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Identification of Genetic Mutations in Japanese Patients with Fructose-1,6-Bisphosphatase Deficiency

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Summary

Fructose-1,6-bisphosphatase (FBPase) deficiency is an autosomal recessive inherited disorder and may cause sudden unexpected infant death. We reported the first case of molecular diagnosis of FBPase deficiency, using cultured monocytes as a source for FBPase mRNA. In the present study, we confirmed the presence of the same genetic mutation in this patient by amplifying genomic DNA. Molecular analysis was also performed to diagnose another 12 Japanese patients with FBPase deficiency. Four mutations responsible for FBPase deficiency were identified in 10 patients from 8 unrelated families among a total of 13 patients from 11 unrelated families; no mutation was found in the remaining 3 patients from 3 unrelated families. The identified mutations included the mutation reported earlier, with an insertion of one G residue at base 961 in exon 7 (960/961insG) (10 alleles, including 2 alleles in the Japanese family from our previous report [46% of the 22 mutant alleles]), and three novel mutations—a G→A transition at base 490 in exon 4 (G164S) (3 alleles [14%]), a C→A transversion at base 530 in exon 4 (A177D) (1 allele [4%]), and a G→T transversion at base 88 in exon 1 (E30X) (2 alleles [9%]). FBPase proteins with G164S or A177D mutations were enzymatically inactive when purified from *E. coli.* Another new mutation, a T→C transition at base 974 in exon 7 (V325A), was found in the same allele with the G164S mutation in one family (one allele) but was not responsible for FBPase deficiency. Our results indicate that the insertion of one G residue at base 961 was associated with a preferential disease-causing alternation in 13 Japanese patients. Our results also indicate accurate carrier detection in eight families (73%) of 11 Japanese patients with FBPase deficiency, in whom mutations in both alleles were identified.

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Introduction

Fructose-1,6-biphosphatase (FBPase; E.C.3.1.3.11), a key enzyme in the regulation of gluconeogenesis, catalyzes the hydrolysis of fructose-1,6-biphosphate to fructose-6-phosphate and inorganic phosphate. FBPase deficiency is inherited as an autosomal recessive disorder mainly in the liver (Gitzelmann et al. 1995) and causes life-threatening episodes of hypoglycemia and metabolic acidosis in newborn infants or young children (Emery et al. 1988). Excretion of glycerol in urine is considered the main abnormality observed in urine organic-acid analysis of patients with FBPase deficiency (Burlina et al. 1990). Furthermore, administration of exogenous glycerol provokes a response such as hypoglycemia, which is quite similar to that observed after fructose administration (Gitzelmann et al. 1995). Hence, the use of glycerol for therapeutic purposes can be potentially fatal. As a rule, the first child with the disease dies or suffers more than the following children, who usually benefit from the experience gained, by parents and physicians, with the first child. Once the diagnosis is established, treatment of FBPase deficiency is simple and prognosis is excellent. Therefore, carrier detection of FBPase deficiency is important for the prevention of sudden unexpected infant death caused by FBPase deficiency (Emery et al. 1988). However, a reliable method to identify such carriers is not available, although enzymatic assay of FBPase in cultured monocytes or leukocytes has been used successfully for this purpose (Ito et al. 1984; Kikawa et al. 1993). In this regard, the diagnosis of FBPase deficiency in leukocytes has been encountered by several difficulties, in part because of the low activity of the enzyme in leukocytes (Gitzelmann et al. 1995). Moreover, there seem to be at least two phenotypes of FBPase deficiency. The first involves deficiency of the enzyme in the liver and leukocytes, whereas in the other only the liver enzyme is affected (Bührdel et al. 1990; Shin 1993; Besley et al. 1994). Therefore, enzyme assay using leukocytes is not reliable for detection of carriers for FBPase deficiency.

Recently, the gene that encodes human FBPase has been identified (El-Maghrabi et al. 1995). FBPase is a

single-copy gene located on chromosome bands 9q22.2-q22.3. The human FBPase transcript unit spans 31 kb and consists of seven exons. However, analysis of genomic DNA in two patients with FBPase deficiency did not show any mutation resulting in the synthesis of an abnormal protein (El-Maghrabi et al. 1995). We have recently shown that, as a source alternative to liver samples, monocytes can be used for mRNA encoding FBPase (Kikawa et al. 1994), and we also have identified homozygosity for an insertion of one G residue at base 961 in a Japanese patient with FBPase deficiency (Kikawa et al. 1995). Our report seems to be the only study that has detected a mutation responsible for FBPase deficiency. Since genomic DNA sequencing is now available, we analyzed the genomic DNA from the same patient reported in our previous study, as well as those of her parents. We confirmed our earlier results obtained by use of mRNA from cultured monocytes. We have also identified mutations responsible for the loss or decrease of FBPase enzyme activities in 9 of 12 other Japanese FBPase-deficient patients, by analyzing genomic DNA and cDNA. These mutations include three new types responsible for FBPase deficiency. In the present study, we report that the insertion of one G residue at base 961 is the most common mutation of FBPase gene among 13 Japanese patients with FBPase deficiency (including the patient from our previous report). This finding should be important for DNA-based diagnosis of carriers of FBPase deficiency.

Patients and Methods

Patients

Molecular analysis was performed in 13 Japanese patients with FBPase deficiency who were from 11 unrelated families. We also recruited 11 carriers or healthy family members for molecular analysis, from 3 of the 11 families. The diagnosis of FBPase deficiency was established on the basis of the lack or presence of $<10\%$ of the normal level of FBPase activity in monocytes or liver tissue samples. Clinical details of some of these patients have been reported elsewhere (Kinugasa et al. 1979; Ito et al. 1984; Nagai et al. 1992; Nakai et al; 1993; Kikawa et al. 1995). Results of analysis of cDNA from patient 1 have been reported in detail elsewhere, as the first case of molecular analysis of a family with FBPase deficiency (Kikawa et al. 1995). Patients 4a and 4b were sisters, and patients 5a and 5b were brothers. Parents of patients 1, parents and maternal grandparents of patients 4a and 4b, and siblings and parents of patient 7 were also included in this study. The clinical profiles of the 13 patients are summarized in table 1. All patients experienced recurrent attacks of metabolic acidosis and hypoglycemia. Eight patients (patients 1, 3, 4a, 4b, 5a, 5b, 6, and 7) were analyzed both at the cDNA and the genomic DNA levels, whereas only analysis of genomic DNA was performed in the other five patients (patients 2, and 8–11). Monocytes and lymphocytes were prepared as reported elsewhere (Kikawa et al. 1993). In brief, monocytes were cultured for cDNA analysis with 1 μM $1,25(OH)_2D_3$ (Wako Chemicals) under 5% CO_2 in air for 4 d. The lymphocytes were used for genomic DNA analysis.

Isolation of RNA and Genomic DNA, PCR Amplification, Sequencing, and Restriction Analysis

Total RNA was isolated, by Isozen (Wako Chemicals), from cultured monocytes. Double-strand cDNA was prepared as described elsewhere (Kikawa et al. 1994). The primer set 1F and 4R (listed in table 2) of complementary human FBPase cDNA permitted amplification of the coding sequence. Genomic DNA was isolated from lymphocytes by standard methods (Maniatis et al. 1989). The promoter and exons 1–7 of the FBPase gene were PCR amplified from genomic DNA on eight DNA fragments. The primers for PCR and the procedures for amplification of genomic DNA were similar to those described by El-Maghrabi et al. (1995). The PCR products from FBPase cDNA or genomic DNA were separated on electrophoresis on a 1% (w/v) agarose gel and were extracted from the gel by use of the Jetsorb kit (Genomed). The PCR products were sequenced by fluorescent-dye terminator–cycle sequencing after being subcloned into Bluescript plasmid vector (Novagen). Alternatively, the PCR products were directly sequenced by use of an automated DNA sequencer (model 373A; Applied Biosystems). The sequencing primers for human FBPase cDNA are listed in table 2. All mutations were confirmed by sequencing after the subcloning. Primers for PCR amplification and M13 universal primers were also used for sequencing.

PCR-SSCP Analysis and Restriction Analysis

The insertion of one G residue at base 961 does not cause any change in restriction-enzyme sites. SSCP analysis of PCR fragments containing the G insertion was performed to detect both the insertion of one G residue at base 961 and the T→C transition at base 974, according to Orita et al.'s (1989) method with minor modifications. In brief, PCR was performed by use of the forward primer 5F and the reverse primer 5R (listed at table 2). The PCR mixture in 20 μl of total volume contained 2 pmol of each unlabeled primer, 4 nmol of each of the four deoxynucleotide triphosphates, 2 μl of PCR product containing the insertion of G961, and 0.5 U of *Taq* DNA polymerase. The PCR condition was 2 min at 94°C (for denaturing), followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C. A 1-μl portion of the reaction mixture was mixed with 9 μl of SSCP loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, and 0.05% xylene cyanol) and was heated at 95°C for 2 min; 2 μl of sample were

Table 1

Clinical Summary of 13 Japanese Patients with FBPase Deficiency

Patient (Gender)	Consanguinity[a]	Age at First Symptom	Age at Diagnosis	First Symptom or Sign
1 (F)	+	1 d	10 years	Vomiting
2 (F)	−	11 mo	4 years	Vomiting
3 (F)	−	1 year	16 years	Drowsiness
4a (F)	−	16 mo	18 mo	Vomiting
4b (F)	−	2 d	11 days	Tachypnea
5a (M)	−	4 years	7 years	Vomiting
5b (M)	−	4 years	4 years	Drowsiness
6 (M)	−	8 mo	3 years	Vomiting
7 (F)	−	Infancy	2 years	Tachypnea
8 (F)	+	1 year	1 year	Vomiting
9 (M)	−	Dead at 1st d of life		Tachypnea
10 (F)	+	Unknown	21 mo	Hepatomegaly
11 (M)	−	4 years	5 years	Vomiting

[a] + = Presence of consanguinity; and − = absence of consanguinity.

loaded onto an SSCP gel. Electrophoresis was performed at 35 W for 3 h at 20°C.

The G→A transition at base 490 in exon 4 and the C→A transversion at base 530 in exon 4 create a new *Alu*I endonuclease restriction site, GGCT→AGCT, and a loss of a *Nco*I endonuclease restriction site, CCATG-G→ACATGG, respectively. Primer sets and PCR conditions for amplification of exon 4 were similar to those described above. The PCR product was a 281-bp fragment. The G→T transversion at base 88 in exon 1 introduces a new *Mae*I endonuclease restriction site, CGAG→CTAG. The primer sets and PCR conditions for amplification of exon 1 were similar to those described above. The PCR product was a 313-bp fragment. Restriction digests of all the PCR products were analyzed on 10% polyacrylamide gel and electrophoresed in 1 × Tris borate–EDTA buffer for 1 h at 100 V. After electrophoresis, the DNA bands were detected by FluorImager (Molecular Dynamics) after being stained with SYBERGreen I (Takara).

In Vitro Mutagenesis and Expression of FBPase cDNA

Among the five types of mutant clones, the clone with one G residue insertion at 961 has already been con-

Table 2

Primers for FBPase cDNA, SSCP Analysis, and In Vitro Mutagenesis

Primer	Type	Sequence	Position in cDNA
For PCR amplification:			
1F	Sense	5′-CCCCGCGCTCTACCCGGTTCA-3′	−24–4
4R	Antisense	5′-TGTGTGAGACAAAAGGTCCA-3′	1056–1075
For sequencing:			
2F	Sense	5′-ACGTGTGTTCTCGTGTC-3′	274–290
3F	Sense	5′-AGCCGGCTACGCACTGTAT-3′	486–504
4F	Sense	5′-CAGATAATTCAGCTCCTTATGG-3′	704–725
1R	Antisense	5′-CATGTTCATAACCAGGT-3′	239–255
2R	Antisense	5′-GCAGTTGACCCCACAGTCCAT-3′	532–552
3R	Antisense	5′-CACCATGGAGCCCACATACC-3′	730–750
For SSCP analysis:			
5F	Sense	5′-CCCGGTCTTGTTTTCCAGCTG-3′	Intron 6–Exon 7
5R	Antisense	5′-GCAGAGTGCTTCTCATACACC-3′	Exon 7
For in-vitro mutagenesis:			
Mutagenic primer for the G164S mutation		5′-GTGCGTAGCTGGCTGCCACC-3′	480–499
Mutagenic primer for the A177D mutation		5′-CAGTCCATGTCAAGGACCAG-3′	520–539
Selection primer		5′-CGTTGTTGCCATTGCCGCGGGCATCGTGGTGTC-3′	

firmed as expressing no FBPase activity (Kikawa et al. 1995). The clone with the E30X mutation is expected to result in premature termination, with approximately one-twelfth of the length of normal FBPase protein with 337 amino acid residues. Therefore, only the effects of the G164S, A177D, and V325A mutations on FBPase activity were tested. One of the 22 alleles harbored both the G164S and the V325A mutations A chimeric clone with only the V325S mutation was prepared for examination of the pathogenicity of the V325A mutation. The clone with both the G164S and the V325A mutations as well as the normal clone, were cut by restriction enzyme *Apa*I, at bases 924–927, and the first part of the normal clone and the latter part of the mutant clone were ligated. This chimeric clone with only the V325S mutation, the site-specific mutagenated clone with the G164S mutation, and a clone with A177D mutation were expressed. The wild-type human liver cDNA (Kikawa et al. 1994) was subcloned into the expression vector pGEX-4T-2 (Pharmacia). In vitro mutagenesis was performed by use of the U.S.E. mutagenesis kit (Pharmacia) and was based on the unique site elimination method (Deng and Nickoloff 1992). To carry the mutations, each mutagenic primer was designed from a complementary sequence corresponding to the G164S and A177D mutations. The selected primer was used to eliminate a unique nonessential restriction-enzyme site in the plasmid, which subsequently served for selection of mutant plasmids. Two mutagenic primers and one selection primer for in vitro mutagenesis are listed in table 2. A mismatch repair–defective (*mut*S) host, *E. coli* BMH71-18*mut*, was used for transformation in vitro mutagenesis. After digestion with the restriction enzymes, *Sma*I and *Sal*I, the expression vectors were ligated and transformed. Constructs containing the normal and mutant FBPase cDNA and the G164S, A177D, and chimeric V325A mutations, were designated as "pGEXFBPN," "pGEXFBPG164S," "pGEXFBPA177D," and "pGEXFBPV325A," respectively, and were overexpressed in *E. coli* JM109 cells.

Purification of the Mutant and Normal FBPase Overexpressed in E. coli

Mutant and normal FBPase proteins were overexpressed as fusion proteins with the C terminus of Sj26, a 26-kD glutathione S-transferase (E.C.2.5.1.18) encoded by the parasitic helminth *Schistosoma japonicum* (Smith and Johnson 1988). Fusion proteins were purified from bacterial lysates by affinity chromatography using glutathione Sepharose 4B contained in the glutathione S-transferase purification kit (Pharmacia). Cleavage of proteins was achieved by use of a site-specific protease, thrombin, whose recognition sequence is located immediately upstream from the multiple cloning site on pGEX plasmids. The purified mutant and normal FBPase proteins were analyzed by SDS-PAGE to estimate their purity and size and were photospectrometrically evaluated for FBPase activity, as reported elsewhere (Mizunuma and Tashima 1986). One unit of enzyme activity represented the amount that catalyzed the hydrolysis of 1 nmol of fructose-1,6-biphosphate/min. Specific activity was defined as units per milligram of purified recombinant FBPase protein. In order to remove AMP contaminating either the solutions or the enzymes used for fructose-1,6-biphosphate assay, 0.1 unit of AMP deaminase was added to the assay mixture, since FBPase shows a high sensitivity to allosteric inhibition by AMP (Mizunuma and Tashima 1986).

SDS-PAGE and Western Blotting Analysis

SDS-PAGE and western blotting of FBPase protein were performed by use of the methods described previously by Mizunuma and Tashima (1986). Antisera against purified recombinant human liver FBPase were raised in rabbits by use of Freund's complete and incomplete adjuvants as reported elsewhere for antisera against purified rat liver FBPase (Mizunuma and Tashima 1986). Recombinant human liver FBPase was expressed and purified as reported elsewhere (Kikawa et al. 1995). The experimental protocol was approved by the ethics-review committees for human and animal experimentation at Fukui Medical School.

Results

Identification of One Known and Four New Point Mutations

Mutation analysis was performed by use of samples from 13 patients with FBPase deficiency (in 11 unrelated Japanese families). One previously characterized and four novel mutations were identified in 16 of the 22 alleles: one G-residue insertion in the cluster of four G's at positions of 957–960 in exon 7, designated "960/961insG" (10 alleles); a Gly→Ser substitution at codon 164 in exon 4 ($G_{490}GC \rightarrow A_{490}GC$), designated "G164S" (3 alleles), a Val→Ala substitution at codon 325 in exon 7 ($GT_{974}G \rightarrow GC_{974}G$), designated "V325A" (1 allele), which harbored in one of the 3 alleles with the G164S mutation an Ala→Asp substitution at codon 177 in exon 4 ($GC_{530}C \rightarrow GA_{530}C$), designated "A177D" (1 allele); and a Glu to stop at codon 30 in exon 1 ($G_{88}AG \rightarrow T_{88}AG$), designated "E30X" (2 alleles). None of these five mutations were found at any of FBPase cDNA samples from 40 healthy donors. Other six alleles that harbored no mutation caused a loss or decrease of FBPase enzyme activity in the coding region and in the exon/intron boundary. The genotype of each patient is shown in table 3.

The 960/961insG mutation was found in patients 1–3, as homozygosity, and in patients 4a, 4b, 5a, 5b, 6, and 7, as heterozygosity, by direct sequencing of PCR-amplified genomic DNA of exon 7 (only data for pa-

tients 3 and 4a are shown in fig. 1*A, top* and *middle,* respectively). This mutation was confirmed both by sequencing of subclones from cDNA and by direct sequencing of cDNA from cultured monocytes, except in patient 2, from whom monocytes were not available for cDNA analysis. The remainder of the genomic DNAs in patients 1–3 were identical to the wild-type sequence. SSCP analysis of exon 7 from patients 1–3, 4a, 4b, 5a, 5b, 6, and 7 and from their family members also confirmed homozygosity or heterozygosity of the 960/961insG mutation. In patients 4a and 4b and their father, the presence of another mutation in exon 7 was suggested on the basis of SSCP patterns different from those of other patients or family members (fig. 2).

Another new mutation in exon 7, the V325A mutation, was found in patients 4a and 4b and their father, as heterozygosity, by direct sequencing of PCR-amplified genomic DNA (only data at the antisense strand for patient 4a are shown in fig. 1*B, top*). This mutation was confirmed as belonging to the second mutant allele different from the one with the 960/961insG mutation, by sequencing of a subclone from PCR-amplified cDNA. The G164S mutation was found in patients 4a, 4b, 5a, 5b, and 6, as heterozygosity, by direct sequencing of PCR-amplified genomic DNA. The G164S and V325A mutations were found to harbor the same second mutant alleles detected in patients 4a and 4b, by sequencing of a subclone from PCR-amplified cDNA. The G164S mutation was also confirmed in the second mutant allele in patients 4a, 4b, 5a, 5b, and 6, by sequencing of subclones from PCR-amplified cDNA (only data at the antisense strand for patient 5a are shown in fig. 1*C, top*).

The A177D mutation was detected in patient 7, as heterozygosity, by direct sequencing of PCR-amplified genomic DNA (data shown in fig. 1*D, top*). This mutation was also confirmed in the second mutant allele, by sequencing of subclones from PCR-amplified cDNA. Both the remainder of cDNA in the coding region and the 3′ UTRs in patients 4a, 4b, 5a, 5b, 6, and 7 were identical to the those of the wild-type sequence.

The E30X mutation was detected in patient 8, as homozygosity, by direct sequencing of PCR-amplified genomic DNA of exon 1 (fig. 1*E, top*). This mutation was confirmed on both alleles of this patient, by sequencing of subclones from PCR-amplified exon 1 of genomic DNA. The remainder of genomic DNA, including exons 1–7 and certain parts of the promoter area, were identical to that of the wild-type sequence.

RFLP Analysis for the G164S, A177D, and E30X Mutations

To confirm the authenticities of the G164S, A177D, and E30X mutations, restriction-enzyme analysis was performed by use of PCR-amplified genomic DNA or cDNA from patients 4a, 4b, 5a, 5b, 6 and 7 and their family members. The G164S mutation created a new *Alu*I endonuclease restriction site, GGCT→AGCT. In the presence of this missense mutation as heterozygosity, the 281-bp PCR fragment was partially cleaved by *Alu*I, into 151-bp and 130-bp fragments; in contrast, this 281-bp fragment remained uncleaved in the absence of G164S mutation (fig. 3). The results of *Alu*I digestion showed that patients 4a and 4b and their father and patients 5a, 5b, and 6 were heteroallelic for this mutation. Heterozygosity of this mutation in the father was confirmed by direct sequencing of the 281-bp PCR fragment. The A177D mutation eliminated a *Nco*I endonuclease restriction site, CCATGG→ACATGG. In the presence of this missense mutation as heterozygosity, the 281-bp PCR fragment was only partially cleaved by *Nco*I, into 191-bp and 90-bp fragments; in contrast, this 281-bp fragment was completely cleaved in the absence of G164S mutation (fig. 4). The results of *Nco*I digestion showed that patient 7 and her father were heteroallelic for this mutation. Heterozygosity of this mutation in her father was confirmed by direct sequencing of the 281-bp PCR fragment (data not shown). Finally, the E30X mutation created a *Mae*I endonuclease restriction site, CGAG→CTAG. In the presence of this nonsense mutation, the 313-bp PCR fragment was cleaved by

Table 3

Genotypes of 13 Japanese Patients with FBPase Deficiency

Patient	Allele 1	Allele 2	Codon	Mutation	Exon(s)
1	InsG960/961	InsG960/961	321/321	Stop	7/7
2	InsG960/961	InsG960/961	321/321	Stop	7/7
3	InsG960/961	InsG960/961	321/321	Stop	7/7
4a	G490A+T974C	InsG960/961	164 and 325/321	G164S and V325A/stop	4, 7/7
4b	G490A+T974C	InsG960/961	164 and 325/321	G164S and V325A/stop	4, 7/7
5a	G490A	InsG960/961	164/321	G164S/stop	4/7
5b	G490A	InsG960/961	164/321	G164S/stop	4/7
6	G490A	InsG960/961	164/321	G164S/stop	4/7
7	C530A	InsG960/961	177/321	A177D/stop	4/7
8	G88T	G88T	30/30	E30X/E30X	1/1
9–11[a]	(No mutation either in coding region or in exon/intron boundary)				

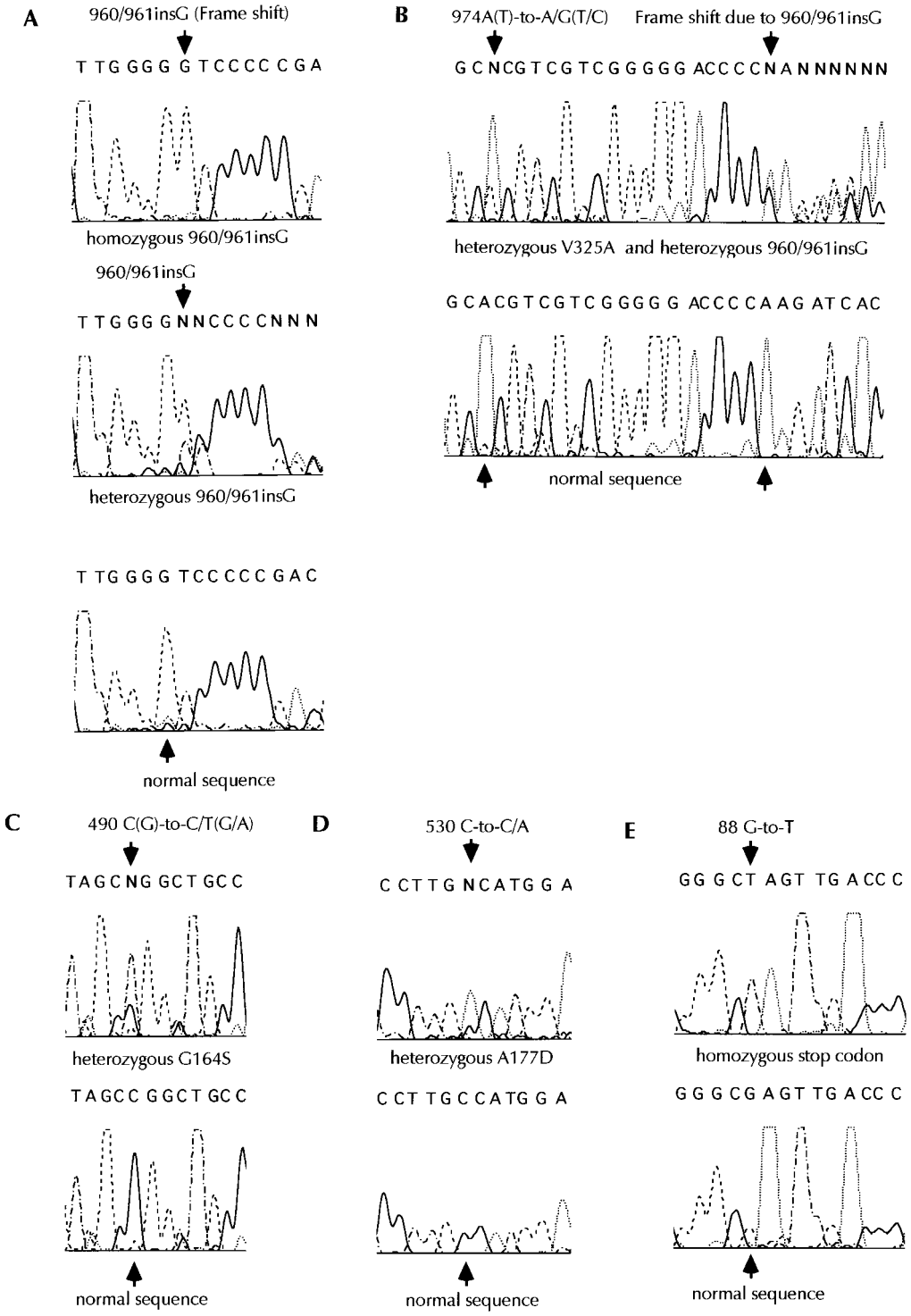

Figure 1 Nucleotide sequence of the PCR-amplified genomic fragment around the observed mutation in patients, and the corresponding region in normal control. Exon 1-, 4-, or 7-specific genomic DNA fragments were amplified, and their nucleotide sequences were directly determined. *A, top,* Nucleotide sequence of the sense strand (exon 7) in patient 3, around the homozygous 960/961insG that leads to a frameshift and the formation of truncated enzyme protein. *middle,* Nucleotide sequence of the sense strand in patient 4a, around the heterozygous 960/961insG. The patient carried the 960/961insG mutation on her maternal chromosome, whereas the paternal chromosome carried the 490G→A transition (G164S missense mutation) and the 974T→C transition (V325A nonsense mutation). *B, top,* Nucleotide sequence of the antisense strand in patient 4a, around the compound heterozygous 974T→C transition and the 960/961insG mutation (exon 7). *C, top,* Nucleotide sequence of the antisense strand in patient 4a, around the 490G→A transition (exon 4; G164S missense mutation). *D, top,* Nucleotide sequence of the sense strand in patient 7, around the 530C→A transversion (exon 4; A177D missense mutation). The patient carried the 530C→A transversion on her maternal chromosome, whereas the paternal chromosome carried the 960/961insG mutation. *E, top,* Nucleotide sequence of the sense strand in patient 8, around the homozygous 88G→T transversion (exon 1; E30X nonsense mutation), which would lead to premature termination of the translation product.

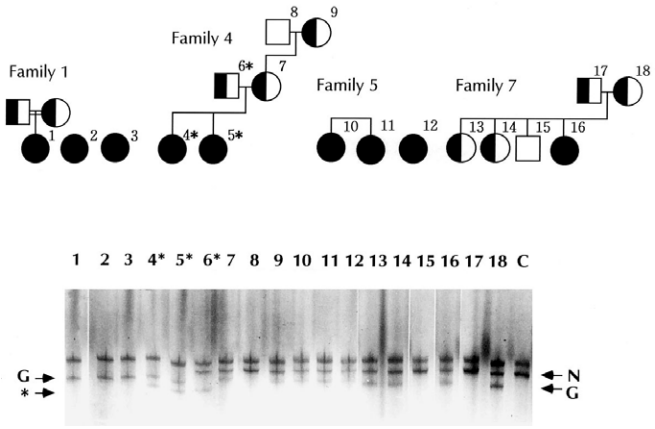

Figure 2 Pedigrees and SSCP analysis of genomic DNA fragments including part of exon 7 from patients and a control, which includes the 960/961insG and V325A mutations. The homoallelic state for the 960/961insG mutation is shown in lanes 1–3 (patients 1–3 are homoallelic for this mutation), by a single strand with the 960/961insG mutation (indicated by the "G" and arrow on the left-hand side of the gel). The heteroallelic states for the 960/961insG and V325A mutations are shown in lanes 4–6 (marked with an asterisk [*]) (patients 4a and 4b were compound heteroallelic for both the mutations, and their father was heteroallelic for the V325A mutation only), by a single strand with the 960/961insG mutation (indicated by the "G" and arrow on the left-hand side of the gel) and by a single strand with V325A mutation (indicated by an asterisk with an arrow). The heteroallelic state for the 960/961insG mutation only is shown in lanes 7, 9, 10, 11, 12, 13, 14, 16, and 18 (representing, respectively, the heteroallelic mother of patients 4a and 4b, their grandmother, patients 5a, 5b, 6, two sisters of patient 7, patient 7, and the father of patient 7), by a normal single strand (indicated by "N" and an arrow, on the right-hand side of the gel) and by a single strand with the 960/961insG mutation (indicated by "G" and an arrow on the right-hand side of the gel). The normoallelic state for these two mutations is confirmed in lanes 8, 15, 17, and C (representing, respectively, the grandmother of patients 4a and 4b, brother and mother of patient 7, and a normal healthy control).

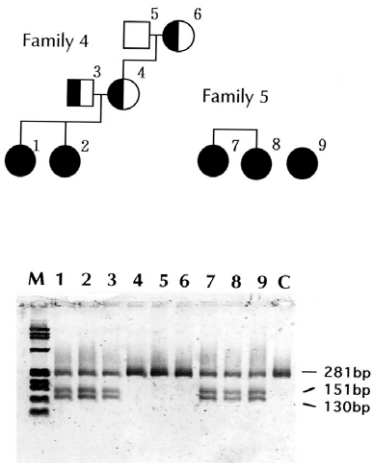

Figure 3 Pedigree of families 4 and 5 and patient 6, and detection of the G164S mutation in a PCR fragment amplified from genomic DNA including exon 4 after digestion by *Alu*I. The 281-bp fragment is partially cleaved into 151-bp and 131-bp fragments only in lanes 1–3 (representing, respectively, patients 4a and 4b and their father) and in lanes 7–9 (representing, respectively, patients 5a, 5b, and 6), indicating a heterozygous state for the G164S mutation. Lane M, Molecular-weight marker PhiX174 digested by *Hae*III. Lane C, Normal control sample.

*Mae*I into 176-bp and 137-bp fragments; in contrast, this 313-bp fragment remained uncleaved in control samples (fig. 5). The results of *Mae*I digestion showed that patient 8 was homoallelic for this mutation.

FBPase Activity of Mutant Proteins Overexpressed in E. coli

The effects of the G164S, A177D, and chimeric V325A mutations on FBPase activity were tested by in vitro expression of the mutant cDNA construct overexpressed in *E. coli.* Overexpressed protein bands in both SDS-PAGE and western blotting analyses were of similar size (~37 kD) in all mutant, chimeric, and normal clones (fig. 6). FBPase activities in purified proteins were 0.1 and 0.2 units/mg protein in mutant clones with G164S and A177D mutations, respectively; in contrast, mean ± SD FBPase activities in purified proteins were 5.3 ± 0.7 units/mg protein and 6.8 ± 0.5 units/mg protein in the chimeric V325A mutant clone and the normal clone, respectively. The mean ± SD value in the normal clone was comparable to that in human liver (9.8 ± 0.8 units/mg protein), as reported by El-Maghrabi et al. (1993).

Discussion

In the present study, we investigated 13 Japanese patients with FBPase deficiency. The major finding of our study was the identification of four novel point mutations and a 960/961insG mutation in 16 of the 22 alleles—that is, an overall detection rate of 73%. Of the

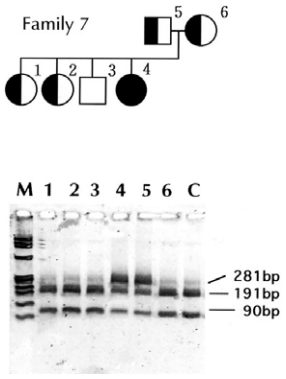

Figure 4 Pedigree of family 7, and detection of A177D mutation in a PCR fragment amplified from genomic DNA including exon 4 after digestion by *Nco*I. The 281-bp fragment is partially cleaved into 191-bp and 90-bp fragments only in lanes 4 and 5 (representing, respectively, patient 7 and her father), indicating a heterozygous state for the A177D mutation. Lane M, Molecular-weight marker PhiX174 digested by *Hae*III. Lane C, Normal control sample.

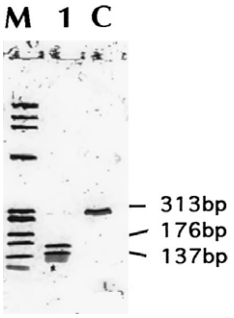

Figure 5 Detection of the E30X mutation in a PCR fragment amplified from genomic DNA including exon 1 after digestion by *Mae*I. The 313-bp fragment is completely cleaved into 176-bp and 137-bp fragments, indicating a homozygous state for the E30X mutation. Lane M, Molecular-weight marker PhiX174 digested by *Hae*III. Lane C, Normal control sample.

four point mutations, the V325A mutation does not seem to be responsible for FBPase deficiency, because the chimeric clone associated with this mutation showed an almost normal FBPase activity. Alternatively, it could represent a rare polymorphism, since this mutation was not found in any of 40 normal healthy donors. The 960/961insG mutation (in 46% of the 22 alleles) was the most common mutation (occurring in 7 of 11 families) among Japanese patients with homozygous or heterozygous FBPase deficiency. We have confirmed recently that such mutation results in the appearance of clinical manifestations of the disease that are due to overexpression of FBPase enzyme protein without enzyme activity (Kikawa et al. 1995). The high frequency of the 960/961insG mutation in the present study may be explained by the homogeneity of Japanese patients. A more plausible explanation is the peculiar DNA sequence around the mutation site, which results in the induction of insertional mutagenesis. Cooper and Krawczak (1991) proposed two separate mechanisms to explain insertional mutagenesis in human genes that causes genetic diseases by inserting DNA fragments <10 bp. The first mechanism involves slipped mispairing mediated by direct repeats or runs of identical bases, whereas the second mechanism involves the templated misincorporation of bases by secondary-structure intermediates whose formation is facilitated by palindromic (inverted repeat) or quasi-palindromic sequences. The insertion of one G residue was found in the wild-type sequence of $TTG_{957}GGG_{960}TCCCCCGACGACG$. The sequence of $G_{957}GGG_{960}$ is a run of identical bases, and that of $G_{957}GGG_{960}TCCC\,CC$ forms a quasi-palindrome. Thus, structures consistent with both mechanisms proposed by Cooper and Krawczak (1991) exist in the sequence containing the insertion of one G residue. Until now, genetic mutations responsible for FBPase deficiency have been examined by our group and El-Maghrabi and coworkers and have been described only in Japanese patients. However, the 960/961insG mutation may be found in other ethnic groups, because the tendency for insertional mutation sequence is supposedly common in other ethnic groups. Confirmation of our speculation must await the results of molecular analysis of patients with FBPase deficiency who are from other ethnic groups.

The other two missense mutations, G164S and A177D, resulted in the formation of FBPase protein with negligible enzyme activity. The glycine at position 164 and the alanine at position 177 are both well conserved in FBPase enzymes of humans, pigs, and rats (El-Maghrabi et al. 1988, 1993). Alanine is conserved even in yeast (Roger et al. 1988), thus suggesting a critical role for these two amino acids in the structure of FBPase protein. The G164S mutation is expected to result in certain conformational changes, since the highly hydrophilic serine substitutes the low-hydrophilic glycine. Similarly, the A177D mutation substitutes the hydrophobic alanine for the hydrophilic negatively charged aspartic acid. The roles of these two amino acids are also suggested by the three-dimensional structure of mammalian FBPase, constructed by crystallography (Hengming et al. 1989). Eight-stranded β-sheets of FBPase enzyme are thought to be responsible for communication between AMP and the active site of the en-

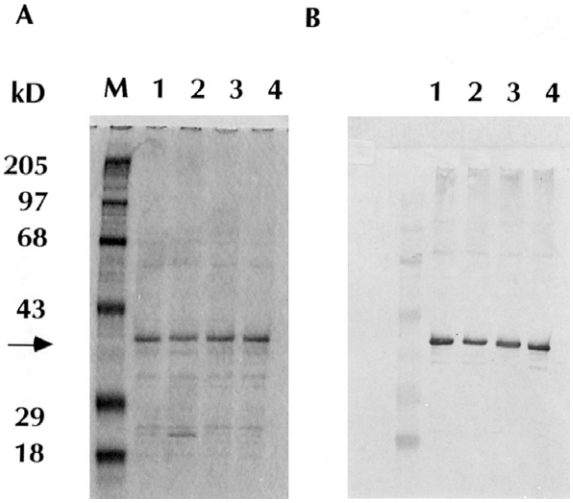

Figure 6 *A,* Analysis of FBPase proteins by SDS-PAGE. *B,* Analysis of FBPase proteins by western blotting. Expression plasmids with normal (from liver) and mutant FBPase cDNA—pGEXFBPN, pGEXFBPG164S, pGEXFBPA177D, and pGEXFBPV325A—were constructed. These FBPase proteins were expressed and purified as described in the text. Approximately 2.5 μg of purified protein was applied in each lane for SDS-PAGE analysis and western blotting analysis. Lane 1, Normal FBPase protein from the expression plasmid pGEXFBPN. Lanes 2–4, Mutant FBPase protein from pGEXFBPG164S, pGEXFBPA177D, and pGEXFBPV325A, respectively. Lane M, Marker standards with sizes (in kD) shown. The arrow indicates the location of purified proteins. Overexpressed protein bands in both SDS-PAGE and western blotting analyses are of a similar size (~37 kD) in all mutant, chimeric, and normal clones.

zyme, and the inhibition of FBPase activity by AMP occurs at the allosteric site (Liang et al. 1993). The glycine at position 164 and alanine at position 177 belong to the β-strands B5 and B6, respectively (Liang et al. 1993). Theoretically, both the G164S and A177D mutations may abolish enzyme activity by affecting communication between AMP and the active site of the enzyme, although this hypothesis remains unconfirmed by crystallographic analysis. Finally, we have not expressed the enzymatic activity of the E30X mutation. This nonsense mutation is expected to result in premature termination resulting in approximately one-twelfth the length of normal FBPase protein, lacking almost all of the crucial amino acid side chains for the active and allosteric sites (Hengming et al. 1989; Liang et al. 1993).

The 960/961insG, G164S, A177D, and E30X mutations were responsible for FBPase deficiency in 8 of the 11 families studied in this report. No mutation of the FBPase protein-coding region was found in the remaining three patients (patients 9–11). El-Maghrabi et al. (1995) also found no genetic mutation responsible for FBPase deficiency. Our results agree, to some extent, with their conclusion, at least with regard to the presence of FBPase-deficient patients without any demonstrable mutation responsible for FBPase deficiency. The same investigators also speculated that the molecular basis of FBPase deficiency in their patients could be due to unidentified mutations in the promoter that decrease the expression or mutations in another gene that indirectly lead to FBPase deficiency. A similar molecular change may be the underlying mechanism in three of our patients. Thus, the results of our molecular analysis suggest heterogeneity of FBPase deficiency. The latter was also suggested on the basis of the characterization of the enzymatic activity in liver samples (Hommes et al. 1985; Gitzelmann et al. 1995) and also was supported by the discrepancy, in enzymatic activity, between the liver and leukocytes (Shin 1993; Besley et al. 1994). A complete characterization of FBPase deficiency, at both the molecular and the enzymatic levels, would be necessary to allow examination of the correlation between genotype and phenotype in FBPase deficiency.

FBPase enzyme is mainly expressed in the liver and kidney, and the most reliable diagnosis of FBPase deficiency is by demonstration of a lack of FBPase activity in a liver biopsy specimen. Other samples, such as leukocytes, may have FBPase activity too low to be sensitive for spectrophotometric measurement (Schrÿer and Hommes 1975), making this method inappropriate for detection of phenotypes with deficient enzymatic activity in samples other than liver specimens. Screening for two relatively common mutations, 960/961insG and G164S, in genomic DNA samples may be useful for identification of FBPase-deficient patients with these two mutations. In this regard, among the 13 Japanese patients, glycerol had been prophylactically used for brain edema associated with hypoglycemia, at least in patients 1 and 9. In these two patients, the clinical condition did not improve even after successful correction of hypoglycemia. At first, the latter patient was diagnosed as having died of sudden unexpected infant death but later, on the basis of an autopsied liver sample, was properly diagnosed to have had deficient FBPase activity. Thus, it is highly likely that the use of glycerol aggravated the clinical condition of these two patients. Carrier detection is, therefore, important as a means of providing appropriate postnatal care for FBPase-deficient babies. The use of molecular tests in the present study allowed carrier detection in eight families of 10 patients—although, unfortunately, not in the family of patient 9—by identifying mutations in the FBPase genomic gene.

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