Carbonic Anhydrase II Deficiency Syndrome —Clinico-pathological, Biochemical and Molecular Studies—

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Summary: We reported on three unrelated Japanese families with carbonic anhydrase II (CA II) deficiency syndrome. In the present study, the CA II gene was sequenced in the family of a patient with hybrid type renal tubular acidosis whose parents were nonconsanguineous, and a T to G transition at exon 2 was identified. The change results in the substitution of the stop codon (TAG) at position 40 for Tyr (TAT). The maternal and paternal mutations were the same suggesting that they were obligate heterozygotes. This is a novel mutation in the CA II deficiency syndrome, which has not been described before.

Key words: carbonic anhydrase II deficiency syndrome — stop codon mutation — osteopetrosis — renal tubular acidosis — cerebral calcification

Introduction

Carbonic anhydrase II (CA II, EC 4.2.1.1.) deficiency is the primary defect in the syndrome of osteopetrosis, renal tubular acidosis and cerebral calcification (Guibaud et al. 1972; Sly et al. 1972; Vainsel et al. 1972). Other features include mental retardation, growth failure, and dental malocclusion (Sly, 1989). CA II deficiency syndrome has been diagnosed in a variety of ethnic backgrounds, including Italian, German, Belgian, French, Hispanic, Afro-American and Middle Eastern countries (Sly et al. 1985; Sly, 1989; Strisciuglio et al. 1990).

CA II is a metalloenzyme containing zinc (Tashian, 1977), which exhibits the highest activity among the CA II isozymes (Koester et al. 1979; Sanyal et al. 1982; Whitney and Briggles, 1982).

CA II is distributed in red blood cells, renal tubular cells, neuroglial cells, osteoclasts, etc. (Tashian et al. 1984; Tashian, 1989). Sly et al. (1983) detected a defect of CA II in patients with osteopetrosis, renal tubular acidosis and cerebral calcification, from the measurement of the enzyme activities and enzyme protein levels in red blood cells. Aramaki et al. (1993) described three unrelated Japanese families with the CA II deficiency syndrome. The number of reported patients was 41 in 25 families.

The cDNA for the human CA II gene was isolated and sequenced (Montgomery et al. 1987; Murakami et al. 1987), and the genomic organization was reported (Venta et al. 1991). This information allowed the development of a general strategy for the analysis of the CA II

gene from CA II deficient patients.

The cloning and sequencing with the restriction enzyme EcoO109 I in a Japanese patient, who has been described as the first patient with CA II deficiency syndrome in Japan (Aramaki et al. 1991), have been investigated. A new mutation with a T to G transition which results in the substitution of the stop codon at position 40 in exon 2 for Tyr (TAT) was found.

Patient and Methods

Patient

A Japanese girl was born after a normal pregnancy at 39 weeks of gestation with a delivery that was uncomplicated and spontaneous. Her birth weight was 2,600 g. She was admitted to Kurume University Hospital because of poor feeding and a low weight at 4 months of age. At 10 months of age osteopetrosis was found by radiography. At 2 years of age a transitional muscle weakness occurred approximately once every six months. At 3 years of age, metabolic acidosis and a short stature were observed. She was given citric acid and its salt orally from 4 years of age. With the medication, her muscle weakness disappeared. At 9 years of age cerebral calcification, mainly in the basal ganglia, was observed with computed tomography. At this time, mental retardation was present (IQ=65) and she had severe caries of the teeth. At 5, 7 and 18 years of age she had bone fractures of the lower extremities. However, the osteopetrosis improved after puberty. The family history was not contributory, with the exception of asthma in her elder

brother.

The findings on physical examination were a height of 152 cm, a weight of 40.5 kg, and a blood pressure of 100/52 mmHg. Her facial appearance was not abnormal. Hepatomegaly and splenomegaly were absent. No neurologic abnormalities were found.

Hypokalemia and metabolic acidosis were present from 3 years of age. Her renal tubular acidosis was diagnosed as the hybrid type by means of bicarbonate infusion and ammonium chloride tolerance tests. At 10 months of age, optical examination showed no abnormalities. Computed tomography of the brain revealed multiple calcified lesions in the cerebrum. At 22 years of age, computed tomography showed calcification in the basal ganglia, the frontal lobe, the occipital lobe, near the junction of the gray and white matter, and the dentate nucleus of the cerebellum. Ultrasonography revealed small nephrocalcinosis of the bilateral kidneys. Urine amino acids were normal.

Renal biopsy with light microscopy revealed normal glomeruli, periodic acid-Schiff stain-positive material, giant cells derived from the epithelium inside the collecting ducts, cell regeneration and degeneration of the epithelium of the tubular duct. At 10 years of age a bone biopsy after double tetracycline labeling was normal except for osteosclerosis.

Her family consisted of her parents, who were non-consanguineous, and her brother. They had approximately 50% of the normal levels of CA II activity and protein indicating that they were heterozygous.

Genomic cloning

Heparinized blood samples from a patient with CA II deficiency syndrome, her parents and a brother were collected. Genomic DNA was isolated by phenol-ethanol precipitation from lymphocytes obtained from blood samples.

Polymerase chain reaction (PCR) and sequencing

The CA II gene, located at q22 on chromosome 8, is composed of seven exons and six introns (Nakai et al. 1987). Exons of the CA II gene were specifically amplified by PCR with 1 μ g of genomic DNA, 100 pmol of each PCR primer, 1.5 mM of each deoxynucleotide triphosphate, 67 mM Tris HCl (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM ammonium sulphoxide, 10% dimethyl sulphoxide (DMSO), 10 mM 2-mercaptoethanol, and 2.5U Taq DNA polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT). The reaction was carried out with an Astec program temperature control system (model PC-700) for exon 3 through 7b, consisting of 30 cycles (94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min). The PCR conditions for exon 1 and exon 2 were different, using 30 cycles (94 °C for 1 min, 60 °C for 2 min, 72 °C for 3 min). For sequencing, the PCR products were isolated in 1% low melting agarose gels, purified with Suprec-01 (TAKARA), and ligated into phage pUC 19 sequencing vectors. The clones were sequenced by the Dye Deoxy Terminator method (Dye Deoxy Terminator Cycle Sequencing Kit, Applied Biosystems) with an auto sequencer (Applied Biosystems). A minimum of 10 clones was sequenced for each exon.

EcoO109 I restriction site analysis

Each PCR sample was digested with restriction enzyme EcoO109 I (Nippon Gene) at 37 °C for 2 hrs. The products were analyzed on 12% polyacrylamide gels, which were stained with ethidium bromide.

Results

Sequence identification of the CA II nonsense mutation

The sequence of the amplified product including exon 2 is shown in Fig.1. A nonsense mutation in exon 2 in this Japanese patient was analyzed. The T to G transition resulted in the substitution of the stop codon (TAG) at position 40 for Tyr (TAT). This nonsense mutation was detected in all the analyzed clones of the patient.

EcoO109 I restriction site analysis

The 372-bp PCR product for exon 2 from the normal allele contains a single EcoO109 I site upstream to the coding region, and EcoO109 I digests it into fragments of 82-bp and 290-bp. Since the T to G transition introduced a new EcoO109 I restriction site into the mutant allele, the 290-bp fragment was further digested into two fragments of 90-bp and 200-bp with EcoO109 I; however the non-mutant fragment was not cut. The existence of the new EcoO109 I restriction site made it possible to ascertain which fragment contained the mutation. Figure 2 shows that the patient, without CA II enzyme protein and activity, had a mutant exon 2 containing a new EcoO109 I site (200-bp fragment).

Fig. 1. Sequence of exon 2 in CA II from normal and mutant alleles. The arrows indicate the nucleotide which is changed from a T in the normal allele to a G.

Each parent and a brother, who exhibited approximately 50% of the normal level of CA II activity and protein, had a normal exon 2 (290-bp fragment) and a mutant exon 2 containing the new EcoO109 I site (200-bp and 90-bp fragments). They were compound heterozygotes of a mutation allele and a normal allele. The presence of the new EcoO109 I site indicated the presence of a nonsense mutation, and was correlated with the levels of CA II enzyme protein and activity.

Fig. 2. EcoO109 I restriction site analysis for the mutant and normal alleles of the father, mother, brother, patient and normal control. PCR products spanning exon 2 in DNA from the immediate family and normal control were digested with the restriction enzyme EcoO109 I. The non-digestion sample was included. The results were electrophoresed on 1.2% agarose gel. The molecular weight standards are indicated.

Discussion

There have been only a few reports on the molecular pathology of the inherited CA II deficiency syndrome. Venta et al. (1991) sequenced the CA II gene in a Belgian patient with consanguineous parents, and identified the mutation that was probably the cause of the CA II deficiency in that family. The change involved a C to T transition, which resulted in the substitution of Tyr (TAT) for His (CAT) at position 107. His-107 appears to have a stabilizing role in the structure of all CA molecules, and its

substitution by Tyr apparently disrupts the critical hydrogen bonding of His-107 to two other similarly invariant residues, Glu-117 and Tyr-194, resulting in an unstable CA II molecule.

Roth et al. (1992) was the first to describe an American family with this syndrome who had inherited a splice acceptor site mutation at the 3' end of intron 5 from their father and a missense mutation in exon 3 (His-107-Tyr) from their mother. The residual activity of the His-107-Tyr mutant enzyme proved to be greatly decreased in comparison with that of the normally expressed CA II enzyme.

Hu et al. (1992) analyzed DNA from members of six unrelated Arabic kindreds and found five to be homozygous and one heterozygous for a novel splice junction (donor site) mutation at the 5' end of intron 2. At 5' of intron 2, a G in the normal sequence was replaced with an A in the mutant. This change created a new Sau3A I restriction site (GATC), which was absent in the normal allele.

Furthermore, Hu et al. (1994) reported that all of seven Hispanic patients were homozygous for a single-base deletion in the coding region of exon 7 that produced a frameshift which changed the next 12 amino acids before leading to chain termination and which also introduced a new Mae III restriction site.

A new mutation, involving a stop codon mutation in exon 2 in a Japanese patient with the CA II deficiency syndrome, has been found. This is the fifth mutation that has been identified in the world in patients with CA II deficiency syndrome (Table 1). The appearance of the new EcoO109 I site indicated the presence of a nonsense mutation, and was correlated with the levels of CA II enzyme protein and activity. It is the first Asian report (Soda et al. 1994).

This syndrome has not previously been reported in the Japanese population except for three patients including this patient (Aramaki et al. 1993). All three patients were habitants of the

TABLE 1.
*Reports on the molecular pathology of the carbonic anhydrase II gene
in patients with carbonic anhydrase II deficiency syndrome*

Race		Gene Mutation	
Venta (1991)	Belgian	CAT→TAT (Exon 3)	His 107→Tyr
Roth (1992)	American	CAT→TAT (Exon 3) GA→CA (3' end of Intron 5)	His 107→Tyr (Maternal) Splice junction Mutation (Paternal)
Hu (1992)	Arabic	GT→AT (5' end of Intron 2)	Splice junction Mutation
Hu (1994)	Hispanic	AAA→AA (Exon 7)	A single-base deletion of an adenosine in the coding region of exon 7
Present case (1994)	Japanese	TAT→TAG (Exon 2)	Tyr 40→Stop codon

northern region of Kyushu island. As Goriki et al. (1984) reported, the gene frequency of the CA II deficiency syndrome in the Japanese population was only two presumed heterozygotes among 3,296 individuals. This indicates that the incidence of CA II deficiency syndrome is one in ten million. CA II deficiency syndrome is a rare inherited metabolic disorder in Japan; therefore it is difficult to describe the common features of this mutation in the Japanese population. Our patient, whose parents were nonconsanguineous, was homozygous for a nonsense mutation in exon 2 in which TAG replaced the TAT at codon 40. Three previous studies (Venta et al. 1991; Hu et al. 1992; Roth et al. 1992) indicated that clinical heterogeneity among CA II deficient patients is due to different structural gene mutations. A mildly affected Belgian patient homozygous for the His-107-Tyr mutation had frequent skeletal fractures and no mental retardation (Venta et al. 1991). The original American kindred included three patients, who were compound heterozygotes for the His-107-Tyr mutation and for a splice junction mutation of intron 5. They resembled the Belgian patient in that they had many skeletal fractures, but were not mentally retarded (Roth et al. 1992).

A 2 1/2-year-old Hispanic girl of Puerto Rican ancestry had no evidence of renal tubular acidosis, even though she did have osteopetrosis, mental retardation and cerebral calcification. Other Hispanic patients had a more severe phenotype, including severe renal tubular acidosis. The clinical phenotype of the Hispanic patients with this mutation is puzzling.

The clinical phenotype of our patient was similar to the Arabic patients (osteopetrosis, severe renal tubular acidosis, cerebral calcification and moderate to severe mental retardation) described by Hu et al. (1992). It is interesting that both the nonsense mutation of exon 2 and the splice junction mutation of intron 2 induce similar clinical features. One can speculate that the pathogenesis of the similarity is as follows: the mutant genes are located close to each other, and the splicing junction mutation at intron 2 results in enzymatic deterioration equivalent to that of the nonsense mutation in exon 2.

The hybrid type with renal tubular acidosis, cerebral calcification and mental retardation might be explained by a total deficiency of the CA II enzyme, but why the osteopetrosis decreased with aging after puberty is unknown. With a total deficiency of CA II, osteopetrosis increased until adolescence. If osteoclasts derived some residual CA II activity from the His-107-Tyr CA II mutant protein, they could resorb the bone minerals under conditions of decreasing bone formation. However, since CA II does not exist in the osteoclast of our patient, the osteoclasts cannot acidify their environment and the released lysosomal enzymes cannot function sufficiently. As a result, osteopetrosis should have existed after puberty. In the present patient, osteopetrosis decreased after puberty and osteomalacia developed. These findings support a mechanism of bone resorption other than by osteoclasts.

CA II is a key enzyme in the regulation of acid-base balance. Further investigations of the biochemical and molecu-

lar aspects of this enzyme are necessary to understand the dysfunctions of kidney, bone, central nervous system and other organs in this syndrome.

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