

Epigenetic inactivation of the *CHFR* gene in cervical cancer contributes to sensitivity to taxanes

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Abstract. A relationship between inactivation of mitotic checkpoint genes and sensitivity of cancer cells to anticancer agents has been reported. We investigated the effect of epigenetic inactivation by aberrant hypermethylation of the mitotic checkpoint gene *CHFR* (*checkpoint with forkhead and ring finger*) on the sensitivity of cervical cancer cells to taxanes. Methylation-specific PCR (MSP) of cervical smears showed aberrant methylation of *CHFR* in 12.3% (2/14) of adenocarcinoma specimens. In contrast, aberrant DNA methylation was not detected in normal cervical cells or squamous cell carcinoma cells. Aberrant methylation of *CHFR* was also analyzed in 6 human cervical carcinoma-derived cell lines and was observed in SKG-IIIb and HeLa cells. These cell lines showed high sensitivity to taxanes, but became taxane-resistant upon treatment with 5-azacytidine. Furthermore, suppression of *CHFR* expression in siRNA-transfected SKG-IIIa cells caused increased sensitivity to taxanes. In conclusion, aberrant methylation of the *CHFR* gene may be useful as a molecular marker for selection of therapy for patients with cervical adenocarcinoma with a poor prognosis, and may also suggest a new therapeutic strategy of targeting *CHFR* in cervical cancer. To our knowledge, this study is the first to examine epigenetic inactivation by aberrant hypermethylation of *CHFR* in cervical cancer.

Introduction

Cervical cancer is the second most common cause of cancer-related mortality in women worldwide: nearly 500,000

women are diagnosed with cervical cancer each year and many die of the disease. In the United States, there were approximately 14,500 new cervical cancer cases and 4,800 cervical cancer deaths in 1997 (1,2). Cervical cancer differs from most other common malignancies in that it is strongly associated with an infection agent, human papillomavirus (HPV). Most studies have focused on the E6 and E7 transforming proteins of oncogenic HPV types, since E6 and E7 interfere with the function of the tumor-suppressor proteins p53 and Rb via protein-protein interactions. By interfering with cell cycle control and DNA repair mechanisms, oncogenic HPVs appear to contribute indirectly to cervical tumorigenesis by promoting genetic instability and the accumulation of mutations in HPV-infected cells (3,4).

In addition to p53 and Rb, p16INK4a and RASSF1A (RAS association domain family protein 1) are candidate tumor suppressor genes in cervical cancer. Inactivation of these genes may be due to aberrant DNA hypermethylation of CpG islands in the promoter region, and a relationship between development of cervical cancer and such epigenetic changes has been proposed (5-7). Inactivation of cell-cycle checkpoint genes in tumor cells by aberrant DNA hypermethylation also has a major effect on sensitivity to specific antitumor agents (8,9). Mitotic checkpoint gene *CHFR* (*checkpoint with forkhead and ring finger*) is located in chromosome 12q24.33 and has the function of delaying chromatin aggregation, leading to delayed progression to mitosis (10). The *CHFR* gene has a forkhead-associated domain in the N-terminal region and a ring finger domain in the center region. Both domains function as a mitotic checkpoint by detecting mitotic stress, and under such conditions *CHFR* induces cell cycle arrest in G2 phase (G2 arrest) to allow repair of damaged DNA.

Taxane is a microtubule depolymerization inhibitor in mitotic cells. Cells with normal *CHFR* expression are arrested in G2 phase to repair damaged DNA and consequently are resistant to taxane. However, cells with a *CHFR* gene inactivated by aberrant hypermethylation cannot detect DNA damage and proceed to mitosis, with subsequent cell death due to mitotic catastrophe; i.e., such cells show high sensitivity to taxane. Therefore, aberrant hypermethylation of the *CHFR* gene is a potential molecular marker for taxane sensitivity. A relationship between aberrant hypermethylation of *CHFR* and sensitivity to taxanes has been reported in

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colon, gastric and endometrial cancer cells *in vitro* (9,11,12), but has not been studied in cervical cancer. Therefore, we investigated this relationship in cervical cancer cells, with the goal of establishing a new molecular marker for selection of therapy for cervical cancer.

Materials and methods

Subjects and cytologic specimens. Samples were obtained from 20 normal cervical smears and 40 cervical cancer smears. After obtaining informed consent, cervical smears were collected using the ThinPrep collecting system (Cytoc Corporation, Boxborough, MA) and kept in preservation fluid (PreservCyt Solution, Cytoc Corp.) (13). Pathological diagnosis was confirmed by cervical histology, and the cytological and histological results were consistent for all 60 smears. Of the 40 cervical cancer smears, 26 were squamous carcinoma and 14 were adenocarcinoma. The histological type and stage were determined according to the General Rules for Clinical Cervical Cancer in Japan published by the Japan Society of Obstetrics and Gynecology.

Cultured cell lines. The human cervical squamous cell carcinoma-derived cell lines, SKG-I, SKG-II, SKG-IIIa and SKG-IIIb, and the human cervical adenocarcinoma-derived cell lines, HeLa and TCO-I, were used in the study. HeLa cells were incubated in DMEM (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS) (Sanko Junyaku Co., Ltd., Tokyo, Japan) and TCO-I cells were incubated in MEM medium (Sigma) with 10% FBS. All other cell lines were incubated in F12 medium (Sigma) with 10% FBS. Cells were incubated in 10-cm dishes at 37°C in a 5% CO₂ atmosphere.

DNA extraction and methylation-specific PCR (MSP) assay of the *CHFR* gene. DNA was extracted from 60 cervical smears and 6 cervical carcinoma-derived cell lines using a Get Pure DNA Kit (Dojin Gloac Corporation, Kumamoto, Japan). DNA (1 µg) extracted from cervical smears was diluted with 50 µl of distilled water and incubated in 5.5 µl of 3 N NaOH at 37°C for 15 min. To this solution, 30 µl of 10 mM hydroquinone (Sigma) and 520 µl of 3 M sodium bisulfite (prepared at pH 5.5 with 10 N NaOH, Sigma) were added with mixing. Mineral oil was laid over the solution to prevent evaporation, and the solution was incubated overnight at 50°C. The lower layer of the reaction solution was mixed with 1 ml of Clean-up Resin (Promega Corporation, Madison, WI) and then injected into a column. After rinsing with 2 ml of 80% isopropanol, the mixture was centrifuged at 15,000 rpm for 3 min to completely remove isopropanol. Hot (70°C) distilled water (50 µl) was added, and the mixture was centrifuged at 15,000 rpm for 2 min to elute DNA. The DNA was then incubated with 5.5 µl of 2 N NaOH at 37°C for 20 min. Next, 66 µl of 5 N ammonium acetate and 243 µl of 95% ethanol were added and the mixture was incubated at -80°C for 1 h and centrifuged at 15,000 rpm for 30 min to precipitate DNA. Supernatant exceeding 50 µl was removed, 1 ml of 60% ethanol was added, and the mixture was centrifuged at 15,000 rpm for 30 min and rinsed. The precipitated DNA was dried in air and dissolved in 20 µl of distilled

water. DNA solution (2 µl) was used as the MSP template. In the PCR assay, AmpliTaq Gold and 10x PCR buffer/MgCl₂ with dNTP (Applied Biosystems, Foster City, CA) were used and the results were analyzed with a GeneAmp PCR System 9700 (Applied Biosystems). The PCR conditions and primer sequence have been described previously (12). DNA extracted from the cultured cell lines was prepared similarly for use in MSP analysis of the *CHFR* gene.

RNA extraction and RT-PCR assay of *CHFR* expression. Total RNA from 6 cervical cancer-derived cell lines was extracted using an RNeasy mini-Kit (Qiagen, Valencia, CA). cDNA was synthesized from 1 µg of total RNA using SuperScriptII Reverse Transcriptase (Invitrogen, Carlsbad, CA). *CHFR* expression was analyzed in an RT-PCR assay using 1 µl of first-strand cDNA as template. AmpliTaq Gold and 10x PCR buffer/MgCl₂ with dNTP were used in the PCR assay, with analysis using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR conditions and primer sequence have been described previously (12).

Demethylation treatment. Cervical carcinoma-derived HeLa cells with aberrant methylation of *CHFR* were plated on a 10-cm dish at 10⁶ cell/dish and incubated for 72 h. 5-aza-dC (Sigma), a demethylating agent, was then added at a final concentration of 1 µM in culture medium. After 48 h of incubation, 5-aza-dC was added again and DNA and RNA were extracted 24 and 72 h after the second addition of 5-aza-dC.

Cell-cycle analysis using flow cytometry. Cervical-carcinoma derived SNG-IIIa and HeLa cells were plated on a 10-cm dish at 5x10⁵ cell/dish and incubated until the cells reached 80% confluence. Paclitaxel (supplied by the Bristol-Myers Squibb Company) was added to the culture medium at a final concentration of 1.0 µg/ml. The cells were trypsinized 48 h later and rinsed twice with PBS. Supernatant was separated from the cell pellets by centrifugation at 15,000 rpm for 5 min, and 500 µl of PBS was added to the pellets and the mixture was pipetted well. As the mixture was vortexed, 1 ml of cool 100% ethanol was added. The mixture was then incubated at room temperature for 30 min for cell fixation. The cells were rinsed twice with PBS and 500 µl of RNase was added to the pellets after supernatant removal. The cells were then incubated at room temperature for 20 min. Subsequently, 500 µl of propidium iodide (PI) solution was added, the mixture was poured into a cell strainer, and the cell cycle was determined by flow cytometry using an EpicsXL MCL (Beckman Coulter, Inc, Fullerton, CA).

***In vitro* test of sensitivity to anticancer agents.** The sensitivity to anti-cancer agents of 6 cervical carcinoma-derived cell lines was determined using the collagen gel droplet embedded culture drug sensitivity test (CD-DST) (14). Cervical carcinoma-derived cells were pretreated with cell dispersion enzyme EZ (Nitta Gelatin Inc., Tokyo, Japan) for 2 h, followed by centrifugation to collect the cells. In a flask containing collagen gel, the cells were pre-incubated for 24 h and surviving cells that adhered to collagen gel were collected. Cellmatrix Type CD solution was added to the

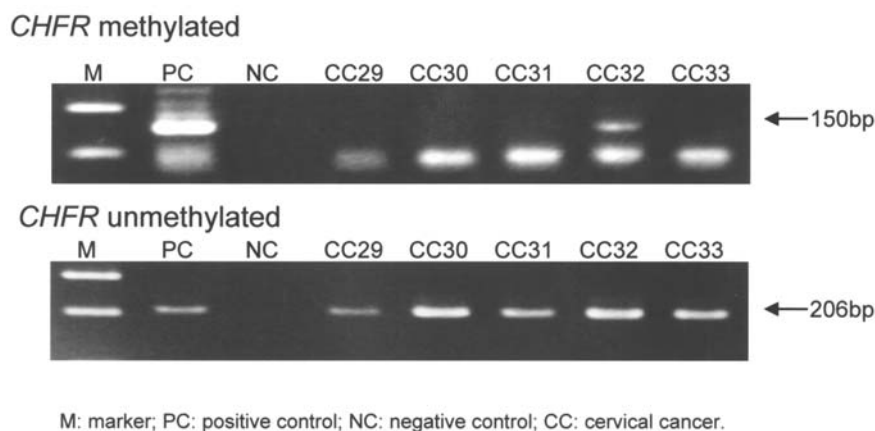


Figure 1. MSP analysis of the *CHFR* gene in cervical cancer cytologic specimens. MSP analysis was examined with DNA extracted from cervical cancer cytologic specimens. A band due to aberrant methylation is observed at CC32.

collected cells, and the suspension of cells and collagen gel was dropped onto a 6-well plate to prepare 3 drops of 30 μ l each. The suspension was left to stand in an incubator at 37°C in a 5% CO₂ atmosphere for 1 h for gelling and then overlaid with 4 ml/well of medium. An anticancer agent was then added to the suspension: cisplatin, doxorubicin, paclitaxel, docetaxel, 5-fluorouracil and etoposide at final concentrations of 2.0, 0.02, 1.0, 0.1, 1.0 and 1.0 μ g/ml, respectively. After 24 h, the drugs were removed by rinsing and the cells were incubated without serum at 37°C in 5% CO₂ for 7 days. The cells were dyed with Neutral Red, fixed with formalin and dried. Images were collected by scanning using an image analyzer and the ratio of surviving cells in the anticancer agent-treated group (T) to that in the non-treated group (C) (T/C ratio) was determined. In general, cells are considered to be highly sensitive to the agent when the T/C ratio is $\leq 40\%$.

Transfection of small interfering RNA (siRNA). SKG-IIIa cells were plated on a 60-mm dish at 4×10^5 cell/dish and transfected 48 h later with siRNA using siFECTOR (B-Bridge International Inc, CA). In this procedure, 4.5 μ l of siRNA stock solution (100 μ M) and 295.5 μ l of serum-free MEM were mixed in a test tube. In another tube, 13.5 μ l of siFECTOR and 286.5 μ l of serum-free MEM were mixed. The solutions from the two tubes were mixed and incubated at room temperature for 30 min. Each dish containing SKG-IIIa cells was rinsed twice with 2 ml of serum-free MEM and 2.4 ml of serum-free MEM was then added. The incubated siRNA mixture solution was added to the dish at 0.6 ml/dish and incubated at 37°C in 5% CO₂ for 6 h. After incubation, 3 ml of MEM containing 20% serum was added to the dish. S20C-0600 (B-Bridge International) was used as negative control siRNA. The siRNA sequence corresponding to the *CHFR* gene was 5'-GGAAAAACAUGUUGACCGAdTdT-3'. The expression levels of mRNA and protein were determined 48 h after siRNA addition. Anticancer agents were added 48 h after siRNA addition and the sensitivity of the cells to each agent was analyzed using the CD-DST.

Immunoblotting. SKG-IIIa cells were rinsed with PBS, trypsinized and centrifuged at 15,000 rpm for 5 min at 4°C.

Protein was extracted using a Mammalian Cell Extraction Kit (Bio Vision Research Products, CA) according to the manufacturer's protocol. The sample (200 μ g of protein) was mixed with sample buffer (Bio-Rad Laboratories, CA) containing the equivalent volume of 5% β -mercaptoethanol (Bio-Rad Laboratories) and the mixture was boiled for 5 min. After boiling, the mixture was electrophoresed on a 10% polyacrylamide gel and the proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were soaked in PBS containing 1% BSA and 0.1% Tween-20 and incubated at room temperature for 1 h for blocking. They were then reacted with anti- β -actin antibody (A5316 Sigma-Aldrich Inc, St. Louis, MO, 5,000-fold diluted) and anti-CHFR antibody (ab13773, Abcam, Cambridge, UK, 500-fold diluted) at 4°C overnight, followed by rinsing three times with PBS containing 0.1% Tween (PBS-T) for 10 min each. The anti- β -actin and anti-CHFR antibodies were reacted with anti-mouse IgG antibody (PK-6102, Vector Laboratories, Inc., CA) and anti-goat IgG antibody (BA-5000, Vector Laboratories, 250-fold diluted), respectively, at room temperature for 1 h. The membranes were rinsed with PBS-T three times and reacted with ABC complex (PK-6102, Vector Laboratories, pre-reacted at 4°C for 30 min) at room temperature for 1 h. The membranes were rinsed with PBS-T twice and PBS once, and visualized with DAB (Sigma).

Results

Results from MSP analysis of cervical cancer cytologic specimens are shown in Fig. 1. Aberrant hypermethylation of the *CHFR* gene in the promoter region was detected in 14.3% (2/14) of adenocarcinoma specimens, whereas there was no aberrant DNA hypermethylation in normal cervical cells and squamous cell carcinoma cells (Fig. 1, Tables I and II). Aberrant hypermethylation of *CHFR* was also analyzed in 6 human cervical carcinoma-derived cell lines and was detected in SKG-IIIb and HeLa cells. RT-PCR analysis confirmed that expression of mRNA for *CHFR* was reduced in SKG-IIIb and HeLa cells (Fig. 2). The sensitivity of the cell lines to paclitaxel and docetaxel was determined using the CD-DST, and SKG-IIIb and HeLa cells showed much

Table I. Aberrant methylation of the *CHFR* gene in cervical cancer cytologic specimens.

| No. | Tissue type | Stage | <i>CHFR</i> |
|------|-------------|-------|-------------|
| CC1 | SCC | Ib1 | U |
| CC2 | SCC | Ib1 | U |
| CC3 | SCC | Ib1 | U |
| CC4 | SCC | Ib1 | U |
| CC5 | SCC | IIa | U |
| CC6 | SCC | IIa | U |
| CC7 | SCC | Ib2 | U |
| CC8 | SCC | IIb | U |
| CC9 | SCC | Ib1 | U |
| CC10 | SCC | Ib1 | U |
| CC11 | SCC | Ib1 | U |
| CC12 | SCC | Ib2 | U |
| CC13 | SCC | Ib1 | U |
| CC14 | SCC | Ib1 | U |
| CC15 | SCC | Ib1 | U |
| CC16 | SCC | IIa | U |
| CC17 | SCC | Ib2 | U |
| CC18 | SCC | Ib2 | U |
| CC19 | SCC | Ib1 | U |
| CC20 | SCC | Ib2 | U |
| CC21 | SCC | Ib1 | U |
| CC22 | SCC | Ib1 | U |
| CC23 | SCC | Ib2 | U |
| CC24 | SCC | Ib1 | U |
| CC25 | SCC | Ib1 | U |
| CC26 | SCC | IIb | U |
| CC27 | MAD | Ib1 | U |
| CC28 | MAD | IIa | M |
| CC29 | MAD | Ib1 | U |
| CC30 | MAD | Ib1 | U |
| CC31 | MAD | Ib1 | U |
| CC32 | MAD | Ib1 | M |
| CC33 | MAD | IIa | U |
| CC34 | MAD | Ib1 | U |
| CC35 | MAD | Ib1 | U |
| CC36 | MAD | Ib1 | U |
| CC37 | MAD | Ib2 | U |
| CC38 | MAD | Ib1 | U |
| CC39 | MAD | Ib1 | U |
| CC40 | MAD | IIb | U |

CC, cervical cancer; SCC, squamous cell carcinoma; MAD, mucinous adenocarcinoma (endocervical type).

Table II. Aberrant methylation frequency of the *CHFR* gene in cervical cancer cytologic specimens.

| | <i>CHFR</i> | |
|-----|-------------|-----------|
| | M (%) | U (%) |
| NCE | 0 (0) | 20 (100) |
| SCC | 0 (0) | 26 (100) |
| MAD | 2 (14.3) | 12 (85.7) |

NCE, normal cervical epithelium; SCC, squamous cell carcinoma; MAD, mucinous adenocarcinoma (endocervical type); M, methylated; U, unmethylated.

following 5-aza-dC treatment was confirmed by RT-PCR (Fig. 3). Changes in the sensitivity of HeLa cells and SKG-IIIa cells (which did not show aberrant *CHFR* hypermethylation) to 6 anticancer agents were determined before and after 5-aza-dC addition, using the CD-DST. Anticancer agents other than taxanes (5-fluorouracil, etoposide, cisplatin and doxorubicin) showed almost no change in the T/C ratio before and after 5-aza-dC addition and regardless of aberrant *CHFR* hypermethylation. In contrast, the T/C ratios of HeLa cells treated with paclitaxel and docetaxel increased significantly after 5-aza-dC addition, indicating a significant decrease in sensitivity (Table IV).

Changes in cell cycle were determined using flow cytometry in SKG-IIIa and HeLa cells treated with paclitaxel alone or a combination of paclitaxel and 5-aza-dC. In SKG-IIIa cells (no aberrant *CHFR* methylation), cells in G2/M phase markedly increased to 73.9% after paclitaxel treatment and G2 arrest was observed. In contrast, in HeLa cells (aberrant *CHFR* hypermethylation), the percentage of G2/M cells remained low (8.3%) after paclitaxel treatment and Sub-G1 cells increased to 13.4%, higher than that of controls, suggesting that paclitaxel treatment induced apoptosis. However, combined treatment with paclitaxel and 5-aza-dC resulted in 73.9% of cells in the G2/M phase and a marked decrease in Sub-G1 cells to 2.2%, showing a similar pattern to paclitaxel treatment of SKG-IIIa cells (Fig. 4).

SKG-IIIa cells were transfected with siRNA for *CHFR* and the expression levels of *CHFR* mRNA and protein decreased to approximately half of the control levels (Fig. 5). Under these conditions, changes in sensitivity to anticancer agents were determined using the CD-DST. The T/C ratios for paclitaxel and docetaxel were significantly decreased compared with those for non-taxane anticancer agents, indicating that reduction of *CHFR* expression specifically increases sensitivity to taxanes (Fig. 6).

Discussion

Aberrant hypermethylation of the *CHFR* gene has been reported in endometrial, gastrointestinal and lung cancers (12,15-17). A similar effect has not been studied in cervical cancer, and the relationship between aberrant *CHFR* hypermethylation and the biological characteristics of cervical

higher high sensitivity to these agents, compared to other cells (Table III).

Recovery of *CHFR* expression by treatment with 5-aza-dC was examined in HeLa cells (which showed aberrant *CHFR* hypermethylation), and increased *CHFR* expression

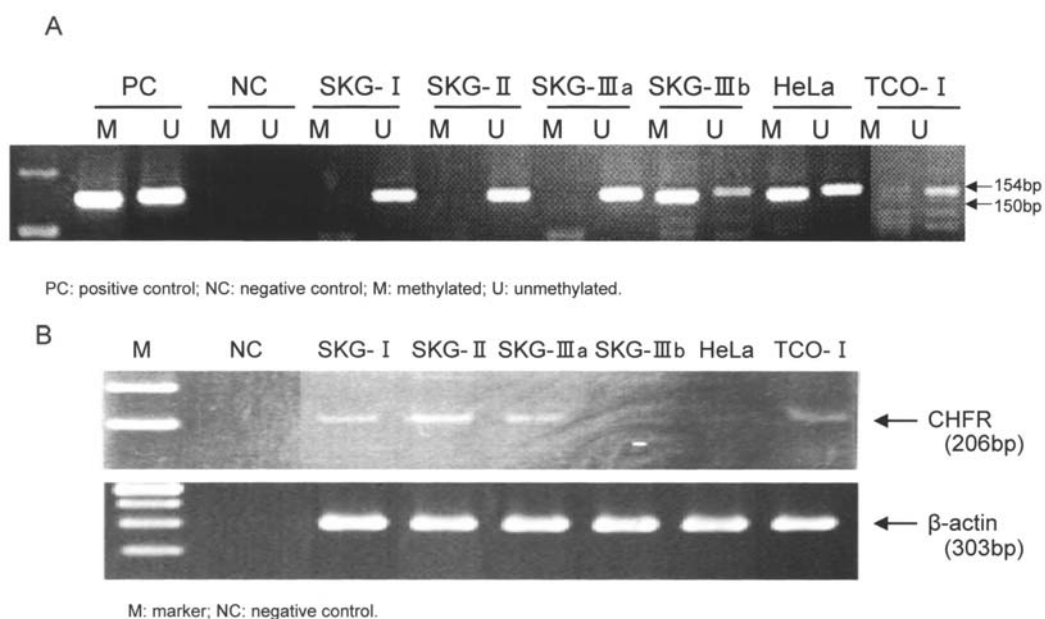


Figure 2. (A) MSP analysis of the *CHFR* gene in cervical cancer-derived cell lines. Aberrant hypermethylation of the *CHFR* gene was observed in SKG-IIIb and HeLa cells. (B) Analysis of *CHFR* expression in cervical cancer-derived cell lines using RT-PCR. *CHFR* expression was decreased in SKG-IIIb and HeLa cells, which had aberrant hypermethylation of the *CHFR* gene.

Table III. Sensitivity (T/C ratio) of cervical cancer-derived cells to various anticancer agents, assessed using the CD-DST.

| Cell line | <i>CHFR</i> | Cisplatin (%) | Doxorubicin (%) | Paclitaxel (%) | Docetaxel (%) |
|-----------|-------------|---------------|-----------------|----------------|---------------|
| SKG-I | U | 75.9 | 89.6 | 39.5 | 41.3 |
| SKG-II | U | 97.8 | 91.6 | 55.5 | 49.6 |
| SKG-IIIa | U | 94.5 | 90.1 | 69.2 | 63.1 |
| SKG-IIIb | M/U | 93.2 | 77.2 | 14.0 | 14.0 |
| HeLa | M/U | 75.2 | 79.1 | 9.8 | 9.7 |
| TCO-I | U | 96.9 | 66.7 | 33.2 | 35.1 |

M, methylated; U, unmethylated.

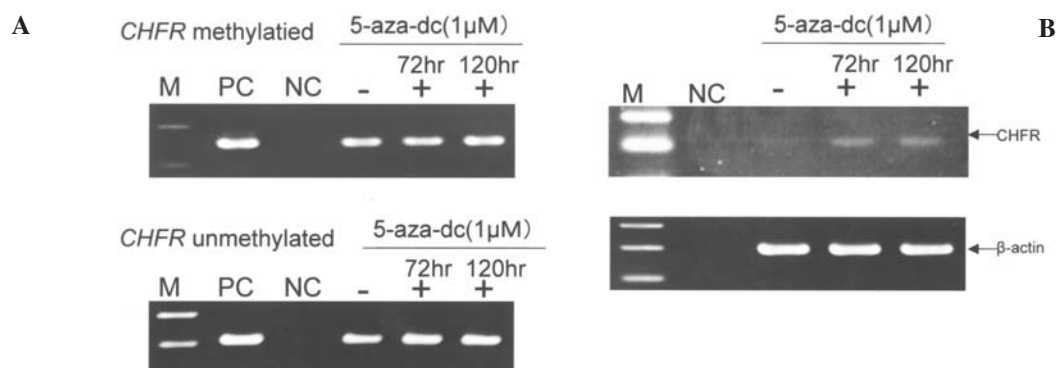


Figure 3. Demethylation analysis of the *CHFR* gene in HeLa cells. (A) MSP analysis after 5-aza-dC treatment. (B) *CHFR* expression recovered 72 h after 5-aza-dC retreatment (RT-PCR).

cancer, including sensitivity to taxanes, is unclear. In this study, aberrant hypermethylation of *CHFR* was observed in

adenocarcinoma cells at a rate of 14.3%, but not in normal cervical cells and squamous cell carcinoma cells. Epigenetic

Table IV. Changes in sensitivity (T/C ratio) of cervical cancer-derived cells to various anticancer agents by treatment with a demethylation agent.

| Cell line | <i>CHFR</i> | 5-FU (%) | | Etoposide (%) | | Cisplatin (%) | | Doxorubicin (%) | | Paclitaxel (%) | | Docetaxel (%) | |
|-----------|-------------|----------|----------|---------------|----------|---------------|----------|-----------------|----------|----------------|----------|---------------|----------|
| | | 5aza (-) | 5aza (+) | 5aza (-) | 5aza (+) | 5aza (-) | 5aza (+) | 5aza (-) | 5aza (+) | 5aza (-) | 5aza (+) | 5aza (-) | 5aza (+) |
| SKG IIIa | U | 88.5 | 83.2 | 76.0 | 84.2 | 94.5 | 80.7 | 90.1 | 98.6 | 69.2 | 87.0 | 63.1 | 88.5 |
| HeLa | M/U | 71.4 | 84.6 | 46.3 | 55.9 | 75.2 | 70.9 | 79.1 | 81.2 | 9.8 | 51.4 | 9.7 | 57.1 |

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

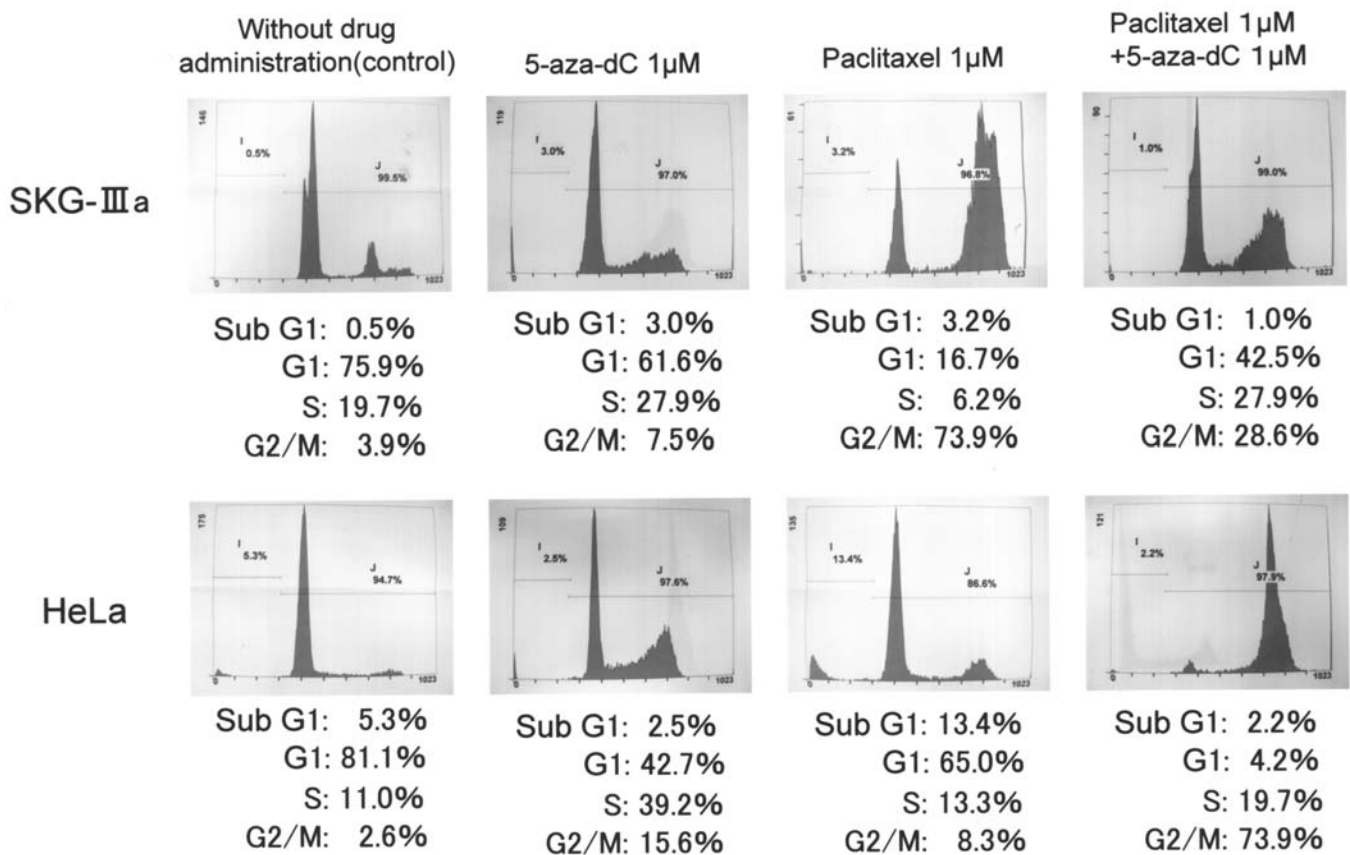
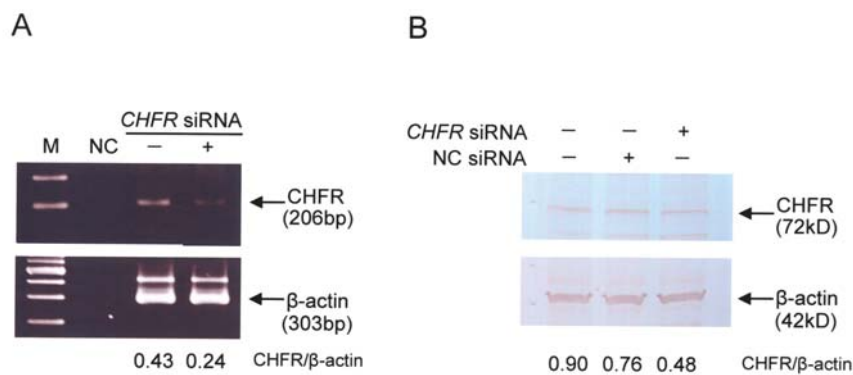


Figure 4. Cell-cycle analysis of SKG-IIIa and HeLa cells using flow cytometry. In SKG-IIIa cells after treatment with paclitaxel alone, the percentage of cells in the G2/M phase was high and that of cells in the Sub-G1 phase did not change markedly. In HeLa cells with paclitaxel alone, the percentage of cells in the G2/M phase was low and that of cells in the Sub-G1 phase increased. In contrast, after treatment with a combination of paclitaxel and 5-aza-dC, cells in the G2/M phase markedly increased and those in the Sub-G1 phase decreased to a level similar to that of the control.

inactivation of *CHFR* has also been observed in endometrial cancer cells, suggesting that aberrant hypermethylation may play an important role in development of uterine cancer, and specifically in adenocarcinoma. There has been a recent increase in cases of cervical cancer, especially in women aged up to 35 years (18-20), and cervical adenocarcinoma has markedly different biological characteristics from squamous cell carcinoma; these characteristics include high nodal metastasis, a refractory nature, poor outcome, and severe malignancy (21-23). The *CHFR* gene negatively regulates the *Aurora-A* gene, a mitotic kinase; hence, suppression of *CHFR* expression increases *Aurora-A* expression (24).

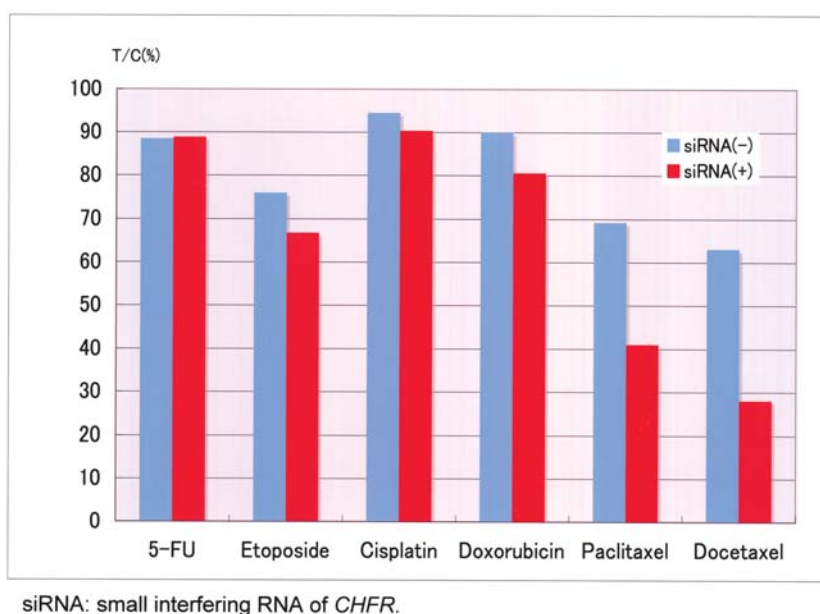
Aurora-A overexpression is reported to induce chromosomal instability (CI) and lead to a poor prognosis in ovarian, breast and bladder cancers (25-27), and a similar mechanism might underlie the characteristics of cervical adenocarcinoma.

Cell-cycle analysis of cervical cancer-derived cells using flow cytometry showed an increase in G2/M cells after paclitaxel treatment in cells with a normal *CHFR* gene. In cells with *CHFR* inactivated epigenetically by aberrant hypermethylation, paclitaxel treatment alone resulted in only a small number of G2/M cells, whereas treatment with a combination of paclitaxel and a demethylation agent caused a marked increase in G2/M cells. These results strongly



M: marker; NC: negative control; siRNA: small interfering RNA.

Figure 5. siRNA-induced suppression of CHFR expression in SKG-IIIa cells. (A) RT-PCR, (B) Western blotting. siRNA suppressed expression of mRNA and protein to approximately 50% of control levels.



siRNA: small interfering RNA of *CHFR*.

Figure 6. siRNA-induced changes in sensitivity (T/C ratio) of SKG-IIIa cells to various anticancer agents. After suppression of *CHFR* expression, the sensitivity to taxanes alone was increased.

support the hypothesis that when tumor cells are treated with taxane, cells with normal *CHFR* expression undergo arrest in G2 phase to repair damaged DNA and are resistant to taxane, whereas cells with an inactivated *CHFR* gene due to aberrant hypermethylation cannot detect DNA damage and proceed to mitosis, thereby showing high sensitivity to taxane. This mechanism was apparent in cells with inactivated *CHFR* genes following paclitaxel treatment, which caused an increase in Sub-G1 cells, rather than G2/M cells, indicating progression to mitosis and subsequent cell death due to the mitotic catastrophe.

In CD-DST analysis of the sensitivity of HeLa cells to anticancer agents, demethylation significantly reduced the sensitivity to taxanes. Treatment with 5-aza-dC is likely to demethylate various genes, in addition to *CHFR*. However, we also confirmed that suppression of *CHFR* in siRNA-transfected SKG-IIIa cells did not alter sensitivity to cisplatin

and doxorubicin, but specifically to taxanes (paclitaxel and docetaxel). This result suggests that epigenetic inactivation of the *CHFR* gene specifically contributed to taxane sensitivity. Therefore, aberrant hypermethylation of *CHFR* may be a molecular marker for prediction of the sensitivity of cervical cancer (and especially cervical adenocarcinoma) to taxane therapy. As discussed above, cervical adenocarcinoma is more refractory and shows a poorer response to anticancer agents compared with squamous carcinoma. Clinical responses of cervical adenocarcinoma are 20% with cisplatin, 14% with 5-fluorouracil, and 12% with etoposide, which are slightly lower than those for squamous carcinoma (28). However, in cervical adenocarcinoma with higher epigenetic inactivation of *CHFR* gene compared to squamous carcinoma, the clinical response to paclitaxel alone is 31%, 17% higher than with any other agent (29), and these findings are consistent with our results.

The CD-DST can be used to test chemosensitivity to anticancer agents using a small number of cells in a three-dimensional culture, and can be analyzed by cultured cells and specimens in the same system. This method has also been reported to show a strong correlation with clinical response in gynecological tumors (14). Therefore, the aberrant hypermethylation of the *CHFR* gene may be useful for a molecular marker for selection of therapy for cervical cancer. Furthermore, transfection of siRNA for *CHFR* increased the sensitivity of cervical squamous carcinoma to taxanes without affecting the sensitivity to other anticancer agents. This approach may be applicable to preoperative chemotherapy for stage Ib and IIb patients, and may offer a new therapeutic strategy for cervical cancer.

Acknowledgments

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