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• BASIC RESEARCH •

Rapid detection of the known SNPs of CYP2C9 using oligonucleotide microarray

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Abstract

AIM: Cytochrome P450 2C9 (CYP2C9) is a polymorphic enzyme responsible for the metabolism of a large number of clinically important drugs. Individuals with mutant enzymes may risk serious side effects under routine therapy with certain drugs metabolized by CYP2C9. In order to facilitate the detection of the known SNPs of CYP2C9, an allele-specific oligonucleotide (ASO) based microarray was made.

METHODS: An oligonucleotide microarray was made to facilitate the SNP (single nucleotide polymorphism) screening and was applied for the detection of CYP2C9 polymorphism in 62 high blood pressure (HBP) patients who received Irbesartan for treatment. Part of the genotyping results was confirmed by direct sequencing. And the relation between CYP2C9 polymorphism and therapeutic outcome of Irbesartan was statistically analyzed.

RESULTS: Heterozygous alleles of CYP2C9*1/*3 were found in 7 out of 62 subjects. No mutant alleles of CYP2C9*2, *4 and *5 and no homozygous mutant alleles were detected. The 7 heterozygous CYP2C9*1/*3 and 13 random wild type DNA samples were subjected to direct sequencing with purified PCR products and same genotyping results were obtained with the 20 DNA samples. There was no significant difference in the odds of effectiveness of Irbesartan between the wild type (normal) group and CYP2C9*1/*3 (mutant) group ($P>0.05$).

CONCLUSION: The oligonucleotide microarray made in this study is a reliable assay for detecting the CYP2C9 known alleles and the heterozygous CYP2C9*1/*3 has no significant effects on the therapeutic outcome of Irbesartan.

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INTRODUCTION

Pharmacogenetics was established on the fact that certain genetic polymorphisms may cause significantly different responses among individuals on exposure to a particular drug[1-3]. Recent advances in the understanding of the molecular genetics of drug-metabolizing enzymes (DME), particularly cytochrome P450, have enabled the molecular basis of many polymorphisms to be elucidated and the genotyping assays to be developed[4-6].

Cytochrome P450 is one of the key enzymatic mechanisms for the metabolism of drugs, pesticides, environmental pollutants, and carcinogens[7-9]. In this superfamily, CYP2C9[10-12] is a polymorphic enzyme responsible for the metabolism of a large number of clinically important drugs such as S-warfarin, phenytoin, tolbutamide, losartan and nonsteroidal anti-inflammatory drugs. To date, 5 alleles of CYP2C9 including the wild type CYP2C9*1 and the mutants CYP2C9*2 (430C-T), CYP2C9*3 (1075A-C), CYP2C9*4 (1076T-C) and CYP2C9*5 (1080C-G) have been found (www.imm.ki.se\cypalleles). In the four mutant alleles, single nucleotide variations in the exon 3 and exon 7 cause amino acid substitutions Arg144Cys, Ile359Leu, Ile359Thr and D360E, respectively, and therefore lead to a slow metabolizing capacity of the enzymes. The altered pharmacogenetics may result in prolonged or shortened effect time. The individuals with mutant enzymes risk serious side effects under routine therapy with certain drugs metabolized by CYP2C9. So frequent variants of CYP2C9 should be analyzed in participants of clinical trials where the enzymes may play a key role.

In order to facilitate the detection of the known SNPs of CYP2C9, an ASO (allele-specific oligonucleotide) hybridization based microarray was made, which could simultaneously screen the 4 mutant alleles of CYP2C9 of 10 individuals, and was applied for the detection of CYP2C9 polymorphism in 62 hypertension patients who received Irbesartan for treatment. Irbesartan is a selective antagonist of the AT1 receptor of angiotensin II receptor (AT1R) used in the treatment of hypertension and congestive heart failure[13,14]. Previous studies indicate that Irbesartan is mainly metabolized by CYP2C9 to the inactive form[15].

MATERIALS AND METHODS

DNA samples

A total of 62 peripheral blood samples were collected from unrelated HBP patients who received Irbesartan for treatment and the therapeutic outcome was classified as outstanding (11 persons), effective (38 persons) and failed (13 persons). Genomic DNA was extracted with the Genomic DNA purification kit (Promega) and quantified by UV spectrophotometer (DU® 640, Beckman coulter).

Oligonucleotides synthesis

Oligonucleotides (primers or probes) were synthesized using automatic DNA synthesizer (ABi 391A). For signal detection, the reverse primers were fluorescein (Cy3) labeled at 5' end. A probe was synthesized with the 3' end amino-modified to have a primary NH_2 group for immobilization onto aldehyde-coated slides, and the NH_2 group was linked by a polyethleneglycol spacer to a specific allele-discriminating sequence, which was 16-17 nucleotides in length with a nucleotide complimentary to either the normal or mutant allele in the middle of the sequence. A list of oligonucleotides used in this study is presented in Table 1.

Table 1 Oligonucleotides used in this study

##	Sequence (5'--3')	Application
C1	CAC ATG GCT GCC CAG TGT CAG CTT C	Primers used to amplify exon3 and exon7 fragments of
C2*	GGC CAC CCC TGA AAT GTT TCC AAG	CYP2C9 containing SNP sites. C2* and C4* were
C3	ACG TGT GAT TGG CAG AAA CCG GAG C	fluorescein (Cy3) labeled at 5' end.
C4*	GGG ACT TCG AAA ACA TGG AGT TGC AG	
C1Tf	ATT GAG GAC TGT GTT CAA GAG GAA GC	Primers used to construct mutant templates.
C1Tr	CTT CCT CTT GAA CAC AGT CCT C	The variant bases (indicated by underlines)
C2Tf	CCT ACA CAG ATG CTG TGG TGC AC	were introduced when synthesized.
C2T1	CTG GTG GGG AGA AGG TCA AGG TAT CTC	
C2T2	CTG GTG GGG AGA AGG TCA GTG TAT CTC	
C2T3	CTG GTG GGG AGA AGC TCA ATG TAT CTC	
P1	TTG AGG ACC GTG TTC AA - spacer- NH_2	Pairs of probes with one base difference (indicated
P2	TTG AGG ACT GTG TTC AA - spacer- NH_2	with underline) for SNP discrimination, immobilized
P3	GAG ATA CAT TGA CCT TC - spacer- NH_2	on aldehyde-coated slides surface with
P4	GAG ATA CCT TGA CCT TC - spacer- NH_2	3' end primary NH_2 group.
P5	GAG ATA CAT TGA CCT TC - spacer- NH_2	
P6	GAG ATA CAC TGA CCT TC - spacer- NH_2	
P7	TAC ATT GAC CTT CTC C - spacer- NH_2	
P8	TAC ATT GAG CTT CTC C - spacer- NH_2	

PCR amplification

The 2 target segments containing the SNP sites to be typed were amplified by PCR in one tube. Asymmetric PCR method was used to generate single-stranded target segments. The ratio of forward primer to reverse primer (fluorescein labeled) was optimized at 1:40 in a PCR reaction (data not shown). Reaction mixtures of 20 μL contained 100 μM dNTP, 0.5 μM forward primer, 20 μM reverse primer, 100 ng of a genomic DNA or 40 ng of a plasmid DNA, 1* PCR buffer and 1 U Taq enzyme. Amplification was conducted in a thermal cycler (PTC-100™ programmable thermal controller, MJ. Research Inc) under the following conditions: initial denaturation (5 min at 94 ℃) followed by 40 cycles of denaturation (30 sec at 94 ℃), annealing (30 sec at 62 ℃) and extension (30 sec at 72 ℃). A final extension step was carried out for 5 min at 72 ℃. The PCR products were analyzed by 2 % agarose gel electrophoresis.

Construction of standard templates

DNA segments of wild type or mutant CYP2C9 were subcloned to PGEM®T4-vector (Promega) according to the protocol. The plasmids were used as standard wild type or mutant templates for the optimization of hybridization conditions and establishment of signal intensity ratio of match to mismatch after verification by sequencing. The artificial heterozygous templates were constructed by mixing equal amounts of wild type and mutant plasimid DNA. To introduce a mutant nucleotide at specific position in a DNA segment, site-directed mutagenesis method[16] with mutant primers was used.

Preparation of DNA microarrays

The 3' end amino-modified probes were diluted to a final concentration of 20 μmol/L in spotting solutions (3*SSC and 0.01 % SDS). The spotting solutions were transferred into 96-well plates in volumes of 10 μL and spotted to aldehyde-coated glass slides with a microarray printer (Cartisan), which deposited 0.5 nL at each spotting site, resulting in spots of 200 μm in diameter. Each probe was spotted in duplicate. The humidity during spotting was 70 % and the temperature was kept at 23 ℃. After spotting, slides were incubated for another 1 h under the same conditions and stored at room temperature for at least 1 day before use. The pattern of slide and array format are shown in Figure 3.

Hybridization and signal detection

Two μL of the single-stranded Cy3-labeled target PCR products was mixed with 10 μL hybridization solution (5*SSC, 0.1 % SDS, 1 % salmon DNA), and the 12 μL final volume was transferred to the hybridization area on the glass slide. The slide was incubated in 40 ℃ water bath for 30 min in a hybridization chamber. After incubation, the slide was washed sequentially in washing solution A (1*SSC, 0.2 % SDS), washing solution B (0.2*SSC) and washing solution C (0.1 *SSC) for 1 min each.

The glass slides were scanned using the confocal Scanarray 3 000 (GSI Lumonics), with excitation at 540 nm and emission at 570 nm (Cy3). Sixteen-bit TIFF images of 10 μm resolution were analyzed. After subtraction of local background, the average signal intensity of the duplicate spots of each probe was used to calculate the signal ratios defining the genotypes.

Direct sequencing with PCR products

Part of the DNA samples was subjected to direct sequencing using DNA sequencer (CEQ™ 2000XL DNA analysis system, Beckman) to confirm the results. PCR reaction was carried out in 50 μL solution and the PCR products were purified to be the sequencing templates with PCR products purification kit (Promega).

Statistical analysis

Statistics was made by χ^2 test.

RESULTS

Determination of the signal intensity ratio of match to mismatch

According to the hybridization result of standard wild type or mutant plasmid templates, under the optimized hybridization and stringent washing conditions, there was a great difference in signal intensities between the perfect match and the single base mismatch probes. The ratio of match to mismatch of signal pairs was above 4 at least. Detection of heterozygous alleles was a hard point for microarray. In the present study, the hybridization results of the heterozygous templates showed that the signal intensity ratio of the probe pair corresponding to heterozygous alleles was below 2.5 (the ratio was always

the stronger to the weaker). Typical results are shown in Figure 1. Repeated experiments with genomic DNA gave a statistically similar result (data not shown). So the ratio value above 4 or below 2.5 was considered sensible for genotype judgement while sample with the ratio value within 2.5-4 should be re-genotyped.

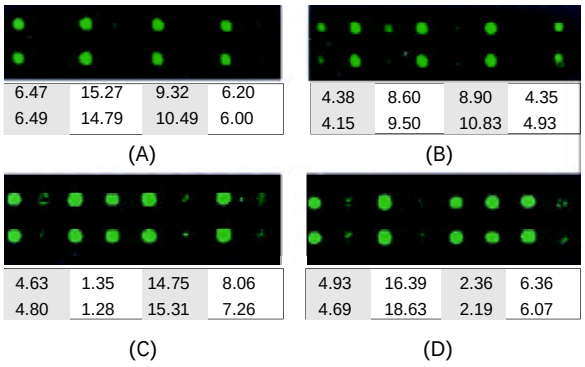

Figure 1 The TIF image of hybridization results of (A) wildtype template, (B) mutant template, (C) heterozygous template of CYP2C9*1/CYP2C9*3, and (D) heterozygous template of CYP2C9*1/CYP2C9*4. The discriminating power of a pair of probes was represented by the ratio of match to mismatch listed below.

Genotype results of 62 DNA samples by DNA microarray

In the genotype result of the 62 samples determined by the microarray, heterozygous alleles of CYP2C9*1/*3 were found in 7 out of 62 subjects. No heterozygous or homozygous mutant alleles of CYP2C9*2, *4 and *5 were detected. Repeated experiments gave the same result. A brief procedure is illustrated in Figure 2 and Figure 3.

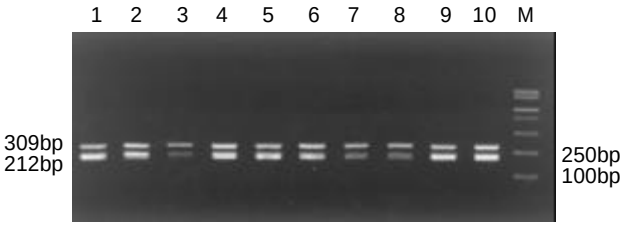

Figure 2 2 % agarose gel electrophoresis of the PCR products of 10 samples. 1-10: PCR products of 10 DNA samples, M: DGL2000 marker.

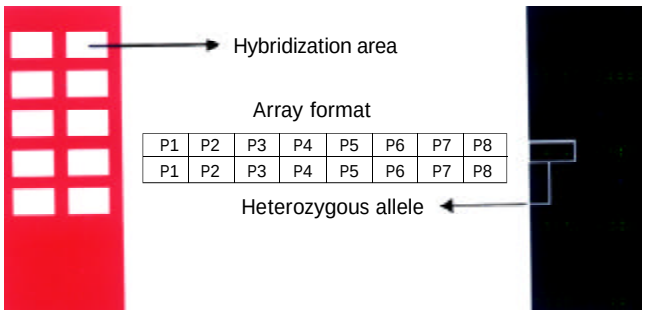

Figure 3 Array format and microarray hybridization result of 10 samples.

DNA samples genotyped by direct sequencing

To confirm the genotype result determined by microarray, the 7 heterozygous CYP2C9*1/*3 and 13 random wild type DNA samples were subjected to direct sequencing with purified PCR products. The same genotype results were obtained with the 20 DNA samples typed with two methods. The typical sequencing results of the heterozygous CYP2C9*1/*3 and wild type are compared in Figure 4.

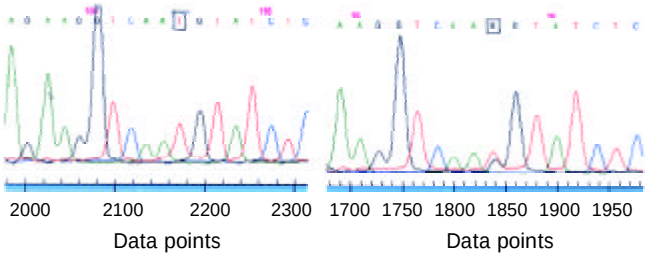

Figure 4 Part of the sequencing results. The letters in square indicate wildtype and heterozygous of CYP2C9*1/CYP2C9*3 (1075A-C) in two samples.

Allele frequency and effects of CYP2C9 polymorphism on therapeutic outcome of Irbesartan

The microarray described here is a reliable assay for detecting the CYP2C9 known alleles. The analysis of 62 HBP patients DNA samples yielded frequencies for CYP known alleles (Table 2) that were in agreement with previous study[7,17,18]. There was no significant difference in the odd of effectiveness of Irbesartan between the wild type (normal) group and the CYP2C9*1/*3 (mutant) group (Table 3, $P>0.05$).

Table 2 CYP2C9 allele frequency in the study population

Allele	Number of alleles	Frequency %
*2	0/124	0
*3	7/124	5.6
*4	0/124	0
*5	0/124	0

Table 3 Therapeutic outcome of Irbesartan in wild type group and CYP2C9*1/*3 group

	Effective	Failed
Wild type	43	12
CYP2C9*1/*3	6	1

$P>1$ (χ^2 test), no significant difference in the odd of effectiveness of Irbesartan between wild type group and CYP2C9*1/*3 group.

DISCUSSION

Single nucleotide polymorphism (SNP) is the most common variation form in human genome[19-21]. The early methods to type SNPs include SSCP, RFLP, AS-PCR, etc[22-24], making genotyping judgement coupled with gel electrophoresis analysis. The methods above are unfit for a large scale screening due to the limitation of detection methods. With the advance of organic fluorescence labeling and detection technology, significant change has taken place in genotyping technology. Molecular beacon[25], TaqMan[26] probe methods, which detect fluorescence signal in homologous reaction, and DNA microarray[27-29], which is based on hybridization coupled with solid phase reaction, make genotyping easier and more accurate. In the newly developed technologies, DNA microarray is more preferable in the genetic linkage and association study between large number of genetic markers and phenotypic traits in pharmacogenomics, disease genomics etc, due to its parallel detection of a large quantity of genetic markers.

Fluorescence-labeled sample preparation is a critical step in microarray genotyping. The quality and amount of genomic DNA used as template in PCR reaction are essential to the hybridization results. In the present study, the two target segments containing the SNP sites to be typed were PCR amplified in one tube. Asymmetric PCR method was used to generate single-stranded target segments complimentary to the probes.

The fluorescence signal intensity and discriminating power (ratio of match to mismatch) of the probe pairs are the key results from microarray for genotyping judgement, which can be affected by many factors. When other conditions (the quality and amount of single stranded PCR products, the quality of aldehyde-coated slides, hybridization/washing conditions, etc) are strictly controlled, the specific sequence context of the probes is the determining factor. Because there was no simultaneous mutation reported in the CYP2C9 known alleles, the probes were designed without consideration of crosslink of SNP sites of 1075A-C, 1076T-C, 1080C-G, any two of which were assumed normal when the other one was set as SNP site to be typed. The assumption reduced the number of probe to be designed and was confirmed by the genotyping result in the present study. Four pairs of probes were designed to discriminate the normal and the four mutant alleles. However, as an improvement to probe redundancy, all possible probe patterns should be fabricated. In probe design, the probes were chosen to have a common algorithmically calculated Tm value of 50±2 ℃ with length of 16-17 nt. Under the optimized hybridization/washing conditions, the P1/P2 and P7/P8 probe pairs had a less intense signal and discriminating power than the P3/P4 and P5/P6 pairs. The signal intensity ratio value above 4 or below 2.5 is considered a critical limit for genotype judgement. When the value fell into 2.5-4, the sample should be re-genotyped.

The allele frequency of CYP2C9 determined in this study was in accordance with the published data. Yoon YR *et al*[17] showed that there were no CYP2C9*2 (430C-T) allele in Asian population. Dickmann *et al*[18] found CYP2C9*5 (1080C-G) only in Black people. In the present study, heterozygous CYP2C9*1/*3 (1075A-C) were detected in 7 out of 62 unrelated Chinese. There were marked ethnic/geographic differences in the distribution of allelic variants of many DMEs[30]. It is of obvious importance that such ethnic/geographic differences be taken into account in routine pharmacogenetic diagnosis or screening. Considering that it is challenging to optimize oligonucleotide probes to achieve global maximum discrimination of many genetic variants simultaneously, it is of importance to reduce the probe number to ensure the reproducibility and reliability of ASO hybridization based-microarray genotyping performance. For example, microarray for genotyping frequent SNP sites (>1 %) in DME-coding gene specific to Chinese should be made and applied in Chinese population.

There were no homozygous mutants detected in the studied population, and there may be other factors that affect the effectiveness of Irbesartan, such as the polymorphism in drug targets (ATIR), other potential mutation sites in CYP2C9, etc. The results of genotyping in this study suggest that there is no significant difference in the odd of effectiveness of Irbesartan between the wild type group and CYP2C9*1/*3 group. Provided that the heterozygous individuals have prolonged effective time, a better therapeutic outcome may be obtained as long as no additional side-effects occur.

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