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AN IMPROVED METHOD FOR GENOTYPING OF *N*-ACETYLTRANSFERASE POLYMORPHISM BY POLYMERASE CHAIN REACTION

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Summary Polymorphic *N*-acetyltransferase in human liver catalyzes *N*-acetylation of various arylamine-containing drugs and environmental chemicals. To accelerate the pharmacogenetic and ecogenetic studies of *N*-acetyltransferase polymorphism, we have developed a rapid and simple method for genotyping using a polymerase chain reaction based restriction fragment length polymorphism. This method distinguishes four kinds of allele of the *N*-acetyltransferase gene using a single polymerase chain reaction starting with a set of primers, followed by successive *Asp*718, *Bam*HI and *Taq*I digestions, and then running the samples on a single electrophoresis lane. This method allows us to determine ten different genotypes easily and reliably.

Key Words genotype, *N*-acetyltransferase, polymorphism, polymerase chain reaction

INTRODUCTION

N-Acetylation polymorphism is one of the most extensively studied pharmacogenetic traits (Weber and Hein, 1985; Evans, 1989). This polymorphism is caused by polymorphic *N*-acetyltransferase (NAT2) that catalyzes the *N*-acetylation of various arylamines and hydrazines. The enzyme is expressed in liver and distinguishable by its substrate specificity from the monomorphic *N*-acetyltransferase (NAT1) (Ohsako and Deguchi, 1990; Grant *et al.*, 1991). *N*-Acetylation polymorphism is related to drug toxicity and it is supposed to be associated with the susceptibility to colorectal cancer (Lang *et al.*, 1986; Ilett *et al.*, 1987) and urinary

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bladder cancer (Mommsen and Aagaard, 1986). NAT2 is produced by a single gene called *NAT2* (Ohsako and Deguchi, 1990; Blum *et al.*, 1990). We have found one 'rapid' human *NAT2* allele (allele 1) which produces the high activity enzyme, and two 'slow' alleles (allele 2 and 3) which produce the low activity enzymes (Deguchi *et al.*, 1990). Three NAT2 phenotypes *i.e.* slow, intermediate, and rapid acetylators were correlated with five genotypes generated by combinations of the 3 alleles (Deguchi *et al.*, 1990). Recently we showed that another allele which has no *Kpn*I site (Hickman and Sim, 1991; Blum *et al.*, 1991; Vatsis *et al.*, 1991) also exists among the Japanese, and is designated as allele 4 (Mashimo *et al.*, 1992). Subsequent study revealed that this allele has the same three nucleotide substitutions *i.e.* nt (nucleotide position according to Ohsako and Deguchi (1990)) 341, 481, and 803 as r_3 reported recently by Vatsis *et al.* (1991) (manuscript in preparation). Allele 4 is referred to as a 'slow' type allele (Vatsis *et al.*, 1991). Consequently *N*-acetylator phenotypes can be predicted by determining which combination of the 4 known alleles an individual has. Blum *et al.* (1991) and Hickman and Sim (1991) have reported different methods for genotyping the *NAT2* using polymerase chain reaction (PCR). However those methods not only seemed to be cumbersome, but also time consuming. In this paper, we describe an improved rapid and simple genotyping method using a PCR-based restriction fragment length polymorphism (RFLP).

MATERIALS AND METHODS

Isolation of Genomic DNA. Genomic DNA was extracted from peripheral leucocytes in 5 ml blood using phenol-chloroform followed by ethanol precipitation (Sambrook *et al.*, 1989).

Determination of the NAT2 Genotype. To amplify the 559 bp *NAT2* genomic sequence containing the polymorphic site near the 3′ end of the coding region (nt-803), sense (5′) TGACGGCAGGAATTACATTGTC (nt342–363) and antisense (3′) ACACAAGGGTTTATTTTGTTCC (nt879–900) oligodeoxynucleotides were synthesized by the Cyclone Plus DNA Synthesizer (Milligen/Biosearch, Burlington, MA). The PCR mixture contained 100 ng genomic DNA, 5 pmol of each oligonucleotide primer, dNTPs (each at 0.2 mM), 10 mM Tris-HCl (pH 8.8 at 25°C), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 0.9 unit *Taq* DNA polymerase (Wako, Osaka) in a total volume of 10 μl. The mixture was incubated for 5 min at 94°C to denature the genomic DNA, annealed for 50 sec at 55°C, and extended for 50 sec at 65°C. Each cycle of amplification was started by denaturing the DNA at 94°C for 50 sec, and the final extension period was for 7 min at 72°C. Twenty-five cycles of amplification were performed using a programmable heat block (Astec PC-700, Fukuoka). After amplification, a mixture of 5 units of *Bam*HI, *Asp*718 (isoschizomer of *Kpn*I) and *Taq*I were added to the PCR product (10 μl) directly. The mixture was incubated at 37°C for 3 hr and then incubated

at 65°C for 1 hr. The digested PCR products (5 μl) were electrophoresed at 7.7 V/cm for 60 min on agarose gel (3% agarose+1.5% Nusieve GTG agarose (FMC Bioproducts, Rockland, ME)) containing 0.5×TBE (1×TBE=90 mM Tris/90 mM boric acid/2 mM EDTA) and ethidium bromide.

Genotyping of NAT2 using a hair root as DNA source. A hair root, instead of extracted leucocyte DNA, was added into 10 μl PCR mixture without extraction of DNA. PCR cycle was increased to thirty, but other conditions were unchanged.

RESULTS

Figure 1 shows that the 4 *NAT2* alleles have the different restriction maps, specifically, *Taq*I, *Asp*718, and *Bam*HI have different sites on each allele. Am-

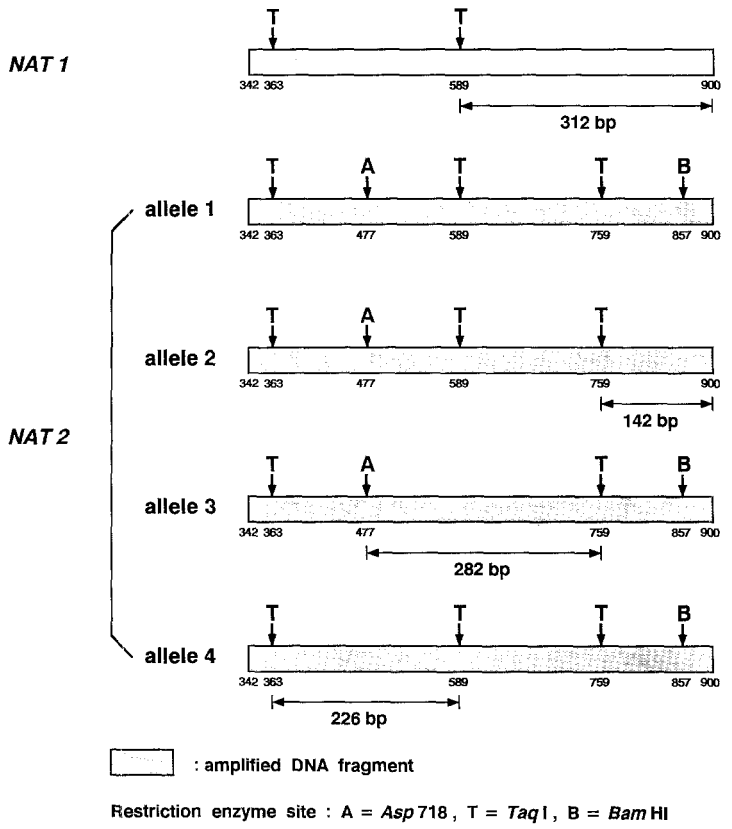

Fig. 1. Restriction enzyme sites of *NAT1* and of 4 types of *NAT2* alleles. Shadowed bars illustrate the 559 bp DNA fragment (nt342–900) of *NAT2* amplified by PCR, and open bar the corresponding region of *NAT1*. *NAT1* structure was cited from the articles of Deguchi (1992). Under the bars, nucleotide numbers are indicated. The arrows under DNA fragments of *NAT2* denote the specific bands for allele 2, 3, and 4.

plification of the *NAT2* genomic fragment with sense and antisense primers resulted in the formation of a 559 bp fragment from nt342 to 900 which includes the RFLP. The figure shows that PCR products would have to be cleaved with *Taq*I, *Bam*HI, and *Asp*718 to determine the types of *NAT2* alleles. Except for allele 1, specific bands for each allele could be easily recognized. For example, the presence of a 142 bp band (*Taq*I^{759} site to 3′ amplified terminal. Superscript number means actual cutting site of restriction enzyme) would signify allele 2, a 282 bp band (*Asp*718^{477} site to *Taq*I^{759} site) for allele 3, and a 226 bp band (*Taq*I^{363} site to *Taq*I^{589} site) for allele 4. If monomorphic NAT is amplified, a 312 bp band (*Taq*I^{589} to 3′ terminal) would emerge.

The electrophorestic pattern of PCR-based RFLP is shown in Fig. 2. We detected ten genotypes expected to appear from a combination of the 4 alleles; the genotype of allele 4/4 was made artificially from the allele 1/4 sample because the allele 4/4 was not found in our Japanese DNA samples. The phenotypes corresponding to the genotypes were indicated; rapid acetylator (the genotype of allele 1/1), intermediate acetylators (the genotypes of the pairs of allele 1 and the other allele) and slow acetylators (the genotypes of the combination of allele 2, 3, and 4). The heterozygotes for allele 1 were distinguished from the homozygotes of allele 2, 3, or 4 by the pattern of non-specific bands. A 312 bp band did not emerge.

We also determined *NAT2* genotyping using a hair root from 12 individuals

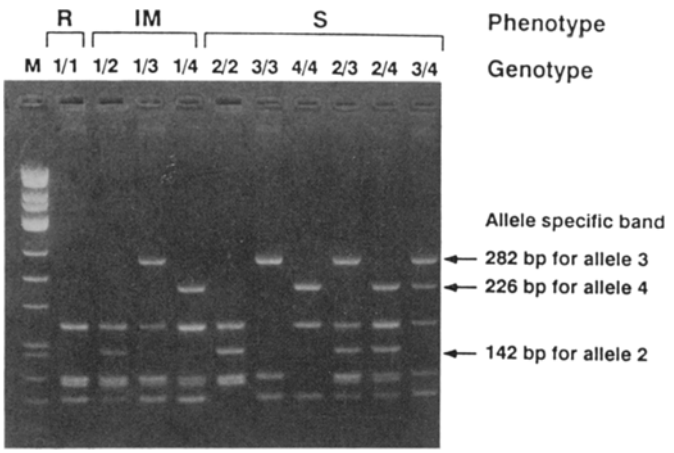

Fig. 2. The electrophoretic patterns of genotyping of *NAT2* by a PCR-based RFLP. A 559 bp *NAT2* genomic sequence (nt342–900) containing the polymorphic site near the 3′ end of the coding region (nt803) was amplified. PCR products were digested with *Bam*HI, *Asp*718 and *Taq*I, and separated on 3% agarose+1.5% Nusieve GTG containing ethidium bromide. Allele 4/4 was prepared artificially from allele 1/4 by digestion with *Asp*718 after amplification. Phenotypes shown in the figure mean acetylator status predicted from genotypes. R, rapid acetylator; IM, intermedate acetylator; S, slow acetylator; M, phaiX DNA markers digested with *Hin*fI.

as genomic DNA source. *NAT2* genotype could be determined in ten, but not in 2 samples because their DNA were not amplified (data not shown).

DISCUSSION

We have developed a *NAT2* genotyping method using a PCR-based RFLP. Recently we published a report using a Southern blot analysis for genotyping (Mashimo *et al.*, 1992). However, the PCR-based genotyping of *NAT2* seems superior to the Southern blot analysis because the PCR doesn't need radioactive materials and it takes only one day to make a PCR mixture and to take an UV irradiated photograph.

Blum *et al.* (1991) distinguished 4 types of *NAT2* using separate PCRs with three sets of allele specific primers specific to each mutation site. In the present study, we have used only one set of primers which amplifies all mutation sites since those sites are included in the recognition site of *Asp*718, *Bam*HI, and *Taq*I. Hickman and Sim (1991) also distinguished *NAT2* using the PCR with two sets of primers, and adopted three separate restriction enzyme digestions of the amplified products. In their study the *Hin*cII digestion was necessary to confirm that the *NAT2* had been amplified. In contrast with their study, our primers were designed to anneal to a less homologous region on *NAT1* which codes for the NAT1 protein. Although *NAT2* is highly homologous to *NAT1* (Blum *et al.*, 1990), the *NAT1* specific band was not identified in our electrophoresis. Our method included successive restriction enzyme digestions without changing the reaction buffer. Since these restriction enzymes worked only moderately in the *Taq* DNA polymerase buffer, we added an excess amount of enzymes. Even when the incubation time was prolonged to one day, the enzymes cut no other site than the correct recognition site. We could determine the *NAT2* genotype by agarose electrophoresis using only one lane per one genomic DNA sample. This method allows us to determine the *NAT2* genotypes rapidly, easily, and reliably. We applied this method to genotype 234 colorectal cancer patients without any trouble (to be submitted).

This method also worked when a hair root was used as genomic DNA source. Thomson *et al.* (1992) reported the superiority of the hair root as the DNA source for PCR. This method sometimes didn't work for an unknown reason, perhaps one trouble is caused by the amount of tissue attached to the hair root. When pupolation study of *NAT2* polymorphism is done, a hair root would be effective DNA source for genotyping.

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