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Prognosis in Thai colorectal-cancer patients with GSTM1 and GSTT1 copy number variation

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Summary. *Background:* Glutathione S-transferase (GST) enzymes including GSTM1 and GSTT1 are involved in the detoxification of carcinogenic compounds. Null genotypes of GSTM1 and GSTT1 may result in reduced enzyme activity and be associated with susceptibility to various cancers. The detection of DNA copy number variation (CNV) by real-time PCR is reportedly very effective for detecting GSTM1 and GSTT1. This study investigated the association between GSTM1 and GSTT1 copy number variations and clinico-pathological parameters and gene copy number variation, and their potential relationship with the survival status of colorectal-cancer patients. *Methods:* Fifty-three Thai colorectal-cancer patients were investigated for GSTM1 and GSTT1 copy number variation by real-time PCR. Correlations between gene copy number variation and clinico-pathological characteristics were analyzed. *Results:* The results showed an association between GSTM1 copy number variation with stage and lymph-node metastasis. Increases in GSTM1 copy number were related to a negative prognosis and reduced survival status of patients. No association was found between GSTT1 copy number variation with patients' clinico-pathological parameters and survival. *Conclusions:* The results demonstrated that GSTM1 copy number variation may be used in guiding the prognosis for Thai colorectal-cancer patients.

Key words: glutathione S-transferase M1, glutathione S-transferase T1, DNA copy number variation, colorectal cancer

«PROGNOSI IN PAZIENTI TAILANDESI AFFETTI DA CANCRO COLONRETTALE CON VARIABILITÀ NEL NUMERO DI COPIE DEL GENE GSTM1 E GSTT1»

Riassunto. *Background:* Gli enzimi glutatione S transferasi (GST), inclusi GSTM1 e GSTT1, sono coinvolti nella detossificazione dei composti cancerogeni. I genotipi nulli di GSTM1 e GSTT1 possono risultare in attività enzimatica ridotta ed essere associati alla suscettibilità a vari tipi di cancro. Il rilevamento della variazione del numero di copie del DNA (CNV) attraverso la PCR in tempo reale è molto efficiente per rilevare GSTM1 e GSTT1. Questo studio ha investigato sulla associazione tra le variazioni numeriche GSTM1 GSTT1 ed i parametri clinico-patologici e la variazione del numero delle copie del gene e il loro potenziale rapporto con lo stato di sopravvivenza dei pazienti con cancro colonrettale. *Metodi:* Cinquantatre pazienti con cancro colonrettale sono stati sottoposti a studio per la variazione del numero delle copie del gene e le caratteristiche clinico-patologiche sono state analizzate. *Risultati*: I risultati hanno mostrato un'associazione tra la variazione del numero di copie del gene GSTM1 con lo stadio della malattia e la presenza di metasta-

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si del linfonodo. Aumenti del numero di copie del gene GSTM1 sono collegati alla prognosi negativa e al ridotto stato di sopravvivenza dei pazienti. Nessuna associazione è stata trovata tra la variazione del numero di copie del gene GSTT1 con i parametri clinico-patologici dei pazienti e la sopravvivenza. *Conclusioni:* I risultati hanno dimostrato che la variazione del numero di copie del gene GSTM1 può essere usata per guidare la prognosi per i pazienti tailandesi con cancro colonrettale.

Parole chiave: : glutatione S-transferasi M1, glutatione S-transferasi T1, variazione numero di copie del DNA, cancro colonrettale

Introduction

Colorectal cancer is the third most common malignancy in Thailand. The incidence in males is 12.9/100,000 and females 9.2/100,000 people (1). The carcinogenesis model for sporadic colorectal cancer has been reported, which involves the sequential accumulation of specific genetic alterations in cells, such as mutation, deletion and gene polymorphism (2-4).

Glutathione S-transferases (GSTs) are a superfamily of enzymes involved in the detoxification of carcinogens including glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1) enzymes. In addition, the null genotypes of *GSTM1* and *GSTT1* may lead to complete loss of enzyme activity and be associated with susceptibility to various cancers (5-7). The null genotypes of *GSTM1* and *GSTT1* have been studied and no association has been found between *GSTM1* and colorectal cancer (8, 9). However, previous reports have only found null genotypes of *GSTT1* to be associated with colorectal cancer (10, 11).

DNA copy number variations (CNV) in *GSTM1* and *GSTT1* have been reported, and have been correlated with altered enzyme activity (12, 13). Most previous studies have investigated DNA copy number variation by direct PCR, which is unable to differentiate deletion, one, two or more than two copies of *GSTM1* and *GSTT1*. The real-time PCR technique has been shown to be more sensitive for detecting genetic DNA copy number variations (14-16).

This study used real-time PCR to investigate DNA copy number variations (CNV) *GSTT1*, *GSTM1* in Thai colorectal- cancer patients. The correlation between *GSTs* gene copy number variation and the clinico-pathological features of colorectal cancer were analyzed by Chi-square test. The prognostic analysis of colorectal cancer depended on the association between *GSTM1* and *GSTT1* copy number variation and survival status, determined by Kaplan-Meier survival curve. Statistical significance was set at P-value ≤0.05. The results of this research may be used to inform prognoses for Thai colorectal-cancer patients with these genes.

Methods

S*ample collection and DNA isolation*

DNA samples were extracted from formalin-fixed, paraffin-embedded, colorectal cancer samples obtained from 53 Thai colorectal-cancer patients. The samples were collected from the National Cancer Institute of Thailand. DNA isolation was performed as described by Chariyalertsak et al. (17).

Detection of GSTM1 and GSTT1 copy number variation

GSTM1 and *GSTT1* copy number variation were quantified by real-time PCR using SsoFast™ EvaGreen® Supermix kit (Bio-Rad Laboratories, USA) with a specific primers and *β*-globin gene as the reference gene copy number. PCR primers for the GSTM1 were 5'-ACC CCA GGG CTC TAT GGG AA-3' and 5'-TGA GGG CAC AAG AAG CCC CT-3', primers for GSTT1 were 5'-ACC CCA GGG CTC TAT GGG AA-3' and 5'-TGA GGG CAC AAG AAG CCC CT-3' (18), and the primers for

reference 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 (20 μl) consisted of SsoFast EvaGreen Supermix reagent 10 μl, 25 ng of genomic DNA and 500 nM of primers. The real-time PCR was performed using a BioRad CFX-96 qPCR (Real Time PCR) System with the following steps: initial denaturation at 98°C for 2 minutes, 50 cycles under denaturation conditions at 94°C for 10 seconds, primer annealing at 54.4°C for 10 seconds, polymerization at 60°C for 20 seconds. Gene copy number was determined by ΔCt method = $Ct_{\text{target gene}} - Ct_{\text{reference gene}}$ (19).

Statistical analysis

The correlation between clinico-pathological parameters [lymph node, epidermal growth factor receptor (EGFR), differentiation, stage, sex and age at diagnosis] of the patients with *GSTM1* and *GSTT1* copy number variation were examined through Chi-square test. Survival was calculated through Kaplan-Meier survival method and log-rank test, with patients followed up for a period of 1-155 months. A P-value ≤0.05 was considered statistically significant.

Results

Detection of GSTM1 and GSTT1 copy number variation

The GSTM1 and GSTT1 copy number was determined by real-time PCR technique for 53 DNA samples of colorectal-cancer patients, the representative fluorescence data of Ct values and melting curves are shown in Fig. 1. The results showed the GSTM1 for genotypes 0/0 45.2%, 1/0 18.9%, 1/1 7.6% and >1/1 28.3% of cases; for the GSTT1 genotypes 0/0 30.3%, 1/0 18.8%, 1/1 5.7% and >1/1 47.2% of cases.

The associations between GSTs copy number and patients' clinico-pathological parameters are summarized in Tables 1 and 2. GSTM1 copy number variation was significantly associated with cancer stage (P=0.038) and lymph-node metastasis (P=0.023). The frequency of high GSTM1 copy numbers was relatively low among patients with early stage and negative node metastasis. No correlations were found with epidermal growth factor receptor (EGFR), differentiation, sex and age at diagnosis (P>0.05), and no statistically significant relationship was found between GSTT1 copy number variation and patients' clinico-pathological parameters (P>0.05). However, GSTT1 copy number may be related to cancer differentiation (P = 0.080) and patient's sex (P = 0.066).

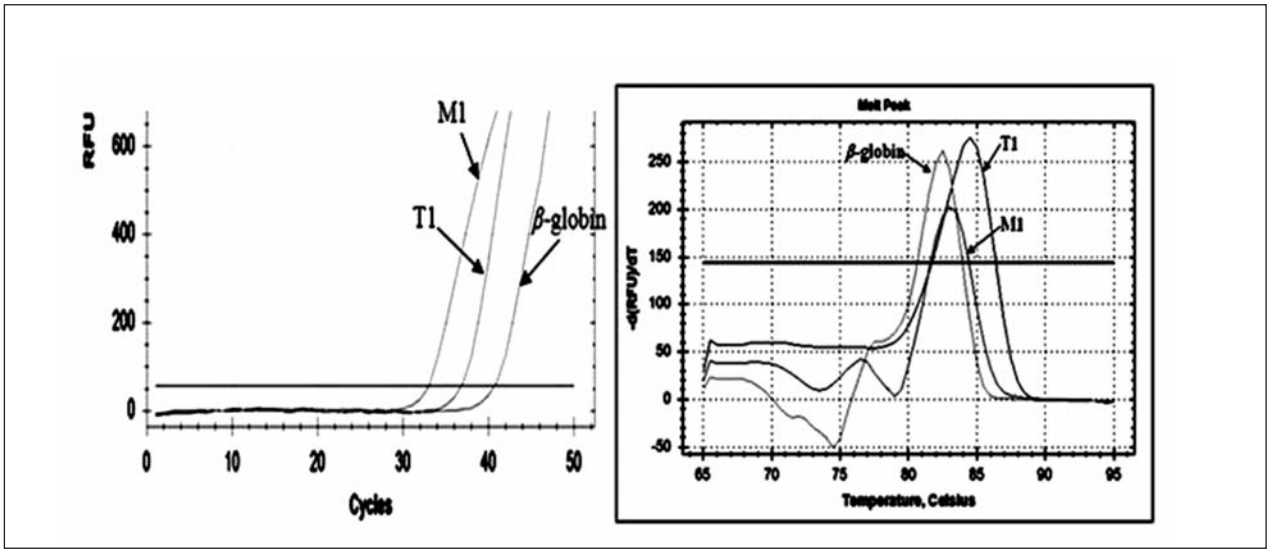

Figure 1. The representative fluorescence data measured by real-time PCR (Left) Ct values of GSTM1, GSTT1 and β-globin; (Right) melting curves of GSTM1, GSTT1 and β-globin

Table 1. GSTM1 copy number variation (CNV) status and clinico-pathological parameters of colorectal cancer patients.

Parameter	CNV status 0/0+1/0 n (%)	CNV status 1/1+>1/1 n (%)	*P*-value
Stage			0.038*
I+II	19 (36)	5 (9)	
III+IV	15 (28)	14 (27)	
Lymph node			0.023*
+	14 (27)	14 (27)	
-	20 (37)	5 (9)	
EGFR			0.801
+	11 (21)	6 (10)	
-	22 (42)	14 (27)	
Differentiation			0.887
WD	15 (28)	8 (16)	
MD+PD	19 (35)	11 (21)	
Sex			0.887
M	19 (35)	15 (28)	
F	11 (21)	8 (16)	
Age			0.990
<50	9 (20)	5 (9)	
≥50	25 (44)	14 (27)	

* statistical significant

Table 2. GSTT1 copy number variation (CNV) status and clinico-pathological parameters of colorectal cancer patients.

Parameter	CNV status 0/0+1/0 n (%)	CNV status 1/1+>1/1 n (%)	*P*-value
Stage			0.139
I+II	14 (27)	10 (19)	
III+IV	11 (21)	18 (33)	
Lymph node			0.224
+	11 (21)	17 (31)	
-	14 (27)	11 (21)	
EGFR			0.111
+	11 (21)	6 (10)	
-	22 (42)	14 (27)	
Differentiation			0.080
WD	14 (27)	9 (16)	
MD+PD	11 (21)	19 (36)	
Sex			0.066
M	17 (31)	8 (16)	
F	12 (23)	16 (30)	
Age			0.460
<50	7 (13)	7 (13)	
≥50	18 (33)	21 (41)	

Survival of patients with GSTM1 and GSTT1 copy number variation

Survival analysis was explained by Kaplan-Meier survival curve. In summary, decrease *GSTM1* copy number (0/0+1/0) correlated with longer patient survival (Fig. 2, *P*=0.034). No association between gene copy number and patient survival for *GSTT1* was observed (Fig. 3, *P*=0.289).

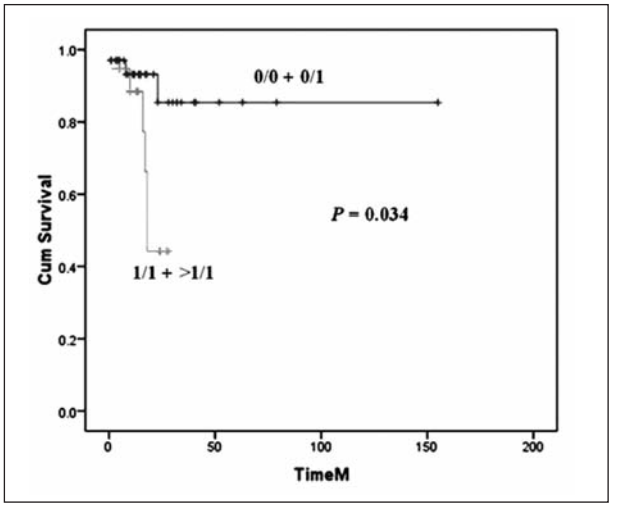

Figure 2. Kaplan-Meier survival curve for the patients with colorectal cancer according to GSTM1 copy number.

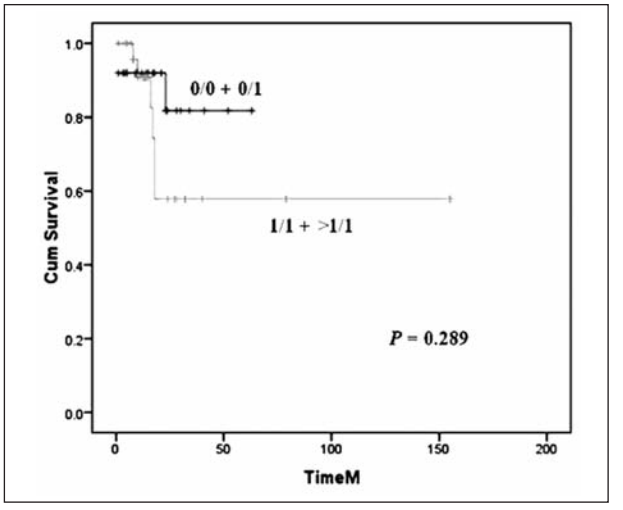

Figure 3. Kaplan-Meier survival curve for the patients with colorectal cancer according to GSTT1 copy number

Discussion

Glutathione S-transferase is a phase-II detoxification protein that protects cells from the cytotoxicity of chemical substances, including cancerous agents. A previous study has indicated that GSTM1 gene polymorphism was associated with resistance to colorectal-cancer chemotherapy (20). In the present study, we determined the gene copy number for GSTM1 and GSTT1 from 53 formalin-fixed, paraffin-embedded colorectal- cancer samples by real-time PCR technique. GSTM1 positive genotypes (1/0, 1/1, >1/1) were found among 54.8% of cases. In comparison, the GSTM1 null genotype (0/0) was found among only 45.2%, while GSTT1 positive genotypes were 69.7% more frequent than the GSTT1 null genotypes, at only 30.3%. These data agreed with other findings among Asian population of colorectal cancer patients, which showed GSTM1 positive genotype at 66.9% and null genotype at 33.1% and GSTT1 positive genotype at 81.1% more than GSTT1 null genotype (18.9%) (21). However, in contrast, a study of an European population found GSTM1 positive genotype at a rate of only 34%, which was lower than for GSTM1 null genotype (66%) (22).

Our experiment showed that, *GSTM1* copy number variation was associated with cancer stage (P= 0.038) and lymph-node metastasis (P=0.023). These observations, indicating that *GSTM1* copy number is associated with progression phenotype in colorectal cancer, according to a previous study that found *GSTM1* polymorphism as significantly associated with lymph-node metastasis in oral squamous-cell carcinoma (23). However, no statistically significant relationship was observed between *GSTT1* copy number variation and clinico-pathological parameters, agreeing with a previous report on colorectal cancer (24).

The key parameters for the progression of cancer are stage and lymph-node metastasis status. These parameters affect patient survival, consistent with the present experiment, which found a significant association between *GSTM1* deletion genotype (0/0+1/0) and colorectal-cancer patients survival (P=0.034), and with strong concurrence with a previous study of bladder cancer (18). The result of our experiment also concurs with reports of the association between null genotype *GSTM1* and better survival among patients with a variety of cancers, including osteosarcoma and breast cancer (25-27). In contrast, some reports have shown that null-genotype *GSTM1* was not associated with the survival of European colorectal-cancer patients (28, 29).

While *GSTM1* copy number was found to be associated with patient survival status, *GSTT1* copy number was not. This finding agreed with a report on prostate cancer, which showed that the *GSTM1* genotype was associated with patient survival, but not *GSTT1* genotype (30). The present study indicates that increase in *GSTM1* copy numbers are related to high patient mortality, which might be explained by *GSTM1* expression in cancer cells. Cancer cells with higher *GSTM1* copy numbers are expressed at a high level in the GSTM1 protein and resist chemotherapeutic agents through enzyme detoxification activity. A previous experiment found elevated *GSTM1* expression in tumor metastases of prostate cancer (31); this was consistent with some other studies, which found high *GSTs* expression in many cancers, resulting in decreasing chemotherapeutic response (32-34).

In conclusion, it was found that *GSTM1* copy number variation was associated with stage and lymph-node metastasis in colorectal cancer. Decreased *GSTM1* copy numbers are related to prolonged survival of the colorectal-cancer patients. No association was found between *GSTT1* copy number variation and clinico-pathological/survival status.

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