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STUDY OF GLUTATHIONE-S-TRANSFERASE GENES POLYMORPHISM BY MULTIPLEX PCR IN PATIENTS WITH CIRRHOSIS ASSOCIATED WITH HEPATITIS C: ONE EGYPTIAN STUDY

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ABSTRACT

The pathogenesis of cirrhosis associated with HCV is a multifactor. Among theories of this disorder is host factors associated with genetic polymorphism. Glutathione –s-transferases enzymes appear to have a role in hepatic fibrosis. The aim of the present study is to determine the distribution of GSTM1 gene and GSTT1 gene polymorphisms in some Egyptian patients with chronic hepatitis C associated with cirrhosis compared to healthy control subjects. This study included seventy one patients complaining of advanced liver cirrhosis associated with HCV infection. In addition, one hundred healthy subjects with cross age and sex were included as healthy control. Blood samples were obtained from each subject and subjected to determination of GSTT1 and GSTM1 genotypes polymorphism by multiplex polymerase chain reaction. Patients have more frequent GSTM1 and GSTT1 null genotypes compared to control subjects (29.6% OR 2.20, 95% CI: 1.05- 4.6, P=0.03) and (28.2% OR 0.2, 95%CI: 0.09-0.53, P=0.03); respectively. The distribution of GSTM1 and GSTT1 according to fibrosis score, revealed high significant association (P=0.0001) between GSTM1 null genotype (85%) ,GSTT1 null genotype (85%) and combined GSTM1 &GSTT` null type (85%) with advanced fibrosis. The present study highlights the prevalence of GSTT1 and GSTM1 polymorphisms in some Egyptian patients with hepatic fibrosis on top of HCV infection. Null genotypes for both GSTT1 and GSTM1 are associated with hepatic fibrosis. Combined null polymorphism is a predisposing risk factor beside older age in development of advanced cirrhosis. Genotypic study of GSTT1 and GSTM1 polymorphism could aid in prediction the high risk patients for advanced liver fibrosis associated with HCV.

KEYWORDS: **HCV, genetic polymorphism, glutathione-s-transferase, cirrhosis**

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INTRODUCTION

Hepatitis C virus infection is a common infection worldwide. This infection is endemic in Egypt affecting around 14.7% of population with age range among the 15–59 years [1]. The progression of HCV infection leads to hepatic fibrosis and HCC. Various factors share in the progression of HCV infection of the liver to hepatic fibrosis. Among those factors are older age at infection, male gender and association of other risk factors such as long excessive alcohol consumption [2]. These host factors may enhance the progression of hepatic fibrosis beside genetic polymorphism in certain genes [3, 4]. Among these genetic polymorphisms, genetic polymorphisms in glutathione-S-transferase (GST) isoenzymes may be a potential risk factor in liver diseases associated with HCV [5-8]. The family of gluthione–s-transferases enzymes contains four classes of enzymes Alpha (GSTA), Mu (GSTM), Pi (GSTP), and Theta (GSTT). These enzymes were mainly involved in the detoxification of xenobiotics substances produced during oxidative stress following many diseases and infectious process. These substances called xenobiotics thus preventing tissue oxidative damage. Specific isoforms of GST enzymes GSTM1 and GSTT1 have been recognized in the cytosol and in mitochondria both in hepatocytes and in Kupffer cells [9, 10]. Their locations in the cytosol and in mitochondria are thought to compact the elaboration of free radicals in those sites [11]. Available data indicate that GST isozymes are indulged in the detoxification of 4-hydroxy-Aquinas compounds such as 9-hydroxynonenal important carcinogens produced during oxidative stress[11].GSTT1 also have glutathione-peroxidase activity [12]. These activities could be linked to the detoxification of reactive species produced during HCV infection of the liver. The genes that control expression of GSTM1 and GSTT1 enzymes are reported to have high incidence of deletions leading to complete absence of the enzymatic activity in tissues in homozygous inheritance. The homozygous polymorphism is known as null genotype [13]. Null genotypes are common finding in general population and due to the speculated role of these isoenzymes in defense mechanism toward progressive HCV infection, it appears reasonable to study the GSTM1 and GSTT1 polymorphisms in relation to chronic hepatitis C associated with hepatic fibrosis to asses if it plays a role to clarify in the cirrhosis associated with HCV infection. Scarce data describes the association of GSTM1 and GSTT1 polymorphisms in HCV infection associated with fibrosis in Egyptian patients [14]. The aim of the present study is to determine the distribution of GSTM1 gene and GSTT1 gene polymorphisms in some Egyptian patients with chronic hepatitis C associated with cirrhosis compared to healthy control subjects.

MATERIALS AND METHODS

This cross sectional study included seventy one patients complaining of advanced liver cirrhosis associated with HCV infection. In addition, one hundred healthy subjects with cross age and sex were included as healthy control. Patients were recruited from Mansoura University hospital from December 2014 till November 2015. Informed consent was obtained from each subject according to Mansoura Faculty of medicine ethical committee. Patients were diagnosed to have cirrhosis by clinical examinations, radiological findings by ultrasound and histopathological findings. The grades of fibrosis was classified based on METAVIR scoring system the grades were (F0, F1, F2, F3, F4) on score from 0-4. Hepatitis C infection was confirmed by positive HCV IgG and positive viremia by real time polymerase chain reaction. Ten milliliter blood was obtained from each subject and divided between three tubes citrated, heparinized and plain. Citrated blood samples were used for plasma separation for measurement of prothrombin time and INR. Sera was separated from plain tubes and subjected to complete biochemical liver functions involving alanine aminotransferase (ALT) aspartate amino transferase (AST), total bilirubin, direct bilirubin and albumin. Also fetoprotein was measured in serum samples. Heparinized blood samples were used to isolate buffy coat and kept frozen at -70ºC for genetic analysis.

DNA Extraction

DNA extraction was performed from buffy coat with a Qiagen DNA extraction kit (Qiagen Inc., Valencia, CA).

Multiplex PCR for GSTM1 and GSTT1 Polymorphisms

A multiplex PCR assay was performed to detect genotype GSTT1 and GSTM1. Dihydrofolate reductase genes (DHFR) were used as an internal control for amplification procedure. The primers used were summarized at table 1. The amplification was performed in total volume 25µl reaction mixture containing 100 ng of extracted DNA, 3.5 pmol of each GSTM1 primer, 2.9 pmol of each GSTT1 primers, 6.2 pmol of each DHFR primer, 0.1 mm each deoxynucleotide triphosphate, 1 × PCR buffer and 2.5 mm $MgCl_2$, and 1.5 units of Taq polymerase (Qiagen Inc., Valencia, CA). The amplification procedure was performed in a thermal cycler with the following conditions, 95°C for 5 min, 30 cycles of 95°C for 30 seconds, 56°C for 45 seconds, and 72°C for 45 seconds and 72°C for 10 minutes. Gel electrophoresis was performed by the use of 1.5% gel to visualize the PCR products. The absence of a 480-bp band or a 215-bp band indicates the GSTT1 null or GSTM1 null genotypes, respectively [15].

Table (1)
Primers used for *GSTT1*, *GSTM1*, *and DHFR* genes

Gene	Primer sequence	Bp
GSTT1	F5′- TTCCTTACTGGTCCTCACATCTC-3′ R5′TCACCGGATCATGGCCAGCA-3′	480-bp
GSTM1	F5′-GAACTCCCTGAAAAGCTAAAGC-3′ R5′-GTTGGGCTCAAATATACGGTGG-3	215-bp
DHFR	F5′-GCATGTCTTTGGGATGTGGA-3′ R5′-GGAATGGAGAACCAGGTCTT-3′	280-bp

Table (2)
Demographic and laboratory data of the studied subjects

	Patients (n=71)	Control (n=100)	P
Age	50.2± 5.4	53.7± 6.4	. .35
Sex Male Female	 55 (77.5%) 16 (22.5%)	 50(50%) 50(50%)	 P=.288
Albumin gm/dl	2.6± 0.9	4.5± 0.5	P=0001
Total bilirubin mg/dl	3.7.0± 1.5	0,9± 0.1	P=0001
Direct bilirubin mg/dl	2.4± 0.5	0.3± 0.1	P=0001
ALT Iu/dl	92.0± 15.5	34.1± 1.5	P=0001
AST Iu/dl	97.0± 16.5	43.1 0.5	P=0001
α fetoprotein	18± 1.5	6.5± 2.8	P=0001
PT time (seconds) INR	17.5 ± 2.9 5.5± 1.5	10.1± 1.2 1.5± 0.8	 P=0.001

Table (3)
Distribution of GSTM1 and GSTT1 genotypes among studied subjects.

	Control (n=100)	Patients (n=71)	Odds ratio	95 % CI:	Significance level
GSTM1 null	16 (16%)	21(29.6%)	2.2050	1.0535 to 4.6153	P = 0.03
GSTM1 active	84 (84%)	50 (70.4%)	0.4535	0.2167 to 0.9493	P = 0.0009
GSTT1 null	8 (8%)	20(28.2%)	0.2217	0.0912 to 0.5391	P = 0.03
GSTT1 active	92 (92%)	51 (71.8%)	0.2217	0.0912 to 0.5391	P = 0.0009
Combined GSTM1 & GSTT1 null	0 (0)	18(25.3%)	69.5	4.1075 to 11.75	P = 0.003

Table (4)
Distribution of GSTM1 and GSTT1 polymorphism among patients according to fibrosis score.

	Patients with fibrosis F1-3 (n=51)	Patients with fibrosis F4 (n=20)
GSTM1 null	4(7.8%)	17(85%)
GSTM1 active	43 (84.3%)	7(35%)
GSTT1 null	3(5.9%)	17(85%)
GSTT1 active	48(94.1%)	3 (15%)
Combined GSTM1 & GSTT1 null	1(1.9%)	17 (85%)

P=0.0001

Table (5)
Logistic regression study of risk factors associated with hepatic fibrosis

Parameter	P
Age	P=.036
Gender	P=.20
GSTT1	P=.020
GSTM1	P= 0.3
GSTT1 & GSTM1 null association	P=.0001

RESULTS

The study was carried out on 71 patients with cirrhosis on top of HCV. The males were more common (77.5%) than females (22.5%) with mean age± SD 50.2± 5.4, table 2. Patients have more frequent GSTM1 and GSTT1 null genotypes compared to control subjects [29.6% OR 2.20 (95% CI: 1.05-4.6), P=0.03] and [28.2% OR 0.2 (95%CI: 0.09-0.53), P=0.03], respectively, and null association genotypes [25.3% OR, 69.5 (95% CI: 4.1-11.8) P = 0.003], table 3. The distribution of GSTM1 and GSTT1 according to fibrosis score, revealed high significant association (P=0.0001) between GSTM1 null genotype (85%), GSTT1 null genotype (85%) and combined GSTM1 & GSTT1 null type (85%) with advanced fibrosis scale F4, table 4. Risk factors analysis for hepatic fibrosis shows that GSTT1& GSTM1 null association GSTT1 and older age is significantly (P=0.0001, P=0.02, P= 0.04 respectively) associated with hepatic fibrosis, table 5.

DISCUSSION

Liver contains large pool of scavengers enzymes protecting human from carcinogenic substances and viruses by detoxification them. Among those enzymes are glutathione-S-transferases enzymes which play a key role in detoxifying xenobiotics and preventing fibrosis of liver [16]. These enzymes are controlled by genes that are known to be polymorphic that results in variation of the level of the enzymes activity [17-19]. In the present study, patients have more frequent GSTM1 and GSTT1 null genotypes compared to control subjects. Previous studies reported similar findings suggest that the GSTT1 and the GSTM1 null genotype and the combined double-null are associated with the risk of chronicity of HCV disease to the chronic phase (Spanish, Tawian, Flippin, Egypt). This finding was explained by the fact that null genotypes are associated with absence of detoxifying the metabolites that result from oxidative stress associated with HCV [20, 21]. The patient group had significantly a high (P=0.003) frequency of GSTM1/GSTT1 double-null genotype than the control group. This polymorphism also was associated with advanced fibrosis score. It is known that GSTT1 plays a vital role in eliminating the reactive oxygen species from hepatic cells and GSTM1 detoxifies the electrophilic elements produced during immune reaction to HCV infection. The combined deficits of both enzymes in the null genotype may be associated with enormous accumulation reactive oxygen species and electrophilic compounds generated during the acute HCV infection that leads to magnify the inflammatory reaction and predispose to the chronic phase of hepatitis C [21]. The mechanism of hepatic cirrhosis is associated by the transformation from the extracellular hepatic matrix into a reticulated and dense matrix [22]. The distribution of GSTM1 and GSTT1 according to fibrosis score, revealed high significant association (P=0.0001) between GSTM1 null genotype (85%) ,GSTT1 null genotype (85%) and combined GSTM1 &GSTT1 null type (85%) with advanced fibrosis scale F4. Previous studies reported also that null variant of both GSTT1 and GSTM1 have considerably increased risk of advanced fibrosis [14, 21, 22]. These observations were support the hypothesis that reduced or absence of the activity of glutathione-s-transferase in null genotypes is associated with advanced hepatic fibrosis on top of HCV infection. This finding may be attributed to suggestion that oxidative stress associated with chronic hepatitis C triggers hepatic fibrogenesis [23-25]. Similar results were obtained by previous studies determining that older age was associated with increased risk of hepatic fibrosis especially at 50 years old [22, 6, 26-27]. The remarkable finding of the present study was GSTT1 & GSTM1 null association polymorphism and GSTT1 null only (P=0.0001, P=0.02 respectively) with hepatic fibrosis. These data supports the hypothesis that GSTT1 null genotype alone may produce a significant deficit in the hepatic antioxidant inactivating mechanisms enhancing fibrosis. This effect could be augmented by simultaneous absence of GSTM1[21]. However, less effect is produced by GSTM1 null alone. The limitation of the present study is the limited patient's samples. However, its results can be used as a screening tool for detecting patients with relative risk for developing advanced cirrhosis. The present study highlights the prevalence of GSTT1 and GSTM1 polymorphisms in some Egyptian patients with hepatic fibrosis on top of HCV infection. Null genotypes for both GSTT1 and GSTM1 are associated with hepatic fibrosis. Combined null polymorphism is a predisposing risk factor beside older age in development of advanced cirrhosis. Genotypic study of GSTT1 and GSTM1 polymorphism could aid in prediction the high risk patients for advanced liver fibrosis associated with HCV.

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