Promoter methylation status and expression of *TMS1* gene in human epithelial ovarian cancer

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Gene silencing associated with aberrant DNA methylation of promoter CpG islands is one mechanism through which tumor suppressor genes are inactivated in human cancers. *TMS1* (*target of methylation-induced silencing*) is a CpG island-associated gene that functions in the regulation of apoptosis. In this study, we examined the DNA methylation status of the TMS1 promoter in ovarian cancer cell lines and tissues by methylation-specific PCR (MSP) and its mRNA expression by reverse transcription and quantitative PCR. Aberrant methylation of TMS1 was present in 7/12 ovarian cancer cell lines and 8/20 primary ovarian cancer tissues. The median value of relative *TMS1* gene expression in cancers with methylation (0.15) was significantly lower than that in cancers without methylation (13.9) ($P<0.001$). The expression of the *TMS1* gene was relatively high (48.5) in the normal ovarian cDNA library. *TMS1* gene expression was restored by treatment with the demethylating agent 5-aza-2′-deoxycitidine in the OV90 cell line, which lacks the TMS1 transcript. Our results suggest that aberrant methylation of TMS1 may play a role in the pathogenesis of ovarian cancer. (Cancer Sci 2004; 95: 40–43)

The cause of epithelial ovarian cancer is unknown. Although BRCA1 and BRCA2 have been identified as being associated with susceptibility to ovarian cancer,[1, 2] mutations in these genes have been found in only 2% to 3% of all ovarian cancers. The remaining cases are considered to be sporadic in nature and to arise as a result of acquired alterations in oncogenes and tumor suppressor genes such as *TP53* and *PTEN*.[3, 4]

DNA methylation has an essential regulatory function in mammalian development, suppressing gene activity by changing chromatin structure.[5, 6] It has become apparent that aberrant DNA methylation of promoter CpG islands may serve as an alternative mechanism to genetic defects for the inactivation of tumor suppressor genes in human cancers.[7, 8] Accordingly, the identification of genes that are inactivated by methylation could lead to the discovery of novel genes involved in the initiation and progression of human neoplasia.

Target of Methylation-associated Silencing-1 (TMS1: also known as ASC and PYCARD) was identified through screening for downstream targets of methylation-mediated silencing induced by ectopic expression of human DNA methyltransferase-1 (DNMT1).[9] TMS1 was also identified in an '*in silico*' screen for mRNAs that were under-represented in breast cancer cDNA libraries.[10] Subsequent studies indicated that loss of TMS1 expression in breast cancer cell lines was correlated with methylation of selected sites in the TMS1 CpG island.[9] TMS1 contains a caspase recruitment domain (CARD), and induces apoptosis through a CARD-dependent aggregation step followed by activation of a caspase-9-mediated pathway.[11] Conway *et al*. reported that TMS1 suppressed the growth of breast cancer cells in a gene replacement study.[9] These data implicate TMS1 as a potential tumor suppressor gene.

TMS1 was demonstrated to be aberrantly methylated and silenced in a significant proportion of human breast cancers, lung cancers, and gastric cancers.[12–14] However, its expression and role in epithelial ovarian cancer have not been examined. Therefore, in this study, we examined the promoter methylation status and gene expression of TMS1 in epithelial ovarian cancer cells.

Materials and Methods

Ovarian cancer cell lines and tissues. OVCAR-3, Caov3, SKOV-3, TOV112d, TOV21G, OV90, and ES2 (adenocarcinoma: OVCAR-3, SKOV-3; serous adenocarcinoma: Caov-3, OV90; clear cell adenocarcinoma: TOV21G, ES2; endometrioid adenocarcinoma: TOV112D) cell lines were purchased from American Type Culture Collection. JHOS2, JHOS3, HTOA, OMC3, and JHOC5 (serous adenocarcinoma: JHOS2, JHOS3, HTOA; mucinous adenocarcinoma: OMC3; clear cell adenocarcinoma: JHOC5) cell lines were purchased from Riken Cell Bank (Tsukuba). Cell lines were maintained in DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin (Invitrogen), and incubated in 5% CO_2 at 37°C. For 5-aza-2′-deoxycytidine (5azaC) treatment, 1×10^6 cells were seeded into T75 flasks, and treated with 0.5 μ*M* or 1.0 μ*M* 5azaC (Sigma) for 72 h.

Twenty primary tumors (11 serous, 4 clear cell, 3 endometrioid, and 2 mucinous adenocarcinoma) were retrieved from surgical pathology files at Tohoku University Hospital, Sendai. These specimens were all fixed in 10% formalin and embedded in paraffin. The research protocol was approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai.

Methylation-specific PCR (MSP). Methylation status of the samples was investigated by MSP as described in the literature.[15] Genomic DNA from ovarian cancer tissue was extracted by using laser capture microdissection and treated with proteinase K (0.5 mg/ml) for 48 h at 37°C. Genomic DNA from ovarian cancer cell lines was extracted using an AquaPure Genomic DNA kit (Biorad). The quality and integrity of the DNA were evaluated in terms of the $A_{260/280}$ ratio. One microgram of genomic DNA was treated with sodium bisulfite using a CpGenome DNA modification kit (Intergen) according to the instructions. Amplification was achieved in a 20-μl reaction volume containing 2 μl of 10× Ex*Taq* buffer, 1.5 μl of 25 m*M* $MgCl_2$, 1 μ*M* of each primer, 1.5 μl of 2.5 m*M* dNTPs, and 1 unit of TaKaRa Ex*Taq* polymerase (TaKaRa). Hot start PCR was performed in a thermal cycler (TaKaRa) for 35 cycles, each of which consisted of denaturation at 96°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 90 s, followed by a final 10 min extension at 72°C. Primers used were 5′-GGTTGTAGTGGGGTGAGTGGT-3′ and 5′-CAAAACATCCATAAACAACAACACA-3′ (191-bp) for the unmethylated reaction, and 5′-TTGTAGCGGGGTGAGCGGC-3′ and 5′-AACGTCCATAAACAACAACGCG-3′ (196-bp) for the methylated reaction.[9] Universal methylated human male genomic DNA (Intergen) was used as a positive control for the methylated reaction. Genomic DNA purified from peripheral blood of a healthy voluntary donor was used as a positive control for the

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unmethylated reaction. A blank control containing all PCR components except template DNA was also included in all PCRs. Reaction products were separated by electrophoresis on a 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination. Specimens with purely unmethylated promoters will have positive PCR products with U primers but not with the M primers. A specimen that contains purely methylated promoter will have PCR products with M primers but not with the U primers. A specimen of heterogenous status, with both methylated and unmethylated promoters, will have PCR products from both U primers and M primers.

Reverse transcription and quantitative PCR. Total RNA was isolated from cell lines by phenol-chloroform extraction using Isogen (Nippon Gene). RNA was treated with RNase-free DNase (Roche Diagnostics) (1 μg/μl) for 2 h at 37°C, followed by heat inactivation at 65°C for 10 min. A RT-PCR kit ("SUPERSCRIPT" II First-strand synthesis system, Invitrogen) was employed and cDNA synthesis was carried out according to the instructions. cDNAs were synthesized from 2 μg of total RNA using random hexamer and reverse transcription was carried out for 50 min at 42°C with SUPERSCRIPT II reverse transcriptase. Real-time quantitative PCR was performed using the "iCycler" (Biorad). For the determination of TMS1 cDNA content, a 25 μl reaction mixture consisting of 23 μl of "iQ" SYBR Green MasterMix, 1 μ*M* of each primer, and 1 μl of template cDNA were prepared. The PCR conditions were as follows: initial duration of 60 s at 96°C, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 63°C (for β-actin, 64°C) for 30 s, and extension at 72°C for 30 s. The fluorescence intensity of the double-strand-specific SYBR Green I, reflecting the amount of formed PCR product, was read at 86°C after the end of each elongation step. Primers used were TMS1: 5′-TGGCTGCTGGATGCTCTGT-3′ and 5′-CAGGCTGGTGTGAAACTGAAG-3′ (116-bp); β-actin: 5′-CCAACCGCGAGAAGATGA-3′ and 5′-GGAAGGAAGGCTGGAAGAGT-3′ (459-bp). β-Actin cDNA fragments were amplified as internal positive controls. A normal human ovarian cDNA library (Stratagene) was used as normal control, cDNA from peripheral blood of a healthy voluntary donor was used as a positive control, and a water blank was used as a negative PCR control. Control reactions in which reverse transcriptase was omitted were amplified under the same conditions to exclude DNA contamination (data not shown). Two independent RT-PCR reactions were performed for each sample.

Results

Methylation status of *TMS1* gene in ovarian cancer cell lines and tissues. Among the 12 ovarian cancer cell lines in which TMS1 promoter methylation was investigated, the presence of methylated TMS1 band was detected in 7 cell lines, 4 of which also contained the unmethylated band, as shown in Fig. 1. The methylated band was detected in all cell lines derived from clear cell adenocarcinoma (TOV21G, ES2, JHOC5), 2/5 from serous adenocarcinoma (Caov3, OV90, JHOS2, JHOS3, HTOA), 1/1 from endometrioid adenocarcinoma (TOV112D), and 0/1 from mucinous adenocarcinoma (OMC-3). We also analyzed the methylation status in microdissected ovarian cancer tissues (Fig. 2). Among the 20 cases examined, the presence of the methylated band was detected in 8 cases (5/11 serous, 1/4 clear cell, 1/3 endometrioid, and 1/2 mucinous adenocarcinomas), 3 of which also contained the unmethylated band.

Expression of *TMS1* gene in ovarian cancer cell lines. The expression of *TMS1* gene in the cell lines is illustrated in Fig. 3. Quantitative RT-PCR was performed and the ratio of TMS1:β-actin was calculated to allow for comparison between the cell lines. The median value of relative *TMS1* gene expression in cell lines with methylation (0.15) was significantly lower than

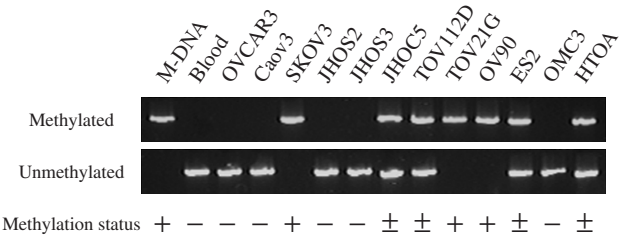

Fig. 1. Methylation status of the *TMS1* gene in ovarian cancer cells. The presence of a visible PCR product in 'Methylated' lanes indicates the presence of methylated alleles (196-bp) of TMS1, and in the 'Unmethylated' lanes, it indicates the presence of unmethylated alleles (191-bp). Methylation status means as follows: +, purely methylated alleles; ±, methylated and unmethylated alleles; −, purely unmethylated alleles. M-DNA, universal methylated human male genomic DNA (positive control for methylated band); blood, positive control for unmethylated band.

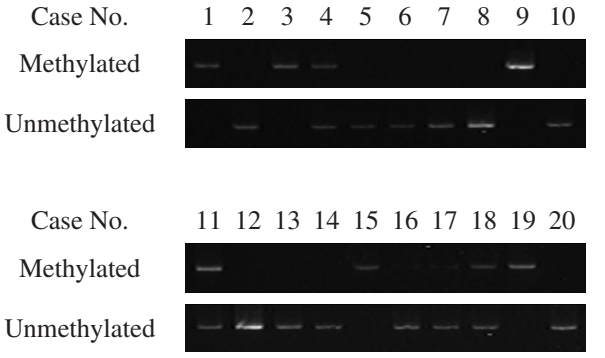

Fig. 2. Methylation status of *TMS1* gene in ovarian cancer tissues. The methylation status of the *TMS1* gene in 20 microdissected ovarian cancer tissues (1–11, serous; 12–15, clear cell; 16–18, endometrioid; 19, 20, mucinous adenocarcinoma) was evaluated by MSP. Definitions of 'Methylated' and 'Unmethylated' lanes are the same as in Fig. 1.

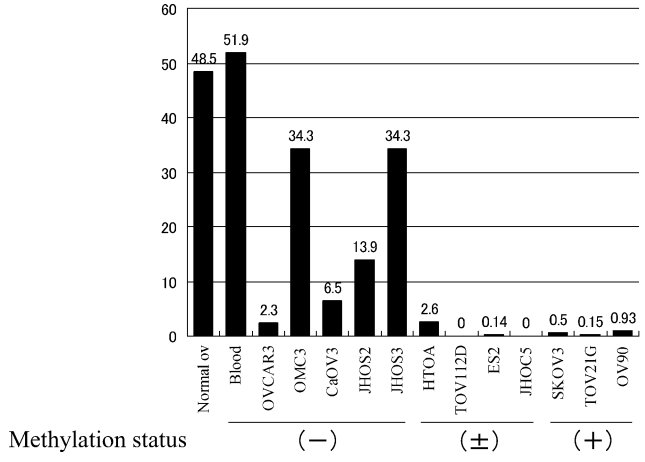

Fig. 3. Expression of *TMS1* gene in ovarian cancer cells. Two independent RT-PCR reactions were performed for each sample, and the ratio of TMS1: β-actin was calculated and normalized. The median values are indicated above each bar. Methylation status is indicated in the same way as in Fig. 1.

that in cell lines without methylation (13.9) ($P<0.001$, Kruskal-Wallis test). The expression of mRNA appeared not to be affected by the existence of the unmethylated band. In OVCAR3, the expression of TMS1 was relatively decreased, although this

cell line does not have methylated TMS1 promoter alleles. The expression of the *TMS1* gene was relatively high (48.5) in the normal ovarian cDNA library. These results indicate that aberrant DNA methylation is the main pathway of transcriptional silencing of the *TMS1* gene in ovarian cancer cells, although it seems not to be the only mechanism of inactivation.

Effect of 5-aza-2′-deoxycytidine treatment. To further confirm that methylation contributed to loss of expression of the *TMS1* gene, we assessed the effect of 5azaC, a demethylating agent, on TMS1 mRNA expression by quantitative RT-PCR. Treatment of OV90 cells with 5azaC for 3 days resulted in the demethylation of the TMS1 CpG island and reexpression of TMS1 (Fig. 4). The amount of expression of mRNA after treatment (10.75) was more than 10-fold higher than that before treatment (0.78), and the difference was significant ($P<0.001$, Kruskal-Wallis test). The decreased expression of TMS1 in this cell line is therefore not attributable to abnormalities at the gene level or to inability to express TMS1, but rather is directly related to the methylation of TMS1.

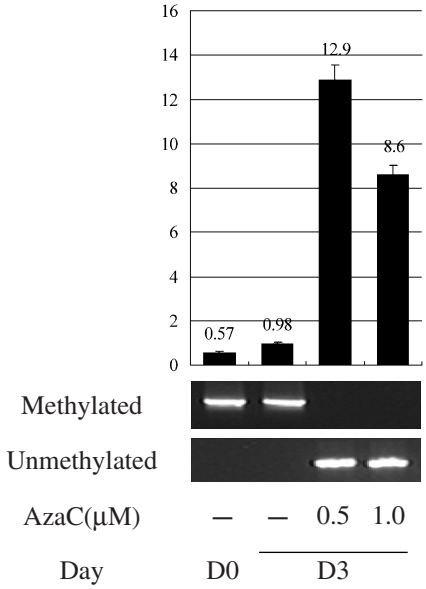

Fig. 4. Reexpression of *TMS1* gene by 5-aza-2′-deoxycitidine (5azaC) treatment. RT-PCR and MSP results for OV90 ovarian cancer cell line with or without 5azaC treatment for 3 days. PCRs were performed under the same conditions as described in "Materials and Methods."

Discussion

Aberrant methylation of promoter CpG islands is well recognized as a mechanism associated with the loss of tumor suppressor gene expression in human cancers, and the list of methylated genes identified in various cancer types continues to grow. To our knowledge, this is the first study to demonstrate aberrant methylation and frequent inactivation of the *TMS1* gene in ovarian cancer cells.

In the present study, a clear correlation between aberrant DNA methylation and silencing of the *TMS1* gene was observed except for one cell line (OVCAR3), which showed an unmethylated pattern despite loss of TMS1 expression. Stimson and Vertino demonstrated that TMS1 expression was not restored with histone deacetylase inhibitors, indicating that histone acetylation is not involved in gene regulation.[16] Also, *TMS1* is the first gene to be identified in a functional screen for methylation-induced gene silencing, and there has been no report that TMS1 is inactivated by loss of heterozygosity (LOH) and/or mutation.[9] Although there are some other routes of gene inactivation, such as loss of transcription factor, these data indicate that aberrant methylation is a primary event in the inactivation of TMS1. Possible involvement of any methylation-independent mechanism remains to be fully investigated.

It is believed that deregulation of programmed cell death, or apoptosis, is involved in the pathogenesis of many human diseases, including cancer.[17] Ectopic expression of TMS1 promotes apoptosis and suppresses the growth of breast cancer cells. Recent evidence suggests that TMS1 interacts with procaspase-1 and regulates the processing of proinflammatory cytokines.[18, 19] Loss of TMS1 through methylation-mediated silencing may be one mechanism through which ovarian cancer cells evade local immune response, and avoid apoptosis.

The incidence of aberrant methylation of the *TMS1* gene in ovarian cancer cells and tissues was similar to that reported earlier in other cancers. Conway *et al*. reported that 4 of 9 breast carcinoma cell lines were completely methylated and did not express TMS1.[9] Virmani *et al*. demonstrated that aberrant methylation of TMS1 was present in 70% of small cell lung cancer (SCLC) cell lines and 48% of non SCLC cell lines, and the methylation status correlated inversely with TMS1 RNA expression.[13] The incidence of aberrant methylation was reported to be relatively low in gastric (1/9) and colorectal (0/13) cancers.[14] On the other hand, *de novo* methylation of 5′CpG islands is a rare occurrence in normal somatic tissues. In primary breast cancers, Levine *et al*. reported that tumor tissues exhibited mixed methylated and unmethylated patterns, whereas adjacent normal tissues exhibited completely unmethylated alleles only.[12] In our study, the expression level of the *TMS1* gene from a normal ovarian cDNA library was relatively high, suggesting that TMS1 is not down-regulated in normal ovarian tissues. However, we could not examine the methylation status of normal ovarian surface epithelial tissues or cells because it is difficult to obtain those tissues and cells. The frequent and direct association between methylation and inactivation for *TMS1* gene suggests that aberrant methylation plays a crucial role in carcinogenesis by precipitating the silencing of TMS1 in these cancers. Although an antibody for TMS1 is not yet available, it will be intriguing to investigate the protein expression of TMS1 by immunohistochemistry or western blotting in future studies.

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