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Original article

Glutathione S-transferase polymorphisms and risk of bladder cancer in Thais

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Abstract:

In this study, we determine the association of glutathione S-transferase Mu-1 gene *(GSTM1)*, glutathione S-transferase Pi-1 gene *(GSTP1)* and glutathione S-transferase Theta-1 gene *(GSTT1)* polymorphisms and the risk of bladder cancer in Thais. One hundred and thirty-nine bladder cancer patients and 278 control subjects were recruited for unmatch case control study. *GSTM1* and *GSTT1* were genotyped by multiplex PCR. Discrimination of *GSTM1* heterozygote was determined by semi-quantitative Denaturing High Performance Liquid Chromatography (DHPLC) analysis. *GSTP1* variants were identified by Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLPs). Null alleles of *GSTM1* and *GSTT1* presented no association with the risk of bladder cancer. Individuals with heterozygous *GSTP1 Ile/Val* (isoleucine/valine) showed the protective effect to bladder cancer (odds ratio (OR) = 0.53 [0.33-0.84], p = 0.006). The combined analysis of *GSTP1* wild type and *GSTT1* heterozygous deletion (OR = 2.39 [1.24-4.59], p = 0.083) or homozygous deletion (OR = 2.44 [1.22-4.90], p = 0.011) demonstrated the increased risk of bladder cancer. In conclusion, risk of bladder cancer was affected by *GST* polymorphisms especially *GSTP1* and the combination of *GSTP1* and *GSTT1*.

Keywords: Bladder cancer; *GSTM1; GSTP1; GSTT1;* Polymorphism; Thais

Introduction

Glutathione S-transferases (GSTs) comprise a superfamily of isoenzymes which play an important role in the detoxification of electrophilic xenobiotics and carcinogens, including environmental pollutants and reactive oxygen species [1, 2]. GSTs catalyze the formation of thioether bond between glutathione (GSH) and the electrophilic compounds and ultimately produce more water-soluble metabolites for elimination [3]. Biotransformation catalyzed by GST is thus determined to be the protective mechanism against carcinogens and also modifying the relative risk of various cancers. At present, gene families which encode GSTs have been grouped into eight classes (Mu, Pi, Sigma, Theta, Alpha, Kappa, Omega and Zeta) and the most extensively studied genes are S-transferase Mu-1 gene *(GSTM1)*, glutathione S-transferase Pi-1 gene *(GSTP1)* and glutathione S-transferase Theta-1 gene *(GSTT1)* [4]. *GSTM1* is responsible for the metabolism of PAHs such as aromatic amines found in cigarette smoke, while *GSTT1* detoxifies smaller reactive hydrocarbons such as ethylene oxide [5]. Null genotype of *GSTM1* and *GSTT1* arise from gene deletion of both alleles showing the absence of enzyme activities [6]. Frequency of *GSTM1* null genotype was reported ranging from 33%-63% in Caucasians and Asians but only 23%-48% in African population [7]. On the other hand, *GSTT1* null genotype found in Caucasians is ranging from 10%-36% while in Asians is more common ranging from 47 to 64% [1]. *GSTM1* null genotype was reported to involve with small to modest increased risk of bladder cancer in some studies [7, 8], extensively in Asians, while the effects of *GSTT1* null genotype are still in contradiction. In contrast to the effect of *GSTM1* and *GSTT* deletion, the substitution of isoleucine (Ile) with valine (Val) in codon 105 of *GSTP1* can enhance the enzyme activity toward specific substrates such as diol epoxide [9]. This variant appears to cause a protective effect against bladder cancer [10]. Although several studies revealed the association of *GST* polymorphisms with the susceptibility to bladder cancer, there were still inconsistently reported among ethnic groups. In addition, most studies did not differentiate the heterozygosity of *GSTM1* and *GSTT1* so the exact null allele frequencies were unable to obtain. This might be an underlying factor of variations in the association of GST genotypes and bladder cancer risk. In Thais, there were some studies regarding GSTs genotypes and disease association but not with bladder cancer [11]. Therefore, this study aims to investigate the associations of *GSTM1*, *GSTP1* and *GSTT1* polymorphisms with bladder cancer susceptibility. Moreover, semi-quantitative analysis of relative gene copy numbers was developed for the discrimination of *GSTM1* heterozygous deletion using Denaturing High Performance Liquid Chromatography (DHPLC).

Materials and Methods

The study consisted of 139 Thai patients with the diagnosis of transitional cell carcinoma of bladder confirmed by histological analysis of endoscopic biopsies and/or surgical resection. Patients with other histological types of bladder cancer or with other cancers were excluded from this study. The control group comprised of 278 healthy volunteers at the age over 40 without current and history of cancer and neither with their family members. This information was given in the questionnaire conducted by trained person from Siriraj Blood Bank Division. The sample size was calculated by using Quanto program version 1.1 [12] and size of control was twofold higher than studied group. The parameters using for calculation are 0.005% for prevalence of bladder cancer in Thais, allele frequency 62.7% and relative risk compared to normal subjects is 1.90. The protocol of this study had been approved by Ethics Committee Board of Siriraj Hospital, Mahidol University.

GSTM1 genotyping

GSTM1 was amplified by multiplex Polymerase Chain Reaction (PCR) using A1 and A2 primers with B1 and B2 primers of *β-actin* as an internal control. PCR reaction was 25 µl containing 100 ng DNA, 20 mM Tris-HCl buffer (pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates (dNTPs), 1 unit of Immolase® (Bioline Ltd., UK), 1% TritonX-100 and 10 pmol of each oligonucleotide primer. It was achieved by 30 cycles of 60 s 95°C, 30 s 61°C, and 60 s 72°C with

final extension 7 min 72 °C. The absence of PCR product was defined as gene deletion while the presence of 273 bp band was *GSTM1* gene. The differentiation between heterozygote and wild type was conducted by semi-quantitative DHPLC analysis. All primer sequences are given in Table 1.

Copy number variation of *GSTM1* was measured by semi-quantitative DHPLC. The triplex products from multiplex PCR reaction consist of *dystrophin* gene (internal one copy control), *β-actin* genes (internal two copy control) and *GSTM1* gene. The triplex-PCRs were optimized by varying each primer concentration in order to have the same efficiency of amplification. PCR cycles were limited to 24 cycles to obtain the product from linear phase of amplification curve. The reaction consisted of 10 pmol of each *GSTM1* primer, 5.5 pmol of each *β-actin* primer, 9.5 pmol of each *dystrophin* primer, 100 ng DNA, 20 mM Tris-HCl buffer (pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of Immolase® (Bioline Ltd., UK) and 1% Triton X-100. PCR cycles were started with an initial denaturation of 10 min 95 °C followed by 24 cycles of 1 min 95 °C, 30 s 61 °C, and 1 min 72 °C with final extension 7 min 72 °C. Amplified products were further injected to DHPLC. The amplicons was eluted from the column using acetonitrile gradients which was a mixture of solution A (0.1 M Tri-ethyl ammonium acetate or TEAA) and solution B (25% ACN in 0.1 M TEAA). The peak heights of products were compared based on relative copy numbers of DNA template.

GSTP1 genotyping

Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLPs) were used to determine *GSTP1* genotype as previously described by Zhao *et al.* [13]. The PCR were carried out in a 25 μl mixture containing 100 ng DNA, 20 mM Tris-HCl buffer (pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 unit of Immolase® (Bioline Ltd., UK), and 10 pmol of each primer. The amplified PCR was 189-bp fragment which was achieved by denaturation of 10 min at 95 °C followed by 30 cycles of 30 s 95 °C, 30 s 60 °C, and 30 s 72 °C with final extension for 7 min 72 °C. Each PCR product (20 μl) was digested with *Bsm*AI and resolved by 2% agarose gel electrophoresis. The presence of the polymorphic *Bsm*AI restriction site yielded 148-bp and 41-bp was indicated the presence of the *GSTP1 (Val)* allele.

Table 1 Primer sequences and product size from PCR reaction in *GST* genotyping

Name	Gene	Sequence	Product size
A1	*GSTM1*	5′-CTG CCC TAC TTG ATT GAT GGG-3′	273 bp
A2		5′-CTG GAT TGT AGC AGA TCA TGC-3′	
B1	*β-actin*	5′-GGC CCC TCC ATC GTC CAC CG-3′	496 bp
B2		5′-GGG CAC GAA GGC TCA TCA TT-3′	
C1	*dystrophin*	5′-TTT TCG GTC TCT CTG CTG GTC AGT G -3′	196 bp
C2		5′-CAA AGC CCT CAC TCA AAC AGT AAG C-3′	
D1	*GSTP1*	5′-CCA GTG ACT GTG TGT TGA TC-3′	189 bp
D2		5′-CAA CCC TGG TGC AGA TGC TC-3′	
E1	*GSTT1*	5′-CAG TTG TGA GCC ACC GTA CCC-3′	1460 bp
E2		5′-CGA TAG TTG CTG GCC CCC TC-3′	
F1	*GSTT1*	5′-CCA GCT CAC CGG ATC ATG GCC AG-3′	466 bp
F2		5′-CCT TCC TTA CTG GTC CTC ACA TCT C-3′	

GSTM1 = glutathione S-transferase Mu-1 gene; *GSTP1* = glutathione S-transferase Pi-1 gene;
GSTT1 = glutathione S-transferase Theta-1 gene

GSTT1 genotyping

Analysis for *GSTT1* gene polymorphism was done by multiplex PCR as described by Sprenger *et al.* [14]. The PCR reaction contained 100 ng DNA, 0.2 mM dNTPs, 10 pmol of each primer and 1 U HotStarTaq polymerase (Qaigen®). PCR was carried out with an initial denaturation of 15 min 95 °C followed by 35 cycles of 30 s 94 °C, 30 s at 60 °C, and 1 min at 72 °C with final extension 7 min 72 °C. The amplified amplicons were analyzed by 1.5% agarose gel electrophoresis. PCR products presenting 1.46 kb were defined as deletion and 459 bp as wild type, heterozygous present both bands. The schematic overview of the *GSTM1, GSTP1 and GSTT1* genes along with methods for detection of polymorphisms is in Figure 1.

Statistical analysis

Genotype proportions between case and control groups were compared by using χ^2 test. Logistic regression model used to estimate the odds ratio (OR) for each genotype with 95% confidence intervals and *P*-value less than 0.05 is considered significant.

Results

In this study, newly developed semi-quantitative-DHPLC based analysis was used to detect heterozygosity of *GSTM1* genes providing the precise null allele frequency distribution in the samples including *GSTT1* and *GSTP1* as shown in Table 2. Among three genes, *GSTM1* (-/-) null genotype was found the most prevalent at 56.83% in controls and 58.99% in case. A slight

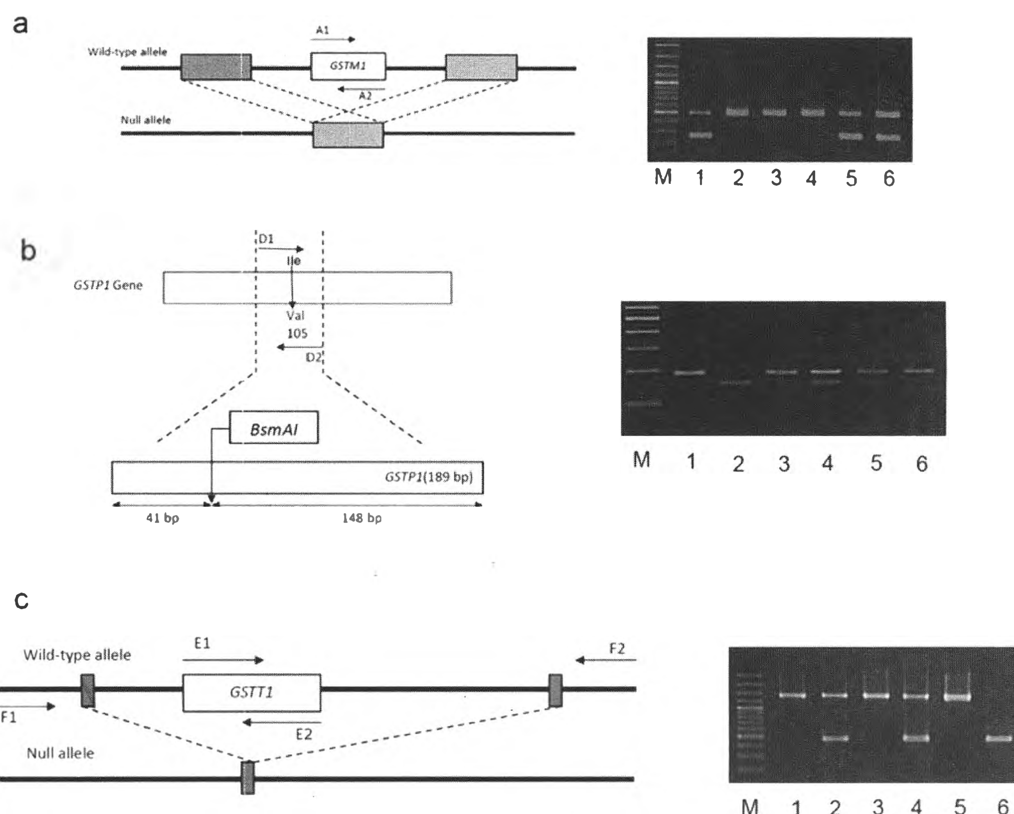

Figure 1 Schematic overview of the *GSTM1, GSTP1 and GSTT1* genes are shown along with methods for detection of polymorphisms. **Panel a** shows *GSTM1* embedded between 4.2 kb repeats region (gray boxes) and A1 and A2 primers are specific to the sequence in *GSTM1* gene. Product band with 278 bp was shown in both wild type and heterozygous mutant. **Panel b** represents polymorphic site of *GSTP1* at codon 105 which was amplified by primer D1 and D2 then PCR product was subjected to *Bsm*AI digestion. The presence of the polymorphic *Bsm*AI restriction site yields 148-and 41-bp fragments indicating *GSTP1 (Val)* allele. **Panel c** demonstrates *GSTT1* embedded between repeats region (gray boxes). Specific primers, E1 and E2 were used to amplify *GSTM1* gene and F1 and F2 for repeats region. The presence of 466 and 1460 bp band are indicated as wild type and mutant, respectively [1]

difference was found with *GSTT1* (-/-) null genotype between cases (35.25%) and controls (28.06%) while *GSTP1* Val/Val genotype frequency was low as presented only 7.19% in case and 6.12% in control group. Insignificant difference in frequencies between cases and controls of *GSTM1* and *GSTT1* were shown while *GSTP1* Ile/Val (isoleucine/valine) genotype demonstrated the decreased risk of bladder cancer (OR = 0.53 [0.33-0.84], p = 0.006).

In double gene interaction analysis, the combination of *GSTM1* with either *GSTP1* or *GSTT1* did not involve with the risk of bladder cancer. In contrast, when *GSTP1* wild type (Ile/Ile) combined with *GSTT1* (+/-) could significantly increase the risk of bladder cancer with OR = 2.39, [1.24-4.59], p = 0.0083. The result is more apparent when *GSTP1* (Ile/Ile) combined with *GSTT1* (-/-) null genotype with OR = 2.44 [1.22-4.90], p = 0.011. There were no significant interactions between *GSTM1*, *GSTP1* or *GSTT1* on modifying the susceptibility of bladder cancer in triple gene analysis as presented in Table 3.

Discussion

This study is clearly presented heterozygosity and allele frequencies of *GSTM1*, *GSTT1* and *GSTP1* in Thai normal subjects and bladder cancer patients. Due to the multi-factorial involvement in cancer etiology, the interaction of numerous genes is the most preference for determination of possible modifying risk of bladder cancer. When consider single gene association, only *GSTP1* Ile/Val genotype demonstrated the protective effect against bladder cancer (OR = 0.53 [0.33-0.84], p = 0.006). This result is in accordant with the study from Coa *et al.* [10] that the substitution of isoleucine with valine in codon 105 of *GSTP1* associated with the decreased susceptibility to bladder cancer. Moreover, the sufficient evidence from Sundberg et al. [9] supported our protective result due to the enhancement of enzyme activity in *GSTP1* valine variant more than wild type. The substitution of bulkier valine was reported to increase affinity toward specific carcinogenic compounds, for example benzopyrene diol epoxide [9]. However, this effect was not observed with *GSTP1* Val/Val which probably resulted from too small numbers of individuals presented Val/Val genotype (Table 2).

In order to see gene interaction effect, double gene analysis was conducted and *GSTP1* (Ile/Ile) in combination with *GSTT1* (+/-) significantly increased bladder cancer risk (OR = 2.39, [1.24-4.59], p = 0.008). This combined effect of two variants (*GSTP1* (Ile/Ile)

Table 2 Distribution of *GST* genotypes in bladder cancer patients and normal controls

GST genotype	Control (N=278)(%)		Patients (N=139)(%)		OR	95% CI	P value
GSTM1							
GSTM1(+/+)	10	(3.60%)	7	(5.04%)	1.0 (ref)	-	-
GSTM1(+/-)	110	(39.57%)	50	(35.97%)	0.6494	0.2337 - 1.8046	0.4048
GSTM1(-/-)	158	(56.83%)	82	(58.99%)	0.7414	0.2722 - 2.0195	0.5569
GSTP1							
GSTP1(Ile/Ile)	153	(55.04%)	94	(67.63%)	1.0 (ref)	-	-
GSTP1(Ile/Val)	108	(38.85%)	35	(25.18%)	0.5275	0.3331 - 0.8353	0.0060*
GSTP1(Val/Val)	17	(6.12%)	10	(7.19%)	0.9574	0.4207 - 2.1880	0.9164
GSTT1							
GSTT1(+/+)	100	(35.97%)	34	(24.46%)	1.0 (ref)	-	-
GSTT1(+/-)	100	(35.97%)	56	(40.29%)	1.6471	0.9907 - 2.7383	0.0534
GSTT1(-/-)	78	(28.06%)	39	(35.25%)	1.4706	0.8510 - 2.5411	0.1659

*p < 0.05; OR = odds ratio; CI = confident interval; *GSTM1*= glutathione S-transferase Mu-1 gene; *GSTP1* = glutathione S-transferase Pi-1 gene; *GSTT1* = glutathione S-transferase Theta-1 gene; (+/+) = wild type alleles; (+/-) = heterozygous mutant alleles; (-/-) = homozygous mutant alleles.

and *GSTT1* (+/-)) that concurrently cause slower detoxification reaction. Likewise, we further see the similar effect in *GSTP1* (Ile/Ile) combined with *GSTT1 (-/-)*.

The protective effect of *GSTP1* (Ile/Val) is in contrast to the results from Indian [15]. They found the highly significant risk of bladder cancer in *GSTP1* Val/Val single gene association (OR = 7.12 [3.14-16.16], p = 0.000) and further with three genes interaction (OR = 7.29 [2.81-18.93], p = 0.000). The conflicts are possibly accounted to the difference of environmental pollutants, industrial chemicals, dietary, and smoking behavior. From one meta-analysis, *GSTP1* (Ile/Val or Val/Val) ascribed higher risk of developing bladder cancer but the odds ratio was not as high as that of Indian [16]. Interestingly, the studies in Chinese were not shown any significant influences. Thai population commonly presented GSTs genotype frequencies comparable to those of Chinese.

Conclusion

In conclusion, *GSTP1* Ile/Val demonstrates the protective effect against bladder cancer while *GSTP1* Ile/Ile combined with either *GSTT1* (+/-) or *GSTT1* (-/-) show the increased risk in Thais. This study provides the effect of *GSTs* polymorphisms with modifying risk to bladder cancer for further clinical research or applications.

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Table 3 Distribution of double and triple *GST* genotypes among bladder cancer patients and normal controls

Double and Triple *GST* genotype	Control (*N*=278)(%)		Patients (*N*=139)(%)		OR	95% CI	*P* value
Double *GST* genotype							
GSTT1 and *GSTP1*							
T1(+/+) and *P1(Ile/Ile)*	58	(20.86%)	19	(13.67%)	1.0000	-	-
T1(+/+) and *P1(Ile/Val)*	38	(13.67%)	12	(8.63%)	0.9640	0.4202 - 2.2117	0.9333
T1(+/+) and *P1(Val/Val)*	4	(1.44%)	3	(2.16%)	2.2895	0.4696 - 11.1609	0.2949
T1(+/-) and *P1(Ile/Ile)*	55	(19.78%)	43	(30.94%)	2.3866	1.2410 - 4.5896	0.0083*
T1(+/-) and *P1(Ile/Val)*	39	(14.03%)	9	(6.47%)	0.7045	0.2890 - 1.7170	0.4397
T1(+/-) and *P1(Val/Val)*	6	(2.16%)	4	(2.88%)	2.0351	0.5186 - 7.9860	0.3011
T1(-/-) and *P1(Ile/Ile)*	40	(14.39%)	32	(23.02%)	2.4421	1.2175 - 4.8985	0.0110*
T1(-/-) and *P1(Ile/Val)*	31	(11.15%)	14	(10.07%)	1.3786	0.6093 - 3.1193	0.4401
T1(-/-) and *P1(Val/Val)*	7	(2.52%)	3	(2.16%)	1.3083	0.3074 - 5.5682	0.7153
Triple *GST* genotype							
GSTM1, *GSTT1* and *GSTP1*							
M1(+/+), *T1(+/+)* and *P1(Ile/Ile)*	49	(17.63%)	25	(17.99%)	1.0000	-	-
M1(+/+), *T1(+/+)* and *P1(Ile/Val or Val/Val)*	42	(15.11%)	12	(8.63%)	0.5600	0.2510 - 1.2492	0.1541
M1(+/+), *T1(-/-)* and *P1(Ile/Ile)*	19	(6.83%)	13	(9.35%)	1.3411	0.5707 - 3.1510	0.4999
M1(+/+), *T1(-/-)* and *P1(Ile/Val or Val/Val)*	10	(3.60%)	7	(5.04%)	1.3720	0.4662 - 4.0375	0.5650
M1(-/-), *T1(+/+)* and *P1(Ile/Ile)*	64	(23.02%)	37	(26.62%)	1.1331	0.6040 - 2.1259	0.6966
M1(-/-), *T1(+/+)* and *P1(Ile/Val or Val/Val)*	45	(16.19%)	16	(11.15%)	0.6969	0.3303 - 1.4704	0.3422
M1(-/-), *T1(-/-)* and *P1(Ile/Ile)*	21	(7.55%)	19	(13.67%)	1.7733	0.8084 - 3.8902	0.1511
M1(-/-), *T1(-/-)* and *P1(Ile/Val or Val/Val)*	28	(10.07%)	10	(7.19%)	0.7000	0.2939 - 1.6675	0.4193

*p < 0.05, OR = odds ratio; CI = confident interval; *GSTM1* = glutathione S-transferase Mu-1 gene; *GSTP1* = glutathione S-transferase Pi-1 gene; *GSTT1* = glutathione S-transferase Theta-1 gene; (+/+) = wild type alleles; (+/-) = heterozygous mutant alleles, (-/-) = homozygous mutant alleles.

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