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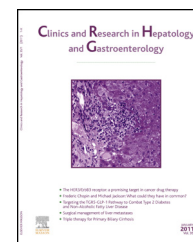
ORIGINAL ARTICLE

Glutathione S-transferase gene polymorphisms in celiac disease and their correlation with genomic instability phenotype

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Summary

Background and objective: Genomic instability and reduced glutathione S-transferase (GST) activity have been identified as potential risk factors for malignant complications in celiac disease (CD). In this study, we assessed the possible influence of GST polymorphisms on genome instability phenotypes in a genetically characterised group of celiac patients from previous studies.
Methods: The deletion polymorphisms in *GSTM1* and *GSTT1* genes and the single-nucleotide polymorphism *GSTP1* c.313A>G were genotyped using PCR in a set of 20 untreated adult patients with a known genomic instability phenotype and 69 age- and sex-matched healthy individuals.
Results: The frequencies of variant genotypes in patients were *GSTM1*-null (30%), *GSTT1*-null (5%), *GSTP1*-AG (60%) and *GSTP1*-GG (15%), and they showed no differences from controls. No significant differences were found in the genotype distribution based on telomere length. Cases with *GSTM1*-null genotype (83%) and microsatellite stability were more frequent than those with genomic instability. Moreover, carriers of *GSTP1*-variant genotype (73%) and stable phenotype were significantly increased compared to unstable patients (27%) ($P = 0.031$). No differences were found according to the clinical-pathological characteristics of celiac cases.
Conclusions: No association between GST polymorphic variants and celiac-associated genomic instability was proven in our cohort. Future studies should explore the usefulness of other biomarkers to distinguish celiac patients who are susceptible to cancer development.
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Introduction

Celiac disease (CD) is a common chronic intestinal inflammatory disorder triggered by dietary gluten whose aetiology involves both genetic and environmental factors. Most patients improve with a gluten-free diet (GFD), but some cases (2—5%) progress to malignancy [1]. CD carcinogenesis has been linked to chromosome instability (CIN) [2,3], telomere shortening [4], microsatellite alterations [5] and chronic intestinal inflammation [6,7]. Recently, Hojsak et al. reported that CIN in CD is not caused by dissemination of abnormal intraepithelial cells, which led to further questions about this phenomenon [8]. To date, the molecular mechanisms underlying genomic instability and cancer onset in CD remain unknown.

Polymorphisms in metabolic enzyme genes that may cause alterations in protein activity have been associated with genomic instability and carcinogenesis [9]. Growing insights suggest that variability within glutathione S-transferase (GST) genes influence DNA stability [10]. Moreover, cancer susceptibility in CD has been associated with lower GST activity in the small intestinal mucosa of untreated patients and an impaired detoxification system [11]. GSTs are phase II detoxification enzymes that are involved in cellular protection against xenobiotics and oxidative stress [12]. Deletion polymorphisms in *GSTM1* and *GSTT1* genes and a single-nucleotide polymorphism (SNP) within the *GSTP1* gene (c.313A>G, rs1695, p.105 Ile>Val) result in complete absence of or a significant reduction in enzyme activity [13—16]. Polymorphisms that alter the detoxification capacity within the small intestinal mucosa of CD patients may induce genomic instability, which in turn, may facilitate malignant transformation. Notably, GST variants could be useful as molecular markers for detecting cancer susceptibility in high-risk CD patients. To our knowledge, the prognostic significance of GST polymorphisms and their relationship to CD-associated genome instability has not been previously explored. We therefore focus on the genetic variability of GST genes to determine its putative modulating role on genome integrity in CD. In this exploratory study, we assessed the genetic profile of GST variants and investigated their possible influence on genome instability in a genetically characterised group of CD patients.

Materials and methods

Patients and controls

Twenty adult patients with CD (14 females/6 males; mean age 35.2 years; range 21—66 years) were recruited in 2003 at the HIGA ''San Martín'' Hospital of La Plata, Buenos Aires, Argentina, to evaluate genomic instability status through the measurement of telomere restriction fragments (TRFs) [4] and microsatellite alterations [5]. Thirteen patients were studied at diagnosis and the remaining 7 had been previously diagnosed, having a mean evolution time of 145 months (range: 11—326 months); however, none of the patients were complying with the GFD at the time of sample collection. Matched peripheral blood lymphocyte (PBL) and small bowel biopsy (SBB) samples were available from each patient. The clinical-pathological data of these patients have been already described [4,5]. CD diagnosis was based on clinical symptoms, positive serology, *i.e.*, antigliadin IgA (AGA-A), antigliadin IgG (AGA-G), antiendomysial IgA (EMA-A) and/or anti-tissue transglutaminase IgA (IgA-tTG) antibodies associated with the typical histopathological lesions sampled during the duodenal biopsy. Intestinal biopsy samples were categorised according to Marsh's criteria [17]. All newly diagnosed and the seven re-biopsied CD patients had a flat duodenal mucosa (III of Marsh's criteria). In addition, PBL samples were obtained from 69 unrelated, healthy blood donors with comparable age (mean age 44.2 years; range: 21—65 years) and gender distribution (46 females and 23 males). Patients and controls were residents of Buenos Aires and the surrounding urban area, which is a central region of Argentina, and had the same ethnicity. All individuals provided their informed consent according to institutional guidelines prior to inclusion. The study was approved by the Institutional Ethical Committee and complies with the International Declaration of Helsinki.

GST genotyping

Genomic DNA was isolated using standard proteinase K/phenol/chloroform or salting out methods. A multiplex PCR assay using previously published primer pairs were used to amplify *GSTM1* and *GSTT1* genes, with *β-globin* as the internal positive control [18]. PCR was performed in a final volume of 25 μL containing the following: $MgCl_2$ (1.5 mM); dNTP (0.1 mM); primers: *GSTM1* (0.3 μM), *GSTT1* (0.4 μM), or *β-globin* (0.6 μM); Taq polymerase (1 U); and 100 ng of genomic DNA. The cycling conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C-59 °C-72 °C for 30 s each; and 5 min at 72 °C. Electrophoresis was performed on 2% agarose gels dyed with ethidium bromide. *GSTP1* c.313A>G genotypes were identified by RFLP-PCR at 55 °C using previously reported primer pairs (0.4 μM) [16]. PCR products were digested with *Alw*261 restriction enzyme and analysed by electrophoresis on 4% 3:1 NuSieve/agarose gels as previously described [19]. All genotypes were independently scored by two reviewers, and 10% of the samples were randomly reanalyzed, yielding identical results.

Statistical analysis

For the statistical analysis, Student's *t*-test, Chi^2 or Fisher's exact tests were performed using GraphPad Prism Version 5.0 (2008). Hardy—Weinberg equilibrium was tested using the Chi^2 test. The 2×2 contingency Fisher's exact test was used for estimating odds ratios (Ors) and their 95% confidence intervals (95% CIs). Values of $P < 0.05$ were regarded as statistically significant.

Results

This study was performed in a cohort of CD patients with previously published features of genomic instability analysed based on telomere length [4] and microsatellite alterations [5]. No deviation from Hardy-Weinberg equilibrium was demonstrated for *GSTP1* c.313A>G in either the controls or patients. Fig. 1 shows the multiplex genotyping of
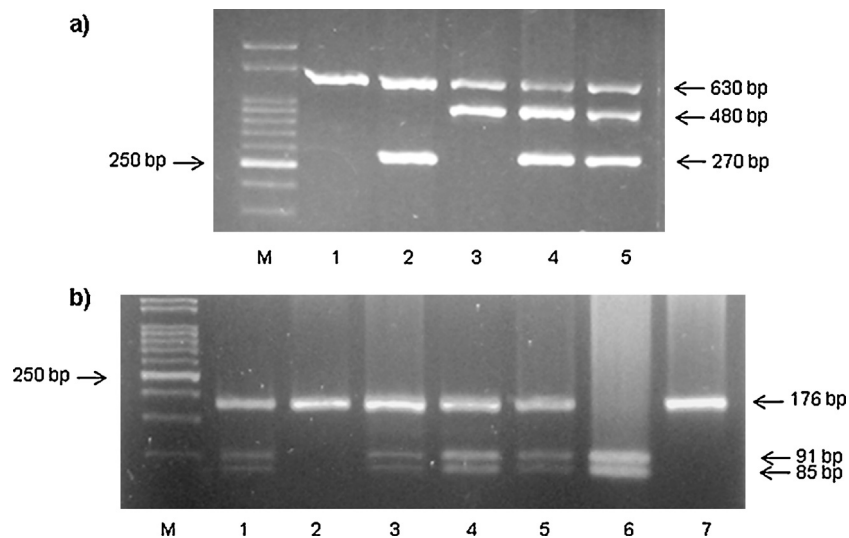
Figure 1 a) agarose gel electrophoresis of multiplex PCR for *GSTM1* and *GSTT1* genotyping. The electrophoretic pattern of CD patients (lanes 1—4) and a healthy control (lane 5) is shown on a 2% ethidium bromide-stained agarose gel. *GSTM1* and *GSTT1* wild-type (wt) genotypes carrying one or two alleles displayed a band of 270 and 480 bp, respectively. Null genotypes were revealed by the absence of a PCR product, despite the presence of β-globin (630 bp) used as an internal positive control. Lane 1: deletion of both *GSTM1* and *GSTT1* genes; lane 2: *GSTT1* deletion; lane 3: *GSTM1* deletion; lanes 4 and 5: *GSTM1* and *GSTT1* wild-type genotypes. b) PCR-RFLP for *GSTP1* c.313A>G separated on a 4% NuSieve/agarose gel (3:1) stained with ethidium bromide for 5 patients (lanes 1—5) and 2 controls (lanes 6 and 7). The 176 bp *GSTP1* product was digested with *Alw*261, which does not cut the wild-type sequence but cleaves the G sequence to yield two restriction fragments of 91 bp and 85 bp. Lanes 1, 3—5: heterozygous genotypes (*GSTP1*-AG); lanes 2 and 7: homozygous wild-type genotypes (*GSTP1*-AA); lane 6: homozygous variant genotype (*GSTP1*-GG). M: molecular weight marker of 50 bp.

GSTM1 and *GSTT1* and the digestion of *GSTP1* c.313A>G. The analysis of the genotype and allele frequencies of GSTs in celiac patients showed no significant differences compared to controls (Table 1). Because the frequencies of the heterozygous *GSTP1*-AG and homozygous *GSTP1*-GG genotypes were slightly increased in patients compared to controls, they were combined (AG + GG) and referred to as the *GSTP1*-variant (*GSTP1*-var) genotype. However, no significant differences were found. Systematic GST gene alterations triggered by the inflammatory process were excluded comparing genotypes of paired PBL and SBB samples from 10 patients. A combined analysis of the three

genes was also performed because GST enzymes are involved in the same metabolic pathway with overlapping substrate specificity, and it is probable that associations exist between different genotypes. No combination of possibly interactive polymorphisms reached statistical significance.

To determine the role of GST polymorphisms in the susceptibility to genetic instability, the *GSTM1* and *GSTP1* c.313A>G genotypes in CD cases were also analysed with respect to the genomic instability status. The *GSTT1* gene was excluded because only one case exhibited a null genotype. The distribution of *GSTM1* or *GSTP1* c.313A>G genotypes according to the mean TRF values showed no significant differences

Table 1 Genotype and allele frequencies of GST polymorphisms in celiac patients and healthy individuals.

Genes	Genotypes/alleles	Controls n = 69 (%)	Patients n = 20 (%)	OR (95% CI)	P
GSTM1	Wild-type	35 (51)	14 (70)	Reference	
	Null	34 (49)	6 (30)	0.44 (0.15—1.28)	0.2
GSTT1	Wild-type	59 (85.5)	19 (95)	Reference	
	Null	10 (14.5)	1 (5)	0.31 (0.04—2.58)	0.44
GSTP1	AA	34 (49)	5 (25)	Reference	
	AG	29 (42)	12 (60)	2.81 (0.88—8.93)	0.10
	GG	6 (9)	3 (15)	3.40 (0.64—18.14)	0.16
	AG + GG	35 (51)	15 (75)	2.81 (0.95—8.91)	0.07
	A	97 (70)	22 (55)	Reference	
	G	41 (30)	18 (45)	1.94 (0.94—3.98)	0.09

GST: glutathione S-transferase; OR: odds ratios; CI: confidence intervals; AA: homozygous wild-type genotype; AG: heterozygous genotype; GG: homozygous variant genotype.
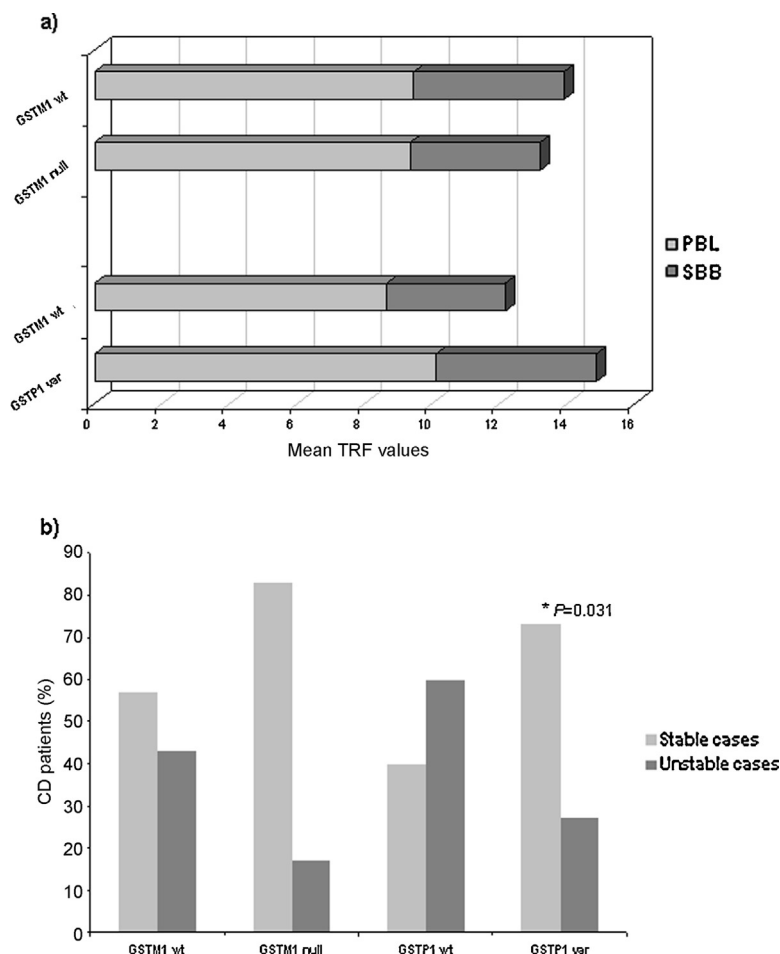
Figure 2 a) the distribution of *GSTM1* and *GSTP1* genotypes according to the telomere length found in peripheral blood lympho-cytes (PBL) and small bowel biopsies (SBB) from CD patients, showing no significant differences between the tested groups. The TRF frequencies (mean $\pm$ SE) in SBB samples were as follows: *GSTM1*-wt (4.44 ± 1.43), *GSTM1*-null (3.84 ± 1.2), *GSTP1*-wt (3.5 ± 1.09) and *GSTP1*-var (4.74 ± 1.32). The mean TRF values in PBL were as follows: *GSTM1*-wt (9.44 ± 1.66), *GSTM1*-null (9.35 ± 1.32), *GSTP1*-wt (8.64 ± 1.36) and *GSTP1*-var (10.10 ± 1.72); b) the distribution of *GSTM1* and *GSTP1* genotypes in CD patients according to genomic instability phenotype defined by microsatellite analysis comparing the percentage of cases with different genotypes as referred in Table 2. A significant difference was observed for cases with the *GSTP1*-variant genotype and the stable phenotype compared to unstable patients ($P = 0.031$). *GSTM1*-wt or *GSTP1*-wt: wild-type genotypes; *GSTP1*-var: variant genotypes.

in both SBB and PBL samples among wild-type (wt) car-riers compared to patients with null or variant genotypes (Fig. 2a). Based on the microsatellite study, 13 geneti-cally stable and 7 unstable cases were analysed according to their genotypes (Table 2). For wild-type carriers, there was no evidence of a difference between patients with

or without genomic instability. Although most cases with the *GSTM1*-null genotype (5/6, 83%) had a stable pheno-type, no significant difference was observed. In addition, most cases with the *GSTP1*-var genotype (11/15, 73%) also exhibited a stable phenotype, showing a significant differ-ence ($P = 0.031$) compared to unstable patients (4/15, 27%)

Table 2 Genotype distribution of *GSTM1* and *GSTP1* genes according to genomic instability phenotype.

	GSTM1		GSTP1	
	Wild-type	Null	Wild-type	Variant
All patients ($n = 20$)	14 (70)	6 (30)	5 (25)	15 (75)
Genomic instability status				
Stable CD ($n = 13$)	8 (57)	5 (83)	2 (40)	11 (73)
Unstable CD ($n = 7$)	6 (43)	1 (17)	3 (60)	4 (27)
P	0.72	0.087	1.00	0.031

CD: celiac disease.

(Fig. 2b). Regarding the clinical-pathological characteristics of these patients, there was no correlation between genotypes and age, sex, clinical form, malabsorption or nutritional status.

Discussion

In this study, the distribution of GST polymorphisms in celiac patients and the putative modulating effect of GST genotypes on CD-associated genome instability were assessed. Indeed, this is a key issue because celiac sprue is a life-long condition that predisposes individuals to malignancy, and genomic instability contributes to cancer. According to previous studies, GST expression is increased in the small intestine of normal individuals [20], but low activity has been reported in celiac patients [11], and an insufficient detoxification capacity of the small intestinal epithelium has been associated with the increased tumour incidence [21,22]. Subsequently, polymorphisms that cause a significant reduction or even complete absence of GST activity may induce genomic instability and malignant transformation in CD patients. However, no association between GST polymorphisms and the instability phenotype was proven in our cohort. No significant differences were found in the genotype distribution between patients and controls or compared with telomere length in either normal lymphocytes or inflamed intestinal mucosa. Moreover, contrary to our hypothesis, the *GSTM1*-null and *GSTP1*-variant genotypes were strongly associated with stable phenotypes.

We recognise that the small number of patients included in this study limits the strength of our findings, but this celiac group is already genetically characterised, and the instability phenotype in these patients is well known. Although studies of polymorphisms usually require a large population, the common finding of CD cases with variant GST genotypes and stable genomes is really striking. Because of this observation, we think that this limitation would be unlikely to have a substantial impact on our interpretation. Therefore, in spite of the cohort size, this study indicates that *GSTM1* and *GSTP1* c.313A>G polymorphisms do not seem to promote genomic instability in CD.

The loss of genome stability is usually accepted as one of the most important features of carcinogenesis through the accumulation of multiple genetic lesions. Alternatively, emerging evidence suggests that germline polymorphisms may influence cancer development [23] as well as modulate genetic instability [9,10,24]. The major sources of genome instability involve exposure to genotoxic agents coupled with failures in DNA replication and the DNA damage response [25]. While there are numerous unknown genetic factors, it is accepted that inherited polymorphisms appear to influence DNA stability. The underlying hypothesis is that individuals with variant genotypes are at an increased risk of DNA mutations due to a lack of protection against carcinogens [26]. Increased chromosomal aberrations and DNA damage was confirmed in subjects carrying *GSTM1*/*GSTT1*-null genotypes who were exposed to carcinogens [27]. *GSTM1* gene deletion was also related to telomere length alterations [28]. In contrast, no significant interaction was observed between GST variants and telomere shortening [29] or chromosomal aberrations [30] by other authors.

Based on these contradictory data, the specific role of GST genes in modulating genome damage remains to be determined.

In summary, the present findings indicate that no association exists between GST polymorphic variants and celiac-associated genomic instability in our cohort. Most likely, mechanisms other than null or reduced GST activity impair DNA integrity during the inflammatory process. Due to the lack of known mechanisms, further studies are needed to identify novel prognostic indicators to distinguish celiac patients who are at a higher risk of cancer development.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgments

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