Association between the Genetic Polymorphism of Glutathione S-Transferase Genes and the Different Stages of Hepatitis B Virus Infection in Egypt

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Abstract: Hepatitis B virus (HBV) infection often leads to chronic hepatitis, cirrhosis and hepatocellular carcinoma. Human cytosolic glutathione-S-transferases (GSTs) include seven distinct classes, namely, Alpha (A), Mu (M), Pi (P), Sigma, Zeta, Omega and Theta (T). In this study, the genotypes of GSTs especially GSTT1, M1, P1 and A1 were determined in the control (C) and the different groups of patients with HBV to investigate the role of host genetic factors in HBV infection. GST activity and liver profile tests were determined. HBV groups were classified according to clinical history, serological tests and histological analysis into normal carriers (N), acute (A), chronic (CH), cirrhosis (CI) and hepatocellular carcinoma (HCC). The results showed that wild GSTM1/GSTT1 genotype, GSTP1 isoleucine / isoleucine and GSTA1 cytosine cytosine (BB) were decreased in N, A, CH, CI and HCC groups compared to the C group. However The genotypes null GSTT1, null GSTM1, double null GSTM1/GSTT1 and GSTP1 valine/valine, GSTP1 valine / isoleucine genotypes and GSTA1 thymine thymine (AA) and GSTA1 cytosine thymine (AB) were increased in all studied groups compared to the C group. This indicates that GSTs genetic polymorphism may be considered as an accurate biomarker for determining and predicting the progression of HBV infection.

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1. Introduction

Hepatitis B is one of the world's most serious and widespread chronic diseases [1]. HBV infection can result in acute hepatitis, HBV carriage, chronic hepatitis, liver cirrhosis, and even primary hepatocellular carcinoma. Progression of hepatitis to cirrhosis and the response to therapy are variable among individuals [2, 3]. Progress of HBV infection might be affected by host genetic susceptibility [4].

Glutathione *S*-transferases represent an important family of phase II drug metabolizing enzymes that play a key role in the protection against oxidative stress that contributes to the development of liver disease [5]. GSTs catalyze the conjugation of a large variety of endogenous and exogenous compounds including carcinogens and anticancer drugs and their metabolites with reduced glutathione [6]. Based on their biochemical, immunologic, and structural properties, human cytosolic GSTs are categorized into seven classes: Alpha, Mu, Pi, Sigma, Zeta, Omega and Theta [6]. The Alpha (A) class family includes five functional genes (GSTA1-GSTA5) and seven pseudo genes [6]. GSTA1 and GSTA2 are the most abundant in the human liver. Theta class functionally has two genes (GSTT1 and GSTT2) and only one pseudo gene [6]. Five Mu genes (GSTM1- GSTM5) have been identified [6]. GSTM1 is abundantly expressed in the liver, while GSTM2-M5 is mainly detected in extrahepatic tissues such as brain and testis [7]. GST-alpha is a dimeric enzyme since the two subunits can form homodimers and heterodimers. The GSTA1 accounts for 80-90% of the GSTA pool in human liver [8]. The previous studies showed that plasma GSTA1 level is an earlier and a more sensitive indicator of hepatocellular damage than ALT and AST [9, 10]. Otherwise, significantly increased expression of GSTP was demonstrated in early hepatocarcinogenesis [11] and HCC specimens [12], compared to their adjacent normal tissues or liver cirrhosis tissues. Loss of GSTP1 has been suggested to increase the risk of DNA damage and mutation [13, 14].

Whalen and Boyer [5] showed a significant association between the null GSTM1 and GSTP1-Val (105) polymorphisms and cryptogenic liver cirrhosis. The frequency of the GST genotypes and the activity of corresponding enzymes vary among different ethnic groups [15]. A relation has also been reported

between GSTs polymorphism and HBV-related hepatocellular carcinoma [16, 17]. In this study, the frequency of GSTM1, GSTT1, GSTP1 and GSTA1 polymorphism in patients with different stages of HBV infection was analyzed to investigate the role of host genetic factors in HBV infection.

2-Materials and Methods

2.1. Chemicals and kits:

GST assay kit was obtained from Cornel lab, USA; *protienase K* was obtained from Amersco, USA; primers and Polymerase chain reaction (PCR) kit was obtained from Microgen, South Korea and Promega, USA, respectively. Agarose was obtained from Bioshop, Germany; BsmAI and EarI was obtained from New England Biolab, England and kits for albumin, AST and ALT were obtained from Borax diagnostic, England, Prodia international, Germany and Crest Biosystem, India, respectively. Kits for bilirubin and total protein were obtained from Diamond, Egypt and Spinreact, Spain, respectively. DNA loading buffer and ladders were obtained from Promega, USA. Phenol equilibrated and chloroform was obtained from Acrosorganic, USA and Applichem, Germany, respectively.

2.2. Patients and control:

The present study was carried out on blood samples of 470 persons (310 males and 160 females) which included 150 healthy persons (control group) and 320 patients: The age of studied cases was (43±13) years. The patients were classified into five groups according to clinical history, serological tests and histological analysis as follow: 67 HBV carriers, 53 acute patients, 78 chronic patients, 62 cirrhotic patients, and 60 hepatocellular carcinoma patients. Complete demographic date and full clinical history were recorded. Different blood samples were collected from different hospitals, medical laboratories and hepatologist clinics in Egypt according to the rules of scientific research ethics.

2.2.1. Serum preparation: Five ml of blood from each person were collected, left at room temperature for 10 min and then centrifuged at 8000 rpm for 5 min at 4 ºC. All serum samples were stored at -80 ºC.

2.3. Biochemical assays:

2.3.1. GST (EC 2.5.1.18) **activity** was determined using kit [18]. The specific activity is defined as U/L.

2.3.2. DNA extraction from serum: Genomic DNA was extracted from different serum samples manually [19]. Briefly, 0.2 ml of serum was added to 0.6 ml of lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na_2EDTA, pH 8.2). Then 0.2 ml of 10% SDS and 20 µl of proteinase K solution were added and incubated for 12 hours at 37 °C. Then 0.6 ml of 6 M NaCl were added and centrifuged at 12000 rpm for 20 minutes at 4 ºC. Then 0.5 ml of the supernatant was added to 1.0 ml of absolute ethanol and incubated for 12 hours at 37 °C, then centrifuged at 12000 rpm for 20 minutes at 4 ºC and the supernatant was discarded. The pellets were washed twice with 0.5 ml of 70 % ethanol and dried in laminar. The dried pellets were dissolved in 70 µl of deionized distilled H_2O and used for determination of DNA concentration and molecular tests. The concentration and the purity of DNA in 10 µl samples was measured using spectrophotometer (A 260 / A 280) [20].

2.4. Molecular assays:

2.4.1. Primers: The specific primers were designed to detect different specific GSTs genes including GSTM1, GSTT1, GSTP1 and GSTA1. The primers sequences, annealing temperatures, annealing times and PCR product sizes were shown in Table 1 [21].

2.4.2. Detection of GSTM1 and GSTT1, GSTP1 and GSTA1:

A triplex PCR was used to detect GSTM1 and GSTT1, GSTP1 and GSTA1 [21]. β- globin gene was used as internal control for DNA integrity. In brief, 2.5 µl (50 ng) of isolated DNA sample was added to 2.5 µl of 2 mmol/L(deoxynucletuide triphosphates) dNTPs, 5 µl of 10X PCR buffer containing 1.5 mmol/L $MgCl_2$, 2µl of 20 pmol from each primers and 0.5 µl (5U/µl) of Taq DNA polymerase. PCR program was 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C and 10 min at 72°C. The PCR products were analyzed electrophoretically on a 2.5 % agarose gel containing 0.5 µg/ml of ethidium bromide with DNA ladder (100-1500 bp).

2.5. HBV DNA titer values were determined [23].

2.6. Liver profile tests were determined using kits [24].

2.7. Statistical analysis: Frequencies and associations between specific genotypes, GST activities and liver function tests were examined by use of logistic regression to calculate the mean ± SD, *P*. values, R square and upper and lower values of 95% confidence intervals (CI). Windows Microsoft office 2007 and SPSS for Windows (version 11.0) statistical package was used for all statistical comparisons. A value of $P < 0.05$ was considered significant.

3-Results

3.1. HBV DNA titer values: Table 2 shows the different stages of HBV infection and their corresponding HBV DNA titer values.

3.2. Agarose gel electrophoresis: The molecular sizes (MS) of GSTT1, GSTM1 and β-globin of PCR products were 480 bp, 215 bp and 268 bp, respectively as compared to DNA marker (Fig. 1 A).

The MS of GSTP1 and GSTA1 of PCR products were 433 bp and 400 bp, respectively, (Fig.

1 B). Also the MS of homozygous wild genotype IIe/IIe was (329 bp and 107 bp), however, the MS of heterozygous mutant genotype Val/IIe was (329 bp, 222 bp and 107 bp) as shown in (Fig. 1 C).

The electrophoresis of GSTA1 shows that, the MS of homozygous wild genotype AA was 400 bp and the MS of homozygous mutant genotype BB was 308 bp and 92 bp. While the MS of heterozygous mutant genotype AB was 400 bp, 308 bp and 92 bp (Fig. 1 D).

3.3. GST activity: GST activities were decreased significantly in N, A, CH, CI and HCC groups by about 40.1 %, 52.9 %, 57%, 54.3 % and 65.8 %, respectively as compared to the control group (Fig. 2). The results in Table 3 show that GST activities within the normal range in the genotypes of wild GSTT1/GSM1, GSTP1 Ile/Ile and GSTA1 AA. However the enzyme activities were decreased gradually in the genotypes of null GSTM1, null GSTT1, null GSTM1/GSTT1, GSTP1 Val/Val, GSTA1 AB and GSTA1 BB.

3.4. Liver profile: Serum TP and albumin levels were decreased significantly in N, A, CH, CI and HCC groups (Fig. 3 A and B respectively) as compared to the control group. However, serum AST and ALT activities and TB level were increased significantly (Fig. 3 C, D and E, respectively).

3.5. Genetic polymorphism of GSTs genes:

Table 1: The primers conditions:

Primer	Primer sequence 5′ → 3′	T_a(ºC) / T(s)	PCR product size (bp)
1-GSTM1F	TTC TGG ATT GTA GCA GAT CA	58 / 60	215
GSTM1R	CGC CAT CTT GTG CTA CAT TGC CCG	58 / 60	
2-GSTT1F	TTC CTT ACT GGT CCT CAC ATC TC	58 / 60	480
GSTT1R	TCA CCG GAT CAT GGC CAG CA	58 / 60	
3-GSTP1F	GTA GTT TGC CCA AGG TCA AG	59 / 90	430
GSTP1R	AGC CAC CTG AGG GGT AAG	59 / 90	
4-GSTA1F	GCA TCA GCT TGC CCT TCA	62 / 60	400
GSTA1R	AAA CGC TGT CAC CGT CCT G	62 / 60	
5-β-GLOBIN F	CAA CTT CAT CCA CGT TCA CC	58 / 60	268
β-GLOBIN R	GAA GAG CCA AGG ACA GGT AC	58 / 60	

Ta: Annealing temperature; T(s): Time (seconds) and bp: base pair

Table 2: Relationship between different stages of HBV infection and HBV DNA load (IU/ml):

Different stages of HBV infection	HBV DNA titer (IU/ml*) (mean ±SD)	*P*.value
Normal carriers (N)	$5X10^3 \pm 3.5\ X10^3$	
Acute infection (A)	$30X10^8 \pm 12\ X10^8$	
Chronic infection (CH)	$60X10^6 \pm 27\ X10^6$	0.002*
Cirrhosis (CI)	$20X10^6 \pm 15\ X10^6$	
Liver cancer (HCC)	$7X10^5 \pm 5.5\ X10^5$	

Values of $P < 0.05$ were considered statistically significant

IU/ml: international unit per ml which equivalent to copies per ml.

Table 3: Logistic regression analysis of association between GST activities and different GSTs genetic polymorphism:

Genotype	%	GST Activity (mean ±SD)	*p*.value	R2	95%CI (upper-lower)
Wild GSTM1/GSTT1	18.5	6.94±2.98			
Null GSTM1	34.4	4.32±1.36			
Null GSTT1	22.6	5.21±0.36			
null GSTM1/GSTT1	24.4	3.35±1.56			
GSTP1 Ile/Ile	17.7	6.41±2.33	0.001	0.33	72.2 - 25.3
GSTP1 Val/Ile	35.9	5.14±0.99			
GSTP1 Val/Val	46.3	5.02±047			
GSTA1 CC	20.3	6.50±1.44			
GSTA1 CT	33.7	3.24±1.84			
GSTA1 TT	38.5	3.03±1.22			

Values of $P < 0.05$ were considered statistically significant, R^2= Regression square and CI= confidence interval.

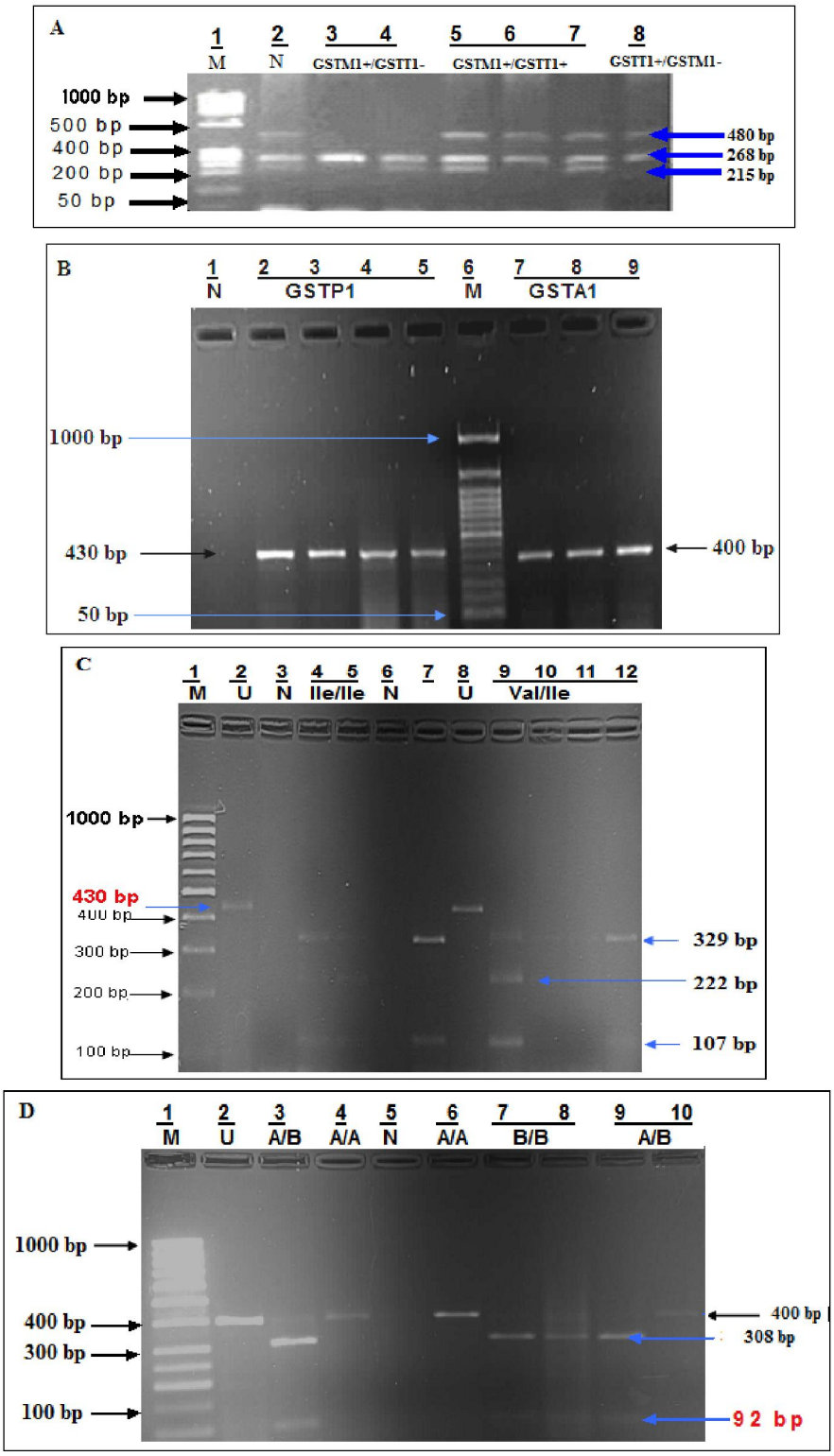


Fig. 1: Electrophoresis of different studied GST genotypes.
(A)GSTM1/ GSTT1. (B) GSTP1 and GSTA1 genes. (C) GSTP1 and (D) GSTA1. Since M ─ marker; N─ negative results; U ─ uncut band; Ile/Ile and Val/ lle represents homozygous and heterozygous wild types respectively.

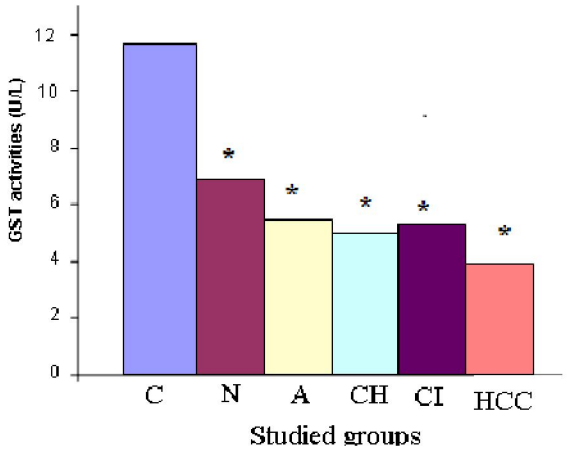
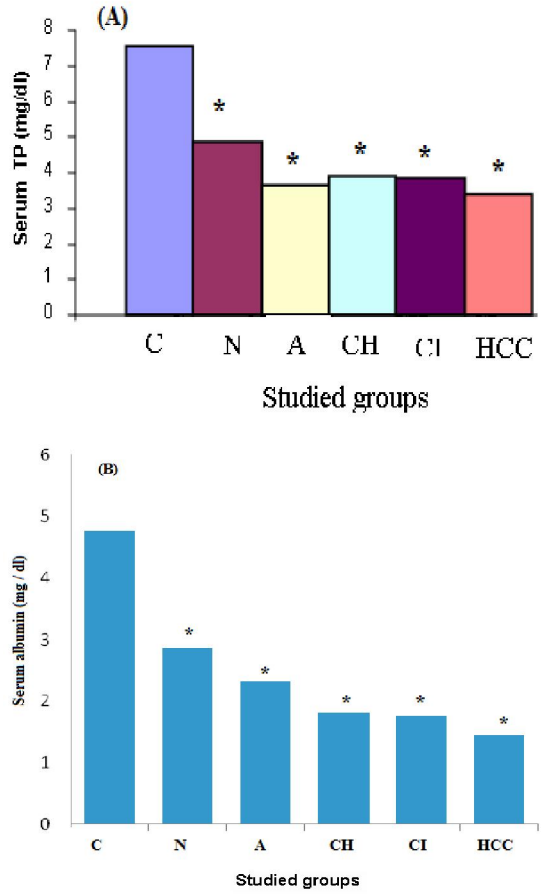


Fig 2: GST activities of different stages of HBV infection.
Where C─ control samples; N ─ normal carriers; A─ acute infection; CH ─ chronic infection; I─ cirrhotic patients and HCC─ hepatocellular carcinoma patients



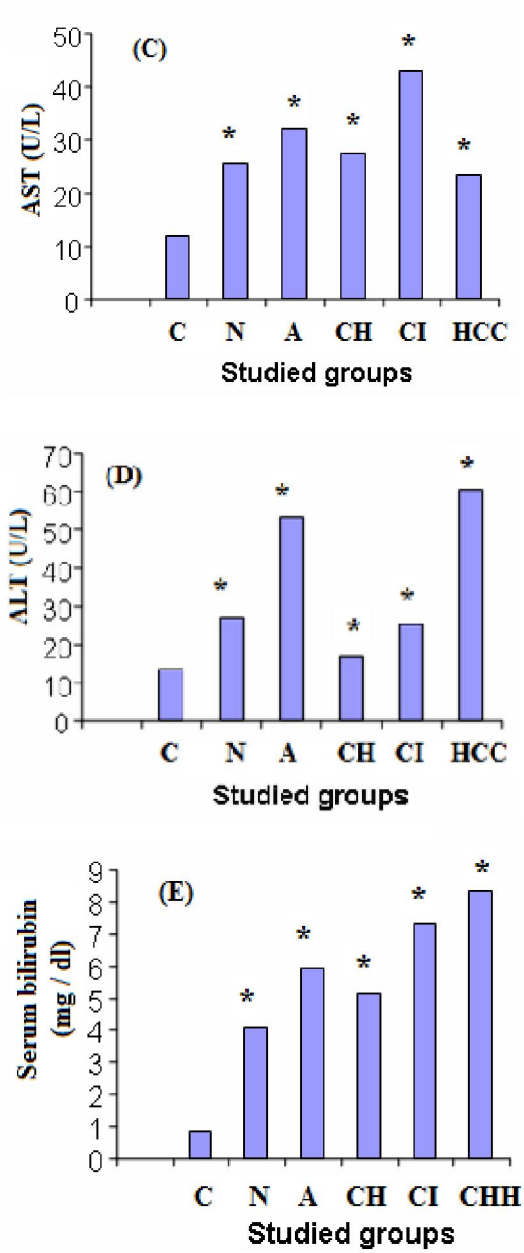


Fig. 3: Liver functions in different stages of HBV infection.
(Fig. 3 A) serum protein levels; (Fig. 3 B) serum albumin levels; (Fig. 3 C) serum AST activities; (Fig. 3 D) serum ALT activites and (Fig. 3 E) serum total bilirubin levels.Where C─ control samples; N ─ normal carriers; A─ acute infection; CH ─ chronic infection; I─ cirrhotic patients and HCC─ hepatocellular carcinoma patients

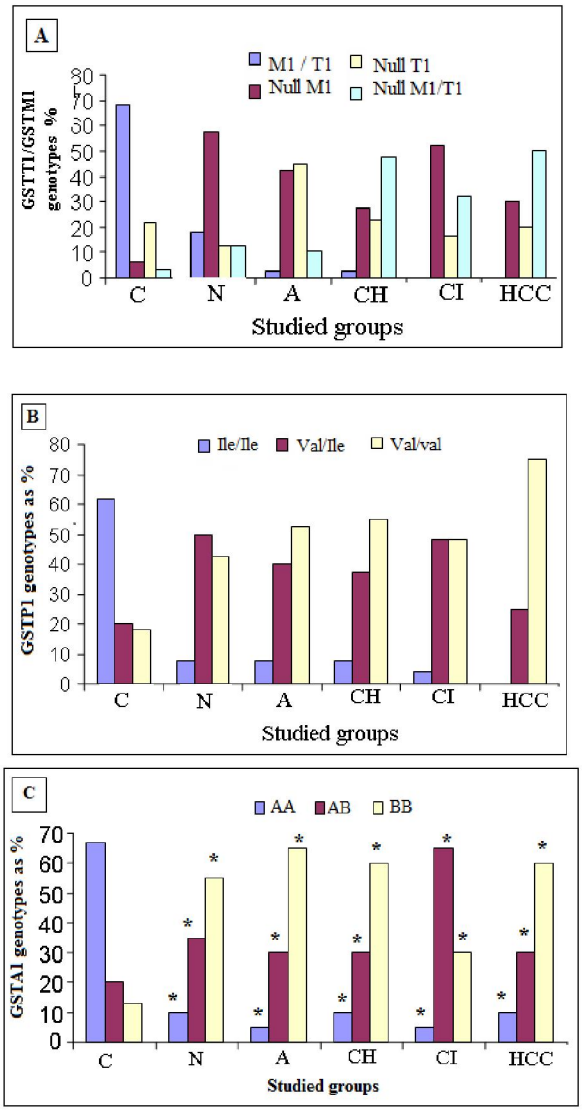


Fig. 4: Frequencies of genetic polymorphism in different stages of HBV infection.
(Fig. 4 **A):** GSTM1/GSTT1 genotypes; **(**Fig. 4 **B):** GSTP1 genotypes and **(**Fig. 4 **C):** GSTA1 genotypes. Where C─ control samples; N ─ normal carriers; A─ acute infection; CH ─ chronic infection; I─ cirrhotic patients and HCC─ hepatocellular carcinoma patients

Wild GSTM1/GSTT1 genotype was decreased significantly in N, A, CH, CI and HCC groups as compared to the control group (Fig. 4 A). While the null GSTM1 genotypes were increased significantly. Null GSTT1 genotypes of N, CI and HCC groups were decreased significantly and increased significantly for A, CH as compared to the control group. Also double null GSTM1/GSTT1 genotype was increased significantly in all HBV groups as compared to the control group (Fig. 4 A).

The results showed that GSTP1 IIe/IIe genotype was decreased significantly in N, A, CH, CI and HCC groups as compared to the control group (Fig. 4 B). However, the GSTP1 Val/IIe and GSTP1 Val/Val genotypes were increased significantly.
The results showed that the GSTA1 AA genotype was decreased significantly in N, A, CH, CI and HCC groups as compared to the control group (Fig. 4 C). While, GSTA1 AB and GSTA1 BB genotypes were increased significantly.

4. Discussion

In this study the relationship between different stages of HBV infection and HBV DNA titer was studied and the data showed that there was no significant relationship between them. Our results agree with the previous studies of Yuen *et al.,* [23], however, other studies reported that patients with less liver damage had higher viral load [25, 26].

Otherwise, the previous studies showed that both GSTP1 and GSTM1 polymorphic variants are associated with altered catalytic function of GST [27]. The current study showed a reduction in GST activities in different HBV groups (N, A, CH, CI and HCC) as compared to the control group. This indicates that GST activity was decreased with the development of HBV infection stages and this may be related to inflammation and hepatocytes injury. So GST activity may be considered as a more accurate biomarker for the diagnosis of HBV infection stages than transaminases (AST and ALT).

In addition, GST activities were within the normal range in the genotypes of wild GSTT1/GSM1, GSTP1 Ile/Ile and GSTA1 AA. However its activities were decreased gradually in the genotypes of null GSTM1, null GSTT1, null GSTM1/GSTT1, GSTP1 Val/Val, GSTA1 AB and GSTA1 BB. This indicates that GST activities were decreased in GST mutant genotypes rather than wild types. Therefore, patients who are genetically predisposed to produce a less active and less specific enzyme might be more prone to develop HBV-related liver disease. Our results agree with Shahrokh *et al.,* [21] who reported that the Val/Val genotype is associated with a lower GST activity compared to the heterozygous and Ile/Ile genotypes. Donna *et al.,* [28] also reported that there is a significant relationship between genetic polymorphisms (GSTP1 and GSTM1) and the development of HBV related liver disease. A decline in the level of GSTA1 AA genotype with elevations in the levels of GSTA1 AB and GSTA1 BB genotypes in HBV groups as compared to the C group indicates that GSTA1 mutant genotypes were increased with increasing the degree of HBV infection. Our results agree with Jie *et al.,* [22] who mentioned that the variant GSTA1 is

associated with attenuated specific GSTA1 activity accompanied by large individual variation. Therefore, it is reasonable to speculate that the variant of GSTA1 might be associated with a deleterious biological consequence in human susceptibility to some diseases and also as a result of using anticancer drugs in the therapy. Consequently, alpha- GST can be considered as a biochemical marker of liver injury, which is more sensitive than ALT due to its zonal distribution, intracellular concentration, and rapid release into plasma [27]. Moreover, Loguercio *et al.,* [29] reported that alpha-GST is a sensitive marker of hepatocellular damage in patients with chronic hepatitis carriers (CHC)—even in apparently HCV "healthy carriers"—and a useful tool for monitoring viral clearance during and after interferon (IFN) therapy.

An elevation in the levels of GSTP1 Val/IIe and GSTP1 Val/Val genotypes with a reduction in the levels of GSTP1 IIe/IIe genotype in HBV groups as compared to the C group indicates that GSTP1 mutant genotypes were increased with the increase in the degree of HBV infection. This means that the patients had mutant GSTP1 genotypes were more susceptible for more HBV disease progression. Our results agree with some previous studies which reported that the particular GST alleles are associated with altered risk or outcome of various diseases including hepatitis B-related hepatocellular carcinoma [30]. Mohammadzadeh *et al.*, [31] reported that both GSTP1 polymorphism and M1 null genotype are significantly more prevalent in patients with cryptogenic cirrhosis. Otherwise, GSTP gene expression is increased during early hepatocarcinogenesis by acetylation of histones H3 and H4 and interaction with the specific transcription factor in the promoter regions of the GSTP gene [32]. Also, it had been reported that normal or increased GSTP1 protein levels or activities contributed to normal hepatocytes against a variety of potentially promutagenic stresses [33], assist detoxification, and inhibit mutagenesis [34].

Elevation in the levels of null GSTM1 and double null GSTM1/GSTT1 genotypes with a decline in the level of wild GSTM1/GSTT1 genotype in all HBV groups as compared to the C group indicates that patients who had mutant GSTT1 and GSTM1 genotypes were more susceptible for more HBV disease progression.

Reduction in the levels of serum TP and albumin and elevation in serum total bilirubin level and AST and ALT activities in all studied groups as compared to the C group may be related to increase in progression of HBV infection and persistent hepatocytes inflammation and injury which led to the hepatocytes rupture and leakage of their contents. These results agree with our previous studies which reported that lipid peroxidation is increased in N, A, CH, CI and HCC groups as compared to the C group [35].

5. Conclusion:

The present study demonstrates that GSTs genetic polymorphism may be considered as an accurate biomarker for determining and predicting the progression of HBV infection. For our knowledge, this is the first report on GSTs genetic polymorphism and association with HBV infection in Egypt.

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