High-Throughput Single-Base Mismatch Detection for Genotyping of UDP-Glucuronosyltransferase (UGT1A1) with Probe Capture Assay Coupled with Modified Allele-Specific Primer Extension Reaction (MASPER)

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We have developed a new method based on specific primer extension reactions coupled with plate hybridization for high-throughput genotyping of single-base mutations. To improve the switching characteristics of the primer extension reaction, we introduced an artificial mismatch two bases upstream of the 3'-terminal base in the detection primers. A set of primers that correspond to wild-type and mutant DNA segments can be used to accurately analyze single-base mutations. The termini of these primers are at the mutation positions. The primer extension products produced by polymerase chain reaction (PCR) were

captured by an oligonucleotide probe immobilized on the surface of microtiter wells and were detected by a colorimetric assay using the streptavidin-conjugated horseradish peroxidase. We used the new method to genotype 96 individuals for 211G>A (G71R) and 119 for 1456T>G (Y486D) in the UDP-glucuronosyltransferase1A1 gene; the results were completely concordant with those found by direct sequencing. The proposed method includes ordinary PCR and a microplate assay format, and may be used in routine laboratory tests. *J. Clin. Lab. Anal.* 24:85–91, 2010. © 2010 Wiley-Liss, Inc.

Key words: UGT1A1; single-base mutation; allele-specific primer; plate hybridization

INTRODUCTION

Single-base mutations are the most common forms of genetic variation, causing many types of serious disease. The detection of single-base mutations for the genetic mapping of complex traits, pharmacogenomics, and clinical diagnostics has been discussed (1–4). However, routine diagnostic testing for these mutations would require new technologies that are high throughput, simple, low cost, and accurate. Toward these aims, many methods have been developed to analyze single-base mutations (5–19).

We propose that the development of a high-throughput genotyping method that requires no special detection instruments would be useful for the detection of these mutations in routine clinical laboratory tests.

UDP-glucuronosyltransferase (UGT1A1) has an important role in the glucuronidation of bilirubin in the liver, and can conjugate phenols, flavonoids, and various therapeutic drugs and their metabolites (20).

Several cases of single-base mutation in the *UGT1A1* gene have been reported (21–25).

In this study, we developed a method for genotyping *UGT1A1* mutations [211G>A (G71R) and 1456T>G (Y486D)] (24) that can be used without any special detection instruments. The method uses an allele-specific primer extension reaction performed using an allele-specific primer to which an artificial mismatched base is introduced at the third base from the 3' end to improve

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the specificity and a probe-capture assay using plate hybridization.

MATERIALS AND METHODS

Chemicals

Ex Taq DNA polymerase and dNTP were purchased from Takara Bio Science (Shiga, Japan). All the oligonucleotide probes and primers were synthesized and purified by Hokkaido System Science Inc. (Sapporo, Japan). DNA-BIND 96-well plates (Corning, Cat. No. 2506) were purchased from Corning Inc. (Corning, NY). Streptavidin-conjugated horseradish peroxidase (StAv-HRP) was purchased from Sigma-Aldrich Co. (St. Louis, MO). All solutions were prepared in deionized and distilled water. Other chemicals were of commercially available analytical grade.

Samples

This work was approved by the Institutional Ethical Review Board. The subjects were Japanese individuals who volunteered in our laboratories and also patients diagnosed with Crigler-Najjar or Gilbert's syndrome (23,25).

Each patient gave informed consent for his/her peripheral blood to be used for the research. All samples were numbered and tested anonymously. Genomic DNA was extracted from 200 μ l of whole blood using a QIAamp DNA Blood Mini Kit (Qiagen, CA). The extracted DNA was purified using the spin column supplied with the kit. The purified DNA was directly analyzed by an allele-specific PCR for the detection of mutations, and no other preamplification steps were performed before the assay.

Assay Platform

The platform for the detection of single-base mutations is shown in Figure 1.

In the first step, genomic DNA was extracted and purified from whole blood. The purified target DNA was subjected to PCR in the presence of the wild type- or the mutant-specific biotin-labeled detection primer and a common reverse primer. PCR was carried out under the conditions indicated in Table 1; this was followed by single-stranded DNA preparation by adding an alkaline solution. The resulting single-stranded DNA was added to the microplate wells, which immobilized a capture-probe DNA. Following the capture reaction, StAv-HRP was allowed to react with the biotin-labeled extension products in the well. Finally, a chromogenic substrate for HRP was added in order to detect the signal corresponding to the match or mismatch at the 3'-terminus of the primer-DNA duplex. The production of sufficient amounts of primer extension products depended on

Fig. 1. Scheme of the assay platform. (A): Outline of assay steps. A biotinylated modified detection primer was used for PCR amplification (Step 1). If the target base in a sample genome was complementary to the 3'-terminus base of the biotinylated-modified detection primer, the detection primer was elongated by the extension reaction. The resulting PCR products were specifically hybridized to the capture probes immobilized on the surface of microplate wells (Step 2). After a first wash to eliminate non-elongated primers, StAv-HRP was reacted with biotin on PCR products captured by completely matched probes. A second washing was performed to eliminate un-reacted StAv-HRP. A chromogenic substrate, OPD- H_2O_2 , was used for signal detection. The genotype was identified by calculating the logarithm of the ratio of the wild type and mutant-primer signals. (B): Design of biotinylated-modified detection primer. The primers have the target base at the 3'-terminus and an artificially introduced mismatch base two bases upstream from the 3'-terminus. The primer extension reaction is regulated by the match or mismatch at the 3'-terminus of the primer.

whether the 3'-terminal base of the modified detection primer was complementary to that of the target DNA.

We have named this assay platform "probe capture assay coupled with modified allele specific primer extension reaction" (MASPER).

Primers

Table 1 shows the sequences of the primers used to detect the mutations 211G>A (G71R) and 1456T>G (Y486D). To prevent mismatched primer extensions, an

TABLE 1. Sequences of Detection Primers, Reverse Primers, and Capture Probes, and PCR Parameters

Mutation	Primer name	Sequence, 5' to 3'	PCR parameters				
			Predenaturation	Denaturation	Annealing	Extension	Number of cycles
211G>A (G71R)	G71R-WD*	Biotin-ttcaaggtgtaaatgc acc	94°C for 5 min	94°C for 30 sec	60°C for 30 sec	72°C for 20 sec	35 cycles
	G71R-MD**	Biotin-ttcaaggtgtaaatgc act					
	G71R-reverse	cccatgctgggaagatactg					
	Capture probe	NH ₂ -(CH ₂) ₆ -cagcagaggggacatgaaat					
1456T>G (Y486D)	Y486D-WD*	Biotin-atcacgtcca aggaatgc ta	98°C for 5 min	98°C for 30 sec	62°C for 30 sec	62°C for 30 sec	32 cycles
	Y486D-MD**	Biotin-atcacgtcca aggaatgc tc					
	Y486D-reverse	ttcataccacagggtttcca					
	Capture probe	NH ₂ -(CH ₂) ₆ -ccgcagccca cgactcacc tggtaaccag					

The boxed base in the primer sequence is the artificially mismatched base, and the bold base is the complementary base to the target base. WD, wild-type detection primer; MD, mutant-type detection primer.

artificial mismatched base was introduced at the third position from the 3' end (18), which was called the modified detection primer.

Primer Extension Reaction

Primer extension and amplification was performed by PCR under the conditions shown in Table 1. One percent aliquot of the total genomic DNA, prepared from 200 µl of whole blood, was used as template DNA. A 30 µl aliquot containing 2 µl template DNA solution, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 50 mM KCl, 6 nmol of each dNTP, 20 pmol of both detection primer and reverse primer, and 1 U Ex Taq DNA polymerase were used for PCR. After primer extension by PCR, single-stranded DNA was prepared by adding 30 µl of 2 M NaOH to the reaction mixture.

Immobilization of Capture Probes

A capture oligonucleotide was chosen from the region extended by the primer extension reaction. We used the modified capture oligonucleotide probe to which an aminohexyl linker was introduced at the 5'-terminal to react with the *N*-hydroxysuccinimide (NHS) ligand on the surface of the microplate well. The sequence of the capture oligonucleotide is shown in Table 1.

Capture oligonucleotide at 20 pmol/100 µl of coupling buffer (500 mM Na₂HPO₄ [pH 8.5] and 1 mM ethylenediaminetetraacetic acid [EDTA]) was incubated in each well of DNA-BINDTR plates (Corning, Cat No. 2506) for 30 min at room temperature. Wells were rinsed three times with 10 mM Tris-HCl [pH 8.0] containing 150 mM NaCl (TBS). The buffer was aspirated and 200 µl of blocking solution (BlockAce^{TR}, Yukijirushi Milk Co. Ltd., Tokyo, Japan) was added to each well. Following overnight incubation at 2–8°C, the blocking solution was aspirated, and the wells were rinsed three times with TBS. The resulting plates were stored with a desiccant at 2–8°C.

Detection of Extension Products

One hundred microliters of hybridization buffer (0.075 M citrate-sodium citrate buffer (pH 7.0), 0.075 M NaCl, and 0.025% sodium dodecyl sulfate) was added to each well, followed by 25 µl of the single-stranded primer extension products. The reaction mixture was incubated for 30 min at 37°C. Then, the wells were aspirated to remove the reaction mixture and were rinsed three times with TBS containing 0.05% Tween20^{TR}. One hundred microliters of the StAv-HRP solution, commercial StAv-HRP diluted to 1:10,000 with 1% bovine serum albumin-containing TBS, was added to all the wells and incubated for 30 min at 37°C.

The wells were then rinsed three times with TBS containing 0.05% Tween20TR. The substrate solution containing H_2O_2 and *o*-phenylene diamine (OPD) was added. Following 15 min incubation at 37°C , the enzymatic reaction was terminated by the addition of 100 μl of 1 M sulfuric acid. Absorbance was measured at 492 nm on a plate reader (model MPR-A4i; Tosoh, Tokyo, Japan).

RESULTS

Switching Characteristics of Modified Primers

To improve the switching characteristics of the detection primers, an artificial mismatch was introduced at the third base from the 3'-terminus of the primer. We studied the effects of this modification on the switching characteristics of primer extensions according to the match status at the 3'-terminus. As shown in Figure 2, the unmodified primer gave signals independent of matching status. On the contrary, the mismatched primer extension signal was less than 1% of the matched primer extension signal. Thus, the primer extension reactions can be perfectly determined by the match status of the 3'-terminus of the modified primer.

Fig. 2. Comparison of primer extensions for wild type and mutant targets. In (A) there is no introduced mismatched base in the primer; while in (B) an introduced mismatched base has been placed at the third position from the 3'-terminus of the primer. This assay was performed by using the detection primer for 1456T>G (Y486D). The amounts of template, primers, and each dNTP in a 30 μl reaction mixture were about 60 μg , 20 pmol, and 6 nmol, respectively. The detailed assay conditions are described in the text.

Sensitivity and Reproducibility

The number of copies of template DNA subjected to PCR was estimated as follows: The genomic DNA prepared from a 200 μl sample of whole human blood, which contains about 5×10^3 leukocytes per microliter, was regarded as about 2×10^6 copies for homozygous, and about 1×10^6 each for heterozygous. Since one percent of the recovered DNA was used for the assay, the template DNA subjected to the assay was about 2×10^4 copies.

MASPER was used to measure all 237 genomes for 211 G>A (G71R) and 312 genomes for 1456T>G (Y486D) in the assay.

We used 10 parallel repetitions to determine the reproducibility of this assay for 1456G>T (Y486D). The reproducibility of the total assay, including PCR, hybridization, and detection of enzyme activity, showed a coefficient variation (CV) of 18.5%. The CV of the hybridization and detection processes without PCR was 7.2–9.1% (data not shown).

Genotype Discrimination

We evaluated MASPER performance in genotype discrimination using 237 individuals for 211G>A (G71R) and 312 individuals for 1456T>G (Y486D), respectively. The results of all the assays are presented numerically as logarithms of signal ratios ($\log [\text{wild primer signal}/\text{mutant primer signal}]$) in Figure 3, in which the values obtained from individuals are plotted in descending order. In keeping with the principle that if alleles in a sample are present as wild type, homozygous mutant (homo-mutation) and heterozygous-mutant (hetero-mutation), the logarithms of their signal ratios would be positive, negative, and zero, respectively, a clear clustering of logarithms of signal ratios corresponding to these three genotypes is observed in Figure 3. Based on the descending plot order of the logarithms of the signal ratios, the following results were obtained; wild type: >0.6 ; hetero-mutation: -0.8 – -0.6 ; and homo-mutation: <-0.8 , for 211G>A (G71R); wild type: >0.7 ; hetero-mutation: -0.7 – -0.7 ; and homo-mutation: <-0.7 , for 1456T>G (Y486D). The genotypes determined by MASPER were as follows: 161 wild type, 71 hetero-mutations, and 5 homo-mutations for 211G>A (G71R) and 301 wild type, 7 hetero-mutations, and 4 homo-mutations for 1456T>G (Y486D). A portion of these samples were analyzed by direct sequencing, and the genotypes obtained were as follows: total 96 individuals containing 20 wild type, 71 hetero-mutations, and 5 homo-mutations for 211G>A (G71R) and total 119 individuals containing 108 wild type, 7 hetero-mutations, and 4 homo-mutations for 1456T>G (Y486D). The genotypes obtained by MASPER and the direct sequencing method were compared, and the results were in complete concordance.

Fig. 3. Identification of UGT1A1 mutations 211G>A (G71R) (A) and 1456T>G (Y486D) (B) from genomic DNA. Signals from the wild type-specific primer and mutant-specific primer were detected by a microtiter plate reader at 492 nm. Results are expressed as logarithms of the signal ratios, (log [wild primer signal/mutant primer signal]). In theory, if two alleles in an individual were wild type, hetero-mutated, or homo-mutated, the logarithm values of the signal ratios would be positive, near zero, and negative, respectively. The values obtained from individuals were plotted in descending order.

Allele Frequency Analysis

Allele frequency analysis was carried out using genomic DNA from anonymous donors from the general Japanese population. In 237 individuals, 474 alleles for 211G>A (G71R), and in 240 individuals, 480 alleles for 1456T>G (Y486D) were genotyped. The resulting 161 wild-type, 71 hetero-mutations, and 5 homo-mutations of 211G>A (G71R) demonstrated a mutant allele frequency of 17.1%, and the resulting 238 wild-type and 2 hetero-mutations of 1456T>G (Y486D) demonstrated an allele frequency of 0.4%. The two individuals with hetero-mutation of 1456T>G (Y486D) did not have the 211G>A (G71R) mutation.

DISCUSSION

The MASPER method of single-base mutation genotyping is based on allele-specific PCR amplification in which the allele-specific primer contains an artificial

mismatched base introduced at the third position from the primer 3'-terminus.

Our method has three useful features: First, it has high specificity. The artificial introduction of a mismatched base into the allele-specific primer improves the switching characteristics of primer extensions at the 3'-terminus. Whether or not mismatched primer extensions occur depends on reaction conditions such as the primer sequence, the particular DNA polymerase used, and the PCR annealing temperature used in the amplification step. The artificial mismatch introduced at the third position from the 3'-terminus of the primer prevents the formation of mismatch primer extension. As shown in Figure 2, primers without a mismatched base all produced similar signals, since they were unable to identify the genotype. The logarithms of the signal ratios are a convenient tool for interpreting genotype results. All samples were successfully genotyped for the 211G>A (G71R) and 1456T>G (Y486D) mutations. The MASPER genotyping results were identical to those

obtained by direct sequencing, demonstrating a high level of specificity.

A second feature of MASPER is its sensitivity. MASPER demonstrates a high level of sensitivity in two ways: (1) the MASPER assay requires a very small blood sample for analysis and (2) the MASPER assay does not require re-amplification of genomic DNA. We estimated the detection limit of genomic DNA by MASPER at about 1 to 2×10^4 copies per assay. We prepared genomic DNA using a QIAamp DNA extraction and purification kit. The instruction manual had stated that about 3–12 µg of genomic DNA can be recovered from 200 µl of whole blood (26), and we were able to perform the MASPER assay on just one percent of this preparation, which corresponds to 2 µl of whole blood. The template DNA subjected to the assay contained 30–120 ng of genomic DNA. On the other hand, the Invader assay is a useful high-throughput single nucleotide polymorphism genotyping method, but one study reported that 6 of 60 samples for 211G>A (G71R) could not be assayed (27). These resistant samples had low fluorescence intensity because of their small genomic DNA content (15–46 ng/10 µl). The author recommended using 100 ng/10 µl of genomic DNA for accurate screening (27). On the other hand, all the tested samples in this study were measurable using MASPER. MASPER has two amplification steps, the first of which is DNA amplification by PCR to detect the mutation in the target genomic DNA and the second is direct enzymatic signal amplification. Using MASPER, it is feasible to detect mutations without pre-amplification; in our study, no samples with undetectably low signals were found. Eliminating the pre-amplification step would help to achieve a cost-effective and high-throughput assay.

A third feature of MASPER is the small number of steps involved. The assay involved a single PCR step, plate hybridization at 37°C, StAv-HRP binding, and an enzymatic reaction; the whole process took less than 3 hr for 96 samples, after preparation of the genomic DNA. Furthermore, our method does not require any special assay equipment except for a thermal cycler for PCR and a colorimetric microplate reader for signal reading. The procedure can be automated by using an automated enzyme-linked immunoassay instrument.

According to in vivo expression studies, the 211G>A (G71R) mutation in the homozygous and heterozygous genetic states decreased the resulting enzyme's activity to 32 and 60% of that of the control, respectively (28). This mutation has been reported to be a significant risk factor for non-physiological hyperbilirubinemia in the Japanese population (29). Recently it was reported that the 211G>A (G71R) mutation was significantly related to irinotecan toxicity (30). In these circumstances,

pharmacogenetics studies may benefit from MASPER genotyping of UGT1A1 mutations, 211G>A (G71R) and 1456T>G (Y486D). Our method for detecting UGT1A1 mutations has the potential to be widely used for the rapid and accurate screening analysis of UGT1A1 mutations. The allele frequencies of these mutations in Japanese individuals in our study were similar to those reported previously for the Japanese population (31,32). Determining the 211G>A (G71R) and 1456T>G (Y486D) genotype might be clinically useful for predicting severe drug toxicity in Japanese populations.

In summary, we have developed a new genotyping method for single-base mutation that is accurate, sensitive, easy, and simple in operation. It could be useful for a number of applications that require high-throughput, such as multiple-candidate gene screens and clinical diagnostics.

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