Short Communication

Direct and Rapid Genotyping of Glutathione-S-transferase M1 and T1 from Human Blood Specimens Using the SmartAmp2 Method

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ABSTRACT:

Clinical studies have suggested that a defect in both glutathione S-transferase (GST) M1 and GSTT1 increases the risk of drug-induced hepatotoxicity. The present study developed the method that enables genotyping of GSTM1 and GSTT1 directly using a small aliquot of blood samples based on an isothermal Smart amplification process version 2 (SmartAmp-2). SmartAmp-2 reaction could complete the genotyping of GSTM1 and GSTT1 within 40 min. The frequency of wild-type, GSTM1 null, GSTT1 null, and double null was 24, 21, 35, and 19%, respectively, consistent with previous reports in the Japanese population. The genotypes of 94 human genomic DNA samples determined by SmartAmp-2 were identical to those determined by the conventional polymerase chain reaction method. SmartAmp-2 was able to determine the genotypes of GSTM1 and GSTT1 even when human blood specimens were used. The SmartAmp-2 method is a rapid and accurate means of identifying the GSTM1 and GSTT1 genotypes, making it less time and more labor efficient in clinical practice than conventional methods requiring preparation of genomic DNA and electrophoresis. This will contribute to evaluate the susceptibility of disease and adverse reactions to drugs caused by deletion of GSTM1 and GSTT1.

Introduction

Idiosyncratic drug-induced liver injury (DILI) is a clinical challenge because of the rarity of its diagnosis and the lack of a standard, which makes determination of causality difficult (Kaplowitz, 2005). Many drugs have been withdrawn from the market and clinical development of new drugs has suddenly been halted because of DILIs (Kola and Landis, 2004). Formation of reactive metabolites, electrophilic intermediates, is attributed to DILI (Walgren et al., 2005). Drugs are metabolized by cytochrome P450 enzymes to reactive metabolites, such as primer-dimer, or inhibiting amplification from nontarget species, as well as self-priming reactions and auto-priming. Boost primer (BP), outer primer (OP)1, and inner primer (IP) participate in extension events, producing an intermediate single-stranded DNA product. These intermediate species then undergo a self-priming reaction to generate long DNA concatemeric products. Boost primer (BP) and turn-back primer (TP) participate in primer extension events, producing an intermediate single-stranded DNA product. These intermediate species then undergo a self-priming reaction to generate long DNA concatemeric products. Boost primer (BP), outer primer (OP1), and inner primer (IP) support functions of FP and TP and therefore increase the reaction speed. The nonspecific amplification can be totally suppressed by preventing generation of unexpected intermediate products, such as primer-dimer, or inhibiting amplification from nontarget sequences.

There is growing interest in GSTM1 and GSTT1 genotyping as risk factors for DILI. GSTM1 and GSTT1 are predominantly expressed in the liver and display polymorphisms in humans. In the double-null variant of GSTM1 and GSTT1, 16 or 52 kilobases (kb) are deleted from chromosome 1 or chromosome 22 by a homologous recombination, respectively (Fig. 1) (Xu et al., 1998; Sprenger et al., 2000). The double-null variant was associated with a higher risk of DILI caused by troglitazone, tacrine, and carbamazepine (Simon et al., 2000; Watanabe et al., 2003; Ueda et al., 2007).

A simple and rapid detection of GSTM1 and GSTT1 genotypes before medication will help to avoid severe DILI in patients in clinical practice. In the present study, we developed a rapid polymorphism detection system, the Smart amplification process version 2 (SmartAmp-2), for GSTM1 and GSTT1 genes. SmartAmp-2 enables genotyping of the genes under isothermal conditions using small aliquots of blood specimens, which could save time and effort by avoiding genomic DNA preparation and electrophoresis (Mitani et al., 2007). In the SmartAmp-2 reaction, the folding primer (FP) and turn-back primer (TP) participate in primer extension events, producing an intermediate single-stranded DNA product. These intermediate species then undergo a self-priming reaction to generate long DNA concatemeric products. Boost primer (BP), outer primer (OP1), and inner primer (IP) support functions of FP and TP and therefore increase the reaction speed. The nonspecific amplification can be totally suppressed by preventing generation of unexpected intermediate products, such as primer-dimer, or inhibiting amplification from nontarget sequences.

In the present study, we designed the five primers for the detection of GSTM1 and GSTT1 genes and validated their specificity and reliability using 94 human genomic DNA specimens.

Materials and Methods

Collection of Genomic DNA and Blood and Preparation of a Template. Peripheral blood was collected from 94 healthy Japanese volunteers, and DNA was extracted by the standard protocol. The samples were numbered, un-
Priming of GSTM1 and GSTT1

Genotyping of GSTT1 and GSTM1 by SmartAmp2 Method

The SmartAmp-2 assay for GSTM1 and GSTT1 polymorphism detection using human blood specimens and genomic DNA is illustrated. Primers were designed for amplification and detection of GSTM1 and GSTT1 genes (Fig. 1). After genomic DNA and blood specimens were denatured at 98°C for 3 and 5 min, respectively, SmartAmp-2 reactions were allowed to take place at 60°C for 60 min. The Mx3000P Real-time polymerase chain reaction (PCR) system (Agilent Technologies, Santa Clara, CA) and LightCycler 480 System II (Roche, Basel, Switzerland) were used for maintaining isothermal conditions and monitoring the change in fluorescence intensity of intercalating SYBR Green I dye (Invitrogen, Carlsbad, CA) during the reaction.

The reaction mixture contains 2.9 μM each of OP1 and OP2, 1.4 mM dNTPs, 5% dimethyl sulfoxide, 20 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄, 0.1% Tween 20, 1/100,000 diluted original SYBR Green I, 6 units of Aae DNA polymerase (DNAFORM K.K., DNAFORM, Yokohama, Japan), and prepared genomic DNA or blood. The quality of control of the reaction was determined using SmartAmp-2 kit for epidermal growth factor receptor. Primers were as follows: TP, 5'-CACCTTCACCTCACAGAGGTACCTGCGAGCCAGGAACG-3'; FP, 5'-GACAGATTTCGGGCTCAGGGTGCAG-3'; OP1, 5'-CACCGACCATGGTTCGCTTGGTGCAGCA-3'; and OP2, 5'-TCCTCTCCTGATGCTTATG-3'.

Genotyping of GSTM1 and GSTT1 of Genomic Samples by the Conventional PCR Method. Genotyping of GSTM1 and GSTT1 was carried out by conventional PCR amplification as described previously (Watanabe et al., 2003; Bernardini et al., 2005). The primers were GSTM1 primers: sense, 5'-GAACCTTCACCTCACAGAGGTACCTGCGAGCCAGGAACG-3'; antisense, 5'-GACAGATTTCGGGCTCAGGGTGCAG-3'; GSTT1 primers: sense, 5'-CACGGTGACCATGGTTCGCTTGGTGCAGCA-3'; and antisense, 5'-TCCTCTCCTGATGCTTATG-3'.

The PCR was carried out for 35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The amplified DNA fragments were electrophoresed in 2.0% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. The amplified fragments detected at 0.46- or 0.23-kb were designated as wild-type GSTM1 or GSTT1 genes, respectively.

Results and Discussion

The primer sets of GSTM1 and GSTT1 were designed to exon 5 and intron 4, respectively, which are not conserved among members of the GST genes. Screening of five primer combinations in primer sets yielded an ideal primer set that completed the amplification within 30 and 40 min for the genomic DNAs and blood specimens, respectively. A representative amplification profile is shown in Fig. 1. The reaction was run for 60 min and after 40 min, the GSTM1 or GSTT1 genes could be detected without nonspecific amplification in the signals produced by self-nonspecific amplification until 60 min.
The genotypes of GSTM1 and GSTT1 were analyzed by both the conventional PCR method and the SmartAmp-2 method. The table shows a comparison of the two methods in 101 genomic DNA samples and in 94 blood samples.

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<tr>
<th>Genomic DNA Samples: SmartAmp-2 Assay</th>
<th>Blood Samples: SmartAmp-2 Assay</th>
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<tr>
<td>Conventional PCR method</td>
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<tr>
<td>M1+/T1+</td>
<td>M1~/T1+</td>
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<td>M1~/T1−</td>
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Specificity of the amplified signal is demonstrated by the complete absence of any product when the genomic DNA of GSTM1 null or GSTT1 null is used as a template for the reaction.

Genotyping of GSTM1 and T1 was carried out with this kit, using a Coriell sample set and blood samples (Table 1). First of all, we checked the accuracy of SmartAmp-2 using the Coriell sample set, and then we validated our method by comparing the genotype data obtained from the SmartAmp-2 method using 94 blood samples directly without any further DNA extraction to the genotype data obtained by a conventional PCR method, using DNA from these blood samples, which was extracted in a separate procedure. The frequency of wild-type, GSTM1 null, GSTT1 null, and double null was 24, 21, 35, and 19%, respectively, consistent with previous reports. Because genotypes of GSTM1 and GSTT1 obtained by a conventional method and the SmartAmp-2 assay were identical in both methods, we can conclude that this new SmartAmp-2 assay is reliable.

Previously, the genotyping of GSTM1 and GSTT1 was carried out by conventional PCR followed by electrophoresis and real-time PCR. The most striking difference in the SmartAmp-2 assay compared with these conventional methods is that SmartAmp-2 can save time and avoid preparation of genomic DNA and electrophoresis because genotyping can be completed within 40 min, including sample preparation. Furthermore, because the reaction in the SmartAmp-2 assay occurs under isothermal conditions, we can perform genotyping with only a simple device for warming and a detection system. Furthermore, because the reaction in the SmartAmp-2 assay occurs under isothermal conditions, we can perform genotyping with only a simple device for warming and a detection system. Furthermore, recently, new primers for SmartAmp-2, called Exciton Primers, were launched. These primers make it possible to visually detect and multiplex label the end products in a single test tube, which makes the SmartAmp-2 method even less time, less cost, and more labor efficient (Lezhava et al., 2010). In the future, these inventions will allow medical staff to determine these genotypes even at the bedside. It should be noted that, due to a lack of detailed information about the exact location of the deletion in GSTM1 and T1, we were unable to design primers for the detection of a null mutation to distinguish homozygotes from heterozygotes. SmartAmp-2 was unable to discriminate the variation in copy number of GSTM1 (McLellan et al., 1999) and the SmartAmp-2 method even less time, less cost, and more labor efficient (Lezhava et al., 2010).

In conclusion, a SmartAmp-2 assay for the genotyping of GSTM1 and GSTT1 was developed in this study, and the reliability of the method was validated using the conventional method. The system will allow evaluation of the susceptibility to disease and adverse reactions to drugs caused by deletion of GSTM1 and GSTT1 in clinical situations.


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