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PRENATAL DIAGNOSIS OF A JAPANESE FAMILY AT RISK FOR TAY-SACHS DISEASE

APPLICATION OF A FLUORESCENT COMPETITIVE ALLELE-SPECIFIC POLYMERASE CHAIN REACTION (PCR) METHOD

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INDEXING WORDS

Tay-Sachs disease; the HEXA gene; hexosaminidase A; fluorescent competitive allele-specific PCR

SYNOPSIS

Tay-Sachs disease (TSD) is caused by mutation of the HEXA gene, which results in a deficiency of the alpha-subunit of hexosaminidase A. The major mutation in Japanese TSD is a G-to-T transversion at the 3'-splice site of intron 5. We established a fluorescent competitive allele-specific polymerase chain reaction (FCAS-PCR) method for detection of the mutation and applied it to prenatal diagnosis of a Japanese TSD family. FCAS-PCR distinguished the wild and mutant alleles clearly, with broad ranges in the amount of template DNA, the dNTP concentration, the MgCl2 concentration and the number of PCR cycles. After obtaining ethics committee approval and informed consent from the parents in the index family, chorionic villus sampling was performed. FCAS-PCR analysis using chorionic villus DNA disclosed that the fetus was

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homozygous for the mutation. To confirm the diagnosis, direct sequencing analysis of the genomic PCR fragment was performed, and showed the same results as those of the FCAS-PCR analysis. FCAS-PCR proved to be helpful for carrier screening and prenatal diagnosis in TSD families in the Japanese population. It would also be a useful DNA-diagnostic method for many other inherited disorders.

INTRODUCTION

Tay-Sachs disease (TSD), an autosomal recessive neurodegenerative disorder with abnormal accumulation of GM2-ganglioside, is caused by mutation of the HEXA gene, which encodes the alpha-subunit of the lysosomal enzyme, hexosaminidase A. The deficiency of the alpha-subunit protein affects hexosaminidase A activity, leading to accumulation of GM2-ganglioside.[14]

The HEXA gene is 35 kb long and contains a coding sequence of 1,587 bp separated into 14 exons.[11, 15, 22] Efforts to identify mutations in TSD initially focused on the Ashkenazic Jewish and French-Canadian populations because of its high incidence in these populations. In Ashkenazic Jewish populations, two major mutations have been found, a G-to-C transversion at the 5'-splice site of intron 12 [1, 12, 13] and a 4-base insertion into exon 11 of the HEXA gene.[8] In French-Canadian populations, a 7.6-kb deletion at the 5' end of the gene is reportedly common.[9, 10] Other mutations responsible for TSD have been reported in families with diverse ethnic backgrounds. In Japan, the incidence of TSD is approximately 1:64,000, and the most common phenotype is the infantile form.[20] A G-to-T transversion at the 3'-splice site of intron 5, resulting in skipping of exon 6 during splicing, has been reported as a major mutation (80% of mutant alleles) in the Japanese population.[20] For detecting the major mutation of Tay-Sachs disease in Japan, a PCR and restriction enzyme method has recently been reported.[19]

We established another rapid and accurate method, fluorescent competitive allele-specific polymerase chain reaction (FCAS-PCR), to detect the major mutation in the HEXA gene in the Japanese population. We successfully used fluorescent forward primers, specific for wild or mutant alleles, that formed a mismatch at the 3'-terminal nucleotide of each primer. In this method, wild- or mutant-allele-specific PCR products can be detected as different fluorescent dye peaks on the electrophoretogram. We report here an application of this method to the prenatal diagnosis of TSD in a Japanese family at risk.

METHODS

Proband

The proband was a Japanese female patient with infantile TSD. She was born as the first child of unconsanguineous parents. Her psychomotor development was almost normal until the age of 12 months, when she could stand up with support. Thereafter, she lost her psychomotor ability rapidly. At the age of 1 year and 5 months, she could not hold her head up and had her first generalized tonic-clonic seizure. Ophthalmologic examination revealed a "cherry-red spot" in the macula. The patient's hexosaminidase A activity (in leukocytes) was 3% of the mean control value (mean ± SD; 430 ± 90 nmol/mg protein/hour; n = 48), while those of her father, mother, sister, maternal grandfather, maternal grandmother and maternal aunt were 66% , 73%, 65%, 80%, 63% and 110%, respectively. She died at the age of 5 years. There were no others in the family with mental or neurodegenerative disorders. Genetic analyses and enzymatic assays were performed after informed consent had been obtained from the family members.

Chorionic villus sampling

After ethics committee approval and informed consent had been obtained, chorionic villus sampling and subsequent genetic analyses and enzymatic assays were performed. Control chorionic villi were obtained from women who were undergoing termination of pregnancy and who volunteered to participate in this study.

Chorionic villus sampling from the mother was performed at 10 weeks' gestation. Villus samples of 20-30 mg were obtained, placed in physiological saline on ice, and taken immediately to the laboratory. Dissection was carried out at room temperature with the aid of a phase microscope, with care to remove all blood and maternal decidua. Samples of 10-15 mg of cleaned villi were washed twice in physiological saline, pelleted by centrifugation and frozen until analyzed. To identify or eliminate maternal cell contamination in the chorionic villus sample, genotyping analysis was performed by the method of Lee et al. .[7)]

Genomic DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes and chorionic villi with the Gene Trapping by Liquid Extraction Kit (Takara

Biomedicals, Kyoto, Japan) and used as a template for PCR. The genomic DNA fragment containing exon 6 and its flanking regions of the HEXA gene was amplified using a primer set, COMf (5'-TGAAACCGGAGAGACTGTGATG-3' [22]) and COMr (5'-GCCACAGCC AGATTCAGACATTG-3' [22]).

Genomic DNA (200 ng) was used in a final concentration of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.001% (w/v) gelatin, 0.15 μM each primer, 1 unit of *Taq* DNA polymerase (AmpliTaq Gold; Perkin-Elmer Applied Biosystems, Foster City, CA, USA) in a final volume of 30 μl. The PCR process was as follows: an initial denaturation step at 94°C for 7 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 65°C for 3 min, and a final extension step at 65°C for 7 min.

The PCR-amplified genomic DNA was directly sequenced. The sequencing reaction of the PCR-amplified fragments was performed using a dye terminator cycle-sequencing kit (Perkin-Elmer Applied Biosystems) according to the supplier's instructions. The sequencing reaction product was electrophoresed on a genetic analyzer (ABI Prim 310; Perkin-Elmer Applied Biosystems) and then analyzed using Sequencing Analysis software (Perkin-Elmer Applied Biosystems).

FCAS-PCR

For the detection of a G-to-T transversion in the patient's family members, FCAS-PCR was performed (Fig. 1). The sequences of the wild- and mutant-allele-specific forward primers, Wf and Mf, were as follows; Wf: 5'-GTGTGCACTTTAACCTACAG-3' and Mf : 5'-GTGTGCACTTTAACCTACAT-3'. The allele-specific primers, Wf and Mf, were labeled with fluorescent dyes HEX (4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein) and FAM (6-carboxyfluorescein), respectively.[21] These fluorescent dye-labeled primers were synthesized by Perkin-Elmer Applied Biosystems. The non-labeled primer COMr was used as a common reverse primer.

Genomic DNA (25-200 ng) was used in a final concentration of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.0-2.5 mM $MgCl_2$, 25-200 μM dNTPs, 0.001% (w/v) gelatin, 0.15 μM each primer, 1 unit of *Taq* DNA polymerase (AmpliTaq Gold; Perkin-Elmer Applied Biosystems) in a final volume of 30 μl. In FCAS-PCR, a set of three primers (two allele-specific primers and a common primer) was added in a single tube. The PCR process was as follows: an initial denaturation step at 94°C for 7 min, 20-35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 65°C for 3 min, and a final extension step at 65°C for 20 min. An aliquot of the FCAS-PCR product was

mixed with a 500-bp marker (GeneScan-500 TAMRA; Perkin-Elmer Applied Biosystems), denatured at 95°C for 2 min, electrophoresed on a genetic analyzer (ABI Prism 310; Perkin-Elmer Applied Biosystems) and then analyzed using the GeneScan analysis software package (Perkin-Elmer Applied Biosystems). The maximum excitation and emission wavelengths for fluorescent dyes, FAM and HEX, were 494 nm and 535 nm, and 517 nm and 553 nm, respectively. The ABI Prism 310 argon laser emits light of the greatest intesty at 488 nm and 514.5 nm. The wavelength centers of detection for FAM and HEX were 531 nm and 560 nm, respectively.

Enzyme assays

Hexosaminidase A activity was ascertained from chorionic villi homogenates with 4-methylumbelliferyl beta-N-acetyl-glucosamine 6-sulfate.[17] The amount of protein was determined according to the method of Watanabe et al.[23] with bovine serum albumin as the standard.

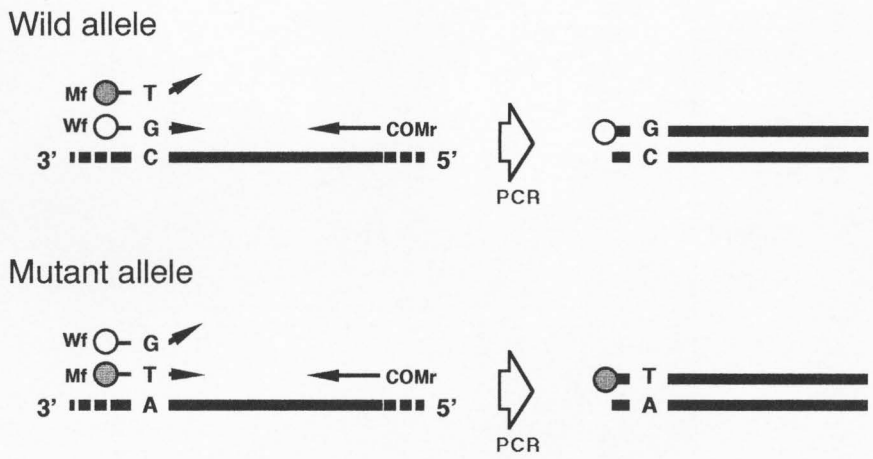

Fig. 1. *Schematic presentation of the FCAS-PCR method.*
To detect the G-to-T transversion, the mutant-allele-specific forward primer (Mf) is designed to form a T (primer):C (template, wild allele) mismatch at the 3'-terminus and the wild-allele-specific forward primer (Wf) is designed to form a G (primer):A (template, mutant allele) mismatch at the 3'-terminus. Primers, Wf and Mf, are labeled with fluorescent dyes HEX and FAM, respectively. In this scheme, HEX is indicated as an open circle and FAM as a closed circle. Primer COMr is a non-labeled common reverse primer. In FCAS-PCR, a set of three primers (two allele-specific primers and a common primer) is added in a single tube. Since competitive annealing of the allele-specific primers occur in FCAS-PCR, each allele is amplified not with its mismatched primer, but solely with its matched primer.

RESULTS

Diagnostic analysis of the patient's DNA

The diagnosis was confirmed by PCR and nucleotide sequencing of the HEXA gene. The patient was homozygous for a mutation involving a single base substitution, G-to-T, at the 3'-splice site of intron 5 of the HEXA gene (Fig. 2).

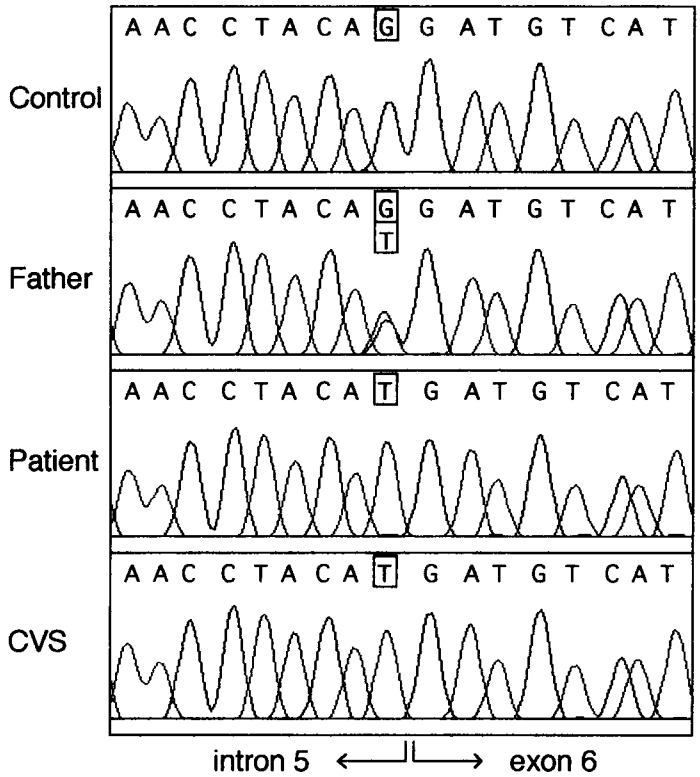

Fig. 2. *Sequence analysis of genomic DNAs.*
The panels show raw data of the nucleotide sequences of the HEXA gene of a control individual, the father, the patient, and the mother's chorionic villi. A single base substitution, G-to-T, occurred on both alleles of the patient and in the chorionic villi (homozygous for the mutant allele). In the father, the G-to-T transversion was detected on one allele, but not on the other, indicating that he was a heterozygote of the wild and mutant alleles.

FCAS-PCR for detection of the mutant allele

For the detection of a G-to-T transversion at the 3'-splice site of intron 5 of the HEXA gene, FCAS-PCR was performed (Fig. 1). After FCAS-PCR, each allele was amplified not with its mismatched primer, but with its matched primer: the wild allele was specifically amplified with wild-allele-specific primer Wf and the mutant allele was specifically amplified with mutant-allele-specific primer Mf. To determine the specificity and sensitivity of FCAS-PCR in various conditions, we changed the variables according to a protocol: the amount of template DNA (25, 50, 100 and 200 ng), the dNTP concentration (25, 50, 100 and 200 μM), the $MgCl_2$ concentration (1.0, 1.5, 2.0 and 2.5 mM) and the PCR cycle number (20, 25, 30, 35 cycles). This method distinguished the wild and mutant alleles clearly in any combination of the variables. For subsequent FCAS-PCR analyses, we decided to add template DNA, 100 ng in 30 μl of the reaction mixture, with a dNTP concentration of 200 μM and $MgCl_2$ concentration of 1.5 mM, and to complete 25 cycles of PCR.

Genomic DNA samples from the patient, her parents, sister, maternal grandparents and maternal aunt, and ten normal controls were analysed using FCAS-PCR. Fig. 3 shows the electrophoretograms of the FCAS-PCR products of the patient, father and a control. The patient's DNA showed amplification of the mutant allele, but no amplification of wild allele, indicating that she was homozygous for the mutant allele. The DNA of her parents, sister and maternal grandparents with moderately low hexosaminidase A activity showed amplification of both alleles, indicating that they were heterozygotes with carrier status. The DNA of the maternal aunt and ten controls with normal hexosaminidase A activity showed amplification of the wild allele, but no amplification of the mutant allele.

Prenatal diagnosis of Tay-Sachs disease

To control for maternal cell contamination in the chorionic villus sample, we performed genotyping analysis using dinucleotide repeat polymorphisms at loci GABRB3 and D15S128. The chorionic villus sample showed only one maternal peak and did not show the other maternal peak at loci GABRB3 and D15S128, which indicated that there was no maternal cell contamination of the chorionic villus sample.

Then, we performed FCAS-PCR using DNA from the chorionic villus sample. The chorionic villus DNA showed amplification of the mutant allele, but no amplification of the wild allele, indicating that the fetus was

homozygous for the mutant allele (Fig. 3). To confirm the diagnosis, direct sequencing analysis and enzymatic assays were performed. Direct sequencing analysis detected a single nucleotide, T, at the position -1 in the 3'-splice site consensus sequence of intron 5, indicating that the fetus was homozygous for the mutation. Hexosaminidase A activity in the index patient's mother's chorionic villus sample was 5% of the mean activity in the control chorionic villus samples (mean ± SD; 575 ± 107 nmol/mg protein/hour; n = 5), which was consistent with the predicted value for homozygous status for the mutation. The pregnancy was discontinued after this prenatal diagnosis.

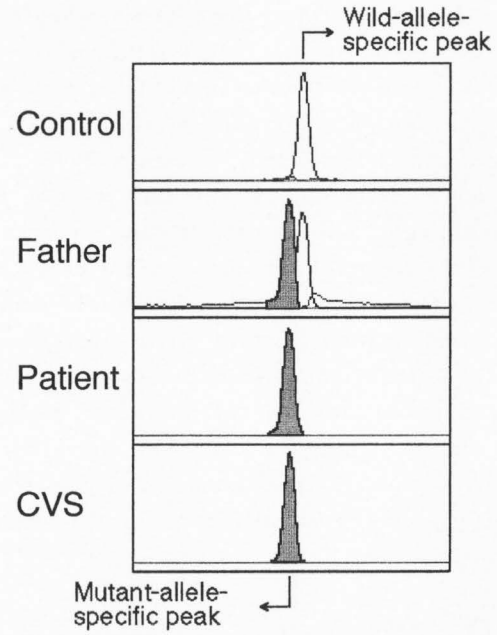

Fig. 3. *Detection of the wild and mutant alleles by FCAS-PCR.*
The mutant-allele-specific PCR product is shown as a shaded peak and the wild-allele-specific PCR product as a non-shaded peak. Although the sizes of the allele-specific products were both 181 bp, their mobility is affected by the fluorescent dyes, leading to slightly different locations of the peaks on the electrophoretogram. The DNA from a control individual showed only the wild-allele-specific peak, but not the mutant-allele-specific peak, indicating that he was a homozygote of the wild allele. The father's DNA showed both peaks, indicating that he was a heterozygote of the wild and mutant alleles. The patient's DNA showed only the mutant-allele-specific peak, but not the wild-allele-specific peak, indicating that she was a homozygote of the mutant allele. The chorionic villus DNA showed the same findings, indicating that the fetus was affected.

DISCUSSION

The mutation in our patient was a G-to-T transversion at the 3'-splice site of intron 5 of the HEXA gene, causing exon 6 skipping of the gene. The transcript with the deletion of 102 nucleotides in exon 6 may generate a truncated alpha-subunit of hexosaminidase A lacking 34 amino acids, leading to a loss of the enzyme activity. Tanaka et al. reported that this transversion was the major mutation among Japanese patients with infantile TSD, since it was detected in 38 mutant alleles out of 48 (80%).[20] They speculated that the mutation might have originated in Japan because of its high frequency in Japan and absence outside of Japan. To detect the mutation in the HEXA gene in the Japanese population, we developed FCAS-PCR, a rapid and accurate method.

Allele-specific PCR has been applied to many inherited disorders as a simple and rapid DNA-diagnostic method.[2, 3, 4, 18] We often encounter nucleotide substitutions which cannot be detected by the PCR and restriction enzyme digestion method because an appropriate restriction enzyme is not available. Conventional allele-specific PCR method has been used to detect such mutations, but optimization of the conventional allele-specific PCR protocols is not easy, because mismatched primer-template pairs are effectively extended when the experimental condition is inappropriate. Important variables in the conventional allele-specific PCR protocol have been reported: the amount of template DNA, the dNTP concentration, the $MgCl_2$ concentration the number of PCR cycles, the annealing temperature, the extension time, etc..[5, 6, 16] In particular, the dNTP concentration has been considered to be the most important factor; the dNTP concentration should be less than 50 μM to maintain specificity of allele-specific PCR.[16]

In our initial experiments, combination of a conventional allele-specific PCR with non-fluorescent primers and agarose gel electrophoresis did not always distinguish the wild and mutant alleles, because mismatched pairs of the allele-specific primers and the allele templates extended effectively. We had to continue changing the variables in the allele-specific PCR protocol until we found an optimum and obtained reproducible results (data not shown). Our conventional allele-specific PCR experiment required a time-consuming optimization procedure.

However, our new allele-specific PCR method, FCAS-PCR, distinguished the wild and mutant alleles clearly with broad ranges of the amount of template DNA, the dNTP concentration, the $MgCl_2$ concentration and the number of PCR cycles. In this study, we labeled the wild- and mutant-allele-specific primers

with different fluorescent dyes and performed a competitive allele-specific PCR in one tube, as described in the methods section. Reducing the dNTP concentration offered little to FCAS-PCR, because both alleles were distinguished at the dNTP concentrations between 25 and 200 μM. The specificity of FCAS-PCR may be due to the competitive annealing of matched and mismatched primers in one tube. Moreover, since the fluorescence technique improves the sensitivity of PCR, the amount of template DNA and the number of PCR cycles could be reduced.

The FCAS-PCR method can now be applied in many laboratories. Use of automated sequencers has become widespread, and primers labeled with fluorescent dyes are now provided by many companies (in this study, our primers were synthesized by Perkin-Elmer Applied Biosystems). To carry out the FCAS-PCR method, a PCR is simply done in a tube, and the PCR product is electrophoresed with an automated sequencer. The whole procedure for our method requires only a few hours, and it is not as complicated or time-consuming as it seems.

We judged that FCAS-PCR is one of the best methods for detecting the G-to-T transversion at the 3'-splice site of intron 5 of the HEXA gene, and applied the method to prenatal diagnosis using chorionic villi. The FCAS-PCR analysis disclosed that the fetus was homozygous for the mutation, indicating that it was affected. This finding was consistent with those of direct nucleotide sequencing analysis and hexosaminidase A activity of the chorionic villi. These consistent results guaranteed the accuracy of the FCAS-PCR method in prenatal diagnosis.

In conclusion, FCAS-PCR could rapidly and accurately identify the major mutation responsible for Japanese TSD. It proved to be helpful for carrier screening and prenatal diagnosis in a high-risk family in a Japanese population. FCAS-PCR could also be a useful DNA-diagnostic method for many other inherited disorders.

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