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ORIGINAL ARTICLE

Association between gastric mucosal glutathione-S-transferase activity, glutathione-S-transferase gene polymorphisms and *Helicobacter pylori* infection in gastric cancer

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Abstract
Aim Helicobacter pylori infection, though common, leads to gastric cancer (GC) in less than 1% individuals, suggesting the role of host factors. We previously reported the role of glutathione–S–transferase (GST) polymorphisms, the gene encoding a carcinogen–detoxifying enzyme, in GC. This study was aimed to evaluate GST enzyme activity, GST polymorphism, glutathione (GSH) levels and *H. pylori* in patients with GC.
Methods GST and GSH levels were estimated in gastric biopsies of 52 patients with GC, 37 functional dyspepsia (FD) and 39 peptic ulcer (PU), and correlated with *H. pylori* (ELISA) infection and GST polymorphisms. GST polymorphisms were separately analyzed in relationship to *H. pylori* in 82 GC, 72 FD, 53 PU and 89 healthy controls (HC).
Results GST activity was lower in patients with GC in comparison to PU (p=0.03), but GSH levels were comparable. GSTT1 null genotype (GSTT1*0) and simultaneous deletion of both GSTT1 and GSTM1 genes was associated with lower enzyme activity (p=0.02 and 0.01, respectively). GST and GSH levels in *H. pylori* positive and negative patients with GC, FD and PU were comparable. Presence of *H. pylori* infection along with GSTT1*0 (p=0.006) and GSTM1*0 (p=0.05) was associated with lower enzyme activity. GSTT1*0 was associated with higher odds ratio (OR) of GC in presence of *H. pylori* (GC vs. HC: p=0.02, OR 2.6 [95% CI=1–6] vs. p=0.7, 1.3 [0.4–5.0]; GC vs. PU: p=0.04, OR 3 [95% CI=1–9] vs. not applicable (OR could not be computed as frequency of GSTT1*0 in *H. pylori* negative patients with PU was zero)].
Conclusions GC is associated with reduced GST activity. Odds ratio of GC associated with GSTT1*0 is enhanced in presence of *H. pylori* probably due to combined effect of both on enzyme activity.

Keywords Detoxification · Gastric neoplasm · Genetic polymorphism · GST enzyme activity · Host factor

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Introduction

Gastric cancer (GC) is the world's second most common and potentially fatal malignancy. Though the incidence of GC is declining, it is still a global health problem. Extensive research on GC is being done for identifying the associated risk factors. However, the exact mechanism of gastric carcinogenesis is still enigmatic.

Helicobacter pylori, which has been classified as group I carcinogen by World Health Organization, is recognized

as one of the most important risk factors for gastric carcinogenesis [1, 2]. However, of 50% to 80% of the world's population infected with *H. pylori,* only about 1% develop GC [3, 4]. Moreover, in some Asian countries such as India, Thailand, Bangladesh, in spite of a high prevalence of *H. pylori*, the incidence rates of GC is low [5]. Studies based on differences in virulence factors of *H. pylori* have failed to explain this enigma [5–7]. This suggests that certain host genetic and environmental factors may modulate the risk of GC in association with *H. pylori* infection [5–7]. However, there is scarcity of data on association of genetic susceptibility of GC in relation to *H. pylori* infection [8].

Recently, a meta analysis revealed that variants of genes encoding metabolizing enzymes exhibit the most consistent association with cancer [9]. Xenobiotic metabolism is the metabolism of toxins and carcinogens entering the body [10]. Glutathione–S–transferases (GSTs) are important phase II enzymes of xenobiotic metabolism. These enzymes catalyze conjugation of mutagenic electrophilic compounds with nucleophilic glutathione (GSH) yielding less toxic and more water-soluble compounds, which are readily excreted via urine or bile [11]. Thus, GSTs and GSH are protective against harmful effects of carcinogens; reduced GST activity therefore, may increase risk of various cancers [12].

GSTM1, GSTT1 and GSTP1 genes of the GST super gene family exhibit polymorphisms, which are associated with reduced enzyme activity [12–14]. We have reported that GSTT1 alone and in combination with GSTM1 null genotype and GSTP1 *ile/val* or *val/val* genotypes acts as risk factor for GC in Indians [15]. However, the results of various studies on association of GST polymorphisms with GC are contradictory. This may be attributed to ethnic variations, [16–19] or differences in expression of GST genes. The frequency of GST gene variants may be higher in patients with GC in comparison to controls but GST enzyme activity may be comparable. This may be because studies relating variant genotypes with reduced activity are expression based studies, that is, cloning and expression of a particular variant genotype [13, 14]. However, enzyme activity is affected by polymorphisms of all genes of the GST super gene family and not of a single gene encoding a particular enzyme isoform. Therefore, in vitro, a particular genotype may be associated with reduced enzyme activity but in vivo it may not lead to significant alteration of total enzyme activity.

Study of polymorphism alone may not be sufficient for assessment of role of GST in carcinogenesis as factors other than genetic variation could also affect its activity. One of the factors known to be associated with reduced GST activity is *H. pylori* infection [20]. Therefore, the presence of *H. pylori* along with variant GST genotypes may enhance the risk of cancer. GSH, which acts as cofactor for GST enzyme, may also act as an important determinant of GST activity. *H. pylori* infection is known to be associated with reduced GSH levels [20, 21]. Therefore, study of GST polymorphism in combination with its enzyme activity, GSH levels and *H. pylori* infection may provide a better evidence for role of this important xenobiotic metabolizing enzyme in carcinogenesis. Therefore, we studied total GST enzyme activity and GSH concentration, and genotype frequencies of GSTM1, GSTT1, and GSTP1 genes, and their association with *H. pylori* infection in patients with GC.

Methods

GST and GSH estimation was done in 52 patients with GC, 37 with functional dyspepsia (FD) and 39 with peptic ulcer (PU). Patients with FD and PU served as diseased controls. *H. pylori* infection was diagnosed in 82, 72 and 53 patients with GC, FD and PU, respectively, which included the patients in whom GSH and GST was estimated. However, there were two patients with PU and one with FD in whom GST and GSH assay was done but tests for *H. pylori* infection were not done, as blood could not be collected from these patients. *H. pylori* infection was also diagnosed in 89 healthy volunteers from community included as healthy controls (HC). All patients and controls were age and sex matched (Table 1). Patients treated with anti-*H. pylori* drugs in past were excluded. Informed consent was obtained from all patients and controls and the study protocol was approved by the Ethics Committee of the Institute.

GSH and GST assay

For GST and GSH estimation multiple biopsies were collected from gastric mucosa away from the tumor (in case of patients with GC) or from antrum (in case of patients with FD and PU). The biopsies were collected in plain micro-centrifuge tube, immediately transferred to liquid nitrogen and stored at −80°C till use.

The total enzyme activity of GST in gastric tissue of patients with GC and diseased controls was determined using commercially available kit (Cayman Chemicals, Michigan, USA) based on the colorimetric method of Habig et al. using 1-chloro 2,4–dinitrobenzene (CDNB) as substrate [22]. Assay was performed as per manufacturer's instructions with minor modifications. In brief, gastric biopsies were first homogenized manually in 85–100 μL of cold buffer (100 mM potassium di-hydrogen phosphate,

Table 1 GST activity and GSH concentration with respect to *H. pylori* positivity

	GC (n=88)[a]		FD (n=77)[a]		PU (n=55)[a]		HC (n=89)[a]
Age in yrs (Mean±SD)	54.3±11.8		52.6±11.7		50.8±14.9		51.8±14.9
Gender [Frequency of males (%)]	64 (72.7)		53 (68.8)		41 (74.5)		66 (74.2)
H. pylori	Positive (n=32)	Negative (n=20)	Positive (n=25)	Negative (n=11)	Positive (n=22)	Negative (n=15)	ND
GST activity (nmoL/min/mg)[b]	57 (12–141)	54 (41–154)	65 (16–133)	76 (41–151)	61 (14–515)	78 (37–224)	ND
GSH concentration (nmoL/mg)[b]	33 (2–105)	39 (3–108)	16 (4–83)	32 (8–92)	30 (3–67)	32 (9–90)	ND

[a] This n refers to total number of patients included in the study. Of these number of patients in whom all the three tests (GST, GSH estimation and *H. pylori* ELISA) was done is as follows: GC (n=52), FD (n=36), PU (n=37)

GC gastric cancer, *FD* functional dyspepsia, *PU* peptic ulcer, *ND* not done

[b] Values expressed in median (range)

P=ns for GST activity and GSH concentration in *H. pylori* negative and positive individuals

pH 7.0, containing 2 mM EDTA) using polypropylene micro pestle. The homogenate was then centrifuged at 10,000 g for 15 min at 4°C and supernatant separated. Twenty microliter aliquot of the supernatant was used for GST assay as per kit's protocol and rest was stored at −80° C for GSH assay and protein estimation. Dilution of the samples was done if required. The absorbance was read immediately at 340 nm using a plate reader.

Total GSH level (both reduced and oxidized forms) in gastric tissue of patients with GC and diseased control was determined using a commercially available kit (Cayman Chemicals, Michigan, USA). Fifty µl aliquot of the supernatant was used in the assay and it was performed as per manufacturer's instructions. Dilution of the samples was done if required. The absorbance was read at 405 nm using a plate reader.

Protein estimation was done using commercially available kit (Pierce, Rockford, USA) based on Bradford's method. GST activity was expressed in nmoL/min/mg of protein. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of one µmoL of product (using CDNB as substrate) per min under the conditions of the specific assay. Specific activity is defined as the units of enzyme activity per mg of protein. GSH concentration was expressed in nmoL/mg of protein.

Diagnosis of *H. pylori* infection

H. pylori infection was diagnosed by enzyme linked immunoabsorbent assay (ELISA) for IgG antibodies (HpIgG ELISA) using commercially available kit (Genesis Diagnostics, Cambridgeshire, UK) as per manufacturer's instructions on sera obtained from 5 mL blood. The sensitivity and specificity of the kit was 91% and 100% respectively.

GSTM1, GSTT1 and GSTP1 genotyping

GSTM1, GSTT1 and GSTP1 genotyping was done in 88 GC, 77 FD and 55 PU and 89 HC. Part of this data has been published and in the present study we analyzed this data with respect to *H. pylori* infection and total enzyme activity [15].

Statistical analysis

Data on GST activity and GSH concentration were expressed as median (range). Continuous data were analyzed using Mann–Whitney *U* test. *p*-values below 0.05 were considered significant. Binary logistic regression was used to estimate risks as odds ratio (OR) with 95% confidence intervals (CI).

Results

One hundred and eight patients with suspected malignancy of the stomach were screened and of these 88 histopathologically confirmed cases were included. All the patients included had non-cardia GC. Fifty (57%) patients had intestinal type tumor, 28 (32%) had diffuse and 8 (9%) had primary gastric lymphoma. In two patients (2%) tumor was unclassified [15].

Of 55 patients with PU, 40 had duodenal ulcer (DU) and 12 had gastric ulcer (GU). In three patients both DU and GU was present; 22 had associated diseases such as cirrhosis of liver (n=5) and ulcerative colitis (n=1) or presented with upper gastrointestinal bleeding (n=16) [15].

GSH concentration and GST activity

The median values of concentration of GSH (nmoL/mg of protein) was 35 (2–108) [n=52], 19 (4–92) [n=37] and 32

(3–90) [n=39] in patients with GC, FD and PU respectively. The concentration of GSH was comparable among patients with GC and controls (Fig. 1a).

The median values for total GST activity (nmoL/min/mg of protein) was 55 (12–154) [n=52], 65 (16–151) [n=37] and 62 (14–515) [n=37] in patients with GC, FD and PU respectively. Total GST activity was lower in patients with GC in comparison to patients with PU (p=0.03, Mann–Whitney U test) (Fig. 1b). However, GST activity in patients with GC and FD was comparable (p=0.06, Mann Whitney U test) (Fig. 1b).

H. pylori infection

Frequency of HpIgG ELISA positivity was similar among patients with GC [53/72 (74%), GC vs. FD, p=0.13], PU [32/53 (60%), GC vs. PU, p=0.8] and HC [65/89 (73%), GC vs. HC, p=0.2].

Median values of GST activity and GSH concentration in *H. pylori* positive and negative individuals are presented in Table 1. GST activity and GSH concentration between *H. pylori* positive and negative individuals was comparable (Fig. 2a and b).

GST polymorphism and GST activity

Total GST activity with respect to GSTT1, GSTM1 and GSTP1 genotypes is presented in Table 2. Deletion of GSTT1 gene (p=0.02) and simultaneous deletion of GSTT1 and GSTM1 genes (p=0.01) was associated with lower enzyme activity. However, GST activity associated with wild (*ile/ile*) and variant GSTP1 genotypes was comparable.

GST enzyme activity with respect to status of both GST genotypes and *H. pylori* infection is summarized in Table 3. Individuals with both deletion of GSTT1 gene and *H. pylori* infection had lower enzyme activity than those with any one of these conditions absent (i.e. individuals with either absence of GSTT1 null genotype or *H. pylori* infection; p=0.006) and both the conditions absent (individuals with both absence of GSTT1 null genotype and *H. pylori* infection; p=0.007). Presence of both GSTT1 null genotype and *H. pylori* infection was also associated with lower enzyme activity in comparison to absence of null genotype but presence of *H. pylori* infection (p=0.02). Similarly, for GSTM1, individuals with both null genotype and *H. pylori* infection had lower enzyme activity in comparison to those with any one of the conditions absent (i.e. either absence of GSTM1 null genotype or *H. pylori* negative; p=0.05) and both the conditions absent (GSTM1 wild and *H. pylori* negative; p=0.03). However, for GSTP1 the enzyme activities were comparable.

GSTM1, GSTT1 and GSTP1 polymorphism in relationship to *H. pylori* infection

Frequency of GSTT1, GSTM1 and GSTP1 genotypes with respect to HpIgG ELISA positivity in different groups is presented in Table 4. GSTP1 *val/val* homozygotes were combined with *ile/val* heterozygotes for analysis. The odds ratio of GC was higher in individuals with both GSTT1 null genotype and *H. pylori* infection, as compared to HC and PU [GC vs. HC: p=0.02, OR 2.6 (95% CI=1–6); GC vs. PU: p=0.04, OR 3 (95% CI=1–9)], whereas when compared to patients with FD, it was comparable [GC vs. FD: p=0.07 OR 2.1 (95% CI=0.9–5)] in comparison to individuals only with null genotype but no infection [GC vs. HC: OR 1.3 (95% CI=0.4–5); GC vs. FD: OR 1.3 (95% CI=0.3–5)]. Odds ratio of GC associated with GSTM1 null genotype was comparable in presence [GC vs. HC: p=0.7, OR 1.2 (95% CI=0.5–3); GC vs. FD: p=0.9, OR 1 (95% CI=0.4–2); GC vs. PU: p=0.8, OR 1.1 (95% CI=0.4–3)] and absence [GC vs. HC: p=0.5, OR 0.7 (95% CI=0.2–2); GC vs. FD: p=0.3, OR 0.5 (95% CI=0.2–2); GC vs. PU: p=0.7, OR 0.8 (95% CI=0.3–2.5)] of *H. pylori* infection. Also, for GSTP1 variant genotypes it was similar.

Discussion

The present study shows that patients with GC have reduced GST activity. GSH does not appear to have effect on odds ratio of GC. Low GST activity observed in the

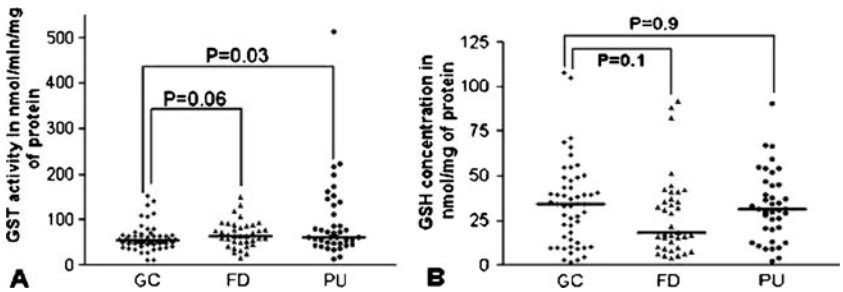

Fig. 1 Glutathione (GSH) level and glutathione-S-transferase activity in patients with gastric neoplasm (GC), functional dyspepsia (FD) and peptic ulcer (PU); **a** GST activity, **b** GSH level

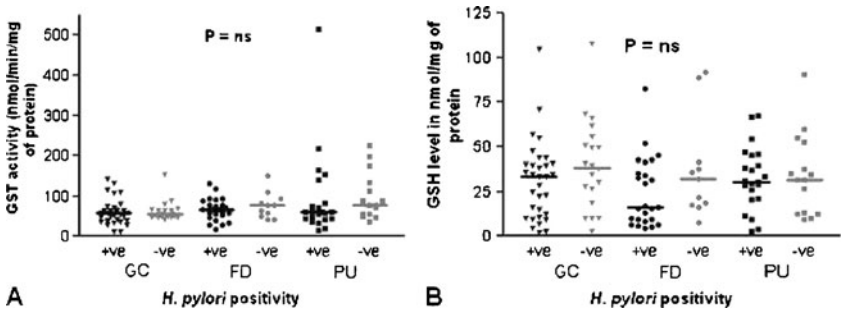

Fig. 2 Glutathione (GSH) level and glutathione-S-transferase activity in *H. pylori* positive and negative individuals [GC: gastric cancer, functional dyspepsia: FD, peptic ulcer: PU] **a** GST activity, **b** GSH level

present study was probably due to the combined effect of both *H. pylori* and GST polymorphism. We could not find differences in enzyme activity and GSH levels with respect to *H. pylori*. However, the enzyme activity was lower in presence of GSTT1 null genotype and simultaneous deletion of both GSTT1 and GSTM1 genes. Enzyme activity was also lower in presence of both GSTT1 and GSTM1 null genotypes and *H. pylori* infection. Moreover, presence of *H. pylori* infection along with GSTT1 null genotype was associated with higher odds ratio of GC in comparison to presence of null genotype alone. GSTP1 gene polymorphism does not appear to modulate the total enzyme activity and odds ratio of GC. To the best of our knowledge, this is the first report in which role of GST enzyme in gastric carcinogenesis has been evaluated in context of its polymorphism, total enzyme activity and *H. pylori* infection.

GST enzyme activity was lower in patients with GC in comparison to patients with PU (p=0.03). The enzyme activity in patients with GC was similar to those with FD. GC is known to be associated with dysplasia and gastric atrophy which may itself lead to reduced enzyme activity. Therefore, one may consider that the low enzyme activity observed in the present study was an effect rather than cause of cancer. However, it can be considered as a cause rather than effect as we also observed higher frequency of variant genotypes, known to be associated with reduced enzyme activity, in patients with GC [15]. Our study is also in accordance with other studies, which reported inverse relationship between GST activity and tumor incidence [20–23].

In the present study, both GST activity and GSH concentration in *H. pylori* positive and negative individuals were comparable. Previous studies have shown that *H. pylori* infection is associated with reduced GST enzyme activity and GSH concentration [20, 21], and that eradication of *H. pylori* restores normal enzyme activity and GSH levels [20, 24]. This indicates that effect of *H. pylori* on GST and GSH is associated with active infection only. In the studies on effect of *H. pylori* on GST activity and GSH levels, *H. pylori* infection was diagnosed by tests which indicate active infection [20–24]. In contrast, we diagnosed *H. pylori* infection by serology, which is indicative of both present as well as past infection. Therefore, some of our patients may not have had active *H. pylori* infection. This might explain comparable GST activity and GSH levels with respect to *H. pylori* infection, observed in the present study. GC is associated with dysplasia, metaplasia and atrophy which make gastric mucosa unfavorable for survival of *H. pylori* and therefore negative biopsy based tests [5]. Besides *H. pylori*, intracellular levels of GSH can also be affected by various other factors such as extent of inflammation, dietary habits and intake of drugs [25].

Though *H. pylori* infection is reported to be associated with reduced GST activity, the present study is the first report of its association with GST polymorphism. In the present study, GST activity in individuals with deletion of GSTT1 alone and simultaneous deletion of both GSTM1 and GSTT1 was associated with lower enzyme activity. However, enzyme activity associated with GSTM1 present or absent genotype was comparable. Similarly, enzyme

Table 2 Association of GSTT1, GSTM1 and GSTP1 genotypes with total GST activity

	GSTM1		GSTT1		GSTT1/GSTM1		GSTP1	
Genotype	Wild (n=80)	Null (n=48)	Wild (n=98)	Null (n=30)	+/+ (n=61)	−/− (n=11)	II (n=75)	IV or VV (n=53)
GST activity (nmoL/min/mg)[a]	63 (12–515)	59 (12–154)	63 (12–515)	53 (12–108)	63 (12–515)	46 (12–90)	59 (12–218)	62 (12–515)
P	0.14		0.02		0.01		0.9	

[a] Median (range)

+/+: Both GSTT1 and GSTM1 present; −/−: Both null; II: *ile/ile*; IV: *ile/val*; VV: *val/val*

Table 3 Combined effect of GST polymorphism and *H. pylori* infection on enzyme activity

	GST enzyme activity (nmol/min/mg[a])					
	Variant *and* positive (A)	Wild *or* negative (B)	Wild *and* negative (C)	Wild *and* positive (D)	Variant *and* negative (E)	*p*-values
GSTT1	46 (12–108) *n*=22	63 (12–515) *n*=105	64 (37–224) *n*=38	61 (12–515) *n*=57	65 (47–87) *n*=8	A vs. B: 0.006
						A vs. C: 0.007
						A vs. D: 0.02
						A vs. E: 0.07
GSTM1	53 (12–142) *n*=31	63 (12–515) *n*=96	64 (37–224) *n*=29	63 (12–515) *n*=48	64 (41–154) *n*=17	A vs. B: 0.05
						A vs. C: 0.03
						A vs. D: 0.2
						A vs. E: 0.1
GSTP1	59 (12–515) *n*=36	63 (12–224) *n*=91	57 (41–198) *n*=30	61 (12–218) *n*=43	69 (37–224) *n*=16	A vs. B: 0.5
						A vs. C: 0.4
						A vs. D: 0.8
						A vs. E: 0.3

[a] Median (range)

Variant Null (for GSTT1 and GSTM1) or *ile/val* or *val/val* (for GSTP1) genotypes

Wild Gene present (for GSTT1 and GSTM1) or *ile/ile* (for GSTP1) genotypes

Positive H. pylori positive

Negative H. pylori negative

activity associated with GSTP1 wild (*ile/ile*) or variant *(ile/val or val/val*) genotype was also comparable. This is in accordance to our previously published data of GST genotype frequencies [15]. In this study we observed that GSTT1 null genotype alone and in combination with GSTM1 null genotype and GSTP1 *ile/val* or *val/val* genotypes was associated with higher risk of GC.

Analysis of combined effect of both polymorphism and *H. pylori* on enzyme activity revealed that presence of null genotype (for GSTT1) and *H. pylori* infection was associated with lower enzyme activity. However, for GSTM1 and GSTP1 the difference was comparable. GSTT1 null genotype was associated with higher odds ratio of GC in presence of *H. pylori* infection. This is expected because in such

Table 4 Frequency of GSTT1, GSTM1 and GSTP1 genotypes with respect to *H. pylori* infection in different groups

	GSTM1 (null vs. wild)		GSTT1 (null vs. wild)		GSTP1 (*ile/val or val/val* vs. *ile/ile carriers*)	
	Hp (+ve)	Hp (−ve)	Hp (+ve)	Hp (−ve)	Hp (+ve)	Hp (−ve)
GC (*n*=82)	19/32	13/18	21/30	8/23	22/29	11/20
FD (*n*=72)	20/33	8/11	13/40	4/15	24/29	7/12
PU (*n*=53)	11/21	10/11	6/26	0/21	14/18	7/14
HC (*n*=89)	22/43	12/12	14/51	5/19	29/36	14/10
			P			
			OR (95%CI)			
GC vs. FD	0.9	0.3	0.07	0.7	0.8	0.9
	1 (0.4–2)	0.5 (0.2–2)	2.1 (1–5)	1.3 (0.3–5)	0.9 (0.4–2)	0.9 (0.3–3)
GC vs. PU	0.8	0.7	0.04	NA[a]	0.9	0.9
	1.1 (0.4–3)	0.8 (0.3–2)	3 (1–9)		1 (0.4–2.4)	1.1 (0.3–4)
GC vs. HC	0.7	0.5	0.02	0.7	0.9	0.09
	1.2 (0.5–3)	0.7 (0.2–2)	2.6 (1–6)	1.3 (0.4–6)	0.9 (0.4–2)	0.4 (0.1–1)

GC gastric cancer, *FD* functional dyspepsia, *PU* peptic ulcer

Hp (+ve) H. pylori positive, *Hp (−ve) H. pylori* negative

[a] OR could not be computed as frequency of null genotype in *H. pylori* negative patients with PU was zero

individuals GST activity will be much lower due to combined effect of both *H. pylori* and polymorphism.

Conclusion

Therefore, we conclude that GST enzyme and *H. pylori* act synergistically in gastric carcinogenesis. *H. pylori* infection and variant GST genotypes may initially lower the GST activity and initiate carcinogenesis. An insufficient carcinogen detoxification may lead to accumulation of mutations and tumor progression on further exposure to carcinogens, even in absence of *H. pylori*. Low frequency of GSTT1 null genotype in India as compared to China and Japan may explain reduced risk of GC despite high *H. pylori* prevalence [7]. However, more studies are warranted to identify other host genetic factors which may modulate the risk of GC due to *H. pylori* infection in order to further explain this enigma.

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