Use of site-directed mutagenesis of allele-specific PCR primers to identify the GSTM1 A, GSTM1 B, GSTM1 A,B and GSTM1 null polymorphisms at the glutathione S-transferase, *GSTM1* locus

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We describe the identification of the GSTM1 null, GSTM1 A, GSTM1 B and GSTM1 A,B polymorphisms at the glutathione S-transferase GSTM1 locus using a single-step PCR method. Target DNA was amplified using primers to intron 6 and exon 7 with site-directed mutagenesis being used to introduce a restriction site in DNA amplified from *GSTM1 *A*, thereby allowing differentiation of this allele and *GSTM1 *B*. The accuracy of this approach in identifying the GSTM1 A, GSTM1 B, GSTM1 A,B and GSTM1 null polymorphisms was confirmed by comparison with, firstly, an established PCR method that distinguishes *GSTM1 *0* homozygotes from individuals with the other *GSTM1* genotypes and, secondly, GSTM1 phenotypes determined using chromatofocusing.

INTRODUCTION

GSTM1, one of at least five genes that comprise the Mu class of the human glutathione S-transferase (GST) supergene family, demonstrates polymorphisms that arise from homo- and hetero-zygotic combinations of the *GSTM1 *0*, *GSTM1 *A* and *GSTM1 *B* alleles [1–5]. *GSTM1 *0* is deleted, and homozygotes (null phenotype) express no protein [6], while *GSTM1 *A* and *GSTM1 *B* differ by a single base in exon 7. The products of these latter two genes combine to form active, homo- and hetero-dimeric, enzymes [2,6].

Interest in GSTM1 has been stimulated by data indicating that homozygosity for *GSTM1 *0* is associated with an increased risk of various pathologies, including some malignancies [6–15]. These studies have largely focused on the hypothesis that the presence or absence of GSTM1 is a determining factor in disease susceptibility. It has been assumed that the products of *GSTM1 *A* and *GSTM1 *B* are equally protective and that disease risk in *GSTM1 *0* heterozygotes and homo- and hetero-zygotes for *GSTM1 *A* and *GSTM1 *B* is the same. These assumptions may not be justified, since studies in patients with primary biliary cirrhosis suggest that, with larger patient numbers, the frequencies of the GSTM1 A phenotype (*GSTM1 *A/GSTM1 *A*, *GSTM1 *A/GSTM1 *0* genotypes) and GSTM1 B phenotype (*GSTM1 *B/GSTM1 *B*, *GSTM1 *B/GSTM1 *0* genotypes) in cases and controls would be significantly different [12]. Further, we failed to identify the GSTM1 A,B phenotype (*GSTM1 *A/GSTM1 *B* genotype) in patients with colorectal and brain cancers, even though this polymorphism has a frequency of 6% in controls [7,8]. This suggests individuals with two positive alleles may be better protected than *GSTM1 *0* heterozygotes.

Several approaches (e.g. starch-gel electrophoresis and chromatofocusing) allow identification of the GSTM1 A, GSTM1 B, GSTM1 A,B and GSTM1 null phenotypes [7,8], but do not discriminate *GSTM1 *0* heterozygotes and corresponding *GSTM1 *A* or *GSTM1 *B* homozygotes. Further, since they depend on the use of GSTM1-expressing tissues, these approaches are not suitable for population screening. Assay of GSTM1 activity in lymphocytes using *trans*-stilbene oxide, though suitable for screening, does not differentiate the products of *GSTM1 *A* and *GSTM1 *B* alleles [9,10]. PCR approaches can be used to identify *GSTM1 *0* homozygotes, but so far published methods do not differentiate different expressing genotypes [10–13].

Since the sequences of GSTM1-M5 are known [1,2,4,6,15,16], PCR using allele-specific primers could allow identification of *GSTM1 *A* and *GSTM1 *B* and testing of hypotheses regarding the relative levels of protection conferred by the products of the different GSTM1 alleles. We describe a PCR method that uses site-directed mutagenesis [17] to introduce a restriction site into the amplified DNA. This allows identification of the GSTM1 null, GSTM1 A, GSTM1 B and GSTM1 A,B polymorphisms in a single PCR step. The accuracy of these assignments has been checked by comparison with an established PCR method that uses primers to exon 4/5 [11,12] and, the GSTM1 phenotype determined using chromatofocusing [8].

MATERIALS AND METHODS

Tissue samples

Tissue samples of human liver were obtained, within 12 h of death, at post-mortem from 29 adults without clinical or histological evidence of malignancy. Blood samples (3 ml) were taken into EDTA with the approval of the Ethics Committee of the North Staffordshire Hospital from a further 84 hospital patients suffering a variety of non-cancer pathologies. Blood and tissue samples were stored at −50 °C until analysis.

Abbreviation used: GST, glutathione S-transferase.

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Table 1 Primers used to identify GSTM1 alleles

The A→G substitution in GSTM1E7A used to generate the *Hae*II restriction site is underlined.

Primer	Sequence	Reference
GSTM1I6	5′-GCTTCACGTGTTATGAAGGTTC-3′	[4]
GSTM1E7A	5′-TTGGGAAGGCGTCCAAGCGC-3′	The present paper
GSTM1E7B	5′-TTGGGAAGGCGTCCAAGCAG-3′	The present paper
GSTM1E4	5′-CTGCCCTACTTGATTGATGGG-3′	[11]
GSTM1E5	5′-CTGGATTGTAGCAGATCATGC-3′	[11]

Identification of GSTM1 polymorphisms

GSTM1 polymorphisms were identified in DNA extracted [12] from the 29 tissue and 84 blood samples by, firstly, PCR-mediated amplification of DNA from intron 6 and exon 7 and, secondly, restriction digestion of amplified DNA to differentiate *GSTM1 *A* and *GSTM1 *B*.

All PCR reactions were performed using three primers to GSTM1. These comprised, firstly, a common GSTM1-specific primer to intron 6 (GSTM1I6) [4] and, secondly, primers to exon 7, one specific to *GSTM1 *A* (GSTM1E7A) and the other (GSTM1E7B) specific to *GSTM1 *B* (Table 1). Pilot studies using DNA from subjects with known GSTM1 A or GSTM1 B phenotypes showed that GSTM1E7A did not anneal to *GSTM1 *B* and vice versa. The *GSTM1 *A* primer differs from the *GSTM1 *A* sequence by an A→G substitution at the 3′ end of the primer, thus introducing an *Hae*II restriction site into the amplified DNA (Table 1). DNA from subjects with positive GSTM1 alleles allowed amplification of a 132 bp fragment. However, while GSTM1E7A and GSTM1E7B effected amplification of a fragment of the same size from *GSTM1 *A* and *GSTM1 *B* respectively, the sequence of the PCR product differed by two bases. *GSTM1 *0* homozygotes failed to amplify target DNA. Success of amplification was confirmed by the presence of a 268 bp DNA fragment using primers, 5′-CAACTTCATCCACGTTCACC-3′ and 5′-GAAGAGCCAAGGACAGTTAC-3′, to β-globin [18].

Reactions were carried out in a solution (50 μl) containing GSTM1I6, GSTM1E7A, GSTM1E7B and β-globin primers (5 × 500 nM), *Taq* polymerase (1 unit), dNTP (4 × 0.2 mM), target DNA (0.5 μg) and buffer [10 mM Tris/HCl (pH 9.0)/ 50 mM KCl/0.1% (v/v) Triton X-100/1.5 mM $MgCl_2$] and overlaid with 50 μl of mineral oil. After initial denaturation (94 °C, 2.5 min), five cycles of denaturation (94 °C, 45 s), primer annealing (57 °C, 1 min) and elongation (72 °C, 2 min), followed by 30 cycles of denaturation (94 °C, 30 s), primer annealing (57 °C, 30 s) and elongation (72 °C, 45 s increasing by 3 s per cycle) were performed.

PCR products (18 μl) were digested (37 °C, 4 h) with *Hae*II (2 units, Promega) in *Hae*II buffer (10 × concentrated; 60 mM Tris/HCl buffer, pH 7.50, containing 500 mM NaCl, 60 mM $MgCl_2$ and 10 mM dithiothreitol). DNA fragments were resolved by electrophoresis in 4%-agarose gels containing ethidium bromide (0.5 μg/ml) and photographed under u.v. light.

Confirmation of positive/negative GSTM1 polymorphisms

All assignments of GSTM1 positivity/negativity made using the intron 6/exon 7 primers were checked by PCR performed with primers to exon 4/5 (Table 1) and, as positive control, primers to β-globin [12]. Fragments were resolved in 2% (w/v)-agarose gels containing ethidium bromide (0.5 μg/ml) [12]. Individuals with positive GSTM1 polymorphisms were similarly identified by amplification of a 273 bp DNA fragment. *GSTM1 *0* homozygotes failed to demonstrate this fragment.

Determination of GSTM1 phenotype

Chromatofocusing was used to determine GSTM1 phenotypes in 29 liver samples [8]. Cytosols (approx. 35 mg of protein) were eluted (20 ml/h, 4 °C) from columns (0.8 cm × 40 cm) of Polybuffer Exchanger PBE94 (Pharmacia LKB Biotechnology) equilibrated with 25 mM imidazole buffer, pH 7.30. The pH gradient was established using Polybuffer 74 (1:10, v/v; pH 4.00) and fractions assayed for GST activity using 1-chloro-2,4-dinitrobenzene and GSH [8]. The GSTM1 A and B phenotypes were identified by peaks of GST activity eluting at pH 6.4 and 5.8 respectively. GSTM1 A,B was identified by the elution of three peaks of activity at pH 6.4, 6.1 and 5.8. Samples with the GSTM1 0 phenotype demonstrated no activity in this pH range.

RESULTS AND DISCUSSION

Identification of GSTM1 polymorphisms

Figure 1 shows the banding patterns for the GSTM1 A, GSTM1 B, GSTM1 A,B and GSTM1 null polymorphisms obtained using primers GSTM1I6 and GSTM1E7A/GSTM1E7B. DNA from subjects with positive GSTM1 genotypes (*GSTM1 *0* hetero-zygotes, *GSTM1 *A* and *GSTM1 *B* homo- and hetero-zygotes) allowed amplification of a 132 bp fragment. Homozygotes for *GSTM1 *0* did not amplify this fragment. The accuracy of these assignments of GSTM1 positivity/negativity was checked by analysis with exon 4/5 primers [12]. DNA from the 113 subjects (72 *GSTM1 *0* homozygotes, 41 GSTM1 positive genotypes) was examined and in each case the two PCR assays were in agreement.

*GSTM1 *A* and *GSTM1 *B* could be identified following *Hae*II digestion; *GSTM1 *A* was cut to give two DNA fragments (112 bp and 20 bp), while *GSTM1 *B* was refractory to digestion.

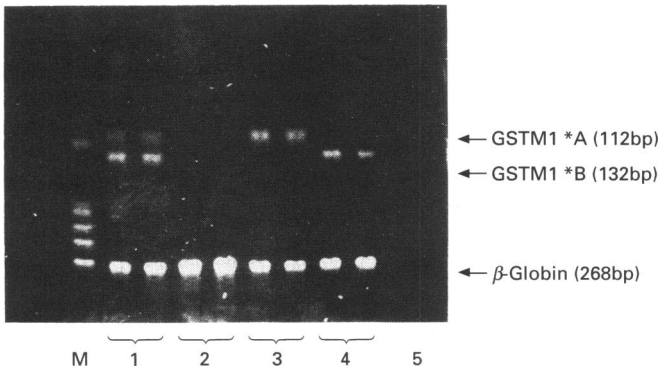

Figure 1 PCR analysis of GSTM1 using allele-specific primers

Agarose-gel electrophoresis of products of PCR amplification using primers to intron 6 (GSTM1I6) and exon 7 (GSTM1E7A and GSTM1E7B) showing the 132 and 112 bp DNA fragments amplified from the gene and, the 268 bp fragment from β-globin. The gel shows: M, molecular-size markers (pBR322 *Hae*III digest), DNA from subjects with the (1) GSTM1 A,B, (2) GSTM1 null, (3) GSTM1 A and (4) GSTM1 B phenotypes; 5, negative control comprising reaction mixture without target DNA. Samples in lanes 1–4 are shown in duplicate.

The expected 268 bp fragment amplified from β-globin was detected in all subjects (Figure 1).

Comparison with phenotype

DNA was prepared from 29 liver samples with known GSTM1 phenotypes (GSTM1 A, nine subjects; GSTM1 B, two subjects; GSTM1 null, 15 subjects; GSTM1 A,B, three subjects) and analysed by PCR using the allele-specific primers. In each case assignments made using chromatofocusing were confirmed by PCR analysis.

Unfortunately it is not presently possible to construct primers that allow positive identification of *GSTM1 *0*, since the size of the deletion is not known. Consequently, the approach we have described cannot distinguish *GSTM1 *0* heterozygotes from the corresponding *GSTM1 *A* and *GSTM1 *B* homozygotes. Such information will be needed to determine whether GSTM1 protection shows a gene-dosage effect, with *GSTM1 *0/GSTM1 *A* and *GSTM1 *0/GSTM1 *B* individuals being at intermediate risk.

Nonetheless, the assay described is as convenient to perform as currently available methods, yet provides considerably more information. Thus confident identification of *GSTM1 *A/GSTM1 *B* heterozygotes can now be made, and it is important to examine the frequency of this polymorphism in patients with various malignancies to confirm our earlier impression that this genotype is protective. Similarly individuals with the GSTM1 A and GSTM1 B polymorphisms can be identified, allowing investigation of the relative protective effects of the two alleles.

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