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Glutathione S-transferase μ polymorphism and gastric cancer in the Portuguese population

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The glutathione S-transferases appear to form part of a protective mechanism against the development of cancer where environmental chemical carcinogens are involved. In humans one member of the *mu* class gene family (GSTM1) has been shown to be polymorphic and is only expressed in ~50% of individuals. Previous studies have shown a possible link between the null phenotype and susceptibility to cancer but have been equivocal regarding stomach cancer. To evaluate any association in Portuguese gastric cancer individuals with GSTM1 variability, we performed *GSTM1* polymorphism by PCR amplification in 148 gastric cancer patients and in 84 healthy control individuals. We found no statistical differences between the gastric cancer and control populations (wild type phenotype: 52%, 48%; null phenotype: 48%, 52%, respectively). A subset analysis into site of tumour also revealed no significant differences between the groups, although we found a slight increase of the wild type phenotype in the samples of the antrum compared with the control population (57% vs 48%, respectively; $\chi^2 = 1{\cdot}18$; $p \leq 0{\cdot}28$) and a slight increase of the null phenotype in the signet ring cells/mucocellular group ($\chi^2 = 1{\cdot}05$; $p \leq 0{\cdot}3$). However, in both cases it did not reach statistical significance. A subset analysis of the histological groups following the WHO criteria revealed a statistically significant difference ($\chi^2 = 3{\cdot}704$; $p \leq 0{\cdot}05$) between the moderately differentiated gastric adenocarcinoma and the presence of the wild type phenotype. These results do not support the hypothesis that the GSTM1 null phenotype predisposes to gastric cancer in the Portuguese population and the moderately differentiated gastric adenocarcinoma seems to be associated with the presence of the *GSTM1* wild type phenotype.

Keywords: glutathione S-transferase, GSTM1 polymorphism, gastric cancer, cancer susceptibility, Portuguese population, cancer epidemiology, null allele.

Introduction

The cytosolic glutathione S-transferases (GSTs) are a complex family of isoenzymes thought to play a significant role in cellular detoxification since they

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catalyse the conjugation of reduced glutathione (GSH) to a wide variety of hydrophobic and electrophilic molecules. Most of the known substrates for the conjugation reactions are environmentally derived and include many known mutagens and carcinogens as well as several therapeutic compounds (Board *et al.* 1990).

The GSTs appear to form a part of a protective mechanism against the development of cancer where environmental chemical carcinogens are involved and are also implicated in drug resistance in cancer chemotherapy (Board 1981, Black and Wolf 1991).

Cytosolic GSTs have been subdivided into four classes: *Alpha, Mu, Pi* and *Theta* (Mannervick *et al.* 1992). Five *Mu* class genes (GSTM1-5) have been localised in a cluster on chromosome 1p (Pearson *et al.* 1993). In humans, a member of the *mu* class gene family (*GSTM1*) has been shown to be polymorphic. Studies on different populations have shown that the fraction of the population with the GSTM1 null phenotype and/or genotype ranges from 35 to 65% (see Mikelsaar *et al.* (1994) for review).

The *GSTM1* null phenotype has been associated with an increased risk for several cancers, namely colon, bladder, lung, skin and stomach (Seidegard *et al.* 1986, Harada *et al.* 1992, Wolf *et al.* 1992, Hirvonen *et al.* 1993, Strange 1993, Zhong *et al.* 1993, Anttila *et al.* 1994, Heagerty *et al.* 1994) and individuals showing the GSTM1 null phenotype seem to be at high risk when exposed to elevated levels of certain electrophilic carcinogens (Board 1981). However, equivocal results regarding this association have been reported, namely for the cancer of the stomach (Strange *et al.* 1991, Harada *et al.* 1992, Deakin *et al.* 1994, 1996).

GST-*Mu* is present in normal and malignant gastric tissues (Howie *et al.* 1990, Peters *et al.* 1990) and in the stomach, the mucosa is in constant contact with noxious chemicals, some of which possess carcinogenic potential (Bruce 1987). It is therefore expected that gastric mucosa would possess endogenous means of inactivating these agents. This paper shows the results of population GSTM1 variability and cancer risk in several different histological types and site of gastric tumours.

The GSTM1 null phenotype has been ascribed to a homozygous deletion of the *GSTM1* gene (Seidegard *et al.* 1988). Two phenotypes have been found: *GSTM1*-null (GSTM1 0/0, null homozygous, corresponding to the absence of the gene and GSTM1 enzyme activity) and *GSTM1*-wild type (+/0, heterozygous and +/+, homozygous for the presence of the gene). In the present study we used a polymerase chain reaction (PCR)-based method to identify nulled and non-nulled individuals.

The question of the validity of these associations namely for gastric cancer, which is the most common cancer in Portugal, has major consequences for public health, cancer prevention, cancer screening and the understanding of mechanisms of carcinogenesis.

Materials and methods

Population samples

The study included 148 patients with histologically verified gastric carcinoma with no family history, from Pulido Valente, S. José and Santa Cruz Hospitals and 84 healthy controls, 65 men (20–60 years old) and 19 women (22–59 years old) obtained in the Blood Centre of the Egas Moniz Hospital, all in Lisbon. Permission was obtained to perform this study. All the analysed individuals

distributed as follows: gastric carcinoma patients, 84 men (ranging from 38 to 88 years old) and 64 women (from 34 to 86 years old); controls, 65 men (ranging from 20 to 60 years old) and 19 women (ranging from 22 to 59 years old).

Samples from formalin-fixed, paraffin-embedded gastric tissue were obtained after surgery or biopsy from gastric cancer patients classified according to the World Health Organization (WHO) histological criteria (Oota and Sobin 1977). Data were obtained regarding grade of differentiation and site of origin of tumour in the stomach. Peripheral blood from controls (2–5 ml), drawn in EDTA as anticoagulant, was taken for analysis .

DNA extraction

Extraction of DNA from tumour tissue was carried out as described by Imprain *et al.* (1987) with the following modifications: the samples were boiled after proteinase K digestion and DNA was resuspended in sterile water. DNA from blood was obtained by the guanidine–HCl extraction method (Sambrook *et al.* 1989), dissolved in 1 × TE buffer (pH 8·0). The DNA samples were stored at –20 ºC until further analysis.

PCR for GSTM1 genotypes

We determined the presence of the *GSTM1* gene by using a differential PCR in which multiple genes are co-amplified in the same reaction tube (Edwards and Gibbs 1994). The amplification reaction was carried out in a total volume of 20 µl containing 20 pmol of each of the GSTM1 primers, as previously described by Comstock *et al.* (1990), 20 pmol of each N-*ras* gene primers flanking codon 12 (N-*ras* 12) (Victor *et al.* 1990); 0·2 mM of each deoxynucleotide triphosphate (Pharmacia); 0·02 U μl^{-1} Taq polymerase (AmpliTaq, Perkin-Elmer); ~50 ng of DNA and 1 × reaction buffer (10 × PCR Buffer, Perkin-Elmer). After an initial denaturation at 94 ºC for 5 min, 37 cycles were run with denaturation at 94 ºC for 20 s, annealing at 55 ºC for 20 s and extension at 72 ºC for 30 s, completed with a final step at 70 ºC for 5 min. From the PCR amplification reaction a 273 and a 109 bp fragment are obtained, corresponding to the GSTM1 and N-*ras* 12 target regions, respectively.

Samples showing no GSTM1 co-amplification product were subject to a second amplification reaction similar to that described above but with no N-*ras* 12 primers to screen for eventual preferential amplification of the N-*ras* 12 PCR product (smaller) versus GSTM1 fragment (larger). All PCR reactions were carried out in a Thermal Cycler 9600, Perkin-Elmer.

After electrophoresis in a 3% (w/v) agarose, ethidium bromide (0·5 mg ml^{-1}) gel in 1 × TBE running buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8·0), PCR products were visualized on a UV transilluminator and photographed on Polaroid film.

Statistical analysis

The *GSTM1* gene distribution in the normal controls and the cancer patients groups were subjected to statistical analysis using chi-square tests and significance was set at $p \leq 0{\cdot}05$.

Results

Population association studies have implicated *GSTM1* null polymorphism with increased risk for certain cancers based on the cellular detoxification role of this enzyme in the noxious chemicals metabolism.

If the GSTM1 enzyme plays an important role in the detoxification of chemicals in the diet, we would expect a different cancer risk associated with *GSTM1* null phenotype. However, we did not find statistical difference between the Portuguese gastric cancer and control population (wild type: 52%, 48%; null: 48%, 52%, respectively) (table 1). A subset analysis into site of tumour also revealed no significant differences between the groups, although we found a slight increase of the wild type phenotype in the samples of the antrum compared with control population (57% vs 48%, respectively) (table 2) but this did not reach statistical significance ($\chi^2 = 1{\cdot}18$; $p = 0{\cdot}28$). A subset analysis of the histological groups revealed a statistically significant difference ($\chi^2 = 3{\cdot}704$; $p = 0{\cdot}05$) between the moderately differentiated adenocarcinoma (MDGA) and the wild type phenotype (table 3). However, signet ring cells/mucocellular gastric carcinoma showed a

Table 1. Frequency in Portuguese population of the presence and absence of *GSTM1* gene in healthy controls and gastric cancer patients (*n* — total number of samples under study).

Individuals	*n*	Presence	Absence
Gastric cancer	148	77 (52%)	71 (48%)
Controls	84	40 (48%)	44 (52%)

Table 2. Frequency of the presence and absence of *GSTM1* gene in different sites of the stomach.

	Localization	*n*	Presence	Absence
1	Body	57	28 (49%)	29 (51%)
2	Antrum	58	33 (57%)	25 (43%)
3	Fundus	20	9 (45%)	11 (55%)
4	Controls	84	40 (48%)	44 (52%)

n — total number of samples under study

Table 3. Frequency of the presence and absence of *GSTM1* gene in histological subsets of gastric cancer patients.

	Histological groups	*n*	Presence	Absence
1	Well differentiated adenocarcinoma	15	7 (47%)	8 (53%)
2	Moderately differentiated adenocarcinoma	46	30 (65%)	16 (35%)
3	Poorly differentiated adenocarcinoma / undifferentiated carcinoma	59	29 (49%)	30 (51%)
4	Signet ring cells / mucocellular	25	9 (36%)	16 (64%)
5	Controls	84	40 (48%)	44 (52%)

2 vs control population: $\chi^2 = 3{\cdot}700$; $p = 0{\cdot}05$.
n — total number of samples under study.

converse result with 64% (16/25) of the analysed samples having homozygous null genotype. However, this was not statistically significant ($\chi^2 = 1{\cdot}05$; $p = 0{\cdot}3$).

In our study we have used the co-amplification of two DNA-target regions: the *GSTM1* gene and an internal control DNA of the N-*ras* 12 gene. Since our DNA samples were extracted from formalin-fixed tissue, degradation is expected and PCR amplification of larger fragments will therefore be more difficult (Jackson *et al.* 1992) So, we have screened all the obtained null genotype samples with only the GSTM1 pair of primers to verify the possible occurrence of preferential amplification of the smaller fragment (N-*ras* 12 target region). We observed in the co-amplification reaction some samples with null genotype which turned out to show the presence of the *GSTM1* gene in the second PCR reaction (figure 1).

Discussion

The finding that individuals with GSTM1 null phenotype apparently express no *mu* activity of proteins (Warholm *et al.*1983, Strange *et al.* 1989) has led to the suggestion that these subjects are at increased risk of developing cancer. In fact, many enzymes are polymorphic and the biological consequences of the phenomenon are largely unknown because it is not generally possible to relate a

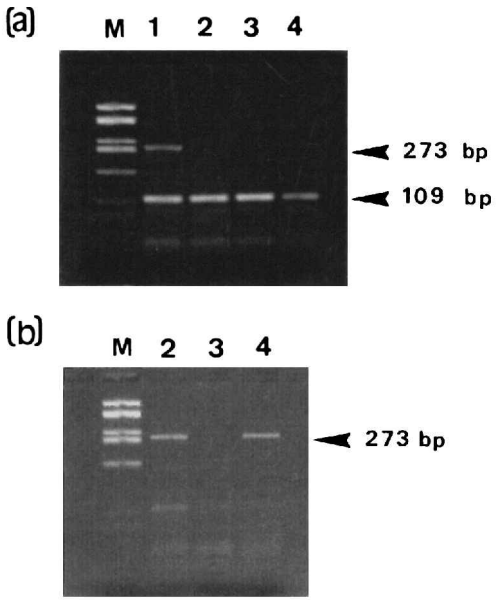

Figure 1. GSTM1 and N-*ras* 12 PCR products (273 bp and 109 bp, respectively) resolved by agarose gel electrophoresis. M is pUC18/HaeIII (Sigma) DNA marker. (a) A 109 bp DNA fragment can be seen in all the PCR reactions and a 273 bp DNA fragment is only present in samples containing GSTM1 gene on the first amplification (lane 1). (b) A 273 bp DNA fragment can be seen only in samples containing GSTM1 gene, on a second amplification procedure with only GSTM1 primers (lanes 2,4). Lanes 1, 2 and 4: wild type phenotype; lane 2: null phenotype. Lanes 2–4 in (a) correspond to lanes 2–4 in (b).

particular allelic variant to a physiological or pathological consequence. Several studies have been reported of the involvement of GSTM1 as a genetic susceptibility marker for cancer including cancer of the stomach (Strange *et al.* 1991). However, some other reports did not support such evidence (Deakin *et al.* 1994).

We assayed for the presence of the GSTM1 gene by using a differential PCR in which multiple genes are co-amplified in the same reaction tube. Primers for the N-*ras* 12 gene region were included as an internal positive reaction control because individuals with the GSTM1 nulled phenotype produce no PCR product. A second amplification was performed for the samples with absence of *GSTM1* gene which proved to be essential for avoiding false null results since preferential amplification of the small fragments appears to exist in some samples.

Although some authors have reported a significant correlation between GSTM1 null phenotype and adenocarcinoma of the stomach (Strange *et al.* 1991, Harada *et al.* 1992) our data for the Portuguese population failed to demonstrate any statistically significant difference in the *GSTM1* phenotype distribution among gastric adenocarcinoma patients and normal controls.

It should be noted that the stomach has distinct physiological regions. Therefore, a subset analysis into site of tumour has been performed (table 2). This analysis also failed to show any statistically significant difference. The samples from antrum showed, however, a slight association with the wild type phenotype but did not reach statistical significance. Further analysis into histological types showed an association between MDGA and the wild type phenotype. This is in marked contrast with data provided by Strange *et al.* (1991)

(1992) who found positive association with the null phenotype. However, these authors did not differentiate their samples either by histological type or by site of tumour in the stomach and a comparison is also not possible. Additionally, Deakin *et al.* (1994) and Deakin *et al.* (1996) published a non-association but a comparison with the MDGA group is not possible since they did not subdivide their samples. It is of interest that signet ring cells/mucocellular tumours showed an increase for homozygosity nulled individuals but statistical significance was not reached. To understand the possible association with the wild type phenotype further studies are needed to clarify the possible lack of activity of some GSTM1 isoenzymes.

It is of utmost importance to define a link between disease susceptibility and individual's polymorphism to provide a basis for a useful screening approach. *GSTM1* null polymorphism is not such a useful marker for susceptibility for cancer of the stomach in the Portuguese population.

In conclusion, these results (i) do not support the hypothesis that the GSTM1 null phenotype predisposes to gastric cancer, (ii) the moderately differentiated gastric adenocarcinoma seems to be associated with the presence of the GSTM1 wild type phenotype, (iii) further studies need to be performed to clarify the GSTM1 enzyme activity in the samples showing wild type phenotype.

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