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Application of polymerase chain reaction-single strand conformation polymorphism analysis to the diagnosis and screening of adenine phosphoribosyltransferase deficiency

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Summary. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis is a rapid and sensitive method used to identify point mutations in a given sequence of genomic DNA. We applied this method to the diagnosis of adenine phosphoribosyltransferase (APRT) deficiency, which is an autosomal recessive hereditary disease leading to 2,8-dihydroxyadenine urolithiasis. Genomic APRT genes were amplified and labeled simultaneously with [α^{-32}P]dCTP (cytidine triphosphate) by PCR. When run in a 6% polyacrylamide gel containing 10% glycerol, two types of mutant genes – APRT*QO and APRT*J – gave bands clearly distinct from those of the equivalent normal APRT genes. Using this method we diagnosed both homozygotes and heterozygotes for defective APRT genes. On screening 80 Japanese individuals for polymorphism or mutations by PCR-SSCP we did not find any alterations leading to a false positive diagnosis. These findings suggest that PCR-SSCP, in addition to being rapid and sensitive, is a useful diagnostic method which is highly specific in detecting mutant APRT genes in the Japanese population.

Key words: Adenine phosphoribosyltransferase deficiency – Polymerase chain reaction – Single strand conformation polymorphism

Adenine phosphoribosyltransferase (APRT) deficiency is one of the most common autosomal recessive hereditary diseases in the Japanese population [8]. Homozygous deficiency of APRT causes 2,8-dihydroxyadenine (DHA) urolithiasis and renal failure [15]. APRT deficiency is classified by APRT activity in hemolysate as either complete deficiency (type 1) or partial deficiency (type 2) [16]. Each type 1 patient possesses two null-type alleles, termed APRT*QO, rather than two normal APRT*1 alleles [16]. Type 2 patients, who are exclusively Japanese, possess at least one mutant allele, APRT*J. This generates the production of a mutant enzyme which has a reduced affinity for the co-substrate 5-phosphoribosyl-1-pyrophosphate [1, 7]. Type 2 patients are either homozygous for APRT*J (APRT*J/APRT*J) or compound heterozygotes with APRT*J and APRT*QO (APRT*J/APRT*QO) [10, 14]. The hemolysate APRT activity of type 2 patients is approximately 25% that of the normal control value. This percentage is also found in heterozygotes APRT*QO/APRT*1 (25%) and APRT*J/APRT*1 (25–100%) [1, 7, 11]. Consequently, type 2 patients who do not form stones of 2,8-DHA cannot be distinguished from heterozygotes by hemolysate APRT activity alone.

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis has recently been documented as a novel method for detecting point mutations in a given sequence of genomic DNA [13]. The technique of PCR-SSCP is a two-step process, involving the amplification of genomic DNA by PCR followed by electrophoresis of the PCR products. Since the conformation of the single-stranded DNA is highly dependent on a particular sequence, the presence of even a single mutation is known to alter the resulting patterns, causing genes with a point mutation to migrate in a polyacrylamide gel at either a slower or faster rate than normal genes. Both APRT*QO and APRT*J are known to be caused by point mutations in Japanese subjects [4, 12]. We report here the diagnosis of APRT deficiency by PCR-SSCP analysis.

Materials and methods

Individuals studied

A Japanese male patient (TAK2) was diagnosed as having APRT deficiency; his genotype had previously been determined to be APRT*J/APRT*QO by DNA sequence analysis [14]. Two other Japanese patients (KT and NS) formed stones composed of 2,8-DHA; no further information regarding their genotype was available but their hemolysate APRT activities were 23% and 25% of

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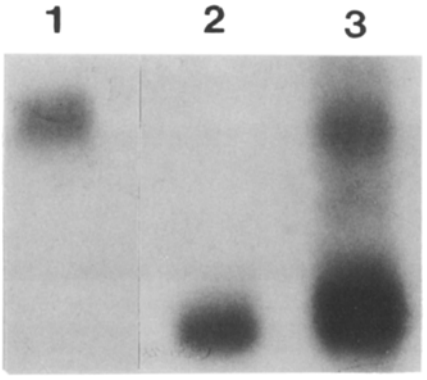

Fig. 1. PCR-SSCP analysis of wild-type exon 5. *Lane 1,* A band of the PCR product amplified with labeled upstream and non-labeled downstream primers; *lane 2,* a band of the PCR product amplified with non-labeled upstream and labeled downstream primers; *lane 3,* bands of the PCR product labeled by incorporating [α^{-32}P]dCTP during amplification

normal values respectively. Both parents of KT and of TAK2 were studied as putative heterozygotes for defective APRT genes.

To screen for polymorphisms or mutant APRT genes in the general population, 80 individuals were randomly selected from outpatients seen at the Division of Urology, Toyooka City Hospital, Hyogo, Japan, where the three patients mentioned above were treated.

DNA extraction

DNA was extracted from blood samples as described by John et al. [5], with slight modifications. A 5-ml blood sample was collected in a Vacutainer tube containing EDTA and stored at −20°C until use. At the time of assay, blood samples were mixed with 5 ml of a solution containing 10 mM TRIS-HCl (pH 7.5), 10 mM KCl and 10 mM $MgCl_2$. One hundred and twenty microliters of Nonidet P-40 was added to the solution to lyse the cells. The mixture was centrifuged at 2000 rpm at room temperature for 10 min. The pellet was resuspended in 800 µl of a solution containing 10 mM TRIS-HCl (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, 0.5 M NaCl, 0.5% $NaDodSO_4$ and 2 mM EDTA, supplemented with 2 µl proteinase K (20 mg/ml). The solution was incubated at 37°C for 4 h and extracted twice with phenol. The aqueous phase was supplemented with two volumes of ice-cold ethanol to precipitate the DNA.

Polymerase chain reaction

Since APRT*J in Japanese individuals has a single point mutation in exon 5 of the APRT gene [4], we used primers flanking exon 5 to detect APRT*J. The sequences of these primers were 5′-CTGCTCTCTGCAGCCCAGGCCA-3′ and 5′-TGGGATCCA-GCTGGAGATGTTG-3′, respectively corresponding to positions 2597–2618 and 2829–2808 of the previously reported wild-type sequence [3]. In order to identify APRT*QO in Japanese subjects, who have been shown to have point mutations in exon 3 [12], we used primers flanking exon 3. The sequences of the primers were 5′-TCACTTACCCTGACAGGCCTAGA-3′ and 5′-GCCTGGGA-AGGCCTTTTAGAACT-3′, respectively, corresponding to positions 1915–1937 and 2148–2126 of the wild-type sequence. Each of the PCR products yielded 0.23 kb fragments. The PCR mixture contained 50 ng each of the primers, 2 nM each of the deoxynucleotides, 200 ng of genomic sample DNA, 0.5 µl of [α^{-32}P]dCTP (cytidine triphosphate; 3000 Ci/mmol, ICN) and 0.2 units of Taq DNA polymerase (Promega, USA) in 20 µl of the buffer supplied with the Taq polymerase. A total of 35 cycles of the reaction were run in a Zymoreacter (Atto, Japan) at 94, 65 and 72°C for periods of 2, 2 and 3 min respectively. In some experiments the primers were end-labeled with [γ^{-32}P]ATP. Five hundred nanograms of each primer was incubated with 50 pmol [γ^{-32}P]ATP (7000 Ci/mmol, ICN) and 5 units of T4 polynucleotide kinase (Takara, Japan) at 37°C for 30 min in 10 µl of 50 mM TRIS-HCl (pH 8.3), 10 mM $MgCl_2$ and 5 mM dithiothreitol. The end-labeled primers were used for PCR without further purification.

SSCP analysis

Aliquots of 5 µl the PCR products were subsequently mixed in 45 µl of solution containing 0.1% $NaDodSO_4$, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol. This solution was then diluted with nine volumes of 95% formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol, heated at 80°C for 5 min and chilled on ice. A 2 µl sample of the diluted solution was applied to a 6% polyacrylamide gel (acrylamide:bis = 49:1, 20 × 40 × 0.035, 0.5 cm per lane) containing 90 mM TRIS-borate (pH 8.3), 2 mM EDTA and 10% glycerol. Electrophoresis was carried out at 10 W for approximately 14 h, which corresponded to the migration of xylene cyanol to 10 cm above the lower margin of the gel. For efficient cooling of the system an aluminum plate was attached to one side of the glass plates and the temperature controlled by use of fans. Gels were dried on filter paper and exposed to X-ray film at −80°C for 12 h using an intensifying screen.

Direct DNA sequencing

PCR products were extracted twice with phenol:chloroform. The amplified DNA fragments were precipitated with isopropyl alcohol. The pellet was diluted in 50 µl of 10 mM TRIS-HCl (pH 8.0) and 1 mM EDTA. The solution was mixed with 30 µl of 20% polyethylene glycol 8000, 2.5 M NaCl and incubated on ice for 1 h to precipitate the DNA fragment again. Template DNA (1 pmol) was denatured by boiling and mixed with an aliquot (5 pmol) of one of the primers used in the PCR. The mixture was incubated at 65°C for 3 min and cooled to 30°C in 30 min to make the template-primer hybrid. The following DNA sequencing reaction was done by the dideoxy method using Sequenase V.2.0 (United States Biochemical Corporation, USA).

Results

PCR-SSCP analysis using labeled primers

The PCR products of wild type exon 5 labeled by [α^{-32}P]dCTP incorporation yielded two bands (Fig. 1). When one of each primer was end-labeled, the PCR products showed only one band. The PCR product amplified with labeled upstream primer and non-labeled downstream primer yielded a band which migrated faster than the band obtained by non-labeled upstream and labeled downstream primers (Fig. 1). These results indicate that each band represents a single-stranded DNA and that the upper band is derived from the upstream strand DNA while the downstream strand DNA formed the lower band. PCR-SSCP analysis of wild-type exon 3 revealed the same results (data not shown). In the following experiments all PCR products were radiolabeled during amplification.

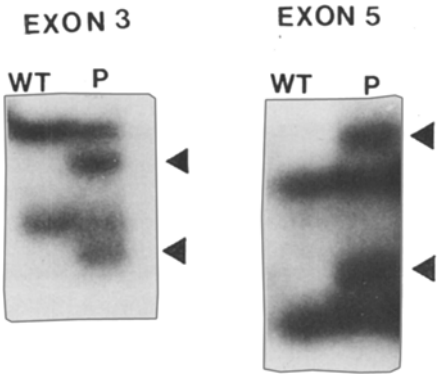

Fig. 2. PCR-SSCP analysis of TAK2. *WT,* Wild-type; *P,* patient (TAK2). TAK2 shows mutant bands following analysis of exons 3 and 5. *Arrowhead* indicates mutant bands

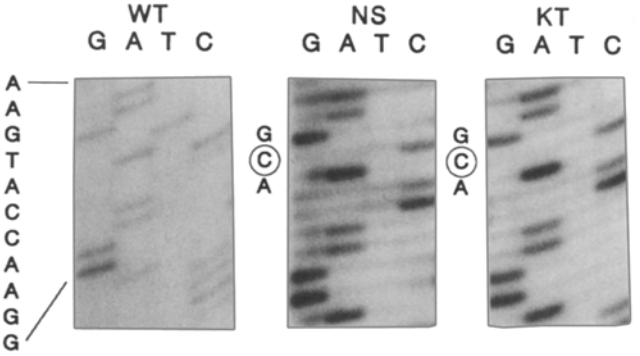

Fig. 4. Autoradiographs of the nucleotide sequence of the mutated region. The sequences of KT and NS are compared with the wild-type sequence. *WT,* Wild-type. Both KT and NS show a single nucleotide mutation: ATG(Met) to ACG(Thr)

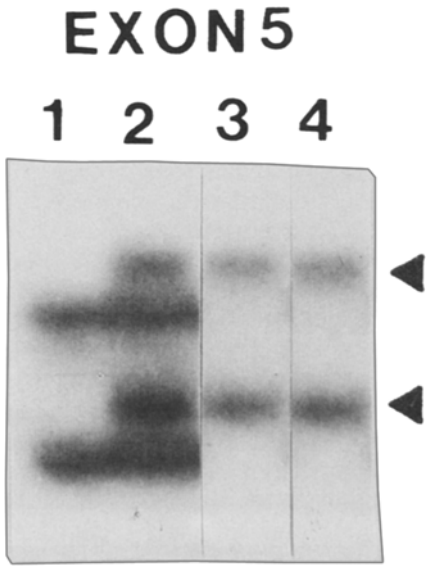

Fig. 3. PCR-SSCP analysis of KT and NS. *Lane 1,* Wild-type; *lane 2,* TAK2; *lane 3,* KT; *lane 4,* NS. Both KT and NS show only mutant bands on analysis of exon 5. *Arrowhead* indicates mutant bands

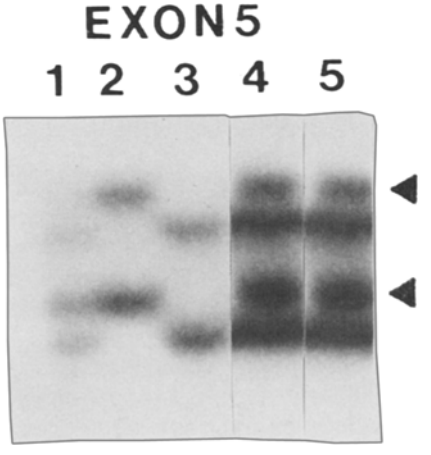

Fig. 5. PCR-SSCP analysis of the parents of KT. *Lane 1,* TAK2; *lane 2,* KT; *lane 3,* wild-type; *lane 4,* father of KT; *lane 5,* mother of KT. Both parents show mutant bands together with normal bands on analysis of exon 5. *Arrowhead* indicates mutant bands

PCR-SSCP analysis of patients with 2,8-DHA stones

Analysis of exon 3 showed that TAK2 yielded four bands following gel electrophoresis; two of the bands were at the same positions as those of the normal controls, but the remaining two were clearly distinct from these bands (Fig. 2). In analysis of exon 5 of patients KT and NS two bands formed the positions of which corresponded with those of the shifted bands of TAK2 (Fig. 3). Examination of exon 3 of patients KT and NS did not reveal mobility shifts of bands when compared with gel analyses of normal individuals (data not shown). These results indicate that both KT and NS are of the same genotype APRT*J/APRT*J.

Direct DNA sequencing of patients with 2,8-DHA stones

Sequencing analysis of KT and NS revealed that they had a single nucleotide substitution: ATG(Met) to ACG(Thr) at codon 136 in exon 5 (Fig. 4). Since this point mutation is reported to be specific for the APRT*J gene [4], the results of DNA sequencing analysis further confirmed that both KT and NS had the genotype APRT*J/APRT*J.

PCR-SSCP analysis of parents

Both parents of KT showed two normal bands together with mutant bands on analysis of exon 5 (Fig. 5). Further,

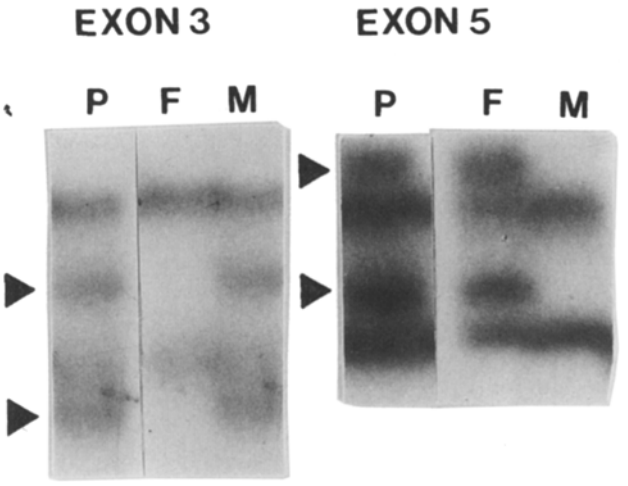

Fig. 6. PCR-SSCP analysis of the parents of TAK2. *P,* Patient (TAK2); *F,* father of TAK2; *M,* mother of TAK2. Analysis of exon 3 reveals that the mother of TAK2 shows mutant bands together with normal bands. Analysis of exon 5 shows that the father of TAK2 has both mutant and normal bands. *Arrowhead* indicates mutant bands

normal bands resulted from analysis of exon 3 (data not shown). These results suggest that both parents were heterozygotes APRT*J/APRT*1. The father of TAK2, however, showed a shift in two bands following analysis of exon 5, while the mother showed shifts in bands of exon 3 (Fig. 6). Thus, the father was considered to be of genotype APRT*J/APRT*1 and the mother to have genotype APRT*QO/APRT*1.

Screening results of a sample of the general population

Eighty individuals studied did not show shifted bands following analysis of exon 3 and exon 5 (data not shown).

Discussion

Recent studies by Kamatani et al. [6] and Hakoda et al. [2] have reported two methods using intact T cells to detect both homozygotes and heterozygotes of mutant APRT enzymes. These methods were shown to be sensitive and to discriminate type 2 patients from heterozygotes. However, such analyses are time-consuming, often taking up to 2 weeks to obtain results. Furthermore, there is little information given about specific genetic changes involved. DNA sequence analysis of five type 1 Japanese subjects (three homozygotes and two heterozygotes) has revealed that the APRT*QO mutation was caused by two nucleotide substitutions common to all affected alleles [12, 14]. These substitutions were TGG(Trp) to TGA(stop) and GCC(Ala) to GCT(Ala) located at codons 98 and 99 in exon 3. In addition, type 2 deficiency has been studied in 11 APRT-deficient lymphoblast cell lines and six Japanese homozygotes by either DNA sequence analysis or RNase mapping [4]. These studies revealed that all APRT*J alleles contained a single nucleotide substitution, ATG(Met) to ACG(Thr), located at codon 136 in exon 5. Thus, both APRT*J and APRT*QO in Japanese subjects studied thus far have specific point mutations at common sites. Therefore, analysis of the specific genomic regions is useful for the diagnosis of APRT deficiency. One of the simplest methods for studying point mutations is the allele-specific oligonucleotide hybridization technique. This method has been used succesfully in the detection of individuals with APRT*J gene [9]. We showed that PCR-SSCP analysis is a simple method that allows detection of both APRT*J and APRT*QO.

Recent studies have shown that there are several polymorphisms at the APRT locus [14]. Moreover, the two mutant bands of APRT*QO and APRT*J could be confused with those of the polymorphic genes if the polymorphic site is present within the regions analyzed by the PCR-SSCP method. Thus, we surveyed a sample of the general population to examine the possibility of false positive diagnosis attributed to such polymorphisms. We found that there were few polymorphisms at the region of the APRT gene. Furthermore, the fact that we did not encounter any mutations in the people sampled in our study confirms the suggested low incidence of the APRT gene mutation (1.2%) in the Japanese population [7]. We therefore concluded that PCR-SSCP is a simple diagnostic method with high specificity for the detection of defective APRT genes in Japanese individuals. By using PCR-SSCP analysis genetic heterogeneity in population, such as compound heterozygotes (ARPT*QO/APRT*J) or heterozygotes, can be detected as well as homozygotes. A definitive diagnosis of putatively APRT deficient individuals such as siblings of 2,8-DHA stone formers or children of heterozygous patients will enable them to be treated before they have symptoms. Moreover, PCR-SSCP analysis can be used for prenatal diagnosis, using cells from the amniotic fluid. When the relationship between the genotypes of patients and severity of their symptoms is revealed, different treatments can be chosen according to genotype. Thus the technique of molecular diagnosis reported here will be used in the clinical diagnosis and treatment of APRT deficiency in the future.

References

1. Fujimori S, Akaoka I, Sakamoto K, Yamanaka H, Nishioka K, Kamatani N (1985) Common characteristics of mutant adenine phosphoribosyltransferase from four separate Japanese families with 2,8-dihydroxyadenine urolithiasis associated with partial enzyme deficiency. Hum Genet 71:171
2. Hakoda M, Yamanaka H, Kamatani N, Kamatani N (1991) Diagnosis of heterozygous states for adenine phosphoribosyltransferase deficiency based on detection of in vivo somatic mutations in blood T cells: application to screening of heterozygotes. Am J Hum Genet 48:552
3. Hidaka Y, Tarle SA, Otoole TE, Kelly WN, Pallela TD (1987) Nucleotide sequence of the human APRT gene. Nucleic Acids Res 15:9086
4. Hidaka Y, Tarle SA, Fujimori S, Kamatani N, Kelly WN, Pallela TD (1988) Human adenine phosphoribosyltransferase defic-

iency: demonstration of a single mutant allele common to the Japanese. J Clin Invest 81:945
5. John SWN, Weitzner G, Rosen R, Scriver CR (1991) A rapid procedure for extracting genomic DNA from leukocytes. Nucleic Acids Res 19:408
6. Kamatani N, Takeuchi F, Nishida Y, Yamanaka H, Nishioka K, Takara K, Fujimori S, Kaneko K, Akaoka I, Tofuku Y (1985) Severe impairment in adenine metabolism with a partial deficiency of adenine phosphoribosyltransferase. Metabolism 34:164
7. Kamatani N, Terai C, Kuroshima S, Nishioka K, Mikanagi K (1987) Genetic and clinical studies on 19 families with adenine phosphoribosyltransferase deficiencies. Hum Genet 75:163
8. Kamatani N, Sonoda T, Nishioka K (1988) Distribution of patients with 2,8-dihydroxyadenine urolithiasis and adenine phosphoribosyltransferase deficiency in Japan. J Urol 140:1470
9. Kamatani N, Kuroshima S, Hakoda M, Pallela TD, Hidaka Y (1990) Crossovers within a short DNA sequence indicate a long evolutionary history of the APRT*J mutation. Hum Genet 85:600
10. Kamatani N, Kuroshima S, Yamanaka H, Nakashe S, Take H, Hakoda M (1990) Identification of a compound heterozygote for adenine phosphoribosyltransferase deficiency (APRT*J/APRT-*QO) leading to 2,8-dihydroxyadenine urolithiasis. Hum Genet 85:500
11. Kelly WN, Levy RI, Rosenbloom FM, Henderson JF, Seegmiller JE (1968) Adenine phosphoribosyltransferase deficiency: a previously undescribed genetic defect in man. J Clin Invest 47:2261
12. Mimori A, Hidaka Y, Wu VC, Tarle SA, Kamatani N, Kelly WN, Pallela TD (1991) A mutant allele common to the type 1 adenine phosphoribosyltransferase deficiency in Japanese subjects. Am J Hum Genet 48:103
13. Orita M, Suzuki Y, Sekiya T, Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5:874
14. Sahota A, Chen J, Asaki K, Takeuchi H, Stambrook PJ, Tischfield JA (1990) Identification of a common nonsense mutation in Japanese patients with type 1 adenine phosphoribosyltransferase deficiency. Nucleic Acids Res 18:5915
15. Simmonds HA, Van Ackar KJ (1983) Adenine phosphoribosyltransferase deficiency: 2,8-dihydroxyadenine lithiasis. In: Stanbury JB, Wyngaaden JB, Fredrickson DS, Goldstein JL, Brown MS (eds) The metabolic basis of inherited disease, 5th edn. McGraw-Hill, New York, p 1145
16. Simmonds HA, Sahota AS, Ackar KJ van (1989) Adenine phosphoribosyltransferase deficiency and 2,8-dihydroxyadenine lithiasis. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) Metabolic basis of inherited disease, 6th edn. McGraw-Hill, New York, p 1029