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A NEW VARIANT OF MUSCLE PHOSPHOFRUCTOKINASE DEFICIENCY IN A JAPANESE CASE WITH ABNORMAL RNA SPLICING

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Summary: A genetic defect was investigated in a newly diagnosed Japanese case with muscle type phosphofructokinase (PFK-M) deficiency. Polymerase chain reaction (PCR) amplification of patient cDNA revealed an in-frame truncation of 165 bases. This was compatible to the complete deletion of exon 19. The rest of the sequence was identical to that of the normal PFK-M cDNA. Sequencing of PCR amplified genomic DNA of the patient revealed a point mutation from G to A at the 5'donor site of intron 19. This mutation resulted in the skipping of exon 19 in the patient mRNA. Homozygosity of this patient was confirmed by allele specific amplification of the genomic DNA. Donor mutations in intron 15 and intron 5 associated with different splicing errors were previously reported to cause this disease. Thus, the human PFK-M gene mutations are heterogeneous, however, the donor mutations and splicing errors would represent one of the frequent causes of this disease.

Phosphofructokinase (PFK, ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is a tetrameric enzyme that plays a key role in the glycolytic pathway (1). There are at least three isozymes of human PFK, known as the muscle (PFK-M), platelet (PFK-P), and liver (PFK-L) types (2, 3, 4, 5). The gene loci for the PFK-M, -P and -L have been assigned to chromosomes 1, 10, and 21, respectively (6). Hereditary PFK-M deficiency (7) is classified as type VII glycogenosis. The disease is characterized by several typical symptoms, such as exercise intolerance, mild hemolysis and hyperuricemia after muscular exercise (8, 9). We have first reported the full-length cDNA and the genomic sequence of PFK-M (3, 10). The first report

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ABBREVIATIONS USED: PFK, phosphofructokinase; -M, muscle type; -L, liver type; -P, platelet type; PCR, polymerase chain reaction.

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on PFK-M mutation has also been made by us on a Japanese patient (11). Very recently, an additional report was made on an Ashkenazi Jewish family (12). In order to investigate whether this disease has molecular heterogeneity and/or a common mechanism, we identified a novel genetic defect in a new case of a Japanese patient with PFK-M deficiency.

MATERIALS AND METHODS

Profile of the patient: The detailed case presentation of this patient has been reported elsewhere (13). In brief, the patient was a 29-year-old female. Her parents had a consanguineous marriage. She suffered from hemolysis and hyperbilirubinemia. She also experienced mild exercise intolerance. Biochemical studies on muscle enzymes showed a complete loss of PFK activity. Red cell PFK activity was also decreased.

Cellular RNA and genomic DNA isolation: Total cellular RNA was extracted from patient peripheral blood red cells by the acid-guanidinium thiocyanate-phenol-chloroform method (14). Genomic DNA was isolated from patient leukocyte using anion-exchange resin (QIAGEN-tube 20, QIAGEN Inc., Chatsworth, CA) as recommended by the manufacturer. Control genomic DNA was also prepared from three normal subjects and from the previously described Japanese patient with PFK-M deficiency (11).

Analysis of PFK-M transcripts: Single-stranded cDNA was synthesized from 5 μg total RNA of the patient blood using random hexanucleotides (TAKARA Shuzo Co., LTD., Kyoto, Japan) and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL; Life Technologies, Inc., Gaithersburg, MD) as reported (15). Four pairs of PCR primers were designed (Table 1) so that the four overlapping fragments would cover the entire coding region of PFK-M mRNA. PCR amplification of the patient mRNA was carried out by 40 cycles of denaturation, annealing and primer extension at 94, 60 and 72°C, respectively, each for 60 sec using AmpliTaq™ (Roche Molecular Systems, Inc., Branchburg, NJ). Reaction conditions were essentially the same as those recommended by the manufacturer. PCR products were sequenced directly using PCR primers on both senses. Sequencing reaction was performed using Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA) according to the supplier's protocol.

Analysis of the patient genomic DNA: The genomic regions from exon 18 to exon 19 and from exon 19 to exon 20 of the patient were amplified with each pair of primers as shown in Table 1. Each PCR product was subcloned into pUC119. Four independent subclones were sequenced from both ends using forward and reverse universal M13 primers. Direct sequencing on the PCR product was also performed as described above. PCR amplification of specific allele (16) was performed on the genomic DNA of the patient and of four control subjects in a stringent condition. The modified PCR cocktail contained 1 unit of AmpliTaq™, 50 nM of allele specific primers (Table 1), 1 mM of magnesium chloride, 100 μM of dNTPs, 50 mM potassium chloride, 10 mM Tris-HCl (pH 8.3) and 0.001% (w/v) gelatin in a volume of 50 μl. Thirty-five cycles of denaturation, primer annealing and the extension reaction were performed each for 30 sec at 95, 67 and 72°C, respectively. The products were then analyzed by agarose gel electrophoresis (2.0%) and visualized by ethidium bromide.

RESULTS AND DISCUSSION

The tissue-specific expression of human PFK-M mRNA was demonstrated in muscle, kidney and reticulocyte (17). Although PCR amplification does not allow a quantitative estimation of PFK-M transcript (18), our preliminary observation suggested that the amount of

Table 1. List of oligonucleotide primers

	Sequence		Location
Primers for cDNA amplifications	5'- GACAATCTGCAAGAAAGCAG -3'	sense	nt -46 -> -27; exon 1
	5'- AGGACAGAGAGGTGACAAGGGCCAGGTAT -3'	antisense	nt 667 -> 639; exon 9
	5'- TGTGGCACTGATATGACCATTGGCACTGAC -3'	sense	nt 508 -> 537; exon 7
	5'- CAAGTTTAGAGCCACCTTGGCCAGTCCAGC -3'	antisense	nt 1402 -> 1373; exon 16
	5'- TCGCACACAGTGGCTGTGATGAAC -3'	sense	nt 1198 -> 1221; exon 15
	5'- AGTGGTATAGTTCTCATTGCA -3'	antisense	nt 1911 -> 1891; exon 21
	5'- TGGAACTGATGGAGGGCAGGAAGCAG -3'	sense	nt 1517 -> 1542; exon 18
	5'- ATGATCAGGTAATCTATTCCCCTCACTCCA -3'	antisense	nt 2380 -> 2351; exon 24
Primers for genomic DNA amplifications	5'- TGGAACTGATGGAGGGCAGGAAGCAG -3'	sense	nt 1517 -> 1542; exon 18
	5'- CTGCAGGTCTCGAATGGTGAAGGGCT -3'	antisense	nt 1818 -> 1793; exon 19
	5'- CTGTGACCGCATCAAGCAGTCAGCA -3'	sense	nt 1656 -> 1680; exon 19
	5'- CTTAACACCAAGCCCCTTTTCACAGTT -3'	antisense	nt 1880 -> 1854; exon 20
Primers for allele-specific amplifications	5'- CTGTGACCGCATCAAGCAGTC -3'	sense	nt 1656 -> 1676; exon 19
	5'- CTCTGGGTGGCCAGCTAC -3'	antisense	specific for wild allele; intron 19
	5'- CTCTGGGTGGCCAGCTAT -3'	antisense	specific for mutant allele; intron 19

Nucleotide numbers and exon/intron numbers correspond to those of PFK-M cDNA and gene as reported[3,10].

PFK-M mRNA of the patient was comparable to that of control materials. We detected a 165-base in-frame deletion from positions 1654 to 1818 (3) in the patient PFK-M transcripts (Fig. 1-a.). The deleted sequence was compatible to exon 19 of the PFK-M gene (10). The rest of the sequence of the patient cDNA was normal. From sequence analysis of the patient genomic DNA, a G to A transition was found at the 5' donor site of intron 19. The rest of the intronic sequences including exon-intron boundaries and a branch point were completely identical to the normal genomic sequence (data not shown). Chromatogram of direct sequencing of the PCR

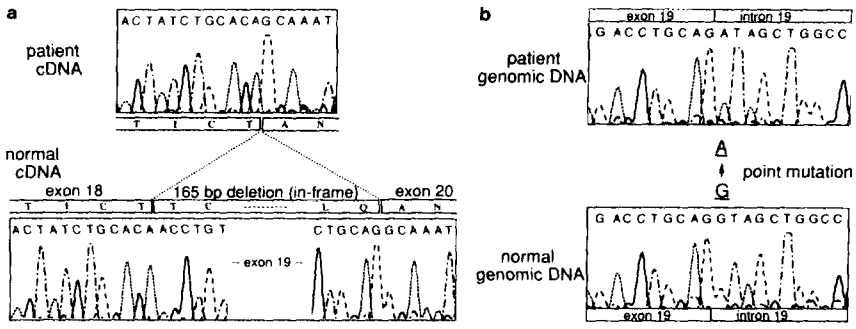

Fig. 1. Identification of PFK-M mutation of the patient.
(a) Direct sequencing of reverse transcription-PCR products showed a 165-base in-frame deletion and skipping of exon 19 in the patient mRNA.
(b) Direct sequencing of the boundary of exon 19-intron 19 revealed a G to A substitution of the 5' donor site in the patient gene.

product of the patient genomic DNA using primer P1700 and primer P1900 exhibited the single signal of A instead of G at the same position (Fig. 1-b). In the consensus sequence of the 5' splice donor site (C/A)AG:GT(A/G)AGT, GT dinucleotide is highly conserved (19). Analyses by site directed mutagenesis and the natural experiments found in some diseases represent the mechanism of exon skipping by the donor inactivation (20, 21, 22). A failure of the recognition of the 5' splice site would delete the preceding exon completely. In some conditions, cryptic splicing phenomenon was also demonstrated (23, 24). The activation of an altered splice site would result in retained intron or deleted exon. Indeed, in the first Japanese patient, a G to T transversion at the 5' donor site of intron 15 activated the cryptic splice site and led to a 75-base in-frame deletion of PFK-M mRNA (11). Although exon 15 involves 150 base pairs in-frame, no other product such as a skipped one was detected by the reverse transcription-PCR (data not shown). On the other hand, in this new case, splice site inactivation by the G to A transition of 5' donor site resulted in aberrant splicing and complete skipping of the exon 19. Exon 19 includes one candidate cryptic site for the consensus sequence, (CGG:GTGTTT), which would cause in-frame cryptic splicing. However, no minor transcript other than the one presented here was detected during PCR amplification of the patient mRNA. Although the precise mechanism for the pre-mRNA splicing has been reported by many investigators (25), the definite mechanism which leads to exon skipping instead of cryptic splicing is not fully understood.

PCR amplification of specific allele was also performed on the genomic DNAs of this patient and four control subjects. The mutant type primer had a T at the 3' end to match the mutated A allele. Under the optimal condition as described in Materials and Methods, successful amplification was obtained by patient DNA only with the mutant type primer, whereas control DNAs were amplified only with the wild type primer (Fig. 2). Since this patient is an offspring of a consanguineous mating, our experimental results strongly support the idea of homozygosity

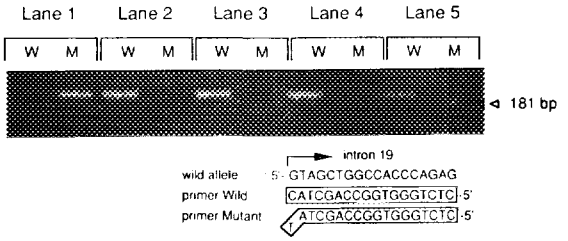

Fig. 2. Allele-specific amplification of genomic DNA.
Genomic DNAs were amplified with wild and mutant primer pairs and were electrophoresed through 2% agarose gel. Symbols W and M represent the result of amplification with the wild type and the mutant type primers, respectively. Lane 1. present patient; Lane 2. previous Japanese patient (10); Lanes 3-5. normal control subjects.

of this patient. A very small possibility that the patient is a compound heterozygote carrying a large deletion in one allele may still exist. Further analyses of the other members of this family would be required to solve this problem. However, no other specimen was available during the study.

Since the deletion in the PFK-M mRNA of this patient does not alter the reading frame of the RNA, the existence of a truncated PFK-M polypeptide lacking 55 amino acids can be expected. The enzymatic assay showed essentially no PFK-M activity in the patient muscle (13), suggesting that if truncated protein was made, it is enzymatically inactive. Similarity of the human PFK-M amino acid sequence to that of the rabbit PFK-M (3, 26) suggests that the truncated region would involve the putative ATP inhibition, fructose-6-phosphate binding and fructose-1,6-bisphosphates binding sites corresponding to those of the rabbit PFK-M (27). Moreover, the deleted region includes stretches of α-helix and β-sheet structures as shown by the method of Chou and Fasman (data not shown) (28). The loss of several active centers and the destruction of the helical structures with a configurational change may contribute largely to the loss of the enzyme activity.

Up to now, including this case, three genetic defects have been reported (11, 12). In the first Japanese patient, the point mutation at the consensus GT motif caused the 75-base in-frame deletion of PFK-M mRNA by the cryptic splicing (11). The second report was made on an Ashkenazi Jewish family (12). A transition from G to A at the 5' donor site of intron 5 led to a skipping of exon 5 and a 78-base in-frame deletion. The present case had another point mutation at a different donor site. These three distinct mutations may indicate the molecular heterogeneity of this disease, however, all of these three variants are characterized by a point mutation at 5' donor site, abnormal RNA splicing and in-frame deletion of PFK-M mRNA. Thus, one of the frequent causes of PFK-M gene mutations could be splicing errors.

Very recently, two other forms of mutations, a missense and a frameshift ones have been identified in the Ashkenazi Jewish patients (29). Further investigations into other unrelated cases would warrant the full resolution of the mutation repertoire of this disease.

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