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Relationship of aberrant DNA hypermethylation of *CHFR* with sensitivity to taxanes in endometrial cancer

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Received August 31, 2006; Accepted October 9, 2006

Abstract. The relationship of aberrant DNA hypermethylation of cell cycle checkpoint genes with the sensitivity of cancer cells to anticancer drugs is a question of current interest. In this study, we investigated the relationship between aberrant hypermethylation of the *CHFR* (checkpoint with forkhead-associated and ring finger) mitotic checkpoint gene and sensitivity to taxanes in endometrial cancer. Methylation-specific PCR (MSP) indicated aberrant hypermethylation of *CHFR* in 12.0% (6/50) of endometrial cancer specimens, and suggested that aberrant hypermethylation is significantly more frequent in poorly differentiated adenocarcinoma (G3) ($p<0.05$). Of six culture cell lines, SNG-II and HEC108 cells showed aberrant hypermethylation and reduced expression of *CHFR*. These cells had high sensitivity to taxanes but became resistant after demethylation. Cancer specimens with aberrant hypermethylation of *CHFR* also exhibited high sensitivity to taxanes. To our knowledge, this study is the first to examine aberrant hypermethylation of *CHFR* in endometrial cancer, and our results suggest that the methylation status of *CHFR* may be a new molecular index that will allow design of personalized treatment in endometrial cancer. This may be particularly important in poorly differentiated adenocarcinoma (G3), which is known to have a poor prognosis.

Introduction

Recent studies have shown that aberrant DNA hypermethylation of cell cycle checkpoint genes in cancer cells has a major effect on specific anticancer drugs (1,2). The *CHFR* (checkpoint with forkhead-associated and ring finger) mitotic checkpoint gene is located in 12q24.33 and has the function of delaying chromatin condensation and progression to the mitotic phase (3). The CHFR protein has a forkhead-associated domain in the N-terminal region and a finger domain in the central region; these two domains act as a sensor for mitotic stress and therefore function as a cell cycle M phase checkpoint. Upon detection of mitotic stress in a cell, CHFR action causes arrest of the cell cycle in G2 phase to allow repair of damaged DNA (G2 arrest).

Taxanes are anticancer agents that act in M phase as microtubule depolymerization inhibitors. Upon administration of a taxane to cancer cells, those cells with normal *CHFR* develop G2 arrest and repair damaged DNA, thereby exhibiting resistance to taxanes. In contrast, cells with inactivated *CHFR* due to aberrant hypermethylation proceed with the cell cycle due to failed detection of damaged DNA and subsequently cannot go on to normal cell division, leading to mitotic catastrophe and cell death; i.e., these cells show high sensitivity to taxanes. Given this background, the methylation status of *CHFR* is likely to be a highly sensitive molecular index for taxane sensitivity of cancer cells.

A relationship between aberrant hypermethylation of *CHFR* and sensitivity to taxanes has been reported in colon and gastric cancer cells in culture (2,4), but not in endometrial cancer. Therefore, we investigated this relationship in endometrial cancer, with the goal of establishing a molecular index that might lead to personalized treatment strategies for endometrial cancer.

Materials and methods

Subjects and specimens for biopsy. The subjects were 69 patients who gave informed consent for collection of endometrial specimens (9 of normal endometrium, 10 of atypical endometrial hyperplasia and 50 of endometrial cancer). Cells obtained from the tissue specimens were examined by liquid-based cytology using the ThinPrep System (Cytyc Corp., Boxborough, MA) with preservation fluid (PreservCyt Solution, Cytyc Corp.) (5). A pathological diagnosis of the endometrial tissue was consistent with cytology results for all 69 subjects. Of the 9 patients with a normal endometrium, 5 were in the secretory phase and 4 were in the proliferative phase, and of the 50 patients with endometrial cancer, 42 had ovarian endometrioid adenocarcinoma (G1, 20; G2, 12; G3, 10) and 8 had adenosquamous carcinoma. The grade of histo-

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Key words: CHFR, endometrial cancer, DNA hypermethylation, taxane, chemosensitivity

Table I. Primer sequences used in MSP and RT-PCR analyses.

Gene name	PCR analysis	Sense	Antisense	Size (bp)	Annealing temperature (˚C)
CHFR	Methylated	GTCGGGTCGGGGTTC	CCCAAAACTACGACGACG	150	60
	Unmethylated	ATATAATATGGTGTTGATT	TCAACTAATCCACAAAACA	206	53
CHFR	RT-PCR	TGGAACAGTGATTAACAAGC	AGGTATCTTTGGTCCCATGG	206	55
ß-actin	RT-PCR	TTATTTGAGCTTTGGTTCTG	CTCCTTAATGTCACGCACGATTTC	303	50

logical differentiation (G1-G3) and the cancer stage at surgery were determined based on the Guidelines for Endometrial Cancer published by the Japan Society of Obstetrics and Gynecology.

Culture cell lines. Six cell strains were used: HEC108 (a human endometrial cancer-derived culture cell line supplied by Dr Hiroyuki Kuramoto), HOOUA and HHUA (supplied by Dr Isamu Ishiwata) and SNG-II, HEC1B and KLE. KLE cells were cultured in a DMEM/F12 (1:1) medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Sanko Junyaku Co., Ltd., Tokyo, Japan), and the other cells were cultured in 10% FBS-supplemented F12 medium (Sigma, St. Louis, MO, USA). The cells were incubated in a 10-cm dish under 5% CO_2 at 37˚C.

DNA extraction and methylation-specific PCR (MSP) analysis of CHFR. DNA was extracted from 69 endometrial specimens and 6 endometrial cancer-derived cell lines using liquid-based cytology with a GetPure DNA Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Distilled water was added to 1 μg of the extracted DNA up to a volume of 50 μl, 5.5 μl of 3 N NaOH solution was added, and after mixing the solution was incubated at 37˚C for 15 min. Following this, 520 μl of 3 M sodium bisulfate (Sigma), which was prepared at pH 5.5 with 30 μl of 10 mM hydroquinone (Sigma) and 10 N NaOH, was added to the solution. After mixing in an upturned position to prevent vaporization, the solution was overlaid with mineral oil and incubated at 50˚C overnight. Next, 1 ml of clean-up resin (Promega Corp., Madison, WI, USA) was added to the lower layer, and the resulting solution was mixed in an upturned position and then injected into a column. After rinsing with 2 μl of 80% isopropanol, the column was centrifuged at 15,000 rpm for 3 min to remove isopropanol completely, after which 50 μl of distilled water (70˚C) was added directly to the column, and the column was centrifuged at 15,000 rpm for 2 min to extract DNA adsorbed on the column. Then, 5.5 μl of 2 N NaOH was added to the resulting DNA solution, and after mixing the solution was incubated at 37˚C for 20 min, after which 66 μl of 5 N ammonium acetate and 243 μl of 95% ethanol were added. The solution was then incubated at -80˚C for 1 h and centrifuged at 15,000 rpm for 30 min to precipitate DNA. Approximately, 50 μl of the supernatant was left in the tube, and the rest of the supernatant was collected, mixed with 1 ml of 70% ethanol, and then centrifuged at 15,000 rpm for 30 min to rinse the DNA. The precipitated DNA was air-dried and dissolved in 20 μl of distilled water; 2 μl of this solution was used as the MSP template solution. AmpliTaq Gold and 10X PCR buffer/$MgCl_2$ with dNTP (Applied Biosystems, Foster City, CA, USA) was used in the PCR analysis, and DNA was analyzed using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR conditions for other genes and primer sequences are shown in Table I. DNA extracted from the culture cell lines was also used in MSP analysis of *CHFR*.

Statistical analysis. Correlations of aberrant DNA hypermethylation of *CHFR* with the grade of histological differentiation and the cancer stage at surgery were analyzed using the χ^2 test and Mann-Whitney test, respectively. Correlation of aberrant DNA hypermethylation of *CHFR* with patient age was also examined, after establishing that the groups of patients with and without aberrant hypermethylation had a normal age distribution. The Mann-Whitney test was used to examine whether the population medians of the two independent groups differed significantly.

RNA extraction and expression analysis of CHFR using RT-PCR. Total-RNA was extracted from 6 endometrial cancer-derived cell lines using a RNeasy mini-Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized with 1 μg of total-RNA using a SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). Synthesized 1st strand cDNA (1 μl) was used as a template solution in RT-PCR analysis of *CHFR* expression. AmpliTaq Gold and 10X PCR buffer/$MgCl_2$ with dNTP (Applied Biosystems) was used in the PCR analysis, and DNA was analyzed using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR conditions and primer sequences are shown in Table I.

Demethylation. SNG-II cells, which are endometrial cancer-derived cells with aberrant hypermethylation of *CHFR*, were plated on a 10-cm dish at 10^6 cells/dish and incubated for 72 h. A demethylating agent, 5-aza-dC (Sigma), was then added until its final concentration in the culture medium was 1 μM. Forty-eight hours after the first addition 5-aza-dC was added again, and DNA and RNA were extracted 24 and 72 h after the second addition, respectively.

Cell cycle analysis using flow cytometry. SNG-II and KLE cells, which are both endometrial cancer-derived cell lines, were plated on a 10-cm dish at $5x10^5$ cell/dish and incubated

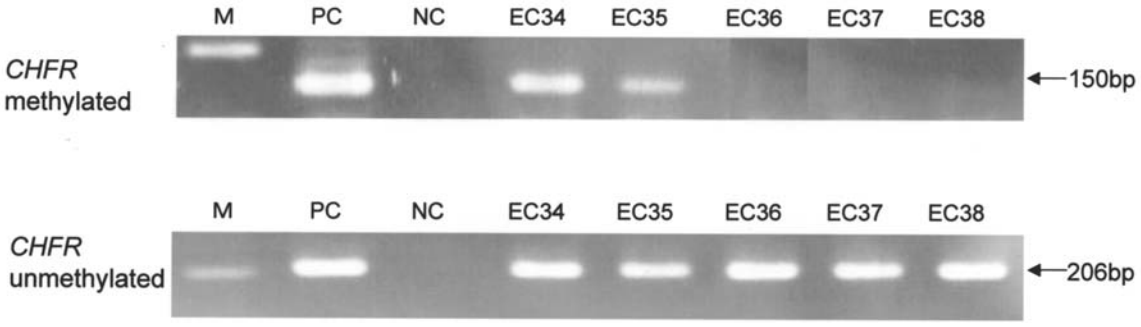

Figure 1. MSP analysis of *CHFR* in endometrial cancer cells obtained using cytology. MSP analysis was conducted with DNA extracted from specimens of endometrial cancer. Bands due to aberrant hypermethylation are found in lanes EC34 and EC35. M, marker; PC, positive control; NC, negative control; EC, endometrial cancer.

to 80% confluence. Paclitaxel (Bristol-Myers Squibb Co., NY, USA) was then added until its final concentration in the culture medium was 1.0 μg/ml. Forty-eight hours later, the cells were treated with trypsin, washed twice with PBS, and then centrifuged at 15,000 rpm for 5 min. The supernatant was removed and the cell pellet was washed with 500 μl of PBS. The vortexed cells were combined with 1 ml of 100% cold ethanol and then incubated at room temperature for 30 min for fixation. After rinsing twice with PBS and removing the supernatant, 500 μl of RNase was added to the cell pellet and the mixture was incubated at room temperature for 20 min. After a further addition of 500 μl of propidium iodide (PI), the cells were strained with a cell strainer and cell cycle status was determined using an EpicsXL MCL flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA).

Anticancer drug-sensitivity test. A collagen gel droplet-embedded culture drug-sensitivity test (CD-DST) was performed using 6 endometrial cancer-derived cell lines and 12 of 50 specimens of endometrial cancer (6). The culture cells and specimens were treated with a cell-dispersing enzyme, EZ (Nitta Gelatin Inc., Tokyo, Japan), for 2 h and centrifuged. The cells were preincubated in a collagen gel flask for 24 h and the living cells that attached to the collagen gel were collected. Cellmatrix Type CD solution was added to these cells, and then 3 drops of a 30-μl/drop collagen gel cell suspension were placed in a 6-well plate. The cell suspension was left to stand for 1 h in an incubator under 5% CO_2 at 37˚C, and after gelation the medium was doubled to 4 ml/well and anticancer drugs were added. Four anticancer drugs, cisplatin, doxorubicin, paclitaxel and docetaxel, were used at final concentrations of 2.0, 0.02, 1.0 and 0.1 μg/ml, respectively. Twenty-four hours after drug administration, the cells were washed to remove the anticancer drugs and then incubated without serum for 7 days under 5% CO_2 at 37˚C. After staining with Neutral Red, the cells were fixed with formalin and dried, and cell images were processed using an image analyzer. In the CD-DST, drug sensitivity is assessed using the ratio (T/C) of the number of living cells cultured in a solution containing anticancer drug (T) to that of control cells cultured in a solution without anticancer drug (C).

Expression analysis of CHFR protein. Specimens of endometrial cancer (G3) from 4 patients with aberrant hypermethylation of *CHFR* were embedded with OCT compound and frozen in liquid nitrogen. Cryostat-sliced sections were applied to slides and fixed with 100% ethanol, and the slides were incubated at 4˚C overnight with primary antibody (anti-*CHFR* antibody; Santa Cruz, Delaware, CA, USA) diluted 100-fold with 1% BSA in PBS. After rinsing three times with PBS, the slides were incubated with secondary antibody (biotin-labeled anti-goat IgG) at room temperature for 30 min, and after three further rinses with PBS the slides were incubated with ABC (avidin-biotin peroxidase) complex at room temperature for 30 min. After further rinsing three times with PBS, the slides were treated with 0.2 mg/ml diaminobenzidine (DAB) for about 5 min as a color reaction. After rinsing twice with PBS, the slides were treated with hematoxylin solution for nuclear staining, then dehydrated and enclosed, and observed microscopically. Immunohistochemical data for staining of *CHFR* protein were assessed using the following criteria: specimens with 30% or more of stained tumor cells were considered positive, and specimens with <30% of tumor cells showing staining were considered negative.

Results

Partial results of MSP analysis of endometrial cancer cells obtained using liquid-based cytology are shown in Fig. 1. Endometrial cancer specimens had a 12.0% (6/50) frequency of aberrant hypermethylation of the promoter region of *CHFR*, whereas specimens of atypical endometrial hyperplasia and normal endometrial cells in the proliferative and secretory phases showed no aberrant hypermethylation of the *CHFR* promoter region (Fig. 1, Tables II and III).

Correlations of aberrant DNA hypermethylation of the *CHFR* promoter with clinicopathological factors were examined in endometrial cancer patients. The frequency of aberrant hypermethylation in G3 adenocarcinoma was significantly higher than in G1 adenocarcinoma ($p<0.05$). Aberrant DNA hypermethylation is also generally thought to increase with age, but no significant difference in mean age was found between patients with and without aberrant hypermethylation of *CHFR*. Therefore, these data do not indicate that aberrant hypermethylation occurs more frequently in elderly patients with endometrial cancer (Table IV).

Of the 6 culture cell lines derived from endometrial cancer, SNG-II and HEC108 cells showed aberrant hypermethylation

Table II. Frequency of aberrant DNA hypermethylation of *CHFR* in specimens of endometrial cancer.

No.	Age	Tissue type	Stage	Differentiation	*CHFR*
EC1	52	EM	Ib	G3	U
EC2	50	EM	Ia	G1	U
EC3	51	EM	IIIc	G1	U
EC4	54	AS	IIIc	G3	M
EC5	51	EM	Ia	G1	U
EC6	61	EM	Ib	G1	U
EC7	70	EM	IIIc	G2	U
EC8	61	EM	IIb	G1	U
EC9	62	AS	IIIa	G2	U
EC10	40	EM	IIa	G1	U
EC11	59	EM	IIa	G3	U
EC12	57	EM	Ib	G3	U
EC13	80	EM	IIIc	G3	U
EC14	54	AS	Ib	G1	U
EC15	53	EM	Ib	G3	U
EC16	42	EM	IIb	G1	U
EC17	71	EM	IIIc	G3	U
EC18	60	EM	Ib	G1	U
EC19	57	EM	IIIa	G2	U
EC20	71	EM	IIa	G1	U
EC21	37	EM	IIa	G2	U
EC22	47	EM	IIIb	G1	U
EC23	67	EM	Ic	G2	M
EC24	53	EM	Ia	G1	U
EC25	69	EM	IIIc	G2	U
EC26	55	EM	IIIc	G2	U
EC27	54	EM	Ia	G1	U
EC28	63	EM	Ia	G1	U
EC29	41	EM	Ib	G1	U
EC30	62	AS	Ib	G1	U
EC31	58	EM	Ib	G2	U
EC32	56	EM	IIIc	G3	M
EC33	71	EM	Ib	G2	U
EC34	53	AS	Ib	G3	M
EC35	50	EM	IIIa	G3	M
EC36	42	AS	IIIc	G3	U
EC37	55	EM	Ic	G3	U
EC38	34	AS	IIIc	G1	U
EC39	61	EM	Ic	G1	U
EC40	61	EM	Ic	G1	U
EC41	61	EM	Ib	G1	U
EC42	59	EM	Ib	G1	U
EC43	55	AS	IVb	G2	U
EC44	54	EM	IIa	G1	U
EC45	78	EM	Ib	G3	U
EC46	65	EM	Ib	G2	M
EC47	68	EM	IIIc	G3	U
EM48	54	EM	IIIc	G2	U
EM49	60	EM	Ib	G1	U
EC50	70	EM	IVb	G2	U

EC, endometrial cancer; EM, endometrioid adenocarcinoma; AS, adenosquamous carcinoma; G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; M, methylated; U, unmethylated.

Table III. Frequency of aberrant DNA hypermethylation of *CHFR* in cells of normal endometrium and atypical endometrial hyperplasia.

No.	Age	Tissue type	*CHFR*
AE1	30	AEH	U
AE2	32	AEH	U
AE3	35	AEH	U
AE4	35	AEH	U
AE5	46	AEH	U
AE6	41	AEH	U
AE7	50	AEH	U
AE8	45	AEH	U
AE9	47	AEH	U
AE10	45	AEH	U
NE1	51	Sec	U
NE2	52	Sec	U
NE3	44	Sec	U
NE4	23	Sec	U
NE5	34	Sec	U
NE6	43	Pro	U
NE7	42	Pro	U
NE8	44	Pro	U
NE9	32	Pro	U

AE, atypical endometrial hyperplasia; NE, normal endometrium; AEH, atypical endometrial hyperplasia; Sec, secretory phase; Pro, proliferative phase; U, unmethylated.

Table IV. Correlation of aberrant DNA hypermethylation of *CHFR* with histological differentiation, stage at surgery and mean onset age.

	CHFR		
	Methylated	Unmethylated	P-value
G1	0	23	
G2	2	11	<0.05
G3	4	10	
Stage			
I	3	22	
II	0	7	NS
III	3	13	
IV	0	2	
Mean onset age	57.5±6.90	57.4±10.34	NS

G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated.

of *CHFR*, and RT-PCR analysis of *CHFR* expression showed reduced mRNA levels in these cells (Fig. 2). Consistent with this observation, the SNG-II and HEC108 cells showed higher

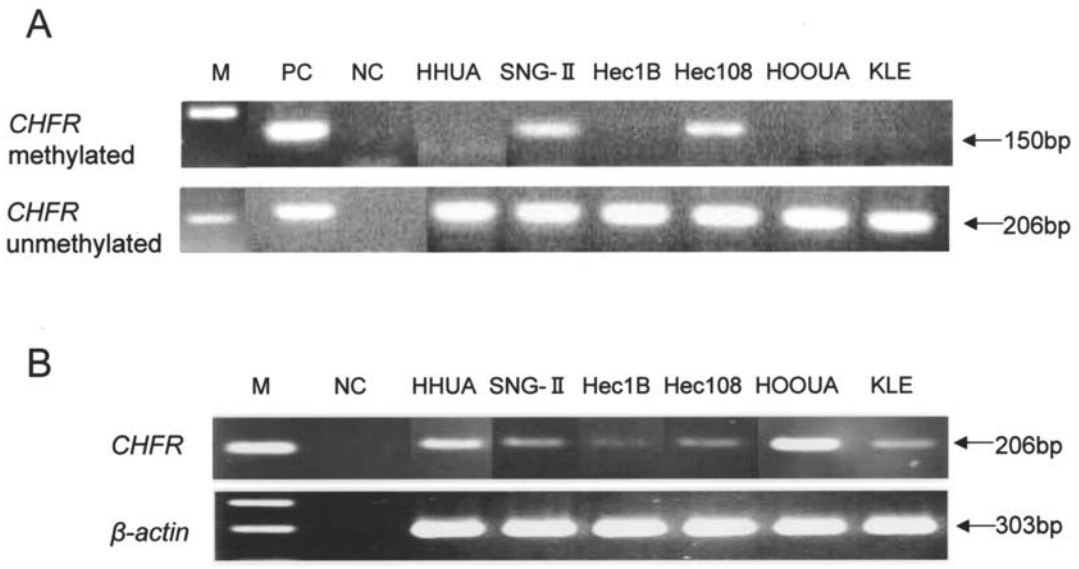

Figure 2. (A), MSP analysis of *CHFR* in endometrial cancer-derived cell lines. Aberrant hypermethylation of *CHFR* was found in two cell lines, SNG-II and HEC108. (B), Expression analysis of CHFR in endometrial cancer-derived cell lines using RT-PCR. *CHFR* expression was reduced in SNG-II and HEC108 cells, which showed aberrant hypermethylation of CHFR. M, marker; PC, positive control; NC, negative control.

Table V. Sensitivity (T/C ratio) of endometrial cancer-drived cells to various anticancer drugs in the CD-DST.

Cell line	*CHFR*	Cisplatin (%)	Doxorubicin (%)	Paclitaxel (%)	Docetaxel (%)
HHUA	U	100	100	22.5	31.0
SNG-II	M	52.0	65.1	16.7	15.1
Hec1B	U	91.1	85.7	71.2	65.9
Hec108	M	56.6	76.6	11.8	20.6
HOOUA	U	94.2	88.4	20.7	50.9
KLE	U	57.6	93.6	60.8	63.9

M, methylated; U, unmethylated.

sensitivity to paclitaxel and docetaxel in the CD-DST, compared to that of other cell lines (Table V).

Following treatment of SNG-II cells with 5-aza-dC, the aberrant hypermethylation band in the MSP analysis was weaker than that before administration of 5-aza-dC, and recovery of *CHFR* expression was shown in these cells (Fig. 3). Differences in sensitivity of SNG-II and HEC108 cells, and of KLE cells (which do not show aberrant hypermethylation of *CHFR*), to four anticancer drugs were examined using the CD-DST before and after 5-aza-dC administration. The T/C ratios of cells treated with cisplatin and doxorubicin did not differ before and after 5-aza-dC administration, regardless of the presence or absence of aberrant hypermethylation of *CHFR*, showing that 5-aza-dC administration had no effect on sensitivity to cisplatin and doxorubicin. Similarly, the T/C ratios of KLE cells treated with paclitaxel and docetaxel were unchanged by 5-aza-dC administration. However, the T/C ratios in SNG-II and HEC108 cells treated with paclitaxel and docetaxel significantly increased after 5-aza-dC administration, showing that these cells initially had low sensitivity to taxanes (Table VI).

Cell cycle changes in SNG-II and KLE cells treated with paclitaxel alone or combined paclitaxel and 5-aza-dC were determined using flow cytometry. The percentages of paclitaxel-treated KLE cells in the G2/M and Sub-G1 phases were 67.3 and 5.1%, respectively; these data were almost the same as those for untreated control cells. KLE cells treated with paclitaxel and 5-aza-dC gave similar results. In contrast, the percentage of paclitaxel-treated SNG-II cells in the G2/M phase was very low (0.2%) and the percentage of these cells in the Sub-G1 phase was higher (13.3%) compared to control cells, indicating that paclitaxel administration induced apoptosis. However, with combined paclitaxel and 5-aza-dC treatment, the percentage of SNG-II cells in the G2/M phase was high (82.7%) and that for cells in the Sub-G1 phase was low (1.8%) compared to control cells; a similar pattern to that seen for paclitaxel-treated KLE cells (Fig. 4).

MSP analysis indicated aberrant hypermethylation of *CHFR* in 12.0% (6/50) of endometrial cancer specimens, with this being particularly common for G3 specimens (4/14, 28.6%). Immunohistochemical analysis was conducted on specimens showing aberrant hypermethylation of *CHFR* from

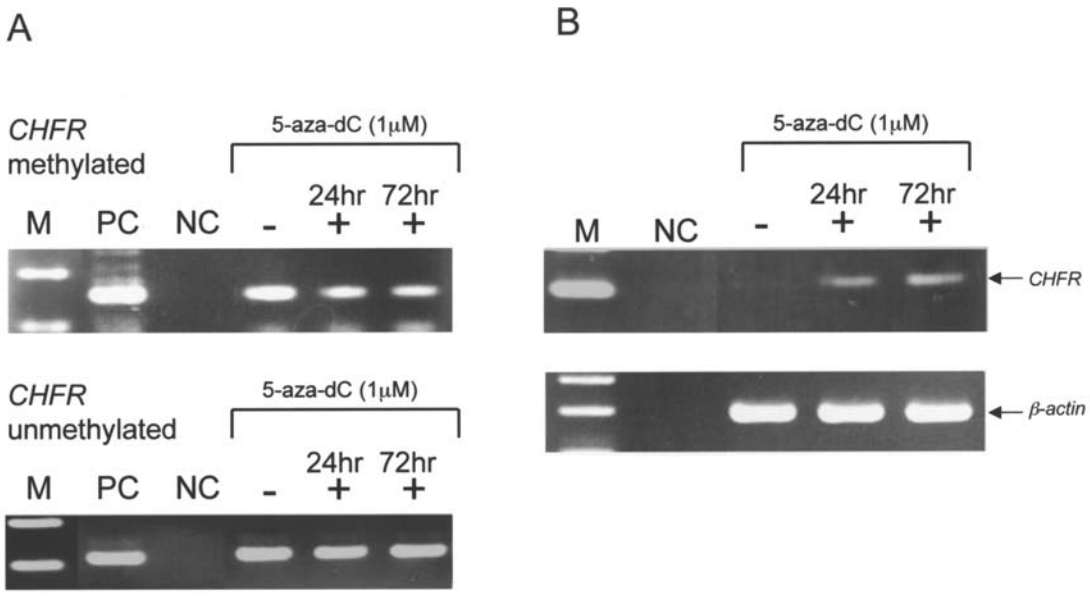

Figure 3. Demethylation analysis of *CHFR* in SNG-II cells. (A), In MSP, bands due to aberrant hypermethylation reduced with time after 5-aza-dC administration. (B), RT-PCR analysis showed recovered *CHFR* expression 24 h after the second 5-aza-dC administration. M, marker; PC, positive control; NC, negative control.

Table VI. Changes in sensitivity (T/C ratio) of endometrial cancer-drived cells to various anticancer drugs after treatment with a demethylating agent.

		Cisplatin (%)		Doxorubicin (%)		Paclitaxel (%)		Docetaxel (%)	
Cell line	*CHFR*	5-aza(-)	5-aza(+)	5-aza(-)	5-aza(+)	5-aza(-)	5-aza(+)	5-aza(-)	5-aza(+)
SNG-II	M	52.0	66.6	65.1	80.5	16.7	60.8	15.1	61.5
Hec108	M	56.6	64.6	76.6	76.7	11.8	70.7	20.6	69.4
KLE	U	57.6	64.5	93.6	100	60.8	75.2	63.9	72.9

5-aza, 5-aza-dC; M, methylated; U, unmethylated.

four G3 patients, and 75.0% (3/4) of these specimens showed reduced *CHFR* expression (Fig. 5). To investigate the relationship of sensitivity to paclitaxel with aberrant hypermethylation of *CHFR*, 12 surgical specimens of endometrial cancer were examined using CD-DST. The T/C ratio of specimens showing aberrant hypermethylation of *CHFR* was the lowest (37.6%), and these specimens exhibited higher sensitivity to paclitaxel compared to specimens without aberrant hypermethylation (Table VII).

Discussion

Aberrant hypermethylation of *CHFR* has been reported in gastrointestinal and lung cancers (7-9), but has not been examined in gynecologic cancer. Furthermore, the relationship between aberrant hypermethylation of *CHFR* and biological and disease characteristics has not been examined in any cancer. Our results indicate that aberrant hypermethylation of *CHFR* occurs in G3 adenocarcinoma significantly more frequently than in G1 adenocarcinoma ($p<0.05$, 28.6%). It is known that *CHFR* negatively regulates *Aurora-A* mitotic kinase: *Aurora-A* is induced by inhibition of expression of *CHFR* (10), and overexpression of *Aurora-A* has been shown to induce chromosomal instability (CI) in various cancers (11-13). Since CI in endometrial cancer is commonly found in G3 adenocarcinoma (65%) (14), it is likely that aberrant hypermethylation of *CHFR* is one cause of induction of CI in endometrial cancer. In addition, a relationship between aberrant hypermethylation of *CHFR* and *hMLH1* has been reported in colon cancer (15), and aberrant hypermethylation of *hMLH1* has also been found in approximately 40% of endometrial cancers and is thought to be involved in the early stage of carcinogenesis (16,17).

In cell cycle analysis using flow cytometry in endometrial cancer-derived cell lines, cells with a normal *CHFR* gene showed cell accumulation in G2/M phase after paclitaxel administration. In contrast, in cells with *CHFR* inactivated by aberrant hypermethylation, cell accumulation in G2/M phase was not observed after paclitaxel administration but was found after combined treatment with paclitaxel and a demethylating

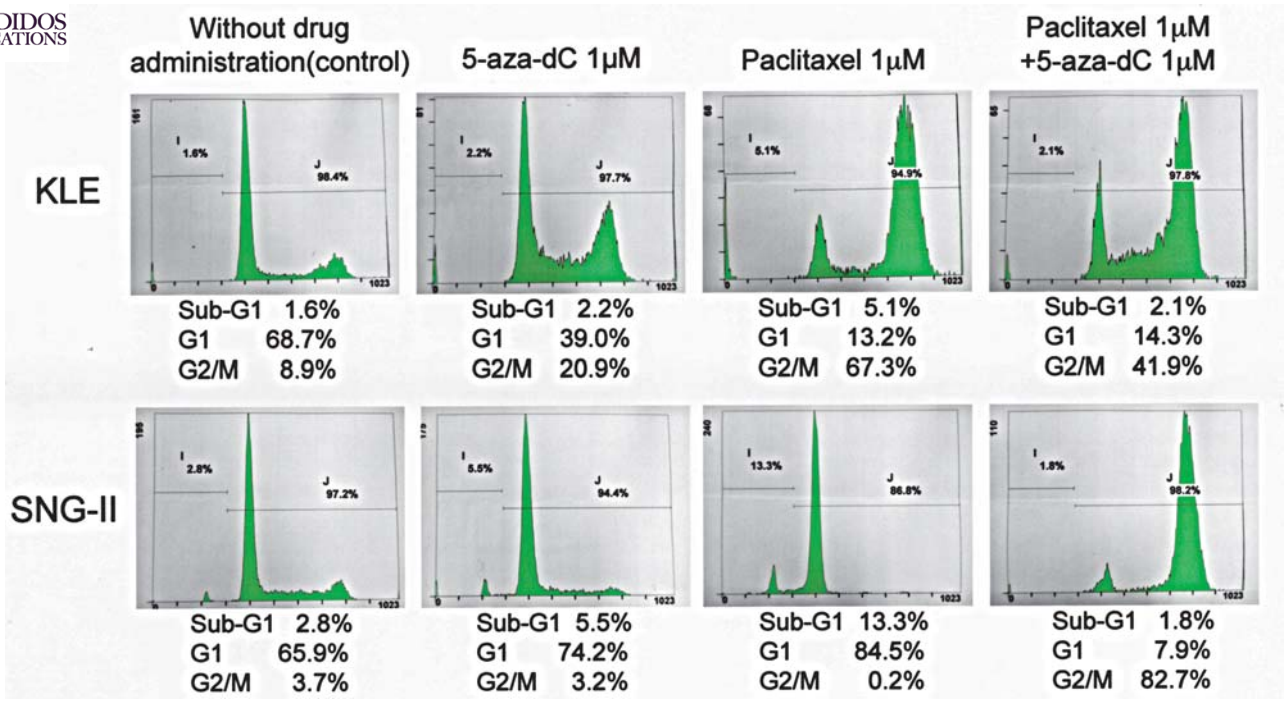

Figure 4. Cell cycle analysis in KLE and SNG-II cells using flow cytometry. KLE cells treated with paclitaxel alone and with combined paclitaxel and 5-aza-dC showed cell accumulation in G2/M phase and no significant change in the number of cells in Sub-G1 phase. SNG-II cells treated with paclitaxel alone exhibited no cell accumulation in G2/M phase, but an increased number of cells in Sub-G1 phase; however, SNG-II cells treated with combined paclitaxel and 5-aza-dC showed significant cell accumulation in G2/M phase and a low number of cells in Sub-G1 phase, similarly to the controls.

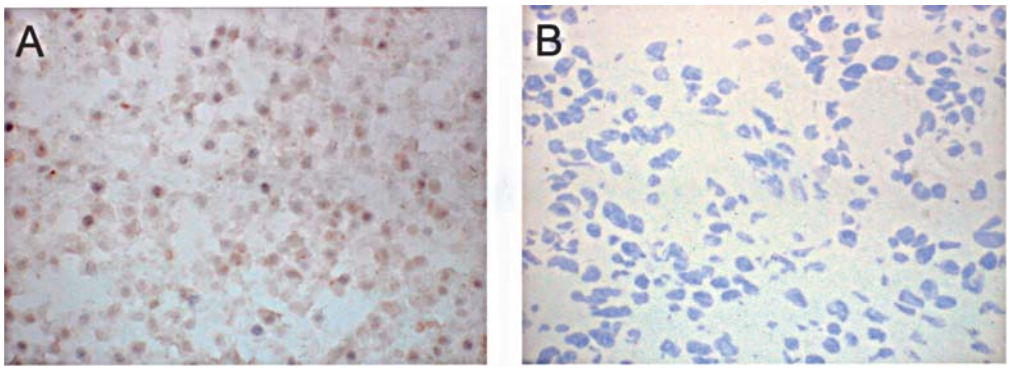

Figure 5. Immunohistochemical analysis of CHFR protein expression in patients with endometrioid adenocarcinoma (G3) with aberrant hypermethylation of *CHFR*. (A), EC35 staining; (B), EC32 staining. The nuclei of one of the four G3 adenocarcinoma patients stained positively (A), but three patients had nuclei with reduced staining (B).

agent. These results suggest that cells with normal *CHFR* undergo G2 arrest and can repair damage when the cells are treated with taxanes, thereby exhibiting resistance to taxanes, whereas cells with *CHFR* inactivated by aberrant hypermethylation cannot detect damage and enter mitosis, thereby showing high sensitivity to taxanes. Based on the increased number of cells in Sub-G1 phase without accumulation of G2/M cells after paclitaxel administration, the high taxane sensitivity of cells with inactivated *CHFR* appears to be due to mitotic catastrophe causing cell death after entry into the mitotic phase.

Cells with aberrant hypermethylation of *CHFR* showed greatly decreased sensitivity to paclitaxel in the CD-DST after demethylation treatment. Although 5-aza-dC administration will result in demethylation of many genes, in addition to *CHFR*, sensitivity to cisplatin or doxorubicin was unaltered after demethylation, whereas sensitivity to paclitaxel and docetaxel increased, suggesting that aberrant hypermethylation of *CHFR* is of importance in sensitivity to taxanes. Thus, aberrant hypermethylation of *CHFR* is a potential index for prediction of cell sensitivity to taxanes.

The CD-DST is an anticancer drug sensitivity test in which tumor cells are cultured three-dimensionally. This method requires only a small number of cells and both culture cell lines and tumor tissues can be analyzed in the same experimental system. Use of the CD-DST for gynecologic cancer has been reported (6), and here we used the CD-DST in analysis of taxane sensitivity of clinical specimens of

Table VII. Aberrant hypermethylation of *CHFR* and sensitivity (T/C ratio) of speciments of endometrial cancer to paclitaxel.

No.	Tissue type	Stage	Differentiation	*CHFR*	T/C ratio (%)
EC30	AS	Ib	G1	U	84.0
EC31	EM	Ib	G2	U	79.6
EC32	EM	IIIc	G3	M	37.6
EC33	EM	Ib	G2	U	86.9
EC36	AS	IIIc	G3	U	59.8
EC37	EM	Ic	G3	U	38.2
EC38	AS	IIIc	G1	U	66.4
EC39	EM	Ic	G1	U	100
EC47	EM	IIIc	G3	U	100
EC48	EM	IIIc	G2	U	84.3
EC49	EM	Ib	G1	U	79.7
EC50	EM	IVb	G2	U	70.3

EC, endometrial cancer; EM, endometrioid adenocarcinoma; AS, adenosquamous carcinoma; G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; M, methylated; U, unmethylated.

endometrial cancer. The CD-DST results for 12 specimens of endometrial cancer showed that cells with aberrant hypermethylation of *CHFR* had high sensitivity to paclitaxel, as also found in cultured cells; this is the first such study of this relationship in endometrial cancer. Immunohistochemical analysis showed that of four patients with G3 adenocarcinoma with aberrant hypermethylation of *CHFR*, three exhibited reduced expression of CHFR protein. This result indicates that aberrant hypermethylation of *CHFR* is involved in reduced protein expression *in vivo*. Overall, our results suggest a new strategy for design of personalized medicine for endometrial cancer using the hypermethylation status of *CHFR* as a molecular index. This strategy may be particularly important in treatment of G3 adenocarcinoma, since it is known to have a poor prognosis.

Acknowledgements

This study was partially supported by the Ministry of Education, Culture, Sports, Science and Technology through a Grant-in-Aid for Young Scientists (B) (17791135), and by the Haraguchi Memorial Cancer Fund charitable trust, and by Keio University Grant-in-Aid for Encouragement of Young Medical Scientists. We are grateful to Cytyc Corp. for provision of ThinPrep.

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