Detection of C1236T, G2677T/A, and C3435T Polymorphism of MDR1 by Amplification Refractory Mutation System PCR

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C1236T, G2677T/A, and C3435T polymorphism of the multidrug resistance (*MDR1*) gene have substantial impact on expression or activity of P-glycoprotein (P-gp). We developed new methods based on amplification refractory mutation system (ARMS) to detect these polymorphisms. Tetra-primers amplification in a single tube was established to detect C1236T and C3435T polymorphism. For G2677T/A polymorphism, a two-step allele-specific amplification method was used. *MDR1* genotypes of 177 Chinese subjects were determined by the methods we established.

The methods we established were verified with gene sequencing. Gene frequencies of 1236C and 1236T were 37.8 and 62.2%, respectively; gene frequencies of 2677G, 2677T and 2677A were 44.1, 38.4 and 17.5%, respectively; the gene frequencies of 3435C and 3435T were 65.0 and 35.0%, respectively. The results were similar with other studies on Oriental subjects. The methods we established are simple, accurate, and economical, and can provide reliable approaches for determining *MDR1* polymorphism. *J. Clin. Lab. Anal.* 23:110–116, 2009. © 2009 Wiley-Liss, Inc.

Key words: multidrug resistance gene (*MDR1*); genotype; amplification refractory mutation system (ARMS); tetra primers amplification; allele-specific amplification (ASA)

P-glycoprotein (P-gp) is an ATP-dependent efflux transporter, which was identified initially in tumor cells associated with an acquired cross-resistance to multiple cytotoxic anticancer agents (1). Subsequently, studies proved that P-gp is also expressed in many normal tissues, including canalicular surface of hepatocytes, the apical surface of proximal tubular cells in kidneys, the brush border surface of the intestine, and endothelial cells at blood–brain barrier (1–4). P-gp protects host from exposure to environmental or dietary toxins by excreting these compounds into the bile, urine, and the intestinal lumen (5,6). P-gp transports a broad range of substrates that include some antibiotics, immunosuppressants, and chemotherapeutic agents and many more (7). Although many of these substrates are structurally unrelated, most of them have lipophilic groups and hydrogen bonds, which are essential in their interactions with P-gp. Recent studies have suggested that P-gp and other transporters play a fundamental role in regulating apoptosis and immunology and are related to susceptibility to certain diseases. P-gp is encoded by the

multidrug resistance gene (*MDR1*), which is located in the human chromosome 7 band q21–21.1 and consists of 28 exons (8). Over 48 single nucleotide polymorphisms (SNPs) of *MDR1* gene have been found from studies of various populations (9). Of these, the C1236T of exon 12, G2677T/A of exon 21, and C3435T of exon 26 are most frequently studied SNPs of *MDR1*. C1236T and C3435T are silent polymorphisms, whereas G2677T/A result from the amino acid exchanges G2677T (Ala893-Ser) to G2677A (Ala893Thr). Hoffmeyer et al. found that individuals homozygous for 3435T had significantly lower P-gp expression levels than homozygous 3435C

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carriers (10). Further studies pointed out that G2677T/A and C1236T also seem to influence P-gp function (11). Genetic polymorphism of *MDR1* is thought to have substantial effect on the pharmacokinetics and pharmacodynamics of many drugs (12). The rate and extent of absorption of digoxin, fexofenadine, and cyclosporine for instance may be influenced by various *MDR1* polymorphisms. It can be inferred that genotyping for *MDR1* polymorphisms may be valuable.

In this study, we developed new methods based on amplification refractory mutation system (ARMS) for rapid and reliable genotyping of the C1236T, G2677T/A, and C3435T polymorphisms in the *MDR1* gene.

METHODS

Subjects

One hundred and seventy seven Chinese volunteers (98 male, 79 female; age: 42.4 ± 13.4 year, weight: 53.3 ± 7.8 kg) were enrolled in this study. The Ethics Committee of Ruijin hospital approved the study. Written informed consent was obtained from each subject. Blood samples of subjects were drawn into sterile tubes containing sodium citrate. Leukocyte DNA was extracted by the phenol-chloroform method and dissolved in TE buffer.

Genotyping

We established tetra-primers amplification methods to detect C1236T and C3435T polymorphism. The primers (synthesized by Invitrogen Co. Ltd, Shanghai, China) were designed using Primer 5 program (Table 1). Twenty five microliter of the reaction system contained 15–50 ng DNA, 0.5 U Heat start Taq DNA polymerase (Bio Basic Co. Ltd, Ont., Canada), $1 \times$ PCR buffer, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.3 μ mol/L of each flanking primers (1236P1 and 1236P2 for C1236T, or 3435P1 and 3435P2 for C3435T), and 0.6 μ mol/L of each allele-specific primers (1236C and 1236T for C1236T, or 3435C and 3435T for C3435T). The reaction was programmed to occur as follows: 15 min at 94°C; followed by 15 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 60 s with a final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis with 2% agarose gels.

A two-step allele-specific amplification (ASA) was established to detect G2677T/A polymorphism. In the first step, 25 μ l reaction mixture contained 15–50 ng genomic DNA, 0.5 U of Taq DNA polymerase, $1 \times$ buffer, 0.2 mmol/L of dNTP mixture, 1.5 mmol/L $MgCl_2$, 0.5 μ mol/L of each primer (2677P1 and 2677P2) (Table 1). The reaction was set to occur as follows: 7 min at 94°C; followed by 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; a final extension at 72°C for 7 min. In the

second step, ASA was developed in three tubes for detection of G2677, T2677, and A2677, respectively. One microliter of diluted (1:10) product of first step was used as template, 0.5 U of Heat start Taq DNA polymerase, 0.5 μ mol/L of primers (general primer 2677P1 and AS primers 2677G, 2677T, or 2677A in each tube) together with other reagent similar with first step. To enhance the specificity of the reaction, 0.04% Tween-20, and 1.25 mmol/L tetramethyl ammonium chloride (TMA) were added in the reaction system. The reaction was carried out as: 15 min at 94°C; followed by 15 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis with 2% agarose gels.

Assay Verification

Samples of various *MDR1* genotypes were selected, and all the samples were analyzed five times to verify the results. At the same time, genotypes of all the samples were confirmed by sequence analysis, including various *MDR1* genotypes. Specific regions containing C1236T and C3435T were amplified using flanking primers. For G2677T/A, specific regions were amplified using the primer 2677P1 and 2677P2. The obtained DNA fragments were sequenced with the help of Invitrogen Co. Ltd.

MDR1 genotypes of three SNPs were determined by the methods we established. Verified samples were used as positive controls and added in each run of PCR. Negative quality controls were generated by inclusion of all reagents except the DNA in a PCR tube for each allele evaluated.

Statistical Analysis

Ninety-five percent confidence intervals (CIs) for the frequency of three SNPs of *MDR1* were determined. The distribution of genotypes expected assuming Hardy-Weinberg equilibrium was calculated from the allele frequencies. χ^2 test was used to compare the observed genotype incidence with the expected genotype incidence. χ^2 test was also used to compare the allele frequencies of three *MDR1* SNPs with previous findings in other ethnic groups. $P < 0.05$ was considered statistically significant.

RESULTS

Detecting C1236T and C3435T Polymorphism Using Tetra-primers Amplification Methods

In the first set of cycles, general amplicons of 508 bp for C1236T and 300 bp for C3435T were obtained. In the second set of cycles, allele-specific primers produced 305 bp amplicon for C1236 and 238 bp amplicon for T1236 (Fig. 1A); 126 bp amplicon for C3435 and 209 bp amplicon for T3435 (Fig. 1B).

Detecting G226T/A Polymorphism Using the Two-Step ASA Method

In the first step, a 225 bp amplicon was obtained and used as the template of the second step. In the second step, one of the three tubes produced a 163-bp amplicon, indicating that the genotype was homogenous for G2677, A2677, or T2677. Obtaining amplicons in two

of the three tubes indicated the presence of the heterozygous genotype GT, GA or AT (Fig. 1C).

Twenty-two subjects with various *MDR1* genotypes were selected for the sequencing. The results of all subjects confirmed the tetra-primers and ASA analysis we established (Fig. 2). The duplicated genotyping of these samples showed perfect consistence (data not shown). The results suggested the reproducibility of the assays is satisfied.

TABLE 1. Primer Sequences for Determining Genotype of C1236T, C3435T, and G2677T/A

Name	Primer sequence	Length	Fragment	T_m
1236P1	5' AAT GTT CAC TTC AGT TAC CCA TCT CG 3'	26		74
1236P2	5' AAT GAT TTC CCG TAG AAA CCT TAC 3'	24	508	66
1236C	5' TGG TAG ATC TTG AAG <u>CGC</u> 3'	18	305	54
1236T	5' TGC ACC TTC AGG TTC <u>TGA</u> 3'	18	238	54
3435P1	5' TGC TGG TCC TGA AGT TGA TCT GTG AAC 3'	27		80
3435P2	5' GGC CAG AGA GGC TGC CAC AT 3'	20	300	66
3435C	5' GTG TCA CAG GAA GAG <u>TTC</u> 3'	18	126	54
3435T	5' TCC TTT GCT GCC CTC <u>TCA</u> 3'	18	209	54
2677P1	5' TAC CCA TCA TTG CAA TAG CAG 3'	21		60
2677P2	5' GAA AAA GAT TGC TTT GAG GAA TGG 3'	24	225	66
2677G	5' GAT AAG AAA GAA CTA GAA <u>GCT</u> G 3'	22	163	60
2677T	5' GAT AAG AAA GAA CTA GAA <u>GCT</u> A 3'	22	163	58
2677A	5' GAT AAG AAA GAA CTA GAA <u>GCT</u> T 3'	22	163	58

Nucleotides with underline indicate deliberate mismatch nucleotide.

Fig. 1. Gel electrophoresis of various *MDR1* C1236T, C3435T, or G2677T/A genotypes. (A) C1236T polymorphism: 508 bp general amplicon; Allele-specific amplification produces 305 bp amplicon for 1236C and 238 bp amplicon for 1236T. (B) C3435T polymorphism: 300 bp general amplicon; Allele-specific amplification produces 126 bp amplicon for 3435C and 209 bp amplicon for 3435T. (C) G2677T/A polymorphism: lower layer: GG, GT, and TT genotype; upper layer: AA, GA, and AT genotype, respectively.

Fig. 2. Gene sequences of *MDR1*, C1236T, C3435T, or G2677T/A genotypes.

Determining the Genotypes of the 3 *MDR1* SNPs in the 177 Subjects

There are 23, 88, and 66 subjects with 1236CC, CT, and TT genotype, the gene frequencies of 1236C and 1236T were 37.8 and 62.2% respectively. There are 39, 48, 32, 4, 30, and 24 subjects with 2677GG, GT, TT,

AA, AG, and AT genotype, respectively, therefore, gene frequencies of 2677G, 2677T, and 2677A were 44.1, 38.4 and 17.5%. Of the subjects, there are 69, 92, and 16 subjects with 3435CC, CT, and TT genotype, respectively. Interpreted as a gene frequency of 65.0% for 3435C and 35.0% for 3435T (Table 2). The distribution

TABLE 2. Gene Frequency of *MDR1*, C1236T, G2677A/T, and C3435T in Various Ethnic Populations

Population	No.	1236		2677			3435		
Present study		C	T	G	T	A	C	T	
		0.38 (0.31–0.45)	0.62 (0.55–0.69)	0.44 (0.42–0.47)	0.38 (0.36–0.41)	0.18 (0.15–0.20)	0.65 (0.58–0.72)	0.35 (0.28–0.42)	
<hr/>									
Oriental									
Chinese	104	0.31	0.69	0.50	0.44	0.06	0.60	0.40	13
Japanese	48	0.35	0.65	0.36	0.42	0.22	0.51	0.49	14
Korean	632			0.44	0.39	0.17	0.61	0.39	15
Vietnamese	142			0.58	0.36	0.06	0.63	0.37	15
Total	926	0.32	0.68	0.46	0.39	0.15	0.61	0.39	
		$\chi^2 = 3.61, P > 0.05$			$\chi^2 = 4.79, P > 0.05$		$\chi^2 = 2.27, P > 0.05$		
Caucasian									
The United States	37	0.58	0.42	0.54	0.46	0	0.46	0.54	11
Germany	461	0.59	0.41	0.56	0.42	0.02	0.46	0.54	16
Germany	67	0.66	0.34	0.56	0.49	0.04	0.49	0.51	17
Total	565	0.60	0.40	0.56	0.43	0.02	0.46	0.54	
		$\chi^2 = 59.73, P < 0.05$			$\chi^2 = 124.84, P < 0.05$		$\chi^2 = 37.31, P < 0.05$		
African	23	0.85	0.15	0.85	0.15	0	0.74	0.26	11
		$\chi^2 = 36.04, P < 0.05$			$\chi^2 = 32.91, P < 0.05$		$\chi^2 = 1.41, P > 0.05$		

of various genotypes was consistent with the Hardy–Weinberg equilibrium ($\chi^2 = 0.924$, $df = 2$, $P = 0.630$ for C3435T; $\chi^2 = 4.228$, $df = 5$, $P = 0.517$ for G2677T/A; and $\chi^2 = 1.246$, $df = 2$, $P = 0.536$ for C3435T).

DISCUSSION

Nearly all SNP genotyping techniques currently in use depend on PCR to obtain target DNA sequence and the subsequent means of differentiating alleles vary among different assays. Commonly used methods include restricted fragment length polymorphism (RFLP), DNA sequencing, Allele-specific oligonucleotide (ASO), LightCycler melting analysis, and ASMS. RFLP, considered the gold standard in detecting genetic polymorphism, is the most frequently used method in the genotyping of *MDR1* (10,11). It involves restriction endonuclease digestion of PCR products. However, this method is time-consuming and labor-intensive as an additional digestion step is needed. Besides, many restriction endonucleases are expensive. DNA sequencing and LightCycler melting analysis are thought to be fast and elegant for genotyping (13,14), but the expensive apparatus needed prevents their wider use. Nakamura et al. designed an electrochemical DNA chip for the genotyping of 4 *MDR1* SNPs simultaneously (15). This method was shown to be accurate and suitable for large-scale studies. However, it also requires special instruments and it is costly.

ARMS employs a series of methods with similar principles. Allele-specific primers are designed comple-

mentary to a given DNA sequence except for 3' residue, which was consistent with the different genotypes. A mismatch between the 3'-OH residue of primer and template compromises the integrity of the sequence generated because of the absence of 3'-exonucleolytic proofreading activity associated with Taq DNA polymerase (16). Tetra-primers amplification and ASA are all based on the principle of ARMS. These methods have been widely used in the genotyping and have been proved to be reliable and robust (17,18). This is the first study that examines the use of ARMS in detecting *MDR1* SNPs. We established tetra-primers PCR method for the genotyping of C1236T and C3435T polymorphism. For each assay, a pair of flanking primers was used together with two allele-specific primers. We designed long-flanking primers and short internal primers. The first set of cycles was performed with higher annealing temperature than subsequent cycles (17). This is valuable in the enhancement of specificity. We attributed specific amplification results obtained in our study to: reduced unspecific amplification by using hot start enzyme and specific reagents; optimized annealing temperature and number of cycles by repeated tests; and finally, a reduced stability of the primer template duplex by designing mismatches in allele-specific primers as some of mismatched pairings at the 3' end of the primer/template duplex (i.e., T:C, T:G, and T:T) are also amplified efficiently. Studies show that primers become increasingly refractory to annealing as the additional mismatch is moved closer to the 3' end. In this study, we designed deliberate mismatch primers at

the third base from the 3' end and obtained specific results. Compared with other methods used for genotyping, the apparatus used in our methods are simple and relatively cheap. At the same time, less time is needed. With the proposed method, results of genotyping can be obtained in about 5 hr. Although the assays we established cannot be used in high throughput genotyping, they are suitable in the small labs without complex apparatuses to study *MDR1* genotypes.

SNPs in exons 26 (C3435T), 21 (G2677T/A), and 12 (C1236T) have been characterized in a number of ethnic populations (11,13,19–22). The populations of the studies can be classified as Orientals, Caucasians, and Africans. We pooled the data of studies with subjects from similar areas. To determine whether available data from each individual study could legitimately be pooled with the other studies, homogeneity across the studies was assessed with a standard χ^2 test on $2 \times n$ -contingency tables. Lack of a significant χ^2 test indicated the homogeneity of eligible data for pooled analysis (23). The results showed that there was a significant homogeneity occurred in Orientals and Caucasians of 3 *MDR1* SNPs. Hence, we compared the gene frequency in our study with the data of Orientals, Caucasians, and Africans. Our studies suggested that the frequency distribution of 1236T is 62%, which is similar to other studies on Orientals (65–69%) ($P > 0.05$) and higher than Caucasians (37.8–42%) or African-Americans (15%) ($P < 0.05$). The frequency of 2677T is 38%, similar to other studies on Orientals (36–44%) and Caucasians (40–46%) and higher than Africans (15%); the frequency of 2677A is comparable with Orientals (18% vs. 6–22%) and significantly higher than Caucasians (0–4%) and Africans (0%). As for the exon 26 C3435T, the frequency of 3435T in this study (35%) is similar to Orientals (37–49%) and significantly higher than Africans (26%). The results suggest that gene frequency of 3 *MDR1* SNPs is comparable with previous studies in Oriental population and has significant difference compared with Caucasians and Africans.

The methods we established are simple, economical, and accurate. They may be useful to carry out the large population-based epidemiologic studies of *MDR1* genotypes. Moreover, as the *MDR1* genotypes are important in pharmacokinetic and pharmacodynamic of many drugs, this method may be helpful in the optimizing therapy.

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