Methylation-associated silencing of the Wnt antagonist *SFRP1* gene in human ovarian cancers

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The *SFRP1* gene on chromosome 8p11.2 encodes a Wnt signaling antagonist, and was recently demonstrated to be a new tumor suppressor that is inactivated by promoter methylation in human colon cancers. Here, we analyzed promoter methylation of the *SFRP1* gene in human ovarian cancers, in which loss of heterozygosity in 8p is frequently observed and involvement of the Wnt signaling pathway has been suggested. Methylation-specific PCR (MSP) analysis showed that four of 13 ovarian cancer cell lines and two of 17 primary ovarian cancers had methylated *SFRP1*, while an immortalized ovarian epithelial cell line, HOSE, and seven ovarian endometrial cyst samples did not. In the four ovarian cancer cell lines with the methylation, *SFRP1* was not expressed at all as determined by quantitative RT-PCR analysis. A cell line with *SFRP1* methylation, MCAS, was treated with a demethylating agent, 5-aza-2′-deoxycytidine, and demethylation of the promoter and re-expression of *SFRP1* were observed. These results show that *SFRP1* is inactivated by promoter methylation in human ovarian cancers, as well as colon cancers. (Cancer Sci 2004; 95: 741–744)

Ovarian cancer is the most lethal gynecological malignancy, and is characterized by the diversity of its histology.[1)] The four major histological subtypes, serous, clear cell, mucinous, and endometrioid, constitute approximately 36, 19, 14, and 15% of the total, respectively, in Japan,[2)] and all of these are considered to be derived from ovarian surface epithelium.[3)] As for genetic alterations, only limited information is available, such as mutations of *β-catenin* in 31% of endometrioid type[4,5)] and c-*erbB-2* amplification in serous type with poor prognosis.[6)] Due to promoter methylation, *BRCA1, hMLH1*, and *RASSF1A* are inactivated (silenced) in 10%,[7)] 12.5%,[8)] and 10–40% of ovarian cancers,[9,10)] respectively. Clarification of molecular alterations is important to identify novel diagnostic and therapeutic targets, and further investigations in ovarian cancers are necessary.

Secreted frizzled-related proteins (SFRPs) are extracellular signaling molecules that antagonize the Wnt signaling pathway.[11,12)] *SFRP1* (8p11.2) is inactivated in colorectal cancers by promoter methylation[13–15)] and also by infrequent mutations.[15)] Other members, *SFRP2* (4q31.3) and *SFRP5* (10q24.1), are also inactivated by promoter methylation in colorectal cancers.[14)] Strikingly, the inactivation of *SFRP* genes enhanced the constitutive Wnt signaling even in the presence of *APC* or *β-catenin* mutations.[14,15)] In ovarian cancers, *SFRP* inactivation could also potentially be present for the following reasons. First, the Wnt signaling pathway can be affected in ovarian cancers, as indicated by the presence of *β-catenin* mutations in cancers of the endometrioid type.[4,5)] Second, in the serous type, in spite of increased β-catenin accumulation,[16)] *β-catenin* mutations are infrequent,[17)] and abnormality in other genes in the Wnt signaling pathway is suggested.

In this study, we analyzed methylation-associated silencing of the *SFRP1* gene on chromosome 8p11.2, which is frequently lost in ovarian cancers.[18,19)]

Materials and Methods

Cell lines, tumor samples, and DNA/RNA extraction. OV-90 (serous), TOV-112D (endometrioid), ES-2 (clear cell), and TOV-21G (clear cell) were purchased from the American Type Culture Collection (Manassas, VA). MCAS (mucinous), RMUG-L (mucinous), RMUG-S (mucinous), RMG-I (clear cell), RTSG (poorly differentiated), TYK-nu (undifferentiated), and KURAMOCHI (undifferentiated) were gifts from the Japanese Collection of Research Bioresources (Tokyo). HTOA (serous) and JHOS-2 (serous) were obtained through the RIKEN Cell Bank (Tsukuba, Japan) through the courtesy of Drs I. Ishiwata and H. Ishikawa. Human ovarian surface epithelial (HOSE) cells were established by immortalizing normal human ovarian surface cells with papilloma virus E6 and E7.[20)] Ovarian cancer samples and endometrial cyst samples were obtained from patients who underwent operation at the University of Tokyo Hospital, with informed consent, and were kept frozen until DNA/RNA extraction. DNA was extracted by a standard phenol/chloroform extraction and ethanol precipitation procedure, and total RNA was isolated using ISOGEN (Nippon Gene, Tokyo).

Bisulfite modification and methylation-specific PCR. Genomic DNA was restricted with *Bam*HI, and purified by phenol extraction. DNA (500 ng) was denatured in 0.3 *N* NaOH, and then subjected to 15 cycles of 30-s denaturation at 95°C and 15-min incubation in 3.1 *M* $NaHSO_3$ (pH 5.3) and 0.5 m*M* hydroquinone at 50°C. The sample was desalted with the Wizard DNA Clean-Up System (Promega, Madison, WI), desulfonated in 0.3 *N* NaOH at room temperature for 5 min, then ethanol-precipitated and dissolved in 20 μl of TE buffer.

Methylation-specific PCR (MSP)[21)] was performed using 1 μl of the sodium-bisulfite-treated DNA. According to the original report,[14)] the primers for the methylated sequence of *SFRP1* were 5′-TGTAGTTTTCGGAGTTAGTGTCGCGC-3′ and 5′-CCTACGATCGAAAACGACGCGAACG-3′ (126 bp; annealing at 60°C), and those for the unmethylated sequence were 5′-GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT-3′ and 5′-CTCAACCTACAATCAAAAACAACACAAACA-3′ (135 bp; 60°C). DNA from HOSE was methylated *in vitro* by using *Sss*I methylase (New England Biolabs, Beverly, MA) and used as a control for M set primers. The number of PCR cycles that gave a minimal band using *Sss*I methylase-treated DNA was determined, and four more cycles were added for actual analysis (total 32 cycles). For U set primers, the number of PCR cycles was determined using DNA from HOSE (32 cycles). For analysis after 5-aza-2′-deoxycytidine (5-aza-dC) treatment, 36 cycles were adopted.

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Abbreviation: *SFRP1*, secreted frizzled-related protein 1.

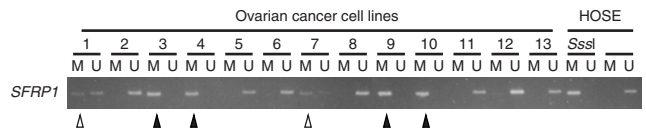

Fig. 1. Methylation of the promoter CGI of the *SFRP1* gene in ovarian cancer cell lines. 1, OV-90; 2, TOV-112D; 3, MCAS; 4, RMUG-L; 5, RMUG-S; 6, ES-2; 7, RMG-I; 8, TOV-21G; 9, RTSG; 10, TYK-nu; 11, KURAMOCHI; 12, HTOA; and 13, JHOS-2. HOSE genomic DNA methylated with *Sss*I methylase (*Sss*I-methylated) was used as a positive control for methylated DNA. Specific amplification of methylated and unmethylated DNA was confirmed by observing a band with M set primers in *Sss*I-methylated DNA and a band with U set primers in HOSE. Four cell lines had only methylated DNA molecules (shown by closed arrowheads), and two cell lines had both methylated (shown by open arrowheads) and unmethylated DNA molecules.

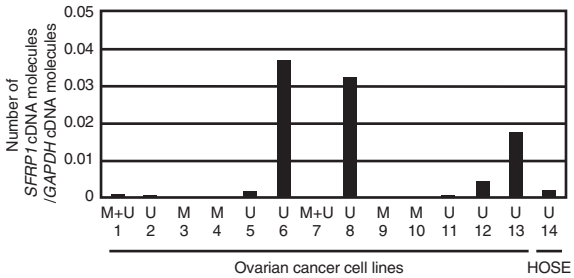

Fig. 2. Expression analysis of *SFRP1* gene. 1–13, ovarian cancer cell lines numbered as in Fig. 1; and 14, HOSE. Numbers of cDNA molecules were measured by quantitative PCR, and normalized to that of *GAPDH*. The methylation status of the *SFRP1* promoter CGI in each cell line is shown by M, U, and M+U. *SFRP1* was not expressed at all in the four cell lines, MCAS, RMUG-L, RTSG, and TYK-nu, which had only methylated DNA. *SFRP1* had low expression in three additional cell lines, OV-90, TOV112D, and KURAMOCHI. *SFRP1* was expressed at levels similar to that of HOSE cells in RMUG-S and HTOA, and was overexpressed in ES-2, TOV-21G, and JHOS-2.

Quantitative RT-PCR analysis. cDNA was synthesized from 3 μg of total RNA treated with *DNase*I (Ambion, Austin, TX) using the random hexamer (Promega) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Primer sequences were based on previous reports[14, 22]: *SFRP1*-F (5′-AGATGCTTAAGTGTGACAAGTTCC-3′) and *SFRP1*-R (5′-TCAGATTTCAACTCGTTGTCACAG-3′) (130 bp; 60°C); *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*)-F (5′-AGGTGAAGGTCGGAGTCAACG-3′); *GAPDH*-R (5′-AGGGGTCATTGATGGCAACA-3′) (102 bp; 62°C). Real-time PCR was performed using SYBR Green PCR Core Reagents (PE Biosystems, Warrington, UK) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of target cDNA molecules was normalized to that of *GAPDH* cDNA molecules.

5-Aza-2′-deoxycytidine treatment. MCAS cells were seeded at a density of 3×10^5 cells/10 cm plate on day 0, and exposed to each dose of 5-aza-dC (Sigma, St Louis, MO) for 24 h on days 1 and 3. The cells were cultured in fresh media after each treatment, and harvested on day 4.

Results

Methylation of the *SFRP1* promoter CGI in ovarian cancer cell lines. The methylation status of the *SFRP1* promoter CGI was first analyzed in 13 ovarian cancer cell lines and in HOSE cells by MSP. Only methylated DNA molecules were detected in four cell lines, MCAS, RMUG-L, RTSG and TYK-nu, and both methylated and unmethylated DNA molecules were detected in two cell lines, OV-90 and RMG-I (Fig. 1). Only unmethylated DNA molecules were detected in other cancer cell lines and HOSE cells.

Silencing of the *SFRP1* gene in ovarian cancer cell lines. *SFRP1* expression was examined in the 13 ovarian cancer cell lines by quantitative RT-PCR. It was found that *SFRP1* was not expressed at all in the four cell lines that had only methylated DNA molecules and in one (RMG-I) of the two cell lines that had both methylated and unmethylated DNA molecules (Fig. 2). *SFRP1* exhibited low expression levels in three additional cell lines, OV-90, TOV-112D, and KURAMOCHI. *SFRP1* was expressed at levels similar to that of HOSE cells in RMUG-S and HTOA, and was overexpressed in ES-2, TOV-21G, and JHOS-2.

To examine the role of the methylation of the *SFRP1* promoter CGI in the loss of expression, the four ovarian cancer cell lines, MCAS, RMUG-L, RTSG, and TYK-nu, which had only methylated DNA molecules, were treated with 5-aza-dC, a demethylating agent. After MCAS cells had been treated with 5-aza-dC at 0.1 μ*M* or higher concentration, it was found that the methylation in the promoter CGI was removed (Fig. 3A), and that *SFRP1* expression was restored (Fig. 3B). This confirmed that *SFRP1* was silenced by methylation of its promoter CGI in ovarian cancers. The other three cell lines showed weaker responses (data not shown).

Methylation of the *SFRP1* gene in primary ovarian cancers. Using 17 primary ovarian cancers, methylation of the *SFRP1* promoter CGI was analyzed by MSP (Table 1). Methylated DNA molecules were detected in two of the 17 samples (12%) while they were not detected in seven endometrial cyst samples (Fig. 4). It was noted that, after four additional cycles of PCR (total 36 cycles), four ovarian cancer samples (cases 8, 14, 15, and 17) and one endometrial cyst sample displayed a band for methylated DNA molecules (data not shown). This demonstrates the presence of trace amounts of methylated DNA molecules in these samples.

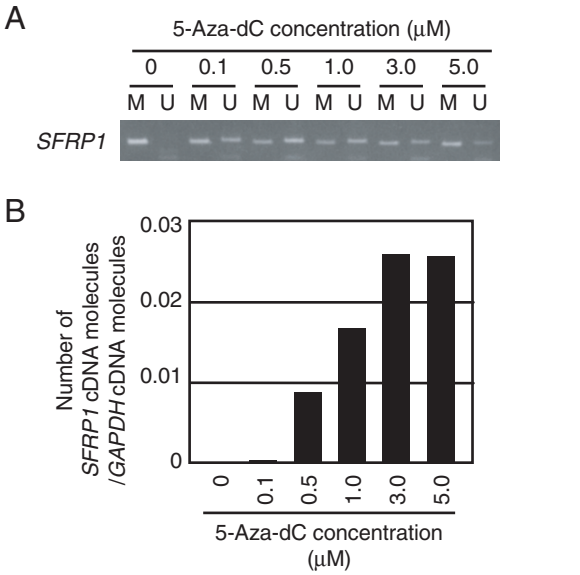

Fig. 3. Demethylation and re-expression of *SFRP1* by 5-aza-dC treatment of a human ovarian cancer cell line, MCAS. (A) Demethylation of *SFRP1* analyzed by MSP. After treatment of MCAS cells with 5-aza-dC at 0.1 μ*M* or higher concentration, unmethylated DNA molecules were detected by MSP. (B) Re-expression of *SFRP1* analyzed by quantitative RT-PCR. Re-expression was observed after treatment with 5-aza-dC at 0.5 μ*M* or higher concentration, and a dose-response relationship was observed between 0.1 μ*M* and 3.0 μ*M*.

Table 1. *SFRP1* methylation in primary ovarian cancers

No.	Age	Stage	Histology	Lymph node metastasis	*SFRP1* methylation
1	55	IIc	clear cell	Nx	–
2	59	IIIc	serous	N0	–
3	33	IIIb	mucinous	N0	+
4	61	IIc	serous	N0	–
5	57	IIIc	serous	N1	–
6	32	Ia	clear cell	N0	–
7	72	Ic	endometrioid	Nx	–
8	71	IIIc	serous	Nx	±
9	53	IIIc	serous	N1	–
10	45	IIIc	serous	N1	–
11	71	IIIc	serous	Nx	–
12	60	IIIc	serous	N1	+
13	75	Ic	serous	N0	–
14	52	Ia	SCC*	N0	±
15	53	IIIc	serous	N1	±
16	50	IV	serous	N1	–
17	52	IIIc	clear cell	N1	±

+, methylated DNA molecules detected; ±, trace amounts of methylated DNA molecules detected. * Squamous cell carcinoma along with mature cystic teratoma with malignant transformation.

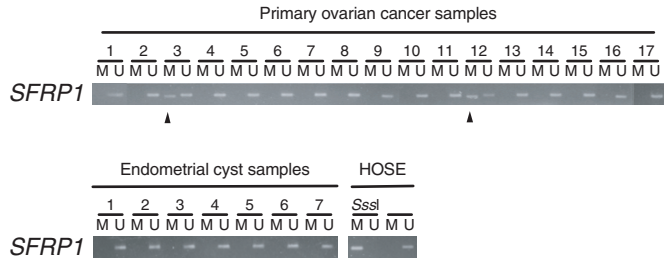

Fig. 4. *SFRP1* methylation in primary ovarian cancers. Two ovarian cancers (shown by closed arrowheads) had methylated DNA molecules. Four cancer and one ovarian cyst samples had trace amounts of methylated DNA molecules, which were detected by addition of four more PCR cycles (not shown here).

Discussion

It was first demonstrated here that *SFRP1* is silenced by promoter methylation in ovarian cancers. An important role of its silencing in the enhancement of Wnt signaling was reported in colon cancers.[14, 15] In ovarian cancers, *SFRP1* inactivation could explain, at least partially, why some ovarian cancers show increased β-catenin protein expression in spite of the absence of gene mutation.[16] Also, the *SFRP1* gene can be considered as a target of frequent chromosomal loss in 8p11.2.[18, 19] These points suggested that *SFRP1* inactivation could also play a role in ovarian cancer development and/or progression.

Among the primary ovarian cancers, one of one mucinous-type cancer and one of 11 serous-type cancers had methylation (Table 1). Among the cell lines, two of three mucinous-type cancers and two of three poorly differentiated or undifferentiated-type cancers exhibited methylation while none of three serous-type cancers did so. Although the number of samples was small, *SFRP1* silencing tended to be observed in mucinous-type ovarian cancers, which are resistant to chemotherapy.[23]

Trace amounts of methylated DNA molecules were detected in four cancer and one ovarian cyst samples. In the cancer samples, the presence of the methylated DNA molecules could have been due to heterogeneity among tumor cells. In the ovarian cyst samples, it could have been due to the presence of a neoplastic lesion in an apparently non-cancerous sample, or to the presence of methylated DNA molecules in non-cancerous cells. Repeated hemorrhage and resultant severe inflammation are involved in the development of endometrial cysts,[24] and chronic inflammation is known to promote methylation.[25] Therefore, it is possible that methylated DNA molecules were present in non-cancerous cells. Considering that *SFRP1* silencing can be present in early lesions of colon cancers and provide a predisposing condition,[14] the methylation in non-cancerous ovarian epithelial cells could also be involved in predisposition to ovarian cancers.

Although RMG-I had unmethylated *SFRP1* DNA molecules, the gene was not expressed at all. This could be due to a mutation on the unmethylated allele that makes *SFRP1* mRNA unstable, or due to depletion of factors necessary for *SFRP1* transcription. Lack of gene expression in cell lines with unmethylated DNA molecules is often observed for genes silenced in cancers.[26, 27] In primary cancer samples, stromal cells are present among cancer cells, and unmethylated DNA molecules are usually detected even when both alleles are methylated in cancer cells.[27]

SFRP1 is expected to be a new target for diagnosis and therapy of ovarian cancers.

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