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ORIGINAL ARTICLE

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Genetic and enzymatic analysis for two Japanese patients with idiopathic infantile arterial calcification

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Abstract Idiopathic infantile arterial calcification (IIAC) is a life-threatening disorder in young infants. Cardiovascular symptoms are usually apparent within the first month of life. The symptoms are caused by calcification of large and medium-sized arteries, including the aorta, coronary arteries, and renal arteries. Most of the patients die by 6 months of age because of heart failure. Recently, homozygous or compound heterozygous mutations for the ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) gene were reported as causative for the disorder. ENPP1 regulates extracellular inorganic pyrophosphate (PPi), a major inhibiter of extracellular matrix calcification. Two Japanese patients with IIAC were studied. One, from first-cousin parents, showed a typical clinical course. The onset in the second patient was late. Both of the patients were clinically compatible for IIAC; arterial calcification was shown, and hypertension was prominent. We sequenced all the exons and exon-intron boundaries of the gene and measured nucleotide pyrophosphohydrolase (NPPH) activity of ENPP1. Homozygous Arg730Stop was detected in the typical IIAC patient. The mutation was a novel nonsense mutation and not detected in 60 healthy controls. His NPPH activity was 4% of normal. On the other hand, the late-onset patient was not shown to have any mutations. NPPH activity in this patient was 70% of normal. We confirmed that ENPP1 was also responsible for the Japanese patient with IIAC. The atypical late-onset phenotype may not be associated with ENPP1 abnormalities. IIAC is considered to be a clinically and genetically heterogeneous disorder.

Key words idiopathic infantile arterial calcification (IIAC) · ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) · inorganic pyrophosphate

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Introduction

Idiopathic infantile arterial calcification (IIAC) (OMIM 208000) is characterized by medial calcification (hydroxyapatite deposition) and intimal proliferation of large and medium-sized arteries throughout the body. In more than 170 cases of IIAC reported, the onset of the disease is usually during the first month of life, and chief clinical symptoms are poor sucking, respiratory distress, hypertension, or signs of heart failure [1–6]. Most of the patients die by 6 months of age due to cardiomyopathy and other complications of obstructive arteriopathy.

IIAC has been considered to be an autosomal recessive disorder. Recently, Rutsch et al. [6] reported that homozygous or compound heterozygous inactivating mutation of the ectonucleotide pyrophosphatase/phosphodiesterase 1 gene (*ENPP1*) was associated with the disorder. ENPP1, formally called PC-1, has nucleotide pyrophosphohydrolase (NPPH) activity that generates inorganic pyrophosphate (PPi) in extracellular fluid [7,8]. PPi is one of the major physiological calcification inhibitors because inactivating mutation of *ENPP1* can promote calcification of extracellular matrix (ECM), including arteries.

Two Japanese IIAC patients were analyzed, and one of the two had a novel homozygous mutation of *ENPP1*.

Materials and methods

Patients

IIAC was diagnosed based on the presence of cardiovascular symptoms associated with evidence of arterial calcification.

Patient 1 was a 1-year-old boy from first-cousin parents. He was born by emergency cesarean section at 36 weeks' gestation because of hydrops fetalis. After birth, he showed systemic edema and hepatomegaly. Pulmonary hypertension and myocardial hypertrophy seen by echocardiography were also observed. At day 3 hypertension (systolic blood pressure 90–120 mmHg) was prominent and his plasma renin activity and aldosterone were 88.0 ng/ml/h (normal range for adults 0.2–3.9 ng/ml/h) and 262.2 ng/dl (normal range for adults 3–21 mg/dl), respectively. Calcification of the aortic root, thoracoabdominal aorta, common carotid artery, bilateral common iliac artery, bilateral renal artery, superior mesenteric artery, splenic artery, and pulmonary artery were detected by computed tomography (CT). Calcification of basal ganglia and periarticular calcification of the right hip joint, knee, and wrist were also detected. Serum calcium, inorganic phosphate, and alkaline phosphatase were 10.0 mg/dl (normal 9.5–10.8 mg/dl), 4.3 mg/dl (normal 4.9–7.1 mg/dl), and 2683 IU/l (normal 528–1183 IU/l), respectively, before treatment.

The following treatments were undertaken to remove the calcification and to reduce blood pressure. Bisphosphonate (etidronate disodium 12–18 mg/kg/day p.o.), was used for calcification from 2 months of age. Three oral antihypertensive agents (a Ca channel blocker, angiotensin-converting enzyme inhibitor, and angiotensin receptor blocker) were dispensed: 0.2 mg/kg/day of amolodipine besilate administered from 2 months of age; lisinopril 0.2 mg/kg/day from 3 months, and valsartan 1 mg/kg/day from 4 months, respectively. The systolic blood pressure at 1 year was reduced to 100 mmHg, and the area of calcification was decreased.

Patient 2 was a 3-year-old girl who was the second children of unrelated parents. She was born at 40 weeks' gestation. She had had no problems during pregnancy or delivery but showed failure to thrive at 3 months of age. At 7 months her liver was palpable 3 cm below the right costal margin, and laboratory data showed liver dysfunction; asparate aminotransferase 173 IU/l (normal 29–63 IU/l), alanine aminotransferase 91 IU/l (normal 13–54 IU/l), lactate dehydrogenase 580 IU/l (normal 200–317 IU/l). Serum calcium, inorganic phosphate, and alkaline phosphatase were 10.1 mg/dl, 6.0 mg/dl, and 2394 IU/l, respectively. Hypertension was apparent at 10 months of age, and the systolic pressure was above 140 mmHg. CT revealed calcification of the hepatic, splenic, superior mesenteric, and renal arteries; but there was no other calcification throughout the aorta. Echocardiography detected thickening of the myocardial and intact coronary arteries. Periarticular calcification was not detected, but subcutaneous calcification of the cheek was found.

She has been treated with intravenous administration of bisphosphonate (pamidronate disodium 9 mg/kg/year, in six doses during the year) since 11 months of age and with a Ca channel blocker (amolodipine besilate 0.1 mg/kg p.o.) since 3 years of age. Calcification of the abdominal arteries decreased with treatment. Hypertension was not completely controlled, and hyperechogenicity of the right and left coronary arteries were prominent when she was 3 years old.

Gene analysis

Genomic DNA was extracted from peripheral blood leukocytes of the two patients, the parents of patient 1, and 60 healthy controls. Both of the patients were analyzed first. All of the coding exons and exon-intron boundaries of *ENPP1* were amplified by polymerase chain reaction (PCR) using primer sets described by Rutsch et al. [6], except for the primer set of exon 1. The sense and antisense primers of the exon 1 were 5′-AGGCGGCCGCGTCCT TGCT-3′ and 5′-AAGAAGCACTCCCGGACCATG-3′, respectively, designed by genomic information. The annealing temperature of the PCR fragment for exon 1 was 65°C. These primers were also used as the sequencing primer. The sequence determination of all the exons was performed on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Single-strand conformation polymorphism (SSCP) was performed for screening a sequence variation of exon 23 (explained later). The fragment for SSCP was 93 bp including exon 23 and exon-intron boundaries of exon 23. The fragments from the two patients, the parents of patient 1, and 60 healthy controls were amplified by PCR. The sense primer and antisense primer were 5′-CTACACTGGCTTC TATCTTGTT-3′ and 5′-GACATTGACACCATTTCTTT CTT-3′, respectively; and the annealing temperature was 57°C. SSCP was performed according to a previous report [9]. Samples that showed an abnormal SSCP pattern were examined for the sequence of exon 23.

Measurement of NPPH activity

Plasma NPPH activity was measured according to a colorimetric assay at alkaline pH using thymidine monophosphate paranitrophenyl ester (TMPNP) as substrate [10]. Controls were selected in an age-matched manner; two children less than 4 years old were controls for the patients, and five adults were controls for the parents of patient 1. The result of colorimetric assay was corrected by total protein, measured according to the Lowry method [11]. NPPH activity was shown by its percentage of that of the age-matched control.

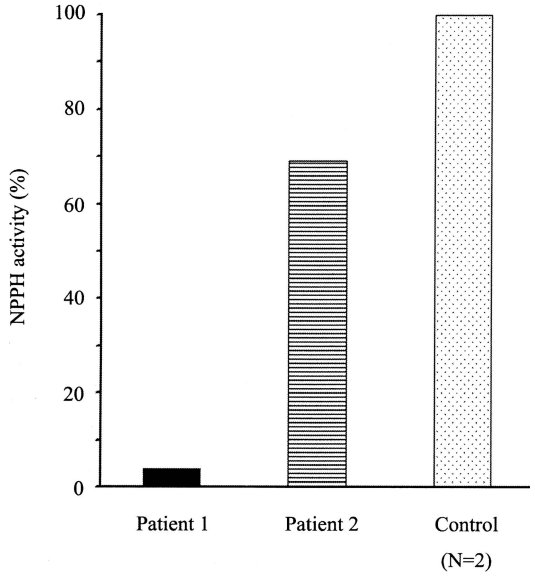

Fig. 1. Nucleotide pyrophosphohydrolase (*NPPH*) activity of *patient 1* and *patient 2*. The control ($n = 2$) was from the same age range of the patients. Patient 1 with homozygous Arg730Stop of *ENPP1* had 4% of normal activity. Patient 2 showed normal NPPH activity but did not have any mutations of *ENPP1*

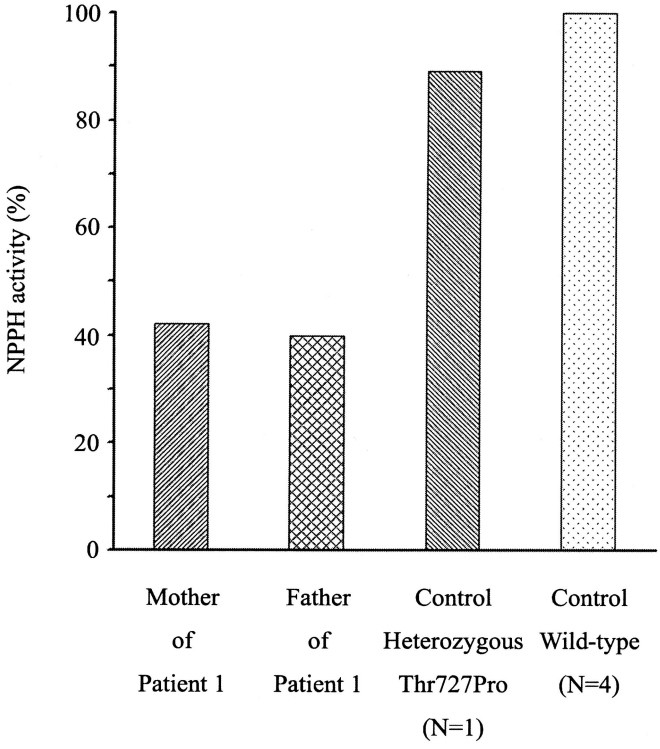

Fig. 2. Nucleotide pyrophosphohydrolase (*NPPH*) activity of the father and mother of patient 1. His mother and father are healthy and heterozygous for Arg730Stop of *ENPP1*. His father also had Thr727Pro substitution and Thr727Pro considered as a polymorphism; see also in the text. NPPH activity of the father was the same as that of the mother who was heterozygous for Arg730Stop. One of five healthy controls had heterozygous Thr727Pro with normal NPPH activity

Results

Gene analysis

Patient 1 had a homozygous mutation of C2188T in exon 23 of *ENPP1*, resulting in an amino acid alteration, Arg730Stop. Both of the patient's parents were heterozygous for the mutation. The mutation was not found in 60 healthy controls by SSCP. The patient's father also had an A2179C substitution in exon 23 that resulted in Thr727Pro in the allele (data not shown) other than one with Arg730Stop. Thr727Pro was found in two of 120 healthy control alleles. Patient 2 did not have any mutations in the exons or exon-intron boundaries.

NPPH activity

The NPPH activity of patient 1 was 4% that of the normal controls, but that of patient 2 was 70% of normal (Fig. 1). The activities of both parents of patient 1 were 40% of normal (Fig. 2). The activity of a healthy control heterozygous for Thr727Pro substitution was sustained (Fig. 2).

Discussion

Idiopathic infantile arterial calcification, characterized by calcification of large and medium-sized arteries, was first described in 1901 [12]. Most of the patients die during early infancy owing to irreversible cardiac failure. Inactivating mutation of *ENPP1* has been causative for 8 of 11 IIAC patients [4,6]. The patients with *ENPP1* mutation showed a homogeneous and early-onset phenotype, except for unusual patients with rickets. In this study, we tried to confirm whether *ENPP1* is also responsible for Japanese patients with IIAC, similar to Turkish and Caucasian patients.

ENPP1, a major factor for producing PPi in extracellular fluid (ECF), also inhibits extraordinary calcification of ECM [7,8]. PPi prevents hydroxyapatite deposition in the ECM, where calcium and phosphate concentrations are near the saturated point [13,14]. PPi is produced by NPPH activity of the phosphodiesterase nucleotide pyrophosphatase (PDNP) family: ENPP1, ENPP2 (autotaxin), and ENPP3 (B10/PDNP3) [7,15–17]. ENPP1 is abundantly expressed on fibroblasts, chondrocytes, osteoblasts, and hepatocytes; and it circulates in soluble form [8]. ENPP2, existing in circulation blood, is a bipotential enzyme. In addition to its weak NPPH activity, ENPP2 promotes calcification through its intrinsic inorganic phosphate-generating threonine-type ATPase activity in chondrocytes [18]. ENPP3 works as regulator of intracellular PPi but causes no increase in extracellular PPi in cultured chondrocytes or osteoblasts [19,20]. Most of the NPPH activity in ECF depends on ENPP1, and an inactivating mutation of ENPP1 reduces PPi production, leading to calcification of ECM.

In the ECF, PPi is also regulated by intracellular to extracellular transportation and degradation other than production by PDNP family [21]. First, PPi was shuttled from intracellular to extracellular fluid by ankylosis progressive homologue (ANKH) [22]; thus, ANKH is potentially important for regulating extracellular PPi. The clinical phenotype of *ANKH* mutations is different from IIAC; activating and inactivating mutations are causative for chondrocalcinosis-2 (OMIM 118600) and craniometaphyseal dysplasia (OMIM 123000), respectively [23,24]. Second, inorganic pyrophosphatases, acid phosphatases, and alkaline phosphatases have pyrophosphatase activity [25–28]. Although these enzymes catalyze PPi in optimal pH and location, the alkaline phosphatase family exerts major effects on extracellular PPi degradation [28]. It is well known that the elevation of PPi in hypophosphatasia (OMIM 146300; OMIM 241500) is caused by inactivating mutations of tissue nonspecific alkaline phosphatase [29]. Symptoms related to reduced PPi by excessive degradation are unlikely because benign familial hyperphosphatasemia is not related to any clinical symptoms [30].

Patient 1 had a novel homozygous mutation of Arg730Stop and was the first case of *ENPP1* mutation in Japanese patients. The nonsense mutation results in premature termination of the protein in the nuclease-like domain. This termination probably disturbs ENPP1 function like other nonsense mutations in this domain reported by Rutsch et al. [6]. NPPH activity of the patient was indeed reduced to 4% of normal. The clinical course and decreased NPPH activity of our patient 1 is quite similar to those of seven typical patients reported by Rutsch et al. [6]. Thus, this mutation is causative for IIAC.

The patient's father was heterozygous for Arg730Stop and Thr727Pro. Although Thr727Pro is a novel substitution, it is considered a polymorphism for the following two reasons. One is that the father is healthy, and he had 40% of normal NPPH activity, like as his mother, who is a heterozygous Arg730Stop carrier. The other is that Thr727Pro was detected in healthy controls with normal NPPH activity.

No mutations of *ENPP1* were detected in patient 2, who had almost normal NPPH activity. Four speculations are possible. First, genetic alteration exists in the promoter region or intron of the gene but is not detected. The second is that one of the other PDNP family genes is causative. The third possibility is that the genetic defect lies in other PPi-regulating factors such as ANKH or the alkaline phosphatase family. The fourth possibility is an abnormality of the PPi independent mechanism in preventing ECM calcification. The first two hypotheses are deniable because of sufficient NPPH activity. Concerning the third speculation, unknown PPi-regulating factors are possible, but ANKH and the alkaline phosphatase family are not causative, as explained above. Thus, we believe that the fourth hypothesis is the most acceptable for explaining why patient 2 had normal NPPH activity.

Patient 2 had late-onset IIAC, with cardiovascular symptoms appearing at 10 months. Many typical patients die by 6 months, so our patient had a different, less severe phenotype of IIAC. Patel et al. [31] described a patient with a similar phenotype whose symptoms were apparent at 33 months of age. This less severe, late-onset phenotype may be due to the genetic heterogeneity of the disease. The cause of the phenotype may be an abnormality of the PPi-regulating factors or the PPi independent calcification regulating pathway, as speculated above.

In conclusion, this is the first report of a mutation of *ENPP1* in a Japanese patient with IIAC. One of our patients with late-onset disease did not have any mutations in this gene. It is thus suggested that IIAC is clinically and genetically a heterogeneous disorder, and the late-onset phenotype may be caused by another gene.

Electronic data access

URLs for data in this article are as follows: Online Mendelian Inheritance in Man (OMIM) at http://www.ncbi.nlm.nih.gov/OMIM (for known syndromes).

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