Mesothelin gene expression and promoter methylation/hypomethylation in gynecological tumors

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Summary

Purpose: Mesothelin is a cell surface glycoprotein that is present on normal mesothelial cells and overexpressed in several cancers. In this study, we investigated the methylation/hypomethylation status in the promoter region of the mesothelin gene in gynecological tumors. *Methods:* Forty-four ovarian tumor specimens and 16 cases of uterine endometrial carcinoma, and normal tissue specimens were used. Monoclonal antibody (5B2) was employed for the immunohistochemical analysis. The methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) technique was used to quantify the methylation/hypomethylation status at 20 CpG sites in the mesothelin promoter region. *Results:* Mesothelin was expressed in 100% of serous cystadenocarcinoma and 100% of serous borderline tumor of the ovary. None of the germ cell tumors and sexcord-stromal tumors was immunoreactive. Fifty percent of endometrial carcinoma was immunoreactive for mesothelin. The average methylation of CpG sites in ovarian tumors ranged from 6-56% (median: 31%) in mesothelin-positive and 13-79% (median: 43%) in mesothelin-negative samples. In endometrial tumors, the average methylation ranged from 5-52% (median: 28%) in mesothlin-positive and from 15-67% (median: 22%) in mesothlin-negative samples. A correlation was found between mesothelin expression and the average methylation/hypomethylation status as well as methylation/hypomethylation status at four of 20 CpG sites in ovarian samples. No correlation was found in endometrial samples. *Conclusion:* We detected diverse levels of methylation/hypomethylation at CpG sites in the mesothelin promoter region in ovarian and endometrial tumors. We speculate that, although methylation/hypomethylation changes may affect its transcription, other mechanisms may synergically operate in tissue-specific expression and tumor-related mesothelin overexpression.

Key words: Mesothelin; Overexpression; Gynecological tumors; Promoter methylation; Hypomethylation.

Introduction

The human mesothelin gene is a 40-kilodalton carboxy-terminal component of a 69-kilodalton precursor protein whose amino portion is the secreted cytokine known as megakaryocyte-potentiating factor. Mesothelin is a glycosy-phosphatidylinositol-linked cytoplasmic membrane glycoprotein whose function has not been clarified, but it may be involved in cell adhesion [1, 2]. In humans, mesothelin is expressed in normal mesothelial cells lining the body cavities and in some epithelial cells of the kidney, tonsil, trachea, and fallopian tube [3]. In immunohistochemical or gene expression studies of human cancers, mesothelin has been reported in mesothelioma, adenocarcinoma of the ovary, pancreas, lung, stomach, colon, rectum, uterus, and some squamous cell carcinomas [4-15]. Mesothelin has been evaluated as a diagnostic marker of ovarian cancer and mesothelioma. The elevation of serum mesothelin in ovarian cancer and mesothelioma has been identified by several groups [9, 14-16]. However, epigenetic mechanisms of mesothelin gene expression were not well studied. Studies of global gene expression and DNA hypomethylation analysis of pancreatic cancer involving 18 genes showed that, in normal pancreatic tissue, the mesothelin gene is hypermethylated, which means it does not express mRNA or protein. On the other hand, mesothelin is overexpressed in most pancreatic cancer cases, and the mesothelin promoter was found to be hypomethylated [17]. The methylation and hypomethylation profile of the CpG sites in the promoter region of mesothelin and the correlation with the expression pattern in various human tissues and tumors are still largely unknown.

Mesothelin overexpression has been reported in subtypes of ovarian and endometrial cancer, but the epigenetic alteration of the mesothelin gene is unknown in these tumors. In the present study, we employed immunohistochemical analysis to investigate the expression of the mesothelin gene in gynecological tumors including several histological subtypes of ovarian tumors, endometrial carcinoma, and normal tissue specimens. Using microdissected tissue DNA, we performed quantitative methylation analysis of the 20 CpG sites in the promoter region of the mesothelin gene, and the correlation with its expression was investigated.

Materials and Methods

Cases

Forty-four ovarian tumor specimens, 42 cases of uterine endometrial carcinoma, and normal tissue specimens were obtained from patients surgically treated between 1993 and 2005 at the Department of Obstetrics & Gynecology of Juntendo University Hospital, Tokyo, Japan. This research project

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was approved by the local ethical committee, and all samples were obtained with the patients' informed consent. A part of the tissue sample was fixed in formalin and embedded in paraffin for histological diagnosis. Histological diagnosis was made when two pathologists specializing in gynecological disease reached a consensus. The age of the patients ranged from 17 to 72 (mean: 43) years old for ovarian tumors and from 35 to 80 (mean: 55) years old for uterine tumors. Analyzed samples are shown in Table 1.

Immunohistochemistry

The expression of mesothelin was assessed by immunohistochemistry using anti-mesothelin monoclonal antibody 5B2 (Novacastra, Newcastle-on-Tyne, UK), an anti-mesothelin antibody that was generated by immunizing mice with a recombinant protein corresponding to 100 amino acids at the NH2 terminus of membrane-bound mesothelin [2]. This antibody has been characterized and used for detection in several types of benign and malignant tumor expression studies. For each case, one to three pathology blocks were selected for human mesothelin gene expression analysis. Immunhistochemical staining was performed as follows: 4-µm-thick sections were deparaffinized, treated for 30 min with 3% hydrogen peroxide to block endogenous peroxidase activity, and then with citric acid (pH 6.0) for 10 min at 100°C in a microwave oven for antigen retrieval. Five percent normal goat serum was applied for 30 min to block nonspecific reactions. Sections were incubated with the mouse anti-human anti-mesothelin antibody 5B2 (1:50) at 4°C overnight. After rinsing in PBS, slides were treated with Envision+HRP System (Dako Cytomation, Glostrup, Denmark, K4000)-labeled polymer anti-mouse immunoglobulin for 60 min. The peroxidase reaction was visualized by incubating sections with 0.02%, 3.3-diaminobenzidine tetrahydrochloride in 0.05M Tris, buffer and then slides were counterstained with hematoxylin. Sections for the negative control were prepared using normal mouse serum instead of the primary antibody. The intensity of the staining was scored from 0-3 (absent, weak, moderate, and strong, respectively). The mesothelin expression was regarded as negative when no cells were stained or only faintly stained over less than 30% of the tumor area. Mesothelin was regarded as positive when more than 30% of the tumor cells were immunostained. Most of the mesothelin staining showed membranous staining, but some cases also showed cytoplasmic staining. No nuclear staining was detected. Most negative cases clearly showed no staining in the majority of tumor cells or normal cells on immunostained slides.

Microdissection and DNA extraction

Serial 8-µm paraffin-embedded tissue sections were cut with a microtome, deparaffinized, and stained with regular hematoxylin-eosin (HE). The first and last sections were cut to 8-µm thick, stained with HE, and cover-slipped for microscopic examination. Tumor and non-tumor portions were microdissected using a 27-gauge needle under an inverted microscope. A laser-assisted microdissection system (Leica laser microdissection system, Leica Microsystems, Wetzlar, Germany) was also used. Visual inspection revealed that at least 95% of the collected cells were tumor cells. Genomic DNA extract was isolated from microdissected tissues using a DNA isolation kit (Qiagen, QIAamp® DNA Micro Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions.

DNA methylation analysis of mesothelin promoter

DNA was modified in 40 µl of water with sodium bisulfite using the EpiTect™ Bisulfite Kit (Qiagen, Hilden, Germany). Sodium bisulfite converts unmethylated cytosine to uracil, which is replicated as thymine in the subsequent PCR step. Methylated cytosines are resistant to deamination by sodium bisulfite and are therefore replicated as cytosine during PCR. Presumed genomic structures (Sequence position 13,501-14,220 of Gene Bank ID: AL031258) of the mesothelin promoter and primers and CpG sites analyzed are shown in Figure 1. The region contains a postulated CpG island, predicted by the CpG island searcher (http://www.uscnorris.com/cpgislands2/cpg.aspx), and an 18-bp upstream enhancer CanScript that contains a transcription enhancer factor-dependent MCAT motif [18]. Ten possible transcription start sites described by Hucl *et al*. [18] are also shown. After bisulfite modification, PCR of the mesothelin promoter was performed with the primers as shown in Figure 1 using the JumpTaqTM REDTaq DNA polymerase (Sigma, Saint Louis, MO, USA) under the following PCR conditions: 40 cycles of 94°C for 30 sec, 58°C for 60 sec, and 72°C for 50 sec. Approximately 1-2 µl of bisulfite-treated DNA was used as a template for strand-specific PCR amplification. PCR products were electrophoresed on a 1.8% ethidium-bromide stained gel and the DNA fragments were purified from agarose gel slices using the Wizard DNA Clean-up system (Promega, Madison, WI, USA).

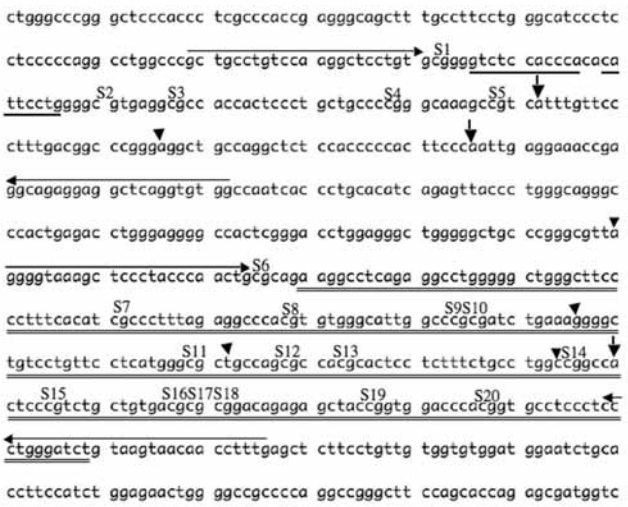

Figure 1. — Genomic region of postulated mesothelin promoter regions (sequence position 13501-14220 of GenBank accession AL031258), postulated CpG islands, and CpG sites analyzed by MsSNuPE. S#, CpG site analyzed for methylation. The position of an 18-bp upstream enhancer CanScript that contains a transcription enhancer factor-dependent MCAT motif is underlined. The promoter CpG island, predicted by the CpG island searcher (http://www.uscnorris.com/cpgislands2/cpg.aspx), is double-underlined. PCR primers for the MsSNuPE template are shown by arrows. The consensus initiator sequence is shown by thick overlying arrows, and the nonconsensus start site is shown by arrowheads.

Quantitation of mesothelin promoter methylation/hypomethylation status at specific CpG sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE)

DNA is treated with sodium bisulfite, followed by PCR amplification of the target mesothelin promoter CpG island sequence to generate a strand-specific DNA template suitable for Ms-SNuPE analysis. The single nucleotide primer extension assay was first described by Kuppuswamy and others for the

Table 1. — *Summary of ovarian and endometrial tumors analyzed for mesothelin expression and their promotoer methylation staus*.

Sample ID (OVA #)	Histology and/or component	Mesothelin expression	Average methylation (%)	Median methylation (%)	Range of methylation (%)
OVA1	Normal stromal component	-	43	48	1-86
OVA2	Mucinous cystadenoma	-	65	93	2-93
OVA3	Serous cystadenoma	+	37	39	1-65
OVA4	Serous cystadenoma	+	18	18	1-38
OVA5	Mucinous cystadenoma	-	49	47	20-79
OVA6	Clear cell carcinoma	+	34	28	2-64
OVA7	Endometrioid adenocarcinoma	-	17	4	1-96
OVA8	Mucinous cystadenocarcinoma	-	28	10	1-88
OVA9	Serous papillary cystadenocarcinoma	+	56	58	1-100
OVA10	Serous papillary cystadenocarcinoma	+	17	14	6-55
OVA11	Clear cell carcinoma	-	24	22	1-66
OVA12	Serous papillary cystadenocarcinoma	+	18	17	2-42
OVA13	Serous papillary cystadenocarcinoma	+	29	27	8-42
OVA14	Serous papillary cystadenocarcinoma	+	34	31	2-90
OVA15	Endometrioid adenocarcinoma	-	36	31	2-69
OVA16	Serous cystadenofibroma	+	31	37	1-83
OVA17	Mucinous cystadenocarcinoma	-	14	14	8-22
OVA18	Mucinous LMP	-	79	92	4-97
OVA19	Endometrioid adenocarcinoma	-	29	24	7-77
OVA20	Clear cell carcinoma	-	18	15	2-47
OVA21	Clear cell carcinoma	-	70	74	46-94
OVA22	Clear cell carcinoma	-	59	67	3-73
OVA23	Clear cell carcinoma	-	37	36	21-58
OVA24	Mucinous LMP	-	24	20	3-71
OVA25	Mucinous LMP	-	64	74	2-100
OVA26	Mucinous LMP	-	34	30	11-80
OVA27	Serous LMP	+	6	4	1-20
OVA28	Serous LMP	+	62	80	3-96
OVA29	Sertoli cell tumor	-	67	93	2-98
OVA30	Normal ovarian stroma	-	48	54	12-82
OVA31	Arterial wall	-	38	37	12-88
OVA32	Normal ovarian stroma	-	13	8	3-63
OVA33	Normal corpus luteum	-	50	58	6-94
OVA34	Immature teratoma, neural component	-	73	95	1-100
OVA35	Immature teratoma, stromal component	-	47	52	1-97
OVA36	Granulosa cell tumor	-	55	68	1-98
OVA37	Immature teratoma, skin	-	42	41	1-88
OVA38	Immature teratoma, cartirage		43	42	18-69
OVA39	Immature teratoma, neural epithelium		42	41	2-93
OVA40	Fibroma	-	60	63	19-86
OVA41	Brenner tumor, epithelium	-	30	24	7-83
OVA42	Brenner tumor, stroma	-	39	33	7-78
OVA43	Yolk sac tumor	-	60	70	1-94
OVA44	Fibroma	-	40	43	1-75
OVA45	Desmoplastic small round cell tumor	-	57	61	12-84
OVA46	Fibrothecoma	-	43	41	22-92
OVA47	Fibroma	-	42	39	15-93
OVA48	Sertoli-Leydig cell tumor, sertoli cells	-	41	32	1-99
OVA49	Mature teratoma, skin & adnexa	-	53	59	2-88
OVA50	Mature teratoma, thyroid	-	32	38	1-69
OVA51	Strumal carcinoid	-	44	42	23-81
OVA52	Brenner tumor, epithelial component	-	51	55	5-89
OVA53	Brenner tumor, stromal component	-	52	56	18-79
OVA54	Yolk sac tumor	-	58	60	19-90
OVA55	Granulosa cell tumor	-	61	62	25-88
EM1	Endometrioid adenocarcinoma	-	25	26	1-44
EM2	Endometrioid adenocarcinoma	+	18	13	5-69
EM3	Normal endometrium	-	16	13	40
EM4	Endometrioid adenocarcinoma	-	28	30	1-73
EM5	Endometrioid adenocarcinoma	+	5	2	1-24
EM6	Endometrioid adenocarcinoma	+	28	24	0-91
EM7	Endometrioid adenocarcinoma	+	27	26	14-50

Sample ID (EM #)	Hystology and/or component	Mesothelin expression	Average methylation (%)	Median methylation (%)	Range of methylation (%)
EM8	Endometrioid adenocarcinoma	-	15	12	3-65
EM9	Endometrioid adenocarcinoma	-	24	18	3-76
EM10	Endometrioid adenocarcinoma	+	40	40	11-74
EM11	Normal endometrium	-	19	18	7-37
EM12	Endometrioid adenocarcinoma	+	52	56	3-82
EM13	Endometrioid adenocarcinoma	-	67	67	27-97
EM14	Endometrioid adenocarcinoma	+	31	21	0-93
EM15	Endometrioid adenocarcinoma	-	18	8	0-73
EM16	Endometrioid adenocarcinoma	-	23	20	1-56
EM17	Endometrioid adenocarcinoma	+	16	14	6-31
EM18	Endometrioid adenocarcinoma	-	22	24	0-46

The methylation status is expressed as the average % methylation at 20 CpG sites analyzed, median % methylation and the range of % methylation. OVA#, ovarian tumor samples or components; EEM#, endometrial tumors and normal endometrial samples; LMP, tumors of low malignant potential (borderline tumor).

detection of mutations in abnormal alleles [19]. Gonzalgo and Jones [20, 21] modified this method for the quantification of DNA methylation differences at specific CpG sites. Briefly, 2 µl of purified bisulfite PCR product was used in each Ms-SNuPE reaction. One µM of Ms-SNuPE primer (each cg site specific 19-27 mers; sequences of primers available upon request) was labeled with 32P-dCTP or 32P-TTP and 1 U of Jump start polymerase was used for the primer extension reaction. The Ms-SNuPE reaction were performed at 94°C for 3 min, 45°C for 2 min, and 72°C for 2 min. Stop solution (10 µl of 0.1% bromophenol blue, 0.1% xylene cyanol, and 95% formamide) was then added to the reaction mixtures, heated at 95°C for 5 min, and samples were loaded on to 23% denaturing polyacrylamide gels. The radioactivity of gel signals was visualized and quantified using the BAS2500 Image analyzer (Fuji, Tokyo, Japan). Different length primers were designed for the multiplex quantitative analysis of methylation at twenty top stand cytosines in the 5´CpG island of the mesothelin gene. The intensities of bands in the C lane were proportional to the percent of methylation at each CpG site being monitored, and band intensities in the T lanes were proportional to the percent of unmethylated cytosine. The percent of methylation at each CpG site is calculated as the signal intensity of C/ (signal intensity of C+T) x 100 (%). Methylase-treated DNA as well as subcloned and sequence-verified DNA from PCR products was used as methylated and unmethylated CpG controls.

Statistical analysis

The Mann-Whitney U test was used to compare differences in the percent of methylation at CpG sites between mesothelin-negative ovarian tumor or normal ovarian tissue samples and mesothelin-positive ovarian tumors. Similarly, in endometrial samples, mesothelin-negative normal endometrial cells, mesothelin-negative endometrial cancers, and mesothelin-positive endometrial cancers were compared.

Results

Mesothelin gene expression in various ovarian tumors and uterine cancers

Immunohistochemical findings are summarized in Table 1 and representative mesothelin immunostaining is shown in Figure 2. Twenty-five percent of ovarian tumors stained positively for mesothelin. Mesothelin was expressed in five of five serous carcinomas (100%), two of two serous cystadenomas (100%), two of two (100%) serous tumors of low malignant potential (borderline serous tumors), one serous cystadenofibroma, and one of six clear cell carcinomas (17%). The staining was mostly detected on the cell surface membrane. Some tumors also showed cytoplasmic staining. None of the mucinous tumors (2 benign, 4 borderline, and 2 malignant tumors), two Brenner tumors, and three endometrioid carcinomas were all negative for mesothelin. None of the germ cell tumors and sex-cord stromal tumors were immunoreactive.

In endometrial samples, endometrioid uterine adenocarcinoma was frequently positive for mesothelin (8 of 16; 50%). None of the normal endometrial glandular cells, endometrial stromal cells and myometrial smooth muscle cells were immunostained for mesothelin.

Quantification of DNA methylation/hypomethylation status at specific CpG sites in the mesothelin promoter

We used the Ms-SNuPE assay to determine the methylation status of multiple CpG sites in the mesothelin promoter in various gynecological tumor specimens. Representative gels of the SNuPE assay are shown in Figure 3. Table 1 shows the average percent of methylation of all CpG sites analyzed and the range of percent of methylation for each sample. Figure 4 shows a box-and-whisker plot of the average percent of methylation and the percent of methylation at individual CpG sites for mesothelin-negative and positive-samples. The average methylation of these 20 CpG sites in ovarian tumors ranged from 6-56% (median: 31%) in mesothelin-positive samples and 13-79% (median: 43%) in mesothelin-negative samples. The lowest level of methylation (hypomethylated) was detected in serous borderline tumor case #OVA27 and the highest was detected in immature teratoma case #OVA35. In endometrial tumors, the average methylation ranged from 5-52% (median: 28%) in mesothelin-positive samples and from 15-67% (median: 22%) in mesothelin-negative samples.

When each CpG site was analyzed separately in ovarian tumor, the lowest level of methylation was detected at the S8 site (median: 7% in mesothelin-positive ovarian tumor and 15% in mesothelin-negative ovarian tumor). The highest level of methylation was detected at the S3 site (median: 74% in mesothelin-posi-

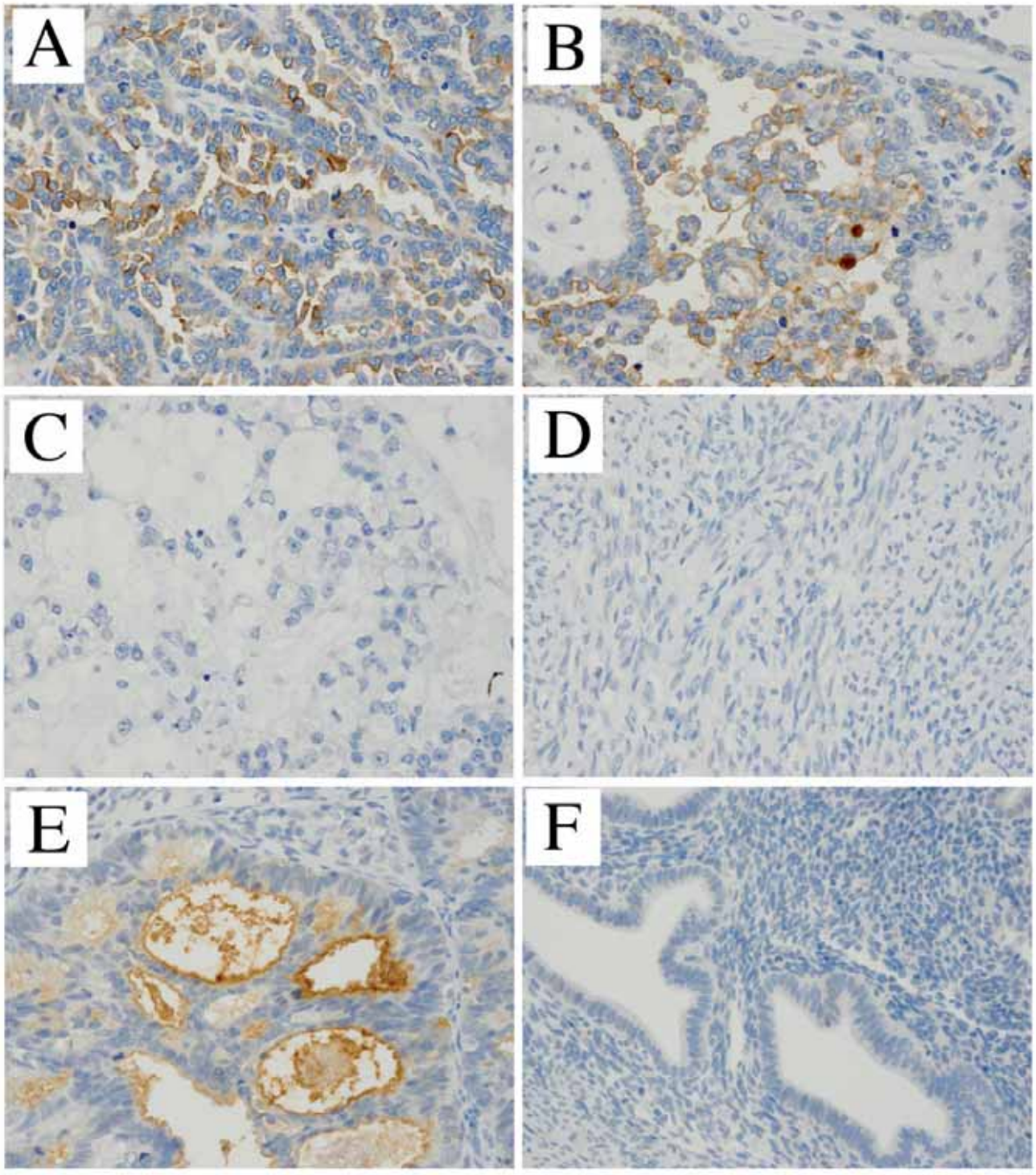

Figure 2. — Immunohistochemical staining of mesothelin in ovarian and uterine endometrial tumors. (A) and (B) Strong mesothelin expression in ovarian serous carcinoma case #OVA9 and OVA10, respectively . Note the predominantly membranous staining. (C) Negative mesothelin staining in ovarian clear cell carcinoma case #OVA21. (D) Negative mesothelin staining in ovarian fibroma case #OVA41. (D) Negative mesothelin expression in normal proliferative phase endometrium. (E) Strong mesothelin expression in endometrial endometrioid adenocarcinoma case #EM6.

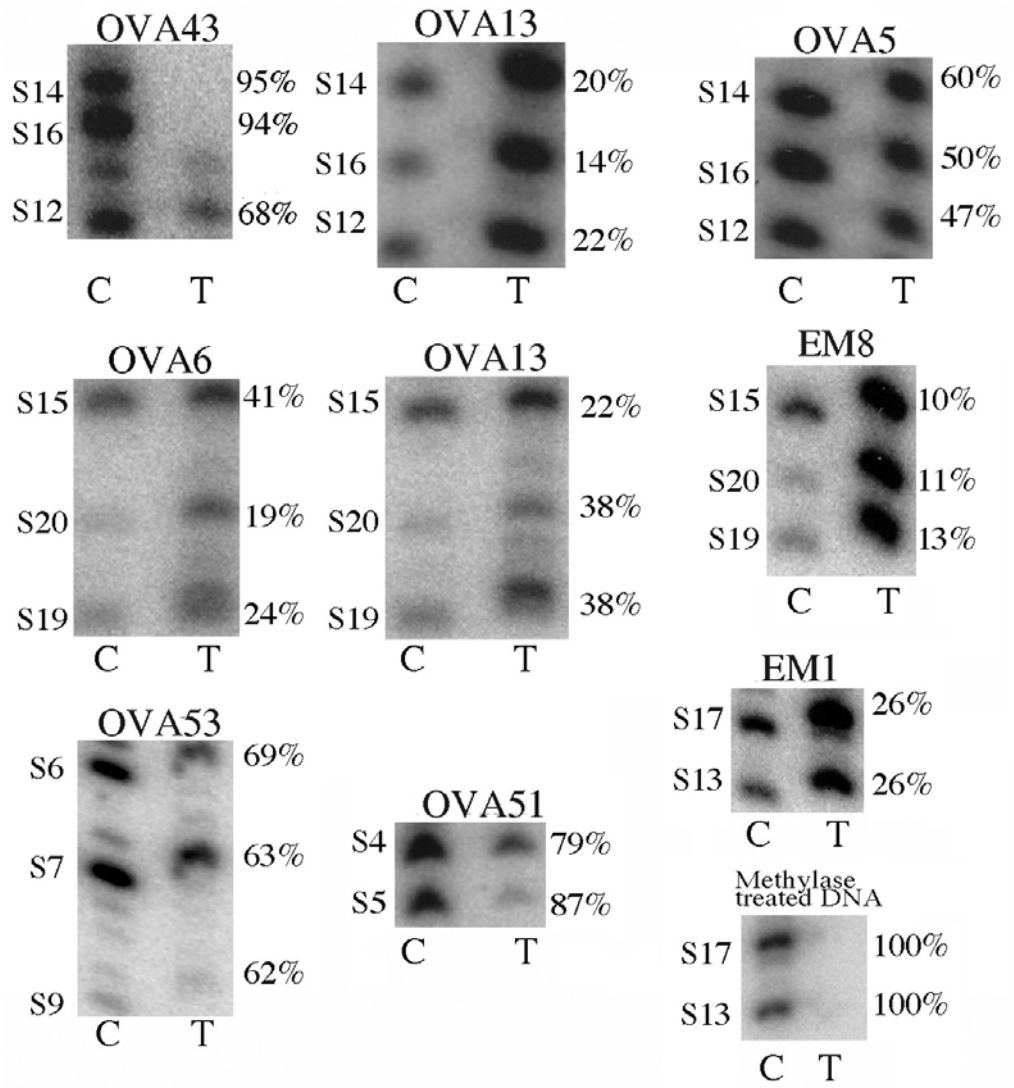

Figure 3. — Representative MsSNuPE gels demonstrating various levels of % methylation at each CpG site in ovarian and endometrial tumors. C represents the signal for MsSNuPE reactions incubated in the presence of [32P]dCTP and T represents the signal for MsSNuPE reactions incubated in the presence of [32P]TTP. S# represents the individual CpG sites analyzed.

tive ovarian tumor and 72% in mesothelin-negative ovarian tumor).

One of the mesothelioma samples and respiratory epithelium showed an average of 20% and 25% methylation, respectively (data not shown).

Correlation of mesothelin expression and promoter methylation in gynecological tumors

By the Mann-Whitney U test, a correlation was found between the mesothelin expression status and the average methylation, as well as, the methylation at the CpG site S9, S14, S15, and S16 in ovarian samples (Figure 4A). No correlation was noted in endometrial tumors (Figure 4B).

Discussion

Mesothelin shows a strong tissue-specific expression. In normal tissue, only mesothelial cells of the body cavities, respiratory epithelium, and tubal epithelium express mesothelin[22]. Many tumors including mesothelioma, lung, pancreatic, ovarian, and endometrial cancer are known to over-express mesothelin [4-15]. However, one of the mechanisms of gene transcription, the methylation/hypomethylation status of the mesothelin promoter, is largely unknown. In this study, in order to evaluate the relationships between mesothelin methylational changes and its expression, we analyzed the level of methylation of the 20 promoter CpG sites in gynecological tumors by quantitative Ms-SNuPE methods.

The results of the immunohistochemical staining of various ovarian tumors and endometrial cancers are comparable to those of previous reports [6, 7, 9, 23-26]. Serous ovarian cancer showed the highest rate of mesothelin immunoreactivity. No mucinous tumors of benign, borderline, and malignant categories were stained for mesothelin. Germ cell tumors and sex-cord stromal tumors were all negative for mesothelin.

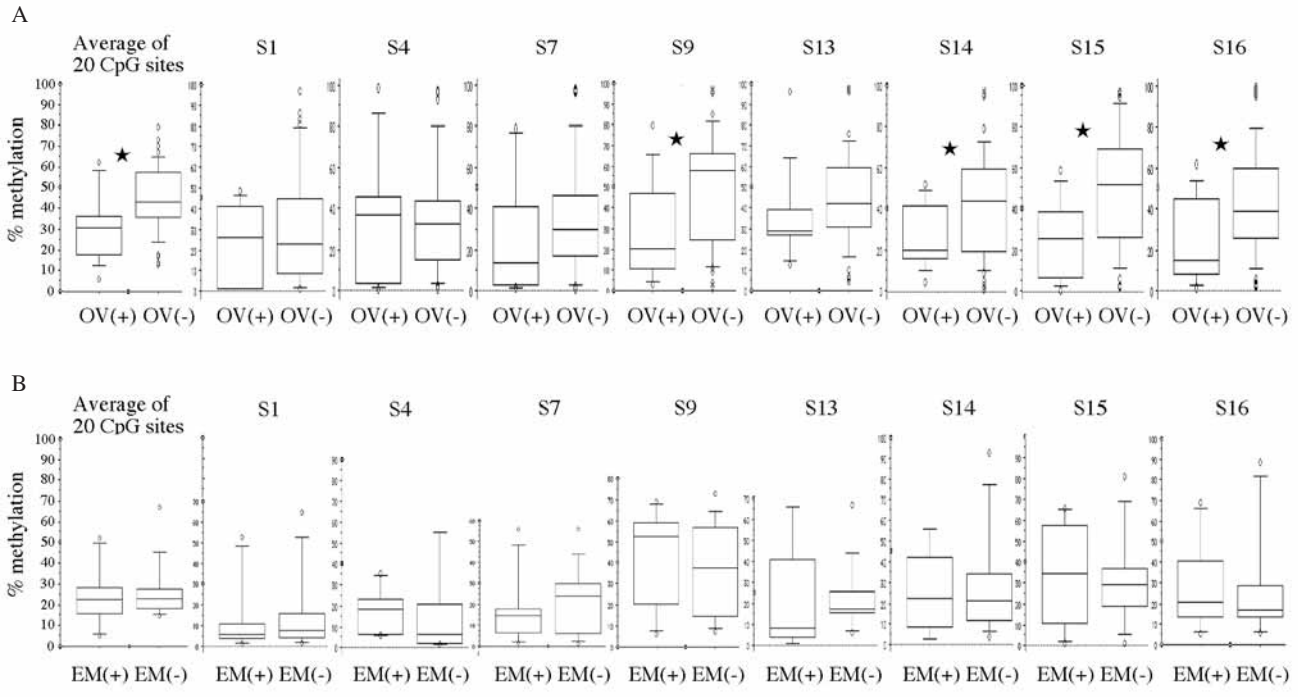

Figure 4. — The extent of mesothelin promoter methylation/hypomethylation. OV(-), mesothelin-negative ovarian tumors or ovarian component; OV(+), mesothelin-positive ovarian tumor; EM(–), mesothelin-negative endometrial cancer and normal endometrial samples; EM(+), mesothelin-positive endometrial cancer. (A) A box-and whisker plot of the average methylation and the methylation at individual CpG sites in ovarian tumors. (B) A box-and whisker plot showing average methylation and the methylation at individual CpG sites in endometrial tumors. The lines within the box indicate the median values, the top and bottom horizontal lines of the box, the 75th and 25th percentiles, respectively, and the top and bottom horizontal lines the 90th and 10th percentiles, respectively. ★, statistically significant differences for percent of methylation in mesothelin-positive and mesothelin-negative samples.

We detected that percentages of methylation/hypomethylation differed from case to case. In serous ovarian cancers showing a strong mesothelin expression (case #OVA10, 12, 13, 14, 16, and 27), we identified predominantly hypomethylated CpG sites. Also, correlation was found between the mesothelin expression status and the average methylation, as well as, the methylation at the four of 20 CpG sites in ovarian samples. We believe that methylation/hypomethylation especially at these 4 CpG sites as well as overall methylation/hypomethylation status of the promoter region may affect its transcriptional machinery in ovarian tumors. A correlation between such a methylational status and mesothelin expression was not evident at the remaining 16 of 20 CpG sites in ovarian tumors.

In pancreatic cancer, hypomethylation of the mesothelin promoter has been described. By systematic analysis of the number of genes for methylation and hypomethylation, Sato *et al*. [17]. found seven genes including mesothelin were overexpressed in pancreatic cancer cell lines and primary pancreatic carcinomas. These genes in pancreatic cancers were strongly hypomethylated in CpG sites in their 5' promoter regions. On the other hand, these genes are normally methylated and not expressed in the non-neoplastic pancreas. Because pancreatic cancers show such a correlation, it is tempting to postulate that varied levels of methylation/hypomethylation in various ovarian and endometrial tumors may also play a certain role in their mesothelin expression.

Although we only analyzed a single case each, we identified strong hypomethylation in mesothelioma and respiratory epithelium, both of which are mesothelin-immunoreactive. Many serous ovarian and endometrial cancers were predominantly hypomethylated. When several components were separately microdissected and analyzed regarding their methylation from a single tumor (immature teratoma, Brenner tumor, etc.), we occasionally detected different levels of methylation among the components (data not shown). We speculate that mesothelin promoter methylation/hypomethylation may play a certain role in the tissue- specific and tumor type-specific overexpression in certain tumors. We are currently evaluating the methylation levels of various tumors and organs including lung cancer, mesothelioma, and related non-neoplastic lesions.

Promoter analysis of mesothelin demonstrated the 18-bp upstream enhancer CanScript that contains a transcription enhancer factor-dependent MCAT motif [18]. CanScript appears to be a modular element for cancer-specific mesothelin transcription. We noted variable levels of methylation at CpG sites in the region close to CanScript,

which may affect the binding of various factors. Other epigenetic mechanisms such as histone acetylation and histone methylation may also operate.

The function of mesothelin in gynecological tumors and normal tissue remains to be elucidated. Mesothelin may play a role in cell adhesion and the metastatic spread of ovarian cancer [27]. On the other hand, high-grade ovarian serous carcinoma with diffuse mesothelin expression has been correlated with prolonged patient survival [26]. The immunological response to mesothelin-expressing tumor cells may be one of the associated mechanisms. Cohesive cell growth in mesothelin-expressing ovarian tumor cells may prevent tumor dissemination and metastasis [26]. The analysis of the methylational profile of ovarian tumors may facilitate the molecular modulation of mesothelin for therapeutic purposes.

Although less frequently than in ovarian tumors, endometrial cancers have been found to express mesothelin. We found that eight of 16 endometrioid carcinomas of the uterus express mesothelin. On the other hand, absent to very faint mesothelin immunostaining was detected in normal endometrial tissue. Interestingly, we detected predominantly unmethylated alleles in most of the 20 CpG sites studied in mesothelin-positive as well as-negative endometrial cancers and normal endometrial epithelial cells. There may be several possibilities to be considered. For the negative scoring of immunohistochemistry, we regarded completely absent-faint immunoreactivity as staining in less than 30% of the tumor cells. A similar criterion has been previously adopted in many related articles. These tumors may actually show certain levels of gene transcription, and exclusive methylation may not be required. Actually, although this has not been reported, we detected very faint mesothelin staining in normal endometrial glandular cells. Thus, the normal counterpart of these tumors may also transcribe mesothelin, but at very low levels. The transcriptional activity of mesothelin mRNA and expression of mesothelin detected by immunohistochemistry may not always be correlated. Alternatively, tissue-specific methylation and mechanisms for gene up-regulation may be involved.

There are at least three mesothelin variants [2, 28-31]. Mesothelin variant 1 is attached to the cell membrane by a glycosylphosphatidyl inositol (GPI) linkage and appears to be the predominant mRNA in both normal and tumor cells. This variant 1 is also a dominantly expressed protein on the cell surface of ovarian carcinoma cells. Also, the variant is currently considered as a major released form detected in the serum as a diagnostic marker [29, 30]. Variant 2 has a 24-bp insert, and variant 3 has an 82-bp insert, which leads to the premature termination of the protein, resulting in the loss of GPI anchorage and its release from the cell. Variants 2 and 3 are expressed and released much less frequently. Thus, it is reasonable that methylation/hypomethylation changes at promoter CpG sites may predominantly affect the expression of variant 1.

There are several potential transcription start sites [18, 32]. We do not know which sites are involved in ovarian and endometrial tumors. The patterns of usage of these transcription start sites may vary from organ to organ and from tumor to tumor. These possible differences may also affect the methylation levels.

Mesothelin is one of the new promising tumor markers for tumor monitoring. Molecular and vaccine therapies targeting mesothelin are currently being investigated [2, 15, 25, 33]. Further analysis of the regulation of mesothelin expression may also advance the future development of molecular therapeutic approaches.

In conclusion, we have detected variable levels of methylation/hypomethylation at CpG sites in the mesothelin promoter region in ovarian and endometrial tumors, but there was some correlation with its protein expression status. We speculate that although methylation/hypomethylation changes may affect its transcription, other mechanisms may synergically operate in tissue-specific expression and tumor-related mesothelin overexpression.

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