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Polymorphism in glutathione-S-transferases: A risk factor in alcoholic liver cirrhosis

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ABSTRACT

In a case-control study, association of polymorphism in glutathione-S-transferases (*GSTM1*, *GSTT1*, *GSTP1*), involved in detoxification of reactive oxygen species (ROS), was studied with alcoholic liver cirrhosis. The study included 175 alcoholic cirrhotic patients (ACPs), 140 non-alcoholic cirrhotic patients (NACPs), visiting Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGI), Lucknow, India, 255 non-alcoholic controls and 140 alcoholic controls. The data showed an increase in risk to alcoholic cirrhosis in ACPs with *GSTM1* (*null*) genotype when compared with non-alcoholic controls (OR: 1.7; 95% CI: 1.15–2.56) or alcoholic controls (OR: 1.7; 95% CI: 1.07–2.73). Significant increase in risk was also observed in ACPs with variant genotype of *GSTP1* when compared with non-alcoholic controls (OR: 1.65; 95% CI: 1.12–2.43). A much higher risk to alcoholic liver cirrhosis was observed in patients carrying combination of null genotypes of *GSTM1* and *GSTT1* (OR: 2.8; 95% CI: 1.3–6.06) or variant genotype of *GSTP1* and null genotype of *GSTM1* (OR: 2.8; 95% CI: 1.58–4.90) or *GSTT1* (OR: 2.16; 95% CI: 1.08–4.28). Likewise, greater risk for alcoholic cirrhosis was observed in patients carrying combination of *GSTM1, GSTT1* (*null*) and variant genotype of *GSTP1* (OR: 5.8; 95% CI: 2.17–15.80). Our data further showed that interaction of *GSTs* with variant genotype of manganese superoxide dismutase (*MnSOD*), which detoxifies free radicals, or cytochrome P450 2E1, which generates free radicals, resulted in several fold increase in risk to alcoholic liver cirrhosis in ACPs when compared with non-alcoholic controls thus demonstrating the role of gene–gene interactions in modulating the risk to alcoholic liver cirrhosis.

1. Introduction

Alcoholic liver diseases (ALD), accounting for most of the chronic liver diseases (Stickel and Osterreicher, 2006), are governed by a complex interplay of numerous genes with several known or unknown environmental factors. Alcohol induced tissue damage is primarily based on the toxicity of its major metabolite, acetaldehyde. In addition, the increased formation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anions have been implicated as causative factors involved in various forms of chronic liver diseases (Parola and Robino, 2001; Stickel and Osterreicher, 2006). However, the vulnerability to ethanol induced hepatotoxicity varies significantly. While about 20% survive the chronic toxicity without developing any pathology of the liver, 20% of the chronic alcoholics develop liver cirrhosis, suggesting that individual predisposition may not be solely explained by exposure to environmental factors (Burim et al., 2004; Ladero et al., 2005; Stickel and Osterreicher, 2006). Twin studies have convincingly shown that individual susceptibility to alcoholic liver cirrhosis is at least partly genetically determined (Hrubec and Omenn, 1981).

Polymorphism in the genes coding for enzymes that catalyze the oxidative metabolism of ethanol were suggested to be involved in determining the susceptibility to ALD, though the association between these polymorphisms and risk of ALD including liver cirrhosis has been inconsistent particularly in the Caucasian population (Borras et al., 2000). However, studies in Oriental populations and our recent study in North Indian populations have shown that polymorphism in cytochrome P450 2E1(*CYP2E1*), which leads to increased formation of reactive oxygen species (ROS), is associated with increased susceptibility to alcoholic cirrhosis (Tsutsumi et al., 1994; Tanaka et al., 1997; Khan et al., 2008 unpublished communication). Likewise, polymorphism in genes coding for enzymes such as glutathione S-transferases (*GSTs*) and manganese dependent superoxide dismutase (*MnSOD*), responsible for detoxifying electrophilic intermediates including free radicals generated as by-products of ethanol metabolism and lipid peroxidation, could also

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be potential risk factors involved in alcoholic liver diseases (Degoul et al., 2001; Burim et al., 2004; Ladero et al., 2005).

GSTs, a family of enzymes, catalyze the conjugation of reduced glutathione (GSH) to the reactive electrophilic intermediates, leading to detoxification of the xenobiotics (Parl, 2005). Though, seven family of *GSTs* are known to exist in humans, *GSTM1* (Mu), *GSTT1* (Theta) and *GSTP1* (Pi) have been found to be associated with alcoholic liver diseases (Brind et al., 2004; Ladero et al., 2005; Parl, 2005). Approximately 40–50% of the Caucasians are homozygous for a deletion that inactivates *GSTM1* gene (Seidegaard et al., 1989). Likewise 15–25% of Caucasians carry deletion of *GSTT1* gene (Pemble et al., 1994). Two genetic variants have been reported in *GSTP1* gene in 40–50% of the Caucasians, leading to Ile to Val polymorphism at codon 105 and Ala to Val transition at codon 114 which leads to significant differences in catalytic activity (Osman et al., 1997). Homozygous gene deletion of *GSTM1*, *GSTT1* and variant genotypes (*Ile*/*Val and Val*/*Val*) of *GSTP1* may lead to the increase in the levels of potential reactive metabolites resulting from chronic ethanol intake (Ghobadloo et al., 2004; Ladero et al., 2005).

GSTM1 null genotype has been found to be associated with alcoholic liver diseases in some studies (Harada et al., 1993; Ladero et al., 2005; Baranov et al., 1996; Savolainen et al., 1996) though there are several reports which do not show significant association of *GSTM1* null genotype with the alcoholic liver disease (Groppi et al., 1991; Rodrigo et al., 1999; Burim et al., 2004). In contrast, there have been fewer studies on *GSTT1* and *GSTP1* which did not observed significant association with alcoholic liver diseases (Frenzer et al., 2002; Brind et al., 2004). However, it has been found that *GSTP1 Val*/*Val* genotype was associated with the development of cirrhosis in patients with hereditary hemochromatosis (Stickel et al., 2005). A higher frequency of *GSTP1 Val*/*Val* genotypes was found in alcoholic pancreatitis as well as cryptogenic liver cirrhosis patients when compared to the controls (Burim et al., 2004; Ghobadloo et al., 2004).

Our recent study has shown that *GSTs* are polymorphic in North Indian population and are associated with susceptibility to oral cancer (Singh et al., 2008). The present case-control study attempted to investigate the association of polymorphism in *GSTs* with alcoholic liver cirrhosis in North Indian population. As liver cirrhosis has been shown to be of multifactorial origin, attempts were also made to investigate the interaction of *GSTs* with other genetic risk factors such as *CYP2E1* and *MnSOD* in determining the susceptibility to alcoholic liver cirrhosis.

2. Materials and methods

A case-control study was initiated to investigate the association of functionally important polymorphisms in *GSTs* (*GSTM1*, *GSTT1* and *GSTP1*), with alcoholic liver cirrhosis. Patients of alcoholic liver cirrhosis ($n = 175$) and non-alcoholic liver cirrhosis ($n = 140$) visiting the OPD facility of Gastroenterology, Department of Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, India were included in the study. Patients with alcoholic and non-alcoholic liver cirrhosis were diagnosed on the basis of their liver biopsy and hepatitis B surface antigen and antibodies against hepatitis C virus. Control group consisted of non-alcoholic ($n = 255$) and alcoholic ($n = 140$) healthy men having no evidence of liver disease as judged by physical examination and normal liver function test. All the patients and controls included in the study belonged to same geographical location (Northern India) and same ethnicity. The average age of non-alcoholic controls, non-alcoholic cirrhosis, alcoholic controls and alcoholic cirrhosis patients were 42 ± 13.9, 47 ± 14.8, 48 ± 15.2 and 52 ± 13.4, respectively. The controls and patients were asked to fill up the detailed questionnaire regarding their family history, medical history, life style habits, etc. The questionnaire also included other details such as frequency of alcohol intake. Subjects who consumed less than 10 g/day of alcohol were classified as non-alcoholics. Subjects who consumed more than 80 g/day of alcohol for more than 10 years were considered as alcoholics (Savolainen et al., 1997; Tanaka et al., 1997; Grove et al., 1998).

2.1. DNA isolation and genotype analysis

One millilitre of blood was collected into citrate containing tubes from all the subjects. DNA was isolated from whole blood with the Flexi Gene DNA kit (Qiagen, CA) following the manufacturers protocol. Isolated DNA was subsequently used for genotyping studies. *GSTM1* and *GSTT1* genotypes were determined by the method of Arand et al. (1996). The method of Harries et al. (1997) was followed for identifying *GSTP1* polymorphism. The method of Liu et al. (2001) was followed for determining the *CYP2E1* RsaI polymorphism, while the method of Akyol et al. (2004) was used for identifying the polymorphisms in *MnSOD*.

2.2. Statistical analysis

All statistical analysis was performed using the SPSS software (Version 11.0). The Chi-square-goodness-of-fit test was used to test the distribution of genotype frequencies for deviations from Hardy–Weinberg equilibrium between the patients and controls. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated to measure the risk associated with variant genotypes by unconditional logistic regression method. Age adjusted risks were also calculated using multivariate logistic regression model. The differences were considered significant if the p-value was less than 0.05 (<0.05). Desired power of the present study was set at >80% as analyzed by power genetic association analysis software (http://dceg.cancer.gov/bb/tools/pga) at the level of significance $\alpha = 0.05$.

3. Results

Table 1 summarizes the distribution of genotype frequency of different forms of *GSTs* in controls and patients suffering from alcoholic and non-alcoholic liver cirrhosis. The null genotype of *GSTM1* was found to be present in increased frequency (44%) in alcoholic cirrhotic patients (ACP) when compared with non-alcoholic controls (31.5%), or alcoholic controls (31.5%) or non-alcoholic cirrhotic patients (NACP, 34.5%). That the crude odds ratio (OR) increase significantly in ACP (OR: 1.7; 95% CI: 1.15–2.56) when compared with non-alcoholic controls (Table 1). A statistically significant increase in the risk to alcoholic liver cirrhosis was observed in ACP when the frequency of null genotype of *GSTM1* in alcoholic liver cirrhotic patients was compared with the alcoholic controls (OR: 1.7; 95% CI: 1.07–2.73). A slight non-significant increase in the risk (OR: 1.5; 95% CI: 0.95–2.38) was observed in the ACP when compared with NACP. No risk to the disease was observed when the frequency of null genotype of *GSTM1* in non-alcoholic controls was compared to the alcoholic controls or NACP or when the frequency of null genotypes of *GSTM1* in alcoholic controls was compared with NACP (Table 1).

As evident from Table 1, the frequency of null genotype of *GSTT1* in ACP (19%) was almost similar to that observed in non-alcoholic controls (17%) or NACP (16.5%) or alcoholic controls (16.5%). No significant change was also observed in the OR associated with the null genotype of *GSTT1* in ACP when compared to non-alcoholic controls or alcoholic controls or NACP (Table 1). The genotype and allele frequency of *GSTP1* in alcoholic and non-alcoholic controls were found to be in Hardy–Weinberg equilibrium. As the frequency of the homozygous mutant (*Val*/*Val*) genotype of *GSTP1* was very rare, the heterozygous and homozygous mutant genotypes were clubbed together and are referred to as variant genotypes (*Ile*/*Val* + *Val*/*Val*) of *GSTP1*. The frequency of the variant genotype of *GSTP1* was found to be higher in ACP (51.5%) when compared to the non-alcoholic controls (39.5%) or NACP (44.5%) or alcoholic controls (43%) and resulted in a significant increase in risk in ACP when compared with non-alcoholic controls (Table 1). A slightly increased OR, though not statistically significant, was also observed when the frequency of variant genotype of *GSTP1* in ACP was compared with NACP or alcoholic controls (Table 1).

The risk to alcoholic liver cirrhosis associated with multiple at-risk genotypes of *GSTs* is summarized in Table 2. When the genotypes of *GSTM1* and *GSTT1* were combined, four combinations were observed. About 11.5% of the alcoholic cirrhotic patients carried the combination of null genotype of both *GSTM1* and *GSTT1*, which increased the risk up to 2.8-fold (95% CI: 1.3–6.06) when compared to non-alcoholic controls or alcoholic controls (OR: 2.7; 95% CI: 1.07–6.70) (Table 2). Likewise, about 24.5% of the alcoholic cirrhotic patients carried the combination of null genotype of *GSTM1* and variant genotype (*Ile*/*Val* + *Val*/*Val*) of *GSTP1*, which

Table 1
Distribution of genotype frequency of *GSTs* (*GSTM1*, *GSTT1* and *GSTP1*) among controls and cirrhotic patients.

	Sample (*n*)	Positive *n* (%)	Null *n* (%)	Crude OR (95% CI)	Adjusted OR[a] (95% CI)	Crude OR (95% CI)	Adjusted OR[a] (95% CI)	Crude OR (95% CI)	Adjusted OR[a] (95% CI)	Allele frequency positive	null
GSTM1	Non-alcoholic control (255)	175 (68.6%)	80 (31.4%)	1 (Ref.)	1 (Ref.)					0.69	0.31
	Non-alcoholic cirrhosis (140)	92 (65.7%)	48 (34.3%)	1.1 (0.73–1.76)	1.1 (0.70–1.70)	1 (Ref.)	1 (Ref.)			0.66	0.34
	Alcoholic control (140)	96 (68.5%)	44 (31.5%)	1.0 (0.64–1.56)	1.0 (0.64–1.57)	0.88 (0.53–1.44)	0.87 (0.52–1.43)	1 (Ref.)	1 (Ref.)	0.69	0.31
	Alcoholic cirrhosis (175)	98 (56%)	77 (44%)	**1.7*** (1.15–2.56)	**1.7*** (1.16–2.61)	1.5 (0.95–2.38)	1.5 (0.96–2.41)	**1.7*** (1.07–2.73)	**1.78*** (1.11–2.85)	0.56	0.44
GSTT1	Non-alcoholic control (255)	212 (83%)	43 (17%)	1 (Ref.)	1 (Ref.)					0.83	0.17
	Non-alcoholic cirrhosis (140)	117 (83.5%)	23 (16.5%)	0.97 (0.55–1.68)	1.0 (0.58–1.81)	1 (Ref.)	1 (Ref.)			0.84	0.16
	Alcoholic control (140)	117 (83.5%)	23 (16.5%)	0.97 (0.55–1.68)	0.99 (0.56–1.73)	1.0 (0.53–1.88)	0.98 (0.52–1.86)	1 (Ref.)	1 (Ref.)	0.84	0.16
	Alcoholic cirrhosis (175)	142 (81%)	33 (19%)	1.1 (0.69–1.89)	1.2 (0.73–2.04)	1.2 (0.65–2.12)	1.23 (0.68–2.22)	1.2 (0.65–2.12)	1.2 (0.66–2.15)	0.81	0.19
		Wild	**Variant**							**Ile**	**Val**
GSTP1	Non-alcoholic control (255)	154 (60.4%)	101 (39.6%)	1 (Ref.)	1 (Ref.)					0.78	0.22
	Non-alcoholic cirrhosis (140)	77 (55%)	63 (45%)	1.24 (0.82–1.89)	1.16 (0.76–1.78)	1 (Ref.)	1 (Ref.)			0.75	0.25
	Alcoholic control (140)	80 (57%)	60 (43%)	1.1 (0.75–1.73)	1.1 (0.73–1.70)	0.92 (0.57–1.47)	0.91 (0.56–1.46)	1 (Ref.)	1 (Ref.)	0.77	0.23
	Alcoholic cirrhosis (175)	84 (48%)	91 (52%)	**1.65*** (1.12–2.43)	**1.55*** (1.02–2.31)	1.3 (0.84–2.06)	1.3 (0.84–2.06)	1.44 (0.92–2.26)	1.46 (0.93–2.30)	0.71	0.29

Ref: reference category; OR: Odds ratio; 95% CI: 95% confidence interval; *$p < 0.05$ is considered statistically significant; OR[a] adjusted with age in multivariate logistic regression models.

Table 2
Risk associated with *GST* genotype combinations in alcoholic cirrhotic patients.

Double/triple *GST* genotype	Alcoholic cirrhosis 175 *n* (%)	Non-alcoholic control 255 *n* (%)	OR (95% CI)	Alcoholic control 140 *n* (%)	OR (95% CI)	Non-alcoholic cirrhosis 140 *n* (%)	OR (95% CI)
GSTM1(+/+) & *GSTT1*(+/+)	85 (48.5%)	144 (56.5%)	1.0 (Ref.)	80 (57%)	1.0 (Ref.)	78 (55.7%)	1.0 (Ref.)
GSTM1(−/−) & *GSTT1*(−/−)	20 (11.5%)	12 (4.7%)	**2.8*** (1.3–6.06)	07 (05%)	**2.7*** (1.07–6.70)	09 (6.5%)	2.0 (0.87–4.74)
GSTM1(+/+) & *GSTP1*(*Ile*/*Ile*)	50 (28.5%)	107 (42%)	1.0 (Ref.)	53 (38%)	1.0 (Ref.)	53 (37.8%)	1.0 (Ref.)
GSTM1(−/−) & *GSTP1*(*Ile*/*Val*+*Val*/*Val*)	43 (24.5%)	33 (13%)	**2.8*** (1.58–4.90)	17 (12%)	**2.7*** (1.35–5.30)	24 (17%)	**1.9*** (1.01–3.57)
GSTT1(+/+) & *GSTP1*(*Ile*/*Ile*)	73 (41.7%)	129 (50.5%)	1.0 (Ref.)	70 (50%)	1.0 (Ref.)	64 (45.7%)	1.0 (Ref.)
GSTT1(−/−) & *GSTP1*(*Ile*/*Val* + *Val*/*Val*)	22 (12.5%)	18 (07%)	**2.16*** (1.08–4.28)	13 (9.3%)	1.6 (0.75–3.47)	10 (07%)	1.9 (0.85–4.37)
GSTM1(+/+) & *GSTT1*(+/+) & *GSTP1*(*Ile*/*Ile*)	45 (25.7%)	88 (34.5%)	1.0 (Ref.)	46 (33%)	1.0 (Ref.)	44 (31.5%)	1.0 (Ref.)
GSTM1(−/−) & *GSTT1*(−/−) & *GSTP1*(*Ile*/*Val* + *Val*/*Val*)	18 (10.3%)	06 (2.4%)	**5.8*** (2.17–15.80)	04 (2.9%)	**4.6*** (1.44–14.65)	05 (3.6%)	**3.5*** (1.2–10.30)

Ref: reference category; OR; Odds ratio; 95% CI; 95% confidence interval; *$p < 0.05$ is considered statistically significant.

significantly increased the risk up to 2.8-fold (95% CI: 1.58–4.90) when compared with non-alcoholic controls or alcoholic controls (OR: 2.7; 95% CI: 1.35–5.30). A slightly increase in risk, though statistically significant was observed in ACP carrying the combination of null genotype of *GSTM1* and variant genotype (*Ile*/*Val* + *Val*/*Val*) of *GSTP1* when compared with NACP (OR: 1.9; 95% CI: 1.01–3.57) (Table 2). Similarly, when genotype combinations of *GSTT1* and *GSTP1* were studied, significantly increased risk (OR: 2.16; 95% CI: 108–4.28) was observed in ACP when compared with non-alcoholic controls (Table 2). When the genotypes of *GSTM1*, *GSTT1* and *GSTP1* were combined, eight possible combinations were observed. Individuals with combination of the null genotypes of *GSTT1*, *GSTM1* and the variant genotype of *GSTP1* were at significantly higher risk to alcoholic cirrhosis when compared with non-alcoholic controls (OR: 5.8; 95% CI: 2.17–15.80) or alcoholic controls (OR: 4.6; 95% CI: 1.44–14.65) or NACP (OR: 3.5; 95% CI: 1.2–10.30) (Table 2).

The distribution of genotype and allele frequencies of *MnSOD* gene in controls and patients suffering from alcoholic and non-alcoholic liver cirrhosis is summarized in Table 3. The frequency of the variant genotype (*Ala*/*Ala* + *Ala*/*Val*) of *MnSOD* was found to be higher in ACP (74%) when compared with non-alcoholic controls (64.5%) or NACP (67.5%) but was almost similar to that observed in alcoholic controls (71.5%). When the frequency of variant genotype of *MnSOD* in ACP was compared with non-alcoholic controls the crude odds ratio was increased to 1.57 (95% CI: 1.03–2.41), which was found to be statistically significant. A slight increase in risk to cirrhosis was also observed in ACP when the frequency of variant genotype of *MnSOD* was compared with NACP (OR: 1.36; 95% CI: 0.83–2.23) or alcoholic controls (OR: 1.15; 95% CI: 0.70–1.90), though the increase in risk was statistically not significant (Table 3).

The frequency of variant genotypes of *CYP2E1*5B* was also found to be higher (8%) in alcoholic cirrhotic patients (ACP) when compared with non-alcoholic controls (2%), or alcoholic controls (2%) or non-alcoholic cirrhotic patients (NACP, 2%) which significantly increased the risk (OR: 4.3; 95% CI: 1.53–12.30) when the frequency of variant genotype in ACP was compared to non-alcoholic controls (Table 4). Similarly, when the frequency of the variant genotype of *CYP2E1*5B* in ACP was compared with NACP or alcoholic controls, an increased risk was observed in ACP (OR: 3.9; 95% CI: 1.11–14.10) which was also found to be statistically significant (Table 4).

The distribution of genotype combinations of *MnSOD* either with *GSTM1* or *GSTT1* or *GSTP1* are summarized in Table 5. Statistically significant increase in risk was observed in ACP carrying the combination of null genotype of *GSTM1* and variant genotype of *MnSOD* when compared with non-alcoholic controls (OR: 2.65; 95% CI: 1.47–4.78) or alcoholic controls (OR: 1.9; 95% CI: 0.99–3.8) or NACP (OR: 2.0; 95% CI: 1.03–3.98) (Table 5). Similarly, 2–2.5-fold increase in the risk was observed in ACP carrying the combination of *GSTT1* (*null*) or variant genotype of GSTP1 and variant genotype of *MnSOD* when compared to non-alcoholic controls (Table 5). Several fold statistically significant risk was observed in the ACP carrying the combination of null genotype of *GSTM1* and variant genotype of *CYP2E1*5B* when compared to non-alcoholic controls (OR: 7.2; 95% CI: 1.98–26.17) or alcoholic controls (OR: 4.0; 95% CI: 1.08–14.47) or NACP (OR: 11.0; 95% CI: 1.43–88.28) (Table 6). Likewise, up to 6-fold increase in the risk was observed in ACP carrying combination of null genotype of *GSTT1* and variant genotype of *CYP2E1*5B* when compared to non-alcoholic controls (OR: 6.1; 95% CI: 1.28–29.38) or alcoholic controls (OR: 6.7; 95% CI: 0.83–54.89). Several fold statistically significant increase in the risk was also observed in the ACP carrying variant genotype combination of *CYP2E1*5B* and *GSTP1* when compared to non-alcoholic controls (OR: 11.0; 95% CI: 2.4–50.56) or alcoholic controls (OR: 5.7; 95% CI: 1.25–26.56) or NACP (OR: 11.0; 95% CI: 1.4–86.25) (Table 6).

Table 3
Distribution of genotype and allele frequency of *MnSOD* among cirrhotic patients and controls.

Sample (*n*)	Val/Val *n* (%)	Ala/Val + Ala/Ala *n* (%)	Crud OR (95% CI)	Adjusted OR[a] (95% CI)	Crud OR (95% CI)	Adjusted OR[a] (95% CI)	Crud OR (95% CI)	Adjusted OR[a] (95% CI)	Allele frequency	
									Val	Ala
Non-alcoholic control (255)	90 (35.3%)	165 (64.7%)	1 (Ref.)	1 (Ref.)					0.57	0.43
Non-alcoholic cirrhosis (140)	45 (32%)	95 (68%)	1.15 (0.74–1.78)	1.1 (0.70–1.72)	1 (Ref.)	1 (Ref.)			0.57	0.43
Alcoholic control (140)	40 (28.5%)	100 (71.5%)	1.36 (0.87–2.13)	1.3 (0.83–2.04)	1.2 (0.71–1.97)	1.2 (0.71–1.99)	1 (Ref.)	1 (Ref.)	0.52	0.48
Alcoholic cirrhosis (175)	45 (25.7%)	130 (74.3%)	**1.57*** (1.03–2.41)	**1.5*** (0.99–2.36)	1.36 (0.83–2.23)	1.34 (0.82–2.20)	1.15 (0.70–1.90)	1.16 (0.70–1.93)	0.48	0.52

Ref: reference category; OR: Odds ratio; 95% CI: 95% confidence interval; *$p < 0.05$ is considered statistically significant; [a]OR adjusted with age in multivariate logistic regression models.

Table 4
Distribution of genotype and allele frequency of *CYP2E1*5B* (RsaI) among cirrhotic patients and controls.

Sample (*n*)	(Wild) *n* (%)	(Variant) *n* (%)	Crude OR (95% CI)	Adjusted OR[a] (95% CI)	Crude OR (95% CI)	Adjusted OR[a] (95% CI)	Crude OR (95% CI)	Adjusted OR[a] (95% CI)	Allele frequency	
									c1	c2
Non-alcoholic control (255)	250 (98%)	05 (02%)	1 (Ref.)	1 (Ref.)					0.99	0.01
Non-alcoholic cirrhosis (140)	137 (98%)	03 (02%)	1.1 (0.25–4.65)	1.1 (0.26–4.90)	1 (Ref.)	1 (Ref.)			0.99	0.01
Alcoholic control (140)	137 (98%)	03 (02%)	1.1 (0.25–4.65)	1.2 (0.28–5.29)	1.0 (0.19–5.04)	0.96 (0.19–4.89)	1 (Ref.)	1 (Ref.)	0.99	0.01
Alcoholic cirrhosis (175)	161 (92%)	14 (08%)	**4.3*** (1.53–12.30)	**4.7*** (1.66–13.74)	**3.9*** (1.11–14.10)	**4.3*** (1.20–15.67)	**3.9*** (1.11–14.10)	**4.6*** (1.27–16.72)	0.96	0.01

Ref: reference category; OR: Odds ratio; 95% CI: 95% confidence interval; *$p < 0.05$ is considered statistically significant; [a]OR adjusted with age in multivariate logistic regression models.

Table 5
Genotype combinations of and *GSTs* and *MnSOD* in controls and cirrhotic patients.

Genotype	Alcoholic cirrhosis (175) *n* (%)	Non-alcoholic control (255) n (%)	OR (95% CI)	Alcoholic control (140) n (%)	OR (95% CI)	Non-alcoholic cirrhosis (140) *n* (%)	OR (95% CI)
GSTM1(+/+) & *MnSOD* (*Val*/*Val*)	26 (14.8%)	63 (24.8%)	1.0 (Ref.)	29 (20.8%)	1.0 (Ref.)	31 (22.2%)	1.0 (Ref.)
GSTM1(+/+) & *MnSOD* (*Ala*/*Val* + *Ala*/*Ala*)	72 (41.2%)	112 (43.9%)	1.55 (0.90–2.68)	67 (47.8%)	1.2 (0.64–2.23)	61 (43.5%)	1.4 (0.75–2.62)
GSTM1(−/−) & *MnSOD* (*Val*/*Val*)	19 (10.8%)	27 (10.5%)	1.7 (0.81–3.58)	11 (7.8%)	1.9 (0.77–4.79)	14 (10.0%)	1.6 (0.68–3.84)
GSTM1(−/−) & *MnSOD* (*Ala*/*Val* + *Ala*/*Ala*)	58 (33.2%)	53 (20.8%)	**2.65*** (1.47–4.78)	33 (23.6%)	**1.9*** (0.99–3.8)	34 (24.3%)	**2.0*** (1.03–3.98)
GSTT1(+/+) & *MnSOD* (*Val*/*Val*)	37 (21.2%)	70 (27.5%)	1.0 (Ref.)	33 (23.5%)	1.0 (Ref.)	37 (26.4%)	1.0 (Ref.)
GSTT1(+/+) & *MnSOD* (*Ala*/*Val* + *Ala*/*Ala*)	105 (60.0%)	142 (55.7%)	1.4 (0.87–2.24)	84 (60.0%)	1.1 (0.64–1.9)	80 (57.2%)	1.3 (0.76–2.25)
GSTT1(−/−) & *MnSOD* (*Val*/*Val*)	08 (4.5%)	20 (7.8%)	0.76 (0.30–1.88)	07 (5.0%)	1.0 (0.33–3.11)	08 (5.7%)	1.0 (0.34–2.94)
GSTT1(−/−) & *MnSOD*(*Ala*/*Val* + *Ala*/*Ala*)	25 (14.3%)	23 (9.0%)	**2.0*** (1.02–4.10)	16 (11.5%)	1.4 (0.63–3.0)	15 (10.7%)	1.7 (0.75–3.65)
GSTP1(*Ile*/*Ile*) & *MnSOD* (*Val*/*Val*)	23 (13.2%)	55 (21.6%)	1.0 (Ref.)	23 (16.4%)	1.0 (Ref.)	23 (16.5%)	1.0 (Ref.)
GSTP1(*Ile*/*Ile*) & *MnSOD* (*Ala*/*Val* + *Ala*/*Ala*)	61 (34.8%)	99 (38.8%)	1.5 (0.82–2.63)	57 (40.7%)	1.0 (0.54–2.11)	54 (38.5%)	1.1 (0.56–2.23)
GSTP1(*Val*/*Val* + *Ile*/*Val*) & *MnSOD* (*Val*/*Val*)	22 (12.5%)	35 (13.8%)	1.5 (0.73–3.09)	17 (12.2%)	1.3 (0.54–3.0)	22 (15.7%)	1.0 (0.43–2.28)
GSTP1(*Val*/*Val* + *Ile*/*Val*) & *MnSOD*(*Ala*/*Val* + *Ala*/*Ala*)	69 (39.5%)	66 (25.8%)	**2.5*** (1.38–4.52)	43 (30.7%)	1.6 (0.80–3.20)	41 (29.3%)	1.68 (0.84–3.37)

Ref: reference category; OR: Odds ratio; 95% CI: 95% confidence interval; *$p < 0.05$ is considered statistically significant.

Table 6
Genotype combinations of and *GSTs* and *CYP2E1*5B* (RsaI) in controls and cirrhotic patients.

Genotype	Alcoholic cirrhosis (175) *n* (%)	Non-alcoholic control (255) *n* (%)	OR (95% CI)	Alcoholic control (140) *n* (%)	OR (95% CI)	Non-alcoholic cirrhosis (140) *n* (%)	OR (95% CI)
*GSTM1(+/+) & CYP2E1*5B(Wild)*	96 (54.8%)	173 (67.8%)	1.0 (Ref.)	95 (67.8%)	1.0 (Ref.)	90 (64.3%)	1.0 (Ref.)
*GSTM1(+/+) & CYP2E1*5B(Variant)*	02 (1.2%)	02 (0.8%)	1.8 (0.25–12.99)	01 (0.7%)	1.98 (0.176–22.19)	02 (1.5%)	0.93 (0.13–6.79)
*GSTM1(−/−) & CYP2E1*5B(Wild)*	65 (37.2%)	77 (30.2%)	**1.5*** (1.0–2.30)	42 (30.0%)	1.5 (0.94–2.47)	47 (33.5%)	1.3 (0.81–2.08)
*GSTM1(−/−) & CYP2E1*5B(Variant)*	12 (6.8%)	03 (1.2%)	**7.2*** (1.98–26.17)	02 (1.5%)	**4.0*** (1.08–14.47)	01 (0.7%)	**11.0*** (1.43–88.28)
*GSTT1(+/+) & CYP2E1*5B(Wild)*	136 (77.7%)	209 (81.9%)	1.0 (Ref.)	115 (82.2%)	1.0 (Ref.)	116 (82.8%)	1.0 (Ref.)
*GSTT1(+/+) & CYP2E1*5B(Variant)*	06 (3.5%)	03 (1.2%)	3.0 (0.75–12.49)	02 (1.4%)	2.5 (0.50–12.81)	01 (0.7%)	5.1 (0.60–43.12)
*GSTT1(−/−) & CYP2E1*5B(Wild)*	25 (14.3%)	41 (16.1%)	0.94 (0.55–1.61)	22 (15.7%)	0.96 (0.51–1.79)	21 (15.0%)	1.0 (0.54–1.91)
*GSTT1(−/−) & CYP2E1*5B(Variant)*	08 (4.5%)	02 (0.8%)	**6.1*** (1.28–29.38)	01 (0.7%)	**6.7*** (0.83–54.89)	02 (1.5%)	3.4 (0.71–16.38)
*GSTP1(Ile/Ile) & CYP2E1*5B(Wild)*	82 (46.8%)	151 (59.2%)	1.0 (Ref.)	79 (56.5%)	1.0 (Ref.)	75 (53.5%)	1.0 (Ref.)
*GSTP1(Ile/Ile) & CYP2E1*5B(Variant)*	02 (1.2%)	03 (1.2%)	1.2 (0.20–7.49)	01 (0.7%)	1.9 (0.17–21.67)	02 (1.5%)	0.91 (0.12–6.65)
*GSTP1(Ile/Val + Val/Val) & CYP2E1*5B(Wild)*	79 (45.2%)	99 (38.8%)	1.46 (0.98–2.19)	58 (41.4%)	1.3 (0.83–2.0)	62 (44.3%)	1.16 (0.73–1.84)
*GSTP1(Ile/Val + Val/Val) & CYP2E1*5B(Variant)*	12 (6.8%)	02 (0.8%)	**11.0*** (2.4–50.56)	02 (1.4%)	**5.7*** (1.25–26.65)	01 (0.7%)	**11.0*** (1.4–86254)

Ref: reference category; OR: Odds ratio; 95% CI: 95% confidence interval; *$p < 0.05$ is considered statistically significant.

4. Discussion

The results of the present study have demonstrated that polymorphism in GST modifies the susceptibility to alcoholic liver cirrhosis. An elevated risk to alcoholic liver cirrhosis was observed in the patients with null genotype of *GSTM1* when compared with the non-alcoholic controls. No significant increase in the frequency of *GSTM1* null genotype in patients with non-alcoholic liver cirrhosis or alcoholic controls when compared with non-alcoholic controls have suggested that *GSTM1* null genotype may be an risk factor important in the development of alcoholic liver diseases associated with chronic ethanol intake. Non-alcoholic cirrhosis has been primarily attributed to hepatitis B and C virus infections which are known to increase the expression of *CYP2E1* in liver resulting in the generation of free radicals (Choi and Ou, 2006) though not to the extent as observed in the alcoholics (Takahashi et al., 1993). That *GSTM1* is important in scavenging the ethanol derived free radicals was further evident by the present data demonstrating statistically significant increase in the risk in the alcoholic cirrhotic patients with null genotype of *GSTM1* when compared with the alcoholic controls. Savolainen et al. (1996) have also earlier reported statistically significant association of *GSTM1* null genotype with alcoholic liver cirrhosis. Null genotype was found to occur more frequently among patients with different features of alcoholic liver disease (Harada et al., 1987, 1993; Baranov et al., 1996; Ladero et al., 2005) though several studies, lacking sufficient sample size, failed to find such an association (Groppi et al., 1991; Frenzer et al., 2002; Burim et al., 2004).

Consistent with earlier reports (Frenzer et al., 2002; Burim et al., 2004), no significant increase in the frequency of null genotype in *GSTT1* was observed in alcoholic cirrhotic patients when compared with the non-alcoholic controls or non-alcoholic cirrhotic patients or alcoholic controls. Ladero et al. (2005), however, reported increase in the frequency of *GSTT1* null genotype in advanced alcoholic liver disease patients when compared with the healthy controls. Burim et al. (2004) also reported an increase in the frequency of *GSTT1* null genotype in alcoholics with pancreatitis than that of the controls or alcoholics without disease. Interestingly *GSTT1* gene is better conserved in humans and has been suggested to have a more relevant functional role for GSTT1 enzyme than for GSTM1 enzyme (Ladero et al., 2005).

The increase in the frequency of variant genotype of *GSTP1* in alcoholic cirrhosis patients when compared with non-alcoholic controls is consistent with earlier reports (Burim et al., 2004; Ghobadloo et al., 2004), indicating that *GSTP1* polymorphism is associated with the development of alcoholic cirrhosis. Burim et al. (2004) earlier reported higher frequency of the *Val*/*Val* genotype of *GSTP1* in patients with alcoholic liver disease or chronic pancreatitis when compared to alcoholics without disease or the healthy controls. *GSTP1 Val*/*Val* genotypes were also found to be associated with the development of cirrhosis in patients with hereditary hemochromatosis (Stickel et al., 2005). Likewise significant association of *Val*/*Val* genotype of *GSTP1* with cryptogenic cirrhosis has implicated this polymorphism as a risk factor for the occurrence of the disease (Ghobadloo et al., 2004). Experimental studies have shown that *GSTP1* polymorphism alter the protein function. Biochemical studies have demonstrated a lower thermal stability of *GSTP1* Val compared with *GSTP1* Ile (Nelson et al., 1995; Alves-Silva et al., 2000) and also lower conjugating activity of Val homozygous compared with Ile homozygous, with heterozygous displaying intermediate activity (Watson et al., 1998). *In vitro* studies in human tissues revealed that *Val*/*Val* genotype is associated with a lower enzyme activity compared to that of the heterozygous and the Ile/Ile genotype. No significant association of variant genotype of *GSTP1* with non-alcoholic cirrhosis or in alcoholic controls when compared to non-alcoholic controls has further suggested that ethanol

derived free radicals are not generated to an extent observed in patients suffering from alcoholic cirrhosis (Takahashi et al., 1993).

As observed with *GSTM1* and *GSTP1*, a statistically significant risk was observed in alcoholic cirrhotic patients with variant genotype of *MnSOD* when compared with non-alcoholic controls. Similar increase in risk in alcoholic cirrhotic patients with variant genotypes of *MnSOD* when compared with non-alcoholic controls has been reported earlier (Degoul et al., 2001; Nahon et al., 2005). The variant form of *MnSOD* (*Ala*/*Ala* + *Val*/*Ala*) is known to facilitate the enhanced translocation of *MnSOD* into mitochondria than the wild form (*Val*/*Val*) resulting in increased lipid peroxidation and mitochondrial damage (Li et al., 1998; Sutton et al., 2003).

Likewise, significant increase in the risk was observed in alcoholic cirrhotic patients with variant genotypes of *CYP2E1*5B* when compared to non-alcoholic controls or non-alcoholic cirrhosis patients or alcoholic controls. Chronic alcohol intake which leads to cirrhosis is essentially associated with the induction of CYP2E1 leading to increased formation of acetaldehyde, reactive oxygen species as well as ethanol derived free radicals (Lieber, 1999; Stickel and Osterreicher, 2006). Since the variant genotype of *CYP2E1*5B* is known to increase the enzyme activity, the increase in frequency of *CYP2E1*5B* may lead to increased metabolism of alcohol resulting in increased formation of reactive oxygen species (ROS) and explain the increased risk observed in alcoholic liver cirrhotic cases when compared to non-alcoholic controls or non-alcoholic cirrhosis or alcoholic controls. Interestingly, Caucasians who have similar frequency of *CYP2E1*5B* variants in controls, do not show any significant association of *CYP2E1*5B* polymorphism with alcoholic liver diseases (Vidal et al., 2004; Wong et al., 2000; Parsian et al., 1998). Few studies, however, exhibit significant association of *CYP2E1*5B* with alcoholic liver diseases (Pirmohamed et al., 1995; Cichoz-Lach et al., 2006). This inconsistency in Caucasians has been partly attributed to the smaller sample size, lower frequency of variant genotype, and ethnic variations amongst the Caucasian studies (Parsian et al., 1998; Wong et al., 2000; Vidal et al., 2004). The power of the study for *CYP2E1*5B* polymorphism was found to be slightly less than 80% when the frequency in ACP was compared with NACP or alcoholic controls. Studies therefore with a larger sample size are needed to demonstrate the association of *CYP2E1*5B* with alcoholic liver cirrhosis.

Our data demonstrating significantly elevated risk (OR: 2.8; 95% CI: 1.3–6.06) of alcoholic cirrhosis in patients with both the *GSTM1* and *GSTT1* null genotypes is consistent with the hypothesis that combinations of various unfavorable deletion genotypes may theoretically confer an even higher risk to alcoholic cirrhosis. Ladero et al. (2005) have shown that individuals with deletions of both genes, *GSTM1* and *GSTT1* are at increased risk of developing the alcoholic liver diseases. The presence of both, *GSTM1* and *GSTT1* null genotypes was four times more frequent in cases with severe alcoholic liver diseases when compared to the controls. Similar increase in risk to alcoholic cirrhosis cases was observed in case who simultaneously carried the variant genotype of *GSTP1* (*Ile*/*Val or Val*/*Val*) and null genotype *of GSTM1* (OR: 2.8; 95% CI: 1.58–4.90) or *GSTT1* (OR: 2.16; 95% CI: 1.08–4.28) when compared to non-alcoholic controls, demonstrating significance of the genotypic combinations in the development of alcoholic liver cirrhosis. That the combination of deletions in *GST* isoforms could be involved in increasing the susceptibility to alcoholic liver cirrhosis was further evident in our study indicating approximately 6-fold increased risk (OR: 5.8; 95% CI: 2.17–15.80) to alcoholic liver cirrhosis in cases carrying combination of null genotypes of *GSTM1*, *GSTT1* and variant genotype of *GSTP1* (*Ile*/*Val or Val*/*Val*) when compared to non-alcoholic controls.

Further evidence for the importance of genotypic combinations in the development of alcoholic liver cirrhosis was provided by the present study indicating significantly increased risk in the cases simultaneously carrying the null or variant genotypes of *GSTs* and *MnSOD* when compared to the patients with the risk genotypes of *GSTs* or *MnSOD* alone. Since polymorphism in *MnSOD* has been shown to lead to the accumulation of reactive oxygen species in alcoholics leading to enhanced mitochondrial damage and lipid peroxidation (Degoul et al., 2001), the patients carrying the risk genotypes of *GSTs* and *MnSOD* simultaneously could be subjected to increased oxidative stress which may account for the increase in risk to alcoholic liver cirrhosis in such cases. Likewise several fold increased risk in the alcoholic liver cirrhosis patients with the combination of variant genotypes of *CYP2E1*5B* and *GSTP1* or null genotype of *GSTM1* or *GSTT1* could be attributed to the increased formation of ROS and ethanol derived free radicals, because of *CYP2E1* polymorphism, which may not be detoxified because of deletion or variant genotype of *GSTs*. Though the number of cases who simultaneously carried variant genotypes of *GSTs* and *CYP2E1*5B* was much higher when compared to the controls, greater magnitude of risk (up to 11-fold) observed in alcoholic cirrhosis patients could also be possible attributed to the rare (1–2%) frequency of *CYP2E1*5B* in the controls (Khan et al., 2008 unpublished observation) because of which the number of individuals with the combination of variant genotypes was very low.

In conclusion, our data suggests that polymorphism in *GSTs* is associated with an increased susceptibility to alcoholic liver cirrhosis. Risk to alcoholic cirrhosis was further found to increase in alcoholic cirrhotic patients carrying the combination of null or variant genotypes of *GSTs*. Likewise, several fold increased risk in the alcoholic cirrhotic cases carrying combinations of risk genotype of *GSTs* and *CYP2E1*5B* or *MnSOD* have suggested that interaction amongst the genes involved in generating oxidative stress may be important in determining the susceptibility to alcoholic liver cirrhosis.

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Contributors

(i) Anwar Jamal Khan: Involved in literature search, experimental study, data analysis and manuscript preparation.
(ii) Gourdas Choudhuri: Involved in clinical studies.
(iii) Qayyum Husain: Involved in standardization of protocol.
(iv) Devendra Parmar: Involved in study design and writing of manuscript.

Conflict of interest

There are no conflicts of interest.

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