Epigenetic Inactivation of TMS1/ASC in Ovarian Cancer

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ABSTRACT

Purpose: The purpose of this work was to explore the role of epigenetic inactivation of apoptotic pathways in ovarian cancer by examining the DNA methylation and expression status of four proapoptotic genes in primary ovarian cancers and cancer cell lines and to correlate those findings with the clinicopathological features of ovarian cancer patients.

Experimental Design: Genomic DNA was isolated from 15 ovarian cancer cell lines, 80 primary ovarian cancer specimens, and 4 normal ovary specimens using phenol-chloroform extraction. The methylation status of the DNA was evaluated using combined bisulfite restriction analysis, gene expression was evaluated using reverse transcription-PCR, and histone acetylation was evaluated using chromatin immunoprecipitation.

Results: Of the four proapoptotic genes studied, expression of TMS1/ASC was absent in six ovarian cancer cell lines. Dense methylation of the 5' region of TMS1/ASC was detected in cells not expressing TMS1/ASC. Treating methylated cells with 5-aza-deoxyctydine restored gene expression, confirming the role of methylation in silencing the gene. Chromatin immunoprecipitation revealed histone to be deacetylated in cells not expressing TMS1/ASC, indicating that histone deacetlylation is also involved in silencing TMS1/ASC. Aberrant methylation of TMS1/ASC was detected in 15 of 80 ovarian cancer tissues (19%) but in none of the normal ovary specimens. Aberrant methylation of TMS1/ASC was observed significantly more often in clear cell-type ovarian cancers than in other tumor types ($P < 0.0001$).

Conclusions: Methylation-mediated silencing of TMS1/ASC confers a survival advantage to tumor cells by enabling them to escape apoptosis. The role for aberrant methylation in human ovarian tumorigenesis may be particularly important for ovarian cancers with the clear cell phenotype.

INTRODUCTION

Ovarian cancer is the most deadly of gynecological malignancies, with an overall 5-year survival rate of <30% (1). In large part, this is because the disease usually presents at an advanced stage because there are no overt symptoms at early stages. In addition, ovarian cancers are morphologically and biologically heterogeneous and associated with distinct genetic alterations (2, 3). For example, ovarian cancers of the serous type often show mutations of p53 (4), whereas ovarian cancers of the mucinous type frequently show K-ras mutations (5), and those of the endometrioid type show mutations of b-catenin (6). Little is known about genetic alterations in clear cell-type ovarian cancer.

Epigenetic alterations, such as aberrant methylation of the CpG island in the promoter region, are also associated with the silencing of tumor suppressor genes in human cancers (7, 8). Methylation-mediated gene silencing contributes to malignant progression by inactivating genes involved in tumor suppression, such as those involved in DNA repair and suppressing genomic instability and metastasis. Aberrant methylation likely also contributes to human tumorigenesis by conferring resistance to cell death signals by silencing genes that promote apoptosis (9, 10). In ovarian cancer, aberrant methylation of such cancer-associated genes as p16INK4A (11), RASSFIA (12), BRCA1 (13), and hMLH1 (14) has been reported. The importance of epigenetic alteration of proapoptotic genes in ovarian cancer is largely unexplored, however (15–17).

In the present study, therefore, we examined the methylation status of four proapoptotic genes previously shown to be inactivated by DNA methylation in various types of human neoplasia (9, 18–20). Death-associated protein kinase (DAPK) is a calmodulin-regulated serine/threonine protein kinase involved in diverse apoptosis pathways and in tumor suppression (9, 21). APAF1 is a cell death effector that acts with cytochrome c and caspase (CASP) 9 to mediate p53-dependent apoptosis (18). TMS1/ASC is a member of the CASP recruitment domain family of proapoptotic mediators and also acts in concert with CASP9 to recruit other activators downstream in this cascade (19). The TMS1/ASC gene was originally identified as a target of methylation-induced silencing using cell lines that overexpress DNA methyltransferase 1 (DNMT1). In another critical pathway mediating cell death via death receptors, CASP8 acts as a key apoptotic enzyme by serving as an "initiator CASP"; moreover, CASP8 was recently shown to be silenced by aberrant methylation (20, 22). However, because the 5' region of CASP8 does not contain a typical CpG island, the relevance of methylation to
its silencing remains unclear (22). Here, we have shown that TMS1/ASC is inactivated by DNA methylation and histone deacetylation in ovarian cancers. In particular, there was a strong correlation between methylation of TMS1/ASC and clear cell-type tumors. These findings shed new light on the molecular basis of this morphological type and could contribute to the development of more specific and effective treatments for ovarian cancer, especially clear cell carcinoma.

MATERIALS AND METHODS

Cell Lines and Specimens. Eight ovarian cancer cell lines (SKOV-3, OVCA, PA-1, Caov-3, TOV112D, TOV21G, SW626, and OV-90) were obtained from the American Type Culture Collection (Manassas, VA); seven others (MH, KURA, AMOC2, MCAS, KF, KFr, and HTBOA) were described previously (23, 24). One cervical cancer cell line (OMC-1) was also examined (25). All cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum and incubated under a 5% CO2 atmosphere at 37°C.

The mixture was then incubated for 16 h at 50°C, and the DNA was isolated from the cell lines and primary tissue samples (cancerous and normal) using the phenol-chloroform method. DNA was isolated from the cell lines and primary tissue samples using the phenol-chloroform method. Primers were designed based on the reverse strand Y and R = G or A.

Reverse Transcription-PCR. Expression of TMS1/ASC, DAPK, CASP8, and APAF1 was analyzed by reverse transcription-PCR. Total RNA was extracted from cell lines using Trizol (Life Technologies, Inc.) according to the manufacturer’s instructions. The reverse transcription reaction was performed on 2 μg of total RNA using a SuperScript II First-Strand Synthesis system (Invitrogen) with random primer. PCR was carried out in solution containing 1× PCR buffer (TaKaRa), 200 μm each deoxynucleotide triphosphate, 2.5 pmol of each primer, 1 unit of ExTaq polymerase (TaKaRa), and 5% (v/v) DMSO. The oligonucleotide sequences and PCR parameters used are shown in Table 1. The housekeeping gene GAPDH served as an internal control to confirm the success of the reverse transcription reaction. The PCR products were subjected to 2.5% agarose gel electrophoresis.

Combined Bisulfite Restriction Analysis. Genomic DNA was isolated from the cell lines and primary tissue samples (cancerous and normal) using the phenol-chloroform method and then treated with sodium bisulfite as described previously (27). Briefly, 2 μg of DNA were denatured for 10 min at 37°C in 2 M NaOH, after which 30 μl of 10 mH hydroquinone (Sigma Chemical Co) and 520 μl of 3 M sodium bisulfite were added. The mixture was then incubated for 16 h at 50°C, and the modified DNA was purified using a Wizard DNA Purification System (Promega, Madison, WI). After treating the DNA with NaOH a second time, the resultant DNA precipitate was resuspended in 20 μl of 10 mM Tris-HCl (pH 8.8) and stored at –80°C until use.

The methylation status of TMS1/ASC and DAPK was examined using combined bisulfite restriction analysis, a semi-quantitative bisulfite-PCR analysis (28). Primers were designed so that both methylated and unmethylated DNA would be amplified equally. PCR was carried out in a 50-μl volume containing 1× PCR buffer (67 mM Tris-HCl (pH 8.8), 16.6 mM MgCl2, 200 μM each deoxynucleotide triphosphate, 2.5 pmol of each primer, 2.5 units of ExTaq polymerase (TaKaRa), and 5% (v/v) DMSO. The oligonucleotide sequences and PCR parameters used are shown in Table 1. The housekeeping gene GAPDH served as an internal control to confirm the success of the reverse transcription reaction. The PCR products were subjected to 2.5% agarose gel electrophoresis.

Table 1. Primer sequences for TMS1 analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing [°C (cycles)]</th>
<th>Size, enzyme</th>
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<tbody>
<tr>
<td>TMS1-set A</td>
<td>F: 5'-GGGGAGTYGGGAGATTAGTG-3'</td>
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<td>166</td>
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<tr>
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<td>R: 5'-AATCTCCAACRCATCAAATAAC-3'</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
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</tr>
<tr>
<td>TMS1-set B</td>
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<td>175</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ACCCRCACTACCTAAACTACCTA-3'</td>
<td>60 (3), 58 (4), 56 (5), 54 (26)</td>
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<tr>
<td>DAPK-set A</td>
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<td>190</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ACTCTCCCAAATATCTAAATCAAC-3'</td>
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<td>TaII</td>
</tr>
<tr>
<td>DAPK-set B</td>
<td>F: 5'-TAGATTTGGTTGTTGGAGGTT-3'</td>
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<td>157</td>
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<tr>
<td>DAPK-set C</td>
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<td>188</td>
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<td></td>
<td>R: 5'-TACCCCCTTTTACCTACCAAATC-3'</td>
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<td>MboI</td>
</tr>
<tr>
<td>ChIP</td>
<td>TMS1 F: 5'-GAGTCGGGAGACCAGTGG-3'</td>
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<td>380</td>
</tr>
<tr>
<td></td>
<td>F: 5'-ACACAGCTTCAGCTGGAATCTG-3'</td>
<td>60 (40)</td>
<td>380</td>
</tr>
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</table>

* RT-PCR, reverse transcription-PCR; DAPK, death-associated protein kinase; ChIP, chromatin immunoprecipitation; CASP, caspase.
(NH₄)₂SO₄, 6.7 mM MgCl₂, and 10 mM β-mercaptoethanol), 0.25 mM deoxynucleotide triphosphate mixture, 0.5 μM each primer, and 1.0 unit of Hot Start Ex-Taq polymerase (TaKaRa). The oligonucleotide sequences and PCR parameters used for bisulfite-PCR are shown in Table 1.

**Bisulfite Sequencing.** For bisulfite sequencing, 2 μl of bisulfite-modified DNA were amplified by PCR using primers TMS1GM1-F and TMS1GM1-R. The amplified products were then cloned into pCR4.0 vector using a TOPO-TA cloning kit (Invitrogen), and at least five clones were sequenced for each cell line analyzed. The plasmid DNA was purified with QIAprep Spin Mini Prep Kit (Qiagen) and then sequenced using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

**Immunofluorescence.** Cells were either mock treated or treated with 2.0 μM 5-aza-dC for 72 h and then fixed in acetone/methanol. D086-3, a monoclonal antibody against human TMS1/ASC developed by Masumoto et al. (29), was used as a primary antibody (MBL, Nagoya, Japan). Alexa 488 antimouse IgG antibody (Cosmo Bio, Tokyo, Japan) was used as the secondary antibody. Finally, the nucleus was stained with Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA), and the cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitation was carried out as described previously (30). Briefly, cells were incubated in 1% formaldehyde for 10 min at 37°C. The nuclei were then collected, sonicated to yield fragments ranging in size from 300 to 2000 bp, and immunoprecipitated with anti-histone H3 antibody (Upstate Biotechnology). This antibody specifically recognizes the diacetylated lysine residues (lysines 9 and 14) of histone H3. The chromatin was recovered using protein A-Sepharose. PCR was then carried out in 50 μl of solution containing 1 μl of chromatin DNA, 2.5 pmol of each primer, and 25 μl of SYBR green PCR mixture (Applied Biosystems). The primers used are shown in Table 1. The PCR cycling protocol consisted of 1 cycle at 95°C for 5 min and 40 cycles at 95°C for 30 s and 60°C for 1 min. Fluorescent signals were detected using an ABI 7000 Prism 7000 (Applied Biosystems), and the accumulation of PCR product was measured in real time as the increase in SYBR green fluorescence. Data were analyzed using ABI Prism 7000 SDS Software (Applied Biosystems). Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified PCR product as a template and used to calculate the DNA copy number in each sample. Ratios of the intensities of the TMS1/ASC and GAPDH signals were used as a relative measure of the level of TMS1/ASC expression in each specimen.

**Statistical Analysis.** The statistical analysis was carried out using StatView software (SAS Institute Inc., Cary, NC). Fisher’s exact test (two-sided) was used to determine the association between TMS1/ASC methylation and clinicopathological features. Values of P < 0.05 were considered significant. Overall survival time was defined as the period between the diagnosis of ovarian cancer and the time of death. Differences between survival curves were analyzed using the log-rank test.

**RESULTS**

We initially examined the expression status of four proapoptotic genes (TMS1/ASC, DAPK, APAF1, and CASP8) previously shown to be epigenetically inactivated in human tumors (9, 10, 18–20) using cDNA from 15 ovarian cancer cell lines and 1 cervical cancer cell line (Fig. 1A). Of the four genes studied, TMS1/ASC expression was lost in six cell lines (AMOC2, SKOV-3, HTBOA, PA-1, TOV112D, and TOV21G) and diminished in two cell lines (OVCAR-3 and KURA), and 1 cervical cancer cell line (Fig. 1A). Of the four genes studied, TMS1/ASC expression was lost in six cell lines (AMOC2, SKOV-3, HTBOA, PA-1, TOV112D, and TOV21G) and diminished in two cell lines (OVCAR-3 and KURA), and expression of DAPK was lost in two cell lines (KF and KFr). Expression of APAF1 and CASP8, by contrast, was readily detectable in all 16 cell lines studied. Epigenetic silencing mediated by DNA methylation was responsible for blocking expression of TMS1/ASC and DAPK, an observation confirmed by the finding that treating the affected cells with a methyltransferase inhibitor (5-aza-dC) restored expression of both (Fig. 1B).

**Fig. 1** A. reverse transcription-PCR analysis of expression of proapoptotic genes in ovarian cancer cell lines. Controls consist of carrying out PCR reactions without reverse transcription and amplification of GAPDH to assess the integrity of the cDNA. Genes examined are shown on the right. B. Restoration of TMS1/ASC expression by treatment with 5-aza-deoxycytidine. Reverse transcription-PCR was carried out with RNA extracted from cell lines before (No treat) or after (Aza-dC) incubation of cells with 1 μM 5-aza-deoxycytidine for 72 h.

**Fig. 2** Immunofluorescence analysis of expression of TMS1/ASC protein in ovarian cancer cell lines. Shown are Caov-3 cells (an unmethylated cell line; top panel), AMOC2 cells (a methylated cell line; middle panel), and AMOC2 cells treated with 5-aza-deoxycytidine (bottom panel).
Moreover, immunohistochemical analysis confirmed that TMS1/ASC protein was not expressed in cells in which the gene was silenced (Fig. 2) and that expression of the protein was also restored by treatment with 5-aza-dC (Fig. 2).

TMS1/ASC and DAPK contain CpG islands that span about 2 kb. To examine in more detail how DNA methylation silences these genes, bisulfite-PCR analysis using primer sets that cover the entire CpG islands of the genes was carried out, followed by restriction digestion (Figs. 3A and 4A). Aberrant methylation of the region around the transcription start site of TMS1/ASC was detected in 9 of 16 cell lines (56%) but in none of the 4 normal ovarian tissue specimens (Fig. 3B). Methylation of exon 2 was detected in some cell lines in which expression of TMS1/ASC was lost or diminished, although five cell lines that expressed TMS1/ASC also showed methylation of exon 2. The fact that demethylation of the 5′ region of TMS1/ASC was observed after treatment with 5-aza-dC is suggestive of the role played by methylation in the gene silencing (Fig. 3C). Among the primary ovarian cancers, aberrant methylation of TMS1/ASC was detected in 15 of 80 (19%) cases (Fig. 3D).

Dense methylation of the region around the 5′ transcription start site, 5′ CpG island, of DAPK was detected in 2 of 16 cell lines (13%) that did not express the gene (Fig. 4B). In contrast, methylation of Alu was detected in all cell lines studied, as well as in the four normal ovarian tissue specimens. Methylation of exon 2 was detected in 7 of 16 (44%) cell lines, including 5 cell lines that expressed the gene and 2 cell lines that did not. Apparently, only methylation of the 5′ region of the transcription start site, not of Alu or exon 2, correlated with loss of expression. None of the 80 primary cancer specimens showed methylation of the 5′ CpG island of DAPK, indicating it to be a rare event in primary ovarian tumors (Fig. 4C).

We confirmed by bisulfite DNA sequencing that the methylation detected by combined bisulfite restriction analysis reflected the overall methylation level of the region analyzed (Fig. 5). In three cell lines (KURA, SKOV-3, and PA-1) shown by bisulfite-PCR to be densely methylated, almost 90% of the CpG dinucleotides analyzed were methylated. By contrast, the normal ovarian tissue and two cell lines (KF and MCAS) that did not show methylation by bisulfite-PCR were unmethylated at the majority of CpG dinucleotides analyzed.

It was shown recently that deacetylation of histone is also involved in methylation-dependent gene silencing (7, 10, 31). Consistent with those findings, we found that in the methylated SKOV-3 cell line, 5-aza-dC and trichostatin A acted synergistically to restore TMS1/ASC expression (Fig. 6A). When we then carried out chromatin immunoprecipitation analysis using an anti-acetylated histone H3 antibody to determine the acetylation status of histone in the TMS1/ASC promoter region, we found that methylated cell lines not expressing TMS1/ASC (AMOC2, KURA, SKOV-3, and PA-I) were indeed unmethylated in their 5′ CpG island compared with those cell lines that expressed the gene (Fig. 6C).
SKOV-3, and HTBOA) showed lower levels of histone acetylation than unmethylated cell lines (KF and MCAS; Fig. 6B).

Finally, we examined the clinicopathological features of ovarian cancers with or without TMS1/ASC methylation (Table 2). Aberrant methylation of TMS1/ASC was detected in 3 of 23 (13.0%) serous tumors, 1 of 16 (6.3%) endometrioid tumors, 9 of 13 (69.2%) clear cell tumors, no mucinous tumors, and 1 of 5 (20.0%) undifferentiated carcinomas. There was a significant correlation between the clear cell phenotype and TMS1/ASC methylation ($P < 0.0001$), but no significant association was seen with any other clinicopathological feature (patient age, the International Federation of Gynecology and Obstetrics [FIGO] clinical stage, and pathological grade). There was also no correlation between the patients’ prognoses and the TMS1/ASC methylation status (data not shown).

**DISCUSSION**

Little is known about the changes in proapoptotic genes in ovarian cancers. TMS1/ASC is a novel proapoptotic gene previously identified as a target of DNA methylation in breast cancer (19), and we have shown here that aberrant methylation of the 5’ region of TMS1/ASC is well correlated with loss of expression in ovarian cancer. Notably, decreasing TMS1/ASC expression reduces sensitivity to chemotherapeutic drugs (29); TMS1/ASC contains a CASP recruitment domain and plays a role in CASP-mediated apoptosis induced by inflammation and chemotherapeutic drugs (32, 33). By enabling them to escape apoptosis, methylation-mediated silencing of TMS1/ASC would be expected to contribute to a survival advantage for tumor cells, which supports a role for aberrant methylation in human ovarian tumorigenesis (31, 33).

Methylation of DAPK also has been reported in several tumors, including B-cell malignancy and lung, bladder, colorectal, and gastric cancers (9, 10, 21, 34). We found DAPK to be methylated in several ovarian cancer cell lines but in 0 of 80 primary ovarian cancers studied, suggesting that it is a rare event in primary ovarian cancers, and another study showed 2 of 23 (9%) cases of methylation in primary ovarian cancers (35). However, methylation of DAPK has been associated with metastasis (34), which suggests that it may be a more common feature of metastatic ovarian cancer. Two cell lines, KF and KFr, did not express DAPK as a result of DNA methylation. These cells were originally established from serous cystadenocarcinoma cell lines and are relatively resistant to chemotherapeutic drugs (24, 36). It is thus plausible that epigenetic inactivation of DAPK provides cancer cells with the ability to escape apoptosis triggered by the DAPK signaling pathway. Obviously, further study will be necessary to determine whether methylation of DAPK plays a role in the progression of ovarian cancer in vivo or whether it is a cell line-specific event.

We examined several regions of CpG islands to identify aberrant methylation of the TMS1/ASC and DAPK promoters. Analysis of the TMS1/ASC CpG island showed that methylation of the region around the transcription start site, but not the edge of island, is important for gene silencing. Indeed, methylation of the edge of the CpG island was detected in virtually all cell lines tested, regardless of gene expression. We found no age-related methylation of the region around the TMS1/ASC transcription start site in normal ovarian tissues from patients.

Of particular interest to us was the finding that methylation of TMS1/ASC was associated with the clear cell type of ovarian cancer. The morphological heterogeneity of ovarian cancer clearly contributes to the difficulty of defining the molecular events associated with its development and prognosis. Four
major types of primary ovarian adenocarcinomas have been identified on the basis of morphological criteria: serous; mucinous; endometrioid; and clear cell. Of these, clear cell carcinoma has a particularly unfavorable prognosis and is classified as a high-grade neoplasm in clinical practice. In the present study, aberrant methylation of TMS1/ASC was detected in 69% of clear cell carcinomas, which is a significantly higher frequency than was seen in the other histological types (serous, 13%; mucinous, 0%; endometrioid, 6.3%; undifferentiated, 20%). Thus, inactivation of TMS1/ASC may play a key role in tumorigenesis of ovarian cancers with a specific etiology and showing the clear cell phenotype.

Evidence suggests that the various histological types of ovarian cancer represent distinct disease entities, and that patterns of gene expression in ovarian cancer reflect both morphological and biological behavior. In fact, ovarian cancer with the clear cell phenotype has a distinctive pattern of gene expression that distinguishes it from other ovarian cancers with poor prognoses (37). For instance, almost all clear cell cancers are resistant to platinum agents, which are key chemotherapeutic agents used in the treatment of ovarian cancer (38). It may be that inactivation of the apoptotic pathway associated with TMS1/ASC and CASP9 contributes to this chemoresistance and to the other biological features of clear cell cancer. Indeed, the prognosis of patients with cancers having the clear cell phenotype are poor, even when the disease is detected at an early stage, and the cancers frequently recur after adjuvant chemotherapy (38). Further study will be necessary to more definitively characterize clear cell ovarian cancer. In that regard, methylation analysis may contribute to the development of diagnostic markers to predict sensitivity to chemotherapeutic drugs; moreover, because inhibitors of DNA methylation and histone deacetylation act synergistically to induce expression of TMS1/ASC, these drugs may be useful for restoring apoptotic signaling pathways and sensitizing cancer cells to chemotherapeutic agents.

In conclusion, methylation-mediated silencing of TMS1/ASC confers a survival advantage to tumor cells by enabling them to escape apoptosis and may thus be a useful target for therapies aimed at treating ovarian cancers resistant to conventional chemotherapy. The role for aberrant methylation in human ovarian tumorigenesis may be particularly important for ovarian cancers with the clear cell phenotype.

ACKNOWLEDGMENTS

We thank Dr. William F. Goldman for editing the manuscript.

REFERENCES


Table 2 Patient characteristics and TMS1 methylation status

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a FIGO, the International Federation of Gynecology and Obstetrics. b Clear cell is significantly different from other types by Fisher’s exact test (P < 0.0001).

Fig. 6 Role of histone deacetylation in silencing TMS1/ASC in ovarian cancer cells. A. SKOV-3 cells were treated with 5-aza-deoxycytidine (5-aza-dC) and trichostatin A and harvested, after which reverse transcription-PCR was carried out using the cDNA prepared from the cells. The cells were mock treated or treated with 300 nM trichostatin A, 0.2 μM 5-aza-dC, 300 nM trichostatin A + 0.2 μM 5-aza-dC, or 2.0 μM 5-aza-dC. B. quantitative analysis of histone acetylation. Chromatin immunoprecipitation analysis was carried out using DNA precipitated with anti-acetylated histone H3 antibody; the bars show the levels of histone acetylation determined by real-time PCR normalized to the GAPDH signal. The cell line of interest is indicated below the panel.


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