

RESEARCH COMMUNICATION

Promoter Methylation and Genetic Polymorphism of Glutathione S-Transferase P1 Gene (GSTP1) in Thai Breast-cancer Patients

Tanett Pongtheerat^{1*}, Supparit Pakdeethai¹, Wichai Purisa², Sunanta Chariyalertsak², Songsak Petmitr³

Abstract

The GSTP1 gene encodes for a detoxification enzyme involved in protecting cells from carcinogens. In breast cancer, GSTP1 polymorphisms may produce lower effective enzyme detoxification properties and GSTP1 promoter hypermethylation may result in inactivation of GSTP1 expression. We therefore hypothesized an influence on progression of breast cancer. To study the effect of GSTP1 polymorphisms and CpG-island hypermethylation on GSTP1 promoter, PCR-RFLP and methylation-specific PCR techniques were used with 41 Thai breast-cancer patients. Associations between the codon 105 (A to G) genetic polymorphism, CpG-island hypermethylation, and clinico-pathological parameters were analyzed. GSTP1 hypermethylation was found in 26% of cases and the GSTP1 polymorphism in 14%. GSTP1 hypermethylation was significantly associated with breast cancer; lymph-node metastasis ($P = 0.02$) while GSTP1 polymorphism status significantly varied with progesterone receptor positivity ($P = 0.04$). No association was found between the GSTP1 polymorphism and methylation status. The results indicated that CpG-island hypermethylation of the GSTP1 promoter is associated with a biologically aggressive phenotype, but may not be related to the codon 105 (A to G) gene polymorphism in breast-cancer patients.

Keywords: Glutathione S-transferase P1 - CpG-island hypermethylation - genetic polymorphism - breast cancer

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Introduction

Breast cancer is the most common malignancy of women. In Thailand, the incidence is 20.9/100,000 population. Among Thai women, the incidence of breast cancer is now higher than cervical cancer (Khuhaprema et al., 2010). Many genetic alterations are involved in the development of breast cancer, such as mutation, deletion, gene polymorphism and CpG-island hypermethylation, leading to gene inactivation and affecting protein function.

Glutathione S-transferase P1 (GSTP1) is in a superfamily of enzymes involved in the detoxification of reactive carcinogen metabolites. GSTP1 is an important anti-carcinogenic protein (Ramos-Gomez et al., 2001). A single nucleotide polymorphism at codon 105 (A to G) in exon5 of the GSTP1 gene causes the substitution of isoleucine with valine (Allan et al., 2001); the valine allele may affect the enzyme function and be associated with susceptibility to breast (Kim et al., 2004) and prostate cancers (Cairns et al., 2001).

The hypermethylation of the CpG islands located in the promoter region of genes may result in decrease gene expression, including GSTP1 (Lin et al., 2001). GSTP1

methylation is most commonly found in prostate, liver, and breast cancers (Esteller et al., 2001). Recent studies have reported that GSTP1 down-regulation in breast cancers is related to CpG-island hypermethylation (Parrella et al., 2004). In addition, breast cancers with GSTP1 CpG-island hypermethylation show an aggressive phenotype (Shinozaki et al., 2005), with a poor prognosis (Arai et al., 2006). Thus, we hypothesized that this GSTP1 polymorphism would support GSTP1 CpG-island hypermethylation and therefore significantly impact on clinicopathological parameters in Thai breast cancer cases.

This study used methylation-specific PCR to examine CpG-island hypermethylation of the GSTP1, and to determine GSTP1 gene polymorphism in breast-cancer patients by PCR-RFLP. The correlation between GSTP1 hypermethylation and GSTP1 polymorphism was observed, and the association between GSTP1 hypermethylation and gene polymorphism with the clinicopathological features of breast cancers was analyzed by Chi-square test (significance set at P -value < 0.05). The results of this research may be used to assess the correlation of this gene and the incidence of breast cancer among Thai patients.

¹Unit of Biochemistry, Department of Medical Sciences, Faculty of Science, Rangsit University, Patumthani, ²Research Division, National Cancer Institute of Thailand, ³Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand *For correspondence: dtanett@yahoo.com

Materials and Methods

Sample collection and DNA isolation

DNA samples were extracted from formalin-fixed, paraffin-embedded, breast-cancer samples obtained from 41 Thai breast-cancer patients. The samples were collected from the National Cancer Institute of Thailand. DNA was isolated as previously described (Chariyalertsak et al., 2007).

Methylation analysis

Genomic DNA sample was subjected to sodium bisulfite modification using an EZ DNA Methylation Gold kit (Zymo Research, Orange, CA). Methylated cytosine and unmethylated cytosine in the GSTP1 alleles were detected by methylation-specific polymerase chain reaction (MSP) and unmethylation-specific polymerase chain reaction (USP). The primers used for the amplification of methylated alleles were 5'-TTCGGGGTGTAGCGGTCGTC-3' and 5'-GCCCCAATACTAAATCACGACG-3'. The primers used for the amplification of unmethylated alleles were 5'-GAT GTT TGG GGT GTA GTG GTT GTT-3' and 5'-CCA CCC CAA TAC TAA ATC ACA ACA-3' (Gonzalzo et al., 2003).

DNA was amplified by PCR in a 25 µl mixture containing 20 pmol primer, 5 µl of 10x buffer, 4 µl 1.25 mM of dNTPs, 2.5 mM of MgCl₂, 1U of Platinum Taq DNA polymerase (Invitrogen) and 2-4 µl of bisulfite template DNA. Following initial denaturation at 94°C for 5 minutes, amplification conditions comprised strand separation at 94 °C for 1 min; the primer annealing temperature used for MSP and USP was 64°C for 1 min, polymerization at 72°C for 1 min, 40 PCR cycles were conducted, with a final elongation step of 72°C for 5 minutes. Universal methylated and unmethylated human DNA standard were used as positive and negative controls for the methylated GSTP1 alleles (Zymo Research, Orange, CA). PCR products were observed on 2% agarose gels and visualized with ethidium bromide staining. Amplification resulted in a 93 bp band for methylated alleles and 97 bp band for unmethylated alleles, visualized by ethidium bromide staining.

DNA sequencing analysis

Direct bisulfite DNA sequencing of the PCR product using methylation and unmethylation-specific primers were conducted using a thermal sequencing system (Bio Basic Inc., Canada).

PCR and RFLP detection of GSTP1 polymorphism

PCR primers for the GSTP1 5' region (5'-ACC CCA GGG CTC TAT GGG AA-3'), and the 3' region (5'-TGA GGG CAC AAG AAG CCC CT-3'), were used to amplify a 176 bp fragment. DNA was PCR-amplified in a 50 µl mixture containing 20 pmol primer, 5 µl of 10x buffer, 8 µl 1.25 mM of dNTPs, 1.5 mM of MgCl₂, 2.5 U of Taq polymerase and 100 ng of template DNA. Following initial denaturation at 94°C for 5 minutes, amplification conditions comprised strand separation at 94 °C for 30 seconds, primer annealing at 55°C for 30 seconds, polymerization at 72°C for 30 seconds, 30 PCR cycles

were performed, with a final elongation step of 72°C for 5 minutes. The amplification band was visualized by ethidium bromide staining (Jerónimo et al., 2002).

The exon 5 polymorphic site in the GSTP1 locus (Ile-105-Val) was detected by restriction fragment length polymorphism of the PCR product (PCR-RFLP). The A to G polymorphism of GSTP1 introduces a restriction site recognized by the Alw26I (BsmA1) restriction enzyme. The PCR products are digested with 1 U Alw26I in 20 µl volume for 18 h at 37°C, which results either in retention of the 176 bp product or complete digestion to 91 bp and 85 bp fragments corresponding to individuals homozygous for the Ile or Val alleles, respectively. The presence of all three fragments corresponds with individuals heterozygous at codon 105. Different detected alleles were separated on 3.5% agarose gel, and visualized by ethidium bromide staining.

Statistical analysis

The association between GSTP1 polymorphism and methylation status, as well as any correlation between clinico-pathological parameters (lymph node, estrogen receptor (ER), progesterone receptor (PR), HER-2, tumor size, and age at diagnosis) of the patients with GSTP1 polymorphism and methylation status were examined by Chi-square test. A P-value < 0.05 was considered significant.

Results

Methylation analysis

The GSTP1 methylation status was analyzed by methylation-specific PCR technique for 41 breast-cancer patients' DNA samples (Figure 1). Eleven (26%) samples were positive for methylation status and 30 (74%) negative for unmethylation status. The methylation study was confirmed by DNA sequencing (Figure 2). Completely methylated (CG) sites were observed in sample 5, with no

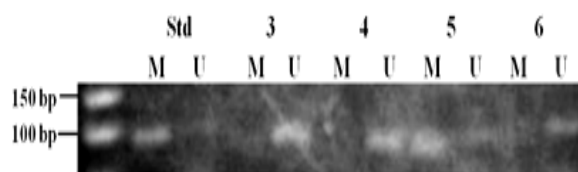


Figure 1. MSP of GSTP1 in Breast-cancer Tissues were Detected by 2.0% Agarose Gel and Visualized by Ethidium Bromide Staining. Universal methylated human DNA standard (Std) as a positive control; M, methylated; U, unmethylated

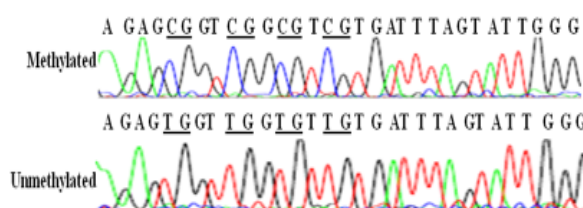


Figure 2. Bisulfite DNA Sequencing of Methylated (top), Unmethylated (bottom) Samples. There was a "C" peak and "T" peak at the CpG sites in methylated and unmethylated samples, respectively

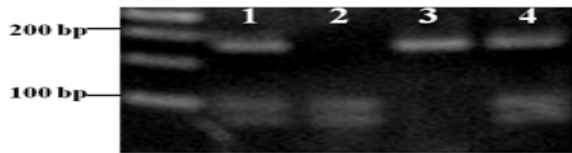


Figure 3. PCR-RFLP product for GSTP1 was detected by 2.0% agarose gel and visualized by ethidium bromide staining; lane 1, 4 are heterozygous genotype(C/T), lane 2 is homozygous mutant genotype (T/T), lane 3 is wild homozygous genotype (C/C)

Table 1. GSTP1 Methylation Status and Clinico-Pathological Parameters of Breast- Cancer Patients

Parameter	Methylation status		P-value *significant
	U (%)	M (%)	
Lymph node			0.02*
+	10(59)	7(41)	
-	19(90)	2(10)	
Estrogen receptor			0.16
+	12(67)	6(33)	
-	18(86)	3(14)	
Progesterone receptor			0.18
+	9(64)	5(36)	
-	20(83)	4(17)	
HER-2			0.61
+	16(57)	12(43)	
-	12(80)	3(20)	
Tumor size (cm.)			0.80
<3	22(79)	6(21)	
>3	9(75)	3(25)	
Age			0.54
≤45	10(71)	4(29)	
>45	20(80)	5(20)	

Table 2. GSTP1 Gene Polymorphism and Clinico-Pathological Parameters of Breast- cancer Patients

Parameter	Polymorphism		P-value *significant
	wild type n(%)	mutant n(%)	
Lymph node			0.43
+	14(82)	3(18)	
-	19(90)	2(10)	
Estrogen receptor			0.72
+	17(85)	3(15)	
-	16(89)	2(11)	
Progesterone receptor			0.04*
+	11(73)	4(27)	
-	12(92)	1(8)	
HER-2			0.87
+	21(86)	3(14)	
-	12(86)	2(14)	
Tumor size (cm.)			0.95
<3	26(87)	4(13)	
>3	7(88)	1(12)	
Age			0.10
≤45	12(36)	21(64)	
>45	0(0)	5(100)	

Table 3. GSTP1 Gene Polymorphism and Methylation Status Among Breast-cancer Patients

Methylation status	Polymorphism		Odds ratio	P-value
	Wild type n(%)	Mutant n(%)		
U	26(87)	4(13)	0.06	0.69
M	9(82)	2(18)		

MSP the CpG sites were completely unmethylated (TG) in sample 3. The correlations between GSTP1 methylation status and patients' clinico-pathological parameters are shown in Table 1. Patients' methylation status was significantly associated with lymph-node metastasis ($P = 0.02$), whereas no significant differences were found for estrogen receptor (ER), progesterone receptor (PR), HER-2, tumor size, or age at diagnosis.

RFLP Detection of GSTP1 genes polymorphisms

GSTP1 genetic polymorphisms were detected by PCR-RFLP technique; the results showed 3 types of polymorphism among breast-cancer patients (Figure 3). The overall frequency of mutant GSTP1 among the cases was 14%. The association between GSTP1 genetic polymorphism and patients' clinico-pathological parameters is shown in Table 2. The results showed that GSTP1 polymorphisms were only significantly associated with progesterone receptor ($P = 0.04$). No statistically significant correlation was observed between GSTP1 polymorphisms and methylation status (Table 3).

Discussion

Glutathione S-transferase is a detoxification enzyme that protects cells from the reactive metabolites of carcinogens. Several studies have indicated that hypermethylation of the CpG-islands located in the GSTP1 promoter region are involved in carcinogenesis (Baylin et al., 2001; Widschwendter and Jones 2002). It has been reported that the silencing of GSTP1 gene expression in breast cancer is induced by CpG-island hypermethylation, observed in 13-30% of breast cancers (Esteller et al., 1998; Shinozaki et al., 2005). In the present study, we investigated the frequency of GSTP1 CpG-island hypermethylation using formalin-fixed, paraffin-embedded breast-cancer samples, and found that 26% of patients showed CpG-island hypermethylation; we confirmed the methylated nucleotides by DNA sequencing technique.

GSTP1 hypermethylation was found to be significantly associated with lymph-node metastasis. These observations indicated that breast tumors with GSTP1 CpG-island hypermethylation may possess have a biologically aggressive phenotype, as suggested in previous studies that reported this condition in Asian and Caucasian breast cancers (Shinozaki et al., 2005; Arai et al., 2006), and other cancers, such as prostate (Bernardini et al., 2004) and endometrial cancer (Chan et al., 2005). However, some research reports have concluded that GSTP1 hypermethylation was not associated with lymph-node metastasis in breast cancers of Asian and Caucasian women (Lee et al., 2007).

GSTP1 (Ile105Val) polymorphism may affect the function of enzymes and decreased enzyme activity (Allan et al., 2001). No previous studies showed that GSTP1 polymorphism was associated with an elevated risk of breast cancer (Helzlsouer et al., 1998; Egan et al., 2004). Our previous study reported that GSTP1 polymorphism was unrelated to increased risk of breast cancer, but found a relationship between GSTP1 genotype and progesterone

receptor protein (Pongtheerat et al., 2009). These results agreed with the results of the current study, which showed a relationship between GSTP1 genotype and progesterone receptor (P = 0.04).

Previous findings indicated that, GSTP1 polymorphism was associated with decreased protein function and that GSTP1 hypermethylation correlated with lack of protein expression, and was found in aggressive-phenotype breast cancer. Thus, it was suspected that the polymorphism may be promoted or linked with GSTP1 hypermethylation status. However, the results of this study showed no association between GSTP1 hypermethylation and GSTP1 polymorphism, agreeing with a previous reported on prostate cancer (Jerónimo et al., 2002).

In conclusion, it was found that 26% of Thai breast cancers had GSTP1 CpG-island hypermethylation. This hypermethylation is associated with lymph-node metastasis, and may be used as an aggressive biological marker in breast cancer. No association was found between GSTP1 hypermethylation and GSTP1 (Ile105Val) polymorphism.

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