP in 100 mmol/l Tris–HCl, pH 8.3, 500 mmol/l KCl, 2 mmol/l $MgCl_2$, 0.01 mg/l bovine serum albumin, 0.2 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, California, USA), using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems).

Amplification involved a denaturation cycle of 95°C for 10 min and 35 amplification cycles followed by a final

Table 1 Sequences of primers used to amplify and sequence each single exon of the *PLG* gene

Exon	Sequences	Amplicon size (bp)	Annealing temperature (°C)
1	5′-ATTCAAGGTAATGTTTGAACC-3′ 5′-AAATGAATTGCACATAAAGCC -3′	304	52
2	5′-AATGCCCGACTGTGTGTTCTTA-3′ 5′-TTACAGATTCCTGCGTAGGTGCA-3′	354	66
3	5′-CTCTGGTCCCTCAAGATATTCA-3′ 5′-ATAACACGATAGTGCCTTCCT-3′	334	58
4	5′-CTCAACGTGACTATGCTG-3′ 5′-GAGGGACAACTTTACAGTAG-3′	244	52
5	5′-CTCCTTCTGCCTTGCTAATA-3′ 5′-AGAGGTGAATACGTTACTC-3′	327	52
6	5′-CATCGGAATGAGAGGCAAGT-3′ 5′-CAGCAAATGTCTGATAGAGC-3′	307	51
7	5′-AGCACACAGCAGGTGCTCAATG-3′ 5′-GGCTGCACTCAGGCACTGACAC-3′	304	63
8	5′-CGCACCTGTAGTCTGAGCTACTCGG-3′ 5′-CCAGAATACTTTATCCTGCGTTCC-3′	438	63
9	5′-CCGTAACGGTTGTTCTCAAAGCGTG-3′ 5′-GCAGGCTTTCTGACCACAATAGC-3′	350	64
10	5′-TAATTCTCAGAGGCTACCGTACTGT-3′ 5′-GGCTTGAAGCATGAAGCATGGC-3′	341	63
11–12	5′-TATTCTCCCACCTCTTGTGA-3′ 5′-TCTGTAACACAGAACCAAT-3′	963	54
13	5′-GAAACACTCCTTTATGTCTTCTA-3′ 5′-TGCTCAGAAGCCCTTTTCCTT-3′	285	58
14	5′-CTTGGAATTTGTCTCGAAT-3′ 5′-GACAGTATAAATCGTTTCT-3′	232	60
15	5′-TTTCTGTACAATGGAGCAG-3′ 5′-TATTTAAGACAAGACTTCA-3′	183	47
16–17	5′-ACCAATTTCATGGCACAGA-3′ 5′-GTAGGTCAAAACCAATTCG-3′	2470	57
18	5′-CTGGAATATCCTCCTGAAT-3′ 5′-CAAGACTAACTTTGGTCTC-3′	244	48
19	5′-GCATCCCATAATAAAAGGC-3′ 5′-TGCTAAATCCCTACCCACG-3′	290	50

Amplicon sizes and annealing temperatures used during the polymerase chain reaction are also shown.

Table 2 Polymorphism genotypes in the proband and his parents

Polymorphism location	Amino acid change	Nucleotide change	NCBI reference[a]	Genotype		
				Proband	Mother	Father
Intron F (14 bp upstream of exon 7)	–	T→G	rs4252109	G/G	T/G	G/G
Exon 9	Gln342Gln	A→G	rs13231	G/G	A/G	G/G
Intron I (63 bp upstream of exon 10)	–	A→G	rs4252117	G/G	A/G	G/G
Intron J (9 bp downstream of exon 10)	–	T→C	rs4252120	C/C	C/T	C/C
Exon 11	Asp453Asn	G→A	rs4252125	A/A	G/A	A/A
Exon 19	Gly743Gly	G→T	rs11060	T/T	G/T	T/T

[a] Polymorphism registration number on the National Centre for Biotechnology Information (NCBI) single nucleotide polymorphisms database (http://www.ncbi.nlm.nih.gov/).

extension of 72°C for 7 min. Each amplification cycle involved a 30 s denaturation step at 95°C, a 30 s or 2 min annealing step, respectively, for a single exon or exons 11–12 and 16–17, at specific temperatures (Table 1), and a 30 s elongation step at 72°C.

Each PCR fragment was purified (QIAquick/PCR purification Kit; Qiagen) and subjected to direct sequencing analysis in both the forward and reverse directions using the Taq dye-deoxy terminator method and an ABI PRISM 310 Genetic Analyser (Applied Biosystems). All detected gene variations have been resequenced and reconfirmed.

Results

Plasminogen functional assay

The assay performed on the affected patient showed a plasminogen functional activity of 26.7% (normal range, 72.9–126.9%). In his parents the plasminogen values were slightly decreased (father, 63.9%; mother, 61.4%).

Molecular analysis

A previously reported missense homozygous A to G substitution in exon 2, leading to the replacement of a Lys by a Glu at codon 19 (K19E), was identified [1]. This mutation is located in the proactivation peptide (NH2-terminal acidic domain of plasminogen), 59 amino acids upstream of the Lys77–Lys78 cleavage site. Both parents were heterozygous for the same mutation. The gene sequence analysis also revealed six additional polymorphisms located in exons 9, 11 and 19, and in introns F, I and J, all previously reported (Table 2).

Discussion

The direct sequence analysis of fragments obtained by PCR generally represents the fastest and simplest strategy to search gene mutations. This strategy is more complex in the case of the *PLG* gene because of the high homology between *PLG*, *LPA* and *PRGs* genes. *PLG* and *PRGs* share about 95% homology in the 5′-untranslated region and intron 5 region, whereas the *LPA* protease domain and *PLG* protease domain sequences share 94% homology [4]. These high homology percentages lead to DNA fragment coamplification problems during PCR, due to the difficulty of drawing specific *PLG* gene sets of primers. Previously, the amplification of *PLG* exons followed by subcloning and sequencing of the M13 vector was used to solve this problem [5,7]. Obviously, this method leads to a certain result, but it is complex and time consuming. In this study, the molecular analysis of the *PLG* gene portion comprised of the 5′-untranslated region and intron E was carried out with a different strategy based on the long PCR method in order to obtain a fragment including exons 1–5. To remove any possible trace of native DNA from the final solution, the product of this reaction was gel extracted and then utilized as a template to perform nested PCRs of each single exon. For the amplification of exons 11, 12, 16 and 17, which could also generate coamplification problems, the forward primer of the upstream exons and the reverse primer of the downstream exons of the couples composed of exons 11–12 and 16–17 were used. The PCR products were gel extracted and sequenced using both forward and reverse primers for each fragment. These strategies led us to identify finally a previously reported mutation, K19E [1], and six polymorphisms (all registered in NCBI: http://www.ncbi.nlm.nih.gov/) in a patient affected by plasminogen deficiency associated with ligneous conjunctivitis, avoiding the coamplification of homologous plasminogen sequences. In the proband, both the mutation and the six polymorphisms were in a homozygous state. His non-consanguineous parents were heterozygous for the K19E mutation, while polymorphism analysis revealed a homozygous pattern for the father and a heterozygous pattern for the mother. We can thus conclude that in this family the K19E mutation was associated with the same pattern of the investigated polymorphisms.

In conclusion, the K19E mutation in the *PLG* gene is a common genetic defect in individuals with hypoplasminogenaemia, including patients with ligneous conjunctivitis, at least in Europe [1]. Surprisingly, this mutation was also reported to be present in a Scottish healthy donor population with a predicted homozygote prevalence of 0.4 in 1 000 000 in the Scottish general population [13].

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