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Expression and methylation status of 14-3-3 sigma gene can characterize the different histological features of ovarian cancer☆

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Abstract

We hypothesize that 14-3-3 sigma gene expression and its regulation by methylation can characterize histological types of primary human epithelial ovarian cancer. To test this hypothesis, ovarian cancer cell lines and 54 ovarian cancer tissue samples were analyzed for expression and methylation of 14-3-3 sigma gene using methylation specific PCR. The results of our experiments demonstrate that 14-3-3 sigma gene was methylated and inactivated in ES-2 ovarian cell line, which was derived from clear cell adenocarcinoma. Treatment of this cell line with demethylating agent 5-aza-2′-deoxycytidine restored the expression of 14-3-3 sigma gene. In human ovarian cancer tissues, the expression of 14-3-3 sigma protein was inactivated in most of the ovarian clear cell carcinoma tissues. Interestingly, 14-3-3 sigma protein expression was positive in significantly higher percentages of serous (89.5%), endometrioid (90%), and mucinous (81.8%) ovarian adenocarcinoma tissues. The ovarian clear cell carcinoma samples with inactivated 14-3-3 sigma protein were highly methylated, suggesting that inactivation of 14-3-3 sigma gene is through DNA methylation. Using direct DNA sequencing, 14-3-3 sigma gene methylation on all the 17 CpG sites was significantly higher in ovarian clear cell carcinoma as compared to other histological types of ovarian cancer (serous, endometrioid, and mucinous). This is the first report suggesting that 14-3-3 sigma gene expression and methylation status can characterize histological features of different types of ovarian cancer.

Keywords: 14-3-3 sigma; Clear cell adenocarcinoma

Epithelial ovarian cancer is one of the major causes of cancer death among women. This type of cancer is divided into four major pathological types (serous, endometrioid, mucinous, and clear cell adenocarcinoma). Because each pathological type of ovarian cancer shows different clinical features, treatment of this cancer is difficult [1]. Therefore, characterization of each pathological type of ovarian cancer is important [2,3].

The 14-3-3 sigma gene was first identified as an epithelial-specific factor, HME1 [4]. Later studies have shown that this gene could regulate cell growth [5,6] and differentiation [7]. Then, Hermeking et al. [8] reported that expression of 14-3-3 sigma gene was induced through the activation of wild-type p53. By these findings, this gene is considered to have tumor suppressive activity. Concordant with this hypothesis, inactivation of this gene through methylation has been reported in human breast cancer [9,10], liver cancer [11], vulval cancer [12], and oral cancer [13]. On the contrary, in pancreas cancer, overexpression of 14-3-3 sigma through hypomethylation was observed [14,15]. Moreover, it was also reported that this gene was commonly methylated in normal and malignant lymphoid cells, which nevertheless express 14-3-3 sigma gene [16]. Thus, the expression and methylation status of this gene should be evaluated depending upon cancer types.

☆ *Abbreviations:* RT-PCR, reverse transcription PCR; MSP, methylation-specific PCR; 5-azaC, 5-aza-2′-deoxycytidine.

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We hypothesize that the expression and methylation of 14-3-3 sigma gene may characterize different histological types of human epithelial ovarian cancer. We tested this hypothesis through the analysis of expression and methylation status in serous, endometrioid, mucinous, and clear cell adenocarcinoma of the ovary. To our knowledge, this is the first report on the expression and methylation status of 14-3-3 sigma gene as factors to characterize differences between human epithelial ovarian cancers.

Materials and methods

Ovarian cancer cell lines and treatment. Human ovarian cancer cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD). OVCAR-3, Caov-3, and ES-2 were used for these experiments and cultured in media as recommended by ATCC in a humidified, 5% CO_2 atmosphere at 37 °C. They were treated with a final concentration of 2 μg/ml of 5-aza-2′-deoxycytidine (5-azaC) on days 1, 3, and 5. On day 6, cells were harvested and nucleic acids were extracted.

Microdissection of human epithelial ovarian cancer samples. Fifty-four paraffin-embedded primary human epithelial ovarian cancer samples, comprising 19 serous, 10 endometrioid, 11 mucinous, and 14 clear cell adenocarcinomas, were obtained under the informed consents from the Department of Gynecology at the Hospital of Hokkaido University, Japan. To collect cancer tissues for DNA extraction, microdissection was carried out as described previously [17].

Nucleic acid extraction. DNA from cell lines and microdissected tissues was extracted using DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA from cell lines was extracted by guanidium thiocyanate–phenol–chloroform extraction using TRI Reagent (Molecular Research Center, Cincinnati, OH).

RT-PCR. Extracted RNA samples from ovarian cancer cell lines were treated with DNase (DNA-free; Ambion, Austin, TX) prior to reverse transcription (RT) reaction. A total of 1 μg of RNA was reverse-transcribed with random hexamers in a 20-μl reaction by Reverse Transcription System kit (Promega, Madison, WI). cDNA was amplified by differential PCR using primers specific for the 14-3-3 sigma gene (5′-GTCTGATCCAGAAGGCCAAG-3′, sense; 5′-CTCCTCGTTGC TTTTCTGCT-3′, antisense) and the G3PDH gene (5′-TCCCATCACC ATCTTCCA-3′, sense; 5′-CATCACGCCACAGTTTCC-3′, antisense). The PCR mixture in a 20 μl volume contained 0.15 μM 14-3-3 sigma primers, 0.05 μM G3PDH primers, 0.2 mM dNTP, 1× REDTaq PCR Buffer, and 4 U REDTaq DNA polymerase (Sigma Chemical, St. Louis, MO). PCRs were performed in a PTC-200 thermal cycler (MJ Research, Watertown, MA) at 94 °C for 3 min; 35 cycles at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s; and an extension step at 72 °C for 5 min. Each of the RNA samples after DNase treatment without RT reaction was also carried out under the same PCR conditions and we confirmed that there were no detectable bands, which might originate from contamination of genomic DNA (data not shown).

Bisulfite modification and methylation-specific PCR. Bisulfite modification of genomic DNA was carried out using CpGenome DNA Modification Kit (Intergen, Purchase, NY) following the manufacturer's protocol. A two-step PCR procedure was utilized. For the first PCR, modified DNA was amplified using the primer, Sigma-Univ-S (5′-AGAAGGTTAAGTTGGTAGAGTAGGT-3′, nt 8660–8684) and –AS (5′-AATAACTATCCAACAAACCCAACAC-3′, nt 8917–8894) (GenBank NCBI Accession No. AF029081). The PCR conditions were 94 °C for 3 min; 40 cycles of 94 °C for 60 s, 50 °C for 60 s, and 72 °C for 60 s; and a final extension at 72 °C for 5 min. These primers were designed not to contain any methylated cytosine in its sequence. Then, 1 μl of first PCR products was subjected to MSP analysis. Primer sets utilized for MSP were the same as those previously published by Ferguson et al. [9]. The PCR conditions for methylated DNA amplification were 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 60 s; and a final extension at 72 °C for 5 min. The PCR conditions for unmethylated DNA amplification were 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 60 s; and 72 °C extension for 5 min.

Sequencing. For confirmation of MSP, the first PCR products were purified by QIAquick PCR Purification kit (Qiagen, Valencia, CA) and 20 ng of PCR products was used as a template for sequencing. Double-strand sequence analysis was performed using each primer set, an ABI 377 Sequencer, and a Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA). The amount of methylcytosine of each CpG dinucleotide was quantitated by comparing the peak height of the cytosine signal with the peak height of the cytosine plus thymine signal, as we reported before [17].

Immunohistochemistry. Immunostaining of 14-3-3 sigma was performed on 5-μm-thick consecutive sections obtained from paraffin-embedded human ovarian cancer blocks. Sections were dried at room temperature, deparaffinized, rehydrated, and then treated with 2% hydrogen peroxide in methanol for 5 min at 37 °C. Antigen retrieval was done by autoclaving the slides for 10 min in 10 mM citrate buffer, pH 6.0. After blocking with 3% normal goat serum, the sections were incubated with primary antibody (MS-1185; NeoMarkers, Fremont, CA) at 1:100 dilution in phosphate-buffered saline overnight at room temperature under a humid chamber with 20 mM Tris, 150 mM NaCl, and 0.025% Tween, pH 7.8, and incubated with the secondary antibody for 30 min. Immunostaining was performed using the avidin–biotin–peroxidase method (Lab Vision, Fremont, CA) with diaminobenzidine as the chromogen and followed by counterstaining with hematoxylin. In each specimen, the percent of positive cytoplasmic stained cells was calculated in four different areas using a 40× objective.

Statistical analyses. All statistical analyses were performed using StatView Statistical Analysis software (SAS Institute, Cary, NC). For comparison of 14-3-3 sigma expression and methylation status in each histological type of epithelial ovarian cancers, Kruskal–Wallis test, followed by Fisher's PLSD test, was performed.

Results

14-3-3 sigma gene methylation in human ovarian cancer cell lines

Using direct bisulfite genome sequencing techniques, we examined 14-3-3 sigma gene methylation in 3 ovarian cancer cell lines (Fig. 1A). A 297-bp region of the 14-3-3 sigma gene locus, encompassing 17 CpG sites, was amplified by PCR from bisulfite-modified DNA. Sequencing results revealed that all 17 CpG sites were methylated in the ES-2 cell line, whereas all of sites were unmethylated in OVCAR-3 and Caov-3 cell lines.

Restoration of 14-3-3 sigma gene in clear cell ovarian cancer cell line after 5-azaC treatment

We determined the expression status of 14-3-3 sigma gene in three ovarian cancer cell lines. Although OVCAR-3 and Caov-3 cell lines showed the expression of 14-3-3 sigma gene, ES-2 cell line originally lacked mRNA for 14-3-3 sigma gene. The mRNA expressions

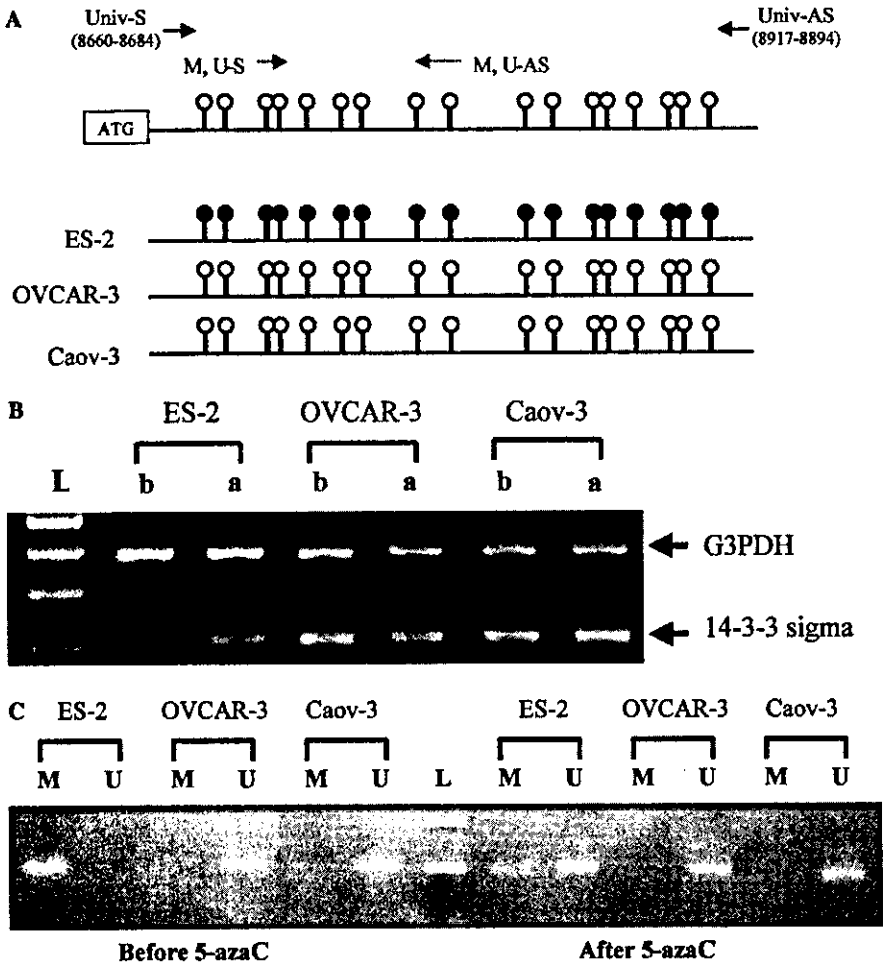

Fig. 1. Methylation and expression status of 14-3-3 sigma gene in ovarian cancer cell lines. (A) Schematic representation of the bisulfite sequencing in ovarian cancer cell lines. 17 CpG sites from the transcription start site were examined. The methylated and unmethylated CpG sites are indicated by closed and open lollipops, respectively. Primer position for first PCR (Univ-S, AS; Accession No. AF029081) and MSP analysis (M, U-S, AS) [9] are indicated. (B) 14-3-3 sigma expression in ovarian cancer cell lines before and after treatment with 5-azaC. 14-3-3 sigma mRNA expressions in ES-2, OVCAR-3, and Caov-3 cell lines before (b) and after (a) treatment with 5-azaC. DNase-treated, total cellular RNA was reverse transcribed, and the resulting cDNA was amplified with differential PCR using primers for the G3PDH (393 bp) and 14-3-3 sigma (203 bp). (C) MSP analysis of 14-3-3 sigma gene before and after treatment with 5-azaC. Unmethylated allele was restored after treatment in ES-2 cell line. M, methylated band (105 bp); U, unmethylated band (107 bp). For all panels: L, 100 bp ladder.

in cell lines were concordant with the methylation studies. These cell lines were then treated with the demethylating agent 5-azaC and analyzed for mRNA expression and methylation status (Figs. 1B and C). After treatment with 5-azaC, this gene was demethylated and mRNA expression of 14-3-3 sigma gene was restored in ES-2 cell line, suggesting that methylation is involved in the inactivation of the 14-3-3 sigma gene in a certain type of ovarian cancer. No significant changes were observed in OVCAR-3 and Caov-3 cell lines.

Expression and methylation of 14-3-3 sigma gene in primary human epithelial ovarian cancer tissue

To evaluate 14-3-3 sigma expressions in primary human epithelial cancer, we analyzed protein expression by immunostaining in 54 primary epithelial ovarian cancer tissues. Representative immunostaining results of each pathological type of ovarian cancer are shown in Fig. 2A. Seventeen of 19 (89.5%) serous adenocarcinoma, 9 of 10 (90%) endometrioid adenocarcinoma, and 9 of 11 (81.8%) mucinous adenocarcinoma tissues showed positive cytoplasmic 14-3-3 sigma immunoreactivity, whereas 11 of 14 (78.6%) clear cell adenocarcinoma showed negative immunoreactivity (Fig. 2B). Methylation status of 14-3-3 sigma was determined by MSP analysis (Table 1). 14-3-3 sigma locus was methylated in 5 of 19 (26.3%) serous adenocarcinomas, 2 of 10 (20.0%) endometrioid adenocarcinomas, 4 of 11 (36.4%) mucinous adenocarcinomas, and 11 of 14 (78.6%) clear cell adenocarcinomas. These results suggested that methylation of 14-3-3 sigma downregulated or silenced

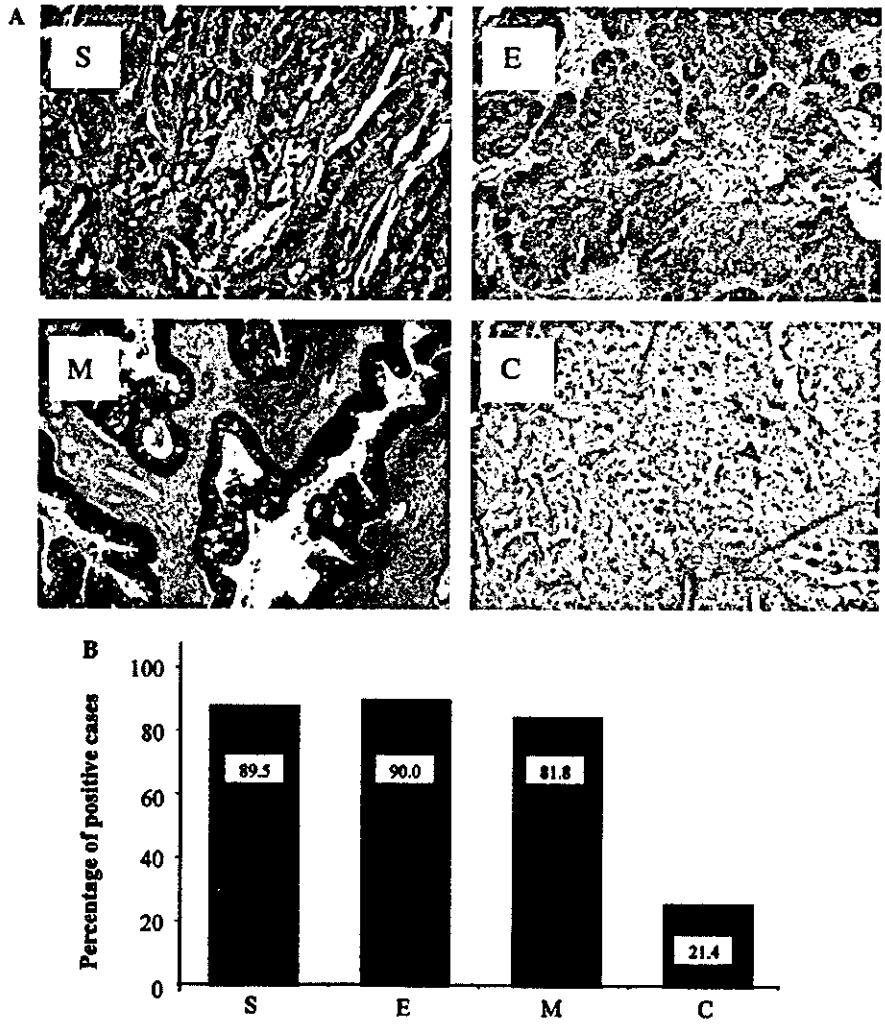

Fig. 2. Expression of 14-3-3 sigma in ovarian cancer tissue. (A) Typical immunostaining of 14-3-3 sigma is shown. Although the majority of cancer cells in serous, endometrioid, and mucinous adenocarcinoma show positive cytoplasmic staining, majority of clear cell adenocarcinoma cancer cells show negative cytoplasmic staining. (B) Relationship of immunostaining data to pathological data is shown. We classified negative as less than 10% positive cells, and positive as more than 10% positive cells depending upon the percent of cytoplasmic stained cells. The percentages of 14-3-3 sigma positive cases are significantly lower in clear cell adenocarcinoma than in serous, endometrioid, and mucinous adenocarcinoma ($P < 0.05$). S, serous adenocarcinoma; E, endometrioid adenocarcinoma; M, mucinous adenocarcinoma; and C, clear cell adenocarcinoma.

Table 1
Methylation profile of various pathological types of ovarian cancers

Pathology	Number of cases (%)
Serous adenocarcinoma	
Methylated	5/19 (26.3)
Unmethylated	14/19 (73.7)
Endometrioid adenocarcinoma	
Methylated	2/10 (20.0)
Unmethylated	8/10 (80.0)
Mucinous adenocarcinoma	
Methylated	4/11 (36.4)
Unmethylated	7/11 (63.6)
Clear cell adenocarcinoma	
Methylated	11/14 (78.6)
Unmethylated	3/14 (21.4)

$P < 0.05$ in C vs. S, E, and M*.
*S, serous; E, endometrioid; M, mucinous; and C, clear cell adenocarcinoma.

the expression of this gene in clear cell adenocarcinoma compared to other pathological types of ovarian cancer.

Methylation-specific DNA sequencing

We confirmed the results for methylation status of 14-3-3 sigma gene by methylation-specific sequencing in each pathological type of ovarian cancer tissues. Fig. 3A shows the percentage of methylation on 17 different CpG sites of 14-3-3 sigma. The methylation rates of 14-3-3 sigma at all 17 CpG sites were significantly high in clear cell adenocarcinoma tissues (overall, 64.0%), although rates were generally low in other pathological types of cancer (31.7% in serous, 25.9% in endometrioid, and 35.5% in mucinous adenocarcinomas). Fig. 3B shows representative results of direct sequencing from

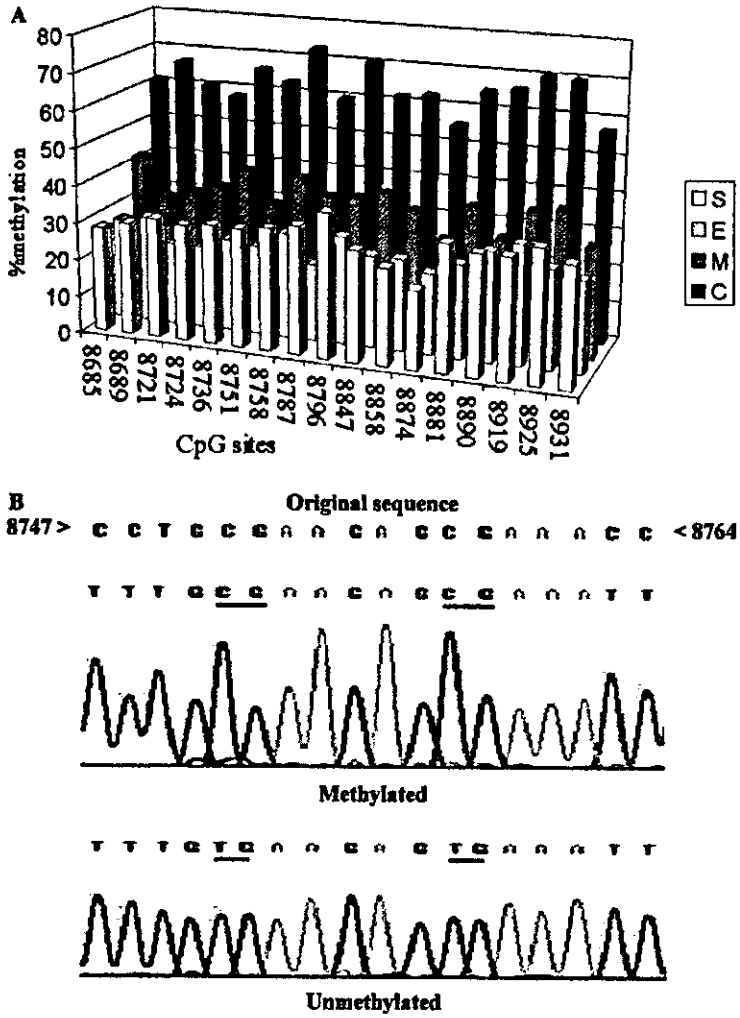

Fig. 3. (A) The percentage of each CpG island methylation of 14-3-3 sigma gene in ovarian cancer tissues. Seventeen CpG sites (genomic positions 8685, 8689, 8721, 8724, 8736, 8751, 8758, 8787, 8796, 8847, 8858, 8874, 8881, 8890, 8919, 8925, and 8931; Accession No. AF029081) were analyzed. The methylation percentage of each CpG site is presented as the average ratio of methylated cytosine to the total cytosine and thymine [17] of all samples in each pathological type of ovarian cancer tissues. Numbers on the *X*-axis are the position of CpG sites. S, serous adenocarcinoma; E, endometrioid adenocarcinoma; M, mucinous adenocarcinoma; and C, clear cell adenocarcinoma. (B) A representation of modified sequence from position 8747 to 8764 is shown and CpG sites are underlined. Upper: methylated sequence as found in clear cell adenocarcinoma tissue. Lower: unmethylated sequence as found in serous adenocarcinoma tissue. Only methylated cytosines are not converted to thymines.

the first PCR product. CpG sites are underlined, and all cytosines are deaminated and converted to thymines in the sample from serous adenocarcinoma, whereas 5-methylcytosines remain unaltered in the sample from clear cell adenocarcinoma.

Discussion

The epithelial ovarian cancers consist of various histological types, such as serous, endometrioid, mucinous, and clear cell adenocarcinoma. Although clinical features of ovarian cancer are also different depending on the histological type [1], the efforts to find the factor to characterize each pathological type of ovarian cancer are limited [2,3]. We thus investigated whether expression statuses of 14-3-3 sigma are different in different pathological types of ovarian cancer. We found that 14-3-3 sigma gene was significantly inactivated through DNA methylation in clear cell ovarian cancer tissues whereas most of serous, endometrioid, and mucinous adenocarcinoma tissues showed strong expression of this gene with little methylation.

Previous reports have shown that 14-3-3 sigma was often methylated and inactivated in human breast cancer [9,10], liver cancer [11], vulval cancer [12], and oral cancer [13]. Since 14-3-3 sigma is located downstream of the p53 pathway, this gene might act as tumor suppressive factor [8]. Therefore, inactivation of this gene could contribute to carcinogenesis of some cancers as

shown above. On the other hand, overexpression of 14-3-3 sigma through hypomethylation was observed in pancreas cancer [14,15]. Moreover, it was also reported that this gene was commonly methylated in normal and malignant lymphoid cells, which nevertheless express 14-3-3 sigma gene [16], and that the status of inactivation of this gene by hypermethylation depends upon the pathological type in lung cancer [18]. In our study, 14-3-3 sigma gene was methylated and inactivated in the ES-2 cell line, whereas this gene was unmethylated and expressed in OVCAR-3 and Caov-3 cell lines. The ES-2 cell line was established from clear cell adenocarcinoma from a tumor of African–American female [19]. Concordant with this observation, methylation of this gene was highly observed in clear cell adenocarcinoma tissues. These observations suggest that inactivation of 14-3-3 sigma might not always be necessary for carcinogenesis in certain types of cancer, and methylation/expression status of 14-3-3 sigma is related with the pathological type of ovarian cancer.

In the present study, clear cell ovarian adenocarcinoma tissues showed significantly higher levels of inactivation of 14-3-3 sigma gene through DNA methylation. Interestingly, other types of ovarian cancers (serous, endometrioid, and mucinous) showed high expression of 14-3-3 sigma gene with little methylation. It is quite possible that differential expression and methylation of 14-3-3 sigma gene can characterize the different histological types of ovarian cancer. In this regard, Rathi et al. [20] reported that the methylation statuses of various genes are different depending upon pathological types of ovarian cancer. These authors examined the methylation statuses of many genes, including RASSF1A, HIC1, E-cadherin, APC, H-cadherin, p16, BRCA1, RARβ, and GSTP1 in ovarian cancer. They showed that not only methylation of these genes was highly observed in ovarian cancer compared to normal tissue, but also was higher in clear cell adenocarcinoma compared to other pathological types of ovarian cancers. Clear cell adenocarcinoma is clinically known to show aggressive features compared to other pathological types of ovarian cancer [1]. Higher frequencies of methylation in clear cell carcinoma, as shown above, might contribute to the clinical features of this type of ovarian cancer.

In conclusion, our study presents a possible mechanism that regulates 14-3-3 sigma expression as it relates to methylation status in ovarian cancer. Moreover, the differential methylation and expression status of this gene may characterize the pathological differences among ovarian cancers.

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GGC and *Stu*I polymorphism on the androgen receptor gene in endometrial cancer patients

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Abstract

Androgens have an anti-proliferative effect on endometrial cells. Human androgen receptor (AR) gene contains two polymorphic short tandem repeats of GGC and CAG, and a single-nucleotide polymorphism on exon 1 that is recognized by the restriction enzyme, *Stu*I. Prior studies have shown that the lengths of the CAG repeat are inversely and linearly related to AR activity and associated with endometrial cancer. However, little is known about the GGC repeat and the *Stu*I polymorphism of the AR gene. Thus, we investigated whether these AR polymorphisms are risk factors for endometrial cancer. To test this hypothesis, the genetic distributions of these polymorphisms were investigated in blood samples from endometrial cancer patients and healthy controls. The allelic and genotyping profiles were analyzed by polymerase chain reaction (PCR), PCR–restriction fragment length polymorphism (PCR–RFLP), and direct DNA sequencing, and analyzed statistically. The GGC repeat was significantly longer in endometrial cancer patients as compared to normal healthy controls. In general, an increased risk of endometrial cancer was found with increasing GGC repeat. The relative risk for the 17 GGC repeat was greater than 4, as compared to controls. However, the *Stu*I polymorphism was not significantly different between patients and controls. The findings suggest that increased numbers of GGC repeat on the AR gene may be a risk factor for endometrial cancer.

Keywords: Androgen receptor gene; Endometrial cancer; GGC repeat; *Stu*I polymorphism

Endometrial carcinoma is the most common malignancy of the female genital tract and its incidence has recently increased [1]. However, the genetic basis of this disease is not well understood. The origin and growth of this tumor is thought to be influenced by a variety of steroid hormones [1,2]. Androgens influence endometrial cancer risk by binding to the androgen receptor (AR) and inhibit endometrial cell growth [2–5]. The AR gene is located on chromosome Xq11-12 and consists of eight exons [6,7]. The AR has two polymorphic repeats on exon 1, characterized by different numbers of GGC and CAG repeats resulting in variable lengths of polyglycine and polyglutamine regions [6,7]. A single-nucleotide polymorphism (SNP) at codon 211 (G1733A) has also been reported between the GGC and CAG repeats on exon 1 that is recognized by the restriction enzyme, *Stu*I [8]. Prior studies have shown that the length of the CAG repeat is inversely and linearly related to AR activity and has significant correlation with several cancers, including endometrial cancer [9–12]. However, little is known about the GGC repeats and *Stu*I polymorphism. Only one paper has been published indicating that the *Stu*I polymorphism was

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associated with prostate cancer [13]. Since androgens act directly on endometrium via the AR to inhibit endometrial cancer, women with decreased AR transactivation (carriers of longer repeat alleles) may have an elevated risk of endometrial cancer. Therefore, we hypothesized that these polymorphisms on the AR gene may be associated with increased risk for endometrial cancers. To test this hypothesis, the allelic and genotypic distributions of the GGC repeat and *Stu*I polymorphisms on the AR gene were investigated in endometrial cancer patients and compared to those of healthy controls by polymerase chain reaction (PCR), PCR–restriction fragment length polymorphism (PCR–RFLP), and direct DNA sequencing.

Materials and methods

Samples. A total of 113 sporadic endometrial cancer blood samples were obtained from the Hokkaido University Hospital (Hokkaido, Japan) between 1992 and 2000. Only Japanese patients, who were diagnosed with histologically confirmed endometrial cancer, were enrolled in the study. The age range among the endometrial cancer patients was 39–87 years, with a median of 61.7 years. Cancer-free control blood samples were obtained from 202 unrelated Japanese healthy volunteers in the same prefecture (100 women and 102 men) at the same time period as the cancer patient samples. No patients or healthy controls of other ethnic groups were recruited, therefore this study was limited to a Japanese population. There were no differences between patients and control groups in regard to race, family history of cancer, indices of body size (height, weight, and body mass index), or income. Appropriate informed consent was obtained from the patients in accordance with Local Ethical Committee guidelines of the hospital.

GGC repeat. The protocol for DNA extraction and PCR methods was previously outlined [2,12]. The primers used in this study were as follows: GGC-f, TCCTGGCACACTCTCTCTTCAC; GGC-r, GCCA GGGTACCACACATCAGGT. PCR amplifications were performed with 10 ng DNA solution containing 1.5 mM $MgCl_2$, 0.8 mM dNTP, and 0.5 U *Taq* polymerase (Cetus) using each primer pair in a total volume of 50 μl. The PCR for GGC consisted of 30 cycles (98 °C for 60 s and 70 °C for 300 s). PCR amplification of CAG consisted of 30 cycles (95 °C for 45 s, 60 °C for 30 s, and 72 °C for 30 s). Aliquots of PCR products (5 μl) were mixed with 1 μl of 10× loading dye and were placed on a denaturing 8% polyacrylamide gel containing 7 M urea and 1× Tris–borate–EDTA buffer. Electrophoresis was carried out at 400 V and ambient temperature. The bands on the gels were visualized by silver staining. Allele designations were performed according to an allelic ladder produced in our laboratory [2,12].

The StuI polymorphism. The PCR–RFLP method was used to detect the *Stu*I polymorphism by a previously published protocol [8,13]. A 416 bp fragment of exon 1 of the AR gene was amplified using primers (5′-CAC AGG CTA CCT GGT CCT GG-3′ and 5′-CTG CCT TAC ACA ACT CCT TGG C-3′) with negative and positive controls [8]. The PCR consisted of 30 cycles of denaturation (94 °C for 60 s), annealing (60 °C for 60 s), and extension (72 °C for 60 s), and was followed by a final incubation at 72 °C for 8 min. DNA fragments were separated on an ethidium bromide-stained agarose gel (4%) and visualized under UV light. Laboratory personnel were blinded to case–control status [2,12].

Sequence analysis. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) [3,4]. Next, double-strand sequence analysis of the PCR products was performed using the first PCR primer, and a ABI 377 Sequencer and Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA) [2,12].

Statistical analysis. Since the AR gene is located on the X chromosome, females have two alleles on their two X chromosomes and males have only one allele on their one X chromosome [12,14]. Homogeneity was tested as described previously [12,14]. χ^2 and t tests that were two-sided for statistical significance were used to test the deviation of allelic and genotype distributions [11,12,14]. Case subjects and control subjects were compared for GGC repeats initially as a continuum. Then the relative risks were estimated using cutoff points, comparing the odds of cancer patients and controls in the category below and above the cutoff point. We used a cut-point of 16 and 17 for GGC repeat, because this divided the distribution of these repeats approximately in half.

Results

GGC repeat

The frequencies of genetic distribution of the GGC repeats on the AR gene in endometrial cancer patients and healthy controls are shown in Table 1. There were no differences in genetic distribution of the GGC repeats between male and female healthy controls (Table 1A). Genotyping of endometrial cancer patients and female controls revealed 11 and 8 distinct alleles, respectively (Table 1B). The mean GGC repeat was 15.9 and 15.4 repeats for cancer patients and female controls, respectively. The length of GGC repeats of the endometrial cancer patients was significantly longer compared to that of female controls ($P < 0.05$). The relative risks for endometrial cancer were estimated using a cutoff point of 16 and 17 repeats, and then comparing the endometrial cancer patients and controls in the category below and above the cutoff point (Table 1C). No significant association was observed for the 16 GGC repeat between endometrial cancer patients and female controls (the relative risks: 1.10). However, the 17 GGC repeat was significantly overrepresented in endometrial cancer patients compared to female controls ($P < 0.001$). Thus, 14.6% of endometrial cancer patients (33 of 226) had the 17 allele while 3.5% of female controls (7 of 226) had it (both: $P < 0.001$) (Table 1B). The relative risks for the 17 repeat of GGC were 4.17 as compared to female controls. Moreover, 5.7% of endometrial cancer patients (13 of 226) had the 18 allele although none of the female controls (0 of 226) did ($P < 0.001$) (Table 1C).

StuI polymorphism

The frequency of distribution of the *Stu*I polymorphism of the AR gene in endometrial cancer patients and the control groups is shown in Table 2. The genotypic distributions of this polymorphism were consistent with the Hardy–Weinberg equilibrium in cancer patients and control groups. No difference was observed for the allelic distribution of this polymorphism between men and women in control groups ($P > 0.75$) (Table 2A).

Table 1
The GGC repeat of androgen receptor gene in endometrial cancer patients and healthy controls

(A) Repeat	Female controls	Male controls[a]	Total controls
10	3/200 (1.5%)	1/102 (1.0%)	4/302 (1.3%)
11	4/200 (1.5%)	2/102 (2.0%)	6/302 (2.0%)
12	10/200 (5.0%)	4/102 (4.0%)	14/302 (4.6%)
13	4/200 (2.0%)	2/102 (2.0%)	6/302 (2.0%)
14	5/200 (2.5%)	3/102 (3.0%)	8/302 (2.6%)
15	21/200 (10.5%)	12/102 (12.0%)	33/302 (10.9%)
16	146/200 (73.0%)	74/102 (74.0%)	220/302 (72.8%)
17	7/200 (3.5%)	3/102 (3.0%)	10/302 (3.3%)
18	0/200 (0.0%)	1/102 (1.0%)	1/302 (0.3%)
19	0/200 (0.0%)	0/102 (0.0%)	0/302 (0.0%)
20	0/200 (0.0%)	0/102 (0.0%)	0/302 (0.0%)
21	0/200 (0.0%)	0/102 (0.0%)	0/302 (0.0%)

(B) Repeat	The patients[b,c]	Female controls
10	1/226 (0.4%)	3/200 (1.5%)
11	1/226 (0.4%)	4/200 (1.5%)
12	6/226 (2.7%)	10/200 (5.0%)
13	4/226 (1.8%)	4/200 (2.0%)
14	4/226 (1.8%)	5/200 (2.5%)
15	17/226 (7.5%)	21/200 (10.5%)
16	160/226 (70.7%)	146/200 (73.0%)
17	20/226 (8.8%)	7/200 (3.5%)
18	11/226 (4.9%)	0/200 (0.0%)
19	1/226 (0.4%)	0/200 (0.0%)
20	0/226 (0.0%)	0/200 (0.0%)
21	1/226 (0.4%)	0/200 (0.0%)

(C) Allele	The patients	Female controls	Relative risk for female controls	95% CI for female controls
≧16	193/226 (85.4%)	153/200 (76.5%)	1.10	1.00–1.21
≧17	33/226 (14.6%)	7/200 (3.5%)	4.17	1.89–9.22

[a] Male healthy controls vs. female healthy controls: $0.95 < P$.
[b] The patients vs. female healthy controls: $P < 0.05$.
[c] The patients vs. total healthy controls: $P < 0.001$.

Table 2
The *StuI* polymorphism of androgen receptor gene in endometrial cancer patients and healthy controls

(A) Allele	Female controls	Male controls[a]	Total controls
G	185/200 (92.5%)	95/102 (93.1%)	280/302 (92.7%)
A	15/200 (7.5%)	7/102 (6.9%)	22/302 (7.3%)

(B) Allele	The patients[b,c]	Female controls	Relative risk for female controls	95% CI for female controls
G	207/226 (91.6%)	185/200 (92.5%)		
A	19/226 (8.4%)	15/200 (7.5%)	1.12	0.59–2.15

(C) Genotype	The patients[d]	Female controls	Relative risk for female controls	95% CI for female controls
G/G	95/113 (84.1%)	86/100 (86.0%)		
G/A	17/113 (15.0%)	13/100 (13.0%)	1.16	0.59–2.26
A/A	1/113 (0.9%)	1/100 (1.0%)	NT	NT

[a] Male healthy controls vs. female healthy controls: $0.75 < P$.
[b] The patients vs. female healthy controls: $0.75 < P$.
[c] The patients vs. total healthy controls: $0.75 < P$.
[d] The patients vs. female healthy controls: $0.75 < P$.

There was no significant difference in allelic distribution in endometrial cancer patients compared to that of female controls ($P > 0.75$) (Table 2B). The relative risk of allele A of this SNP was calculated as 1.1.2 compared to allele G for female controls. There was also no significant difference in genotypic distribution in endometrial cancer patients compared to that of female controls ($P > 0.75$) (Table 2C). The relative risk of the genotype

G/A of this SNP was calculated as 1.16 compared to allele G.

Discussion

Androgens have an anti-proliferative effect on endometrial cells. The GGC repeat encodes polyglycine region, which has a length that is inversely and linearly related to AR activity [9,10]. It has been observed that shorter repeats impose a higher transactivation activity on AR and have an increased binding affinity for androgens [15,16]. In contrast, longer repeats lead to lower androgen responsiveness, weaker androgen inhibition, and increased proliferative activity, and promote carcinogenesis of the uterine endometrial cells [2,12,17–19].

Our data show that the length of GGC CAG repeat in the endometrial cancer patients was significantly longer compared to that of total controls. Moreover, increased risks of endometrial cancer were found with increasing GGC repeat. Based on a previous report, it was confirmed that even small differences in these repeats cause a significant difference in transcriptional activation of the AR gene [20]. An appreciable decrease in the transcriptional activity of an AR with a long polyglycine tract could lead to a reproductive lifetime of functional hyperestrogenicity in the endometrium, and this might contribute to initiation and/or progression of endometrial cancer. We observed no significant differences in allelic or genotypic distribution of the *Stu*I polymorphism between endometrial cancer patients and control groups. These data may reflect the fact that the *Stu*I nucleotide change is silent and does not induce any amino acid change in the AR protein. In conclusion, our findings suggest that the GGC repeat in the AR gene is a risk factor for endometrial cancer. Therefore, these polymorphic tandem repeats of the AR gene can serve as biomarkers to identify a population with higher risk for endometrial cancer.

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