Multiplex PCR Detection of *GSTM1*, *GSTT1*, and *GSTP1* Gene Variants

Simultaneously Detecting GSTM1 and GSTT1 Gene Copy Number and the Allelic Status of the GSTP1 Ile105Val Genetic Variant

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The glutathione S-transferase (GST) genes *GSTM1*, *GSTT1*, and *GSTP1* are involved in the detoxification of a broad range of toxic substances. Genetic polymorphisms in these genes have been studied intensively for their potential role in cancer susceptibility and drug response. In Caucasians, the enzyme activity of *GSTM1* and *GSTT1* is absent in approximately 50 and 15% of the population, respectively, due to deletions of both chromosomal copies of the genes. A trimodal phenotype pattern exists in which individuals with two, one, or no functional genes are fast, intermediate, or slow “conjugators,” respectively. Most studies investigating the effect of the *GSTM1* and *GSTT1* deletions do not distinguish between fast and intermediate conjugators because the applied genotyping assays only detect if at least one copy of either gene is present. We present a multiplex PCR assay that detects if an individual has none, one, or two copies of the *GSTM1* and *GSTT1* genes and simultaneously detects the allelic status of the *GSTP1* Ile105Val genetic variant. A total of 200 Danes, 100 Somalis, and 100 Greenlanders were genotyped. This multiplex PCR assay enables future large-scale studies to investigate the role of GSTs. (*J Mol Diagn* 2007; 9:612–617; DOI: 10.2353/jmoldx.2007.070030)

and drugs. In humans, five classes of GST genes exist: α (*GSTA*), μ (*GSTM*), π (*GSTP*), θ (*GSTT*), and ζ (*GSTZ*) with one or more genes in each class. The enzymes have different, but sometimes overlapping, substrate affinity. The three most intensively studied GSTs are *GSTM1*, *GSTT1*, and *GSTP1*.

Genetic polymorphisms resulting in lack of enzyme activity due to homozygous deletion of the *GSTM1* and *GSTT1* genes have been described. The frequencies of the deletions vary between populations. In Caucasians, the frequencies of homozygous deletions are approximately 0.50 for *GSTM1* and 0.10 to 0.20 for *GSTT1*.^{1–3} Most studies investigating the effect of genetic polymorphisms in *GSTM1* and *GSTT1* do not distinguish between individuals with one or two copies of the genes. However, a trimodal phenotype pattern exists in which individuals with two, one, or no functional enzymes are fast, intermediate, and slow “conjugators,” respectively.^{2,4}

The *GSTP1* gene displays a polymorphism at codon 105 (Ile105Val), resulting in an enzyme with altered substrate affinity.⁵ Approximately 10% of Caucasians are homozygous for this mutation and 40% are heterozygous.^{6–8} The *GSTP1* enzyme is associated with resistance to certain anticancer drugs and to the risk of developing some types of cancer.^{9–12}

In recent years, many groups have investigated the possible effects of genetic variants of *GSTM1*, *GSTT1*, and *GSTP1* genes in relation to various factors, especially cancer susceptibility and the effectiveness of cancer treatment.^{9,12,13} Analyses that are able to distinguish between individuals with two, one, or no functional *GSTM1* and *GSTT1* enzymes can be used to investigate whether the genes influence the studied topic in a dose-dependent manner. We have developed a PCR multiplex method that in a fast, inexpensive, and reliable manner can detect whether a person has two, one, or no *GSTM1*

The glutathione S-transferase (GST) genes code for a superfamily of enzymes that are involved in phase II drug metabolism by conjugation of electrophilic substances with glutathione. The GSTs detoxify a broad range of substances including carcinogens, environmental toxins,

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Figure 1. Amplification strategy for the detection of the *GSTT1/GSTM1* deletion. If the *GSTT1/GSTM1* is present, the DNA fragment between the forward and reverse primers is too long for amplification. If the *GSTT1/GSTM1* is deleted, amplification will occur.

and *GSTT1* genes and at the same time can detect the allelic status of the *GSTP1 Ile105Val* genetic variant.

Materials and Methods

Samples

A total of 200 unrelated healthy Danes (100 males and 100 females), 100 unrelated healthy Somalis (50 males and 50 females), and 100 unrelated healthy Greenlanders (50 males and 50 females) were included in the study. The protocol was approved by the Danish Ethical Committee (KF-01-118/02).

Purification and Measurement of DNA

Genomic DNA was extracted from 200 μ l of peripheral blood using QIAamp DNA Blood Mini kit (Qiagen, Ballerup, Denmark) according to the manufacturer's protocol. DNA concentrations were measured by the Quantifiler Human DNA quantification kit (Applied Biosystems, Naerum, Denmark) using the ABI Prism 7900HT sequence detecting system according to the manufacturer's protocol.

PCR Assay

A multiplex PCR assay was developed for the simultaneous detection of the allelic status of *GSTM1*, *GSTT1*, and *GSTP1*. The assay includes five primer pairs that simultaneously amplify fragments of the *GSTM1*, *GSTT1*, and *GSTP1* genes and fragments specific for the deletions in the *GSTM1* and *GSTT1* genes. The *GSTM1* and *GSTT1* deletions are believed to be caused by an unequal crossover between two highly homogeneous regions at each side of the genes (Figure 1). Because of the

high degree of similarity between the repeated regions on each side of the genes, it is necessary to amplify long DNA fragments to achieve bands specific for the deletions. To make the multiplex PCR as robust as possible, the lengths of the amplifications were kept as short as possible. The primer design was performed using Primer 3.0 v. 0.2¹⁴ and Oligo Analyzer v. 1.0.3 (Freeware, Teemu Kuulasmaa, Finland). Reverse-phase high-performance liquid chromatography purified primers and probes were purchased from DNA-Technology A/S (Aarhus, Denmark). Table 1 shows the primer sequences. The multiplex PCR was optimized to achieve balanced reactions with all possible combinations of amplicons present.

The multiplex PCR was conducted in a 25- μ l reaction volume containing 50 ng of DNA, 1 $\times$ buffer (DyNAzyme EXT buffer), 1.5 mmol/L $MgCl_2$, 240 μ mol/L of each dNTP, 0.04 to 0.4 μ mol/L of each primer (Table 1) and 0.7 U of DyNAzyme EXT DNA Polymerase (Medinova, Glostrup, Denmark). The PCR was performed in an Eppendorf MasterCycler gradient thermal cycler with an initial denaturation time of 3 minutes at 94°C followed by 35 cycles with denaturation at 94°C for 30 seconds and annealing/extension at 68°C for 7 minutes. The final extension was performed at 72°C for 10 minutes. The separation of amplicons was done using 1.5% agarose gels in TBE buffer. The gels were stained with ethidium bromide (0.07 μ g/ml) and photographed on an UV transilluminator (VWR, Albertslund, Denmark).

To verify the *GSTP1* genotyping, singleplex PCR was performed using the primers PiF2306 (5'-GTAGTTTGC-CCAAGGTCAAG-3') and PiR2721 (5'-AGCCACCT-GAGGGGTAAG-3') from Watson et al.¹⁷ The positions of the primers did not overlap with those of the primers in the multiplex. The PCR was conducted in a 25- μ l reaction volume containing 25 ng of DNA, 1 $\times$ buffer (AmpliTaQ Gold Buffer), 1.5 mmol/L $MgCl_2$, 160 μ mol/L of each dNTP, 0.4 μ mol/L of each primer, and 1 U of AmpliTaQ Gold Polymerase (Applied Biosystems). The PCR was performed in an Eppendorf MasterCycler gradient thermal cycler with an initial denaturation time of 10 minutes at 94°C followed by 30 cycles with denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 68°C for 1.5 minutes. The final extension was performed at 72°C for 10 minutes.

Single-Base Extension

A multiplex single-base extension (SBE) reaction was developed to detect the *GSTP1 Ile105Val* polymorphism

Table 1. Multiplex PCR Primers

Amplicon	Forward primer	Reverse primer	μ mol/L	Amplicon size (bp)
<i>GSTT1</i> gene	5'-TCTTTTGCATAGAGACCATGACCAG-3'	5'-CTCCCTACTCCAGTAAGTCCCGACT-3'	0.30	969
<i>GSTT1</i> deletion	5'-GAAGCCCAAGAATGGGTGTGTGTG-3'	5'-TGTCCCATGGCCTCCAACATT-3'	0.04	3106
<i>GSTM1</i> gene	5'-CAAATTCTGGATTGTAGCAGATCATGC-3'	5'-CACAGCTCCTGATTATGACAGAAGCC-3'	0.22	625
<i>GSTM1</i> deletion	5'-AAGACAGAGGAAGGGTGCATTTGATA-3'	5'-ACAGACATTCAATCCCAAAGCGACCA-3'	0.40	4748*
<i>GSTP1</i> A/G	5'-TCCTTCCACGCACATCCTCT-3'	5'-AGCCCTTTCTTTGTTTCAGC-3'	0.20	294†

*Kerb et al.¹⁵

†Šarmanová et al.¹⁶

Table 2. Single-Base Extension Primers

Polymorphism	Neutral sequence	Target-specific sequence	μmol/L	Primer size (bp)
<i>GSTT1</i> gene	5'-ACTAGGTGCCACGTCGTGAAAGTCTGACAA-3'	5'-GACCCACCATATAAGCAGAA-3'	1.0	50
<i>GSTT1</i> deletion	5'-AGGTGCCACGTCGTGAAAGTCTGACAA-3'	5'-GCCTTTTGACGCTGAGATTC-3'	0.2	47
<i>GSTM1</i> gene	5'-TGCCACGTCGTGAAAGTCTGACAA-3'	5'-ACAGCCAGGAGTGAGAGGAA-3'	3.0	44
<i>GSTM1</i> deletion	5'-CGTGAAAGTCTGACAA-3'	5'-AAGGCCCTCAGAACAAAGGT-3'	0.6	36
<i>GSTP1</i> A/G	5'-CTGACAA-3'	5'-TGGAGGACCTCCGCTGCAATAC-3'	0.1	30

and simultaneously detect the allelic status of the *GSTM1* and *GSTT1* genes. SBE primers were designed for the detection of the *GSTP1* variant and for the detection of the *GSTM1* and *GSTT1* genes/deletions using the program Oligo Analyzer v. 1.0.3 (Freeware). The lengths of the template-specific parts of the primers were between 20 and 23 bp. To distinguish between the signals from the SBE primers, the lengths were adjusted by the addition of pieces of "neutral" sequences at the 5' end of the primers (Table 2). These sequences did not match any human sequence in the National Center for Biotechnology Information database.¹⁸ The SBE primer (5'-GTCT-GACAATAGTTGGTGTAGATGAGGGAGA-3') that aligns to the opposite strand in regard to the primer applied in the multiplex SBE reaction was used to verify the *GSTP1* genotyping.

The purification of the PCR products, minisequencing, and subsequent purification of the minisequencing reaction were performed in accordance to the SNaPshot protocol of the manufacturer (Applied Biosystems). One microliter of the purified SBE reaction was analyzed on an ABI Prism 3130 Genetic Analyser with a 36-cm capillary array, POP-4 polymer and 10 seconds at 3000-V injection. GeneScan-120 LIZ was used as internal size standard. The data were analyzed using GeneScan Analysis software v. 3.7 (Applied Biosystems). All samples were tested in duplicates.

Statistical Analysis

Testing of deviations from Hardy-Weinberg proportions among the populations was made using the exact test by the Markov chain method. Testing of genetic differentiation between the populations was performed by the Fisher's exact test. All statistical analyses were made using the software GENEPOP version 3.1c updated from Raymond and Rousset,¹⁹ as implemented for online access by E. Morgan (<http://genepop.curtin.edu.au/>).

Results

The first genotyping of each sample was made using agarose gels and minisequencing, whereas the second typing was made using only minisequencing. In total, 16.5% of the samples were typed three times and 1.5% were typed four times because of weak or inconclusive signals. Concordant profiles were obtained in all 400 samples.

Figure 2 shows the electrophoresis of 10 samples on a 1.5% agarose gel. The *GSTM1* and *GSTT1* genotypes were detected by the presence or absence of PCR products. The amplified fragment containing the *GSTP1* polymorphism was not always visible. This has no influence on the minisequencing reaction.

Figure 3 shows the electropherogram of the minisequencing reactions of two individuals. Both individuals were heterozygous (A/G) for the *GSTP1* polymorphism. Subject 1 was heterozygous for *GSTM1* and *GSTT1*, and subject 2 was homozygous for the deletion in *GSTM1* and *GSTT1*. In both electropherograms, some background was present. The SBE primers were designed to prevent the noise from overlapping with the signals from the extended SBE primers. Some of the noise may originate from primer dimers and primers that had not been totally digested by the SAP/Exol treatment. When the Qiagen MinElute PCR Purification kit (Qiagen) was used to purify the PCR products before minisequencing, a significant decrease in noise was obtained (data not shown).

Table 3 shows the distribution of *GSTM1*, *GSTT1*, and *GSTP1* alleles among the investigated populations. No significant deviation from Hardy-Weinberg proportions was found if adjusting the *P* values for multiple testing

Figure 2. Multiplex PCR products of 10 samples electrophoresed on a 1.5% agarose gel. For each individual, it was possible to determine the *GSTM1* and *GSTT1* genotypes by the presence or absence of the relevant amplification products. The DNA of the individual in lane 1, for example, was heterozygous for *GSTM1* and homozygous for the deletion in *GSTT1*. The DNA of the individual in lane 2 had two copies of both *GSTM1* and *GSTT1* because amplification products for both genes were present, whereas no amplification product was present corresponding to the deletions. The 1-kb Plus DNA Ladder (Invitrogen) was used as reference.

Figure 3. Electropherograms of *GSTT1*, *GSTM1*, and *GSTP1* typing in two individuals. The first individual was heterozygous for *GSTM1*, *GSTT1*, and *GSTP1*. The second individual was homozygous for the deletion in *GSTM1* and *GSTT1* and heterozygous for *GSTP1*.

using sequential Bonferroni adjustments.²⁰ Without adjustments, the locus *GSTP1* showed significant deviation from Hardy-Weinberg proportions in the Danish population ($P = 0.030$) due to an excess of heterozygotes, and the locus *GSTT1* showed significant deviations in the Greenlandic population ($P = 0.046$) due to an excess of homozygotes. Table 4 shows the combined genotype frequencies.

Table 3. Genotype Frequencies of *GSTT1*, *GSTM1*, and *GSTP1*

Gene/genotype	Frequencies		
	Denmark	Somalia	Greenland
<i>GSTT1</i>			
+/+	0.400	0.110	0.170
+/-	0.460	0.450	0.370
-/-	0.140	0.440	0.460
<i>GSTM1</i>			
+/+	0.070	0.160	0.080
+/-	0.405	0.440	0.450
-/-	0.525	0.400	0.470
<i>GSTP1</i>			
Ile/Ile	0.375	0.530	0.420
Ile/Val	0.535	0.390	0.520
Val/Val	0.090	0.080	0.060

Table 4. Combined Genotype Frequencies of *GSTT1*, *GSTM1*, and *GSTP1*

<i>GSTT1</i>	<i>GSTM1</i>	<i>GSTP1</i>	Frequencies		
			Denmark	Somalia	Greenland
+/+	+/+	Ile/Ile	0.0	0.0	1.0
		Ile/Val	2.5	0.0	1.0
		Val/Val	0.5	0.0	0.0
	+/-	Ile/Ile	5.0	1.0	4.0
		Ile/Val	9.0	3.0	3.0
		Val/Val	1.5	1.0	1.0
+/-	-/-	Ile/Ile	10.5	1.0	4.0
		Ile/Val	9.5	3.0	3.0
		Val/Val	1.5	2.0	0.0
	+/+	Ile/Ile	1.5	3.0	1.0
		Ile/Val	1.5	3.0	1.0
		Val/Val	1.0	0.0	0.0
	+/-	Ile/Ile	7.5	17.0	5.0
		Ile/Val	10.0	4.0	8.0
		Val/Val	0.5	1.0	2.0
	-/-	Ile/Ile	8.0	7.0	9.0
		Ile/Val	12.5	9.0	11.0
		Val/Val	3.5	1.0	0.0
-/-	+/+	Ile/Ile	0.0	7.0	0.0
		Ile/Val	0.0	3.0	3.0
		Val/Val	0.0	0.0	1.0
	+/-	Ile/Ile	3.0	8.0	9.0
		Ile/Val	3.5	8.0	12.0
		Val/Val	0.5	1.0	1.0
	-/-	Ile/Ile	2.0	9.0	9.0
		Ile/Val	5.0	6.0	10.0
		Val/Val	0.0	2.0	1.0

Discussion

Most studies investigating the possible effects of genetic variants in the *GSTM1* and *GSTT1* did not distinguish between genotypes with one or two copies of *GSTM1* and *GSTT1*. Previously developed assays testing the trimodal phenotype pattern only tested for one gene at a time.^{2,15} To our knowledge, these assays have only been used in small-scale studies probably because of the amount of work involved in the genotyping. Recently, multiplex real-time quantitative PCR-based assays for quantification of *GSTM1* and *GSTT1* gene copy numbers based on the TaqMan platform were developed allowing typing of one gene at a time.^{21,22} In this study, we developed a multiplex PCR assay that enables the distinction between no, one, or two copies of *GSTM1* and *GSTT1* and at the same time allows the detection of the allelic status of the *GSTP1* Ile105Val genetic variant. This makes large-scale studies of the effect of functional gene copy number of GSTs possible.

The locus *GSTP1* showed deviation from Hardy-Weinberg proportions in the Danish population ($P = 0.030$) when the P values were not adjusted by sequential Bonferroni adjustments. To investigate whether this deviation could be due to genotyping errors, all 200 Danish samples were retyped in singleplex PCR. There was no discrepancy in the results between the two assays, suggesting that the deviations from Hardy-Weinberg proportions in the *GSTP1* locus are not due to genotyping errors. In addition, in the Greenlandic population, the *GSTT1* locus showed deviation from Hardy-Weinberg proportions ($P = 0.046$) when the P values were not adjusted by sequential

Bonferroni correction. The Greenlandic population is genetically an admixture consisting of the original Greenlandic Inuits and Caucasians of mainly Scandinavian descent.²³ Significant differences in the allele frequencies of GST genotypes among various ethnic populations exist.²⁴ We believe that the deviation from Hardy-Weinberg in the *GSTT1* locus in Greenlanders is caused by the fact that the Greenlandic population consists of two major subpopulations and that the deviation is not due to genotyping errors.

In the present study, the distribution of GST alleles in the Danish population was significantly different from those in the Somali and Greenlandic populations ($P < 0.01$ for both comparisons), but the distribution of GST alleles in the Somali and Greenlandic populations were not significantly different ($P = 0.15$). The most marked difference between the ethnic groups concerned the frequencies of the *GSTT1* deletion. Among the Danes, we found a significant ($P < 0.0001$) lower frequency of double deletion of *GSTT1* (0.14) than among the Somalis (0.44) and Greenlanders (0.46). The frequencies of the investigated polymorphisms among the Danes and Greenlanders are similar to those observed in related populations.^{24–27} To our knowledge, no population closely related to the Somalis has been typed for *GSTM1*, *GSTT1*, and *GSTP1* alleles. The frequency of the *GSTM1* double deletion among Somalis (0.40) was intermediate to those found in sub-Saharan Africans and African Americans (0.28) and Arabs (0.55). In contrast, the frequency of the *GSTT1* double deletion among Somalis (0.44) was higher than those in the sub-Saharan Africans and African Americans (0.26) and Arabs (0.25). In addition, the frequency of the *GSTP1* Val/Val polymorphism was lower in Somalis (0.08) than in sub-Saharan Africans (0.12 to 0.21) and in Arabs (0.13).^{28,29}

Therapy regimens based on clinical trials in Europe and the United States often become the “standards” of treatment in many parts of the world. However, genetic differences between ethnic groups can be important in drug response, and therapy regimens should ideally be adjusted accordingly. We believe that the method described in the present study, allowing investigations of GST gene variants in a trimodal manner in large-scale studies, will be of relevance in both clinical and research settings.

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