We identified four distinct point mutations in homozygous pyruvate kinase (PK) variants in Japanese and Chinese patients with chronic nonspherocytic hemolytic anemia. All gene abnormalities were missense mutations that caused single amino acid substitutions. 1261A (G421K) and 1436A (R436H), which were identified in PK Sendai and PK Shinshu, had been found in unrelated Japanese and Amish PK variants, respectively. The clinical severity and extremely low residual erythrocyte PK activity of PK Shinshu as well as of the Amish PK might be caused partly by aberrant splicing, because the 1436A mutation changes a nucleotide at the last nucleotide in the exon 10. Recently, we diagnosed a 42-year-old Japanese woman with chronic nonspherocytic hemolytic anemia as having a homozygous PK deficiency. DNA sequencing of the variant PK gene showed a homozygous missense mutation at 1403GCT → GTT, resulting in a single amino acid substitution from 468Ala → Val. The gene mutation is likely to impair the allostericity of this enzyme, speculated from the tertiary structure. A homozygous missense mutation in PK Hong Kong, a boy of a non-Han southern Chinese minority group, was identified in exon 7 of the human L-PK gene, 941ATT → ACT, resulting in a single amino acid substitution from 314Leu → Thr. The R-PK activity is expected to be severely affected, because the mutated amino acid residue is located between the 313 Lys and the 315 Glu, which are very important for acid-base catalysis and magnesium binding, respectively. Both the R- and MZ-type PK were shown by polyacrylamide gel electrophoresis of the PK Hong Kong erythrocyte lysate, and this is the first report of a homozygous individual whose erythrocytes contain the immature (MZ)-type isozyme.

ERYTHROENZYMOPATHIES are a category of hereditary metabolic disorders that affect the energy production machinery of erythrocytes. A few metabolic pathways are involved, such as glycolysis, the hexose monophosphate shunt, nucleotide metabolism, or glutathione synthesis. Two types of hemolytic anemia have been noted in erythroenzymopathies; one is the chronic nonspherocytic type, and the other is drug-induced. A pyruvate kinase (PK) deficiency is the most common type of hereditary nonspherocytic hemolytic anemia caused by a glycolytic enzyme defect, and over 300 cases have been reported. In our experience, only 10 Japanese PK variants among 64 affected families were found to be homozygous. Molecular analysis of the homozygous PK variants is considered to be useful not only for studying the structure-function relationship of the enzyme, but also for understanding the genotype-phenotype relationship of PK deficiency. Because the International Committee for Standardization in Haematology established standard methods for characterizing variant PK in 1979, enzymatic characteristics determined by various investigators could be compared. However, enzymatic characterization of variant PK is problematic. Variant PK often loses its activity during purification, and the procedure requires more blood than do molecular biologic methods. To avoid contamination with normal enzyme, blood samples must be collected several months after the last transfusion. Identical nucleotide changes have been shown in some variants that has been considered to be distinct by enzymatic properties. Recent progress in understanding the tertiary structure of PK and genomic structure of human PK gene enabled estimations of the likely effects of structural changes on enzymatic properties to be determined. Polymerase chain reaction (PCR)-related genetic diagnosis information about the relationship between gene mutation and clinical severity of homozygous PK deficiency can provide useful tools not only to establish prognostic criteria for PK-deficient hemolytic anemia but also to discover a PK deficiency among patients who require frequent transfusion or among infants. In this report, we describe the molecular analysis of three Japanese and one Chinese homozygous PK variants associated with hereditary hemolytic anemia and discuss the relationship between the mutations and the clinical manifestations of a homozygous PK deficiency.

MATERIALS AND METHODS

Clinical features of Japanese and Chinese PK-deficient subjects. Case reports, laboratory data, and the enzymatic properties of PK Sendai, PK Shinshu, and a Chinese PK variant have been reported elsewhere. Because of the consanguineous marriages of the parents, these PK variants were most probably homozygous for mutant PK alleles.

Recently, we measured the red blood cell (RBC) enzyme activities of a 42-year-old Japanese woman (T.T.) who had been diagnosed at 16 years of age as having congenital chronic hemolytic anemia from unknown causes. The proband had suffered from severe jaundice and convulsions at the neonatal period. At 5 years of age, she experienced meningitis, and, since then, epilepsy and mental retardation have developed. She received RBC transfusion once a
month, and the blood sample was taken just before transfusion. The hematologic data at 42 years of age were a RBC count of 1.31 × 10^12/L, hemoglobin (Hb) of 114 g/L, hematocrit of 36.2%, and reticulocytes of 11%. Serum indirect bilirubin was 1.1 mg/dL, and lactate dehydrogenase was 1,398 IU. The parents were first cousins, and the father is dead. Erythrocyte enzyme activities and glycolytic intermediates were measured by protocols standardized by the International Committee for Standardization in Haematology or as described by Minakami et al. Polyacrylamide gel electrophoresis and staining for PK activity were performed as described.

Materials. Blood samples from the probands were obtained under informed consent. Reticulocyte RNA and high molecular weight genomic DNA were purified by the standard protocol. Restriction endonucleases and modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, MA). Taq DNA polymerase (AmpliTaq) was obtained from Perkin Elmer-Cetus (Norwalk, CT). NuSieve GTG agarose and SeaKem HGT agarose were obtained from FMC Bioproducts (Rockland, ME). DNA was automatically sequenced (Model 373A; Applied Biosystems, Foster City, CA). The computer software, DNASIS (Hitachi Software Engineering, Yokohama, Japan), was used to analyze the hypophosphibody properties and secondary structure of variant PK.

Amplification and sequencing of the human R-type PK cDNA and L-PK gene. The R-type PK cDNAs of PK Shinshu and PK Sendai were synthesized by avian myeloblastosis virus reverse transcriptase and were amplified by PCR as described. The human L-PK gene was amplified by PCR as described. The amplified cDNA and genomic DNA were subcloned into pBluescript (Stratagene, La Jolla, CA) by restriction sites within the DNA or primers. Exons and adjacent intron sequences of exons 1 through 12, except exon 2 (L), were sequenced by dideoxy-chain termination with fluorescent primers. To confirm the nucleotide changes detected by sequencing, we used the PCR primers and restriction endonucleases as listed in Table 1 and Table 2.

Determination of the 1705 A/C polymorphism among the PK variants. To test the polymorphic nucleotide at no. 1705, the exon-12 region was amplified by PCR with primers PK-6A and -6B under the conditions previously described and was digested with BspHI. A new homozygous PK variant associated with chronic hemolytic anemia. The RBC PK activity of the proband (T.T.) was 5.6 IU/g Hb, about 40% of the mean value of normal controls (normal range, 12.0 to 15.6), and the extent of the decrease of PK activity was almost equal to that of the control at a lower substrate concentration, meaning that this variant enzyme had normal substrate affinity. The PK activity of the mother, 8.2 IU/g Hb, was about half that of normal controls and was consistent with the fact that she was heterozygous with an abnormal PK gene. Glycolytic intermediates such as 2,3-diphosphoglycerate, 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate (PEP) were accumulated to twofold to fourfold of the normal concentration in the proband’s erythrocytes, which is compatible with a PK deficiency (data not shown). The DNA of this variant was amplified, and the human PK L-gene was sequenced. A single nucleotide substitution was identified in exon 10, 1403GCT → GTT, resulting in a single amino acid substitution from 468Ala → Val. PCR-restriction fragment length polymorphism analysis showed that the proband is homozygous with this mutation and that the mother and a niece were found to be heterozygotes. Because this missense mutation has not been reported, we designated this variant as PK Hadano.

The persistent expression of immature type PK isozyme in erythrocytes caused by a homozygous missense mutation of PK gene. In 1991, Wei et al. reported two Chinese PK variants with severe congenital hemolytic anemia. We analyzed the PK gene of a non-Han Chinese boy, described as patient no. 1 in the report, and found a missense mutation in exon 7 of L-PK gene at no. 941ACT → TCT, causing a single amino acid substitution, 314Le → Thr (I341T). The PCR-restriction fragment length polymorphism analysis showed that the proband was homozygous and that the parents and a sister were heterozygous with this mutation (Fig 1). The R-PK activity was considered to be severely affected, because the mutated amino acid residue is located between the 313Lys and the 315Glu, which are very important for acid-base catalysis and magnesium binding, respectively. Both R- and M2-type PK were shown by polyacrylamide gel electrophoresis of the proband’s erythrocyte lysate (Fig 2), suggesting that this is the first true homozygous PK variant in whose erythrocytes the immature (M2)-type isozyme persists.

Demonstration of point mutations in PK Sendai and PK Shinshu. In PK Sendai and PK Shinshu, the missense mutations, 1261A (Glu → Lys; Q421K) and 1436A (Asp → His; R436H) have been identified. These mutations are iden-

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**Table 1. PCR Primers Used in This Study**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Pair</th>
<th>DNA Length</th>
<th>Restriction Enzyme</th>
<th>Normal Allele</th>
<th>Mutant Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>1403T</td>
<td>PK-1A/PK-1B</td>
<td>88 bp</td>
<td>Fnu4Hl</td>
<td>49, 21, 18 bp</td>
<td>49, 39 bp</td>
</tr>
<tr>
<td>941C</td>
<td>PK-2A/PK-2B</td>
<td>351 bp</td>
<td>Dde I</td>
<td>259, 92 bp</td>
<td>143, 116, 92 bp</td>
</tr>
<tr>
<td>1261A</td>
<td>PK-3A/PK-3B</td>
<td>285 bp</td>
<td>Fnu4Hl</td>
<td>130, 122, 33 bp</td>
<td>262, 33 bp</td>
</tr>
<tr>
<td>1436A</td>
<td>PK-4A/PK-4B</td>
<td>201 bp</td>
<td>Hpa II</td>
<td>132, 39, 27, 3 bp</td>
<td>171, 27, 3 bp</td>
</tr>
</tbody>
</table>
Fig 1. A pedigree of the PK Hong Kong family and PCR-restriction fragment length polymorphism of the 941T→C mutation. The PCR products flanking the exon 7 of human PK gene were tested to determine whether a new Dde I site is created by the mutation. The samples were applied to a 3% NuSieve-1%HGT agarose gel and were stained with ethidium bromide. M, pBR322 digested with Msp I; 1, the father; 2, the mother; 3, the proband; 4, the sister; 5, the sister; and C, normal control.

tical with those in the unrelated variants, PK Fukushima, PK Maebashi, or the Amish PK deficiency, respectively. In addition to a change of the encoded amino acid, the 1436A mutation altered the last nucleotide of exon 10, resulting in an alteration of the 5' splice site consensus sequence, C/AAAGta/gag to C/AAAGta/gag. To test whether splicing of the PK gene transcript had been impaired, we performed reverse transcription-PCR of the R-type PK mRNA in reticulocytes. Using primers PK-SA and -5B (Table 1) that flank the exon-7 through exon-11 region, only normal 745-bp fragments were shown by ethidium bromide staining of agarose gel, meaning either that the 1436A mutation did not affect the splicing efficiency or that the aberrant transcripts could not be amplified in this system (data not shown).

Identical PK gene mutations with or without same nucleotide polymorphism. To investigate the origin of the identical PK gene mutations in an unrelated Japanese family or distinct ethnic group, we checked the 1705 A/C polymorphism of the PK variants. As shown in Table 3, all PK variants with the 1261A were homozygous with cytosine at no. 1705. In contrast, PK Shinshu (1705 A/A) and the Amish PK variant (1705 C/C) had different polymorphisms.

DISCUSSION

Since the first report of PK deficiency, over 300 PK-deficient hemolytic anemia cases have been described. The degree of anemia varied from a mild compensated anemia to a severe anemia that can be life-threatening during the neonatal or infantile periods. Various gene mutations associated with PK-deficient hemolytic anemia have been reported. Progress in understanding the structure-function relationship of this enzyme has allowed this inherited disorder to be diagnosed at the gene level. To date, 21 missense, 1 nonsense, and 2 splicing mutations and 2 insertions and 3 deletions have been documented. Accumulated knowledge of PK gene mutations have provided useful information for both the diagnosis and prognosis of PK-deficient hemolytic anemia.

The missense mutation found in PK Shinshu was identical to that of the Amish PK variant. Although there were some discrepancies in the enzymatic characteristics between PK Shinshu and the Amish variant, it should be emphasized that both variant enzymes showed a lower response to the allosteric activator, fructose-1,6-diphosphate, and normal (increased in the Amish variant) affinity for the substrate, phosphoenolpyruvate. This was accounted for by the occurrence of the amino acid substitution in the C domain of PK, as previously discussed. Clinical records describing PK-deficient patients with a 1436A mutation show that both variants were severely affected during the neonatal or infantile period. Although the aberrant transcripts could not be detected in reticulocytes of PK Shinshu, splicing might be disturbed by the 1436A mutation because of the following. First, statistical analysis of the 5' splice sequences for 97 donor sites of human genes and 542 donor sites of primate genes has shown that 100% (human) or 78% (primates) of the nucleotides at the -1 location of the 5' splice site (last nucleotide in the preceding exon) were guanine. Second, there are several reports that the G-to-A transition at this position causes exon-skipping or cryptic site utilization, and in most cases aberrant mRNAs were produced.

Fig 2. Polyacrylamide gel electrophoresis for detecting PK activity. C, normal control; P, PK Hong Kong. M2 and R show the position of M2- and R-type PK, respectively. In the proband erythrocyte lysate, both R- and M2-type PK were identified.
Table 3. Structure-Function Relationship of the PK Variants With Homozygous Missense Mutations

<table>
<thead>
<tr>
<th>PK Variants</th>
<th>Base Change</th>
<th>Amino Acid Substitution</th>
<th>Affected Domain</th>
<th>Substrate Affinity</th>
<th>Allosteric Activation</th>
<th>1705 A/C Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hong Kong</td>
<td>941ATT → ACT</td>
<td>314Ile → Thr</td>
<td>Aβ5</td>
<td>ND</td>
<td>ND</td>
<td>C/C</td>
</tr>
<tr>
<td>Tokyo</td>
<td>1151ACG → ATG</td>
<td>384Thr → Met</td>
<td>Aα7</td>
<td>ND</td>
<td>ND</td>
<td>A/A</td>
</tr>
<tr>
<td>Nagasaki</td>
<td>1261CAG → AAG</td>
<td>421Gln → Lys</td>
<td>Aα8</td>
<td>ND</td>
<td>ND</td>
<td>C/C</td>
</tr>
<tr>
<td>Fukushima</td>
<td>1277CGG → CAG</td>
<td>426Arg → Lys</td>
<td>Cβ1</td>
<td>ND</td>
<td>ND</td>
<td>C/C</td>
</tr>
<tr>
<td>Maebashi</td>
<td>1403GCT → GTT</td>
<td>468Ala → Val</td>
<td>Cr3</td>
<td>ND</td>
<td>ND</td>
<td>C/C</td>
</tr>
<tr>
<td>Sendai</td>
<td>1436CGC → CAC</td>
<td>479Arg → His</td>
<td>Cr3</td>
<td>ND</td>
<td>ND</td>
<td>C/C</td>
</tr>
</tbody>
</table>

During genetic diagnosis of PK deficiency, we identified 1151T and 1436A mutations in unrelated families of different ethnic origins. Although PK Shinshu and Amish PK had an identical missense mutation at 1436A, they had a distinct DNA polymorphism at no. 1705. These results indicated that the mutation had occurred independently at the same nucleotide. On the other hand, 1261A might have originated from a common ancestor, because the families of PK Fukushina, PK Maebashi, and PK Sendai were derived from the northern part of the main island of Japan, and they shared the same 1705 C/C. Although the 1529A (R510Q) mutation is prevalent in the PK variants of the United States and Europe, we were unable to find this mutation in Japanese PK variants. Although the 1261A and 1151T seemed to be dominant in the Japanese homozygous PK variants, preliminary results provided by the restriction endonuclease digestion of PCR products showed that those missense mutations found in homozygotes were not necessarily common in compound heterozygotes. Therefore, we considered that an efficient screening system for detecting the PK gene mutation is required to clarify the genetic basis of PK deficiency.

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Hereditary hemolytic anemia caused by diverse point mutations of pyruvate kinase gene found in Japan and Hong Kong

H Kanno, DC Wei, LC Chan, H Mizoguchi, M Ando, T Nakahata, K Narisawa, H Fujii and S Miwa