Detection of Mutations in Adenine Phosphoribosyltransferase (APRT) Deficiency Using the LightCycler System

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We have applied an established technique, the polymerase chain reaction (PCR) with LightCycler technology, to a single disease with well-defined mutations. This assay produces results within only 30 min by combining PCR and fluorescence detection in one tube without electrophoretic band detection. In this study, we found 2,8-dihydroxyadenine (DHA) lithiasis in Japanese patients who were heterozygous for Japanese-type (type II) adenine phosphoribosyltransferase

(APRT) deficiency (APRT*J). These patients, from a family with 2,8-DHA lithiasis, had a heterozygous mutation in the J region of the APRT gene. We demonstrated that the present system, using LightCycler technology, was simple, rapid, and reliable for detecting known mutations, and capable of identifying heterozygous and homozygous mutations in this family with APRT deficiency. *J. Clin. Lab. Anal.* 14:274–279, 2000. © 2000 Wiley-Liss, Inc.

Key words: PCR; adenine phosphoribosyltransferase (APRT) deficiency; 2,8-dihydroxyadenine (DHA) lithiasis; LightCycler technology

Rapid advances in the field of molecular genetics have led to the need for a highly sensitive, robust assay for detection of mutations. A number of methods have been devised for this purpose, the most widely used being PCR-SSCP (polymerase chain reaction–single-stranded conformation polymorphism) (1) and automated DNA sequencing (2). PCR-SSCP is favored because of its simplicity. Although some automated DNA-sequencing methods require steps for cloning the PCR products, they offer the advantage of better sensitivity and specificity. However, these methods are not able to detect all mutations, and are time-consuming and expensive. Other methods for detecting mutations, such as denaturing gradient gel electrophoresis (DGGE), have been reported (3), but these have not improved the sensitivity of SSCP for gel electrophoresis analysis. Recently, a new online fluorescence polymerase chain reaction (PCR) detection system and HybProbe chemistry were developed for use in the LightCycler system (4). This system for online PCR detection became available to researchers for the quantification of bcr/abl fusion transcripts (5) and for the detection of hemochromatosis mutations (6) and plasminogen inhibitor-1 mutations (7). The system has been used to perform rapid PCR in less than 30 min, while simultaneously quantifying and analyzing the amplification results. Detection of mutations by TaqMan chemistry and other methods has been developed to discriminate polymorphic alleles that differ by a single base nucleotide

(8). Although there are limitations to many mutation detection systems, we used the LightCycler system in the present study to detect known mutations in a family with adenine phosphoribosyltransferase (APRT) deficiency and obtained good results quickly and simply.

APRT catalyzes the synthesis of AMP from adenine and 5-phosphoribosyl-1-pyrophosphate (PRPP). In the absence of APRT activity, adenine is oxidized by xanthine oxidase to 2,8-dihydroxyadenine (DHA). Deficiency of this enzyme results in the accumulation of both adenine and its insoluble derivative 2,8-DHA, which can cause nephrolithiasis (9). The APRT gene encodes 180 amino acids and is located on chromosome 16 (10). Two types of APRT deficiency leading to 2,8-DHA lithiasis have been identified (9). One group of patients (type I) is homozygous for null alleles, designated APRT*Q011. Another group (type II) has variable APRT activity and is considered to be homozygous for the mutant allele APRT*J, found only in Japan (12). Both types of APRT deficiency are found in the Japanese population, but type II is the main cause of 2,8-DHA lithiasis. A patient heterozygous for type II deficiency has also been reported to have 2,8-DHA

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lithiasis (13). Thus, determination of the genotype of individuals with APRT deficiency is clinically useful.

In this study, we designed and established a rapid and reliable system capable of detecting mutations of the APRT gene using the LightCycler system. We then applied the system to clinical samples from patients with APRT deficiency.

MATERIALS AND METHODS

Patients

A 14-year-old Japanese girl presenting at Tohoku University Hospital for plastic surgery in January 1999 was unexpectedly found to have a brownish sediment in her urine. No kidney stones were demonstrated by abdominal X-ray, and ultrasound examination showed no shadows suggestive of lithiasis. However, the brownish sediment detected in her urine was identified as 2,8-DHA. The hemolysate APRT activity was 1 nmol/hr/mg protein (mean $\pm$ SD = 20 ± 6 , $N = 10$). Family members were also examined with their informed consent. The pedigree structure of this family is shown in Fig. 1a. Not shown are several apparently unaffected siblings in generation I whose blood samples were not available. None of the family members had a medical history of urolithiasis. A 50-year-old man with 2,8-DHA urolithiasis, who had al-

ready been proved to have the APRT *QO type, was also examined at Fukuoka University Hospital as a control.

APRT Assay

APRT was assayed in cell extracts according to a method described previously (14). The final concentration of [14 C]adenine (Amersham International plc, UK) was 0.2 mM, and that of 5-phosphoribosyl-1-pyrophosphate (PRPP, Na salt) was 1 mM. About 5 μ g protein was used per assay, and the incubation time was 15 min. The reaction was terminated with perchloric acid, followed by neutralization with KOH. The substrate and products were separated on cellulose TLC sheets (Kodak) in 5% NaH_2PO_4 . Radioactivity was determined with an AMBIS radioanalytic image analyzer. Specific enzyme activity was defined as nanomoles of adenine nucleotides formed per hour per milligram of protein.

Samples

Blood samples were collected from the patient and family members with 2,8-DHA sediments, a patient with 2,8-DHA urolithiasis, and 10 healthy individuals. DNA was extracted from the blood samples by phenol-chloroform and ethanol precipitation.

Fig. 1. (a) Pedigree and mutational analysis of the proband (arrow) in the family. Square symbols, males; circles, females. (b) Genomic organization of adenine phosphoribosyltransferase (APRT) and the positions of the amplified fragments used for LightCycler analysis (arrow shows the amplified

regions). The nature and position of the mutations that were identified are shown below the figure. (c) Design of the LightCycler system using two hybridization probes for detection of APRT mutations.

PCR Amplification

PCR amplification of the APRT gene (GenBank accession no. Y00486) was carried out employing the primer sequences described previously (14) (Fig. 1b). The primers for APRT PCR correspond to the nucleotides in the J and Q regions of the APRT gene (15): the sequence of the upstream primer in exon 5, including the J region, was 5'-CTGCTCTTCTGCAGCCCAGGCCA-3'; the downstream primer in the J region was 5'-TGGGATCCAGCTGGAGATGTTG-3'; the sequence of the upstream primer in exon 3, including the Q region, was 5'-CCGAGTCACTCCTGTCACTTA-3'; the downstream primer in the Q region was 5'-GATCCAGCCTGGAGATGTTGGGCT-3'. The PCR reaction was performed in a reaction mixture of 50 µl that contained genomic DNA (1 µg), 2.5 units of *Taq* DNA polymerase (Toyobo, Japan), 1 µl of each primer (0.5 µM), 1.25 mM each dNTP (ATP, GTP, CTP, TTP), 5 µl of 10× reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1% v/v Triton X-100), 2.5 mM MgCl₂, and distilled water. Each mixture was subjected to 30 cycles of amplification at 94°C for 2 min, 55°C for 30 sec, and 72°C for 90 sec using a DNA thermal cycler. The amplified PCR products were electrophoresed on 1.6% agarose gel and then visualized with UV light as a single band of 462 bp by staining with ethidium bromide.

Genotyping

To obtain a melting curve by monitoring the denaturation profile of the PCR product in the presence of hybridization probes, we used a LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany). This may also be used to identify the relative contributions of mutated versus wild-type sequences to signal levels. For sequence-specific fluorescence detection, oligonucleotide hybridization probes coupled at the 3' end (anchor) to a 5'-fluorescein label and at the 5' end (mutation) to 3'-LightCycler-Red 640 were used. The sequences of the two hybridization probes for the APRT*J type were LightCycler-Red 640-labeled 5'-AGGCAGCGTCGTTTCATGGTTCC-3' and fluorescence-labeled 5'-GCAGGCGGCCAGCAGCTCA-3'. The sequences of the two hybridization probes for the APRT*Q0 type were LightCycler-Red 640-labeled 5'-CACTCTGTGGCCTCCTAT-3' and fluorescence-labeled 5'-GGGGGAAGCTGCCAGGCC-3'. This melting curve analysis was performed using a PCR mixture of 20 µl including 50 ng of template (genomic) DNA (0.5 µl), 0.4 unit of *Taq* DNA polymerase (0.8 µl), 0.5 mol/l each PCR primers (each 1 µl), MgCl₂ (25 mM, 1.6 µl), 50 mmol/l Tris-HCl (pH 8.3, 2 µl), a master mix of deoxynucleotides (dNTPs, 2 µl), 0.5 µmol/l mutation hybridization probe (0.4 µl), 0.1 µmol/l anchor hybridization probe (0.8 µl) and distilled water (9.9 µl) in each capillary. These mixtures for PCR using the LightCycler system were subjected to denaturation at 95°C for 60 sec, 45 cycles of amplification consisting of 94°C for 0 sec, 65°C for 10 sec,

and 72°C for 5 sec, and 95°C for 30 sec, 40°C for 15 sec, and 85°C for 0 sec as a melting step, and 40°C for 30 sec for cooling. After amplification, melting curves were converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature against temperature (T_m , °C).

PCR restriction fragment length polymorphism (PCR-RFLP) was performed by digestion with *Nla*III (New England Biolabs, Inc., Beverly, MA). PCR was performed under the conditions described previously, and then the PCR products were incubated with *Nla*III and NE reaction buffer at 37°C for 2 hr. The digested PCR products were electrophoresed on 6% polyacrylamide gel and then visualized with UV light by staining with ethidium bromide. *Nla*III is a restriction enzyme that recognizes the CATG sequence at codon 136 in exon 5 of the APRT gene. When a mutation (ACG) in this position was present, APRT*J had fragments of 233 bp compared with fragments of 177 and 56 bp for the normal (wild) type.

DNA Sequencing

Mutations in PCR products that showed abnormality upon LightCycler analysis were confirmed by sequencing analysis. The PCR products were subcloned into the pGEM-T vector (Promega, WI), and at least three independent clones were sequenced with a cycle sequencing kit using 7-deaza-ddGTP (Amersham) with an automated DNA sequencer (DSQ-1000L, Shimadzu Co., Japan).

RESULTS

Genotyping Using the LightCycler System

A schematic representation of the adjacent fluorescent hybridization probes used for genotyping to distinguish wild or mutant loci is shown in Fig. 1c. These probes were designed so that each formed a mismatch with the wild-type allele extended by the downstream primer during hybridization. Genotyping for the mutation was done at a different melting temperature, which began at 55–70°C. Melting curve analysis with the LightCycler system was done for three variants in exon 5 at an adequate melting temperature (Fig. 2a). These variants consisted of wild, heterozygous, and homozygous types, which were shown as a pattern of clearly separated peaks. One peak in exon 5 was revealed in DNA from the patient's mother and younger sister, who were heterozygous (J*/1), compared with a different peak for the known homozygous genotype (J*/J*) and also a different peak for the wild type. On the other hand, melting analysis of exon 3 for the APRT*Q0 type showed only one peak for the wild type (Fig. 2b).

The results of genotyping using the LightCycler were compared with those of conventional PCR restriction fragment length polymorphism (PCR-RFLP) analysis (data not shown). PCR-RFLP analysis of the APRT*J type revealed one frag-

Fig. 2. Derivative melting curves showing three genotypes by LightCycler analysis. (a) Fluorescence data for APRT*J regions from all patients, generated by the LightCycler instrument. The relative change in the fluorescence curve is given on the cycles of the Y axis. Genotyping by derivative melting curves for three alleles is shown. Shown are seven samples with different

genotypes: heterozygous (traces 1, 3, and 5), homozygous (p), wild (traces 2, 4, and N), and background (DDW). (b) Fluorescence data for APRT*Q0 regions from all patients, generated by the LightCycler instrument. Numbers of samples in figures are the same as in Table 1.

ment which was not digested by *Nla*III, whereas two fragments were shown in the wild type. The genotypes of all samples typed by LightCycler analysis agreed with those determined by PCR-RFLP analysis. The size of the PCR products obtained from LightCycler analysis also coincided with those of the fragments from PCR-RFLP analysis.

APRT Activity

APRT activity in the patients and family is shown in Table 1. All showed reduced activity except for the elder sister. The

patient and younger sister had clinical 2,8-DHA lithiasis, but the others did not. As shown in Fig. 2, the heterozygous patterns were not associated with APRT activity. The genotypes determined by mutation analysis and APRT activity are indicated in Table 1. APRT activity in the patient, mother, and younger sister possessing the heterozygous type *J mutation was 7%, 63%, and 8% lower than the control mean (mean $\pm$ SD), respectively. In comparison, APRT activity in the father and elder sister who had the wild type of *J was 25% and 100%, respectively. Thus, although the patient and younger

TABLE 1. Patient's profile

	Age	Sex	APRT activity (%) ^a	Genotypes	J mutation
1. Patient	14	F	1.0 (7)	Presumed mixed heterozygote	ATG→ACG
2. Father	48	M	3.5 (25)	Presumed heterozygote ^c	ATG
3. Mother	41	F	8.9 (63)	Heterozygote	ATG→ACG
4. Elder sister	18	F	15.1 (100)	Wild ^b	ATG
5. Younger sister	8	F	1.2 (8)	Presumed mixed heterozygote	ATG→ACG

^aNormal range is 14–26 nmol/hr/mg protein.

^bThe genotype APRT*1/APRT*1 means without J mutation.

^cThe genotype in the father did not include a J or Q mutation; however, he had low-level APRT activity. The proband and younger sister were indicated as presumed heterozygotes by the same reasoning.

sister were heterozygous for the *J mutation, they may have been compound heterozygotes for APRT in view of their very low level of enzyme activity. Furthermore, although the father had also the wild type genotype for *J, he may have been heterozygous for APRT because of his low enzyme activity (Fig. 1a).

Sequencing Analysis

Sequence analysis showed a point mutation resulting in a T to C transversion at nucleotide 136 of exon 5 in the APRT*J allele (Fig. 3). This mutation caused a change from ATG to ACG, resulting in an amino acid substitution change from methionine to threonine. No mutations were found at codon

98 in exon 3 for the APRT*Q0 type. The results for this family are summarized in Table 1.

DISCUSSION

The newly available LightCycler system offers a number of methodological advantages. We used two labeled hybridization probes that hybridized to an internal sequence of the amplified fragment during the annealing phase in the LightCycler system. This system has already been used for quantification analysis by comparing the fluorescence of a PCR product of unknown concentration with the fluorescence of several dilutions of an external standard (5). In other applications, this system has detected mutations by analysis of

Fig. 3. Sequencing analysis. Nucleotides in the samples from patients identified as heterozygous and homozygous from the pattern revealed by the LightCycler system.

the melting characteristics of a PCR product. Unlike conventional PCR, in which cycling programs take several hours, PCR analyses conducted with the LightCycler take only 30 min. The ABI PRISM 7700 system for detecting mutations is also useful for two hybridization probes during PCR (8). In this study, we were able to detect a single base-pair mutation by melting curve analysis with the LightCycler system. The different melting behavior of each sample corresponded to that of the wild-type, the homozygous mutant, and the heterozygous mutant.

We analyzed the mutant alleles in the present family with APRT deficiency. A missense mutation was detected in three patients with clinical APRT deficiency. Two different mutations of APRT have been identified among the Japanese population; the most common mutant allele APRT*J with a missense mutation at codon 136 resulting in conversion of ATG to ACG is known to represent about 70% of all the mutant alleles, and in patients with 2,8-DHA urolithiasis bearing the mutant allele there is a partial enzyme deficiency (16). However, the family examined in this study had 2,8-DHA sediments in their urine but no detectable urolithiasis, and APRT activity in the sister and father was lower than that in the controls. It may be suggested that the father and sister, who appeared to be the homozygous wild type, had decreased enzyme activity due to other mutations. From these results, it is suggested that the patient and younger sister were compound heterozygotes and that the father was a heterozygote. It is suspected that he had unknown mutations in other J or Q regions.

In summary, the present one-step system using LightCycler technology is simple and rapid. The system may save time because rapid temperature ramping and high-speed cycling of PCR reduces the time required for a typical experiment to less than 30 min. The key advantages are that it is ideal for detection of the difference in mutations between homozygous and heterozygous genotypes because different genotypes exhibit different melting behaviors in melting curve experiments. Therefore, we were able to obtain good results in the detection of APRP gene mutations with this system. Further studies should be done to optimize and standardize the system to further increase its applications in the detection of known mutations.

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