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Allele-Specific Polymerase Chain Reaction for Genotyping Human Cytochrome P450 2E1

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Allele-specific polymerase chain reaction (AS-PCR) was applied to investigate the cytochrome P450 2E1 (CYP2E1) genotype. AS-PCR is a competitive multiplex PCR method in which PCR amplification is successfully performed only by using the sequence of 3′ oligonucleotide ends as a DNA template in order to obtain an absolutely complementary product. I was able to produce allele-specific primers whose 3′ ends had the base specific to Pst I polymorphism located within the 5′-flanking region of the CYP2E1 gene. Electrophoresis of the products showed that bands derived from common PCR products, allele C1 and C2, were clearly separate from each other due to the difference in the size of the products. I tested 102 unrelated Japanese individuals, and the results of both restriction fragment length polymorphism (RFLP) by Pst I or Rsa I and direct sequencing were in complete agreement with those of AS-PCR. These results lead me to conclude that AS-PCR is a simple and useful technique for investigating CYP2E1 genotype. J. Clin. Lab. Anal. 13: 205–208, 1999.

Key words: allele-specific PCR; CYP2E1; mutation analysis; Pst I polymorphism; C1 allele; C2 allele

INTRODUCTION

Cytochrome P450 2E1 (CYP2E1) is well known as an ethanol-metabolizing enzyme (1). The enzyme is also of critical importance in the metabolic activation of many low-molecular-weight carcinogens, including *N*-nitrosamines (2). Since polymorphism in the 5′-flanking region of the CYP2E1 gene was reported by Hayashi et al. in 1991 (3), several investigators have studied the relationship between CYP2E1 genotypes and carcinogenesis in various organs (4–9). However, controversy still remains as to which of the genotypes of CYP2E1 is actually related with carcinogenesis.

To date, the genotype and allele usage of CYP2E1 have been analyzed by means of restriction fragment length polymorphism (RFLP) which is polymerase chain reaction (PCR) in combination with Rsa I or Pst I digestion. However, enzymatic digestion in the process of RFLP often gives rise to ambiguous results, possibly due to partial digestion or the presence of heteroduplexes within the PCR products that are refractory to digestion. To overcome this problem, a novel method needed to be developed. With this in mind, I focused my attention on allele-specific PCR (AS-PCR).

AS-PCR is a competitive multiplex PCR method. Successful PCR amplification requires the sequence of 3′ oligonucleotide ends to be absolutely complementary to the DNA template. AS-PCR appears to be a simple and accurate method which allows genotyping to be undertaken more quickly and in a less costly manner. AS-PCR has been extensively studied within the field of gene mutation research (10,11). In the current study, I applied this simple method to an investigation of CYP2E1 genotype.

MATERIALS AND METHODS

Samples

Blood samples were obtained from 102 unrelated Japanese people. Genomic DNA was extracted from whole peripheral blood using the High Pure PCR Template Preparation Kit (Boeringer Mannheim, Germany). The nucleic acid-extraction procedure was performed according to the manufacturer's recommendations. Three μl of the sample were then directly used for each mutation analysis.

Allele-Specific PCR

The procedure of AS-PCR is shown in Figure 1. Four different oligonucleotide primers were used, i.e., J9: 5′-CCA GTC GAG TCT ACA TTG TCA-3′, C1-R: 5′-TGC TGC ACC TAA CAC TGC AC-3′; C2-F: 5′-CCT TCT TGG TTC AGG AGA GC-3′; and J8: 5′-TTC ATT CTG TCT TCT AAC TGG-3′. The allele-specific primers, C1-R and C2-F, were designed

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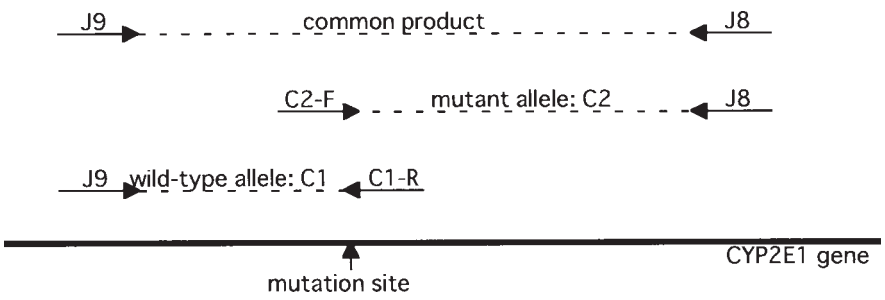

Fig. 1. AS-PCR. A common PCR product is amplified from both wild-type and mutant alleles by the primers J9 and J8. The primers, C1-R and C2-F, recognize the wild-type and mutant-allele sequence, respectively. Therefore, the PCR product by J9 and C1-R indicates the presence of a wild-type allele, while the product by C2-F and J8 is derived from a mutant allele. Each allele is depicted by its product size.

so that their 3′ ends would have the base specific to Pst I polymorphism located within the 5′-flanking region of the CYP2E1 gene. The C1-R primer recognizes "allele C1" which is a normal allele; the other primer, C2-F, recognizes "allele C2," the mutated allele.

Twenty-five µl of PCR reaction mixture consisting of 2.5 µl GeneAmp® 10 × PCR buffer (Perkin Elmer, Branchburg, NJ), 0.2 M of each of the dNTPs (Perkin Elmer), 500 nM J9, 500nM J8, 1,000 nM C1-R, 2,000 nM C2-F, 0.2 U Amplitaq Gold™ (Perkin Elmer), and sample DNA as a template were placed in each tube for the hot-start PCR. PCR was performed with an initial denaturation at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C for 80 seconds, annealing at 60°C for 60 seconds and extension at 72°C for 90 seconds, before a final extension at 72°C for 10 minutes.

PCR Restriction Fragment Length Polymorphism

According to the method reported by Hayashi et al. (3), conventional PCR was carried out using the same PCR reaction mixtures under the same cycling conditions as those used for AS-PCR, except that the allele-specific primers, C1-R and C2-F, were not used in the conventional PCR. PCR products were purified using DNA purification cartridges, SUPRECTM-02 (Takara Biochemicals, Japan), according to the manufacturer's recommendations. Purified PCR products were then suspended in 10 µl of distilled water, and 5 µl of each DNA sample were then digested by incubation with 1 µl of Rsa I (Nippon Gene, Japan) or 1 l of Pst I (Nippon Gene, Japan) plus their reaction buffer in 20 µl of reaction mixture at 37°C overnight. The samples were electrophoresed through a 2%-agarose gel at 100 V for 25 minutes, and visualized by staining with ethidium bromide.

PCR Direct Sequencing

To confirm these results, I then analyzed these individual mutations by PCR direct sequencing on an ABI PRISM™ 310 Genetic Analyzer (Perkin Elmer). Primer J8 was used to label fluorescent pigments. The procedure was performed according to the manufacturer's protocol.

RESULTS

The results of electrophoresis are demonstrated in Figure 2 that shows three discrete bands of differing sizes. The band of 413 bp is a common PCR product amplified by a primer pair of J9 and J8, the 319-bp band is derived from allele C2 and the 133-bp band is derived from allele C1. Therefore, Cases 1, 2, and 3 indicate the genotypes C1/C1, C2/C2 and C1/C2, respectively.

I applied the AS-PCR to 102 unrelated Japanese people in order to discern their genotypes. I found the C1/C1 genotype in 72 people (71%), C1/C2 in 26 (25%), and C2/C2 in 4 (4%). Normal and mutant alleles were estimated to be 0.83 and 0.17 in frequency, respectively. These results were compared with those of the PCR-RFLP method. In a case which demonstrated C1/C1 genotype by AS-PCR, the PCR product amplified by a primer pair of J9 and J8 was not digested by the restriction enzyme Pst I, but was completely digested by Rsa I, whereas a C2/C2 case genotyped by AS-PCR showed complete digestion by Pst I but no digestion by Rsa I. A C1/C2 case demonstrated incomple digestion by both Pst I and Rsa I. The results obtained by PCR-RFLP were in completely agreement with those obtained by AS-PCR (Fig. 2).

In addition, I analyzed the three genotypes by PCR direct sequencing in order to confirm these results (Fig. 3). All three

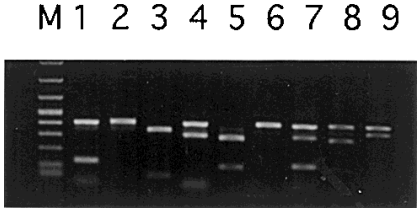

Fig. 2. AS-PCR analysis for CYP2E1 genotype. The PCR products from Case 1 show the C1 allele and common product (lane 1), whereas the PCR products from Case 2 show the C2 allele and common product (lane 4), while those from Case 3 show the C1 allele, C2 allele and common product (lane 7). These results were then compared with the results of RFLP analyses. Lanes 2, 5, and 8 are the results of Pst I digestion, and lanes 3, 6, and 9 are the results of Rsa I digestion, indicating that Cases 1, 2, and 3 have the genotypes C1/C1, C2/C2, and C1/C2, respectively.

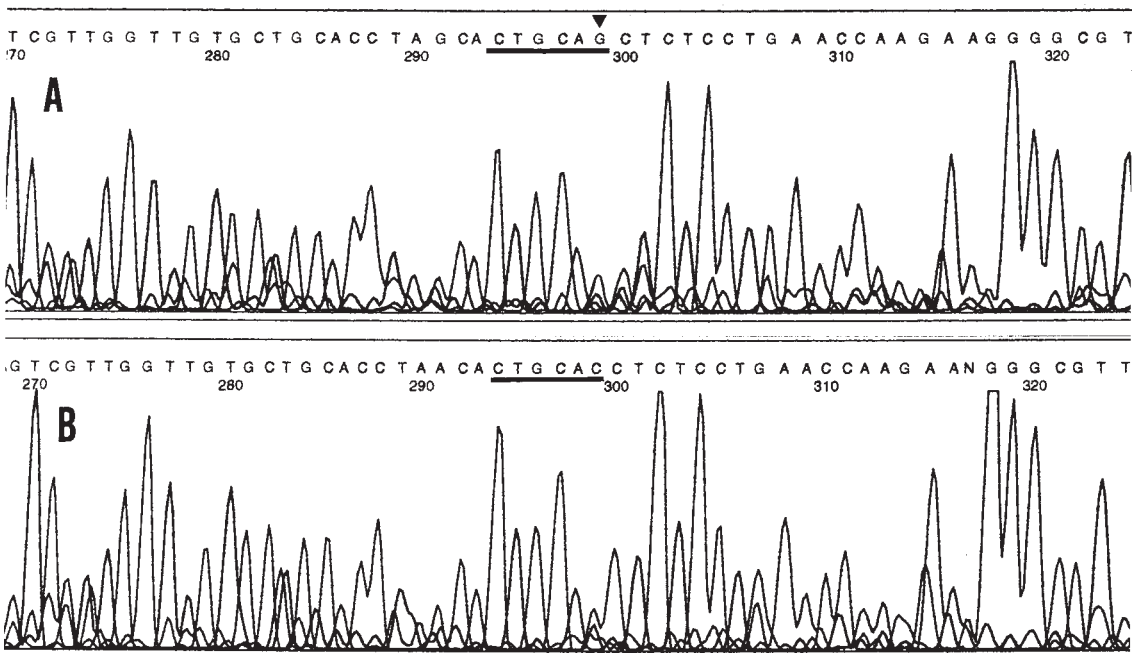

Fig. 3. Sequence of the PCR product. **A**, Direct sequence of the PCR product in a case with homozygous mutation of CYP2E1. The point mutation indicated by an arrow is involved in the Pst I site (underlined); **B**, Normal control.

methods described above, i.e., AS-PCR, PCR-RFLP, and PCR direct sequencing, were comparable and gave rise to the same results.

DISCUSSION

AS-PCR is a competitive multiplex PCR method. Although the procedure is easy, confirmation of successful PCR amplification is essential. Because a common PCR product is the largest one among the three bands and can be expected to be amplified in all cases, confirmation that PCR has been successfully performed should be established by the presence of this common PCR product.

To discriminate each PCR product by size, I designed allele-specific primers that can recognize the Pst I, but not the Rsa I, polymorphism. Using these primers, three bands derived from common PCR products, allele C1 and C2, were clearly distinguishable from each other according to the difference of their product size. Thus, a case that shows two bands derived from a common PCR product and allele C1 indicates genotype C1/C1, whereas bands from a common PCR product and allele C2 indicate C2/C2. When all three bands are derived from common PCR products, the presence of allele C1 and allele C2 indicates C1/C2, the heterozygote.

Genotyping of CYP2E1 was completed by a one-step-only PCR procedure. I compared the results obtained by AS-PCR with those obtained by PCR-RFLP, a standard method, in 102 individuals. The results were completely identical using the two methods, and were similar to the results of previous reports (12–14). I am thus convinced that AS-PCR, a novel method for the genotyping of CYP2E1, is of practical use in that it can be performed quickly, at little expense.

In conclusion, AS-PCR was found to be a novel but simple technique for investigating CYP2E1 genotype. This technique will no doubt be useful for research into the mechanisms behind alcoholic organ damage and chemical carcinogenesis.

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