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A Search for the Primary Abnormality in Adult-Onset Type II Citrullinemia

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Summary

Deficiency of argininosuccinate synthetase (ASS) causes citrullinemia in human beings. Type II citrullinemia is found in most patients with adult-onset citrullinemia in Japan, and ASS deficiency is found specifically in the liver. Previous studies have shown that the decrease of hepatic ASS activity is caused by a decrease in enzyme protein with normal kinetic properties and that there were no apparent abnormalities in the amount, translational activity, and gross structure of hepatic ASS mRNA. In the present work, we show by sequencing analysis that there was no mutation in the ASS mRNA from two patients with type II citrullinemia. We also report RFLP analysis of a consanguineous family with type II citrullinemia, by using three DNA polymorphisms located within the ASS gene locus. In spite of having consanguineous parents, the patient was not a homozygous haplotype for the ASS gene. The RFLP analysis of 16 affected patients from consanguineous parents showed that 5 of 16 patients had the heterozygous pattern for one of the three DNA probes and that the frequency of the heterozygous haplotype was not different from the control frequency. These results suggest that the primary defect of type II citrullinemia is not within the ASS gene locus.

Introduction

Citrullinemia is an autosomal recessive disease caused by deficiency of argininosuccinate synthetase (ASS; E.C.6.3.4.5), which catalyzes the conversion of citrulline, aspartate, and adenosine triphosphate into argininosuccinate, adenosine monophosphate, and pyrophosphate. In ureotelic animals such as man, the enzyme is expressed at high levels in the liver, where it functions as part of the urea cycle. The ASS in the kidney plays a role in the synthesis of arginine, and the activity is lower but detectable in most other tissues.

The clinical, biochemical, and molecular aspects of citrullinemia have been reviewed elsewhere (Beaudet et al. 1986; Saheki et al. 1987*b*; Brusilow and Horwich 1989), and heterogeneity is seen clinically, biochemically, and at the molecular level. Saheki et al. (1987*b*) analyzed the enzyme abnormalities in Japanese citrullinemia patients and classified them into three types. The classic neonatal and infantile form was assigned to type I (with abnormal kinetics of the enzyme) and type III (with undetectable or extremely low amounts) and is characterized by hyperammonemia, mental retardation, and early death when untreated. The enzyme defect in the classic form is found in all tissues and/or cells where ASS is expressed. More recent studies by sequencing analysis of the amplified cDNA from 14 neonatal/infantile citrullinemia patients (Jackson et al. 1989; Kobayashi et al. 1990, 1991; Su and Lin 1990) have identified nine single-base missense mutations, four other mutations associated with large deletions in the mRNA, and one mutation with small deletions in the mRNA that result from abnormal splicing. Mutations causing the classic neonatal citrullinemia are extremely heterogeneous, and all nonconsanguineous individuals studied to date are compound heterozygotes.

On the other hand, we have also described a different type of citrullinemia, which occurs in association with

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decreased ASS activity and protein in the liver but normal ASS levels in other tissues, such as kidney, brain, and fibroblasts (Saheki et al. 1981, 1982, 1983, 1985, 1987*b*; Imamura et al. 1987), in spite of the same genetic origin of the enzyme. Most patients suffer from sudden disturbance of consciousness, such as disorientation, restlessness, and coma. This unique citrullinemia was classified as type II by Saheki et al. (1987*b*), and most patients with adult-onset citrullinemia belonged to type II. In type II citrullinemia, decreased hepatic ASS with normal kinetic properties and heat stability is accompanied by almost normal levels of ASS mRNA in the liver, normal translational activity, and no gross structural abnormality (Akaboshi et al. 1983; Sase et al. 1985; Kobayashi et al. 1986; Saheki et al. 1987*a*, 1987*b*). The results on ASS mRNA were obtained from experiments with a cell-free translation system, dot and northern blotting, and S1 nuclease analyses. In these experiments, aldolase B, which is located in the cytosol of the liver and has a molecular weight similar to that of ASS, was used as an internal control because constant recovery of mRNAs from liver taken under various conditions is impossible and because the aldolase B activities in type II citrullinemia patients were almost the same as those in controls. The activity ratio of ASS to aldolase B in type II citrullinemia liver was 0.044 $\pm$ 0.008 (n = 8), compared with 0.40 $\pm$ 0.11 (n = 7) in controls. The ratio of the mRNA level of ASS to aldolase B was 0.063 $\pm$ 0.014 in eight type II citrullinemia patients and was 0.077 $\pm$ 0.012 in seven controls (Kobayashi et al. 1986). In the present paper, we show that there are no mutations in the whole coding and noncoding sequences of ASS mRNA from two patients with type II citrullinemia, except for 20 bases at both the 5′ end and the 3′ end, which are included in the oligonucleotide used as a primer for PCR.

We have analyzed over 80 patients with adult-onset type II citrullinemia, since Saheki et al. reported the first cases in 1981, and the proportion of type II citrullinemia patients from consanguineous parents is approximately 20%. The proportion of inbreeding in the Japanese population is considered to be about 1%–2% (Imaizumi et al. 1975). Therefore, the frequency of type II citrullinemia is calculated to be approximately 1 in 100,000, according to Dahlberg's (1929, 1948) equation. This suggests that type II citrullinemia is a relatively rare autosomal recessive disorder. Lander and Botstein (1987) showed that homozygosity mapping is an efficient way to map human recessive traits by using the DNA of inbred children. It is very important to know whether the primary defect of type II citrullinemia is in the ASS gene locus. Northrup et al. (1989) reported three RFLPs from within the ASS gene that map on human chromosome 9q34-qter. In the present paper, we present the results of homozygosity tests with the ASS gene by RFLP analysis using consanguineous patients with adult-onset type II citrullinemia.

Patients, Material, and Methods

History of Patient AP-61

Patient AP-61, a 35-year-old Japanese man, is the second son of healthy first-cousin parents. He has a normal brother and sister and is the father of a healthy daughter. His blood ammonia level was 264 nmol/ml (normal <39 nmol/ml), and his serum citrulline and arginine levels were 544 $\pm$ 204 and 205 $\pm$ 110 nmol/ml, respectively (normal 20–40 and 80–130 nmol/ml, respectively), as given in table 1. The hepatic ASS activity in patient AP-61 was very low, but the activities of the other urea-cycle enzymes were within their normal ranges (Todo et al. 1992). In the present study, we performed an enzyme assay on the patient fibroblasts by the method of Su et al. (1982). The ASS activity in the cultured fibroblasts of the patient was 0.88 mU/mg protein (control range 0.5–2.0 mU/mg protein). On diagnosis of type II citrullinemia, liver transplantation was performed at the University of Pittsburgh in 1988 (Todo et al. 1992). The concentration of plasma citrulline decreased from a very high level (400–800 nmol/ml) before therapy to the normal level (30 nmol/ml) after the treatment. The patient is now under rehabilitation and has no symptoms such as hyperammonemia and abnormal pattern in plasma amino acids (R. Kumashiro, unpublished data). These results strongly suggest that the ASS defect in type II citrullinemia is found only in the liver tissue and that ASS deficiency is not seen in any other tissues or cells.

Other Adult-Onset Type II Citrullinemia Patients

The patients involved in this report are 17 adults (16–48 years old) whose blood, surgical or autopsy liver specimens, and/or fibroblasts were sent to our laboratory. We diagnosed them as type II citrullinemia patients under the criteria described previously (Saheki et al. 1981, 1982, 1983, 1986, 1987*b*, 1987*c*): high concentrations of serum citrulline, a slight increase of serum arginine, an increase in the ratio of threonine to serine in serum, and a decrease in the hepatic ASS activity (but without abnormal kinetic properties), together with no decrease in renal and/or fibroblast ASS activity. Table 1 summarizes age at onset, sex, serum citrul-

Table 1

List of 17 Type II Citrullinemia Patients Described in Present Paper

Source	Age (years)	Sex	Serum			Liver	Reference(s)
			Citrulline (nmol/ml)	Arginine (nmol/ml)	Threonine/Serine ratio	ASS Activity (% of control)	
Patients:							
AP-9	21	M	173	...	...	17	Saheki et al. 1982, 1983, 1985; Yagi et al. 1988
AP-16	16	F	240	139	...	8.0	Saheki et al. 1985; Yagi et al. 1988
AP-18	33	M	582±570	365±173	2.16	14	Saheki et al. 1985, 1986; Sase et al. 1985; Kobayashi et al. 1986
AP-27	40	M	364	122	3.0	11	Kobayashi et al. 1986; Saheki et al. 1986
AP-30	19	F	478±215	302±140	...	20	Kobayashi et al. 1986; Saheki et al. 1986, 1987*c*; Yagi et al. 1988
AP-31	32	M	289±87	138±33	2.07	37	Saheki et al. 1986, 1987*c*
AP-36	48	M	309	190	4.16	17	Present paper
AP-37	42	M	289	195	2.01	24	Saheki et al. 1986; Yagi et al. 1988
AP-38	35	M	508	200	1.64	11	Saheki et al. 1986; Yagi et al. 1988
AP-39	31	M	403	198	1.90	16	Saheki et al. 1986; Yagi et al. 1988
AP-45	36	F	641	283	3.45	7.4	Present paper
AP-48	27	M	497	197	2.47	7.0	Present paper
AP-61	35	M	544±204	205±110	1.7	5.2	Todo et al. 1992
AP-69	34	F	442	188	2.87	27	Present paper
AP-72	21	M	489	302	2.1	27	Present paper
AP-80	19	M	301	143	1.56	9.4	Present paper
AP-81	30	M	451±116	261±28	2.05	...	Present paper
Control			20–40	80–130	1.17±.13		

line and arginine levels, ratio of threonine to serine in serum, and hepatic ASS activity in 16 citrullinemia type II patients from consanguineous parents and in 1 citrullinemia type II patient from nonconsanguineous parents. Hepatic ASS activity was determined as described by Saheki et al. (1981) or Su et al. (1982) and was expressed as a percentage of the adult control value. All patients showed normal kinetic properties of ASS. Saheki et al. (1982, 1983, 1985) have already reported that the ASS activity of a citrullinemia type II patient, AP-9, was normal in cultured fibroblasts, kidney, and brain. In the present paper, we report that ASS activity is normal in the fibroblasts of patient AP-16; in the kidney of patient AP-38; in the fibroblasts, kidney, and brain of AP-39; and in the fibroblasts of patient AP-61.

Sequencing Analysis of Amplified cDNA

The first strand of cDNA was synthesized by reverse transcription of RNA from both a liver specimen and cultured skin fibroblasts of citrullinemia type II patient AP-61, who was from consanguineous parents, and from the fibroblasts of citrullinemia type II patient AP-16, who was from nonconsanguineous parents. By PCR, cDNA containing the entire coding and noncoding region was amplified using oligonucleotides SF (−89 to −70 of ASS mRNA; the nucleotides are numbered by denoting the A residue of the initiation codon as +1) and TB-2 (1452 to 1433 of ASS mRNA) as the PCR primer, as described elsewhere (Kobayashi et al. 1991). The amplified cDNA was digested with *Bam*HI and *Pst*I and was ligated into M13 vector for cloning. Eight to 10 clones from each patient sample were sequenced according to the method of Kobayashi et al. (1990).

DNA Extraction and RFLP Analysis

Genomic DNA was isolated from blood, cells, or tissues of patient AP-61 and his family, other citrullinemia patients from consanguineous parents, and control individuals. DNA preparation, restriction-enzyme digestion, agarose gel electrophoresis, and Southern blotting were performed as described by Jackson et al. (1989), to analyze three DNA polymorphisms within the ASS gene locus, which maps to human chromosome

9q34-qter. Three DNA fragments within a 30-kb region were found to detect polymorphisms and to be free of human repeat sequences (Northrup et al. 1989). The ASSg1 probe derived from the 14th intron is a 450-bp *Eco*RI/*Pvu*II fragment that detects a single two-allele polymorphism with *Hin*dIII. The ASSg2 probe derived from the fifth intron is a 1.8-kb *Pvu*II/*Eco*RI fragment that detects a two-allele polymorphism with *Sph*I. The ASSg3 probe is a 4.4-kb *Eco*RI/*Hin*dIII fragment that detects a two-allele polymorphism with *Pst*I and is derived from a region between the other two probes from the vicinity of the 11th exon. The *Pst*I polymorphism is the most informative, whereas the *Hin*dIII polymorphism is the least informative, in Caucasians (Northrup et al. 1989).

Results

Sequencing Analysis of Amplified cDNA

Analyses of the ASS cDNA prepared from citrullinemia type II patients were performed as described in Patients, Material, and Methods. The amplified PCR products from two patients, AP-61 and AP-16, were the same size as that in the control, even when we used PCR-primer sets such as SF and TB-2 (−89 to 1452 of ASS mRNA, from the first exon to the last exon), SF and TB-1 (−89 to 1275 of ASS mRNA, including the entire coding region), SF and $E_{13}B$ (−89 to 970 of ASS mRNA, from exon 1 to exon 13), and $E_{13}F$ and TB-2 (839 to 1452 of ASS mRNA, from exon 13 to exon 16) (Kobayashi et al. 1990, 1991). No mutation was detected in ASS mRNA (from −89 to 1452, including the entire coding and noncoding region), from the liver specimen and fibroblasts of patient AP-61 and from the fibroblasts of patient AP-16, by the sequencing analysis with the amplified cDNA (data not shown).

RFLP Analysis of Family of Patient AP-61

In order to understand the pathogenesis of adult-onset type II citrullinemia, we performed RFLP analysis on the consanguineous family with type II citrullinemia. This analysis is also important to find out whether the primary abnormality is seen in the ASS gene locus. As shown in figure 1, Southern blot analysis for the family of patient AP-61 detected two of the DNA polymorphisms with the ASSg2 and ASSg3 probes but detected no polymorphism with the ASSg1 probe. In spite of having consanguineous parents, patient AP-61 was not a homozygous haplotype for the ASS gene, as shown in the lower part of figure 1. This result suggests that the primary abnormality of this disease is not on the ASS gene locus.

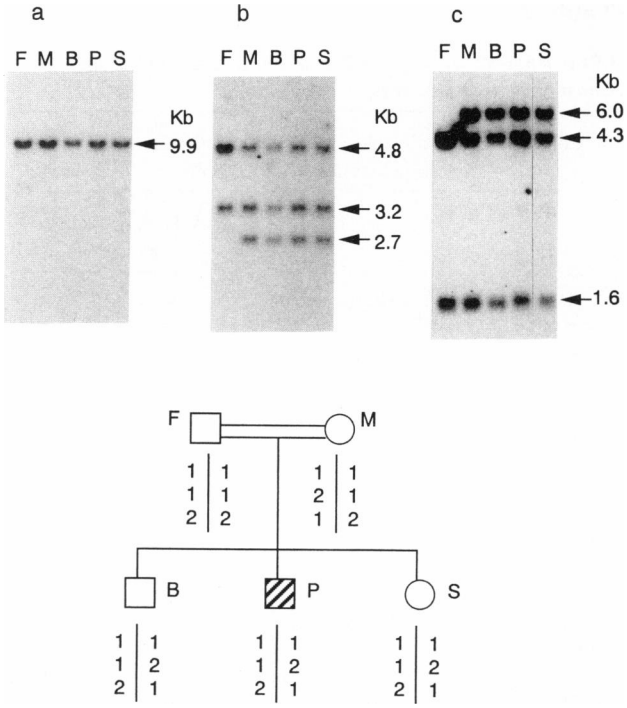

Figure 1 Southern blot analysis and RFLP haplotyping of the consanguineous family of citrullinemia type II patient AP-61. *Top,* Southern blot. DNA was (*a*) digested with *Hin*dIII and hybridized with probe ASSg1, (*b*) digested with *Sph*I and hybridized with probe ASSg2 (the polymorphic bands are 4.8 kb and 2.7 kb), and (*c*) digested with *Pst*I and hybridized with probe ASSg3 (the polymorphic bands are 6.0 kb and 4.3 kb). Results are shown for the father (lanes F), mother (lanes M), brother (lanes B), patient (lanes P), and sister (lanes S). *Bottom,* RFLP haplotyping. RFLP-1 (1) and RFLP-2 (2) indicate, respectively, absence and presence of each restriction-enzyme site. Individuals are identified by the same letters as are used for the lanes in the Southern blot (*top*).

Homozygosity Mapping in the ASS Gene Locus for 16 Patients from Consanguineous Parents

The main advantage of homozygosity mapping is that it provides a way to map a rare recessive disease (Lander and Botstein 1987). The allele frequency of type II citrullinemia, q, is considered to be about 1/300. The coefficient of inbreeding, F, is high ($F = {}^1/_{16}$ for 12 patients, and $F = {}^1/_{64}$ for 2 patients), with a few exceptions (table 2), and the mutant allele frequency, q, is very small compared with F. This indicates that the Lander-Botstein requirement is fulfilled.

The frequency of the heterozygous haplotype was investigated by homozygosity mapping in the ASS gene locus by using the 15 remaining patients from consanguineous parents. The results for *Sph*I and *Pst*I polymorphisms are summarized in table 2. Especially in the

Table 2

RFLP Analysis of Type II Citrullinemia Patients from Consanguineous Parents

A. Consanguinity and Haplotypes of Type II Citrullinemia Patients from Consanguineous Parents

Patient	Consanguinity	Haplotype with RFLP Probe	
		ASSg2	ASSg3
AP-9	Second cousin	1,1	[1,2]
AP-18	First cousin	1,1	2,2
AP-27	First cousin	1,1	2,2
AP-30	First cousin	1,1	2,2
AP-31	First cousin	1,1	2,2
AP-36	First cousin	1,1	[1,2]
AP-37	First cousin	1,1	1,1
AP-38	First cousin	1,1	2,2
AP-39	First cousin	1,1	2,2
AP-45	Second cousin	1,1	2,2
AP-48	First cousin	1,1	2,2
AP-61	First cousin	[1,2]	[1,2]
AP-69	Fourth cousin	1,1	2,2
AP-72	First cousin	1,1	[1,2]
AP-80	Fifth cousin	[1,2]	[1,2]
AP-81	First cousin	1,1	2,2

B. Frequency of Heterozygous Haplotype Expressed as [1,2]

Group	Frequency of [1,2]	
	ASSg2	ASSg3
Type II citrullinemia patients from consanguineous parents	2/16 (13%)	5/16 (31%)
Other Japanese tested	7/34 (21%)	9/30 (30%)

case of ASSg3 probe, 5 of the 16 patients showed the heterozygous pattern (expressed as [1,2] in table 2), and the frequency was not different from the value (about 30%) observed in other Japanese tested. The RFLP analysis of these patients from consanguineous parents supports the above result—that the primary defect of type II citrullinemia is not located on the ASS gene locus.

Discussion

Adult-onset type II citrullinemia with liver-specific deficiency is a unique recessive inherited disorder. The symptoms—such as high concentration of serum citrulline, slight increase of serum arginine, and hyperammonemia—resulted from the decrease of only the hepatic ASS level (Saheki et al. 1987*b*) and, in one patient, were corrected by liver transplantation (Todo et al. 1992). The analysis of mRNA from type II citrullinemia patients revealed that hepatic ASS mRNA has (*a*) a translatable activity in vitro when a cell-free translation system is used, (*b*) no decreased amount, (*c*) no detectable structural abnormality by northern blotting and S1-nuclease digestion, and (*d*) no mutation by sequencing (Sase et al. 1985; Kobayashi et al. 1986; Saheki et al. 1987*a*, 1987*b*; present paper). On the basis of these findings, we performed homozygosity mapping within the ASS gene locus by RFLP analysis using DNA samples from 16 type II citrullinemia patients from consanguineous parents. It was not possible to obtain DNA samples from their parents and to analyze their families by RFLP linkage mapping, because type II citrullinemia is adult onset. Therefore, homozygosity mapping (Lander and Botstein 1987) is expected to be an efficient and useful strategy to identify the primary abnormality in hepatic ASS deficiency. In the present study, RFLP analysis of the affected patients from consanguineous parents suggested that the abnormality is not within the ASS gene locus and that the primary defect is located on another gene.

The primary abnormality in adult-onset type II citrullinemia is still unknown. There are two possible causes for the disease. One is a rapid degradation of the enzyme protein; the other is a low rate of protein synthesis.

In type II citrullinemia, the decrease of hepatic ASS activity was caused by a decrease in the hepatic amount of ASS protein as determined by both single radial immunodiffusion (Saheki et al. 1981, 1983) and enzyme-linked immunosorbent assay (Imamura et al. 1987; Saheki et al. 1987*a*). The ASS protein had no abnormal kinetic properties (Saheki et al. 1981, 1982, 1983, 1987*b*) and was as thermostable as the controls (Akaboshi et al. 1983). The protein synthesized in vitro with mRNA from patients' liver was found to possess immunological and physicochemical properties similar to those of purified human ASS (Sase et al. 1985). On the basis of these results and the sequencing data of patient cDNA in the present work, it would seem that instabilities caused by mutations in the structural gene of the ASS locus are unlikely in the case of type II citrullinemia. Some factor that is required to keep ASS protein stable by an unknown mechanism may be missing or specifically decreased in type II citrullinemia patients' liver, and, as a result, the enzyme protein may be degraded easily. A defect in the protective or stabilizing protein has been identified as the cause of the lyso-

somal storage disorder galactosialidosis (Hoogeveen et al. 1983; Palmeri et al. 1986; Nanba et al. 1987). A similar mechanism may be operative in type II citrullinemia. On the other hand, it may be (1) that some liver-specific factor required for ASS translation is defective or decreased and/or (2) that either an RNA species, such as an antisense RNA, or some other factor, such as an RNA-binding protein that stops the translation of ASS, is produced in the liver of type II citrullinemia patients. We are examining these possibilities, but no positive evidence has yet been obtained.

It is very difficult to say whether the ASS deficiency in such type II citrullinemia patients can be detected in early life before they exhibit symptoms. We have found that the decreased ASS activity detected in the liver of a few type II citrullinemia patients did not change for 3–5 years after the first enzyme assay (authors' unpublished data). However, the possibility of an acquired component in this disorder remains.

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