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Original Article

Association of glutathione S-transferase T1 and M1 genotypes with chronic liver diseases among Filipinos

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Abstract: The glutathione S-transferase (GST) supergene family is made up of four gene families responsible for the biotransformation of drugs and other xenobiotics. Genetic variations in this supergene family influence individual detoxification levels and may contribute to the development of cancer. A hospital-based case-control study was conducted to evaluate the association between GST polymorphism among Filipino patients positive for hepatitis B virus (HBV DNA) and clinically diagnosed as either with chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma as well as normal individuals negative for HBV infection. Multiplex PCR was used to detect the presence or absence of the GSTT1 and GSTM1 polymorphisms in peripheral blood. DNA sequencing of the S gene region of the virus was used to determine the predominant genotype found among HBV-infected patients. Our results showed that the odds of having a chronic liver disease is only 0.95 (95% CI 0.58-1.57) among those with GSTT1 null genotype compared to those with GSTT1+ genotype. On the other hand, the odds of chronic liver disease is 17.85 times (95% CI 7.34-43.45) for those with GSTM1 null genotype compared to those with GSTM1+ genotype. Using the GSTT1+/GSTM1+ genotype as the reference, both GSTT1+/GSTM1- (OR 16.61; 95% CI 6.69-41.22) and GSTT1-/GSTM1- (OR 11.91; 95% CI 4.48-31.66) genotypes seem to be risk factors for chronic liver disease. From our observations, we conclude that polymorphism in GSTM1 null genotype (OR 17.85; 95% CI 7.34-43.45) seem to be associated with an increased risk of chronic liver disease among Filipinos.

Keywords: GSTT1, GSTM1, chronic liver disease, hepatitis B virus, polymorphism, Filipino

Introduction

Glutathione S-transferase (GST) enzymes are involved in the metabolism of a wide variety of carcinogenic compounds. GSTT1 and GSTM1 subfamilies exhibit homozygous deletions (null genotype) that have been considered as modifiers of individual risk of environmentally-induced cancers. These deletions are easily detected by molecular-based assays such as multiplex PCR [1].

The prevalence of the null genotype of GSTT1 and GSTM1 has been reported to vary among different ethnic populations. The GSTT1 is absent in 20% of Caucasians and 60% of Asians while GSTM1 is absent in 30% to 70% of the population [2-4]. Both genes are inherited independently from one another. The GST null genotype has been shown to be associated with various malignancies including cancers of the blood, bladder, colon, and lung [5-8].

Several studies have investigated the role of GSTT1 and GSTM1 in HBV-related liver cirrhosis (LC) and hepatocellular carcinoma (HCC) but the findings are inconsistent across studies [9-14]. It has been reported that individuals with dual null genotypes (GSTT1-/GSTM1-) were particularly susceptible to develop HCC. However, these findings were not found among Caucasians but only among Asian populations [15].

Here we aimed to determine the distribution of GSTT1 and GSTM1 null genotypes in patients with chronic liver disease and in normal individuals. Additionally, we evaluated the association between GSTT1 and GSTM1 gene polymorphisms and chronic liver disease among Filipinos and compared the frequency among vari-

Table 1. Primers used for GSTT1, GSTM1, β-globin, and HBV DNA amplification.

Gene	Polarity	Nucleotide Sequence (5' to 3')	Reference
GSTT1	Sense	TTC CTT ACT GGT CCT CAC ATC TC	[5]
	Antisense	TCA CCG GAT CAT GGC CAG CA	
GSTM1	Sense	GAA CTC CCT GAA AAG CTA AAG C	[5]
	Antisense	GTT GGG CTC AAA TAT ACG GTG G	
β-globin	Sense	GAA GAG CCA AGG ACA GGT AC	[5]
	Antisense	CAA CTT CAT CCA CGT TCA CC	
HBV PrS2	Outer sense	GGG ACA CCA TAT TCT TGG	[16]
HBV S1R	Outerantisense	TTA GGG TTT AAA TGT ATA CCC A	
HBV YS2	Inner sense	GCG GGG TTT TTC TTG TTG A	[16]
HBV YS1	Inner antisense	GGG ACT CAA GAT GTT GTA CAG	

ous populations. To the best of our knowledge, this is the first report of the incidence of GSTT1 and GSTM1 polymorphisms among Filipino patients positive for HBV DNA and clinically diagnosed as either with chronic active hepatitis (CAH), LC, or HCC.

Materials and methods

Samples and patients

Blood samples from 107 Filipino patients infected with hepatitis B and 127 control subjects at St. Luke's Medical Center-Quezon City were retrospectively studied. The cases include patients positive for HBV DNA and clinically diagnosed as either with CAH, LC, or HCC. A total of 52 samples positive for HBV were genotyped. The control group was composed of normal individuals negative for HBV DNA or non reactive to hepatitis B surface antigen. There were 138 (59%) males and 96 (41%) females with ages ranging from 15 to 76 years old.

Multiplex PCR amplification of GSTT1 and GSTM1 genes

Genomic DNA from normal individuals and HBV-infected patients was obtained from peripheral blood using the QIAamp DNA Blood Mini kit from Qiagen according to manufacturer's instructions. GST gene amplification was carried with 1.0 pmol each of the primers, 1X Phusion HF Buffer (Finnzymes), 200 μM dNTPs, 0.02 U of Phusion DNA polymerase and DNA template was made up to a volume of 25 μl. Multiplex PCR step was carried out at 98°C for 1 minute, 35 cycles at 98°C for 1 minute, 63°C for 1 minute, 72°C for 1 minute and a final elongation at

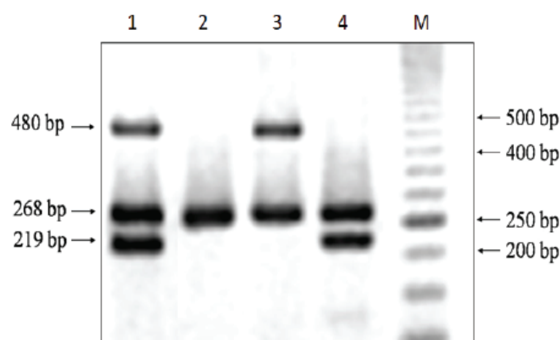

Figure 1. Ethidium bromide-stained 2% agarose gel showing the amplified products of a multiplex PCR used in the detection of GSTM1 (219-bp) and GSTT1 (480-bp) genotypes. The 268-bp fragment corresponds to the β-globin gene that was used as an internal control. Lane 1: GSTT1+/GSTM1+, Lane 2: GSTT1-/GSTM1-, Lane 3: GSTT1+/GSTM1-, Lane 4: GSTT1-/GSTM1+, Lane 5: 50-bp ladder.

72°C for 10 minutes. Multiplex PCR was done using the sense and antisense primers (**Table 1**). Amplicons were analyzed on 2% agarose gel followed by staining with ethidium bromide (**Figure 1**).

Genotyping of hepatitis B virus by direct DNA sequencing

The viral DNA from HBV-infected patient plasma was extracted from peripheral blood using the QIAamp DNA and Blood Mini kit from Qiagen. The S gene amplification was carried with 10 pmol each of the primers, 1.5U HotStar Taq DNA polymerase and DNA template was made up to a volume of 50 μl. Nested PCR was carried out at 95°C for 15 minutes, 35 cycles at

Table 2. Distribution of subjects by GSTT1 and GSTM1 genotypes.

Genotype	Chronic liver disease	Normal individuals	Total	p-value
	N (%)	N (%)	N (%)	
GSTT1				NS
GSTT1-	26 (24)	32 (25)	58 (25)	
GSTT1+	81 (76)	95 (75)	176 (75)	
Total	107 (100)	127 (100)	234 (100)	
GSTM1				<0.001
GSTM1-	103 (96)	75 (59)	178 (76)	
GSTM1+	4 (4)	52 (41)	56 (24)	
Total	107 (100)	127 (100)	234 (100)	

NS = not significant

Table 3. Distribution of GSTT1 and GSTM1 genotypes in patients with chronic liver disease and normal individuals

	GSTT1+ GSTM1+	GSTT1+ GSTM1-	GSTT1- GSTM1+	GSTT1- GSTM1-	Total
	N (%)	N (%)	N (%)	N (%)	
Chronic liver disease	4 (4)	77 (72)	0 (0)	26 (24)	107
Normal individuals	44 (35)	51 (40)	8 (6)	24 (19)	127
Total	48 (21)	128 (55)	8 (3)	50 (21)	234

94°C for 1 minute, 53°C for 45 seconds, 72°C for 1 minute and a final elongation at 74°C for 7 minutes. First round PCR was done using the outersense and outer antisense primers [16]. One μl of the first round PCR product was reamplified with internal primers for another 35 cycles under the same conditions. The 585-bp amplicon was purified prior to direct DNA sequencing (Macrogen, Korea). The sequence data were aligned with the consensus sequences of confirmed genotypes. The DNA sequences were then compared for identity with sequence from NCBI using the BLAST program.

Statistical analysis

The data were analyzed using OpenEpi 2.2. The chi-square (X^2) test was used to determine significant differences in the demographic and genetic characteristics between cases and controls. The odds ratios (OR) with their corresponding 95% confidence intervals (CI) and p-values were calculated simultaneously as an estimate of the risk of having chronic liver disease given a particular variable or polymorphism.

Results

The distribution of subjects by GSTT1 and GSTM1 genotypes as well as the distribution of GSTT1 and GSTM1 genotypes in patients with chronic liver disease and normal individuals are shown in Tables 2 and 3. Out of the 107 cases, 90 (84%) were classified as CAH, 12 (11%) were LC and 5 (5%) were HCC. Most individuals were found to be GSTT1+/GSTM1- (55%). Among GSTT1+/GSTM1- individuals, 50% were CAH, 6% LC, 4% were HCC, and 40% were normal. For GSTT1-/GSTM1-, 44% were CAH, 8% were LC, and 48% were normal. Individuals who were GSTT1-/GSTM1- comprised 21% of the study subjects. Also, 21% were genotyped as GSTT1+/GSTM1+ and 3% were GSTT1-/GSTM1+. Among GSTT1+/GSTM1+ individuals, 8% had liver diseases and 92% were healthy. All GSTT1-/GSTM1+ individuals were normal.

The association of age, sex, GSTT1, and GSTM1 genotypes was examined (Table 4). There is a significant difference in age distribution (p-value 0.007) but not in sex between the two groups. The odds of having a chronic liver disease is only 0.95 (95% CI 0.58-1.57) among those with GSTT1 null genotype compared to those with GSTT1+ genotype. On the other hand, the odds of chronic liver disease is 17.85 times (95% CI 7.34-43.45) for those with GSTM1 null genotype compared to those with GSTM1+ genotype. Using the GSTT1+/GSTM1+ genotype as the reference, both GSTT1+/GSTM1- (OR 16.61; 95% CI 6.69-41.22) and GSTT1-/GSTM1- (OR 11.91; 95% CI 4.48-31.66) genotypes seem to be risk factors for chronic liver disease.

Out of 107 HBV positive samples, only 52 (49%) were genotyped. Sequence analysis of the S

Table 4. Odds ratio and 95% confidence interval of GSTT1 and GSTM1 genotypes.

Parameters	Chronic liver disease N=107 (%)		Normal individuals N=127 (%)	p-value
Age				0.007
≤ 40	56 (52)		49 (39)	
41-64	46 (43)		57 (45)	
≥65	5 (5)		21 (16)	
Sex				NS
Male	68 (64)		70 (55)	
Female	39 (36)		57 (45)	
GSTT1				NS
Null	26 (24)		32 (25)	
Positive	81 (76)		95 (75)	
OR (95% CI)		0.95 (0.58-1.57)		
GSTM1				<0.001
Null	103 (96)		75 (59)	
Positive	4 (4)		52 (41)	
OR (95% CI)		17.85 (7.34-43.45)		
GSTT1+/GSTM1+*	4 (4)		44 (35)	<0.001
GSTT1+/GSTM1-	77 (72)		51 (40)	
OR (95% CI)		16.61 (6.69-41.22)		
GSTT1-/GSTM1+	0 (0)		8 (6)	
OR (95% CI)		0 (0)		
GSTT1-/GSTM1-	26 (24)		24 (19)	
OR (95% CI)		11.91 (4.48-31.66)		

NS = not significant; *used as a reference

gene region showed that 24 (46%) belonged to genotype HBV-A, 14 (27%) belonged to genotype HBV-B, and 14 (27%) was of genotype HBV-C. No HBV genotypes D to H were noted.

Discussion

Polymorphism in GSTT1 and GSTM1 genes were identified through multiplex PCR and the β-globin gene was used as an internal control. It has been suggested that internal controls such as β-globin, dihydrofolate reductase (DHFR), or p53 gene must be included in order to exclude false negative reactions [9, 11]. This approach was effective in accurately identifying GST null genotypes since PCR inhibition can be ruled out through a positive β-globin band when a negative result for GSTT1 and GSTM1 is obtained. Heterozygous genotypes for GSTT1 and GSTM1 cannot be distinguished using current PCR techniques including multiplex PCR [10, 17]. Thus, Hardy-Weinberg equilibrium (HWE) cannot be determined in this study. As previously described, Hardy-Weinberg tests in case-control studies are not available that can be applied to dominant and recessive markers with only two alleles such as GST [18].

The frequency of the GSTT1 and GSTM1 null genotype has been reported in various ethnic backgrounds worldwide. Deng et al., and Munaka et al., have shown that GSTT1 null genotype among healthy Chinese and Japanese populations were absent in 42.7% and 47.8%, respectively. In another study, Sun et al., reported that the frequency of GSTT1 and GSTM1 null genotypes among healthy Taiwanese were 60.2%. In this study, the GSTT1 null genotype was observed in 24.2% of HBV-infected patients and 25.2% among normal individuals. On the other hand, GSTM1 null genotype was observed in 96.3% of HBV-infected patients and 59.0% among normal individuals (Table 5 and 6).

Several associations of GSTT1 and GSTM1 polymorphisms with HBV-related LC and HCC have been reported. However, the results are conflicting among different ethnic populations and do not provide consistent evidence for an association of genetic polymorphisms on GST enzymes with the development of HCC [9-12, 19-21]. One of the possible reasons for this may be explained by the selection of study participants using cohort, hospital, or community-based subjects [22]. Another possible reason is that not

Table 5. GSTT1 null genotype distribution in patients with chronic liver disease and normal individuals.

	Method	Case			Control			Reference
		N	Null	(%)	N	Null	(%)	
Filipino	Multiplex PCR	107	26	(24.2)	127	32	(25.2)	Present study
Chinese	PCR	181	108	(59.7)	360	154	(42.7)	[9]
Italian	Multiplex PCR	67	30	(16.0)	400	72	(18.0)	[10]
Spanish	Multiplex PCR	184	53	(28.8)	329	76	(23.1)	[11]
Japanese	Multiplex PCR	78	39	(51.3)	138	66	(47.8)	[19]
Taiwanese	PCR	67	30	(44.8)	128	77	(60.2)	[20]

Table 6. GSTM1 null genotype distribution in patients with chronic liver disease and normal individuals.

	Method	Case			Control			Reference
		N	Null	(%)	N	Null	(%)	
Filipino	Multiplex PCR	107	103	(96.3)	127	75	(59.0)	Present study
Japanese	PCR	83	51	(61.4)	101	43	(42.5)	[4]
Chinese	PCR	181	117	(64.6)	360	172	(47.8)	[9]
Italian	Multiplex PCR	200	99	(49.5)	400	215	(53.8)	[10]
Spanish	Multiplex PCR	184	88	(47.8)	329	149	(45.3)	[11]
Japanese	Multiplex PCR	78	29	(38.5)	138	68	(49.3)	[19]
Taiwanese	PCR	69	26	(37.7)	128	77	(60.2)	[20]

all study check for HWE, which can affect the result especially of case-control studies [10, 17].

The present study did not look into the association between GSTT1 and GSTM1 genotypes with CAH, LC, and HCC because of the limited number of LC and HCC cases. Ideally, GSTT1 and GSTM1 association should be determined in relation to the progression of chronic liver disease to HCC as previously described [9-12, 19-21]. Gene-environment interactions were also not addressed in this study due to the limited reported data. Further studies in combination with other genes and environmental factors are recommended. For instance, N-acetyltransferase-2 (NAT-2) slow acetylator genotype in combination with GSTT1 null genotype further increase the risk of chronic liver disease among cigarette smokers [10, 23].

Sobel *et al.*, reported that approximately seven million Filipinos are chronically infected with HBV. Considering the importance of determining the HBV genotypes, several methods have been developed for genotyping of HBV strains such as restriction fragment length polymorphism, line probe assay, and quantitative real-time PCR [16, 25-30]. Here we report the prevalent genotype found among HBV-infected Filipino patients using DNA sequencing. Results showed that HBV-A (46%) is the predominant genotype found among 52 samples analyzed in this study. Our data corroborates the findings that HBV-A and HBV-C are the common genotypes found among patients with chronic HBV infection in Cebu City, Philippines [31]. HBV genotyping have major implications for molecular epidemiologic studies, as well as investigating clinical outcomes particularly among patients infected with HBV-A, HBV-B, or HBV-C. It has been reported that patients infected with genotype HBV-C have been found to have more severe liver disease than genotype HBV-B and a lower response rate to treatment in comparison with those individuals infected with genotypes HBV-A and HBV-B [31-33]. However, the present study did not look into the disease progression and treatment response. Thus, we cannot confirm such findings. We are also aware of the limitation in sample size and other data in this study which prevents the analysis of confounding factors and detection of significant effects in some statistical tests.

From our observations, the GSTT1 did not show significant association with chronic liver disease in the available data, but the genetic polymorphism in GSTM1 null genotype seem to be associated with an increased risk of chronic liver disease among Filipinos. The limitation of this conclusion is due to the fact that spurious association cannot be easily ruled out with the available data at hand. However, our findings may

have prognostic value to identify high risk groups and possible genetic factors in future studies on chronic liver disease.

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