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Original articles

Impact of CpG site specific methylation on silencing protein expression of GSTP1 in breast cancer

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ABSTRACT

Introduction: From previous study with Methylation specific polymerase chain reaction (MSP), there were cases of DNA methylation showing positive protein expression. Some CpG sites of the primer were speculated to have no effect on protein expression.

Methods: Forty-six cases were selected including the two problem cases from our previous study samples of 88. The DNA extracts were sequenced after bisulfite conversion for the epigenetic part of the GSTP1 gene, 298 base pairs, which comprised 38 CpG sites. Methylation status at the sites 10-13 and 20-22 representing in the primer used in MSP study was correlated with the expression of protein to detect the active and inactive sites.

Result: The two problem cases revealed methylation of CpG sites no.11, 12, 13 and no. 21, 22, suggested that these were inactive sites. The sites no. 10 and 20 were regarded as active sites. The validity was confirmed on the methylation data of the rest of 44 cases. There were 11 cases showing methylated cytosine at either CpG site no. 10 or 20. All cases showed negative for the protein. On the other hand, of the 21 cases with positive protein, none elicited methylation at CpG sites no.10 and 20.

Conclusion: The impact of location of CpG sites on the silencing of the protein expression of GSTP1 is present. There are at least two active sites at no.10 and 20. Further studies to verify the other active sites are encouraging.

Keywords: DNA methylation, CpG site, Protein expression, GSTP1, Breast cancer

INTRODUCTION

DNA methylation is a well-established epigenetic mechanism in regulation of protein expression[1]. The conjoint mechanism is unclear whether the inhibition is dependent on level of methylation or site specific of CpG that is methylated [2, 3]. Regarding our finding in the previous study, there were some cases of hypermethylation found and protein present for GSTP1 in invasive breast carcinoma[3]. We speculated that this event might be due to positive methylation in "inactive" CpG sites. To prove this concept of some sites when methyl-

ated will affect the gene silencing on encoding protein while other sites when methylated will not, we carried out the study with Bisulfite sequencing technique to reveal the methylation status of the CpG sites that were comprised in the primer of former MSP study and verify the concept statement.

MATERIAL AND METHOD

Sample collection

From our previous study, the correlation between hypermethylation by Methylation specific PCR (MSP) technique and protein expression by immunohistochemistry were categorized into four groups of samples (shown in table1). This studied samples were randomly drawn from each group with an approximate ratio; they comprised 2 cases of hypermethylation with protein expression, 8 cases of hypermethylation with non-protein expression, 21 cases of unmethylation with protein expression and 15 cases of unmethylation with non-protein expression.

DNA isolation

Fresh tumor tissues of breast cancer kept in the refrigerator (-80°C) were thawed. The source and specimen data had mentioned in the previous paper. DNA was isolated by phenol-chloroform method; in brief, incubate the tissue samples with proteinase K for 16-18 hours before phenol extraction and sodium acetate-isopropanol precipitation. Concentration of DNA was verified by Nanodrop 2000c Thermo Scientific.

Bisulfite sequencing

- Bisulfite reaction

Genomic DNA 400 ng was performed using EZ DNA Methylation-Gold kit™ (Zymo Research). CT conversion reagent consisted of 900 µl distill water, 300 µl M-dilution buffer and 50 µl M-dissolving buffer. Each reaction used 130 µl of the CT conversion reagent to 20 µl of DNA sample in a PCR tube. The tubes were placed in Thermal Cycler, incubated at 98°C for 10 minutes and 64°C for 2.5 hours. Products could be stored at 4°C. More details are elaborated elsewhere[3].

- PCR reaction

PCR was performed in Vertiri 96 well thermo cycler Applied Biosystem™ for 45 cycles. The PCR program started with activation of the polymerase at 95°C for 15 min followed by denaturation at 95°C for 1 min, annealing at 64°C for 1 min and extension at 72°C for 1 min followed by a final 4 mins extension at 72°C and cooling at 4°C.

The reaction was assembled in a final volume at 30 µl, containing 0.6 µl of dNTP, 3 µl of 10x buffer, 0.3 µl of primer, 2 µl of bisulfite treated

Table 1 Number of cases in the strata of methylation and protein expression of GSTP1 in breast cancer of the previous and present studies

Methylation status by MSP	Number of cases from previous study	Number of cases used in this study
Hypermethylation		
- with protein expression	6	2
- with non-protein expression	13	8
Unmethylation		
- with protein expression	42	21
- with non-protein expression	26	15

DNA and 21.1 µl of water.

PCR products were run on 8% polyacrylamide gel to verify the correct size.

Before the sequencing reaction, PCR products were purified with ExoSAP™ for cleanup of PCR products that eliminates unincorporated primers and dNTPs.

- Sequencing reaction

The sequencing reaction contains DNA template from purified PCR product, reverse primer 0.8 pmol/µl, sequencing buffer and dNTP/ddNTP substrate from the BigDye terminator v3.1 Cycle Sequencing kit (Applied Biosystem, USA). The mixture was performed on Thermocycler and program started with 95°C for 1 minute and 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 minutes for 25 cycles and 60°C for 4 minutes in the final step.

DNA bisulfite sequencing was analyzed by automate capillary electrophoresis and the reaction was purified by 3M sodium acetate precipitation before capillary electrophoresis using Genetic analyzer ABI3130.

Immunohistochemistry and Methylation specific PCR

Data were obtained from the previous study and the method descriptions were referred to the paper[3]. In order to verify the result of immunohistochemistry, the relevant stained slides were reviewed, scanned with Image Scope®, and 400 tumor cells were counted for the positive staining. The cut off was used at 10%.

RESULTS

1. CpG sites

The Bisulfite sequencing method successfully revealed 32 CpG sites, the numbers started from 5' end of the PCR products were CpG sites no. 1, 2 …. till 32 in sequence. From the mapping to the gene bank data, the primer used in the previous MSP study was composed of CpG sites no. 10, 11, 12, 13, 20, 21 and 22 (totally 7 sites).

2. Candidate active and inactive CpG sites

The candidate inactive CpG sites were derived from the group of hypermethylation with protein expression. The methylated sites in the two samples comprised sites no. 3, 7, 11, 12, 13, 14, 16, 17, 19, 21, 22, 23, 24, 25, 26 and 27 were not able to silencing the gene on encoding the protein (Table 2). Five of which, CpG sites no. 11, 12, 13, 21 and 22, were components of the primer of MSP.

The candidate active CpG sites were the rest of the CpG sites of the primer of MSP. They were CpG sites no. 10 and 20.

3. Verified active CpG sites

From Table 3, the samples of the group of MSP-related hypermethylation and non-protein expression revealed 6 out of 8 cases were found methylation involving either CpG no.10 or 20.

Table2 Methylation status by Bisulfite sequencing technique in the group of MSP-related hypermethylation and protein expression

Hypermethylation with protein expression

No.	IHC	MSP	CpG site																														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	+	m	N/A	N/A		N/A	N/A	N/A		N/A	N/A						N/A			N/A											N/A	N/A	N/A	N/A
2	+	m	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A						N/A		N/A	N/A										N/A	N/A	N/A	N/A

Methylation; Partial methylation; Unmethylation; N/A Inconclusive result; CpG included in MSP primer

Table3 Methylation status by Bisulfite sequencing technique in the group of MSP-related hypermethylation and non-protein expression

Hypermethylation with non-protein expression

No.	IHC	MSP	CpG site 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
3	-	m	Partial	Partial	Partial	N/A	Partial	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Methylation	Unmethylation	Partial	Methylation	Unmethylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	N/A	N/A	N/A	N/A
4	-	m	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Partial	N/A	N/A	N/A	N/A
5	-	m	Partial	N/A	Methylation	Methylation	Methylation	Methylation	N/A	N/A	N/A	Partial	Methylation	Partial	N/A	Methylation	Partial	Methylation	Methylation	Unmethylation	Methylation	N/A	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	N/A	Methylation	N/A	Methylation	Methylation	Methylation
6	-	m	Unmethylation	Partial	N/A	N/A	Unmethylation	Methylation	Unmethylation	Unmethylation	N/A	Unmethylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Partial	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	N/A	N/A	N/A	N/A	N/A
7	-	m	Unmethylation	N/A	Partial	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Methylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Methylation	N/A	Unmethylation	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	-	m	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	N/A	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Unmethylation	N/A	N/A	N/A	N/A
9	-	m	Unmethylation	N/A	Methylation	Methylation	Methylation	Partial	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	Unmethylation	Unmethylation	Methylation	Unmethylation	Unmethylation	Unmethylation	Methylation	Methylation	Unmethylation	Unmethylation	Partial	Methylation	Partial	N/A	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation	Methylation
10	-	m	Methylation	Methylation	Methylation	Methylation	Unmethylation	Methylation	Unmethylation	Methylation	Methylation	Methylation	Partial	N/A	Partial	Unmethylation	N/A	Unmethylation	Unmethylation	N/A	Unmethylation	Unmethylation	N/A	Unmethylation	Unmethylation	Unmethylation	N/A	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A

Methylation; Partial methylation; Unmethylation; N/A Inconclusive result; CpG included in MSP primer

From Table 4, the samples of the group of MSP-related unmethylation with protein expression showed none of the 16 cases had methylation at CpG no. 10 and 20.

From Table 5, there were 15 samples of the group of MSP-related unmethylation with no protein, three cases of which were confirmed by bisulfite sequencing as truly unmethylation. Three cases out of the 12 cases that disclosed methylated cytosine by Bisulfite sequencing technique elicited either CpG 10 or 20.

DISCUSSION

DNA methylation is an epigenetic mechanism that is associated with many phenomena such as gene expression, genomic imprinting, and X-chromosome inactivation[4]. The mechanism on the protein expression is likely complicated; the

Table4 Methylation status by Bisulfite sequencing technique in the group of MSP-related unmethylation and protein expression

Unmethylation with protein expression

No.	IHC	MSP	CpG site 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
11	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Partial	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	Unmethylation	N/A	N/A	N/A	N/A	N/A	Unmethylation	Unmethylation	N/A
12	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Methylation	N/A	N/A	Unmethylation	N/A	N/A	N/A	N/A	N/A
13	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A	N/A
14	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A
15	+	u	N/A	Unmethylation	N/A	N/A	N/A	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	Unmethylation	N/A	N/A	N/A	Methylation	N/A	N/A	N/A	Unmethylation	Unmethylation	N/A	N/A	Unmethylation	Unmethylation	Methylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A
16	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A
17	+	u	Unmethylation	N/A	Partial	N/A	N/A	Unmethylation	Unmethylation	Methylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	Methylation	N/A	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A
18	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Methylation	N/A	N/A
19	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A
20	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Methylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A
21	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A
22	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A
23	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation
24	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A	N/A
25	+	u	Unmethylation	Unmethylation	Methylation	N/A	N/A	Unmethylation	N/A	N/A	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	Partial	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	Unmethylation	N/A	N/A	Unmethylation	Methylation	Methylation	Methylation	Unmethylation	N/A	N/A	N/A	N/A
26	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A
27	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	Unmethylation	N/A
28	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A
29	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	N/A	N/A	N/A
30	+	u	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Partial	N/A
31	+	u	Unmethylation	Unmethylation	Partial	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Partial	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Methylation	Methylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	Unmethylation	N/A	Methylation	Methylation	Unmethylation	N/A	N/A	Unmethylation	N/A

Methylation; Partial methylation; Unmethylation; N/A Inconclusive result; CpG included in MSP primer

Table5 Methylation status by Bisulfite sequencing technique in the group of MSP-related unmethylation and non-protein expression

Unmethylation with non-protein expression

No.	IHC	MSP	CpG site 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
32	-	u	N/A	N/A	■		N/A		N/A	■														N/A	N/A	N/A	■	■	N/A		N/A	N/A	N/A	N/A
33	-	u																														N/A		
34	-	u																												■	N/A	N/A	N/A	N/A
35	-	u	■	■	■	■	■	■	N/A	N/A	N/A	■	N/A	■		N/A		■		■	N/A	N/A	N/A	■	■	■	■	■	■	■	■	■	■	N/A
36	-	u		■	■	N/A	■	N/A	N/A	N/A	N/A	■	N/A	N/A	■	■	N/A	■	■	■			■	■	■	■		■	■		N/A	N/A	■	N/A
37	-	u																			■					■	■	N/A		N/A	N/A	N/A	N/A	N/A
38	-	u		■	■	■	■	■	■	■	■	■	■		■			■	■	■	N/A	■		■	■	N/A	■	■	■	N/A	N/A	■		N/A
39	-	u	■		■	N/A	■	N/A	N/A	N/A	N/A		■				N/A			■	N/A	N/A	■	■	N/A	■	N/A	■	■		N/A	N/A	N/A	N/A
40	-	u	N/A	■	■	N/A		N/A	■	N/A	N/A		■	N/A	■	■	N/A	■	■	■	N/A	N/A	■	■	■	■	■	■	■	■	■	■	■	■
41	-	u						■																		N/A					N/A	N/A	N/A	N/A
42	-	u																													N/A	N/A	N/A	N/A
43	-	u																						N/A	N/A		N/A			N/A	N/A	N/A		N/A
44	-	u																									N/A	N/A		N/A	N/A	N/A	N/A	N/A
45	-	u																								■	N/A	N/A	■		N/A	N/A	N/A	N/A
46	-	u				N/A	N/A			N/A		N/A	N/A		N/A	N/A	N/A	■		N/A	■	N/A	N/A	N/A	■	N/A	N/A	■	■	N/A	N/A	N/A	N/A	N/A

■ Methylation Partial methylation Unmethylation N/A Inconclusive result

CpG included in MSP primer

attributed factors might include level and location of methylation[2].

DNA methylation is the adding of methyl group into 5-carbon position of cytosine that interferes the binding of transcription factor and thus protein expression[5,6]. As natural regulation, the level of methylation can be found on reducing or inhibiting the expression of consequent protein[1,6]. In pathologic process, DNA methylation is attributable to carcinogenesis as well as survival prognosis and treatment response[1, 5, 7, 8, 9, 10]. Jain S. et al used Bisulfite sequencing to study methylation status in individual CpG sites on promoter GSTP1 gene in various diseases of the liver and found that there are CpG site specific for disease subtypes and cancer[7]. Lin et al. reported the correlation between CpG island specific hypermethylation and disease severity of prostate cancer[11]. It is possible that some sites are active and some sites are not active. In addition, active sites may work in quantitative effects.

There were papers that quoted MSP positive and protein expression, though existed in a small percentage[3,12,13,14]. The previous study was shown by our group for the GSTP1[3] and there were for MGMT, GSTP1 and ATM by other authors [12,13,14]. These discordances between DNA methylation and the protein expression were probably from the MSP-based detections of some inactive methylated CpG sites. The individual positive sites could be only viewed with a DNA sequencing technique. In this study, we conducted with Bisulfite sequencing (BS) method to disclose the methylation in individual CpG sites. The MSP-based CpG sites are corresponding to the sites no. 10-13 and 20-22, totally 7 sites. We can find that the sites no. 10 and 20 are likely to be the active and all the other five CpG sites are inactive.

BS method is laborious , not suitable for use as a routine medical test. It is essential when individual CpG methylation status need known. In addition, the method is able to reveal a long segment of DNA region and to show whether the site is partially or fully methylated. In this study, we could demonstrate the CpG sites as far as 32 sites and with some partially methylated sites and much more sites with full methylation.

Although we experimented with a limited number of samples, the findings are adequate for the

explanation; however repeated studies in a larger sample size is encouraging in order to achieve a detailed solution. From our findings, the methylated CpG sites that protein GSTP1 existed are regarded as inactive. There are 16 sites namely no. 3, 7, 11, 12, 13, 14, 16, 17, 19, 21, 22, 23, 24, 25, 26 and 27. The discovery of CpG no. 10 and 20 as active sites is not explicit since the other 14 sites that were not included in the candidate inactive group are await verification. The effect of the level of methylation is also not exhibited herewith since there was not any partial methylation of the CpG no.10 and 20 belonging to the protein presence group. The only partially methylated cytosine found at CpG no.10 was associated with fully methylated CpG sites outside the candidate inactive CpG sites.

MSP is the commonly used technique due to high sensitivity, specificity and l [3, 13, 15]. It is feasible to routine. Nevertheless, the reliability is dependent greatly on the primer design. We had tried our best to search the primer that had been used for the MSP to detect DNA methylation on the promoter of GSPT1 gene and found that all the published works quoted the primer from one single source that the specificity was unknown [16]. Based on our protein expression results, the new primer design to cover more active CpG sites may need. This can be accomplished if we know the impact of the remaining CpG sites including CpG sites no. 1,2,4,5,6,8,9,15,18,28-32. On the other hand, in our current experiment, many inconclusive results of CpG sites as well as the unble sequenced CpG sites that were the no. 33-38 were probably limitation of the BS. Pyrosequencing might be the technique of choice for further investigation [17].

In conclusion, the impact of location of CpG sites on the silencing of the protein expression of GSTP1 is present. There are at least two active sites and sixteen inactive sites out of the 32 CpG sites that were investigated. Further studies to design a new primer for the MSP are encouraging.

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