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New mutations in the *PPBG* gene lead to loss of PPCA protein which affects the level of the β-galactosidase/neuraminidase complex and the EBP-receptor

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Abstract

We describe the clinical findings, and the molecular and biochemical studies in an Italian family with recurrent hydrops fetalis due to galactosialidosis (GS). GS is a rare lysosomal storage disorder caused by a deficiency of the protective protein/cathepsin A (PPCA). This protein forms a high-molecular-weight complex with the hydrolases β-galactosidase (GLB1) and neuraminidase (NEU1). By virtue of this association these two enzymes are correctly compartmentalized in lysosomes and protected against rapid proteolytic degradation. Controversial data show that PPCA is also present in a second complex, including the Elastin Binding Protein (EBP) the EBP-receptor, which is involved in elastogenesis, and NEU1. We investigated the potential role of the PPCA in both complexes. Two new genetic lesions (c60delG and IVS2 + 1 G > T) that lead to a frameshift and a premature stop codon were detected in the PPCA cDNA and genomic DNA of the patient. The deleterious effect of such mutations was confirmed by the complete absence of the PPCA protein on Western blots. Thus, we examined the effect of the loss of PPCA on the two protein complexes in the patient's fibroblasts. Interestingly, a reduced amount of both GLB1 and EBP proteins was detected. These data confirm that PPCA is present in two functional complexes one with GLB1 and NEU1 in the lysosomal lumen and the other with EBP at the cell surface. The reduction in GLB1 and EBP confirms that PPCA is essential for their integrity.

Keywords: Protective protein/cathepsin A; PPCA; *PPBG* gene; Lysosomal storage disease; Hydrops fetalis

Introduction

Galactosialidosis (GS; McKusick 256540) is a rare lysosomal storage disorder with an autosomal recessive inheritance caused by a defect of in protective protein/cathepsin A (PPCA). This protein forms a high-molecular-weight multicomplex with the hydrolases β-galatosidase (GLB1; E.C. 3.2.1.23) and neuraminidase (NEU1; E.C. 3.2.1.18), defending them against rapid proteolytic degradation and allowing them to take on the correct folding [1]. The GLB1 gene, involved in G_{M1} gangliosidosis, gives rise to two alternative spliced mRNAs corresponding to the GLB1 protein and to the Elastin Binding Protein (EBP) [2,3]. A primary deficiency of the NEU1 gene gives rise to sialidosis. The PPCA deficiency leads to a secondary combined deficiency of both GLB1 and NEU1 proteins. PPCA is also required for transport of NEU1 to lysosomes [4]. Besides protective function, PPCA has a cathepsin A-like enzymatic activity at acid pH and a deamidase/esterase activity at neutral pH [5]. PPCA plays an important role also in the EBP-receptor, the major component of the non-integrin cell surface receptor. The EBP-receptor is composed of three subunits: EBP, PPCA, and NEU1 [6,7]. In this complex, EBP allows the secretion and assembly of tropoelastin monomers into elastic fibers, NEU1 attaches the complex to the membrane surface, while PPCA carries out its protective function of EBP and NEU1 [6–9].

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The *PPBG* gene encoding PPCA protein, mapped on chromosome 20q13 [10], is organized into 14 exons and one additional 3′ untranslated region. The *PPBG* mRNA contains 1815 nucleotides and encodes a 54 kDa protein precursor which includes the N-terminus signal sequence of 28 amino acids [11]. The signal peptide is cleaved by proteases, producing the mature heterodimer of 32 and 20 kDa chains [1,11]. These subunits are connected by disulfide bridges. Based on the age of onset and on the degree of clinical manifestations, GS has been classified into early infantile form, late infantile form, and juvenile/adult form [12]. Patients with early infantile form show hydrops fetalis, hepatosplenomegaly, edema, telangiectasias, and skeletal dysplasia. So far, only 18 mutations have been identified in the *PPBG* gene, most of them in Japanese patients affected by the juvanile/adult form [13–20]. Determination of the atomic model of PPCA [13] and characterization of mutations have helped to clarify the molecular mechanism of wild type and mutant PPCA proteins. Here we report clinical, biochemical, and molecular studies of the second Italian case of early infantile form of GS in a family with recurrent hydrops fetalis.

Family history

The patient was the second-born from unrelated Italian parent. The first-born, a female, was delivered at the 31st week of pregnancy complicated by gestosis and hydrops fetalis recognized by ultrasound at the 25th week of gestation. She showed severe generalized hydrops, sepsis, and disseminated intravascular coagulation and died on the 16th day of age. Autopsy revealed multiorgan dysfunction syndrome and congenital infection was suspected despite vacuolization of the liver.

The proband was delivered by cesarean section at the 26th week for ingravescent hydrops that was diagnosed at the 18th week of gestation. The Apgar score was 2–3. Immediately after birth he was intubated and resuscitation procedures were instituted. Physical examination showed hepatosplenomegaly, telangiectasias, and generalized hydrops. During the first day of his life evacuative thoracentesis and abdominal paracentesis were necessary. The patient's clinical condition rapidly deteriorated and mechanical ventilation was required. He died 52 days after birth. Since recurrent hydrops fetalis was present in the history family and intrauterine infections or iso-immunization were excluded, genetic and metabolic investigations were performed. The karyotype was normal. Biochemical findings showed abnormal oligosaccharidiuria, vacuolated lymphocytes, abnormal pale eosinophils with gray-greenish granules and neutrophils with basophilic cytoplasmic granules in peripheral blood smear. These data indicated a lysosomal storage disease. Enzymatic assays of GLB1 and NEU1 showed the patient's cultured fibroblasts has reduced activity while his parents' were normal (Table 1). The proband's clinical course and the laboratory findings allowed the diagnosis of the early infantile form of GS. Prenatal diagnosis was performed in a third pregnancy by biochemical and molecular analysis. The fetus resulted as a normal female.

Table 1
Enzymatic activities in fibroblasts from the patient and from his parents

Enzyme (nmol/mg/h)	Patient	Mother	Father	n.v.
GLB1	79	510	344	300–525
NEU1	0	20	24	17–68

Materials and methods

Cell culture

Skin fibroblasts from patient, his parents, and normal controls were cultured in Ham's F-10 medium supplemented with 10% fetal bovine serum and antibiotics.

Enzyme assays

Total protein concentration was determined in triplicate by the Lowry method [21]. GLB1 and NEU1 enzymatic assays on fibroblasts were performed in triplicate as previously described [22] using artificial fluorogenic substrates (Koch-Light Laboratories, Colnbrook, UK).

PCR amplification of genomic DNA

Genomic DNA was extracted from the patient's fibroblasts and from his relatives' peripheral blood lymphocytes using standard methods. The genomic fragments covering all 14 exons and the exon/intron boundaries of the *PPBG* gene were amplified by a set of primers located in flanking intronic sequences (Table 2). PCR amplification was performed under the following conditions: initial denaturation at 94 °C for 4 min was followed by 30 cycles with denaturation at 94 °C for 30 s, annealing temperature of 63 °C for 30 s and extension at 72 °C for 1 min. All the amplification reactions were performed in a total volume of 25 μl containing 2.5 U PolyTaq DNA polymerase (Polymed, Sambuca-Firenze, Italy), 25 mM of dNTPs, 200 ng forward primer, 200 ng of the reverse primer, and 1× PCR reaction buffer.

DNA sequencing

PCR fragments were separated on a 2% agarose gel containing ethidium bromide and the bands were

Table 2
PCR primers for genomic DNA amplification and for sequencing analysis of the *PPBG* gene

Forward	Reverse	Fragment length (bp)	N° exon
5′-gaggcagcacgttcagctca	5′-gaggagaaagagctgggctg	336	Signal sequence Ex 1
5′-gaggaagactgtcacgtggc	5′-gctgttgactcgctcctcag	268	Signal sequence Ex 2
5′-ctgaggagcgagtcaacagc	5′-cctcatccgtcatctcttctc	286	Ex 1
5′-ctggaaagggcccctccaac	5′-ctctgagaggtacaggcagc	314	Ex 2
5′-ctctcatggtggccctttcc	5′-tcatgccatggatggaggacc	341	Ex 3-4
5′-gggtgggttaatgtcattatctc	5′-taactgtaaggccacagcctc	301	Ex 5
5′-gaaggtctgggtggtagggt	5′-aggacagacctgagatcatgtg	235	Ex 6
5′-cctcctctgccttcctcttc	5′-gagggaatgtggtcctgttc	215	Ex 7
5′-caacttggtggggtcgtggag	5′-ccacctcaccaggaggaagc	353	Ex 8–9
5′-cagggaagcagaggccctga	5′-aagccccagcaacccaccac	231	Ex 10
5′-gagagaaggtctgatctgttga	5′-cttgtcctgacaggaagtctc	247	Ex 11
5′-gactgggcttgttccacacc	5′-tgggtcctgctcctttgcctc	496	Ex 12–13
5′-ctgggaagaataaagggtttgg	5′-gggcactttgcttagaagagg	273	Ex 14

visualized using a UV transilluminator. DNA products were purified by Nucleospin Extract kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. The double-stranded purified products were used for direct sequencing with the same PCR amplification primers. The sequencing reactions were performed by Big Dye Terminator Cycle Sequencing Ready Reaction Kit reagents (Applied Biosystems, Foster City, USA). The reactions were run on an ABI PRISM 310 sequencer and were analyzed using Sequencing analysis software, version 3.3.

RNA isolation and synthesis of cDNA

Total RNA was extracted using a commercial kit, (Perfect RNA Mini Eppendorf, Westbury, New York, USA) and treated with RNase-free DNase. RNA integrity was verified by 0.8% agarose gel electrophoresis, and concentration was determined by OD 260. The full length *PPBG* cDNA was directly synthesized, according to the manufacturer's instructions, using the Display Thermo-RT Kit (Display Systems Biotech, Vista, CA, USA) with an oligo-30dT primer. RT-PCR on the *PPBG* cDNA was carried out by the following primer pairs 5′-CCAGTACTCCGGCTACCTCA (PPRNA1 sense) and 5′-TGTTCCCCAGAAGGCCATGG (PPRNA6 antisense) spanning the first six exons. cDNA amplification was done under the following conditions: 1 μl of *PPBG*-cDNA was added to 2.5 units of ampliTAQ DNA polymerase (Polymed, Sambuca-Firenze, Italy), 30 pmol of both forward and reverse primers, and 1× PCR reaction buffer in a total volume of 25 μl. Cycling conditions were the same as those used for genomic DNA amplification. The RT-PCR products were directly sequenced by automatic DNA sequencer.

GeneScan analysis

A fragment containing the leucine-repeat region was amplified using the forward fluorescent primer (1–2 F) and the reverse normal primer (1R). One microlitre of each PCR sample was mixed with 1 μl of the molecular weight marker TAMRA-500 (Applied Biosystems, Foster City, USA) and 15 μl of deionized formamide. After denaturation for 2 min at 95 °C, samples were chilled on ice. The products were separated on POP-4 gel by an ABI 310 Genetic Analyzer. The size determination was calculated using the software GeneScan 3.1.

Western blot analysis

About 20 μg of total proteins from the patient's fibroblasts were used in the blots. Western blots were prepared from 12.5% SDS–polyacrylamide gels and probed as described [23]. Blots were developed with colorimetric substrates [24]. Following electrophoresis, proteins were transferred to nitrocellulose (Bio-Rad, Hercules, CA). Western blot was carried out with: an anti-85 GLB1 antibody and anti-54 PPCA antibody [5,25], the anti-EBP antibody provided by Igtech, (Perdifumo Savona, Italy) and the anti-actin antibody (Sigma, Milano, Italy) as control protein. The results of the blots were visualized by reaction with the secondary antibody anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma, Milano, Italy), using the AP Conjugate Substrate kit (Bio-Rad, Hercules, CA).

Immunostaining

Ten-day-old cultures from normal control's and patient's fibroblasts were fixed at −20 °C in 100% methanol for 30 min, as previously reported [2]. Each culture was incubated separately for an hour with polyclonal human tropoelastin (Elastin Products, Missouri, USA) and fibronectin (Sigma, Milano, Italy) antibodies, developed in rabbits. These cultures were incubated for an additional hour with the secondary antibody anti-rabbit fluorescein isothiocyanate conjugated (Sigma, Milano, Italy). Fibroblasts were incubated for 30 min with propidium iodide (10 μg/ml) to stain nucleic acids.

Results

Sequencing analysis of the PPGB gene

Mutation analysis was performed by direct sequencing of all the 14 exons and the intron/exon boundaries of the patient's *PPBG* gene. The patient resulted as a compound heterozygote for two new genetic lesions. The first mutation, present in the paternal allele, was IVS2 + 1 G > T, which alters the highly conserved splice donor consensus site (Fig. 1B). Amplification of the patient's cDNA by the exonic primers PPRNA1 and PPRNA6 revealed an additional band of 436 bp besides the normal 548 bp product. The shorter fragment was sequenced and was found to be missing an exon 2 (Fig. 2). The skipping of this exon results in an out-of-frame deletion, giving rise to a premature stop codon. In addition, the new polymorphism L102L was identified in the patient's and in his father's genomic DNAs. This polymorphic site is located in exon 2 (c306G > A) close to the IVS2 + 1 G > T mutation (Fig. 1B). The second mutation, inherited from his mother, was a single base deletion in exon 1 (c60delG), leading to a premature stop codon within 273 bases (Fig. 1A). In his mother's and maternal grandfather's DNAs a deletion of a leucine in the nine-leucine-repeat region was also detected at heterozygous level, while in his father's DNA both alleles had eight leucines. The single leucine-deletion was in exon 1 of the *PPBG* gene, a few bases upstream of the c60delG genetic lesion. In order to investigate the benign nature of this mutation, 200 chromosomes from unaffected Italian individuals were analyzed by GeneScan. The leucine repeat region in exon 1 was highly polymorphic with a frequency of nine repeats in 34.5% of the population, eight repeats in 38.5%, and ten repeats in 1% (Fig. 4).

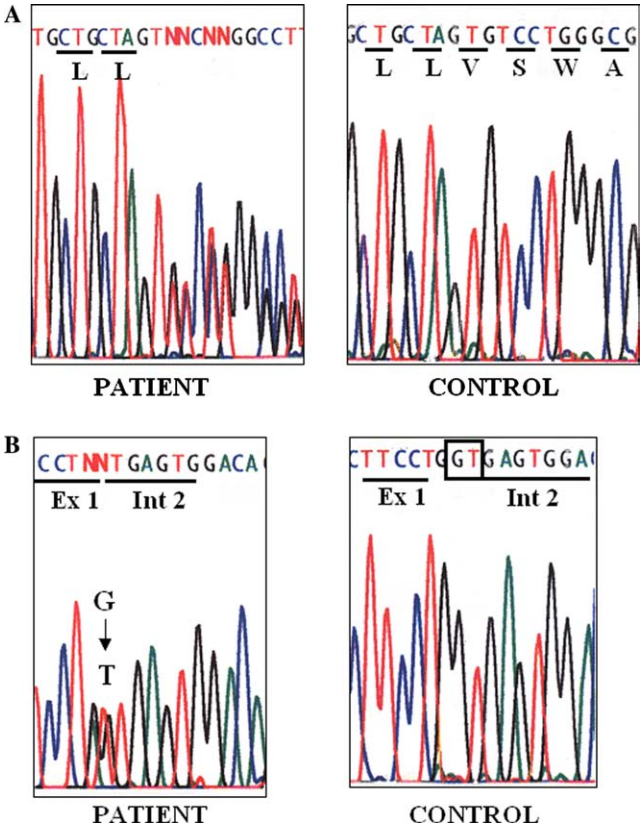

Fig. 1. Partial nucleotide sequence of the *PPBG* gene. Genomic DNA isolated from the patient's fibroblasts was amplified by PCR. (A) In exon 1 of the patient's *PPBG* gene, the new c60delG genetic lesion was identified at the heterozygous level. A normal control is shown lower. (B) The first base of intron 2 (IVS2 + 1 G > T), which alters the highly conserved splice donor consensus site, was mutated in the patient's DNA. The splice donor site in the control sequence (lower) is boxed. Moreover, the last nucleotide of exon 2 c306G > A was a polymorphic site which is adjacent to the IVS2 + 1 G > T mutation (panel B).

Western blotting

Western blot analysis revealed the absence of the PPCA mature form in the lane corresponding to the patient's fibroblasts. Western blots using GLB1 and EBP antibodies showed a decreased amount of both GLB1 and EBP proteins in the patient's fibroblasts compared to the control lane. The anti-actin antibody was used in order to check the amounts of loaded proteins (Figs. 3A–C).

Immunostaining

Immunostaining with anti-tropoelastin in normal fibroblasts showed the long elastic fibers forming a continuos net in comparison with the patient's fibroblasts where no deposition of organized elastin fibers was present (Fig. 5). The parallel cultures immunostained with anti-fibronectin antibody showed the patient's fibroblasts deposit more fibronectin than the control's (Fig. 5). This effect seems to be due to the failure of the EBP to inhibit the stimulation of fibronectin synthesis, as previously reported [2,26].

Discussion

Our patient was a typical case of early infantile form of GS. Clinical findings such as hydrops fetalis, severe generalized edema at birth, hepatosplenomegaly, telangiectasias, vacuoles in peripheral blood lymphocytes, and oligosaccharidiuria, led us to suspect a lysosomal storage disorder. In particular, a blood smear with eosinophils displaying unusually pale cytoplasm with coarse and gray-greenish stained granules indicated G_{M1} gangliosidosis, sialidosis or galactosialidosis [27].

The characteristics of this case are represented by recurrent hydrops fetalis in two subsequently pregnancies and by the identification of two new severe genetic lesions. Hydrops fetalis may be an extreme presentation of several lysosomal storage disorders as well as other

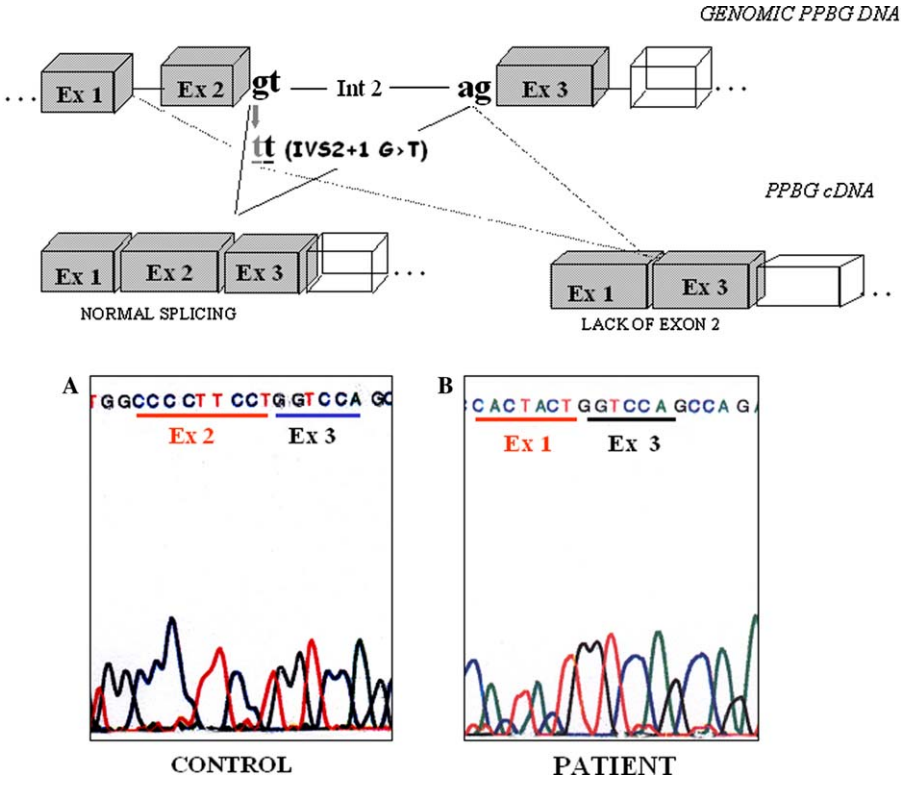

Fig. 2. Sequencing analysis of the *PPBG* cDNA. RT-PCR products obtained from the total RNA and spanning exon 1–3 showed an aberrant splicing lacking exon 2 in the patient's fibroblasts (B) that was absent in the fibroblasts from the control (A).

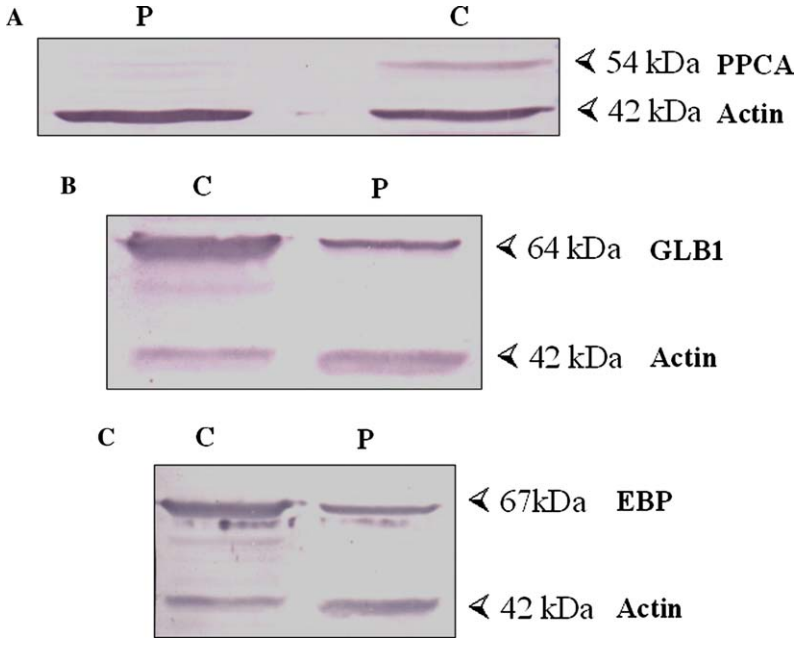

Fig. 3. Western blotting of PPCA, EBP, and GLB1. Fibroblasts from the patient and from a normal control were lysates and total proteins were separated by SDS–PAGE. (A) The nitrocellular filter was incubated with the α54 anti PPCA and the anti-actin antibodies as control protein (42 kDa). In the control lane two bands corresponding of the precursor form of PPCA (54 kDa) and of actin (42 kDa) are present. In the patient no band of 54 kDa molecular weight has been identified, in agreement with genetic lesions found. (B,C) The Western blot using anti-GLB1 and anti-EBP antibodies demonstrates a decreased amount of both GLB1 and EBP proteins in the patient's fibroblasts, compared to the control lane. The control actin protein shows equal intensity in all lanes.

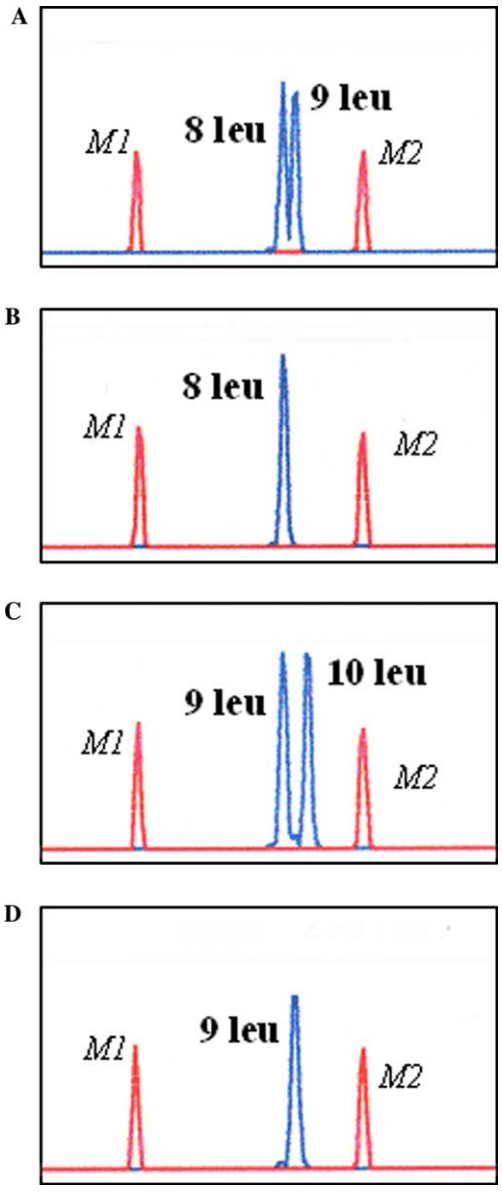

Fig. 4. GeneScan analysis of the frequency in the Italian population of polymorphisms in the rich-leucine region of the *PPBG* gene. GeneScan analysis showed a double peak, corresponding to a 281 bp and a 284 bp fragment in 48% of samples (A); only the first peak of 281 bp (B) appeared in 21%; a double peak of 281 bp and 287 bp in 2% (C); only the second peak of 284 bp (D) in 29%. The frequency was based on the analysis of 200 control chromosomes. Sequencing analysis revealed that samples with double peaks of 281 and 284 bp were heterozygotes for the one leucine deletion, samples with double peaks of 281 and 287 bp had a leucine insertion in one allele and a leucine deletion in the other allele, samples with the 281 peak were lacking a leucine at the homozygous level and samples with the 184 peak were normal.

metabolic diseases, such as CDG syndrome, Pearson disease, LCHAD defect, and Greenberg skeletal dysplasia [28,29]. On the other hand, lysosomal storage disorders represent 1–2% of the non-immune hydrops fetalis [30]. All patients affected by the early infantile form of galactosialidosis present hydrops fetalis, but only two families with recurrent hydrops fetalis were reported in the literature and none of them have been characterized at a molecular level [12].

The severe clinical findings of our patient are strictly correlated with his serious *PPBG* genetic lesions. Molecular analysis of the patient's and of his parents' DNA identified the two new *PPBG* genetic lesions: c60delG and IVS2 + 1 G > T.

The c60delG, which leads to an out reading frame and to a premature stop codon after 273 bases, with the c517delTT are the only deletions reported in the *PPGB* gene [16]. The other mutation is the IVS2 + 1 G > T splicing defect, which causes the skipping of exon 2 and introduces a premature stop codon. Two other abnormal splicing mutations are in the *PPBG* gene have been reported previously (IVS7 + 3 A > G, IVS8 + 9 C > G). The mutation in the 5′ splicing donor site of intron 7 (IVS7 + 3 A > G) has been described as a common mutation in Japanese patients, [17]. This mutation at the homozygous state correlates with a relatively mild juvenile/adult phenotype related to a residual normal mRNA. In compound heterozygosity with missense mutations, IVS7 + 3 A > G was correlated with more severe clinical presentation [12,20]. The other previously reported intronic mutation (IVS8 + 9 C > G) was identified in a late infantile Caucasian patient with a deletion of two nucleotides (c517delTT). The small amount of PPCA detected in the fibroblasts of this patient suggests that a few mRNA molecules were normally spliced [18]. On the other hand, immunoblot analysis revealed that no residual PPCA protein was present in our patient's fibroblasts. The onset and severity of the symptoms in GS patients correlate closely with the amount of residual PPCA in lysosomes [19]. Thus, the complete absence of PPCA protein in our patient explains his severe clinical condition with *exitus* at 52 days of age.

A molecular prenatal diagnosis for a third pregnancy was carried out on a chorionic villi sample. Direct sequencing of fetal DNA identified no paternal or maternal genetic lesions. In addition, the fetus resulted to be homozygous for the deletion of one leucine (eight instead of nine). Since the maternal c60delG mutation was inherited from his grandmother who had both alleles with nine leucine, the mutant maternal allele had nine leucine while the other maternal allele with eight leucine was normal. These data confirmed that the fetus inherited a normal allele from his mother.

In order to check the effect of mutant PPCA in the lysosomal complex and also in the EBP-receptor, the immunoblots with anti-GLB1 and anti-EBP antibodies were performed. Interestingly, the Western blot analysis revealed, in the patient's fibroblasts compared to the normal control, a reduced amount of both GLB1 and EBP proteins. These data confirm that PPCA is present in two functional complexes inside lysosomes with GLB1 and on the cell surface with EBP. Impaired elastogenesis due to the disruption of EBP function has been

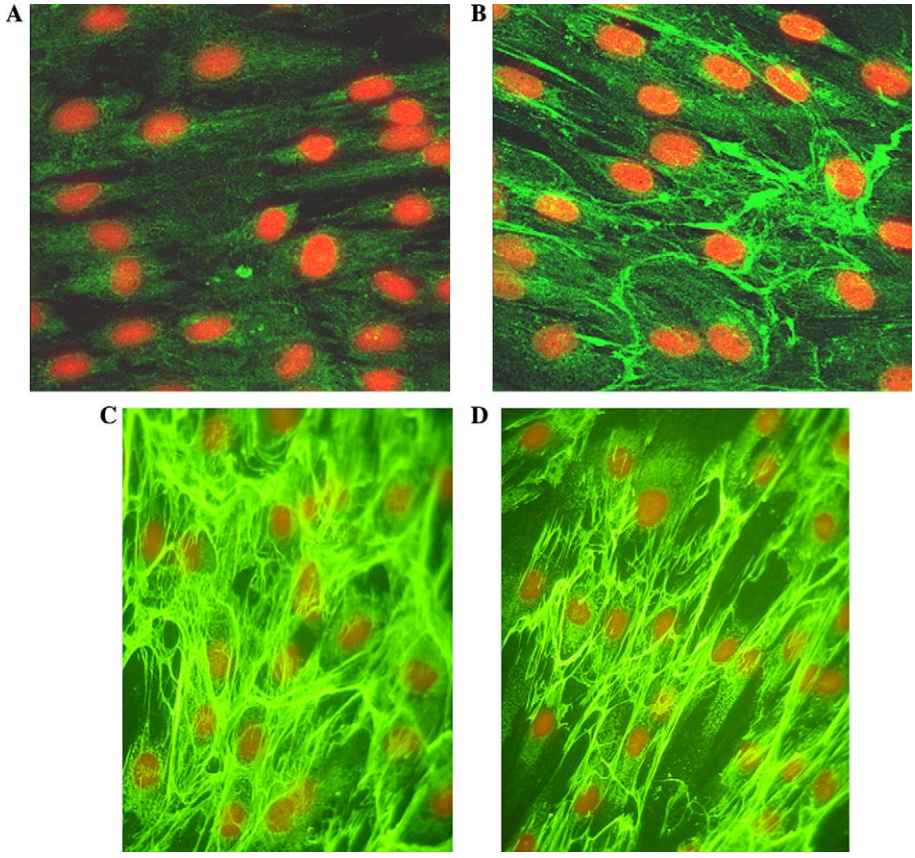

Fig. 5. Photomicrographs of ten-day-old cultures immunostained with anti-tropoelastin and anti-fibronectin antibodies. Immunostaining with anti-tropoelastin antibody show an absence of elastic fibers deposition in the patient's fibroblasts (A) in comparison with the normal control's that produce long branching elastic fibers (B). Immunostaining with anti-fibronectin antibody show an increased deposition of fibronectin in the patient's fibroblasts (C) in comparison with the normal control (D).

reported in Costello, Morquio B syndrome, and Hurler disease [31,32].

In order to determinate more precisely the functional role of PPCA in the elastin binding protein, immunostaining experiment was performed. The cell cultures treated with anti-elastin and anti-fibronectin antibodies showed the existence of impaired elastogenesis in the patient's fibroblasts. These data confirm that PPCA is essential for GLB1 and EBP integrity and suggest that some of the clinical manifestations of lysosomal disorders such as skeletal deformities, loose skin, ligamentous laxity, and heart involvement could be caused by impaired elastogenesis [30]. We hypothesized that skeletal deformities and ligamentous laxity found in our patient could be linked to a secondary defect of EBP caused by the loss of the protective function of PPCA. Cardiac involvement, frequently reported in GS patients [12,33], was probably not observed in the proband because of his short life span (52 days).

The knowledge that some metabolic inherited diseases are responsible for hydrops fetalis can assist in the diagnosis of cases where an immunological cause is excluded. In families at high risk of recurrent hydrops fetalis it is important to make the diagnosis early in order to provide genetic counseling.

Acknowledgments

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