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Protective Protein/Cathepsin A Loss in Cultured Cells Derived from an Early-Infantile Form of Galactosialidosis Patients Homozygous for the A1184-G Transition (Y395C Mutation)

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Galactosialidosis is a human autosomal recessive lysosomal storage disease caused by a genetic defect of protective protein/cathepsin A (PPCA). The patients in a Japanese family with the severe early-infantile form of galactosialidosis were revealed to be homozygous for the A1184-G transition in the PPCA gene in both alleles, which leads to the Y395C substitution. The acid carboxypeptidase (cathepsin A) and lysosomal neuraminidase activities were markedly decreased in cultured fibroblasts and chorionic villus cells derived from the patients, although the decrease in β-galactosidase activity was less. Immunoblot and immunocytochemical analyses showed that neither the precursor nor the mature form of the PPCA gene product was present in the cultured cells. The Y395C mutation was revealed to cause the loss of the translated product, that determines the severity of the clinical phenotype.

Lysosomal protective protein/cathepsin A (EC 3.2.1.14) (PPCA) is a multifunctional glycoprotein that regulates the expression of α-N-acetylneuraminidase (EC 3.2.1.18) and β-galactosidase (EC 3.2.1.23) through the formation of a multienzymic complex in lysosomes (protective function) (1-3), and also itself exhibits serine esterase activities (acid carboxypeptidase and neutral esterase/deamidase) independent of the protective function (4-6). Galactosialidosis is an autosomal recessive disease due to a primary defect of PPCA, which secondarily causes simultaneous deficiencies of the two glycosidases as well as serine esterase activities (acid carboxypeptidase and neutral esterase/deamidase), and consequent storage of sialylated oligosaccharides in tissues and urine, and putative abnormal metabolism of some bioactive peptides (7-11).

The clinical phenotypes of this disease are quite heterogeneous, and are classified into three forms based on the age of onset and severity (7,8, 12-14). Patients with the most severe manifestations, occurring in early infancy, have fetal hydrops, edema, marked ascites, skeletal dysplasia, cherry-red spots, renal dysfunction, visceromegaly, and coarse facies (early infantile form or type I). Juvenile/adult form (or type II) cases, mainly of Japanese origin, develop loss of vision as an initial symptom at age 10-15 years, followed by neurological abnormalities, such as action myoclonus, cerebellar ataxia, skeletal dysplasia, cherry-red spots, and angiokeratoma. A small group of patients have the milder late infantile form (or type III), in which symptoms develop at 12-24 months of age, and then gradually progress with visceromegaly, dysostosis multiplex, heart involvement, and no or minor neurological abnormalities.

PPCA is synthesized as a 54-kDa glycosylated precursor/zymogen. The precursor undergoes dimerization at neutral pH and is then transported to an acidic subcellular compartment through binding with a mannose-6 phosphate receptor. The zymogen is further proteolytically processed to a catalytically active 32/20-kDa two-chain mature form (2,15).

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Abbreviations: PPCA, protective protein/cathepsin A; FCS, fetal calf serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; Z-Phe-Leu, N-carbobenzoxy-L-phenylalanyl-L-leucine; anti-PP32N12, an antibody against the amino-terminal peptide (amino acids #1-12) of the 32-kDa subunit of the human mature PPCA; anti-PP20C12, an antibody against the carboxy-terminal peptide (amino acids #441-452) of the 20-kDa subunit of the human mature PPCA; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; SV-40, simian virus-40.

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In this study, we diagnosed the first patients homozygous for an A1184-G transition, and characterized the expression of the mutated gene in cultured fibroblasts or villus cells derived from the patients, in which only the Y395C mutant PPCA protein is considered to be expressed.

MATERIALS AND METHODS

Patients. Figure 1A shows the family pedigree of a Japanese family with the early-infantile form of galactosialidosis. A female patient (II-2, the second child, who died at 5 months of age) and an affected fetus (II-3, the third child, who spontaneously aborted at 14 weeks of gestation) with non-consanguineous Japanese parents were diagnosed as previously reported (16). The fourth offspring (II-4) was also prenatally diagnosed at the parents' request (Miharu et al., in preparation). Chorionic villus tissues were obtained from the fetus at 11 weeks gestation with the permission of the parents. The diagnosis of galactosialidosis was made by enzyme assaying with cultured villus cells as samples. The affected fetus was artificially aborted at the parents' request. Skin tissue was also obtained from the aborted fetus and a cultured fibroblast line was established with permission. The experiments were performed according to local institutional guidelines.

Cell culture. Villus cells from the affected fetus and a control fetus were cultured in AmnioMax-C100 medium (Gibco-BRL, Grand Island, NY, USA), and then in Ham's F-10 medium (Gibco-BRL), with 20% fetal calf serum (FCS) and antibiotics, at 37°C in a humidified incubator continuously flushed with a mixture of 5% CO_2-95% air. Fibroblasts from the patients and control subjects were cultured in Ham's F-10 medium, with 10% FCS, under the same conditions as for the cultured villus cells. Lymphoblastoid cells from the mother (Fig. 1, I-2) and the brother (the first child of the parents) (Fig. 1, II-1) were also cultured in RPMI-1640 medium (Gibco-BRL) with 10% FCS.

Gene analysis. Genomic DNAs were isolated from cultured fibroblasts, lymphoblastoid cells and villus cells from the affected patients (Fig. 1A, II-2 to 4), their mother (Fig. 1A, I-2), the brother (Fig. 1A, II-1) and normal subjects as described previously (12). Polymerase chain reaction (PCR) amplification of reverse transcribed cDNA from the patient (II-2) was performed as described previously (12), and then sequenced. Only the A1184-G transversion causing the Tyr395-Cys substitution (Y395C mutation) was detected in the PPCA cDNA of this patient. For confirmation of this mutation, the genomic DNAs were amplified by means of the PCR using a pair of primers, 5′-TATCTGAAGCTGCTTAGC-3′ (sense) and 5′-ATCTGCCCCCAC-CAGGAAAGA-3′ (antisense), as described previously (12,13). The amplified product around this region was directly sequenced by the fluorescent dye deoxy terminator cycle sequencing method using an automated DNA sequencer (Model 373A; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's method.

Preparation of cell extracts and PPCA precursor fractions. Cell extracts and PPCA precursor fractions were prepared as previously described (17) with some modification. Briefly, fibroblasts or villus cells grown to confluency were washed with phosphate-buffered saline (PBS), harvested by scraping, and then suspended in distilled water containing 0.1 mM leupeptin. The suspension was sonicated, and then centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was stored as the cell extract at −80°C. A transformed Chinese hamster ovary (CHO) cell line stably expressing a human PPCA cDNA was cultured in serum-free Ham's F-10 medium supplemented with 10 mM NH_4Cl (5). After three days' culture, the culture medium was collected, concentrated, de-salted, and then stored as the precursor fraction at −80°C before use.

Enzyme assays. The activities of acid carboxypeptidase, α-neuraminidase, β-galactosidase, and β-hexosaminidase in the cell extracts were assayed as described previously, using N-carbobenzoxy-L-phenylalanyl-L-leucine (Z-Phe-Leu) or 4-methylumbelliferyl glycosides as substrates, respectively (18,19). Protein determinations were performed with a DC protein assay kit (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (BSA) as a standard.

Immunoblotting and immunofluorescence analysis of the human PPCA protein. Previously, we raised rabbit antibodies against the amino-terminal oligopeptide of the 32-kDa subunit of the human mature PPCA (anti-PP32N12) and against the carboxy-terminal oligopeptide of the 20-kDa subunit of the mature protein (anti-PP20C12), which recognize both the mature and precursor forms of the protein on immunoblotting (17).

Immunoblotting was performed as previously described (17). Briefly, an aliquot of a cell extract or precursor fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10-20% gradient gel. Proteins were visualized by immunostaining with a mixture of anti-PP32N12 and anti-PP20C12 antibodies. Biotinylated SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used as molecular mass standards.

Immunofluorescence analysis with anti-PP32N12 was performed as previously described (16). Briefly, villus cells and fibroblasts were cultured on chamber slides (Lab-Tek; Nunc, IL, USA), and fixed with 2% (w/v) paraformaldehyde in PBS and then methanol. After blocking nonspecific binding with 5% (v/v) normal goat serum and 1% (w/v) BSA in PBS, they were treated with affinity-purified anti-PP32N12 overnight at 4°C. After washing with PBS, they were then treated with fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-rabbit immunoglobulin G (BioSource, Camarillo, CA, USA). The stained cells were examined under a confocal laser scanning microscope (MRC-600; Bio-Rad, Hemel Hempstead, UK).

RESULTS

Genotypes of the Galactosialidosis Patients and Lysosomal Enzymes Activities in Cultured Cells

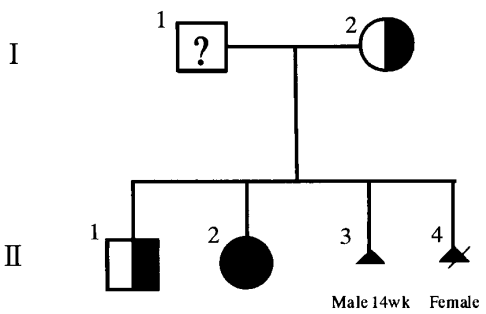
As shown in Fig. 1B, the acid carboxypeptidase activity in cultured cells derived from the patients was below 1% of the control value. α-Neuraminidase activity was also markedly decreased in all cases. β-Galactosidase activity was decreased compared with in the control subjects, although the residual activity, especially in fetal cells, was rather high (27-37% of the control value). The activity of β-hexosaminidase, as a reference lysosomal enzyme, was normal (data not shown).

PCR amplification of reverse transcribed cDNA from the patient (II-2) showed that an A1184-G transition, leading to the Y395C mutation, was the only one transition detected in the PPCA cDNA. Direct sequencing analysis revealed that the second and fourth children of the non-consanguineous Japanese parents were homozygous for the A1184-G transition (Y395C mutation) in both alleles (Fig. 2A and B). Gene analysis of the third child who was spontaneously aborted could not be performed because samples were not available for this purpose. The mother (Fig. 1A, I-2) and the brother (Fig. 1A, II-1) were also shown to be heterozygous for the A1184-G transition in one allele (data not shown).

Immunofluorescence and Immunoblot Analyses with Anti-PPCA Antibodies of Cultured Cells

In control cultured fibroblasts, remarkable granular fluorescence was observed, which suggested the exis-

A.



B.

Cell type	Lysosomal enzyme	II-2	II-3	II-4
Fibroblasts	Carboxypeptidase	1%>	-	1%>
	Neuraminidase	N.D.	-	N.D.
	β-Galactosidase	5%	-	37%
Villus cells	Carboxypeptidase	-	1%>	1%>
	Neuraminidase	-	8%	N.D.
	β-Galactosidase	-	31%	27%

FIG. 1. (A) Pedigree of a Japanese galactosialidosis family. (B) Relative lysosomal enzyme activities in cultured cells derived from the patients. The values are expressed as percentages of the control means, which were obtained for 7 cases for fibroblasts, and 3 cases for villus cells, respectively, N.D., not detectable.

tence of a sufficient amount of the mature PPCA in lysosomes (Fig. 3A). On the other hand, granular immunofluorescence was hardly detected in the cultured fibroblasts from the second patient (Fig. 3B). The same results were obtained for the villus cells from the third and fourth offspring affected with galactosialidosis (data not shown).

For control cultured fibroblasts, immunoreactive bands corresponding to the 32-kDa and 20-kDa subunits of the mature PPCA were detected on immunoblotting, while a 54-kDa precursor band was hardly observed (Fig. 4, lane 1). In contrast, neither the mature nor the precursor bands were observed for the extracts of cultured fibroblasts derived from the affected fourth offspring (Fig. 4, lane 2).

DISCUSSION

We previously performed gene analysis on 17 galactosialidosis patients from unrelated Japanese families (12-14). The Y395C substitution was identified as a common mutation in two patients (compound heterozygotes) of the four families with the early-infantile form of the disease, and three of the seven patients with the juvenile form [all compound heterozygotes having SpDEx7 (A-to-G substitution at position 3 of the 5′ splice donor site of intron 7)(12-14) on the opposite allele]. However, it was not detected in any of the eight patients with the adult form of the disease. Thus, the Y395C mutation was detected in severer cases. The amounts of the mutant mRNA were not also decreased as compared with that of the normal one in the control subjects. SpDEx7 is another common mutation in Japanese galactosialidosis patients, that causes skipping of exon 7 in PPCA mRNA (12-14). The homozygotes with the SpDEx7 mutation showed a milder clinical phenotype than the compound heterozygotes. The SpDEx7 mutation induces alternative splicing of the PPCA mRNA, and the presence of a small amount of normal mRNA probably reflects mild phenotypic expression.

The previous transient expression study on the Y395C mutant cDNA in SV40-adenovirus transformed galactosialidosis fibroblasts did not reveal the expres-

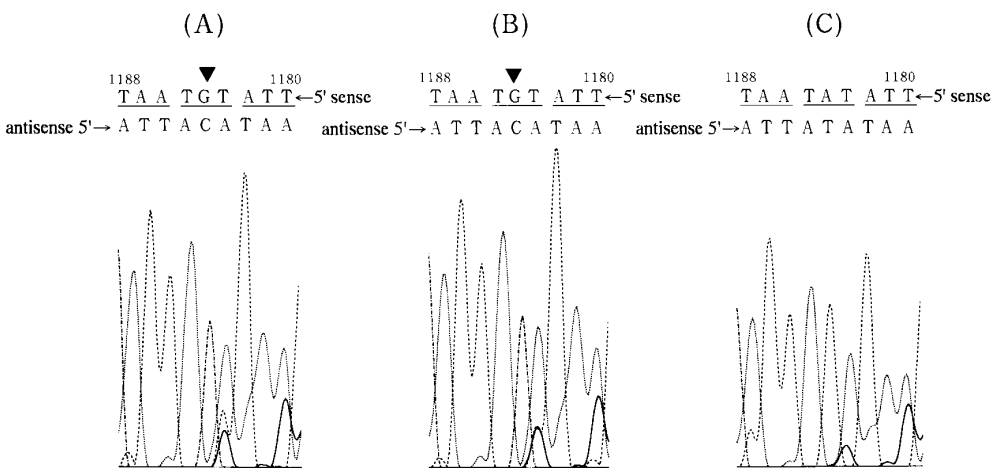


FIG. 2. A single-base substitution in the PPCA gene from the patients. DNA sequences were analyzed by direct sequencing as described under "MATERIALS AND METHODS", using DNAs prepared from villus cells derived from the affected fetus (fourth offspring) (panel A), fibroblasts from the second child patient (panel B), and fibroblasts from a control subject (panel C).

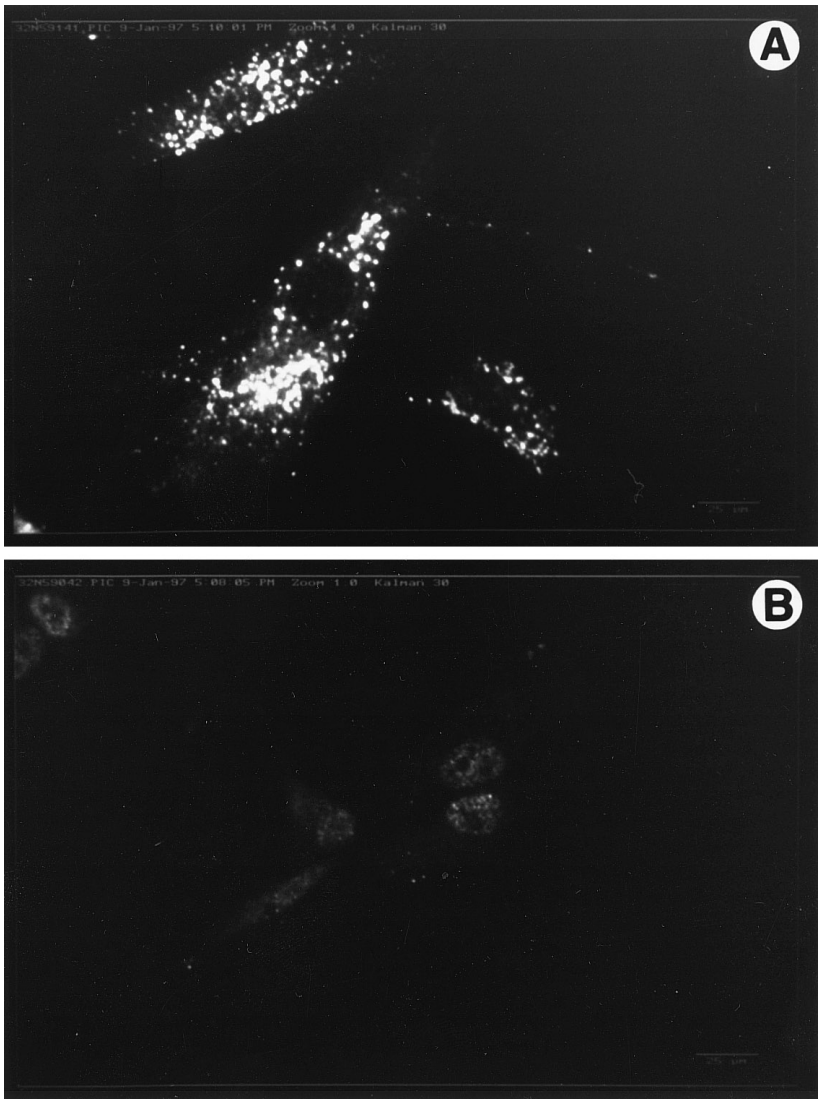


FIG. 3. Immunofluorescence pattern of human intracellular PPCA. (A) Cultured fibroblasts from a control subject, (B) cultured fibroblasts from a patient (second offspring). Bar = 50 μm.

sion of any acid carboxypeptidase activity nor a protective function against lysosomal neuraminidase and β-galactosidase (13). Pulse-chase analysis revealed that the synthesized mutant precursor might have been degraded before it was processed to the mature form in the cells transfected with the mutant cDNA. The expressed precursor was also unphosphorylated (Shimmoto et al., unpublished data).

In this study, we experienced Japanese cases with an early-infantile form of galactosialidosis, who are homozygous for the Y395C mutation in both alleles of the PPCA gene. It was an advantage that only the Y395C mutant PPCA product is naturally translated in tissues and cultured cells derived from patients. So, we characterized the translation product of the mutated gene in cultured fibroblasts and villus cells derived from the patients.

Immunoblotting and immunofluorescence analyses revealed that the immunoreactivity with anti-PPCA antibodies was totally absent in the affected cells. Therefore, the marked defects in the catalytic and protective functions of PPCA in the cells reflect loss of the translation product of the Y395C mutant gene. Taking into account the previous results of an expression study, it is suggested that the Y395C gene product is synthesized as a precursor protein in the endoplasmic reticulum, but is immediately degraded and thus not transported to further subcellular compartments. Recently, several amino acid substitutions identified in mutant PPCAs from severer clinical forms of galactosialidosis patients, including Y395C mutation, had been modeled in the three-dimensional structure of the wild-type enzyme (20). The Y395C substitution was located in the conserved core domain, and was suggested to

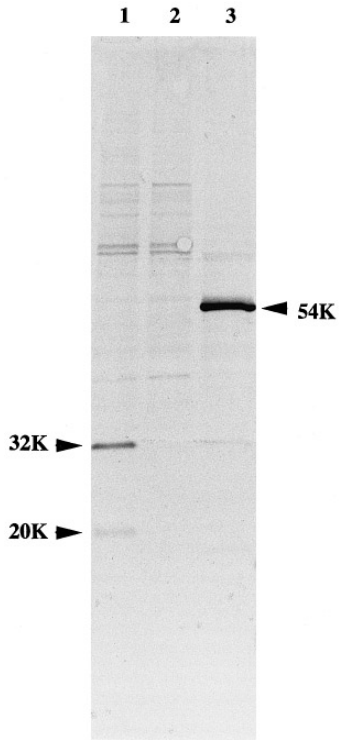


FIG. 4. Immunoblotting pattern of human intracellular PPCA. Extracts of human fibroblasts or recombinant precursor fractions were subjected to electrophoresis on a 10-20% gradient gel in the presence of SDS after reduction with 2-mercaptoethanol, and then immunostained. Lane 1, normal subject: lane 2, galactosialidosis (fourth offspring); lane 3, recombinant human precursor expressed in a CHO cell line.

contribute to drastic alteration of folding and to protein destabilization. The introduction of an extra free cysteine residue might also have promoted the formation of improper disulfide bonds and concomitant misfolded intermediates. The mutant protein is probably recognized as conformationally altered by the 'quality control' system and is degraded in the endoplasmic reticulum (21).

Residual acid carboxypeptidase and neuraminidase activities were hardly detected in the cultured cells derived from the patients in this study. However, the β-galactosidase activity was rather high (about 30% of the control value), especially in the cells derived from the affected fetus. Expression of lysosomal neuraminidase cDNA in deficient human fibroblasts revealed that the enzyme protein is localized in lysosomes but the restoration of neuraminidase activity is dependent on association with PPCA (3). Therefore, the degree of neuraminidase deficiency was correlated with that of PPCA. On the other hand, expression of the β-galactosidase activity itself is not necessarily dependent on co-existence of the functional PPCA, although the intralysosomal stability of β-galactosidase protein is decreased in PPCA-deficient cells (22). Zhou et al. reported that lysosomal β-galactosidase activity varied considerably in different tissues from the PPCA gene knockout mouse, being around 20% of the normal level in murine fibroblasts, but even higher than normal in liver (23). It is suggested that the decrease in β-galactosidase activity may not necessarily contribute to the pathogenesis of the severe manifestations of the early-infantile phenotype. We concluded that the mutation, Y395C, causes drastic losses of the translation product and PPCA functions, leading to severe phenotypic expression of galactosialidosis.

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