

RESEARCH ARTICLE

Distribution of Glutathione S-Transferase Omega Gene Polymorphism with Different Stages of HBV Infection Including Hepatocellular Carcinoma in the Egyptian Population

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Abstract

Background: Infection with hepatitis B virus (HBV) is a major global public health problem, with a wide spectrum of clinical manifestations. Human cytosolic glutathione-S-transferases (GSTs) include several classes such as alpha (A), mu (M), pi (P), sigma (S), zeta (Z), omega (O) and theta (T). The present study aimed to investigate the role of GST omega genes (GSTO1 and GSTO2) in different groups of patients infected with HBV. **Materials and Methods:** 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) cases. The study focused on determination of the genotypes of GST omega genes (GSTO1 and GSTO2) and GST activity and liver function tests. **Results:** The results showed that GSTO1 (A/A) was decreased in N, A, CH, CI and HCC groups compared to the C-group, while, GSTO1 (C/A) and GSTO1(C/C) genotypes were increased significantly in N, A, CH, CI and HCC groups. GSTO2 (A/A) was decreased in all studied groups as compared to the C-group but GSTO2(A/G) and GSTO2(G/G) genotypes were increased significantly. In addition, GST activities, albumin and TP levels were decreased in all studied groups compared to the C-group, while the activities of transaminases were increased to differing degrees. **Conclusions:** The results indicate that GSTO genetic polymorphisms may be considered as biomarkers for determining and predicting the progression of HBV infection.

Keywords: HBV - infection - cirrhosis - HCC - GST - omega genes - genetic polymorphisms

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Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione (Shaban et al., 2003, 2013, 2014 a & b). In general, GSTs protect cellular macromolecules from damage caused by several xenobiotics (Shaban et al., 2013). GSTs are divided into two distinct super-family members: the membrane-bound microsomal and cytosolic family members.

Microsomal GSTs are structurally distinct from the cytosolic in that they are homo- and heterotrimerize rather than dimerize to form a single active site. Microsomal GSTs play a key role in the endogenous metabolism of leukotrienes and prostaglandins. The cytosolic GSTs are categorized into several classes according to their genetic and biochemical properties such as alpha (A), mu (M),

omega (O), pi (P), sigma (S), theta (T), and zeta (Z) families (Strange et al., 2001, Li et al., 2003, Shaban et al., 2014c & d).

The GST omega class (GSTO) contains two expressed genes GSTO1 (12.5 kb) and GSTO2 (24.5 kb) beside a pseudo gene GSTO3p. GSTO1 and GSTO2 are 12.5 and 24.5 kb, respectively, and lie 7.5 kb on chromosome 10q25.1 (Polimantia et al., 2011). Members of this family do not display activity with "classical" GST substrates such as 1-chloro-2,4-dinitrobenzene, dichloromethane, or ethacrynic acid, but they do exhibit several enzymatic characteristics of glutaredoxins (Board et al., 2000, Whitbread et al., 2003). Although most GSTs have a serine or tyrosine residue at the active site, the GSTOs have cysteine, as well as a 19-amino acid extension at the N terminus that is not present in other cytosolic GSTs (Board et al., 2000). The genes encoding GSTO1 and GSTO2 map to chromosome 10 and are separated by approximately 1.5 kb (Whitbread et al., 2003). Both GSTO1 and

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GSTO2 catalyze the reduction of monomethyl arsenate from an oxidation state of +V to +III (Schmuck et al., 2005, Mukherjee et al., 2006). GSTO1 and GSTO2 have been identified in many organisms, including humans, and have poor activity with common GST substrates (Whitbread et al., 2003, Pongstaporn et al., 2008). GSTO1 plays a role in apoptosis and is a potential reservoir of intracellular glutathione (GSH), which protects against cellular oxidative stress (Ada et al., 2013). The gene polymorphisms of GSTO1 have been associated with the risk of human cancers, such as hepatocellular carcinoma, cholangiocarcinoma, and breast cancer (Marahatta et al., 2006). While GSTO2 polymorphism has been reported in ovarian cancer (Pongstaporn et al., 2006). Genetic studies of common complex diseases have focused on identifying risk genes as targets for developing new treatments and improved diagnoses. This approach led to the identification of several genes (Li et al., 2003). Genetic variation in GSTs has been reported to represent a risk factor for a variety of cancers (Strange et al., 2001).

Hepatitis B virus, abbreviated HBV, is species of the genus Orthohepadnavirus, which is likewise a part of the Hepadnaviridae family of viruses. HBV causes hepatitis B (Hassan et al., 2008) and leads to cirrhosis and hepatocellular carcinoma (Chisari and Ferrari, 1995, Hassan et al., 2008). The prevalence of HBV infection varies widely among different parts of the world and this variability is due to the primary cause of disease and inter-individual differences in target proteins and drug metabolism (Chisari and Ferrari, 1995). Progress of HBV infection may be affected by host genetic susceptibility (Wang, 2003). However, hepatitis is a systemic infectious process, and HBV virus may travel through the bloodstream and be deposited in non liver tissue- for example, kidney, skin, and vessel walls (Locarnini and Shaw, 2004). So it has been suggested that it may increase the risk of pancreatic cancer (Hassan et al., 2008).

Previous studies demonstrated that GSTs genetic polymorphism may be considered as an accurate biomarker for investigation, prediction and progression of HBV infection in Egypt (Polimantia et al., 2011, Shaban et al., 2014 d). Till now, there are no studies evaluating how much of known GSTO polymorphism can contribute to the development of HBV cirrhosis in human. In this study, the incidence of GSTO1 and GSTO2 polymorphism in patients with different stages of HBV infection was analyzed to investigate the role of host genetic factors in HBV infection.

Materials and Methods

Chemicals and kits

Kits for albumin, AST and ALT kits 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. GST assay kit was obtained from Cornel lab, USA; protienase K was obtained from Amersco, USA; primers and Polymerase chain reaction (PCR) kits were obtained from Microgen,

South Korea and Promega, USA, respectively. Agarose was obtained from Bioshop, Germany; Cac8I and MboI restriction enzymes were obtained from New England Biolab, England . DNA loading buffer and ladders were obtained from Promega, USA. Phenol equilibrated and chloroform was obtained from Acrosorganic, USA and Applichem, Germany, respectively.

Patients and Control:

Different blood samples were collected from different hospitals, medical laboratories and hepatologist clinics in Egypt according to the rules of scientific research ethics under supervision of Prof. Dr. Ehab Hassona (Internal Medicine Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt). Complete demographic data and full clinical history were recorded. Blood samples of 470 persons (160 females and 310 males) 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 normal HBV carriers, 53 acute patients, 78 chronic patients, 62 cirrhotic patients, and 60 hepatocellular carcinoma patients.

Sample 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 °C. All serum samples were stored at -80 °C.

Molecular Assays:

DNA extraction from serum: Genomic DNA was extracted from different serum samples manually (Miller et al., 1988). Briefly, 0.2 ml of serum were added to 0.6 ml of lysis buffer (10 mM Tris-HCl, pH8) H₂O 400 mM NaCl, 2 mM Na₂EDTA, pH 8.2) and mixed well. Then 0.2 ml of 10% SDS and 20 μ l of proteinase K solution were added and incubated for 2 hours at 37 °C. Then 0.6 ml of 6 M NaCl were added, mixed well and centrifuged at 12000 rpm for 20 minutes at 4 °C. Then 0.5 ml of the supernatant was added to 1.0 ml of absolute ethanol, mixed well and incubated for 12 hours at -20 °C, then centrifuged at 12000 rpm for 20 minutes at 4 °C 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 μ l of TE buffer (10 mM EDTA, 10 mM Tris-HCl). The concentration and the purity of DNA in 10 μ l samples was measured using spectrophotometer (A 260 / A 280) (William, 1997).

Primers: Specific primers were designed to detect GSTO1 and GSTO2. The primers sequences, annealing temperatures, annealing times and PCR product sizes were shown in Table 1 (Pongstaporn and Pakakasam 2009).

Detection of GSTO1: Specific PCR was used to detect GSTO1. In brief, 2.5 μ l (50 ng) of isolated DNA was added to 2.5 μ l of 2 mmol/L dNTPs, 5 μ l of 10X PCR buffer containing 1.5 mmol/L MgCl₂, 2 μ l (20 pmol) of GSTO1 primers , 0.5 μ l (5u/ μ l) of Taq DNA polymerase and 10.5 μ l deionized distilled H₂O (d.d H₂O). PCR program was 1 min at 94°C followed by 35 cycles of (1 min at 94°C, 1 min at 61°C, and 1 min at 72°C, and 10 min at 72°C) .The PCR products were analyzed electrophoretically on

a 2% agarose gel containing 0.5 µg/ml ethidium bromide with 1.5 kb DNA ladder. Five µl of the PCR product was digested with 1.5 µl (1U/µl) of *Cac8I*, 2.5 µl of 10X reaction buffer and 11 µl of d.d H₂O, mixed well and incubated at 37°C for 18 hours. The digested fragments were analyzed electrophoretically on 3% agarose gel containing 0.5 µg/ml ethidium bromide with 1.5 kb DNA ladder (Pongstaporn and Pakakasam 2009).

Detection of GSTO2: It was detected as described before in GSTO1 gene. Five µl of the PCR product was digested with 1.5 µl (1U/µl) of *MboI* as described before in the condition of GSTO1 gene (Pongstaporn and Pakakasam 2009).

HBV DNA titer values: HBV DNA titer values of the different stages of HBV infection were determined by Real Time PCR (Roch Diagnostic, USA) (Chu et al., 2002).

Biochemical Assays

GST (EC 2.5.1.18) activity: It was determined using kit (Habig et al., 1974). The specific activity is defined as U/L.

Liver Function Tests: Total protein (TP), albumin, total bilirubin (TB), transaminases [aspartate transaminase (AST) and alanine transaminase (ALT)], were determined using specific kits (Danil et al., 1999).

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). SPSS for Windows (version 11.0) statistical package was used for all statistical comparisons. A value of P < 0.05 was considered significant.

Results

Agarose gel electrophoresis: The electrophoresis of GSTO1 gene, lane 2, represents negative sample. The molecular sizes (MS) of GSTO1 PCR products were 254 bp as compared to DNA marker (lane M) as shown in (Figure 1A). Electrophoresis of GSTO2 gene, lane 6 represents negative sample. The molecular sizes (MS) of GSTO2 PCR products were 185 bp as compared to DNA marker (lane M) as shown in (Figure 1B). For GSTO1 genotypes, the MS of C/C wild type homozygote was 186 and 68 bp fragments, the MS of A/A polymorphic homozygote exhibited 254 bp respectively, (Figure 2). For GSTO2 genotypes, the MS of G/A heterozygote exhibited 185, 122, and 63 bp fragments, the MS of A/A wild type homozygote was 185 bp fragment, the MS of G/G polymorphic homozygote presented the expected 122 and 63 bp fragments (Figure 3A & 3B, respectively).

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 (Table 2).

Liver Function Tests: Serum (TP) and albumin levels were decreased significantly in N, A, CH, CI and HCC groups by about (35.4%, 51.3%, 48.2%, 49.2% and 54.8%, respectively, for TP and (39.6%, 51.3%, 62%, 62.8% and 69.6%, respectively, for albumin, (Table 2) as compared to the control group. Serum AST and ALT activities in N,

A, CH, CI and HCC groups were increased significantly by about (114.8%, 169.8%, 128.9%, 261% and 97,% respectively, for AST) and (98.9%, 288.6%, 24.7%, 85.4% and 341.9%, respectively, for ALT) as compared to the control group. Also serum TB levels were increased significantly in N, A, CH, CI and HCC groups by about 393.9%, 615.6%, 520.4%, 780.7%, 906%, respectively, as compared to the control group (Table 2).

Genetic polymorphism of GSTO genes

GSTO1: GSTO1 (A/A) was decreased significantly in N, A, CH, CI and HCC groups by about 83.3%, 72.2%, 88.9%, 77.7% and 83.3% respectively, however, GSTO1 (CA) and GSTO1 (C/C) genotypes were increased significantly {by about 77.7%, 122.2%, 107.3%, 62.9% and 48.1% respectively, for GSTO1 (C/A) and (by about 185.7%, 90.4%, 166.6%, 185.7% and 223.7% respectively,

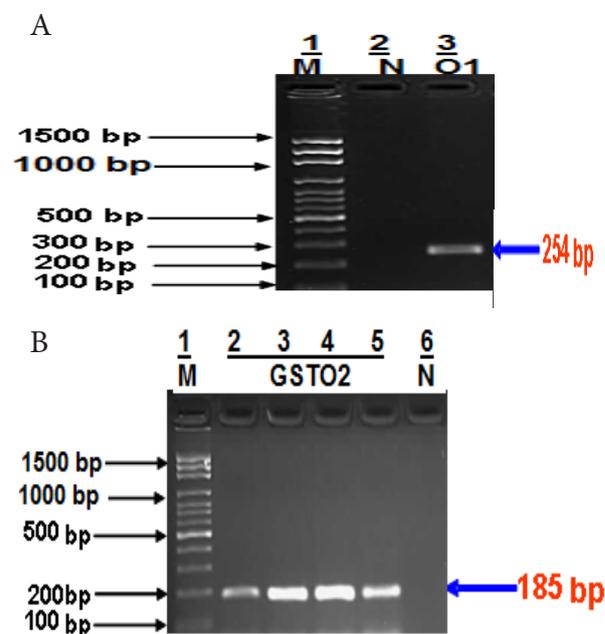


Figure 1. Agarose Gel Electrophoresis Findings. (A) of GSTO1 PCR product. Lane 1 (M): 1.5 kb DNA marker, Lane 2: negative control and Lane 3: 254 bp PCR fragment amplified from GSTO1 gene. B): Agarose gel electrophoresis of GSTO2 PCR products. Lane 1 (M): 1.5 kb DNA marker, Lanes 2, 3, 4&5: 185 bp PCR fragments amplified from GSTO2 gene and Lane 6: negative control

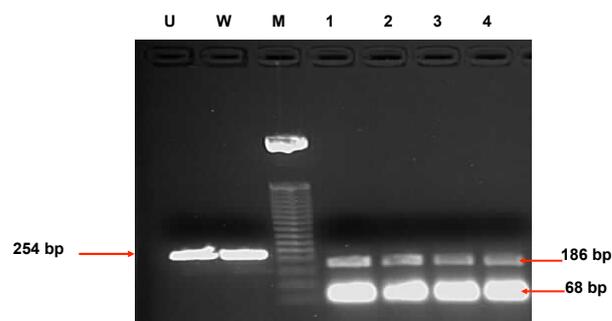


Figure 2. Agarose Gel Electrophoresis Findings for GSTO1 PCR-RFLP Products. Lane U, uncut band (254 bp).lane W, A/A polymorphic homozygous (254 bp).lane M, .50 bp DNA ladder. Lanes 1-4, C/C wild type (186 bp and 68 bp)

Table 1. Primer Details

| Primers | Primer Sequence | Ta(C) / T (s) | PCR product size (bp) |
|---------|----------------------------|-----------------|-----------------------|
| GSTO1F | GAA CTT GAT GCA CCC TTG GT | 61 / 60 | 254 |
| GSTO1R | TGA TAG CTA GGA ATA ATT AC | | |
| GSTO2F | AGG CAG AAC AGG AAC TGG AA | 61 / 60 | 185 |
| GSTO2R | GAG GGA CCC CTT TTT GTA CC | | |

Ta:annealing temperature; T(s):Time per seconds; bp:base pairs

Table 2. GST Activities and Liver Function Tests in Different Studied Groups

| Studied groups | GST(U/L) | TP(mg/dl) | TB(mg/dl) | AST(U/L) | ALT(U/L) | ALB(mg/dl) |
|----------------|----------|-----------|-----------|----------|----------|------------|
| control | 11.6 | 7.55 | 0.83 | 11.88 | 13.65 | 4.77 |
| Normal | 6.94 | 4.87 | 4.1 | 25.53 | 27.16 | 2.88 |
| Acute | 5.46 | 3.67 | 5.94 | 32.06 | 53.05 | 2.32 |
| Chronic | 4.98 | 3.91 | 5.15 | 27.2 | 17.03 | 1.81 |
| Cirrhosis | 5.3 | 3.83 | 7.31 | 42.9 | 25.32 | 1.77 |
| HCC | 3.96 | 3.41 | 8.35 | 23.41 | 60.33 | 1.45 |
| P.value | 0.004 | 0.003 | 0.001 | 0.02 | 0.008 | 0.01 |

*values of P <0.05 were considered statistically significant

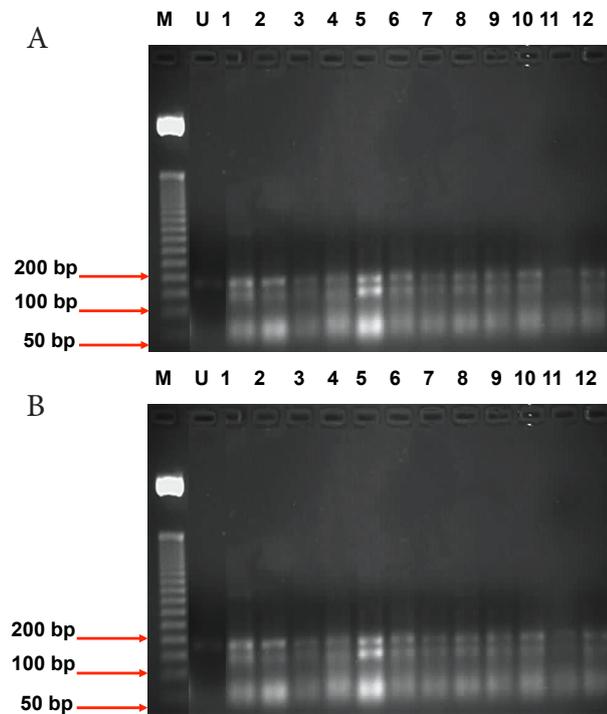


Figure 3. Agarose Gel Electrophoresis Findings. A): GSTO2 PCR-RFLP product. lane M ,50 bp DNA ladder lane U ,uncut band (185 bp). Lanes 1-12 G/A heterozygous type (185 bp , 122 bp and 63 bp). B): Agarose gel electrophoresis of GSTO2 PCR-RFLP product. lane M ,50 bp DNA ladder. lane U ,uncut band (185 bp).lane 1,2. A/A homozygous of wild type (185 bp). Lanes 3-8 , G/G homozygous of polymorphic type (122 bp, 63 bp)

for GSTO1 (C/C) } as compared to C group (Figure 4A).

GSTO2: GSTO2 (A/A) was decreased significantly in N, A, CH, CI and HCC groups by about 50%, 50%, 94.4%, 88.9% and 94.4% respectively, However, GSTO2 (A/G)and GSTO2 (G/G) genotypes were increased significantly by about 105.8%, 93.9%,57.5%,57.5% and 45.4% respectively, for GSTO2 (A/G)and (6.4%, 33.2%,326.6%300% and 353.2% respectively, for GSTO2 (G/G)) as compared to C group (Figure 4).

HBV DNA titer values: The results showed that the

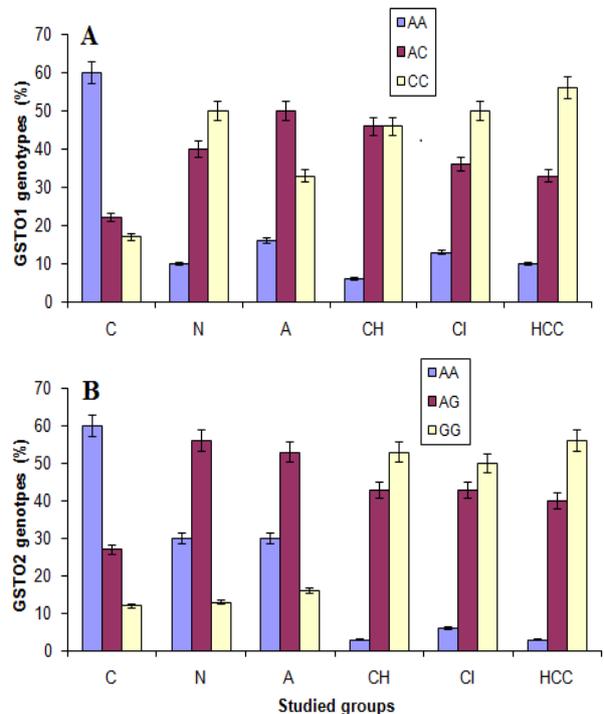


Figure 4. Relationship between Genotype Frequency and Studied Groups: A) GSTO1; B) GSTO2

HBV DNA titer values (as mean ±SD and p value = 0.002) for N, A, CH, CI and HCC groups were $5 \times 10^3 \pm 3.5 \times 10^3$, $30 \times 10^8 \pm 12 \times 10^8$, $60 \times 10^6 \pm 27 \times 10^6$, $20 \times 10^6 \pm 15 \times 10^6$ and $7 \times 10^5 \pm 5.5 \times 10^5$ IU/ml, respectively. IU/ml is international unit per ml which equivalent to five copies per ml. The values of P <0.05 were considered statistically significant

Discussion

The results showed that there was no significant relationship between different stages of HBV infection and HBV DNA titer. These results agree with the previous studies (Lindh et al., 2000, Yuen et al., 2004, Shaban et al., 2014 d). In contrast, other studies reported that patients who had increased liver damage had lower viral load (Chu

et al., 2002, Heo et al., 2003). The current study showed a reduction in GST activities in different HBV groups (N, A, CH, CI and HCC) as compared to the C 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. On the other hand, our previous studies showed that oxidative stress and lipid peroxidation and GSR were increased, while GSH level and activities of GST, SOD and GST were decreased in all different HBV groups (Shaban et al., 2014c). The study also showed that lipid peroxidation which lead to hepatocytes injury was increased with the development of HBV infection stages.

Otherwise, previous studies showed that GSTA1, GSTT1, GSTP1 and GSTM1 polymorphic variants are associated with altered catalytic function of GST (Becket et al., 1997, Shaban et al., 2014 d).

In this study GST activities were within normal ranges in wild GSTO genotypes GSTO1 AA and GSTO2 AA, while they were decreased significantly in mutant GSTO genotypes GSTO1 AC, GSTO CC, GSTO2 AG and GSTO2 GG). These data agree with previous study which showed that GST activities were within the normal range in the genotypes of wild GSTT1/GSM1, GSTP1 Ile/Ile and GSTA1 AA. However GST activities were decreased significantly in the genotypes of null GSTM1, null GSTT1, null GSTM1/GSTT1, GSTP1 Val/Val, GSTA1 AB and GSTA1 BB (Shaban et al., 2014 d). 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 susceptible to develop HBV-related liver disease. The widespread distribution of GSTO1 suggests that it has important biological functions. It is possible that the glutathione-dependent dehydroascorbate-reductase and thiol-transferase activities of GSTO1 may be generally required for normal cellular process and protection against cellular oxidative stresses (Whitbread et al., 2005). In addition, previous studies reported that GSTO1 may have a role in protection against calcium-induced apoptosis in cells containing ryanodine receptors (Dulhunty et al., 2001). Till now, there are no studies discussing the relationship between the genetic polymorphism of GSTO1 and different stages of HBV infection. However, the current study showed that the frequency of GSTO1 mainly A/C and C/C genotypes increased in N, A, CH, CI and HCC as compared to C group. These results agree with previous study which reported that a significantly higher risk of breast cancer among heterozygous or homozygous carriers of GSTO1*D140, but absent in carriers of GSTO2*D142 (Marahatta et al., 2006). Also, the previous results demonstrated that homozygous carriage of GSTO1*D140 was significantly associated with higher risk, especially for estrogen- receptor-positive breast cancer (Oslen et al., 2008). Several studies have shown that the polymorphism of GSTO2 has a role in the colorectal, gastric and hepatocellular carcinoma (Marahatta et al., 2006, Masoudi et al., 2009 and 2011). It has been reported that there is no association between GSTM1 and GSTT1 polymorphism and acute rejection

of liver transplantation (Azarpira et al., 2010). In addition, augmentation of GG allele can increase the risk of hepatic failure that leads to liver transplantation (Mukherjee et al., 2006). Our previous study demonstrated that there is an elevation in the levels of GSTP1 Val/Ile, GSTP1 Val/Val, null GSTM1, double null GSTM1/GSTT1, GSTA1 AB and GSTA1 BB genotypes with a reduction in the levels of GSTP1 Ile/Ile, wild GSTM1/GSTT1 and GSTA1 AA genotype in N, A, CH, CI and HCC as compared to C group (Shaban et al., 2014 d).

The current study showed that GSTO2 A/G and GSTO2 G/G mutant genotypes were increased in N, A, CH, CI and HCC as compared to C group. This indicates that GSTO2 mutant genotypes were increased with increasing the degree of HBV infection and the patients had mutant GSTO2 genotypes such as (A/G, and G/G) were more susceptible for more HBV disease progression. These results agree with the previous study which reported that there is an association between N142D polymorphism of GSTO2 and susceptibility to several types of cancers. Other studies demonstrated that GSTO2, XRCC1 has an additive effect on the risk of breast cancer (Azarpira et al., 2010, Masoudi et al., 2011). We suggest that the wild type genotype GSTO2 (A/A) can detoxify the active compounds and free radicals better, more rapidly and efficiently than the mutant genotypes GSTO2 (A/G, G/G), thus, a person with the mutant genotype is more sensitive to these compounds. People with A/G and G/G genotype for GSTO2 are more prone to develop hepatic failure.

Otherwise, the present study showed a reduction in the levels of TP and albumin but elevation in total bilirubin level in serum of N, A, CH, CI and HCC groups as compared to the C group. In addition the ALT activities were increased in all studied groups with different degrees but AST activities were increased in all studied groups except HCC group which was decreased as compared to the C group and this may be due to liver injury. So GSTO genetic polymorphism may be considered as a more accurate biomarker for the diagnosis of HBV infection stages than transaminases (AST and ALT).

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