Promoter Methylation and Differential Expression of π -Class Glutathione S-Transferase in Endometrial Carcinoma

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π -Class glutathione S-transferase (*GSTP1*), located on chromosome 11q13, codes for a phase II metabolic enzyme that detoxifies reactive electrophilic intermediates. The protein also interacts with steroid hormones in the human body. The role of *GSTP1* in endometrial carcinoma has not been reported. In this study, we aimed at determining the expression of *GSTP1* in relation to the epigenetic and genetic changes of the gene in endometrial carcinoma. The *GSTP1* protein and mRNA expression was assessed by immunohistochemistry on tissue microarray and quantitative real-time reverse transcriptase-polymerase chain reaction, respectively. Its methylation status was studied by methylation-specific polymerase chain reaction and bisulfite sequencing. Possible mutations in coding region of *GSTP1* were assessed by cDNA sequencing. Ninety-seven cases of endometrial carcinoma with available tissue blocks and clinical data were studied. Our results showed that 68.0% (66 of 97) of the cases showed reduced protein expression while 64% (16 of 25) showed reduced mRNA expression; 30.9% (30 of 97) of the cases demonstrated methylated alleles in at least one of the six methylation-specific polymerase chain reaction reactions. The methylation status significantly correlated with reduced protein expression ($P = 0.008$) and reduced mRNA expression ($P = 0.003$). Methylation at non-CpG sites including CpCpG trinucleotides and CpT dinucleotides were also observed. cDNA sequencing did not reveal genetic alterations in coding region of the gene. The extent of myometrial invasion was found to be significantly correlated with both the methylation status ($P = 0.009$) and the protein expression ($P = 0.036$) of the *GSTP1* gene. We postulated that hypermethylation of the *GSTP1* gene promoter region may act as a dynamic regulation

mechanism contributing to reduced *GSTP1* expression, which is associated with myometrial invasion potential of the endometrial carcinoma. (*J Mol Diagn* 2005, 7:8–16)

Endometrial carcinoma is the most common cancer of the female genital tract with the incidence rate of 25.6 per 100,000 females in the United States in 1999.¹ The incidence of this cancer in Asia is relatively lower than that in North America. In Hong Kong, endometrial carcinoma is the third leading gynecological cancer with an incidence rate of 9.4 per 100,000 females in the same year.² Endometrioid adenocarcinoma is the most common histological subtype in endometrial carcinoma, accounting for ~80% of all of the cases.³ Like most malignancies, endometrial carcinoma results from the accumulation of genetic and epigenetic alterations in oncogenes, tumor suppressor genes, and genes involved in metabolism or DNA repair.⁴

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of phase II metabolic enzymes, which detoxify a wide variety of reactive electrophilic intermediates by conjugating them with glutathione.⁵ Moreover, GSTs may also be engaged in the intracellular transport of steroid hormones.⁶ There are at least four gene superfamilies (α , π , μ , and θ) coding for soluble GSTs in human.⁷ Combination of subunits from the same gene family results in the formation of a large number of enzymes that are homodimers or heterodimers with different kinetic properties. Among all these GSTs, the π -class enzyme *GSTP1* is the major GST isoform expressed consistently in a wide range of tissues including prostate, placenta, breast, colon, brain, esophagus, lung, and spleen.^{8,9} Inactivation of

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the *GSTP1* expression was found to be associated with methylation of the CpG dinucleotides in the promoter region of the gene.¹⁰

In normal cells, CpG islands in gene promoter regions facilitate the expression of housekeeping genes, tumor suppressor genes, and genes involved in cellular metabolism.¹¹ When methylation of CpG islands occurs, CpG island-dependent gene expression is affected. As a result, such epigenetic changes provide an important alternate pathway to gene deletion or mutation in coding regions for gene silencing.

Methylation status of *GSTP1* has been studied in some human cancers especially the prostatic cancers.^{8,12} It has been reported that hypermethylation in the 5' region of *GSTP1* occurs frequently (~90%) in prostatic cancers including prostatic intraepithelial neoplasia, and this methylation is accompanied by gene silencing. Both prostatic and endometrial carcinomas are cancers with steroid hormone-related carcinogenesis and may share similar genetic alterations.¹³ We therefore hypothesized that hypermethylation of CpG dinucleotides in the *GSTP1* promoter region may also be an important event in endometrial cancers.

In this study, we aimed at evaluating the expression level, methylation status, and genetic profiles of *GSTP1* in endometrial carcinoma. We performed immunohistochemical staining on tissue microarrays (TMAs) of endometrial carcinoma to determine the *GSTP1* protein expression, real-time reverse transcriptase-polymerase chain reaction (RT-PCR) to quantify mRNA expression of the gene, methylation-specific polymerase chain reaction (MS-PCR), and bisulfite sequencing to evaluate methylation status of its promoter and cDNA sequencing to screen for genetic aberrations. Genetic and epigenetic alterations were then correlated with clinicopathological parameters of the patients.

Materials and Methods

Clinical Samples and Cell Line

The tissue samples of 97 endometrial carcinomas were collected from hysterectomy specimens from patients managed in Queen Mary Hospital, the University of Hong Kong, from 1993 to 2001. The pathological diagnosis was made using established criteria.^{14,15} Clinical data were also retrieved. Patients' age ranged from 31 to 97 years (mean age, 56.9 years). Sixty-nine of the cases were in stage I, 12 in stage II, and 16 cases in stage III. Histologically, there were 70 pure endometrioid adenocarcinomas, 12 endometrioid adenocarcinomas with focal squamoid differentiation, 3 with mucinous differentiation, and 2 with secretory change as well as 7 high-grade papillary serous carcinoma, and 3 clear cell carcinoma. For comparison, formalin fixed paraffin-embedded tissues from 8 samples of proliferative endometrium, 20 cases of simple hyperplasia of endometrium, 11 cases of complex hyperplasia, and 20 cases of atypical hyperplasia, some with focal secretory changes were also recruited for immunohistochemical study.

Selective tissue blocks were snap-frozen in liquid nitrogen soon after surgical removal with additional representative blocks fixed in formalin and embedded in paraffin. The nontumor counterparts, including uninvolved endometrial tissue or fallopian tube, were also sampled. Histopathology reports and slides as well as clinical data of these patients were reviewed by gynecological oncologists and pathologists before the study.

The human endometrial cancer cell line *HEC-1A* (derived from endometrial adenocarcinomas) was purchased from the American Type Culture Collection (Manassas, VA). It was maintained in McCoy's 5a medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum.

RNA and DNA Extraction

Before extraction, 5- μ m-thick frozen sections were cut from each tissue block for hematoxylin and eosin (H&E) staining to confirm histological diagnosis and define purity of cancer in the blocks. If necessary, two 24-gauge needles were used to microdissect tissues from the sections such that each sample used in this study contained more than 80% of histologically confirmed cancer cell population. The corresponding nontumor blocks were also examined to ascertain absence of tumor. Total RNA was extracted using Trizol (Life Technologies, Inc.).¹⁶ Genomic DNA of tissue samples and cell line were extracted by digestion with 100 μ g/ml of proteinase K followed by standard phenol-chloroform-isoamyl alcohol extraction (25:24:1) and ethanol precipitation.¹⁷ The quality and quantity of the extracted DNA were checked under gel electrophoresis and measured by spectrophotometry.

Construction of TMA

The paraffin-embedded tumor tissues were used for constructing TMA blocks. Selected cancer foci were marked on H&E-stained sections. The TMAs were constructed as previously described.¹⁸ A tissue-arraying instrument (Beecher Instruments, Silver Spring, MD) was used to acquire cylindrical core tissue biopsies with a diameter of 0.6 mm from histologically representative areas of the donor blocks. Forty-eight tissue cores were composited into a single recipient paraffin block at defined array positions. Two core tissue biopsies were obtained from each specimen and represented in duplicate on the array in this study. Four- μ m sections were cut from the TMA block and mounted on 2% aminopropyltriethoxysilane-coated glass slides. The presence of tumor tissue on the arrayed samples was verified on H&E section to confirm tissue morphology (Figure 1A).

Immunohistochemical Study

Five- μ m-thick sections were cut from paraffin-embedded whole tissue blocks or blocks of TMA, dewaxed in xylene, and rehydrated in a graded series of alcohol washes down to water. The sections were tested with primary

Figure 1. A: TMA was constructed containing 48 cases of endometrial carcinoma in each block. B and C: Immunohistochemical staining of the *GSTP1* protein in endometrioid adenocarcinoma. Two representative cases showing reduced cytoplasmic and nuclear staining in tumor cells (T) compared with residual normal endometrial glands (NT) present. D: Strong immunoreactivity was observed in foci of squamous differentiation (Sq).

mouse anti-human *GSTP1* monoclonal antibody (1:100 dilution; DAKO, Glostrup, Denmark) overnight at 4°C, then with biotinylated secondary rabbit anti-mouse antibody (1:200 dilution, DAKO) for 40 minutes at room temperature.¹⁶ Color development was performed using streptavidin-biotin peroxidase and diaminobenzidine-hydrogen peroxide. The sections were then counterstained with hematoxylin. Normal thyroid tissue section stained with *GSTP1* antibody was used as positive control, whereas section with rabbit serum replacing the *GSTP1* antibody was used as negative control.

Real-Time Quantitative RT-PCR

The mRNA level of the *GSTP1* gene in 25 cases of endometrial carcinomas, including 21 endometrioid adenocarcinoma, and 4 nonendometrioid cases, was assessed by real-time RT-PCR using 5' nuclease assay. First strand cDNA of each sample was synthesized from 2 µg of total RNA by reverse transcription using the SuperScript II RNase H⁻ reverse transcriptase system (Invitrogen, Life Technologies Inc., Rockville, MD). Synthesized cDNA was diluted to 10 ng/µl for the real-time RT-PCR that was performed using the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) in a total volume of

25 µl that contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.34 µmol/L each forward and reverse primers (Table 1), and 0.14 µmol/L of TaqMan probe (Table 1) labeled with the reporting dye FAM (6-carboxy-fluorescein) and the quencher dye TAMRA (6-carboxytetramethyl-rhodamine). Linearized pGEM-T vector containing the insert of *GSTP1*, the housekeeping genes *TBP* (GenBank accession no. NM_003194) or *GAPDH* (GenBank accession no. NM_002046) served as standards, and the calibration standard curve was set up with three serial 10-fold dilutions. Primers and TaqMan probes for each of the genes (Table 1) were designed to span over two exons to prevent amplification of residual genomic DNA. All of the standards and samples for real-time quantitative RT-PCR were duplicated to ensure the reproducibility of the measurements.

Methylation-Specific PCR

MS-PCR study was performed using 29 frozen clinical samples and 97 paraffin-embedded formalin-fixed clinical samples as well as a human endometrial carcinoma cell line (HEC-1A). Sodium bisulfite conversion of unmethylated cytosine residues to uracil in genomic DNA samples was performed as previously described.^{19,20} Six

Table 1. Sequences of Primers and TaqMan Probes Used in Quantitative Real-Time PCR

Reaction	Primers	Sequences (5' to 3')	Location
GSTP1	Forward	ACCTCACCTGTACCAGTCCAA	Exon 3
	Reverse	CGCCGTCATTACCATGTC	Exon 4
	TaqMan probe	TCACCTGGGCGCCGACCCCTTG	Exon 3
TBP	Forward	ACGAACCACGGCACTGAT	Exon 4
	Reverse	AACCCAACTTCTGTACAACCTCTAGCA	Exon 5
	TaqMan probe	ACAGTCCAGACTGGCAGCAAGAAAATA	Exon 5
GAPDH	Forward	TCCATGACAACCTTTGGTATCGTG	Exon 7
	Reverse	ACAGTCTTCTGGGTGGCAGTG	Exon 8
	TaqMan probe	ACCACAGTCCATGCCAT	Exon 8

sets of MS-PCR (MSP1 to MSP6) covering 20 CpG dinucleotides were performed. Five of the reactions, MSP1 to MSP5, were performed on DNA extracted from fresh frozen tissue, whereas MSP6 reaction, which involved smaller sized PCR products, was performed on DNA extracted from paraffin-embedded tissues. For each MS-PCR reaction, one pair of primers was specifically designed to detect methylated allele whereas another pair was designed for unmethylated allele. Details of the primer sequences and the size of PCR products were summarized in Table 2. PCR amplifications were performed in a 25- μ l reaction containing 25 ng of bisulfite-treated genomic DNA, 200 μ mol/L dNTPs, 0.24 μ mol/L of each of the primers, 1 $\times$ reaction buffer, 2 mmol/L MgCl₂, and 0.5 U of FastStart TaqDNA polymerase (Roche, Indianapolis, IN). Water was substituted for DNA as a PCR-negative control. DNA of term placenta treated with SssI methylase (New England Biolabs, Beverly, MA) was used as a positive control. DNA samples were amplified for 45 cycles with an initial cycle at 94°C for 15 seconds, annealing for 30 seconds, and 72°C for 30 seconds, and ending with a 4-minute extension at 72°C. The PCR products of MSPCR1 to and MSPCR6 were resolved using 2% agarose gel electrophoresis and 8% polyacrylamide gel electrophoresis, respectively.

Bisulfite Sequencing

Bisulfite-treated DNA of 12 samples showing methylated alleles in MSP1 to reactions were sequenced using the ABI Prism 377 automated DNA sequencer (Applied Biosys-

tems). PCR products, resulting from amplification using the primers in Table 2, were cloned into pGEM-T Easy vector (Promega, Madison, WI) following the protocol provided by the manufacturer and subsequently sequenced using the universal T7 primer provided on the vector.

cDNA Sequencing

Twenty-seven fresh frozen cases of endometrial tumors were studied. cDNA was amplified with the forward primer 5'-GAGTTTCGCCGCCGAGTCTT-3' and the reverse primer 5'-CAAACCTCTGCCTCCCGCTCAGAGT-3', and was directly sequenced using ABI Prism 370 automatic DNA sequencer (Applied Biosystems).¹⁹

Statistical Analysis

Software of GraphPad Prism 3.02 for Windows (GraphPad Software, San Diego, CA) was used for data analysis. Comparisons were made by χ^2 and Fisher's exact test as appropriate. *P* values of <0.05 were taken to be statistically significant with two-tailed test. Correlation with clinical and pathology data were performed using SPSS 10.1 for Windows (SPSS Inc., Chicago, IL).

Results

GSTP1 Expression Analysis

From immunohistochemical study, we observed that the endometrial glandular epithelial cells showed both nu-

Table 2. Primer Sequences and Size of the Amplicons in Each MSP Reaction and Bisulfite Sequencing

Reactions	Forward (5'→3')	Reverse (5'→3')	Size of amplicon (bp)
MSP1	U: GATGTTTGGGTGTGTTAGTTTGTGTTG	U: CCCCATACTAAAACTCTAAACCCC	392
	M: ATGTTTCCGCCGCTTAGTTCCG	M: CCCCATACTAAAACTCTAAACCCC	391
MSP2	U: GAAAGGTTTTTTGGTTAGTTGTGTGGTG	U: CCCCATACTAAAACTCTAAACCCC	294
	M: TTTTTCGGTTAGTTGCGCGGC	M: CCCCATACTAAAACTCTAAACCCC	290
MSP3	U: GATGTTTGGGGTGTAGTGGTTGTT	U: CCCCATACTAAAACTCTAAACCCC	232
	M: TTCGGGGGTGTAGCGGTCTGTC	M: CCCCATACTAAAACTCTAAACCCC	228
MSP4	U: TTTTGTAGAAGAGTGGTTGGTGTGTTG	U: CCCCATACTAAAACTCTAAACCCC	179
	M: TAGAAGAGCGGTCTGGCGTCCG	M: CCCCATACTAAAACTCTAAACCCC	174
MSP5	U: GAAAGGTTTTTTGGTTAGTTGTGTGGTG	U: CCAACAAAAACCTCACACCTCCA	211
	M: TTTTTCGGTTAGTTGCGCGGC	M: AAACCTCAACGAAAACTCTGCGACCTCCG	212
MSP6	U: GAAAGGTTTTTTGGTTAGTTGTGTGGTG	U: AACCAACCACTACACCCCAACATCAA	86
	M: TTTTTCGGTTAGTTGCGCGGC	M: GACGACCGCTACACCCCGAAC	82
Bisulfite sequencing	TTTGGGAAGAGGGAAAGGTTTT	CCCATACTAAAACTCTAAACCCC	307

U, unmethylation-specific primer sets; M, methylation-specific primer sets.

Figure 2. The scatterplot showing overall distribution of the normalized results of the real-time quantitative RT-PCR assay. Each dot represented a normalized mRNA expression of *GSTP1* for each of the sample cases, and the horizontal lines showed the mean values. Reduced expression of *GSTP1* was observed in tumor tissues of endometrial carcinomas.

clear and cytoplasmic immunoreactivity for *GSTP1*. According to the previous evaluation system for the *GSTP1* protein expression,^{9,21,22} immunoreactivity of our tissue samples was assessed with respect to intensity of staining and the area of tumor tissues being stained. The strongest *GSTP1* expression was observed in proliferative endometrium, followed by simple hyperplasia, complex hyperplasia, and atypical hyperplasia. Endometrium with glandular cells in secretory phase showed reduced immunoreactivity (data not shown). In 66 of the 97 (68.0%) cancer cases, weak or absent *GSTP1* protein expression (Figure 1, B and C) was detected compared with the noncancer endometrium. Strong *GSTP1* protein expression was observed in foci of squamous differentiation in endometrioid adenocarcinoma (Figure 1D).

The standard curve of each run of real-time RT-PCR was generated with a high linear coefficient value of 0.99 to 1.00. For validation, measurements of each case were repeated when there was more than 5% difference observed between the duplicates. The mRNA expression of *GSTP1* was normalized by the expression of either the *TBP* gene or the *GAPDH* gene of each sample. The values between the tumor and nontumor of each case were then compared. The overall distribution of the *GSTP1* mRNA expression normalized by the *TBP* gene (Figure 2) and *GAPDH* gene showed that 64% (16 of 25) and 60% (15 of 25) of the cases showed reduced *GSTP1* expression in tumor samples, respectively. Moreover, seven cases (28%) showed more than a fourfold reduction. However, such reduction of the *GSTP1* mRNA expression in endometrial cancers did not reach statistical significance ($P = 0.061$ for the *TBP* gene and 0.1305 for the *GAPDH* gene; Wilcoxon signed rank test), probably related to the sample size.

Promoter Methylation Analysis

In the MS-PCR study, six pairs of primers were designed for detecting the methylated and unmethylated alleles, taking into consideration the bisulfite conversion of sequences. Methylated alleles were demonstrated in 13.8%

Figure 3. Representative results of MS-PCR on CpG clusters of *GSTP1* promoter region. Horizontally, each panel represented one sample case showing results of the six MS-PCR reactions. PCR products of MSP1 to MSP5 were resolved on 2% agarose gel; whereas the product of MSP6 was resolved on 8% PAGE. Lanes U, amplification with primers recognizing unmethylated sequence; lanes M, amplification with primers recognizing methylated sequence. DNA treated with *SssI* methylase was used as a positive control.

(4 of 29) cases in MSP3 reaction, 17.2% (5 of 29) cases in MSP4 reaction, and 24.1% (7 of 29) tumor cases in MSP5 reaction (Figure 3). None of the 29 cases showed methylated alleles in MSP1 and MSP2 reactions. MSP6 reaction demonstrated methylated allele in 24.4% (19 of 78) tumor cases. Overall, 30.9% (30 of 97) of endometrial carcinomas showed aberrant promoter hypermethylation in at least one of the six tested sites. Only the unmethylated alleles were detected in the HEC-1A endometrial carcinoma cell line in all of the six MS-PCR reactions. Bisulfite sequencing analysis (Figure 4) performed on the 12 endometrial carcinomas with fresh-frozen DNA and positive MSPCR reactions, confirmed that all these samples had methylated CpG islands in tumor blocks, but not in the corresponding nontumor blocks. In addition, methylation at non-CpG sites including CpCpG trinucleotides and CpT dinucleotides were found in 6 of these 12 samples.

Genetic Analysis

Direct sequencing of the cDNA from 27 cases of endometrial carcinoma showed no somatic mutations in coding regions of the *GSTP1*.

Statistical Correlations of Expression, and Methylation Status, with Clinical Data

Results of immunohistochemistry and MS-PCR analysis on 97 cases of endometrial carcinoma were summarized in Table 3. χ^2 analysis showed statistically significant correlation between methylation of the *GSTP1* promoter and its reduced protein expression ($P = 0.008$), and mRNA expression ($P = 0.003$) in endometrial cancer. Moreover, mRNA expression was statistically correlated with protein expression ($P < 0.001$). The extent of myometrial invasion of the cancer statistically correlated with the *GSTP1* methylation status ($P = 0.009$) and with the protein expression ($P = 0.036$), suggesting that hypermethylation of the *GSTP1* promoter may account for its reduced expression, which may contribute to more advanced myometrial invasion of the cancer. Age of the patients did not statistically correlate with the extent of myometrial invasion of the cancer ($P = 0.2163$, χ^2), nor

Figure 4. Bisulfite sequencing of the *GSTP1* promoter. Three representative sequences showed methylation status of cytosines at the target sites of MS-PCR primers. Methylated cytosines found in tumor cases remained unchanged as cytosines after bisulfite modification, whereas unmethylated cytosines were converted and sequenced as thymine.

with the methylation status of the gene ($P = 0.709$, χ^2). No correlation between histological types and methylation status of *GSTP1* could be demonstrated in our study ($P = 0.430$, χ^2), possibly related to small number in the samples of nonendometrioid type of endometrial cancer in the series. There were no correlations between grading and cervical invasion of the cancer with methylation status ($P = 0.182$ and 0.058 , respectively; χ^2) nor with protein expression ($P = 0.121$ and 0.383 , respectively; χ^2). No correlations could be demonstrated between stages and the survival status of patients ($P = 0.118$, log rank test).

Discussion

GSTP1 is known to play a role in detoxification of potential carcinogens. Its ability to bind steroid hormones noncovalently also allows the protein to act as intracellular buffer minimizing the effects of short-term fluctuations in extracellular hormone levels.⁷ Reduced expression and hypermethylation has been frequently reported in prostatic carcinoma, a cancer known to be related to androgen stimulation. Because the majority of endometrial can-

cers, especially the endometrioid type, is associated with estrogen hyperstimulation, we decided to conduct a study to assess the role of *GSTP1* in endometrial carcinogenesis.

Approximately two-thirds of the endometrial carcinoma in our study showed reduced expression of *GSTP1* at both the mRNA and protein levels. The *GSTP1* protein, interestingly, was found to be strongly expressed in foci of squamous differentiation in endometrioid adenocarcinoma. Terrier and colleagues⁹ have reported this phenomenon in cancer of the uterine cervix. Such difference of the *GSTP1* gene expression in distinct epithelial types may be related to the physio-structural differences between the squamous and glandular cell in uterine endometrial and cervical tissues.

There were statistically significant correlations between reduced *GSTP1* mRNA and protein expression and hypermethylation of the gene in endometrial carcinoma. Our findings suggest that the *GSTP1* gene expression was altered at the transcriptional level and CpG methylation may play a role in such regulation in human endometrial cancer cells. Indeed, in endometrial cancers, hypermethylation of gene promoter regions have been reported recently to be contributing to the inactivation of the corresponding genes, including *hMLH1*,²³ *ER-alpha-C*,²⁴ *PR-beta*,²⁵ *p16*,²⁶ and *E-cadherin*.²⁷ However, the precise roles of such aberrant methylation in endometrial carcinogenesis are still not fully understood.

We found that approximately one-third of our endometrial carcinoma cases showed aberrant promoter hypermethylation in at least one of the six tested sites while two-thirds displayed reduced *GSTP1* expression. A much higher percentage of *GSTP1* hypermethylation has been reported in prostatic cancers, approaching 90%, in association in more than 95% of prostate cancer showing reduced expression.^{12,28} Our relatively lower incidence

Table 3. Protein Expression Level and Methylation Status of the *GSTP1* Gene in Endometrial Adenocarcinoma

Histological subtype	Methylation expression*		Unmethylation expression	
	Strong/normal	Reduced	Strong/normal	Reduced
Endometrioid	4	24	24	35
Nonendometrioid	0	2	2	6
Total cases	4	26	26	41

* Samples showing positive methylation in at least one of the six MS-PCR.

of methylation in endometrial cancers was actually also noted in an earlier report involving smaller number of samples.²⁹ In that study, no aberrant methylation could be detected in all 10 endometrial cancers being studied. Molecular carcinogenic pathway of these two steroid-sensitive cancers may be different.

In this study, both methylated and unmethylated alleles were often found in the same cancer sample whereas the nontumor tissue was always free of methylated alleles. Hypermethylation of the *GSTP1* was associated with reduced expression of the gene and extent of myometrial invasion. We believe that such heterogeneous methylation of gene promoters may reflect a flexibility of phenotype with respect to cancer invasion. It has been suggested that low expression of certain genes such as E-cadherin could allow dissociation of individual cells from the primary tumor mass to facilitate invasion or metastasis, and restoration of E-cadherin expression in the invasive or metastatic cells could allow these cells to re-establish cadherin-mediated intercellular adhesion and signaling.³⁰ This flexible epigenetic control of gene expression allows adaptable regulation of cancer cell phenotype in different steps of tumor progression.³¹

We have chosen -307 bp to +7 bp in the promoter region for our methylation study because the relatively high density of CpG dinucleotides in this region should allow adequate sensitivity for our MS-PCR approach. The detection rate for individual MS-PCR reaction varied from 0 to 24.4%. By combining the results of the six MS-PCR reactions, the cumulated rate of detecting methylated alleles was raised to 30.9%. Therefore, effective detection of aberrant methylation of the *GSTP1* gene in endometrial carcinoma may require a panel of markers spanning along a wide region of the promoter, rather than using a single marker. On the other hand, we also observed a trend of increasing methylation density along the core CpG island: from 0% at -308 bp and -209 bp, 13.8% at -147 bp, 17.2% at -94 bp, to 24.1% at +1 bp. Hypermethylation in the later regions may have more effect on the *GSTP1* gene expression.

Interestingly, our bisulfite sequencing also revealed methylation at non-CpG sites in tumor samples. These include methylation of both cytosines in CpCpG trinucleotides, and CpT dinucleotides contiguous to CpG dinucleotides. Although there are few reports on such methylation phenomenon, our findings concurred with those reported by Millar and colleagues³² who demonstrated that CpCpG methylation was relatively common in prostate cancer in addition to typical CpG dinucleotide methylation. As early as in 1996, such unusual methylation was found to be related to over-activity of DNA-methyltransferase and enhanced mutagenesis in prokaryotic system.³³ Ramsahoye and colleagues³⁴ later reported that expression of the methyltransferase Dnmt3a correlated with the presence of non-CpG methylation in embryonic stem cells. Our detection of such methylation pattern in endometrial tissues was not surprising because this tissue is probably stem cell enriched in association with its perpetual cyclic proliferation.³⁵ Nevertheless, the significance of such non-CpG methylation in cancers is not fully understood. We noticed that

such non-CpG methylation usually occurred in the region where high level of CpG dinucleotide methylation was observed and believe that its occurrence in tumor cells may be, to a certain extent, dependent on the high level of CpG dinucleotide methylation. Such phenomenon may reflect the loss on control or the specificity of methylation in the process of tumor progression. Alternatively, non-CpG methylation may also be contributing to repression of the *GSTP1* gene transcription in endometrial carcinoma. More investigations are needed to assess the significance of such non-CpG methylation in human cancers.

Mechanisms underlying methylation-dependent gene silencing are still controversial, and two hitherto models have been proposed. Watt and Molly³⁶ suggested that methyl-CpG dinucleotides would directly hinder the normal binding of transcription factors on their specific sites in the promoter region. This may not be applicable to the *GSTP1*. The AP1 recognition site and the two SP1 binding sites on the *GSTP1* proximal promoter region are the essential *cis*-elements required for the basal activity of the genes.³⁷ Moreover, AP1 site does not possess CpG dinucleotide sequence, and the binding of SP1 to its recognition site is not sensitive to cytosine methylation.^{38,39} Another model proposed by Boyes and Bird,^{40,41} and Nan and colleagues⁴² stated that methylated genes may attract methyl-CpG binding proteins such as MeCP1 and MeCP2 that mediate repression via themselves and/or other proteins (histone deacetylases) and affect chromatin compaction, provided that the density of methylated sites exceeds a threshold level. These indirect processes may be more applicable to methylation-dependent silencing in the *GSTP1*. Our results demonstrated that no particular methylated CpG sites were particularly associated with markedly reduced *GSTP1* expression and suggested that it was the overall high density of methylation along the promoter attracting MeCP proteins that repressed expression at the transcriptional level.

There are several detecting systems to determine methylation status of CpG islands. The MS-PCR approach has several advantages. It requires small quantities of DNA and is sensitive to as little as 0.1% methylated alleles of a given CpG island locus.¹⁸ It is feasible to almost any sites of genes containing CpG dinucleotides. The system can also be performed on DNA extracted from paraffin-embedded tissues. We have performed one of the MS-PCR reactions on DNA extracted from paraffin blocks of 78 cases of endometrial carcinoma and they were all successfully amplified during PCR reaction. Quantitative assessment of promoter hypermethylation using, for example, the real-time PCR-based approach should give a more accurate reflection and evaluation on methylation status. Our data based on the six MS-PCR reactions designed to cover a range of promoter region of *GSTP1*, showed that 30 of the 97 cases of endometrial carcinoma had methylated alleles, of which only 3 cases showed positive reactions in two to three of the six MS-PCR reactions, although all of the 3 cases exhibited reduced expression. Such data on endometrial carcinoma suggested that the methylated CpG sites scattered throughout the promoter region of the *GSTP1* gene. It was

the overall methylation density on several scattered CpG dinucleotides scattered along the promoter region of the *GSTP1* gene rather than focused and dominant site-specific methylation that was critical for the reduced expression. The lack of hot-spot CpG dinucleotides for methylation made design of quantitative methylation assessment less feasible and hence, such quantitative approach was not adopted in this study.

Our cDNA sequencing did not show any genetic mutations in coding region of *GSTP1*. In fact, earlier comparative genomic hybridization study on our samples showed regional gains on chromosomes 1q31-qter, 3q25.3, 5p, 6q23.3, 8, 12, 19, 20q, and losses on chromosomes 9q, 16q, 22, and X, but not on 11q where the *GSTP1* gene is located. Thus, both the CGH and cDNA sequencing data showed that the reduced expression of *GSTP1* was most probably not caused by somatic genetic changes.

On the other hand, reduced expression of the *GSTP1* gene was observed in some endometrial cancers with no demonstrable methylated alleles. These observations suggested that reduction or loss of *GSTP1* expression may be caused by mechanisms other than hypermethylation of its promoter region. Histone deacetylation, or other epigenetic factors such as non-CpG methylation may play a role in repressing transcription of this cancer and remains to be investigated.

To conclude, we demonstrated reduced expression and hypermethylation of the *GSTP1* gene in a significant portion of endometrial carcinoma and such *GSTP1* gene alterations was related to extent of myometrial invasion by the carcinoma. Hypermethylation of the *GSTP1* gene may be a flexible and dynamic mechanism in the control of gene expression as measure to affect cancer invasion property.

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