

Papers

Association of p53 genomic instability with the glutathione S-transferase null genotype in gastric cancer in the Portuguese population

A R Conde, G Martins, C Saraiva, J Rueff, C Monteiro

Abstract

Aims—p53 gene mutations are the most common genetic changes known to occur in human cancer. In previous studies, the presence of alterations to the p53 gene has been linked to the null phenotype of the glutathione S-transferase μ gene (GSTM1). GSTM1 appears to be part of a protective mechanism against the development of cancers in which environmental chemical carcinogens are involved. To screen for such an association in stomach cancer, p53 allelic loss and genomic instability and GSTM1 genotypes were investigated in gastric tumour DNA samples from 113 patients.

Methods—The polymerase chain (PCR) reaction was used to amplify a (CA) repeat array in the p53 locus; electrophoresis, genotyping, and allele quantification were performed using an automated DNA sequencer and Genescan software. The presence of the GSTM1 gene was determined by means of a differential PCR in which multiple genes were co-amplified in the same reaction tube.

Results—Loss of heterozygosity (LOH) of the p53 gene was found in 36 of 87 informative cases and genomic instability was present in eight of 113 cases. Further analysis into histological subtypes and sites of tumours did not show any positive association with p53 loss. An association between the presence of LOH and the GSTM1 null genotype was not seen; however, all the samples with genomic instability of the p53 gene (eight of 113) also showed a GSTM1 null genotype.

Conclusion—This study does not support the hypothesis of an association between LOH in the p53 gene and the GSTM1 null genotype, but suggests that the GSTM1 null genotype might influence p53 genomic instability.

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Keywords: gastric cancer; GSTM1; p53; genomic instability

leads to the progressive disturbance of cell growth and differentiation control. Despite the recent decline in incidence in most countries, gastric carcinoma still remains a common worldwide cause of cancer related deaths. The prognosis of gastric cancer is extremely poor, with a five year survival rate ranging from 10% to 25%.¹ Several molecular genetic studies are underway in an attempt to clarify the mechanisms of carcinogenesis in these tumours.

The human p53 gene, located on chromosome 17p13, is a tumour suppressor gene whose inactivation is involved in carcinogenesis in various organs.² Functional inactivation of this gene by mutation and allelic deletion is thought to play an important role in the development of many human cancers.

Some reports on the association between alterations in the p53 gene and glutathione S-transferase μ (GSTM1) genotypes have been published but the issue still remains controversial.³⁻¹⁰ GSTM1 appears to be part of a protective mechanism against the development of cancer in which environmental chemical carcinogens are involved. Human GSTM1 is polymorphic and various studies have shown that the percentages of different populations with the GSTM1 null genotype range from 35% to 65%.¹¹ We undertook DNA analysis of 113 gastric carcinomas to investigate p53 allelic loss and genomic instability and GSTM1 genotypes to check for an involvement of this carcinogen metabolising enzyme in p53 gene disturbance.

Materials and methods

We obtained gastric carcinoma samples, including all locations and histological types using World Health Organisation criteria,¹² from 113 patients; in addition, blood was drawn from 84 healthy individuals. We extracted DNA from paraffin wax embedded tissues from biopsies or surgical specimens using a phenol/chloroform method,¹³ and obtained DNA from blood by means of the guanidine/HCl extraction method.¹⁴ Polymerase chain reactions (PCRs) were carried out in a Perkin-Elmer thermocycler model 9600.

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The development of cancer requires the accumulation of multiple genetic lesions, which

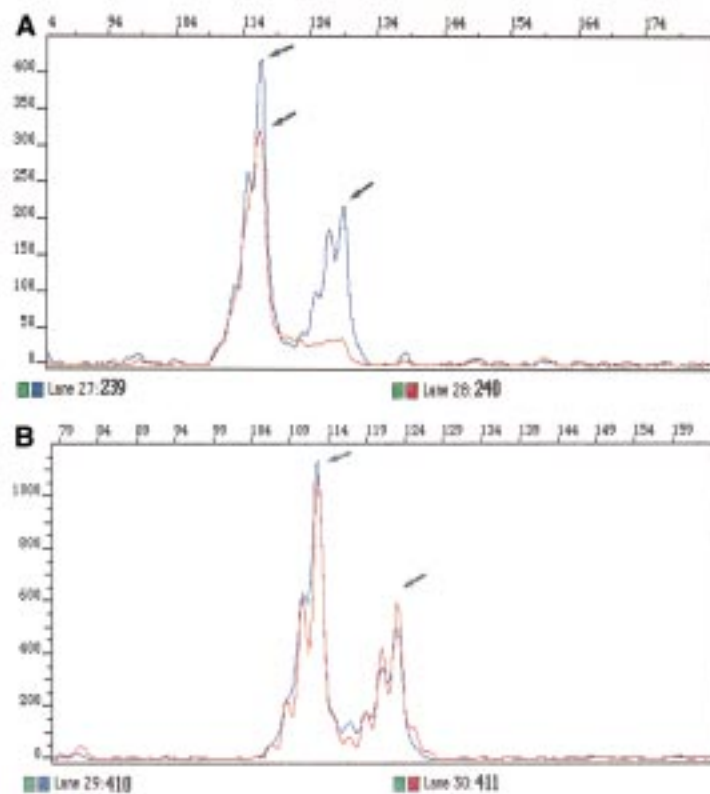


Figure 1 (A) Electrophoretogram showing p53 loss of heterozygosity (sample number 240). Normal DNA alleles are shown in blue; DNA alleles of the matched tumour sample are shown in red. Constitutional alleles are indicated by the arrows. A reduction of one of the constitutional alleles can be seen in tumour DNA when compared with the matched normal sample. (B) Electrophoretogram showing retention of heterozygosity of p53 (sample number 411). Normal DNA alleles are shown in blue; DNA alleles of the matched tumour sample are shown in red. The allelic profiles are identical.

p53 ANALYSIS

In the DNA samples obtained from tumours and adjacent tissues, we amplified a (CA)_n flanking marker by the PCR using the fluorochrom TET (Perkin-Elmer ABD, Branchburg, New Jersey, USA) attached to the 5' end of one of the PCR primers. The PCRs contained ~ 100 ng genomic DNA, 5 pmol of each primer,¹⁵ 0.2 mM dNTPs, and 0.5 U Taq DNA polymerase (AmpliTaq-PE; Perkin-Elmer ABD) in 1× reaction buffer (Perkin-Elmer ABD). The PCR amplification protocol

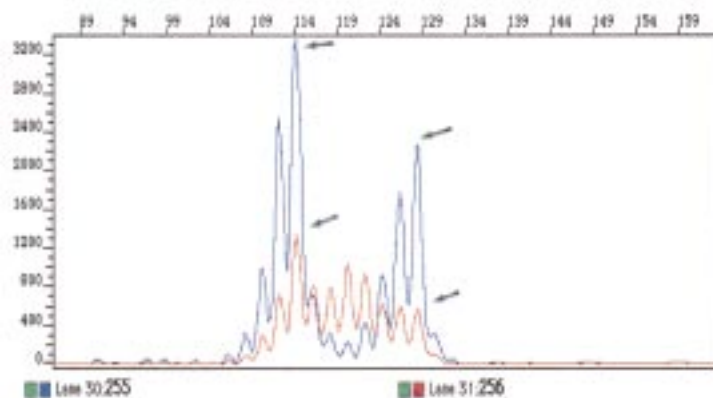


Figure 2 Electrophoretogram showing p53 microsatellite instability (sample number 256). Normal sample DNA alleles are shown in blue; DNA alleles of the matched tumour sample are shown in red. Constitutional DNA alleles are indicated by the arrows. The areas of the constitutional alleles are reduced in the tumour sample in favour of new ones.

was as follows: an initial denaturation step at 95°C for five minutes, followed by 35 two step cycles of two minutes at 60°C and one minute at 94°C, with a final extension step at 72°C for five minutes.

We determined loss of heterozygosity (LOH) of the p53 gene by size separation of PCR products in 6% polyacrylamide gels containing 8.3 M urea in the presence of 350-TAMRA size standard (Perkin-Elmer ABD). Electrophoresis was performed for three to four hours in an automated DNA sequencer 373A (Perkin-Elmer ABD, Foster City, California, USA) and the results were analysed with Genescan 672 software (Perkin-Elmer ABD).

GSTM1 ANALYSIS

In the DNA samples obtained from gastric tumours and from healthy individuals we determined the presence or absence of the GSTM1 gene using a protocol in which the sequences of two genes (GSTM1 and N-ras) are co-amplified in the same reaction tube. The PCR amplification protocol was as follows: 94°C for five minutes, followed by 37 cycles of 94°C for 20 seconds, 55°C for 20 seconds, 72°C 30 seconds, and a final extension at 72°C for five minutes. We detected GSTM1 genotypes using oligonucleotide primers to the 5' region of exon 4 and to the 3' region of exon 3 of this gene.¹⁶

Primers flanking codon 12 of the N-ras 12 gene were included as an internal positive reaction control because individuals with the GSTM1 null phenotype produce no PCR product. We performed a second amplification of the samples in which the GSTM1 gene was absent, and this proved to be essential for avoiding false null results, because preferential amplification of the small fragments (N-ras 12) occurred in some samples.¹⁷

PCR amplified products were electrophoresed on a 3% agarose gel and visualised using ethidium bromide and UV light.

Results

We tested gastric primary tumours and corresponding normal mucosal tissues for retention or loss of the p53 gene, p53 instability (replication errors phenotype) and for GSTM1 polymorphism.

We evaluated LOH by comparing the areas and the shapes of the allele peaks in normal and matched tumour samples. In addition, we used the following formula to assess the eventual allele reduction: $(T1/T2)/(N1/N2)$, where T1 and N1 are the areas of the shorter length allele product peaks for the tumour and normal samples, respectively, and T2 and N2 are the areas of the longer length allele product peaks for the tumour and normal samples, respectively.¹⁸ Tumour samples were considered to have LOH when a 34%¹⁹ or more area of reduction was detected in one of the alleles compared with the normal sample. Eighty nine of the 113 individuals assayed were heterozygous (informative); of these, 36 of 87 patients showed LOH in their tumour samples. Figures 1A and B show examples of LOH and retention of het-

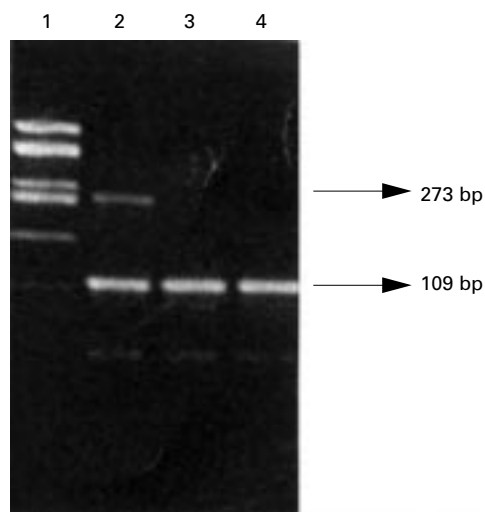


Figure 3 Glutathione S-transferase μ (GSTM1) and N-ras 12 PCR products. Electrophoresis of co-amplified products GSTM1 (273 bp) and N-ras 12 (109 bp). Lane 1, pUC18/HaeIII DNA marker; lane 2, GSTM1 wild-type phenotype; lanes 3 and 4, GSTM1 null phenotype.

erozygosity, respectively. There was no significant variation in p53 LOH frequency among different histological types or different tumour sites.

Microsatellite instability within the p53 gene was identified by: (1) the presence of novel (smaller and/or larger) alleles in the tumour samples that were not seen in matched normal samples; and (2) a reduction in the areas of the constitutional alleles in favour of the areas of the stutter bands, with no evidence of other new alleles, but with the persistence of the constitutional ones. Instability was present in eight of 113 cases (7.1%). Figure 2 shows an example of microsatellite instability. Size separation of p53 PCR products using an automated DNA sequencer and Genscan analysis demonstrated microsatellite instability of the p53 gene.

The enzyme encoded by GSTM1 plays an important role in the detoxification of chemicals in the diet, and a higher cancer risk is reported to be associated with the GSTM1 null phenotype. However, in a previous study we found no significant difference between a group of Portuguese patients with gastric cancer and a control population (wild-type genotype: patients, 52%; controls, 48%; null genotype: patients, 48%; controls, 52%).¹⁷

In our study, we used the co-amplification of two DNA target regions: the GSTM1 gene and an internal control of the N-ras gene (fig 3). Because our DNA samples were extracted from formalin fixed tissue, some DNA degradation would probably have occurred, so that

Table 1 Results of p53 allele profiles and GSTM1 genotypes

		GSTM1 positive	GSTM1 negative
p53 LOH	36/87	18/36	18/36
p53 no LOH	43/87	21/43	22/43
p53 genomic instability	8/113	0/8	8/8

GSTM1, glutathione S-transferase μ ; LOH, loss of heterozygosity.

PCR amplification of larger fragments would be more difficult. Therefore, we screened all the samples that initially gave null genotype results with the GSTM1 pair of primers only, in case preferential amplification of the smaller fragment (N-ras 12 target region) had occurred, to avoid false null GSTM1 genotypes.

Recent studies have revealed that genetic instability and LOH are two of the most important predisposing factors for human multistep carcinogenesis.²⁰⁻²² We found no association between the presence of LOH and the GSTM1 null genotype in our study (table 1). In addition, subset analysis of tumour site and histological subtypes did not reveal any association between p53 LOH and instability profiles and the GSTM1 genotype. However, the samples with genomic instability of the p53 gene (eight of 113) also showed a GSTM1 null genotype. Therefore, because the GSTM1 null phenotype has a frequency of one in two in the Portuguese population,¹⁷ the probability that all eight tumour samples show this phenotype is one in 256 (one in 2⁸).

Discussion

Functional inactivation of the p53 gene through mutation or allelic deletion might play an important role in the development of a variety of human tumours. Several studies have shown that many tumours with allelic deletion of chromosome 17p had point mutations of the p53 gene in the remaining allele and that the presence of p53 LOH might be an important factor involved in the association between p53 gene abnormality and the development of cancer.²³

Population studies have associated the presence of the GSTM1 null polymorphism with an increased risk for certain cancers, based on the role of this enzyme in the cellular detoxification of noxious chemicals.

Our study of the Portuguese population shows an incidence of p53 LOH of 36 of 87 (41%) in human gastric carcinomas. Microsatellite instability was detected in eight of 113 cases (7.1%). We have previously described the frequency of the different GSTM1 genotypes in Portuguese patients with gastric cancer and in a control population (wild-type genotype: patients, 52%; controls, 48%; null genotype: patients, 48%; controls, 52%).¹⁷

A comparison of the instability profile of gastric cancer with the histological classification of Laurén²⁴ has been attempted by several authors but their data are still debatable. Although some authors found no correlation between microsatellite instability and histological subtype,²⁵⁻²⁸ others did find such a correlation.²⁹⁻³³ In our study, we analysed the samples with both microsatellite instability in the p53 gene and the GSTM1 null genotype for any correlation with histological subtypes according to the Laurén classification. We found no such association with any of the subgroups (four of eight intestinal type; four of eight diffuse type).

Until now, controversial results regarding the association of alterations in the p53 gene and GSTM1 genotypes have been reported. Perrett

et al described studies in pituitary tumours and found no association between GSTM1 genotype and altered p53 expression.⁴ Ohshima and Xu reported that there was no significant relation between p53 mutations and GSTM1 genotypes in lung cancer.⁹ Studying oral carcinomas, Lazarus *et al* found that the highest p53 mutation prevalence was seen in individuals with a GSTM1 positive genotype.¹⁰ However, these reports contrast strongly with the data provided by McGlynn *et al*, who found that individuals with the GSTM1 null genotype might be at greater risk of developing p53 mutations when exposed to the human hepatocarcinogen aflatoxin B1.³ Furthermore, Kawajiri *et al* reported a remarkably high risk of having a mutation of the p53 gene when combined with the GSTM1 null genotype.⁶ In addition, Sarhanis and colleagues⁷ found a strong association between overexpression of p53 and the GSTM1 null genotype in epithelial ovarian cancer and Brockmoller and colleagues⁵ reported that, in bladder cancer, low/deficient activities of conjugating enzymes, such as GSTM1, might influence acquired mutations in the p53 gene.

Our data show that all the gastric cancer samples with the instability profile also have the GSTM1 null genotype. Our study does not support an association between LOH in the p53 gene and the GSTM1 null genotype, but suggests that the GSTM1 null genotype may influence p53 genomic instability.

The influence of GSTM1 and other polymorphic carcinogen metabolising enzymes on mutational events is not yet well understood and, no doubt, will have important implications in disease prognosis or prevention.

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