RESEARCH COMMUNICATION

Glutathione S-Transferase P1 Variant Plays a Major Contribution to Decreased Susceptibility to Liver Cancer in Thais

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Abstract

Glutathione S-transferases (GSTs) play important roles in carcinogenic biotransformation processes, which vary among individuals. Polymorphisms of the encoding genes are associated with alteration of detoxification capacity, resulting in a variable risk of cancer development. The present study was performed to determine the effects of polymorphisms in GST (M1, P1, and T1) genes on susceptibility to liver cancer in Thais. We recruited 140 hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) patients and 280 healthy volunteers for our unmatched case-control based association study. GSTM1 deletion and heterozygous deletion were determined and discriminated by semi-quantitative denaturing high performance liquid chromatography (DHPLC). A polymerase chain reaction - restriction fragment length polymorphisms (PCR-RFLPs) approach was utilized to detect the GSTP1 Ile105Val variant, while the GSTT1 null allele was detected by multiplex PCR. With results for single locus associations, only GSTP1 Ile/Val showed a significant decrease in the risk of liver cancer (OR=0.58; 95% CI: 0.36-0.90; p-value=0.016). GSTP1 (Ile/Val) interacted with the GSTT1 wild type to further decrease susceptibility to liver cancer (OR=0.41; 95% CI: 0.18-0.93; p-value=0.029). Moreover, three locus interactions of GSTP1 (Ile/Val or Val/Val) with either wild type or null alleles of both GSTM1 and GSTT1 decreased risk of liver cancer. In conclusion the GSTP1 null genotype apparently causes decreased risk of liver cancer in Thais. The findings point to GSTP1 Ile105Val as a possible protective allele against liver cancer risk.

Key Words: Glutathione s-transferases (GSTs) - hepatocellular carcinoma (HCC) - GSTP1 - GSTM1 - GSTT1

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Introduction

Glutathione S-transferases (GSTs) are multigene family encoding enzymes that play a major role in phase II biotransformation of drugs and numerous carcinogenic compounds. This enzyme family is divided into classes according to >40% identity of amino acid constituents designated as alpha, kappar, mu, omega, pi, sigma, theta and zeta (Parl FF, 2005). Among eight major classes of GSTs, three isozymes (GSTM1, GSTT1 and GSTP1), are considered to contribute mainly on detoxification process. Most of industrial chemicals and pollutants, such as polycyclic aromatic hydrocarbon (PAH), benzo [a] pyrene, ethylene oxide and methyl halides are substrates in GST metabolic pathway (Hayes et al., 2005; Bolt and Their, 2006). The polymorphisms of these genes are a significant factor contributed to individual difference in detoxification capacity (Bolt and Their, 2006). GSTT1 and GSTM1 whole gene deletion was shown to have a relevant gene-dose effect correlated to its phenotypes; therefore, null allele presumably conveyed none of enzyme activity (Bolt and Their, 2006). The polymorphic GSTP1 was extensively reported on the base substitution (A1404G) in exon 5 resulting in the exchange of isoleucine to valine in codon 105. This change causes a reduced enzyme activity, while with certain substances such as diol epoxide, it appeared to be more active (Sundberg et al., 1998). Consequently, the polymorphisms of GSTs are recognized to affect both deactivation or activation of toxic compounds and for some degree influence the susceptibility of individuals toward toxicity or cancers. Polymorphisms of GSTs gene differ in allele frequencies among ethnics. For instance, GSTM1 null allele was detected at 42-60% in Caucasians, 16-36% in African-American and 42-54% in Asians (Garte et al., 2001). Interestingly, GSTT1 null allele is approximately twofold higher in Asian (~47%) than in Caucasians (~20%) (Garte et al., 2001). GSTP1 Ile105Val variant was found at 31% and 54% in Caucasians and African-Americans, repectively, and slightly lower at 17 % in Asians (Harris

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et al., 1998). Due to its role of toxic deactivation, the deficiency of these enzymes in person with null genotype was therefore investigated for the possible risk of individual susceptibility to cancers. Most studies determined only two locus interaction, GSTM1 and GSTT1, with various types of cancer such as lung, head and neck, bladder and liver cancers (Cao W et al., 2005; Covolo et al., 2005; Deng et al., 2005; Tiwawech et al., 2005; Czeczot et al., 2006; Ladero et al., 2006; Ye et al., 2006). As yet, hardly any studies regarded the interaction of combined three GSTs (P1, M1 and T1) genotypes with liver cancers.

Liver is well known for bearing important detoxification process of both endogenous and exogenous compounds including carcinogens. Liver cancer was ranked the fifth most common cause of cancer death worldwide (Vatanasapt et al., 2002). Epidemiological studies have shown that risk factors of liver cancer are related to familial history, environmental factors (e.g., alcohol, hepatitis B and/or C virus, and aflatoxin B1 exposure) and genetic variations (Sun et al., 2001; Covolo et al., 2005). It is interesting to study genetic polymorphisms for consideration of explaining in any parts of worldwide cancer epidemiology. The association of GST polymorphisms and liver cancer has been studied but not that extensively in the country with low incidence. There are some reports from the high prevalent region such as sub-Saharan Africa and Eastern Asia but the results appeared to be contradictory.

There is yet a need to establish more evidences to ascertain the association between GST polymorphisms and liver cancer in various ethnics. Thus, this study investigated the effect of GSTM1, P1 and T1 polymorphisms with the interaction of liver cancer in Thais. Moreover, new technique was developed for an advantageous discrimination of heterozygous from homozygous active GSTM1 gene. The results apparently show the protective effect of GSTP1 Ile105Val variant to liver cancer in studied population.

Materials and Methods

This unmatched case-control (1:2) study comprised of 140 liver cancer subjects (HCC or CCA patients) as a case group. Two hundred and eighty healthy volunteers (blood donors) who are over 40 years old and having no cancer and no history of cancer in their family members were recruited for control group. All patients and volunteers were informed and given consent. Study protocol was approved by the Ethics Committee Board of Siriraj Hospital, Mahidol University.

Blood sample recruitment

Blood samples were collected from Thai patients who underwent major hepatectomy followed the diagnosis of HCC or CCA at Siriraj Hospital. Ten ml of venous blood samples were collected and transferred into sterile screw cap tube containing 200 µl of sterile 10% EDTA for DNA extraction. For the control group, 4 ml buffy coat blood samples were obtained from Division of Blood Bank, Siriraj Hospital with complete non-identification.

Genotypic determination

GSTM1 deletion was detected and differentiated for wild type, heterozygous and homozygous deletion by DHPLC-based analysis of its relative gene copy numbers. PCR-RFLP was utilized for GSTP1 variant detection. GSTT1 null allele were determined by multiplex PCR amplifications for the presence or absence of active gene using beta-actin as an internal control. All primer sequences were given in Table 1.

GSTM1 deletion

GSTM1 was genotyped by triplex-PCR reaction containing primers for GSTM1, dystrophin (x-linked gene for a reference of one copy in men) and b-actin (a reference of two copies) gene amplification. The optimized tri-plex reaction mixture contained 100 ng DNA, 10x PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 10 pmol of each GSTM1 primer, 5.5 pmol of each b-actin primer and 9.5 pmol of each dystrophin primer, 1% TritonX-100 and 0.2 units of Immolase™ (Bioline Ltd., UK). The amplification conditions were 95°C for 10 min, and then 24 cycles of 95°C 1 min, 61°C 30 s, and 72°C 1 min followed by final extension at 72°C 7 min. The amplified 3-plex products were subsequently injected to DHPLC column. The amplicons were eluted from the column with acetonitrile gradients in a mixture of solution A (0.1 M TEAA) and solution B (25% ACN in 0.1 M TEAA). Five µl of 3-plex PCR products were eluted with a linear gradient solution of A (0.1 M TEAA) and B (0.1 M TEAA, 25% acetonitrile) from 50% to 85% B with a separation time of 4.0 min.

Table 1. Primer Sequences and Product Size from PCR Reaction

Gene	Primer	Sequence	Product size
GSTM1	GSTM1F	5'-CTGCCCTACTTGATTGATGGG- 3'	273 bp
	GSTM1R	5'-CTGGATTGTAGCAGATCATGC-3'	
α-actin	ACTBF	5'-GGCCCCTCCATCGTCCACCG-3'	496 bp
	ACTBR	5'-GGGCACGAAGGCTCATCATT-3'	
Dystrophin	DYSF	5'-CAAAGCCCTCACTCAAACAGTAAGC-3'	196 bp
	DYSR	5'-TTTTCGGTCTCTCTGCTGGTCAGTG -3'	
GSTP1	GSTP1F	5'-CCAGTGACTGTGTGTTGATC-3'	189 bp
	GSTP1R	5'-CAACCCTGGTGCAGATGCTC-3'	
GSTT1	LGSTT1F	5'-CAGTTGTGAGCCACCGTACCC-3'	1,460 bp
	LGSTT1R	5'-CGATAGTTGCTGGCCCCCTC-3'	
GSTT1	SGSTT1F	5'-CCAGCTCACCGGATCATGGCCAG-3'	466 bp
	SGSTT1R	5'-CCTTCCTTACTGGTCCTCACATCTC-3'	

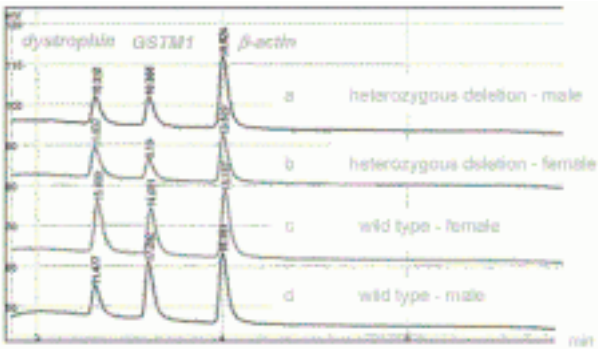

Figure 1. Determination of GSTM1 Deletion. Discriminated for the wild type and heterozygous deletion was by semi-quantitative DHPLC analysis. The resulting peak height of GSTM1 gene can reflect proportionally to its initial gDNA copy numbers when compare to its reference gene. For dystrophin (x-linked gene) peak height is exhibited as half of autosomal gene (b-actin) peak height in male (a and d) and with the same height in female (b and c). Likewise, the heterozygous deletion of GSTM1 can be interpreted from the peak presented only half of b-actin peak height (a and b). Digits over the peak are calculated peak heights from DHPLC analyzing program and min is elution time in minutes.

Flow rate was set 0.9 ml/min and at an oven temperature of 50°C. The resulting peak height of GSTM1 was compared to its internal reference for the relative copy numbers as shown in Figure 1.

GSTP1

GSTP1 genotypes were determined by the PCR-RFLP described by Zhao et al (2001). GSTP1F and GSTP1R primer were used to amplify GSTP1 in 25 µl reaction mixture containing 100 ng of DNA, 10x PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 10 pmol of each primer and 0.15 units of Taq polymerase Immolase™ (Bioline Ltd., UK). PCR condition started with initial denaturating at 95 °C for 10 min followed by 30 cycles of 95°C 30 s, 60°C 30 s, 72°C 30 s and final extension at 72°C for 7 min. Amplified products (378 bp) were then digested with BsmAI (BioLabs Inc., UK) for 16 hrs at 55°C. The digested fragments were diagnosed as wild type when presented 189 bp band.

GSTT1 deletion

We optimized multiplex PCR methods described prior by Sprenger et al (2000) for detecting the presence or absence of GSTT1 gene. SGSTT1F and gene region while LGSTT1F and LGSTT1R annealed to intergenic flanking region of GSTT1 (Figure 2). The 25 µl reaction mixture contained 100 ng of DNA, 10X PCR buffer, 0.2 mM dNTPs, 10 pmol of each primer and 0.2 units of Immolase™ (Bioline Ltd., UK). PCR conditions were initially denaturating at 95 °C for 15 min followed by 35 cycles of 94°C 30 s, 68°C 30 s, 72°C 1 min and final extension at 72°C for 7 min. The amplified products were analyzed by 1.5% agarose gel. PCR product with 1.46 kb was presenting deletion and 459 bp was wild type as demonstrated in Figure 2.

Statistical analysis

In single-locus analysis, the distributions of allele frequencies were tested for confirmation to Hardy-Weinberg equilibrium by Pearson $\chi 2$ test or Fisher's exact test. Each polymorphism was analyzed by comparing allele and genotype frequencies between those in the case and the control population using Pearson $\chi 2$ and odds ratio (OR) tests with p-value less than 0.05 within 95% confidence interval (CI). In multi-locus analysis, we tested for gene-gene interactions by multivariate analysis with MedCalc v9.6.2.0 (MedCalc Software, Belgium).

Results

Among three genes, GSTM1 allele was found at highest frequency in both case and control groups for 77.8% and 76.6%, respectively. GSTT1 was shown to be in second prevalence as 43.2% in case and 46.3% in control group. GSTP1 allele was found at the lowest frequency among them only 21.8% in case and 25.5% in control group (Table 2). For the gene-disease association study, single, double, and triple gene interaction were analyzed for the impact of association/interaction on the risk of having disease. For single gene analysis, we found a significant difference in frequency between case and control group only with heterozygous GSTP1 allele (Ile/Val) which showed the decreased risk effect, OR=0.57; 95% CI: 0.36-0.90; p-value=0.016. When GSTP1 (Ile/Val) combined with GSTT1 wild type, they also gave the significantly reduced risk, OR=0.41; 95% CI: 0.18-0.93; p-value=0.029 (Table 3). Surprisingly, when GSTP1 (Ile/Val or Val/Val) combined with any genotypes of GSTM1 and GSTT1, they further presented the protective effect against the susceptibility to liver cancer in various degree of significance (Table 4).This association study between

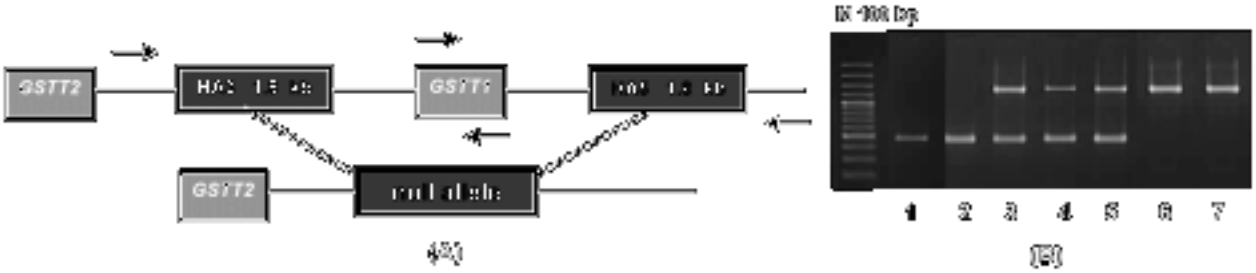

Figure 2. Genotypes. (A): GSTT1 is embedded in a region with extensive homology of region HA3 and HA5. Deletion (null allele) of GSTT1 gene is arised by the recombination of the central of HA3 and HA5 which results in a 54 kb deletion. (B): GSTT1 genotypes were determined by multiplex-PCR methods. The fragment of 459 bp indicates wild type (no 1 and 2) while 1.46 kb is interpreted as deletion (no 6 and 7). Number 3, 4 and 5 are heterozygous deletion. Arrows indicate annealing primers. M 100 bp is 100 basepair DNA ladders.

Table 2. GST Genotype Frequencies Among Liver Cancer Patients and Controls in Thais

Single GST Genotypes	Controls N=280 (%)	Patients N=140 (%)	OR	95% CI	p-value
GSTM1					
wild type (+/+)	10 (3.6)	7 (5.0)	1.00 (ref)	-	-
heterozygous (+/-)	111 (39.6)	48 (34.3)	0.62	0.22-1.72	0.35
homozygous (-/-)	159 (56.8)	85 (60.7)	0.76	0.28-2.08	0.60
GSTP1					
wild type (Ile/Ile)	154 (55.0)	91 (65.0)	1.00 (ref)	-	-
heterozygous (Ile/Val)	109 (38.9)	37 (26.4)	0.57	0.36-0.90	0.016*
homozygous variant (Val/Val)	17 (6.1)	12 (8.6)	1.20	0.55-2.61	0.66
GSTT1					
wild type (+/+)	100 (35.7)	52 (37.1)	1.00 (ref)	-	-
heterozygous (+/-)	101 (36.1)	55 (39.3)	1.05	0.66-1.67	0.85
homozygous (-/-)	79 (28.2)	33 (23.6)	0.80	0.47-1.36	0.41

OR = odds ratio; CI = confidence interval; ref = reference category; * statistically significant (p-value<0.05); (+/+) = wild type; (+/-) = heterozygous deletion and (-/-) = homozygous deletion

Table 3. Two Combinations of GST Genotypes Among Liver Cancer Patients and Control in Thais

Double GST Genotypes	Controls N=280 (%)	Patients N=140 (%)	OR	95% CI	p-value
GSTT1 and GSTP1					
T1 (+/+) and P1 (Ile/Ile)	58 (20.7)	37 (26.4)	1.00 (ref)	-	-
T1 (+/+) and P1 (Ile/Val)	38 (13.6)	10 (7.1)	0.41	0.18-0.93	0.029*
T1 (+/+) and P1 (Val/Val)	4 (1.4)	5 (3.6)	1.96	0.49-7.77	0.33
T1 (+/-) and P1 (Ile/Ile)	56 (20.0)	35 (25.0)	0.98	0.54-1.77	0.94
T1 (+/-) and P1 (Ile/Val)	39 (13.9)	16 (11.4)	0.64	0.36-1.31	0.22
T1 (+/-) and P1 (Val/Val)	6 (2.1)	4 (2.9)	1.05	0.28-3.95	0.22
T1 (-/-) and P1 (Ile/Ile)	40 (14.3)	19 (13.6)	0.75	0.38-1.48	0.40
T1 (-/-) and P1 (Ile/Val)	32 (11.4)	11 (7.9)	0.54	0.24-1.20	0.126
T1 (-/-) and P1 (Val/Val)	7 (2.5)	3 (2.1)	0.67	0.16-2.76	0.58

OR = odds ratio; CI = confidence interval; ref = reference category; * statistically significant (p-value<0.05); (+/+) = wild type; (+/-) = heterozygous deletion and (-/-) = homozygous deletion

Table 4. Three Combinations of GST Genotypes Among Liver Cancer Patients and Control in Thais

Triple GST Genotypes	Controls N=280 (%)	Patients N=140 (%)	OR	95% CI	p-value
M1(+/+), T1(+/+) and P1(Ile/Ile)	51 (18.21)	38 (27.14)	1.0 (ref)	-	-
M1(+/+), T1(+/+) and P1 (Ile/Val or Val/Val)	43 (15.36)	12 (8.57)	0.38	0.17-0.81	0.011*
M1(-/-), T1(+/+) and P1(Ile/Ile)	63 (22.50)	54 (38.57)	1.15	0.66-2.01	0.62
M1(-/-), T1(+/+) and P1(Ile/Val or Val/Val)	44 (15.17)	6 (4.29)	0.18	0.07-0.47	0.0002*
M1(+/+), T1(-/-) and P1(Ile/Ile)	20 (7.14)	13 (9.29)	0.87	0.39-1.97	0.74
M1(+/+), T1(-/-) and P1(Ile/Val or Val/Val)	11 (3.93)	1 (0.71)	0.12	0.06-0.99	0.022*
M1(-/-), T1(-/-) and P1(Ile/Ile)	20 (7.14)	8 (5.71)	0.54	0.21-1.35	0.18
M1(-/-), T1(-/-) and P1(Ile/Val or Val/Val)	28 (10.00)	8 (5.71)	0.39	0.16-0.94	0.032*

OR = odds ratio; CI = confidence interval; ref = reference category; * statistically significant (p-value<0.05); (-/-) = homozygous deletion; (+/+) = wild type included heterozygous deletion

GST polymorphisms and liver cancer appeared to show the protective effect against the susceptibility to liver cancer.

Discussion

The whole gene deletion of GSTT1 was successfully detected by multiplex PCR showing short fragment of GSTT1 gene (459 bp) and/or region that flank the deletion gap (1460 bp) in Fig 2. This technique applied from Sprenger et al (2000) is useful to distinguish the heterozygous deletion out of wild type for GSTT1. However, this technique cannot simply apply with GSTM1 since the definite break point deletion of GSTM1 was unable to find and the possible long-range PCR product covering the deletion gap was almost 15 kb. The long-range PCR is time consuming and it requires high integrity of DNA to obtain the amplified products successfully. Most of the studies therefore conducted only an absence or a presence of the active gene to define the deletion (Zhou et al., 1997; Covolo et al., 2005; Deng et al., 2005; Ladero et al., 2006). Hence, the optimized semi-

quantitative DHPLC-based analysis is very useful for the discrimination of heterozygous deletion from wild type in our experiment. The DHPLC peaks of triplex genes were easily interpreted by the comparison of GSTM1 peak height with its reference peak height. GSTM1 peak that shows a half of b-actin peak height can reflect heterozygous deletion as shown in Figure 2. We found GSTM1 at slightly higher frequencies (~ 77%) among the reported ranges in Asians (42-54%) and Caucasians (42-60%) (Garte S et al., 2001). The higher numbers in our study might result from the discrimination of heterozygous deletion out of wildtype which actually give more precise calculation of allele and genotype frequency.

However, GSTM1*0 frequency (wild type included heterozygote) is relatively comparable to that of a previous report in Thailand ranging from 30.2 to 62.7% (Sangrajrang et al., 2006). In addition, GSTT1*0 and GSTP1 Ile105val were in accordant with the frequencies found in Asians (Harris et al., 1998). GSTT1*0 (~ 46%) was apparently two-fold higher than those reported in Caucasians (13-26%) (Garteet al., 2001). In single gene association, GSTP1 Ile/Val genotype solely showed significant decreased risk of liver cancer (OR=0.57; 95%CI: 0.36-0.91; p-value=0.016) which could be explained from an increased metabolic activity for certain compounds. There was an evidence that the replacement of valine in codon 105 of GSTP1 was suggested for being more efficient in modification of benzypyrene diol epoxide (BPDE). It has been known that BPDE was the ultimate carcinogenic metabolite of benzo[a] pyrene (Sundberg et al., 1998). In contrast, the lower activity was observed with anti-diol epoxides since the bulkier valine possibly disturbed hydrophilic character of H-site (Sundberg K et al., 1998). The protective results of GSTP1 Ile105Val that constantly found with other GST gene interactions would be attributable to the higher detoxification from GSTP1 variant (Val/Val) for some carcinogenic substances. In addition to the high frequency of GSTM1 and T1 null allele which were presumably lacked of activity, GSTP1 probably play a major contribution to this metabolic compensation mechanism.

For double-gene interaction, GSTT1 wild type combined with GSTP1 (Ile/Val) was surprisingly found to reduce risk of liver cancer (OR=0.41; 95%CI: 0.18-0.93; p-value=0.029). As a matter of fact, GSTP1 variant (Val/Val) should accordingly present the protective effect but it was not shown likewise because of too few numbers of individuals (N<6) having Val/Val genotype (Table 3). Furthermore, in triple-gene interaction analysis, heterozygous deletion of each GSTM1 and GSTT1 was included in their wild type (+/+) in order to have adequate samples for statistical calculation. GSTP1, either Ile/Val or Val/Val, combined with any genotypes of GSTM1 and GSTT1 can give variable association significance for protective effect against liver cancer (Table 4). Remarkably, the presence or absence of both GSTM1 and GSTT1 did not influence the reduced risk of liver cancer. Therefore, GSTP1 appears to play a major contribution on the decreased susceptibility to liver cancer. From meta-analysis by White et al, only one study showed a small and nonsignificant decrease in risk of HCC in GSTP1 Ile105Val genotype (OR=0.75; 95%CI: 0.50-1.15) (White et al., 2008). but the sample size was smaller than our study., Taken together, the factor that contributes to the high prevalence of liver cancer in Thais are probably attributed to the higher hepatitis B virus infected carriers or the infestation of *O. viverrini* (OV) in CCA rather than the effect of genetic polymorphisms of GSTs. Nevertheless, we have a limitation on retrieving individual hepatitis B antigen result so we cannot calculate the frequency of hepatitis B virus carriers in studied population.

In conclusion, the GSTP1 Ile105val variant apparently plays a major contribution to the decreased risk of liver cancer in Thais. This result possibly derived from the enhanced activity of the enzyme to detoxify hepatotoxic compounds. Our finding supports the gene-gene interaction of genetic polymorphisms to modifying risk of cancer development.

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