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GLUTATHIONE-S-TRANSFERASE M1 AND T1 GENE POLYMORPHISMS AND SUSCEPTIBILITY TO THE PROGRESSION OF LIVER FIBROSIS IN HCV-INFECTED PATIENTS IN TAIWAN

GENETSKI POLIMORFIZMI GLUTATION-S-TRANSFERAZE M1 I T1 I PODLOŽNOST PROGRESIJI FIBROZE JETRE KOD PACIJENATA ZARAŽENIH VIRUSOM HEPATITISA C NA TAJVANU

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Summary

Background: This study was conducted to evaluate the influence of glutathione-S-transferase (GST) M1 and T1 polymorphisms in 184 patients with different stages of liver fibrosis and hepatitis C virus infection and 173 healthy control subjects.
Methods: DNA samples were extracted from whole blood, and the polymorphisms of GSTM1 and GSTT1 were determined with PCR using fluorescence-labeled Taq Man probes. Associations between specific genotypes and progression of liver fibrosis were examined by use of the logistic regression analysis to calculate the odds ratio (OR) and 95% confidence intervals (CI).
Results: Results show that no differences were found between the frequencies of GSTM1 (49.8% versus 50.2%) and GSTT1 (52.2% versus 47.8%) null genotypes in HCV-infected patients and healthy controls, respectively. In addition, there was also no significant relation between the frequency of GSTM1 or GSTT1 gene polymorphisms and fibrosis stage as classified by the METAVIR group.
Conclusions: The combined GSTM1 and GSTT1 null genotypes showed an association between GSTM1 [-]/GSTT1 [-] and progression of liver fibrosis.

Keywords: glutathione-S-transferase, gene polymorphism, HCV, liver fibrosis

Kratak sadržaj

Uvod: Ova studija sprovedena je sa ciljem da se utvrdi uticaj polimorfizama M1 i T1 glutation-S-transferaze (GST) kod 184 pacijenta sa različitim stadijumima fibroze jetre inficiranih virusom hepatitisa C i 173 zdravih kontrolnih osoba.
Metode: Uzorci DNK ekstrahovani su iz pune krvi a polimorfizmi GSTM1 i GSTT1 određeni su pomoću PCR uz korišćenje fluorescentno obeleženih Taq Man sondi. Povezanost između specifičnih genotipova i progresije fibroze jetre ispitana je pomoću logističke regresije da bi se izračunala verovatnoća (odnos šansi) i 95% intervali poverenja.
Rezultati: Rezultati pokazuju da nisu pronađene razlike između učestalosti nultih genotipova GSTM1 (49,8% prema 50,2%) i GSTT1 (52,2% prema 47,8%) kod pacijenata inficiranih virusom hepatitisa C i zdravih kontrolnih subjekata. Pored toga, nije utvrđena značajnost odnosa između učestalosti genetičkih polimorfizama GSTM1 ili GSTT1 i stupnja fibroze određenog na osnovu klasifikacije METAVIR grupe.
Zaključak: Kombinovani nulti genotipovi gena GSTM1 i GSTT1 ukazali su na povezanost između GSTM1 [-]/GSTT1 [-] i progresije fibroze jetre.

Ključne reči: glutation s-transferaza, genetički polimorfizam, virus hepatitisa C, fibroza jetre

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Introduction

Hepatitis C is a global health problem that affects a significant portion of the world's population. Hepatitis C virus (HCV) is the primary cause of chronic hepatitis, and is one of the risk factors for development of liver cirrhosis and hepatocellular carcinoma (1–3). Chronic infection with HCV typically induces injury and inflammation of the liver, which appear to be responsible for the associated fibrogenesis. The mechanism of fibrogenesis is non-specific, and is characterized by the transformation from a normal extracellular matrix into a reticulated and dense matrix. Hepatic fibrosis assessment is based on liver biopsy. The histology activity index (HAI) and the METAVIR scoring systems are frequently used for assessment of liver fibrosis (4, 5). The METAVIR scoring system has better intra- and interobserver reproducibility than the HAI score (6).

Liver fibrosis progression does not always have a fixed speed or follow a linear pattern; it can occur at a slow or a rapid rate (7). Fibrogenesis is a complex dynamic process, and the rate at which fibrosis progresses varies markedly between patients. Three independent factors known to be associated with a faster fibrosis progression rate are older age at infection, male gender and long-term excessive alcohol consumption (8). Such host factors seem to play a role in HCV infection. However, during the last few years, numerous association studies have indicated that a number of gene variations influence both the nature of HCV infection and the progression of hepatic fibrosis in patients with chronic disease and hepatitis C virus (7, 9); thus, genetic factors play a particularly important role in the pathogenesis. Variations in the genes involved in the alcohol-metabolizing enzyme system and inflammatory response system have been identified, such as CYP2E1 and TNF-alpha gene (10). Genetic polymorphisms in glutathione-S-transferase (GST) isoenzymes could also be potential risk factors in liver and various cancer diseases (11–14).

The glutathione-S-transferases (GSTs) are an important family of enzymes involved in the biosynthesis and metabolism of many substances. They comprise four classes of enzymes: Alpha (GSTA), Mu (GSTM), Pi (GSTP), Theta (GSTT), responsible for detoxification of xenobiotic compounds and carcinogens generated by products of oxidative stress, and prevention of tissue oxidative damage. GSTM1 and GSTT1 are the main GST genes described as polymorphic in humans. They can be categorized in two classes: the homologous deletion genotype (referred to as null genotype) and one or two undeleted alleles (refered to as non-null or present genotype). The sequence variation of the GSTM1 and GSTT1 genes may be associated with different GST gene expression, affecting enzyme activity and increasing susceptibility to cancer (15–21). Therefore, to study the polymorphism of GSTM1 and GSTT1 genes is important for the evaluation of disease progression.

In chronic hepatitis C, oxidative stress can trigger hepatic fibrogenesis (22–24). Thus, genetic polymorphisms of antioxidant enzymes could modify the capacity to alleviate oxidative stress and thereby influence the progression of liver damage. However, there have been few studies on the effects of GST gene polymorphism on the disease progression of HBV infection (25, 26), but so far we have not seen any reports about that association in the investigation of the relationship between GST gene polymorphism and stage of liver fibrosis in HCV-infected patients in Taiwan, only one report in Spain (27).

In the current study, we analyzed the genetic polymorphisms of GSTT1 and GSTM1 genes in a group of patients with hepatitis C virus infection, as compared to control subjects, in Taiwan.

Methods

Study subjects and specimen collection

Cases of HCV infection were identified through the division of Hepato-gastroenterology at Tungs' MetroHarbor Hospital, Taichung, Taiwan, between August 2009 and July 2010. After informed consent was obtained, a total of 184 outpatients (105 males and 79 females) with HCV infection were enrolled, and 146 of them were treated by pegylated interferon alfa 2a plus ribavirin. The diagnoses of HCV infected patients were based on the results of clinical, biochemical, and viral tests, such as alanine transaminase (ALT), aspartate transaminase (AST), Alpha-fetoprotein (AFP), HBsAg and anti-HCV. All subjects who had an elevated level of ALT ($\geqq$45 IU/mL), AST ($\geqq$40 IU/mL) or AFP ($\geqq$20 ng/mL), and positivity for anti-HCV were included in this study; hepatitis B virus (HBV) and human immunodeficiency virus (HIV) infection patients were excluded. The patients had various liver fibrosis levels as obtained from liver biopsy and estimation of fibrosis progression rates based on the ratio of fibrosis stage by the METAVIR scoring system. Using standard assessment, liver fibrosis was classified into five stages on a scale 0–4 (F0, F1, F2, F3, F4). The 173 control subjects, who visited the division of family medicine for health examination, were selected based on no evidence of liver disease and similar demographic characteristics to the patients in the experimental group. All study subjects gave informed consent to participate in this research under a protocol approved by the Committee for Studies on Human Subjects at Tungs' MetroHarbor Hospital, Taichung, Taiwan. Whole blood specimens, collected from healthy controls and HCV patients, were placed in tubes containing EDTA for the extraction of genomic DNA.

DNA collection and extraction

Venous blood from each subject was collected into EDTA tubes and immediately centrifuged and the buffy coats were stored at –70 °C in a freezer. Genomic DNA was extracted by Genomaker DNA blood kits (GenePure Tech Co., Ltd, Taichung City, Taiwan) according to the manufacturer's instructions. DNA was dissolved in TE buffer (10 mmol/L Tris pH 7.8, 1 mmol/L EDTA) and DNA quality was assessed by calculating the absorbance ratio $OD_{260nm/280nm}$ (28) using a spectrophotometer model DU–80°C (Beckman Coulter). The samples of isolated DNA were stored at –80 °C and used as templates for genotyping.

Glutathione-S-transferase M1 and T1 genotyping

The genotypes of the patients were determined with the ABI TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, ABI assay C_8717770_20 for GSTT1 and C_26020680_10 for GSTM1). The PCR reactions were performed in 96-well microplates with ABI Step One plus real-time PCR (Applied Biosystems) and allele discrimination was achieved by the detection of fluorescence with Step One software V2.0.

Statistical analysis

The SPSS-17.0 for Windows statistical package was used for all statistical comparisons. The Chi-square statistic was calculated to test the distribution trend of index by genotype. The t-test was used to evaluate the distribution difference in age between patients and controls, while the relationships between genetic polymorphisms and liver fibrosis were examined by logistic regression analysis. Differences less than 0.05 were regarded as significant.

Results

The distributions of demographical characteristics and gene polymorphisms in normal controls and HCV-infected patients are shown in *Table I*. This study included 184 (105 male and 79 female) cases and 173 (106 male and 67 female) controls. The mean age of HCV-infected patients and controls was 56.7±10.7 years and 51.9±15.3 years, respectively. Except for age ($p<0.001$), the GSTT1 and GSTM1 gene deletions were not significantly different between the HCV-infected patients and the healthy controls. The p values of the GSTT1 and GSTM1 gene deletions are 0.818 and 0.419, respectively.

Furthermore, we divided the necroinflammatory global score (items F1, F2, and F3 of METAVIR) and the fibrosis score (item F4 of METAVIR) in two categories, and the observed distribution frequencies of GSTM1 and GSTT1 genotypes were not significantly different among the groups (*Table II*). Additionally, except for age ($P<0.001$), liver viral load and weight were not significantly associated with the development of fibrosis in HCV-infected patients ($p=0.853$ and $p=0.631$, respectively; *Figure 1*).

The final multivariate logistic regression model is shown in *Table III*. Except for age ($P<0.001$), the result revealed that there was a significant association of liver fibrosis with the GSTM1 and GSTT1 genotype. The presence of at least one of the GSTM1 or GSTT1 genes was associated with a lower risk of liver fibrosis after adjusting the age and weight. The group GSTM1[-]/GSTT1[+] has the lowest observed risk [OR=0.342; CI 95% (0.124–0.943); $p=0.038$].

Table I Odds Ratio and 95% Confidence Intervals (CIs) of HCV Infected patients and healthy control subjects associated with genotypic frequencies and demographic characteristics distributions.

Variable	Controls (n=173) (%)	Patients (n=184) (%)	OR (95% CI)
GSTT1			
Null	75 (47.8%)	82 (52.2%)	1.05 (0.691–1.596)
Present	98 (49.0%)	102 (51.0%)	1.00
GSTM1			
Null	106 (50.2%)	105 (49.8%)	0.84 (0.55–1.282)
Present	67 (45.9%)	79 (54.1%)	1.00
Gender			
Female	67 (45.9%)	79 (54.1%)	1.19 (0.78–1.817)
Male	106 (50.2%)	105 (49.8%)	1.00
Age (yrs)			
Mean±SD	51.9±15.3	56.7±10.7	P = 0.001*

*Statistically significant effect.

Table II The distribution of gender and genotype frequencies in patients with HCV-related fibrosis stage.

Variable	F1~F3 (n=84) (%)	F4 (n=62) (%)	P value
Gender			0.934
Female	36 (57.1%)	27 (42.9%)	
Male	48 (57.8%)	35 (42.2%)	
GSTM1			0.673
Null	49 (59%)	34 (41%)	
Present	35 (55.6%)	28 (44.4%)	
GSTT1			0.252
Null	34 (52.3%)	31 (47.7%)	
Present	50 (61.7%)	31 (38.3%)	

Note: 38 patients without liver biopsy records were excluded from this analysis.

Table III Results of multivariate logistic regression analysis of glutathione-S-transferase genotypes in patients with HCV-related fibrosis.

Variable	N(F4/F1~F3)	OR (95% CI)	P-Value
GSTM1[-][a]/GSTT1[-]	34(19/15)	1.00	-[c]
GSTM1[+][b]/GSTT1[-]	31(12/19)	0.588 (0.200–1.726)	0.334
GSTM1[-]/GSTT1[+]	49(15/34)	0.342 (0.124–0.943)	0.038*
GSTM1[+]/GSTT1[+]	32(16/16)	0.947 (0.323–2.773)	0.921
Age		1.095 (1.049–1.143)	<0.001*
Weight		1.025 (0.995–1.057)	0.103

*Statistically significant effect.
[a][-] = null, [b][+] = positive, [c]- reference group

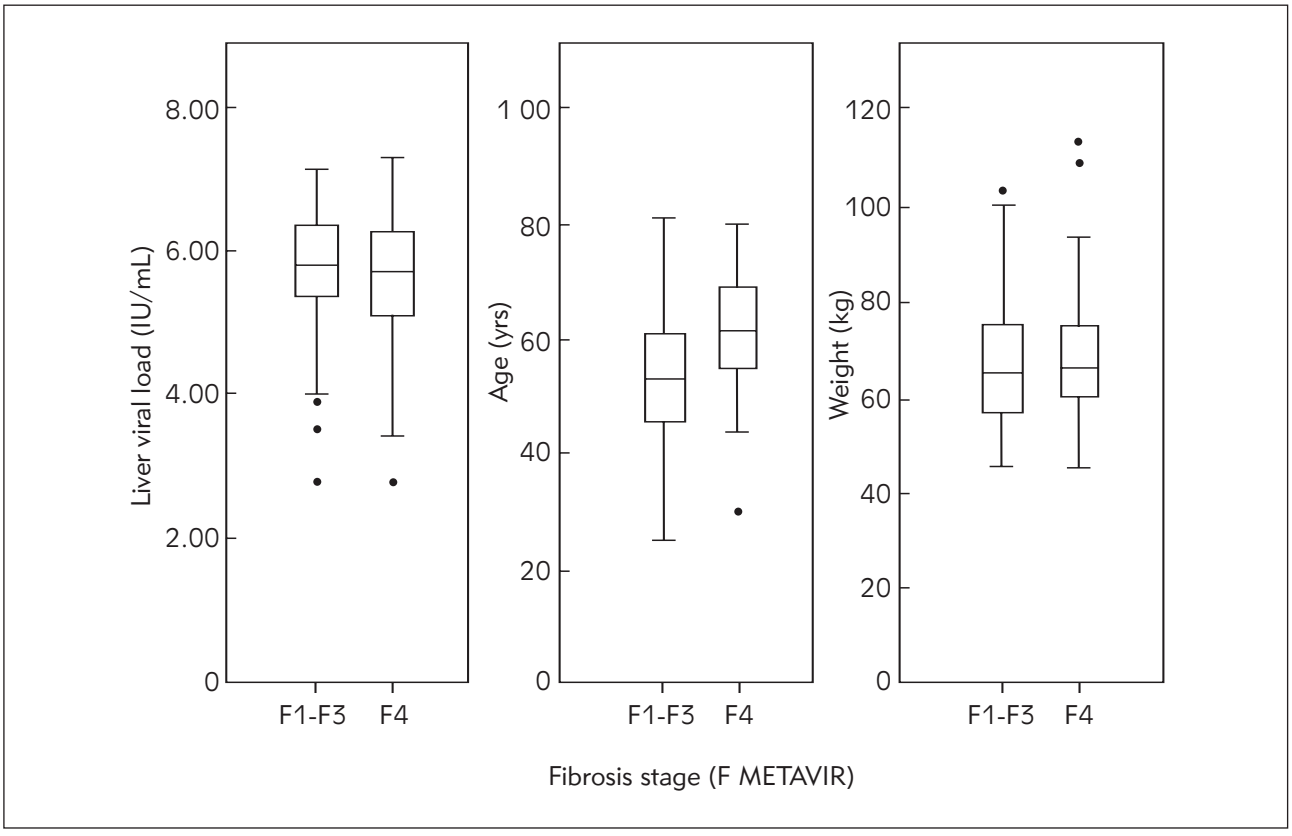

Figure 1 The box plot for level of liver viral load, age and weight in HCV-infected patients with different fibrosis staging by METAVIR system.

Discussion

Most endogenous and environmental substrates interact deleteriously with an organism, causing toxic and sometimes carcinogenic effects (29). The detoxification is usually kept by liver enzyme activity with the scavenging of toxic compounds and carcinogens, but many enzymes involved in carcinogen metabolism exhibit genetic polymorphisms resulting in variability in both their level of expression and activity (30–32). The glutathione-S-transferases have multiple forms distributed among different tissues, which play a key role in phase II enzymes for the detoxification of xenobiotics and prevention of tissue damage (33). Numerous studies have shown polymorphic GST alleles to be associated with altered risk or outcome of various diseases. The GST variants modify the catalytic function of enzymes. Therefore, the individuals who produce less specific detoxification enzymes may be at a higher risk of adverse disease outcome.

In this study, we found the age was significantly associated with the susceptibility to progression of liver fibrosis in HCV-infected patients after adjusting for other variables ($p<0.001$). Our results are identical to the previous reports (6, 34, 35). Thus, the findings indicate that two parameters, age and duration of infection, are potential risk factors and should be applied to evaluate the rate of progression of fibrosis in hepatitis C and the progression of liver fibrosis. Besides, we also found that 42% of HCV patients aged over 50 years have developed cirrhosis (*Table II*). The result was supported again by the previous studies indicating that the progression of liver fibrosis begins to accelerate at 50 years of age, regardless of the duration of infection (6, 34, 35). In addition, we investigated whether GSTT1 and GSTM1 gene variations play a role in liver fibrosis caused by hepatitis C virus and found that individuals with the null variant of both GSTT1 and GSTM1 have considerably increased risk of advanced fibrosis. This result was in agreement with a previous study in Spain (27). Martinez et al. recruited 139 HCV-infected Spanish patients and 329 healthy controls to estimate the association between both gene polymorphisms and chronic phase of HCV-induced liver disease. They found a significant difference between the frequencies of genotypes and the clinical course of HCV infection. These observations revealed that the progression of liver fibrosis in HCV-infected patients was associated with GST gene polymorphisms in the Taiwanese and Spaniards, regardless of the racial difference. In addition, both GSTM1 and GSTT1 null genotypes are also associated with the occurrence of cancer or disease, and an association has been reported between GSTM1 and GSTT1 null genotypes and cancer of the lung, bladder and colon. Therefore, patients who are genetically predisposed to produce less specific enzyme activity might be prone to develop hepatic liver disease with HCV infection.

It has been reported that high serum levels of ALT, AST, AFP and a high ratio of AST to ALT are highly associated with the progression of hepatocarcinogenesis in HCV or HBV-infected and liver cirrhosis patients (36–39). In this study, we found the clinical serological markers ALT and AFP were associated with liver fibrosis in HCV-infected patients ($p<0.05$ and $p<0.001$, respectively), but AST was not associated ($p=0.695$). It may be that the serum AST level has correlated weakly with disease activity and little or not at all with hepatic fibrosis in cross-sectional studies (6).

To our knowledge, this is the first genetic study of HCV-related liver fibrosis in the Taiwan population. We found that either GSTM1 or GSTT1 gene polymorphism was significantly associated with HCV-infected liver fibrosis and showed that the null alleles of GSTT1 and GSTM1 are predisposing risk factors for HCV-infected liver fibrosis. Despite the limited number of patient samples, the results of this study may be of particular relevance to clinical practice and can be used as a screening tool for finding HCV-infected patients at risk.

Conclusions

In this work, we investigated the risk of progress of liver fibrosis in a Taiwanese population via genetic polymorphisms in the GST genes. Both GSTM1 and GSTT1 null genotypes polymorphisms were associated with the risk for progression of liver fibrosis among subjects infected by hepatitis C virus.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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