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SHORT COMMUNICATION

Single tube genotyping of *GSTM1*, *GSTT1* and *TP53* polymorphisms by multiplex PCR

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Abstract
Glutathione S-transferases (GST) are enzymes involved in the conjugation of a number of human carcinogens, while *p53* tumour suppressor gene is the most frequently mutated gene identified till now in human neoplasias. Typically, *GSTM1* and *GSTT1* genotyping are performed together, with several different protocol described and sometimes with the risk of misclassification due to "false negative", depending on the internal positive control employed. Here, we report a modification of the classical multiplex polymerase chain reaction (PCR) method, allowing the genotyping of *GSTM1*, *GSTT1*, together with a polymorphism within the intron 3 of *TP53* tumour suppressor gene (a 16 base pairs (bp) duplication) in a single tube, with an appropriate internal positive control. To test the applicability of the method, the frequencies of the deleted alleles of *GSTM1* and *GSTT1* (null genotypes), and the 16 bp duplication of *TP53* gene were assayed in a series of Caucasian DNA samples.

Keywords: *Multiplex PCR*, GSTM1, GSTT1, TP53, *genotyping*

The glutathione S-transferase (GST) family of phase II metabolizing enzymes catalyzes the conjugation of glutathione to a wide variety of endogenous and exogenous molecules, including mutagens, carcinogens and chemotherapeutic agents (Strange et al. 2001). The resulting glutathione adducts have increased solubility and can then be either excreted or further metabolized. *GSTM1*, a member of the GSTμ family, is polymorphic with both alleles being deleted in approximately half of the population, while homozygotes for the *GSTT1* (class GSTθ) deletion were found in 20% of Caucasian population (Garte et al. 2001). For a quarter of a century the gene *TP53* has attracted close attention of scientists who deal with problems of carcinogenesis and maintenance of genetic stability. Mutations in this tumor suppressor gene are the most common genetic alteration in human malignancies, and different mutations are associated with different response to therapy (Aas et al. 1996). Within *TP53* gene, IVS3 + 47 16 bp duplication is one of the most studied polymorphism. This genetic variation consists of a duplication of 16 bp (GGGC-TGGGGACCTGGA) within the intron 3 (dbSNP ID: rs12720058). The wild-type allele (called A1) bears only one repetition. The variant allele (called A2) bears two repetitions, shows a frequency of about 0.12 in Caucasians, and it has been suggested to be associated with slightly reduced levels of *TP53* mRNA (Gemignani et al. 2004). This allelic variant was previously associated with several types of cancer, as shown in Table I.

Over 500 studies have examined the association of genetic variants of *GST*and *TP53* polymorphisms with various malignancies. In general, *GSTT1* and *GSTM1* are analysed together by multiplex PCR, whereas *TP53* requires an *ad hoc* genotyping method. In spite of the multiplicity of the studies on *GSTM1* and *GSTT1*, there is not a standardized protocol.

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Table I. OR and CIs for carriers of the 16 bp insertion in the intron 3 of the *TP53* gene (IVS3 + 47ins), as described in literature.

Tumor site (ca/co)	Codominant model					Dominant model			Citation
	OR for A1/A2	95% CI	OR for A2/A2	95% CI	p_{trend}	OR for carriers	95% CI	p_{assoc}	
Colorectal (374/322)	1.54	1.08–2.20	1.62	0.57–4.62	0.014				Gemignani et al. (2004)
Breast (448/249)						0.86	0.63–1.16	NS	Suspitsin et al. (2003)
Lung (517/544)	1.59	1.17–2.15	1.63	0.72–3.72	<0.001				Wu et al. (2002)
Breast* (116/167)						2.4*	1.3–4.2	0.004*	Powell et al. (2002)
Breast† (577/579)	1.30	1.00–1.70	1.70	0.80–3.40	0.03				Wang-Gohrke et al. (2002)
Cervical‡ (181/181)					NS				Kim et al. (2000)
Ovary¶ (310/364)						1.71	1.17–2.49	0.003	Wang-Gohrke et al. (1999)
Breast (107/305)						1.31	0.71–2.42	0.16§	Wang-Gohrke et al. (1998)
Breast (65/117)						2.19	1.21–3.97	0.01	Weston et al. (1997)
Breast (212/689)					NS				Sjalander et al. (1996)
Ovary (82/100)					NS				Lancaster et al. (1995)
Bladder (101/100)					NS				Lancaster et al. (1995)
Ovary (62/424)			8.64	2.97–25.2	<0.001				Runnebaum et al. (1995)
Lung (142/76)					NS				Birgander et al. (1995)
Colorectal (155/206)	0.50	0.29–0.89			<0.05	0.49	0.13–1.95	NS	Sjalander et al. (1995)

A1, wild type allele, no insertion; A2, variant allele carrying the IVS3 + 47ins allele. ORs are calculated setting the A1/A1 genotype as 1. ORs were reported when available. All studies, when not differently specified, were carried out on Caucasians. NS, statistically non significant; CI, confidence interval; ca, number of cases; co, number of controls.

* Association between poorly differentiated tumors vs. well/moderated differentiated tumors. † For age <50 years old. ‡ In women from Korea. ¶ Cases with *BRCA1* or *BRCA2* negative. § Associated with cancer when intron 3 + makes the haplotype with intron 6 MspI A1 allele.

Table II. Oligonucleotide sequences of primers and amplification product lengths.

Multiplex PCR	Size (bp)	Primer sense	Primer anti-sense
CYP2A6	570	AGCGGAACTGTTTCGGAGA	TGTGAGACATCAGAGACAACTTTC
GSTT1	233	caaccatccatccccagtc	TCACCGGATCATGGCCAGCA
GSTM1	189	GCTTCACGTGTTATGAAGGTTC	CAAAGAGAAAGGAGGATGGG
TP53	114 or 130	Tgctcttgtctttcagacttcct	gagcagtcagaggaccaggtc

For example, the employed primers differ among the studies and an appropriate internal positive control is often lacking. In particular, the internal positive control is not appropriate when it gives an amplification product shorter than those of *GSTT1* or *GSTM1*.

In fact, when a PCR reaction runs sub-optimally, the amplicons with a high molecular weight may undergo fainting, whereas the short-size amplicons are less affected by this technical artefact. When the internal positive PCR product is shorter than those of *GSTT1* and *GSTM1*, it is not possible to discriminate whether the lack of *GSTT1* or *GSTM1* bands is due to a real null genotype or to a malfunction in the PCR reaction, leading to possible false–null *GSTM1* or *GSTT1* calls.

Since, *GSTM1*, *GSTT1* and *TP53* are important candidate genes for performing case-control association studies to study the susceptibility to various types of cancer, and extensively studied, we propose here a one-tube multiplex polymerase chain reaction (PCR) method for the simultaneous detection of the three polymorphisms. The method is a modification of a classical standard protocol for genotyping *GSTM1* and *GSTT1* null alleles. With this method we assay the presence/absence of the *GSTM1* and *GSTT1* null deletions and the *P53* A1/A2 genotypes in one reaction. Multiplex PCR is performed using three primer pairs for the three variants and an additional primer pair for amplification of a *CYP2A6* positive control amplicon. The oligonucleotide sequences of primers and the lengths of the amplification products are given in Table II.

Ten-μM primers working solution is employed. The amount of sense and anti-sense primers for *CYP2A6*, *GSTT1* and *TP53* amplicons is 7.5 pmol (0.75 μl) each, whereas 15 pmol (1.5 μl) each are used for *GSTM1* primers.

Genomic DNA (20 ng) is mixed with primer solution, HotFire® Reaction Buffer BD 10X ($MgCl_2$ free), 200 nM dNTPs, 3 mM $MgCl_2$ and 1 U of HotFire® Taq polymerase (Solis Biodine, Tartu, Estonia) in a final volume of 20 μl. After initial denaturation at 94°C for 10 min, amplification is accomplished using a touchdown protocol with 20 cycles of 30 s at 94°C, 30 s at 68°C (− 1°C/cycle) and 30 s at 72°C followed by a final extension of 10 min at 72°C. The fragment sizes of the PCR products are: 570 bp for *CYP2A6* (the amplicon for positive control), 233 bp for *GSTT1*, 189 bp for *GSTM1* and 114 (for A1) or 130 bp (for A2) for *TP53* (Figure 1). The fragments are analysed in 3% agarose gel in a standard electrophoretic apparatus.

We have tested this method by genotyping the status of *GSTM1*, *GSTT1* and *TP53* IVS3 + 47dup in ten cell lines whose T1/M1/p53 status was known, obtaining the same results (data not shown). Moreover, we performed a hospital-based case-control association study on 74 controls and 65 colorectal cancer patients, to show the applicability of this method to a larger sample set. Cases were patients with a new diagnosis collected between 2000 and 2005. All cases had histological confirmation of their diagnosis. Controls were healthy blood donor volunteers collected during the same period of time. To avoid selection bias, only incident cases were selected and all subjects had the same ethnicity (Caucasian). This criterion was used to avoid inclusion of patients with chronic diseases. The ethical committee

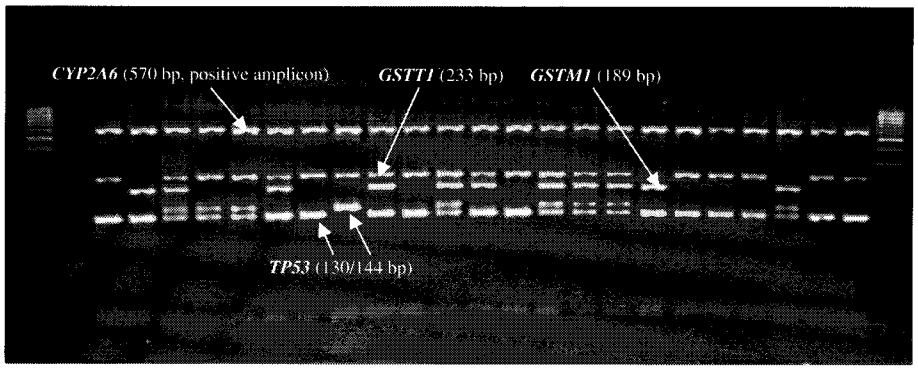

Figure 1. Picture of a 3% agarose gel showing various *GSTT1*/*GSTM1*/*TP53* genotypes and PCR product sizes.

Table III. Calculation of the ORs for *GSTM1*, *GSTT1* and *TP53* polymorphisms.

	Controls		Cases		OR	95% CI	Chi-sq.	Pass
	Positive	Null	Positive	Null				
GSTT1	34	9	52	13	0.944	0.364–2.451	0.014	0.906
GSTM1	24	19	41	24	0.739	0.337–1.621	0.570	0.450

	Controls			Cases			Dominant model: (+/− & −/−) vs. +/+			
	A1/A1	A1/A2	A2/A2	A1/A1	A1/A2	A2/A2	OR	95% CI	Chi-sq.	Pass
TP53	55	16	3	40	21	4	1.809	0.879–3.726	2.615	0.106

approved the study and subjects gave the informed consent. Statistical analyses was performed by the use of a logistic regression model, and odds ratio (OR) and 95% confidence intervals (95% CI) were calculated in order to assess the statistical significance of the association. The results are reported in Table III, showing no statistical association between cancer and *GSTM1* or *GSTT1* status. For *TP53*, the results show a positive association between A2 allele and cancer, when the eterozygotes were pooled with the A2/A2 homozygotes (dominant model). Carriers of the A2 allele show an OR of 1.81 as compared to the A1/A1 homozygotes. This association is of the same extent as described in larger studies (Gemignani et al. 2004), but the low number of subjects analysed here does not allow reaching the statistical significance.

The use of an amplicon with the highest molecular weight as positive control (i.e. the *CYP2A6*-570 bp band) enables us to check for the performance of the reaction and/or the quality of DNA sample. In fact, when PCR does not perform efficiently the long-size amplicons are affected more than the short-size amplicons. Since the presence or the absence of the band is crucial for assigning the correct genotype for *GSTM1* and *GSTT1* genes, the use of a positive control band with a higher molecular weight is highly recommended to avoid false negative calls. This simple and rapid method allows genotyping a high number of individuals in short time, increasing the throughput, as compared to the standard *GSTM1/GSTT1* genotyping. The assay is cost effective and provides the analysis of three genes at once.

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