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journal or publication title	Drug Metabolism and Pharmacokinetics
volume	33
number	6
page range	258-263
year	2018-12
URL	http://hdl.handle.net/10097/00128365

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Abstract

Genetic polymorphisms contribute to inter-individual variability in the metabolism of multiple clinical drugs, including warfarin, thiopurines, primaquine, and aminoglycosides. A rapid and sensitive clinical assessment of various genome biomarkers is, therefore, required to predict the individual responsiveness of each patient to these drugs. In this study, we developed a novel genotyping method for the detection of nine pharmacogene variants that are important in the prediction of drug efficiency and toxicity. This genotyping method uses competitive allele-specific PCR and a single-stranded tag hybridization chromatographic printed-array strip (STH-PAS) that can unambiguously determine the presence or absence of the gene variant by displaying visible blue lines on the chromatographic printed-array strip. Notably, the results of our STH-PAS method were in 100% agreement with those obtained using standard Sanger sequencing and KASP assay genotyping methods for *CYP4F2* gene deletion. Moreover, the results were obtained within 90 min, including the PCR amplification and signal detection processes. The sensitive and rapid nature of this novel method make it ideal for clinical genetic testing to predict drug efficacy and toxicity, and in doing so will aid in the development of individualized medicine and better patient care.

Keywords: genetic polymorphisms; warfarin; thiopurine; G6PD; mitochondrial DNA

Introduction

Differences in an individual's response to a drug and the occurrence of adverse reactions have been associated with various factors, such as age, sex, concomitant drugs, diet, smoking, and genetic polymorphisms [1, 2]. It is widely known that genetic polymorphisms in genes that encode proteins involved in drug absorption, distribution, metabolism, and excretion often cause issues with drug efficacy or increase toxicity [3]. Indeed, single nucleotide variations at the loci for cytochrome P450 (CYP) 2C9, vitamin K epoxide reductase complex 1 (VKORC1), CYP4F2, nudix protein 15 (NUDT15), ATP-binding cassette sub-family C member 4 (ABCC4), glucose-6-phosphate dehydrogenase (G6PD), and mitochondrial DNA (mtDNA) have been shown to influence the drug response [4-8]. The field of pharmacogenetics largely focuses on determining the influence of these gene variations and uses this information to customize drug class, optimal dose, and dosage regimen for individual patients.

Before drug treatment, it is extremely desirable to use bedside genotyping methods to identify drug responders or non-responders as well as to identify patients with an increased risk of toxicity. Currently, numerous polymerase chain reaction (PCR)-based genotyping methods have been developed, including PCR-restriction fragment length polymorphism, allele-specific PCR, and TaqMan PCR [9-12]. However, each of these

techniques requires expensive instrumentation and extensive technical expertise to obtain reliable, reproducible results.

Recently, Tian *et al.* developed a single-stranded tag hybridization chromatographic printed-array strip (STH-PAS) genotyping method [13]. This method allows multiplex DNA signals to be visualized in a single reaction with high sensitivity in a markedly short time. Moreover, it does not require the preparation or staining of cumbersome gels. Furthermore, Saito *et al.* recently reported a rapid and sensitive multiplex single-tube nested PCR method using STH-PAS for the identification of five human Plasmodium species [14]. While this technique has been investigated for various applications, it has not been evaluated for its use as a pharmacogene identification method.

In this study, we developed a multiplex single-tube PCR assay to genotypically identify common pharmacogene variants, including warfarin (WRF)-related gene polymorphisms (*CYP2C9*3*, *VKORC1* -1639G>A, *CYP4F2*3*, and *CYP4F2* gene deletion), thiopurine-related gene polymorphisms (*NUDT15*3* and *ABCC4* c.2268G>A), *mtDNA* 1555A>G, and *G6PD* gene polymorphisms (c.202G>A and c.376A>G). To our knowledge, this is the first time this novel method has been applied to rapidly and sensitively analyze multiple genotype variations.

Materials and Methods

Sample collection

Subject samples of each desired genotype were collected from unrelated Japanese (n = 180; 60 for WRF-related, 60 for thiopurine-related, and 60 for mtDNA). Additionally, Kenyan samples (n = 52) containing the polymorphisms 202G>A and 376A>G were obtained in order to verify the functionality of STH-PAS. Genomic DNA was isolated from peripheral blood samples (Japanese individuals) or three blood spot-containing discs (6 mm in diameter) (Kenyan individuals) using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Genomic DNAs with rare variants were also prepared from an Epstein-Barr virus-immortalized cell line established in Tohoku Medical Megabank Organization using a Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) [15]. This study was conducted in accordance with the Declaration of Helsinki and the participants provided written informed consent according to the protocols approved by the Osaka City University committee (permission number 3206), the Tohoku University Graduate School of Pharmaceutical Sciences committee (permission number 15-03), and the Tohoku Medical Megabank Organization committee (permission number 2017-4-26, 2017-4-58, and 2017-4-090).

Primer design

The following GenBank genomic sequences were used as reference sequences: CYP2C9, NG_008385.1; VKORC1, NG_011564.1; CYP4F2, NG_007971.2; NUDT15, NG_047021.1; ABCC4, NG_050651.1; mtDNA, NC_012920.1; and G6PD, NG_009015.2. Primers were designed to specifically amplify each gene (Table 1). Primer pairs were checked using a BLAST search to confirm the absence of nonspecific amplification. For all primer pairs, a single primer was labeled with a tag-spacer sequence at its 5′ end for detection as shown in Figures 1A−1D, while the others were labeled with biotin at their 5′ ends.

Genotyping of WRF-related genes polymorphisms

Multiplex single-tube PCR amplification was performed with genomic DNA samples (10 ng), 2× KAPA2G Fast Multiplex Mix (Kapa Biosystems, Wilmington, MA, USA), and a mixture containing each primer at a concentration of 0.1 μM (VKORC1_Fw_Wt_G) or 0.2 μM (VKORC1_Fw_Vr_T, VKORC1_Rv, CYP2C9_Fw_Wt_C, CYP2C9_Fw_Vr_G, CYP2C9_Rv, CYP4F2_Fw_Wt, CYP4F2_Fw_Vr, CYP4F2_Rv, CYP4F2_Del_Fw, and CYP4F2_Del_Rv). The PCR conditions included an initial denaturation step at 95°C for 2 min; 30 cycles of

denaturation at 95°C for 30 s, annealing at 61°C for 20 s, and extension at 72°C for 30 s; and a final extension at 72°C for 7 min.

STH-PAS was performed in a 20 µL reaction system containing 10 µL of developing solution (Tohoku Bio-Array, Sendai, Japan), 1 µL of PCR product, and 1 µL of streptavidin-coated blue latex suspension (Tohoku Bio-Array). The C-PAS12 membrane stick (Tohoku Bio-Array) was dipped into the mixture for 10 min at 20–25°C. During this time, the blue latex particles coated with streptavidin interacted with the biotinylated terminal amplicons. A total of seven complementary oligonucleotides designed to specifically recognize the PCR amplicons through hybridization with the 5′ terminus tags were immobilized on the dipstick strips. The appearance of a visible blue test line indicated the presence of the target DNA sequence tagged with the complementary oligonucleotide. A biotin-immobilized flow control line was arranged at the end of each strip and functioned as an internal control to ensure the chromatographic migration of the solution.

Genotyping of thiopurine-related genes polymorphisms

Multiplex single-tube PCR amplification was performed with genomic DNA samples (10 ng), 2× KAPA2G Fast Multiplex Mix, and a mixture of primers at a

concentration of 0.1 µM (NUDT15_Fw_Wt, NUDT15_Rv, ABCC4_Fw_Wt, and ABCC4_Rv) or 0.2 µM (NUDT15_Fw_Vr and ABCC4_Fw_Vr). The PCR conditions included an initial denaturation step at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 7 min. STH-PAS was then performed as described above. The C-PAS8 (Tohoku Bio-Array) membrane stick was dipped into the mixture for 10 min at 20–25°C.

Genotyping of G6PD gene polymorphisms

Multiplex single-tube PCR amplification was performed with genomic DNA samples (1 ng), 2× KAPA2G Fast Multiplex Mix (Kapa Biosystems), and a mixture of primers at a concentration of 0.05 µM (G6PD_202_Fw_Wt and G6PD_376_Fw_Vr) or 0.1 µM (G6PD_202_Fw_Vr, G6PD_202_Rv, G6PD_376_Fw_Wt, and G6PD_376_Rv). The PCR conditions included an initial denaturation step at 95°C for 3 min; and then 30 cycles of denaturation at 95°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s. STH-PAS was then performed as described above only with 2 µL of the streptavidin-coated blue latex suspension. The C-PAS4 membrane stick was dipped into the mixture for 5 min at 20–25°C.

Genotyping of mtDNA gene polymorphisms

PCR amplification was performed with genomic DNA samples (10 ng), 2× SYBR Premix Ex Taq II (TaKaRa, Shiga, Japan), and a mixture of primers at a concentration of 0.25 μM Mt_Fw_Wt_T; 0.25 μM Mt_Fw_Vr_T; and 0.25 μM Mt_Rv). The PCR conditions involved an initial denaturation step at 94°C for 2 min; 28 cycles of denaturation at 94°C for 30 s, annealing at 62°C (increasing 0.2ºC per cycle) for 20 s, and extension at 72°C for 30 s; and a final extension at 72°C for 7 min. STH-PAS was performed as described above. The C-PAS4 (Tohoku Bio-Array) membrane stick was dipped into the mixture for 5 min at 20–25°C.

Statistical analysis

The Kompetitive Allele Specific PCR (KASP) genotyping assay (LGC Genomics, Hertz, UK) for the CYP4F2 gene deletion variant [16] and Sanger sequencing-based genotyping were performed to evaluate the concordance rate for each genotyping method with all multiplex single-tube nested PCR methods. KASP assay was utilized as a unique form of allele-specific PCR that enables accurate bi-allelic scoring of single nucleotide polymorphisms, insertions, and deletions. The KASP genotyping reaction comprises sample DNA, KASP Assay mix (containing the target-specific primers), and KASP

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Master mix. Unlike other PCR-based genotyping assays, KASP requires no labeling of

the target-specific primers and probes, giving it a clear cost advantage. The 95%

confidence intervals (CIs) were calculated using R version 3.0.3 (R core team (2014); R

Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/).

Results

Representative results for our STH-PAS detection method-based genotyping are shown in Figure 2. There were no false positives or false negatives observed. There was a 100% match in the genotyping results for nine variants: *CYP2C9* (*CYP2C9*3*, rs1057910), *VKORC1* (-1639G>A, rs9923231), *CYP4F2* (*CYP4F2*3*, rs2108622 and *CYP4F2* gene deletion allele), *NUDT15* (*NUDT15*3*, rs116855232), *ABCC4* (c.2268G>A, rs3765534), *G6PD* (c.202G>A, rs1050828 and c.376A>G, rs1050829), and mtDNA (1555A>G, rs267606617). Using sanger-sequencing and the KASP assay as the standard genotyping methods to detect *CYP4F2* gene deletion, the concordance rates of STH-PAS genotyping for WRF-related genes (n = 60), thiopurine-related genes (n = 60), G6PD gene (n = 52), and mtDNA gene (n = 60) were 100% (95% CI = 94.0–100.0%, 94.0–100.0%, 93.2–100.0%, and 94.0–100.0%, respectively).

Discussion

Variations in pharmacogenes that encode proteins involved in drug absorption, distribution, metabolism, and excretion have been known to influence inter-individual variability in drug efficacy and adverse drug reactions [1, 3]. These genetic polymorphisms can, therefore, be used as genome biomarkers to predict the responsiveness of each patient to specific drugs prior to treatment. This allows more effective and safer individualized drug treatment to be administrated as the therapeutic agents can be selected based on the gene polymorphisms present in each patient. While various methods have attempted to do this, they typically involve expensive equipment or significant expertise with the method [9-12]. In this study, we developed genotyping methods for WRF-related, thiopurine-related, G6PD (c.202G>A and c.376A>G), and mtDNA 1555A>G gene polymorphisms based on allele-specific PCR and STH-PAS, a recently established detection method.

The STH-PAS has several advantages when compared to PCR-RFLP and other allele-specific PCR methods, including that in order to detect genetic variations, STH-PAS requires little technical interpretation and provides results within 90 min, enabling rapid genotyping. Moreover, the cost of STH-PAS ranges from 7–8 dollars per sample, making it more economical for visualizing multiplex DNA. Generally, ethidium bromide

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staining following gel electrophoresis is used to visualize PCR amplicons because of its simplicity and low cost. However, it poses the expected safety risks that accompany the use of a carcinogenic agent [17, 18]. Therefore, the STH-PAS genotyping method would allow for rapid genetic testing in a clinical setting.

WRF, a widely prescribed anticoagulant, is administered as a racemic mixture of (S) and (R)-WRF for the long-term treatment and prevention of thromboembolic events [19]. Unfortunately, this drug is associated with significant inter-individual variability, particularly with regards to the dose required to produce a therapeutic effect [20, 21]. Recent genome-wide analyses have provided strong evidence that genetic polymorphisms can predict bleeding or the risk of thrombotic events, which occur with either an excessive or insufficient dose, respectively [22, 23]. In the Japanese patients in one study, the primary polymorphisms were *CYP2C9*3*, *VKORC1* -1639G>A, and *CYP4F2*3* [4]. As an anticoagulant, (S)-WRF, which is metabolized to 7-hydroxywarfarin by CYP2C9, is 3–5 times more potent than (R)-WRF [24-26]. The *CYP2C9*3* allelic variation appears to cause low (S)-WRF clearance, consequently increasing the risk of bleeding [27, 28]. The *VKORC1* -1639G>A polymorphism in the promoter region decreases the enzymatic activity of the protein, which catalyzes the reduction of vitamin K epoxide to vitamin K [29]. Vitamin K is known to play an important role in blood coagulation [30]. In fact,

vitamin K hydroxylation, which is catalyzed by CYP4F2, has been shown to decrease coagulation [31]. It is, therefore, not surprising that the *CYP4F2*3* mutation, which influences the oxidation activity of CYP4F2, also has an effect on WRF-mediated anticoagulation. Moreover, a novel *CYP4F2* deletion variant was found in a Japanese study that accounts for 1.6% of this population [16]. Thus, analyzing the genetic polymorphisms that affect WRF-related anticoagulation therapy would be beneficial in attaining an approximate dosing regimen in Japanese patients.

Thiopurines, including 6-mercaptopurine (6-MP), thioguanine, and azathioprine, are commonly used to treat acute lymphoblastic leukemia and inflammatory bowel diseases [32, 33]. Notably, 6-thioguanine nucleotides (6-TGNs) are the main cytotoxic metabolites of thiopurines [34]. Recent studies have reported that *NUDT15* and *ABCC4* genetic variants are significantly associated with thiopurine-induced toxicity in the Asian population [5], with *NUDT15*3* (10.5%) and *ABCC4* c.2268G>A (14.7%) being the most significant in the Japanese population reference panel [15, 35]. Almost all patients carrying these mutations showed severe 6-MP-associated toxicities [5, 36, 37]. Therefore, before selecting a therapeutic 6-MP dose, it would be beneficial to analyze *NUDT15* and *ABCC4* variants in the patients to prevent severe adverse effects.

Malaria is caused by widespread prevalence of protozoan parasites of the genus

Plasmodium, and leads to health and economic burden in the Americas, the Western

Pacific region, and South-east Asia [38]. A considerable number of people suffering from

acute *Plasmodium vivax* malaria cannot safely undergo primaquine therapy to prevent

recurrent relapses because of the risk of severe primaquine toxicity in patients with an

inherent deficiency of G6PD [39-41]. G6PD deficiency affects more than 400 million

people, approximately 8% of the general population in malaria-endemic nations [42]. The

202G>A and 376A>G mutations are considered the most common G6PD deficiency

alleles in sub-Saharan Africa [8]. In addition to the genotypic method developed in the

present study to identify these G6PD genetic polymorphisms, a malaria species-specific

diagnostic method using STH-PAS involving a multiplex single-tube nested PCR

targeting *Plasmodium* mitochondrial cytochrome c oxidase III has also been recently

reported [14]. Combining these G6PD polymorphism genotyping and malaria species-specific diagnosis methods would greatly enhance the selection of therapeutic agents and

dose adjustments to suit individual needs. Moreover, it might be possible to achieve a

more appropriate antimalarial therapy by incorporating representative G6PD genetic

polymorphisms to this genotyping method.

Aminoglycosides have been used as effective antibiotics for both gram-positive and

gram-negative infections [43]. The characteristic side effect associated with these drugs

is irreversible hearing loss observed in patients [44]. Recently, mtDNA mutations, including 1555A>G, have been shown to be responsible for aminoglycoside-induced deafness [6, 7]. The frequency of this mutation in patients who have aminoglycoside-induced deafness was identified in various ethnic groups: Brazilian (1.3–3%), Japanese (3%), Danish (2.4%), German (0.7%), Polish (5.3%), and Southeast Asian (5.3%) [45]. However, in a previous study focused on 279 Asian individuals, aminoglycoside-induced deafness only occurred in patients with the 1555G mutation; all individuals with this mutation presented aminoglycoside-induced deafness [44]. Therefore, using our genotyping method before drug administration could enable prevention of this serious adverse effect in patients carrying the 1555G mutation. Moreover, because mtDNA exhibits a maternal inheritance pattern in mammals, genotyping results could also be relevant for blood relatives, including the patient's brothers and sisters.

In conclusion, we have described and tested an allele-specific PCR and STH-PAS method for the detection of well-known genetic polymorphisms involved in the efficacy and toxicity of various drugs. This method would be valuable in the improvement of pharmacotherapies with the use of WRF and thiopurine in Japanese patients. Simultaneously, this study also shows that it is possible to perform mtDNA genotyping by using this method in much the same way as its potential application in G6PD

genotyping using representative G6PD mutations, in malaria-endemic nations. Additionally, the STH-PAS method would be expected to be developed for other clinically important genetic polymorphisms such as *CYP2C19* (*2, *3*) and *UGT1A1* (*6, *28*). Notably, the genotyping results obtained using our method were 100% similar to those obtained using standard Sanger sequencing. However, one limitation of our method is that the combination of all genotypes cannot be confirmed because of low allele frequency. While a large sample size is needed to validate all of the genotype combinations, this study highlights the simplicity and reliability of this method to predict drug efficacy and toxicity. Further improvements and application of this method will ultimately allow medical staff to quickly and easily evaluate patient genotypes, resulting in more effective individualized drug therapy.

Acknowledgements

We warmly thank Evelyn Marie Gutierrez, Tohoku University, for proofreading the manuscript.

Author Contributions

Participated in research design: Kumondai, Ito, Hishinuma, Kikuchi, T Satio, Takahashi, Tsukada, and Hiratsuka

Conducted experiments: Kumondai, Ito, Hishinuma, Kikuchi, T Satio, Takahashi, and Tsukada

Contributed new reagents or analytic tools: Hirasawa, S Saito, Yasuda, Nagasaki, Minegishi, Yamamoto, Kaneko, Teramoto, Kimura, and Hiratsuka

Performed data analysis: Kumondai and Hiratsuka

Wrote or contributed to the writing of the manuscript: Kumondai, Ito, and Hiratsuka

Conflict of Interest

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Figure Legends

Fig. 1 Schematic diagram of a single-stranded tag hybridization chromatographic printed-array strip (STH-PAS). (A-D) Test line positions on the STH-PAS. Red lines indicate positional markers.

Fig. 2 Representative PCR amplicon signals using STH-PAS. Data represent independent replicates. Top panels show representative images of the STH-PASs, while the bottom graphics provide a summary of the genotype by lane. NC, negative control; Wt, template non-carrying mutation; Vr, template carrying mutation; Del, template carrying *CYP4F2* gene deletion allele.

Table 1. PCR primers

Target gene	Position	Primer name	Primer sequence (5′ to 3′)	Size (bp)
VKORC1	-1639G>A (rs9923231)	VKORC1_Fw_Wt_G	[Tag 1]-X-CCTGAAAAACAACCATTGGCGG	348
		VKORC1_Fw_Vr_T	[Tag 4]-X-CCTGAAAAACAACCATTGGCTA	
		VKORC1_Rv	[Biotin]-CACCAAGACGCTAGACCCAATG	
CYP2C9	42614A>C	CYP2C9_Fw_Wt	[Tag 5]-X-GCACGAGGTCCAGAGATACA	180
	(CYP2C9*3, rs1057910)	CYP2C9_Fw_Vr_G	[Tag 8]-X-GCACGAGGTCCAGAGATAGC	
		CYP2C9_Rv	[Biotin]-ACCCGGTGATGGTAGAGGTTT	
CYP4F2	18000G>A	CYP4F2_Fw_Wt	[Tag 10]-X-CATCACAACCCAGCTG	470
	(CYP4F2*3, rs2108622)	CYP4F2_Fw_Vr	[Tag 12]-X-CATCACAACCCAGCTA	
		CYP4F2_Rv	[Biotin]-AGGACCAACCCAACCG	
	Deletion	CYP4F2_Del_Fw	[Tag 11]-X-TTCCCGCCAAACCACTC	395

		CYP4F2_Del_Rv	[Biotin]-TGCTCTTGAACAACCAATGG	
NUDT15	415C>T	NUDT15_Fw_Wt	[Tag 1]-X-CTTTTCTGGGGACTGC	403
	(*NUDT15*3*, rs11655232)	NUDT15_Fw_Vr	[Tag 2]-X-CTTTTCTGGGGACTGT	
		NUDT15_Rv	[Biotin]-GCAAGCTAGTAATGACAAACTGCAC	
ABCC4	2268G>A (rs3765534)	ABCC4_Fw_Wt	[Tag 7]-X-GGAGGAGGAAATGTAACCG	433
		ABCC4_Fw_Vr	[Tag 8]-X-GGAGGAGGAAATGTAACCA	
		ABCC4_Rv	[Biotin]-ATTGGAACCATCCGAACCAC	
G6PD	202G>A (rs1050828)	G6PD_202_Fw_Wt	[Tag 1]-X-GCCCGAAAACACCTTCATCG	249
		G6PD_202_Fw_Vr	[Tag 2]-X-GCCCGAAAACACCTTCATCA	
		G6PD_202_Rv	[Biotin]-GGGCTGGTAATGGGGGTCTC	
	376A>G (rs1050829)	G6PD_376_Fw_Wt	[Tag 3]-X-GCCTCAACAGCCACATGA	145
		G6PD_376_Fw_Vr	[Tag 4]-X-GCCTCAACAGCCACATGG	

		G6PD_376_Rv	[Biotin]-GGCAACGGCAAGCCTTAC	
mtDNA	1555A>G (rs267606617)	Mt_Fw_Wt_T	[Tag 1]-X-CCAGTACACTTACCATGTTACGACTT<u>T</u>T	146
		Mt_Fw_Vr_T	[Tag 4]-X-CCAGTACACTTACCATGTTACGACTT<u>T</u>C	
		Mt_Rv	[Biotin]-TAAGAGTAGAGTGCTTAGTTGAACAGGG	

"X" represents "spacer C3". The spacer C3 [three-carbon spacer, $(CH_2)_3$, using Phosphoramide C3 spacer] is inserted between the Tags and the respective forward primer. According to each genetic polymorphism, the nucleotides at the 3′ end of each forward primer were changed to match each sequence. The underlined nucleotides just before the 3′ end of these primers (VKORC1_Fw_Wt_G, VKORC1_Fw_Vr_T, CYP2C9_Fw_Vr_G, Mt_Fw_Wt_T, and Mt_Fw_Vr_T) indicate the differences made regarding the reference sequences in order to avoid non-specific amplification.