Original Article

GSTM1 and GSTT1 copy number variants and the risk to Thai females of hepatocellular carcinoma

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Background: Hepatocellular carcinoma (HCC) is a common malignancy found throughout the world that most often occurs in males. The cancer is associated with many risk factors such as viral infection, cirrhosis, alcohol, smoking, and fungal toxins. GSTM1 and GSTT1 are detoxification enzymes activated by the cleansing of carcinogenic compounds. Low DNA copy numbers of Glutathione S-transferases M1 and T1 result in a loss of enzyme activity, which causes carcinogenesis factors. DNA copy number variants (CNVs) were determined to compare the differences between the frequencies of GSTM1 and GSTT1 in a control group and patients. Then, the association of these genes with the pathological/survival status of HCC patients was investigated.

Methods: Forty-nine Thai HCC patients' DNA and the genomic DNA of 66 healthy controls were investigated for GSTM1 and GSTT1 CNVs by real-time polymerase chain reaction (PCR). Then, the correlations between GSTM1 and GSTT1 patients' CNVs, the control group, and clinico-pathological parameters were determined.

Results: The results show that were no differences between the CNVs of GSTM1 and GSTT1 in the controls and patients ($P \geq 0.05$). Only GSTT1 genotypes 0/0 correlated to an increase in the risk of hepatocellular carcinogenesis (OR value was 1.88). GSTM1 CNVs were associated with the gender of patients ($P=0.002$). However, no correlations were found between GSTT1 CNVs and any of the clinico-pathological parameters.

Conclusions: The results suggest that only GSTT1 CNVs are associated with increased risk factors of HCC in Thais. GSTM1 copy numbers had a dominant correlation with female HCC patients.

Keywords: Glutathione S-transferase M1 (GSTM1); glutathione S-transferase T1 (GSTT1); DNA copy number variant; hepatocellular carcinoma (HCC)

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy found in the world and 85% of cases occur in men (1). Overall, an estimated 748,300 new cases of HCC and 695,900 deaths occurred in 2008 (2). In Thailand, HCC is the most common cancer found in males with an incidence rate of 33.9/100,000 and it is the third most common cancer found in females 12.9/100,000 population (3).

However, the diagnosis and prognosis of HCC are still poor. The 5-year overall survival (OS) rate is about 26–44% after resection because patients who are diagnosed

with HCC are at an advanced stage (4). The HCC DNA marker is necessary for the diagnosis and prognosis of the disease. HCC risk factors include chronic hepatitis, viral infections, cirrhosis, alcohol, smoking, and consuming food contaminated with the following fungal toxins: Aspergillus flavus and Aspergillus parasiticus (5).

Glutathione S-transferases (GSTs) are a superfamily of enzymes that play an important role in the detoxification of the carcinogens glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1) (6). The null genotypes of GSTM1 and GSTT1 might lead to a complete loss of enzyme activity. GSTM1 null genotypes have been associated with susceptibility to lung cancer, bladder cancer and colorectal cancer (7-9). The GSTM1 null genotype was detected in 60% of the population in western countries (10,11). While the GSTT1 null genotype was detected in only 20% of the population in western countries (12) and it was associated as a risk factor for susceptibility to colorectal cancer and endometrial cancer (13,14).

Real-time polymerase chain reaction (PCR) was used to investigate the DNA copy number variants (CNVs) of GSTM1 and GSTT1 in Thai HCC patients. The correlations between the frequencies of the GST genes' CNVs in the control group and the patients were examined by Binary Logistic Regression and the clinico-pathological features of HCC were analyzed by Chi-square test. The CNVs for the GSTs and the survival status were determined by the Kaplan-Meier survival curve. A P value of ≤0.05 was set as the criteria for statistical significance. It is hoped that the results of this research will serve as fundamental knowledge on the effect of these genes' CNVs on Thai HCC patients.

Methods

Sample collection and DNA isolation

Forty-nine HCC DNA samples were collected from the National Cancer Institute of Thailand. The DNA was extracted from formalin-fixed, paraffin-embedded tissues. Sixty-six healthy individuals with no prior history of cancer were selected as the healthy control group. The DNA from the control group was extracted from peripheral blood using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany).

Detection of GSTM1 and GSTT1 CNVs

The GSTM1 and GSTT1 CNVs were determined by multiplex real-time PCR using the Luna® Universal qPCR Master mix kit (Bio-Rad Laboratories, USA) and the β-globin gene was used as a reference gene. The PCR primers for GSTM1 were 5'-GAA CTC CCT GAA AAG CTA AAG C-3' and 5'-5'-CTT GGG CTC AAA TAT ACG GTG G-3', the primers for GSTT1 were 5'-GCC ATC CTG CTC TAC CTG AC-3' and 5'-TGC CAG GTA CTC ATC CAC AC-3', and the primers for the β-globin gene were 5'-AAC TTC ATC CAC GTT CAC C-3' and 5'-GAA GAG CCA AGG ACA GGT AC-3'.

The master mix (10 µL) consisted of 1× Luna® Universal qPCR Master mix, 25 ng of DNA sample and 0.5 µM of primers. Real-time PCR was performed by the BioRad CFX connect qPCR (real-time PCR) system using the following steps: initial denaturation at 95 ℃ for 1 minute, 40 cycles under denaturation conditions at 95 ℃ for 5 seconds, and primer annealing and polymerization at 60 ℃ for 30 seconds. The gene copy numbers were determined by the ΔCt method = $Ct,_{\text{target gene}} - Ct,_{\text{reference gene}}$ (15).

Statistical analysis

The frequencies of the CNVs in the control group and the patients were compared using the Chi-square test. Binary logistic regression was used to evaluate the relationship between the control and disease genotypes. The relationships between the patients' clinico-pathological parameters (gender, stage, size of the tumor, differentiation, and age at diagnosis) to the GSTM1 and GSTT1 CNVs were examined by Chi-square test. Survival status was determined by the Kaplan-Meier survival method and the log-rank test. The patients were followed up for a period of 1–155 months. A P value of ≤0.05 was considered statistically significant.

Results

Detection of GSTM1 and GSTT1 CNVs

The GSTM1 CNVs were determined using the real-time PCR technique to analyze 49 DNA samples obtained from HCC patients and 66 DNA samples obtained from the control group. The fluorescent data in *Figure 1* represents the Ct value and the melting curve. The results show that the GSTM1 for genotypes was 0/0 69.3% and 1/0 22.6%, 1/1 8.1% of cases. The GSTT1 for genotypes was 0/0 24.5% and 1/0 42.8%, 1/1 10.2%, >1 22.5% of cases.

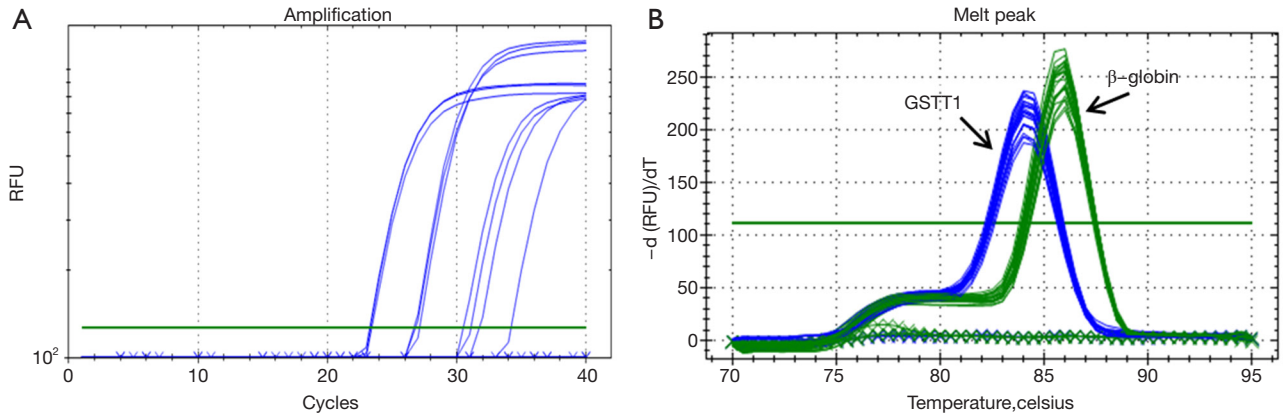

Figure 1 The representative fluorescence data measured by real-time PCR (A) Ct values of *GSTT1* and *β-globin*; (B) melting curves of *GSTT1* and *β-globin*. PCR, polymerase chain reaction.

Table 1 *GSTM1* and *GSTT1* CNV in hepatocellular carcinoma patients and control group

CNV status	*GSTM1*		*GSTT1*	
	Control	HCC	Control	HCC
0/0	40 (61%)	34 (69%)	25 (38%)	12 (24%)
1/0+1/1+>1	26 (39%)	15 (31%)	41 (62%)	37 (76%)
Total	66	49	66	49
Odds ratio (95% CI)	0.67 (0.31–1.49)		1.88 (0.83–4.26)	
P value	0.331		0.129	

CNV, copy number variants; HCC, hepatocellular carcinoma; CI, confidence interval.

The relationship between the frequencies of GSTM1 and GSTT1 CNVs in the control group and patients

The GSTM1 and GSTT1 CNVs were analyzed. The results show that the difference between the controls and the patients was not statistically significant (P≥0.05). Overall, the frequencies of the CNVs for the GSTM1 genotypes 0/0 between patients and controls were 60.6% and 69.6%, respectively and the GSTT1 genotypes were 24.4% and 37.8%, respectively. Only the GSTT1 genotypes 0/0 were associated with an increased risk of hepatocellular carcinogenesis (OR value was 1.88). While the GSTM1 genotypes were not associated with the risk factors of HCC (OR value was 0.67). *Table 1* shows a summary of the data.

Statistical analysis of GSTM1 and GSTT1 CNVs and patients' clinico-pathological parameters

The correlation between the patients' clinico-pathological parameters and the CNVs of GSTM1 and GSTT1 were analyzed, as shown in *Tables 2,3*. The GSTM1 CNVs were associated with the gender of the patients (P=0.002). Stage, the size of the tumor, differentiation, and the patient's age at diagnosis showed no significant difference (P>0.05). However, in the case of the GSTT1 CNVs, no correlations were found to any of the clinico-pathological parameters (P>0.05).

Survival of patients with GSTM1 and GSTT1 CNVs

Survival analysis was determined by the Kaplan-Meier survival curve. In summary, there was no correlation found between GSTM1 and GSTT1 CNVs and patient survival (*Figures 2,3*, P=0.444 and 0.803, respectively).

Discussion

GSTM1 and GSTT1 genes are involved in the detoxification of activated carcinogen and other toxins. When this happens, the loss of enzyme catalytic activity from the deletion genotype leads to a decrease of cell protection from various toxic agents (16) causing an association with susceptibility to many cancers (7-9).

While, GSTM1 has been associated with an increased risk of lung (7) and colorectal cancer (17) in previous research. We found no significant difference between controls and patients for the GSTM1 CNVs in HCC; however, we found that GSTT1 genotypes 0/0 increased the risk of susceptibility to HCC (OR =1.88). This is in parallel with other research on lung, breast, colon and endometrial cancer, which concluded that GSTT1 genotypes 0/0 or null

Table 2 GSTM1 CNV status and clinico-pathological parameters of hepatocellular carcinoma patients

Parameter	CNV status		P value
	0/0, n [%]	1/0+1/1+>1, n [%]	
Stage			0.882
I + II	20 [63]	8 [25]	
III	3 [9]	1 [3]	
Tumor size			0.077
≤3	6 [13]	0 [0]	
>3	27 [56]	15 [31]	
Differentiation			0.975
WD	3 [9]	1 [3]	
MD	17 [53]	7 [23]	
PD	3 [9]	1 [3]	
Sex			0.002*
Female	15 [31]	0 [0]	
Male	19 [38]	15 [31]	
Age			0.105
≤50	8 [16]	7 [14]	
>50	26 [54]	8 [16]	

*, significant. CNV, copy number variants; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

Table 3 GSTT1 CNV status and clinico-pathological parameters of hepatocellular carcinoma patients

Parameter	CNV status		P value
	0/0, n [%]	1/0+1/1+>1, n [%]	
Stage			0.217
I + II	8 [25]	20 [63]	
III	0 [0]	4 [12]	
Tumor size			0.516
≤3	2 [4]	4 [8]	
>3	9 [19]	33 [69]	
Differentiation			0.264
WD	2 [6]	2 [6]	
MD	6 [18]	18 [58]	
PD	0 [0]	4 [12]	
Sex			0.814
Female	4 [8]	11 [22]	
Male	8 [16]	26 [64]	
Age			0.094
≤50	6 [13]	9 [18]	
>50	6 [13]	28 [56]	

CNV, copy number variants; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

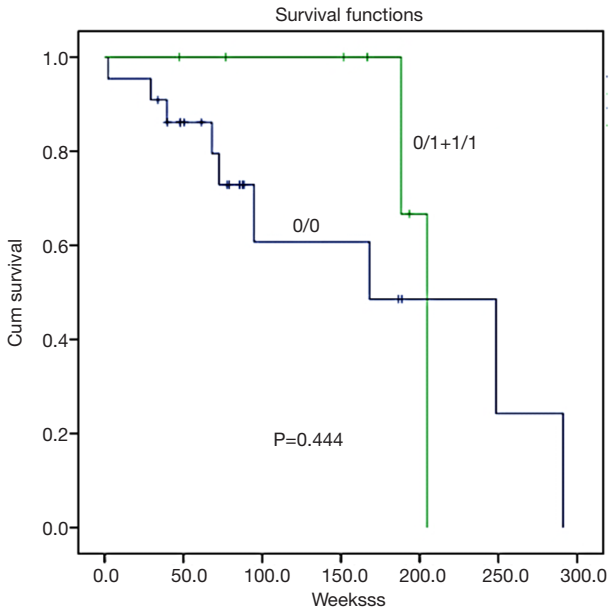

Figure 2 Kaplan-Meier survival curve for the patients with hepatocellular carcinoma according to *GSTM1* copy number.

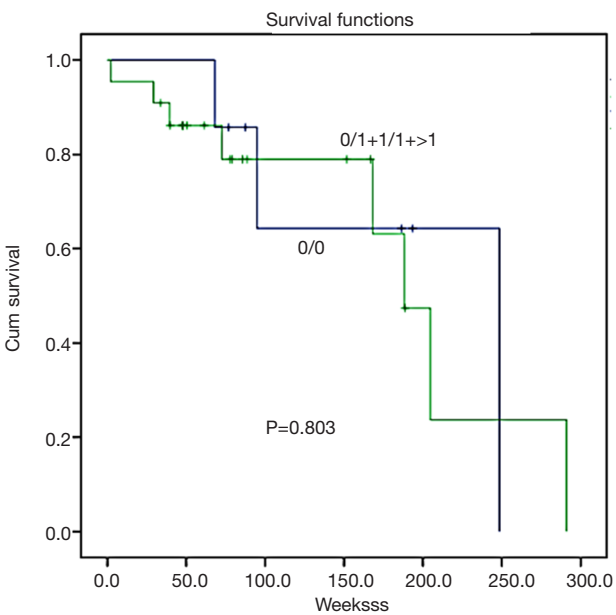

Figure 3 Kaplan-Meier survival curve for the patients with hepatocellular carcinoma according to *GSTT1* copy number.

genotypes resulted in a loss of GSTT1 enzyme activity and reduced toxin detoxification leading to an increased risk of susceptibility to carcinogenesis (13,14,18,19).

First, the gene copy numbers for GSTM1 and GSTT1 were determined from 49 HCC samples by the

real-time PCR technique. Then, the associations with clinico-pathological parameters were analyzed. The results show that only the GSTM1 CNVs were associated with the gender of patients (P=0.002), the frequency of genotypes 0/0 was more dominant in females than males (male 55.9%, female 100.0%). The results of this research concur with a study on Japanese lung cancer patients (20).

This phenomenon allows sex hormones to play a role in protecting organs such as the lung and liver from inflammatory substances and preventing HCC development in females (21). The protective effect of estrogen inhibited proinflammatory cytokines such as IL-6 contributed to the causes of liver disease (22) and the risk of female HCC incidence increased at the menopausal age (23). In addition, inflammation resulting from oxidative substances may be prevented by the GSTM1 function; however, the data assumed that the null genotype of the GSTM1 gene would increase the risk of inflammatory diseases (24). Prior research, which supports our results, identified GSTM1 CNVs and sex hormones as two factors that were associated with liver inflammation and carcinogenesis. GST polymorphisms were found to influence the efficiency of estrogen metabolism in women (25).

According to previous research, GSTM1 CNVs have been associated with the progression of phenotypes in colorectal cancer (26) and oral squamous cell carcinoma (27). GSTM1 CNVs tended to be related to tumor size (P=0.077). However, we found no correlation between the GSTT1 CNVs and clinico-pathological status.

In conclusion, the results of this research indicate that only the GSTT1 CNVs are associated with an increased risk of susceptibility to HCC. GSTM1 copy numbers were also associated with the gender of patients more dominantly in females than in males and tended to be related to the progression phenotype. No association was found between the GSTT1 CNVs and the clinico-pathological status of patients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the Rangsit University Ethics Committee (No. RSPE 20/2560) and Ethics Committee of National Cancer Institute, Thailand (No. EC COA 005/2017). As this was a retrospective study, informed consent is not required.

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