Evaluation of a Fully Automated, Rapid Detection System for *CYP2C19* and *UGT1A1* Genotyping

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Background: The need for genotyping single nucleotide polymorphisms (SNPs) in genes encoding drug-metabolizing enzymes is increasing. Therefore, the recent focus has been on developing fully automated methods for the rapid and accurate measurement of SNPs.

Methods: We used the quenching probe (QP) method and i-densy IS-5310 to genotype 200 DNA specimens from 200 healthy Koreans and 100 whole blood from another 100 for the SNPs *CYP2C19**2 and *CYP2C19**3. We also performed genotyping of *UGT1A1**6 and *UGT1A1**28 with the above mentioned 200 DNA samples and 81 whole blood samples. The results of the assay were then compared to conventional direct sequencing.

Results: The allele frequencies of *CYP2C19* were 25.7% for *2 and 10.3% for *3, and those of *UGT1A1* were 17.3% for *6 and 11.2% for *28. These results are similar to those reported in previous studies on Korean populations. The *CYP2C19* and *UGT1A1* genotypes determined by the QP method perfectly matched (100.0%, $K=1.000$, $P<0.001$ for *CYP2C19*, and 99.6%, $K=0.992$, $P<0.001$ for *UGT1A1*) those determined by direct sequencing, barring a single exception for the *UGT1A1* genotype in 1 DNA specimen.

Conclusions: Our results suggest that the QP method, owing to its speed and ease of use, will enable rapid and sensitive diagnosis in clinical laboratories.

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Key Words : Genotyping, *CYP2C19*, *UGT1A1*, Single nucleotide polymorphism

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INTRODUCTION

Individualized drug therapy based on patients' genetic information has the potential to improve drug efficacy and reduce adverse drug reactions [1]. The knowledge of genetic polymorphisms in genes encoding drug-metabolizing enzymes, transporters, receptors, and other drug targets can be clinically applied to help overcome the variability of drug responses among individuals. For example, drugs such as proton pump inhibitors are eliminated through the hepatic route, and the drug-metabolizing enzyme *CYP2C19* is primarily involved

in their metabolism [2]. *CYP2C19**2 and *CYP2C19**3 are null alleles of the *CYP2C19* gene that encode non-functional enzymes. They are responsible for almost all the cases of poor metabolism involving *CYP2C19* [3]. The single nucleotide polymorphisms (SNPs) *CYP2C19**17, a fast metabolic subtype, have been reported to reduce the healing rate for peptic ulcers and gastro-oesophageal reflux disease [4]. Therefore, we believe that the choice and dosage of proton pump inhibitors can be easily determined with an improved knowledge of *CYP2C19* polymorphisms. The knowledge of *CYP2C19* variants is also important for patients treated with phenytoin and

clopidogrel [5,6].

The enzyme *UGT1A1* is particularly notable because of its relationship with irinotecan, a camptotecin-derived anti-neoplastic drug [7]. Commonly observed genetic SNPs in *UGT1A1*, namely *UGT1A1*6* and *UGT1A1*28*, are associated with adverse effects of its action on irinotecan. The *UGT1A1*6* allele mutation is found in 20% to 30% Asians, but it is rarely observed in Caucasians [7]. Bilirubin glucuronidation is reduced by up to 32% by the *UGT1A1*6* mutation and up to 30% by the *UGT1A1*28* mutation [8]. Gilbert's syndrome is strongly associated with homozygosity for allele 7 (7/7 genotype) and is present in 11% to 13% of the Eastern Scottish population [9].

The direct sequencing method, which is both time- and labour-consuming, has been traditionally used to examine SNPs. It has been previously reported that the quenching probe (QP) method is an effective technique for detecting target genes by employing the fluorescence-quenching phenomenon [10]. Hence, we evaluated a rapid, fully automated SNP-detection system based on fluorescence quenching for the accurate measurement of SNPs.

METHODS

1. Subjects

We genotyped genomic DNA from 200 healthy Koreans as well as 100 peripheral whole blood specimens from 100 others for *CYP2C19*2* and *CYP2C19*3* using the QP method and i-densy IS-5310 (ARKRAY Inc., Kyoto, Japan). We also performed genotyping of *UGT1A1*6* and *UGT1A1*28* using the same 200 DNA samples and 81 of the 100 whole blood samples. Samples were obtained from healthy volunteers. The protocol used in this study was approved by the institutional review board at Seoul National University Hospital (IRB no. 1112-016-388).

2. Quenching Probe Method

The i-densy IS-5310 is a fully automated genotyping system that detects SNPs in extracted DNA samples as well as whole blood specimens. A DNA fragment containing the potential SNP is amplified by PCR and

hybridized with a probe having a complimentary sequence. On the basis of the knowledge that the dissociation temperature differs according to the conformity of the complimentary sequences, and by detecting the fluorescence emitted upon dissociation, the SNP was measured by using the QP method.

This system uses the Qprobe (J-Bio21, Tokyo, Japan) containing a fluorescently labelled cytosine-base terminal. Qprobe fluorescent emission is quenched by products of DNA hybridization. The fluorescence emitted increases upon dissociation. Currently, available i-densy kits include gene probes for cancer, coagulation, transplantation, and risk prediction.

After loading a sample into a cartridge, the i-densy system automatically performs pre-treatment, DNA amplification, and mutation detection via melting temperature analysis. The test results are provided in a single platform. SNP testing by i-densy IS-5310 takes 65 minutes for purified DNA samples and 90 minutes for whole blood specimens. The method of operation varied depending on the type of sample. An operator first indicated the type of sample—purified DNA or whole blood. The required number of tips, reaction tubes, reagent packs, and specimens were arranged at their designated places. The genotypes of *CYP2C19* and *UGT1A1* were detected by PCR using published primers (Table 1) [11]. For the *CYP2C19* gene, initial denaturation was performed at 95°C for 1 minute, followed by 50 cycles of denaturation at 95°C for 1 second, and annealing and extension at 64°C for 15 seconds. For the *UGT1A1* gene, PCR was performed with initial denaturation for 1 minute at 95°C, followed by 50 cycles of denaturation for 1 minute at 95°C for 1 second, and annealing and extension at 59°C for 15 seconds. Upon completion of the PCR, melting temperature analyses were performed. Subsequently, SNPs were identified by the differences in temperature and fluorescence.

3. Direct Sequencing

Genomic DNA was extracted from blood using the PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). Direct sequencing of the *CYP2C19*

Table 1. Primer pairs used for the detection of single nucleotide polymorphisms in *CYP2C19* and *UGT1A1*

Genotype	Primer	Sequence
<i>CYP2C19</i> *2	Forward	5'-TAAATATTGTTTTCTCTTAGATATGCAATAATTTTCCCA-3'
	Reverse	5'-CCCGAGGGTTGTTGATGTCCATC-3'
	QProbe	5'-TCCCAGGAACCCATAAC-(BODIPY FL)-3'
<i>CYP2C19</i> *3	Forward	5'-TGATGGAAAAATGAATGAAAACATCAGGATTGTA-3'
	Reverse	5'-TCAAAAATGTACTTCAGGGCTTGGTCAATA-3'
	QProbe	5'-(TAMRA)-CCCTGAATCCAGGTAAG-P-3'
<i>UGT1A1</i> *6	Forward	5'-TGAAATAGTTGTCCTAGCACCTGACGC-3'
	Reverse	5'-CAAAAGACTCTTTTCACATCCTCCCTTTGG-3'
	QProbe	5'-GAGACAGAGCATTTTACAC-(TAMRA)-3'
<i>UGT1A1</i> *28	Forward	5'-AGCTTTTTTATAGTCACGTGACACAGTCAAAC-3'
	Reverse	5'-CGCCTTTTGCTCCTGCCAGAG-3'
	QProbe	5'-(BODIPY FL)-CCATATATATATATATAAGTAGGAGAG-P-3'

and *UGT1A1* genes was performed. The amplified products were analysed by capillary electrophoresis with the ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing reaction mixtures were prepared according to the manufacturer's instructions (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, http://tools.invitrogen.com/content/sfs/brochures/cms_081527.pdf). The forward and reverse primer sequences used for direct sequencing were the same as those used for PCR (Table 1).

4. Statistical Analysis

Kappa statistics were applied to determine the agreement between quenching probe method and direct sequencing. Cohen's kappa coefficient (K) was calculated using IBM SPSS ver. 19.0 (IBM Co., Armonk, NY, USA). *P*-values < 0.05 were considered significant.

RESULTS

The allele frequencies of *CYP2C19* were 25.7% for c.681G>A in *2 allele and 10.3% for c.636G>A in *3, whereas those of *UGT1A1* were 17.3% for c.211G>A in *6 allele and 11.2% for [thymine-adenine (TA)]₇ promoter polymorphism in *28 mutant allele. The genotype frequencies of *CYP2C19* were 6.0% for *2/*2, 3.7% for

Table 2. Comparison of genotype frequencies of *CYP2C19* and *UGT1A1* observed in Korean individuals

Genotype	Catalytic activity	Frequency (%) (95% confidence interval)	
		This study	Roh et al. (1996) [12]
<i>CYP2C19</i>			
*1/*1	Normal	39.0 (33.5–44.5)	46.6
*1/*2	Moderately reduced	35.7 (30.2–41.1)	41.7*
*1/*3	Moderately reduced	14.3 (10.4–18.3)	
*2/*2	None	6.0 (3.3–8.7)	11.7 [†]
*2/*3	None	3.7 (1.5–5.8)	
*3/*3	None	1.3 (0.0–2.6)	
<i>UGT1A1</i>		This study	Yea et al. (2008) [13]
*6			
W/W	Normal	69.8 (64.4–75.1)	80.0
W/m	40% reduced	26.0 (20.9–31.1)	18.0
m/m	70% reduced	4.2 (1.9–6.6)	2.0
*28			
6/6	Normal	78.6 (73.9–83.4)	78.0
6/7	Reduced up to 80%	20.3 (15.6–25.0)	16.0
7/7	Reduced up to 80%	1.1 (-0.1–2.3)	6.0

Abbreviations: W/W, wild-type; W/m, c.211G>A heterozygote; m/m, c.211G>A homozygote; 6/6, (TA)₆ dinucleotide-repeat homozygote (wild-type); 6/7, (TA)₇-repeat heterozygote; 7/7, (TA)₇-repeat homozygote; TA, thymine-adenine.

*For the sum of *1/*2 and *1/*3. [†]For the sum of *2/*2, *2/*3, and *3/*3.

Table 3. Comparison of the QP method and the DS method for the detection of single nucleotide polymorphisms in *CYP2C19* and *UGT1A1*

QP								QP				QP				
CYP2C19								UGT1A1*6				UGT1A1*28				
	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3		W/W	W/m	m/m		W/W	W/m	m/m		
DS	*1/*1	117	0	0	0	0	DS	W/W	196	0	0	DS	W/W	220	1*	0
	*1/*2	0	107	0	0	0		W/m	0	73	0		W/m	0	57	0
	*1/*3	0	0	43	0	0		m/m	0	0	12		m/m	0	0	3
	*2/*2	0	0	0	18	0										
	*2/*3	0	0	0	0	11										
	*3/*3	0	0	0	0	0										
All subjects (N)		300						281				281				

Abbreviations: QP, quenching probe; DS, direct sequencing; W/W, wild-type; W/m, c.211G>A heterozygote; m/m, c.211G>A homozygote.

*The discordant result is in bold font.

*2/*3, and 1.3% for *3/*3 (Table 2), whereas those of *UGT1A1* were 4.2% for the *6 homozygote and 1.1% for the *28 homozygote. We performed Fisher's exact test to compare our results with those of previous studies. The frequencies reported in this study were statistically similar to those reported previously for the Korean population ($P=0.333$ for *CYP2C19*, 0.321 for *UGT1A1*6*, and 0.056 for *UGT1A1*28*) [12,13].

In this study, 200 purified DNA specimens and 100 whole blood specimens were genotyped for *CYP2C19*2* and *CYP2C19*3* by using both QP and direct sequencing methods. For *UGT1A1*6* and *UGT1A1*28*, 200 DNA specimens and 81 whole blood specimens were analysed by both methods. We measured Cohen's kappa coefficient to evaluate the agreement between the 2 methods. The genotypes of *CYP2C19* detected by the QP method showed matched perfectly (100.0%, $K=1.000$, $P<0.001$) with those determined by the direct sequencing method (Table 3). *UGT1A1* genotypes determined by the QP method were concordant with those determined by the direct sequencing method with the exception of 1 of the 281 specimens (200 DNA samples and 81 whole blood samples; 99.6%, $K=0.992$, $P<0.001$). We believe that the single discordant result (obtained from a DNA specimen during genotyping of *UGT1A1*28*) was observed due to a misinterpretation of a noise peak by the built-in analysis software of i-densy (Fig. 1). The problem was resolved by conducting a rerun of the sample thereafter, producing

results that matched with the results obtained by the direct sequencing method.

DISCUSSION

The accuracy of the QP method has been proven by several previous studies [10,11,14-17]. The agreement between the results obtained from the QP and the direct sequencing methods in previous studies ranged between 72% and 100%. The result showing 72% agreement originated from the QP system identifying mutations in 7 out of 25 cases that were not identified by the direct sequencing method but were confirmed by allele-specific PCR analysis [10].

The sample size analysed in this study is larger than that in previous studies. Our results (100.0% correlation for *CYP2C19* and 99.6% correlation for *UGT1A1*) also demonstrated good correlation between the QP and direct sequencing methods. Additionally, the QP method using the i-densy system enabled a one-step analysis and provided results within 90 minutes for whole blood samples. Direct sequencing methods usually provide results in about 1 week. Thus, our results suggest that the QP method is equally accurate, more convenient, and less time- and labour-consuming than the direct sequencing method.

We also compared the results of genotype frequencies of *CYP2C19* and *UGT1A1* in the healthy Korean population

Fig. 1. The single case of a discordant result between the quenching probe and direct sequencing methods. The i-densy IS-5310 system software misinterpreted the melting curve for genotyping *UGT1A1**28 ((TA)₆(TA)₇), as a 6/7 (wild-type/mutant) heterozygote due to a false noise peak at 52°C. (A) Melting curve generated by the system. The x-axis represents temperature (T) and the y-axis represents arbitrary FU. (B) Differential plot of the melting curve. The x-axis represents temperature and the y-axis represents differential fluorescence intensity with respect to temperature (dF/dT). (C) Melting curve indicating a routine positive case. (D) Differential plot of the melting curve indicating a routine positive case. Abbreviations: TA, thymine-adenine; TA, thymine-adenine.

of this study with those reported previously. In this comparative analysis, the results of *CYP2C19* did not show significant statistical differences with those of a previous study conducted with 103 healthy Korean subjects [12]. Our study also yielded results for the genotype frequencies of *UGT1A1* similar to those reported in a previous study

using the direct sequencing method for determination of the genotype frequencies in samples obtained from 50 healthy Koreans [13]. These observations suggest that the technology used in this study, the QP method, gave the same results as those obtained by conventional methods.

The cause of the single discordant, however, result

remains unclear. We believe that the discordant result was due to the detection sensitivity of the system for the peak. The detection sensitivity can be modified using the built-in analysis software of the i-densy system. Hence, if false noise peaks give rise to misinterpretations and to false mutation peaks, the detection sensitivity of the system can be reduced to obtain results that are more accurate. The melting curve and differential curve of each sample can also be inspected by the personnel or supervisor conducting the analysis. By comparing them to true positive peaks, discordant or ambiguous results can be resolved. In addition, we suggest that a quality control plan to monitor the built-in analysis procedure should be established by clinical laboratories to verify the accuracy of these results.

In conclusion, we believe that the QP method will enable a rapid and sensitive diagnosis of SNPs in clinical laboratories. This, in turn, will ensure the availability of a convenient and rapid customized therapy for patients who are treated with drugs targeting specific molecular targets. The QP method can be adapted for SNPs in any gene with appropriately designed PCR primers and QProbes. This could increase the number of drugs covered by the i-densy system and could be a promising SNP-detection system.

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CYP2C19과 UGT1A1의 신속한 유전형 분석을 위한 자동화 장비의 평가

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배경: 약물대사효소를 암호화하는 유전자에서 단일염기다형성(single nucleotide polymorphism, SNP)의 유전형 분석의 필요성은 증가하고 있다. 그러므로 SNP의 신속하고 정확한 자동화 분석법이 최근 주목받고 있다.

방법: 저자들은 200명의 정상 한국인의 DNA 검체와 100명의 전혈에서 i-densy IS-5310을 이용한 소광소식자법(quenching probe, QP)으로 CYP2C19*2와 CYP2C19*3 유전자의 유전형을 분석했다. 또한 200개의 DNA와 81개의 전혈 검체에 대해 UGT1A1*6과 UGT1A1*28 유전자의 유전형을 분석했다. 분석결과는 전통적 염기서열분석법과 비교되었다.

결과: CYP2C19의 대립유전자 빈도는 *2에 대해 25.7%, *3에 대해 10.3%였으며, UGT1A1에서는 *6에 대해 17.3%, *28에 대해 11.2%로 기존의 한국인에서의 보고와 유사했다. QP법에 의한 CYP2C19 유전형은 직접서열분석법과 완벽히 일치했다(100.0%, K=1.000, P<0.001). 281검체 중, QP법에 의한 UGT1A1 유전형은 한 개의 검체를 제외하고 역시 일치했다(99.6%, K=0.992, P<0.001).

결론: QP법은 그 검사속도와 사용 면에서 임상검사실에서 빠르고 정확한 진단에 도움을 줄 것으로 판단된다.

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