A novel mechanism for acquired cisplatin-resistance: Suppressed translation of death-associated protein kinase mRNA is insensitive to 5-aza-2'-deoxycitidine and trichostatin in cisplatin-resistant cervical squamous cancer cells

TAO BAI[1], TETSUJI TANAKA[1], KAZUNORI YUKAWA[2] and NAOHIKO UMESAKI[1]

Departments of [1]Obstetrics and Gynecology, [2]Physiology, Wakayama Medical University, 811-1 Kimi-idera, Wakayama 641-0012, Japan

Received August 12, 2005; Accepted September 29, 2005

Abstract. The molecular mechanism for cisplatin (CDDP)-resistance of cancer cells has not yet been clarified, despite extensive studies. Here, we investigated whether death-associated protein (DAP) kinase, an apoptosis modulator, was involved in CDDP-resistance by examining the ME180 human cervical squamous cancer cell line and 6 monoclonal ME180-derived CDDP-resistant subclones. Co-treatment with CDDP and 5-aza-2'-deoxycytidine (5-aza-CdR), a demethylating agent, significantly enhanced the CDDP-sensitivities of the parent cells and CDDP-resistant subclones. Subsequent removal of 5-aza-CdR rapidly reversed the CDDP-sensitivity of the CDDP-resistant subclones to their original levels, whereas the parent cells retained the enhanced CDDP-sensitivity for at least 24 h. Quantitative RT-PCR revealed that the CDDP-resistant subclones expressed higher DNA methyltransferase (DNMT) mRNA levels than the parent cells, suggesting that increased DNMT expressions easily restored the CDDP-resistance of the CDDP-resistant subclones following 5-aza-CdR removal. Although the parent cells showed hypermethylation in the DAP kinase promoter region, corresponding methylated bands were not detected in 2 of the 6 CDDP-resistant subclones by methylation-specific PCR. All 6 CDDP-resistant subclones expressed higher DAP kinase mRNA levels than the parent cells, as evaluated by quantitative RT-PCR. Although DAP kinase protein expression was strongly suppressed in the parent cells and CDDP-resistant subclones, 5-aza-CdR treatment of the parent cells dose-dependently stimulated the DAP kinase protein expression, and this was synergistically enhanced by inhibiting histone deacetylation via trichostatin treatment in addition to 5-aza-CdR. However, DAP kinase protein expression in the CDDP-resistant subclones was not stimulated by treatment with 5-aza-CdR and/or trichostatin. These results indicate that post-transcriptional translation of DAP kinase mRNA is strongly suppressed and insensitive to treatment with 5-aza-CdR and trichostatin in the CDDP-resistant subclones established from ME180 human cervical squamous cancer cells. This CDDP-resistance is accompanied by molecular changes that disturb the post-transcriptional translation of the DAP kinase mRNA, and these molecular changes are transiently restored by demethylation.

Correspondence to: Dr Tetsuji Tanaka, Department of Obstetrics and Gynecology, Wakayama Medical University, 811-1 Kimi-idera, Wakayama 641-0012, Japan
E-mail: tetanaka@wakayama-med.ac.jp

Key words: death-associated protein kinase, cisplatin-resistance, methylation, squamous cell carcinoma, cervical cancer

Introduction

Combination chemotherapy remains the predominant treatment approach before and after surgery for advanced malignant tumors although both *de novo* and acquired anticancer drug-resistance limits further treatment clinically. Among the important chemotherapeutic agents, cisplatin (CDDP) is particularly effective and clinically useful against various types of malignant tumor (1,2). The ability of CDDP to become incorporated into DNA, where it forms intra- and inter-strand crosslinks, is generally considered to be one of its main anticancer effects (3,4). However, a variety of observations reported for certain tumor types and cell lines suggest that the mechanism of CDDP-resistance is multifactorial, and involves alterations in drug-uptake and -efflux, defective mismatch repair due to hypermethylation of the human mut-L homologue 1 (hMLH1) gene promoter (5,6), p53 mutations (7,8), elevated cellular glutathione (GSH) levels and a concomitant increase of multidrug resistance protein 2 (MRP2) expression (9). Based on these potential mechanisms, a variety of approaches have been adopted to reverse or overcome CDDP-resistance, including demethylation (6). 5-aza-2'-deoxycytidine (5-aza-CdR), a demethylating agent, has been shown to induce demethylation by inhibiting DNA methyltransferases (DNMTs) and synergistically potentiating the cytotoxicity of CDDP due to DNA topologic changes (10,11). It was reported that treatment of ovarian and colon carcinoma xenografts with 5-aza-CdR and CDDP *in vivo*

could reverse drug-resistance by restoring hMLH1 expression, which is silenced by promoter methylation (6). 5-aza-CdR inhibits DNA methylation by reducing DNMT enzymatic activity via stable complex formation between the enzyme and 5-aza-CdR-substituted DNA (12). In addition to DNA methylation, histone deacetylation is also involved in methylation-induced gene silencing (13). Furthermore, inhibition of histone deacetylation was reported to act synergistically with inhibition of DNA methylation to induce gene expression (14).

Death-associated protein (DAP) kinase is a cytoskeleton-localized Ca^{2+}/calmodulin (CaM)-regulated serine/threonine kinase which modulates the cell death induced by interferon-γ, tumor necrosis factor-ß, Fas, transforming growth factor-ß, the oncogenes c-myc and E2F, ceramide and detachment from the extracellular matrix (15-20). Conversely, several lines of evidence have indicated that DAP kinase functions as an anti-apoptotic factor in cells under normal growth conditions, depending on the apoptotic stimuli (21,22). Hypermethylation of normally unmethylated CpG islands in the promoter regions of many genes, including DAP kinase, during the development of various malignancies results in transcriptional inactivation and loss of protein expression, which serves as an alternative mechanism to genetic alterations (23-28). Our previous study revealed that DAP kinase expression was strongly reduced, possibly via aberrant methylation, in many ovarian and uterine carcinoma cell lines (29). Recently, we established 6 monoclonal CDDP-resistant subclones from a cervical squamous cell carcinoma cell line, ME180 (30), in order to investigate the molecular mechanisms of CDDP-resistance in cervical cancer and squamous cell carcinoma. In the present study, we examined the effects of 5-aza-CdR treatment on DAP kinase expression, DAP kinase promoter methylation status and the CDDP-sensitivity of the CDDP-resistant subclones in order to investigate the potential therapeutic applications of regulating the DAP kinase expression and CDDP-sensitivity of CDDP-resistant cancers.

Materials and methods

Cell lines and culture. The human cervical squamous cell carcinoma cell line, ME180, was purchased from the JCRB Cell Bank (Japan Collection of Research Bioresources Cell Bank, Tokyo, Japan). All cells were cultured in OPTI-MEM (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FCS; Equitech Bio Inc., Ingram, TX, USA), 100 U/ml penicillin (PC), 100 µg/ml streptomycin (SM) and 0.25 µg/ml fungizone (Invitrogen) in 5% CO_2/95% air at 37°C.

Establishment of CDDP-resistant subclones from ME180 cells. To establish CDDP-resistant subclones, ME180 cells were cultured with various concentrations of CDDP (courtesy of Nihon-Kayaku Co. Ltd., Tokyo, Japan) for 3-5 weeks, and the surviving cells were collected. This collection procedure after CDDP exposure was repeated 4 times. Finally, 6 single cell-derived CDDP-resistant subclones, designated CDDPrA, CDDPrB, CDDPrC, CDDPrD, CDDPrE and CDDPrF, were established by the limiting dilution method. The monoclonality of each CDDP-resistant subclone was confirmed by chromosome analysis (data not shown). The establishment of these CDDP-resistant subclones took 1 year.

CDDP-sensitivity assay. Cell viability was assayed using a non-RI colorimetric assay kit (XTT; Boehringer-Mannheim, Mannheim, Germany). The inhibitory effects of CDDP on cell growth were assayed as follows. Cells in the log-phase were detached using 0.25% trypsin/1 mM EDTA (Invitrogen), and cultured overnight in 96-well plates ($5x10^3$ cells/well). On day 2, various concentrations of CDDP were added to the cells. On day 4, the numbers of viable cells were evaluated using the XTT kit and expressed as the percentage of viable cells (%) relative to the mean number of viable unstimulated cells. All experiments were performed 3 times to verify the results. The data are shown as the mean ± SD, and comparative data (n=6) were analyzed by ANOVA.

DNA fragmentation assay. ME180 parent cells and CDDP-resistant subclones in the log phase were detached using 0.25% trypsin/1 mM EDTA, and then cultured overnight in culture dishes ($3x10^6$ cells/dish) containing OPTI-MEM/5% FCS/PC/SM. On day 2, CDDP (final concentrations: 4 and 20 µg/ml) was added to the cells. On day 4, genomic DNA was extracted from all cells, including the dead ones, using a SepaGene DNA extraction kit (Sankyo-Junyaku Co. Ltd., Tokyo, Japan) and treated with 100 µg/ml of RNase A (Sigma, St. Louis, MO, USA) in TE buffer (10 mM Tris, pH 8.0, 2 mM EDTA) for 90 min at 37°C to remove any contaminating RNA. Then, approximately 20 µg of the genomic DNA isolated from $5x10^5$ cells was electrophoresed in a 1.4% agarose gel at 50 V for approximately 2 h, stained with 5 µg/ml of ethidium bromide and visualized by UV fluorescence.

Effects of the methyltransferase inhibitor, 5-aza-CdR, on the CDDP-sensitivity of the parent cells and CDDP-resistant subclones. Approximately $5x10^3$ ME180 parent cells or ME180-derived CDDP-resistant subclones were seeded in 0.1 ml OPTI-MEM in 96-well culture plates and incubated for 24 h. The medium was then replaced with medium containing the designated concentrations of CDDP with or without 1 µM 5-aza-CdR (Sigma) and the cells were incubated for a further 48 h. Then, the viable cell numbers were determined using Cell Counting Kit (Dojindo Chemical Laboratory Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a 96-well plate reader (Dainihon-Seiyaku Co., Osaka Japan). The absorbance obtained from control cells without drug administration was set as 100% viability. All experiments were performed 3 times to verify the results. The data are shown as the mean ± SD, and comparative data (n=4) were analyzed by ANOVA.

Effects of 5-aza-CdR pretreatment on the CDDP-sensitivity of the parent cells and CDDP-resistant subclones. To examine the effects of sequential administration of 5-aza-CdR and CDDP, $5x10^3$ ME180 parent cells or ME180-derived CDDP-resistant subclones were plated in 0.1 ml OPTI-MEM in 96-well culture plates and incubated for 24 h. Then, the cells were exposed to 1 µM 5-aza-CdR or left untreated for a further 24 h, followed by treatment with the indicated

Table I. Primer sequences for DAPK-MS-PCR analysis.

PCR analysis	Primer sequence	Amplicon size (bp)	Refs.
Stage I PCR	Forward 5'-GGTTGTTTCGGAGTGTGAGGAG-3' Reverse 5'-GCTATCGAAAACCGACCATAAAC-3'	209	(33)
Stage II PCR			
Unmethylation	Forward 5'-GGAGGATAGTTGGATTGAGTTAATGTT-3' Reverse 5'-CAAATCCCTCCCAAACACCAA-3'	106	(33)
Methylation	Forward 5'-ATAGTCGGATCGAGTTAACGTC-3' Reverse 5'-AAAACTAACCGAAACGACGACG-3'	153	(33)

concentrations of CDDP for 48 h. Finally, the viable cell numbers were determined as described above. All experiments were performed 3 times to verify the results. The data are shown as the mean ± SD, and comparative data (n=4) were analyzed by ANOVA.

Western blot analysis of DAP kinase protein expression. The ME180 parent cells and CDDP-resistant subclones were incubated with 1 μM 5-aza-CdR for 96 h and/or with 300 nM trichostatin (TSA), a histone deacetylase inhibitor (Wako-Junyaku Co. Ltd., Tokyo, Japan), for 48 h, after which they were harvested and lysed with 0.3 ml lysis buffer (Sigma). For dose-dependency experiments, ME180 parent cells were treated with various concentrations of 5-aza-CdR (0.5-10 μM) for 96 h and then lysed as described above. The protein contents of the cell lysates were quantified using a Coomassie Plus Protein assay (Pierce Biotechnology Inc., Rockford, IL, USA) and aliquots (25 μg total protein) were dissolved in Laemmli SDS-PAGE sample buffer prior to separation by 7.5% SDS-PAGE. The separated proteins were transferred to a polyvinylidene fluoride membrane (ATTO Corp., Tokyo, Japan) by using a wet transfer method. The membrane was blocked with 5% skim milk for 1 h at room temperature and subsequently incubated with a mouse monoclonal anti-human DAP kinase antibody (clone 55, 1:5000 dilution; Sigma) for 1 h at room temperature. After washing with TBS-T (20 mM Tris, pH 7.6, 0.3 M NaCl, 0.1% Tween-20), the membrane was incubated with a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Sigma) for 1 h at room temperature. The bound antibodies were detected using an ECL plus kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and the membrane was scanned using a Luminocapture (type AE6955; ATTO Corp.).

Genomic DNA extraction and bisulfite modification for DAP kinase methylation-specific polymerase chain reaction (DAPK-MS-PCR). Genomic DNA was isolated from cultured cells using a SepaGene kit (Sanko-Junyaku Ltd.) according to the manufacturer's instructions. The DNA concentrations were calculated from the UV absorptions at 260 and 280 nm. Genomic DNA was modified by chemical treatment with sodium bisulfite (Sigma) as described previously (31,32). In this reaction, all unmethylated cytosines were converted to uracils, while methylated cytosines remained unaltered. Briefly, 2 μg/50 μl of DNA was denatured by adding freshly prepared sodium hydroxide (final concentration: 0.3 M) and incubating the mixture for 20 min at 37°C. Then, 30 μl of freshly prepared 10 mM hydroquinine (Sigma) and 520 μl of 3 M sodium bisulfite (pH 5.0) were added to the DNA solutions, mixed and incubated for 16 h at 55°C. The DNA samples were desalted using a Wizard DNA Clean-Up System (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol, redissolved in 50 μl of autoclaved distilled water, desulfonated with 0.3 M NaOH for 20 min at 37°C and precipitated with ethanol. The bisulfite-modified genomic DNA was resuspended in 20 μl of autoclaved distilled water and either used immediately or stored at -70°C.

DAP kinase-methylation-specific polymerase chain reaction (DAPK-MS-PCR). DAPK-MS-PCR was performed as described previously (33). Briefly, the bisulfite-modified DNA was used as a template for stage I PCR amplification to generate a 209-bp fragment of the DAP kinase gene that included a portion of its CpG-rich promoter region. The stage I PCR primers recognized the modified DNA but could not discriminate between methylated and unmethylated alleles. Stage I PCR amplification was carried out as follows: 95°C for 15 min; 35 cycles of 94°C for 1 min for denaturation, 58°C for 150 sec for annealing and 72°C for 150 sec for extension; followed by a final extension at 72°C for 10 min. The stage I PCR products were diluted 50-fold, and 5 μl was subjected to stage II PCR amplification using primers specifically designed for methylated or unmethylated DNA in the promoter region of the DAP kinase gene. The primers used for stage I and II PCR amplification are summarized in Table I. For stage II PCR, the annealing temperature was increased to 65°C and the annealing time was reduced to 90 sec for 40 cycles. Stage II PCR amplified 153-bp and 106-bp products from methylated and unmethylated DAP kinase genes, respectively. Finally, these PCR products were electrophoresed in a 2.0% agarose gel at 100 V for approximately 30-40 min, and visualized by staining with 5 μg/ml ethidium bromide.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR of DAP kinase and DNMT genes. Total RNA was isolated from cultured cells using

Table II. Primer sequences for RT-PCR and quantitative real-time RT-PCR analysis.

Gene	Primer sequence	Amplicon size (bp)	Refs.
DAP kinase	Forward 5'-TGGATCCACCAGCAAAGCAC-3' Reverse 5'-GTGTTGGTTAGTGAGGTTTC-3'	350	(34)
DNMT-1	Forward 5'-GTTCTTCCTCCTGGAGAATGTCA-3' Reverse 5'-GGGCCACGCCGTACTG-3'	138	(35)
DNMT-3A	Forward 5'-CCTGTGGGAGCCTCAATGTTA-3' Reverse 5'-TTCTTGCAGTTTTGGCACATTC-3'	72	(35)
DNMT-3B	Forward 5'-GACTCGAAGACGCACAGCTG-3' Reverse 5'-CTCGGTCTTTGCCGTTGTTATAG-3'	97	(35)
ß-actin	Forward 5'-ATTGCCGACAGGATGCAGAA-3' Reverse 5'-GCTGATCCACATCTGGTGGAA-3'	150	Original

TRIzol reagent (Invitrogen) for RT-PCR and an RNeasy mini kit (Qiagen Inc., Valencia, CA, USA) for real-time RT-PCR. Aliquots containing 1 μg of total RNA were pretreated with DNase I (Invitrogen) and then used for cDNA synthesis with a reverse transcriptase kit (Bio-Rad, Hercules, CA, USA) in a reaction volume of 20 μl. Each cDNA product was diluted to 100 μl. The PCR reaction mixture (25 μl) contained 5 μl of diluted cDNA, 0.125 μl Hotstart polymerase (Qiagen Inc.), 0.2 mM dNTP, 1xQ solution and 0.5 μM primers. The primers used for DAP kinase, DNMT 1, DNMT 3A and DNMT 3B are summarized in Table II. ß-actin was used as a positive control for the mRNA amount. For RT-PCR, an initial hot start at 95°C for 15 min was followed by 35 amplification cycles (30 sec at 94°C, 30 sec at the annealing temperature and 60 sec at 72°C). The annealing temperatures were 55°C for DAP kinase and 60°C for DNMT 1, DNMT 3A and DNMT 3B. The PCR products were electrophoresed in a 1.5-2.0% agarose gel at 100 V for approximately 30-40 min and visualized by staining with 5 μg/ml ethidium bromide. Quantitative real-time RT-PCR was performed using an iCycler (Bio-Rad). The PCR reaction mixture (25 μl) contained 2.5 μl of diluted cDNA, 12.5 μl of iQ™ SYBR-Green Supermix (Bio-Rad) and 0.5 μM of the above-described primers. An initial hot start at 95°C for 3 min was followed by 40 cycles of amplification (95°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec). The relative values of DAP kinase mRNA in the ME180 parent cells and CDDP-resistant subclones were calculated based on the $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\ CDDP\text{-}resistant\ subclones} - \Delta C_{T\ ME180\ parent\ cells} = (C_{T\ DAP\ kinase} - C_{T\ actin})_{CDDP\text{-}resistant\ subclones} - (C_{T\ DAP\ kinase} - C_{T\ actin})_{ME180\ parent\ cells}$.

Results

Establishment of 6 monoclonal CDDP-resistant subclones from the ME180 human cervical squamous cell carcinoma cell line. To investigate the mechanisms involved in anticancer drug-resistance, we established 6 independent monoclonal CDDP-resistant subclones from the ME180 cervical squamous [illegible] using the limiting dilution method. The CDDP-sensitivities of these subclones were determined using XTT assays. As shown in Fig. 1, all 6 CDDP-resistant subclones showed significantly lower CDDP-sensitivities than the parent cells. To confirm the resistance of the subclones against CDDP-induced apoptosis, we carried out DNA fragmentation assays. The parent cells and CDDP-resistant subclones were exposed to two different concentrations of CDDP (4 and 20 μg/ml), based on the XTT assays shown in Fig. 1. After incubation with CDDP for 48 h, DNA was extracted and chromatin fragmentation was examined by agarose gel electrophoresis. Typical DNA ladder patterns were detected in the parent cells treated with both 4 and 20 μg/ml CDDP, whereas DNA ladders were hardly seen in any CDDP-resistant subclones examined after culture with 4 μg/ml of CDDP (Fig. 2).

Effects of demethylation on the CDDP-sensitivity of the CDDP-resistant subclones. To address whether the acquired CDDP-resistance of the 6 subclones was caused by aberrant methylation of gene promoters, we analyzed the effects of demethylation on the CDDP-sensitivity of the CDDP-resistant subclones (Fig. 3). Preliminary culture experiments with 5-aza-CdR (0-20 μM) treatment showed no growth-inhibitory effects on the ME180 parent cells (data not shown). Next, we treated the parent cells and CDDP-resistant subclones with various concentrations of CDDP and 10 μM 5-aza-CdR for 48 h to examine the effects of concurrent treatment with 5-aza-CdR and CDDP on the CDDP-sensitivity. As shown in Fig. 3, parent cells co-treated with CDDP and 5-aza-CdR showed significantly increased CDDP-sensitivity compared to parent cells treated with CDDP alone. Among the 6 CDDP-resistant subclones, 3 subclones (B, D and F) showed a much higher CDDP-sensitivity, while the other 3 subclones (A, C and E) showed a slightly, but significantly, higher CDDP-sensitivity when co-treated with CDDP and 5-aza-CdR compared with the same subclones treated with CDDP alone. To further elucidate the reversibility of the CDDP-sensitivity after demethylation in CDDP-resistant malignancies, we investigated the persistence of the demethylation-mediated sensitization

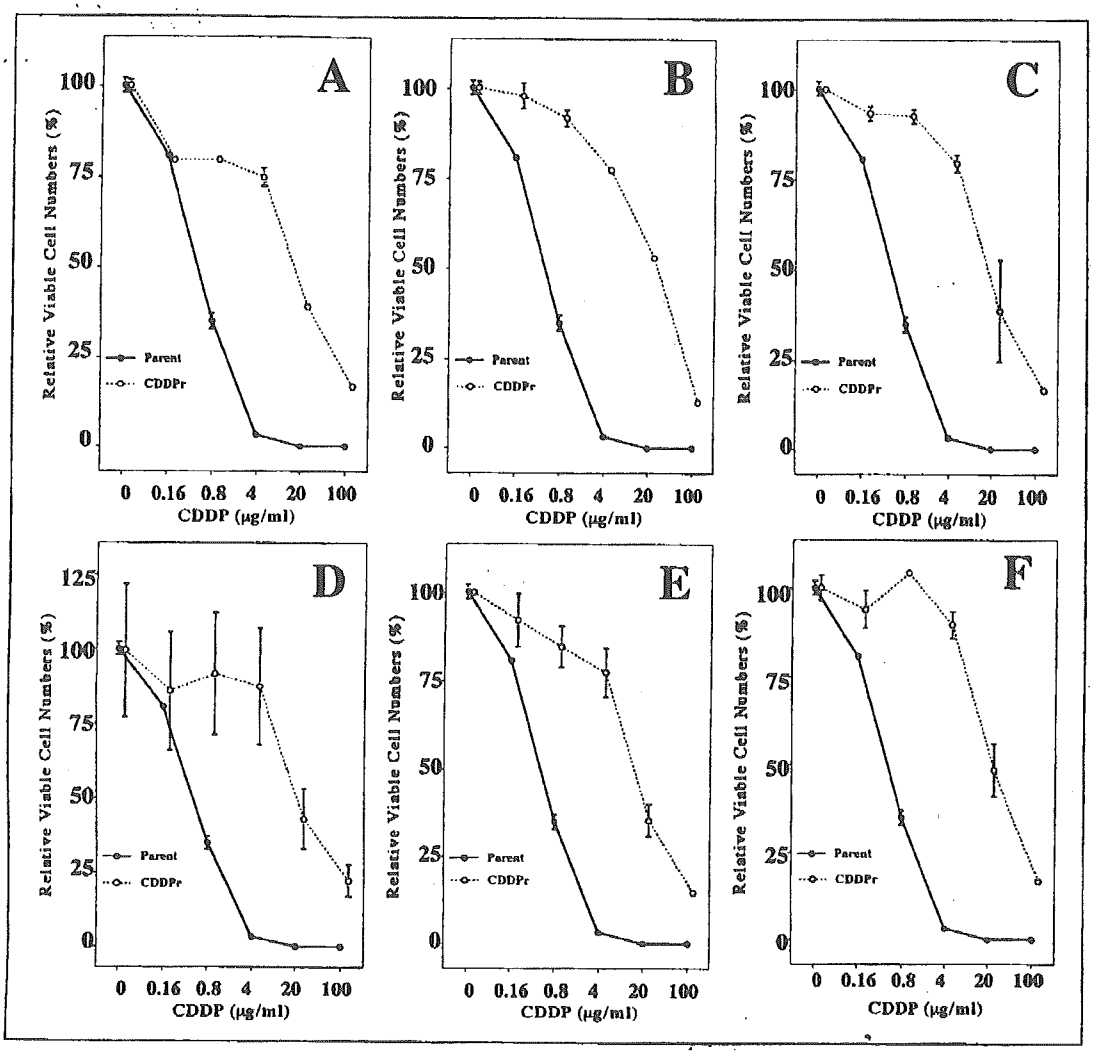

Figure 1. CDDP sensitivities of the CDDP-resistant subclones established from ME180 cells. Six monoclonal subclones (CDDPrA, B, C, D, E and F) were established. The solid lines with closed circles are CDDP-sensitivity curves of the parent cells and the dotted lines with open circles are CDDP-sensitivity curves of the CDDP-resistant subclones. All the subclones are clearly more resistant to CDDP-induced inhibition of cell growth than the parent cells.

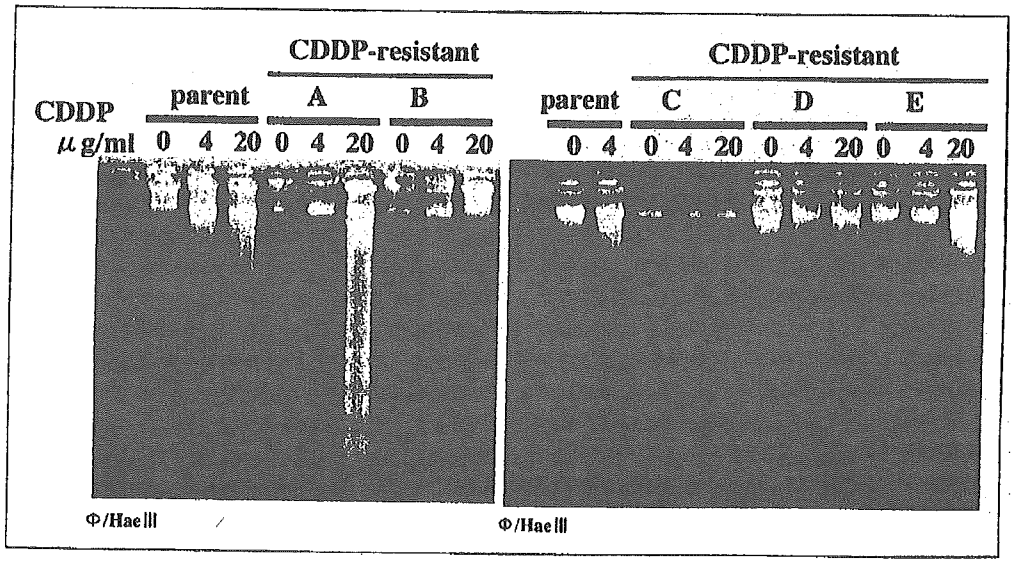

Figure 2. DNA fragmentation assay of the CDDP-resistant subclones. CDDP-induced DNA fragmentation is inhibited in the CDDP-resistant subclones (CDDPrA, B, C, D and E).

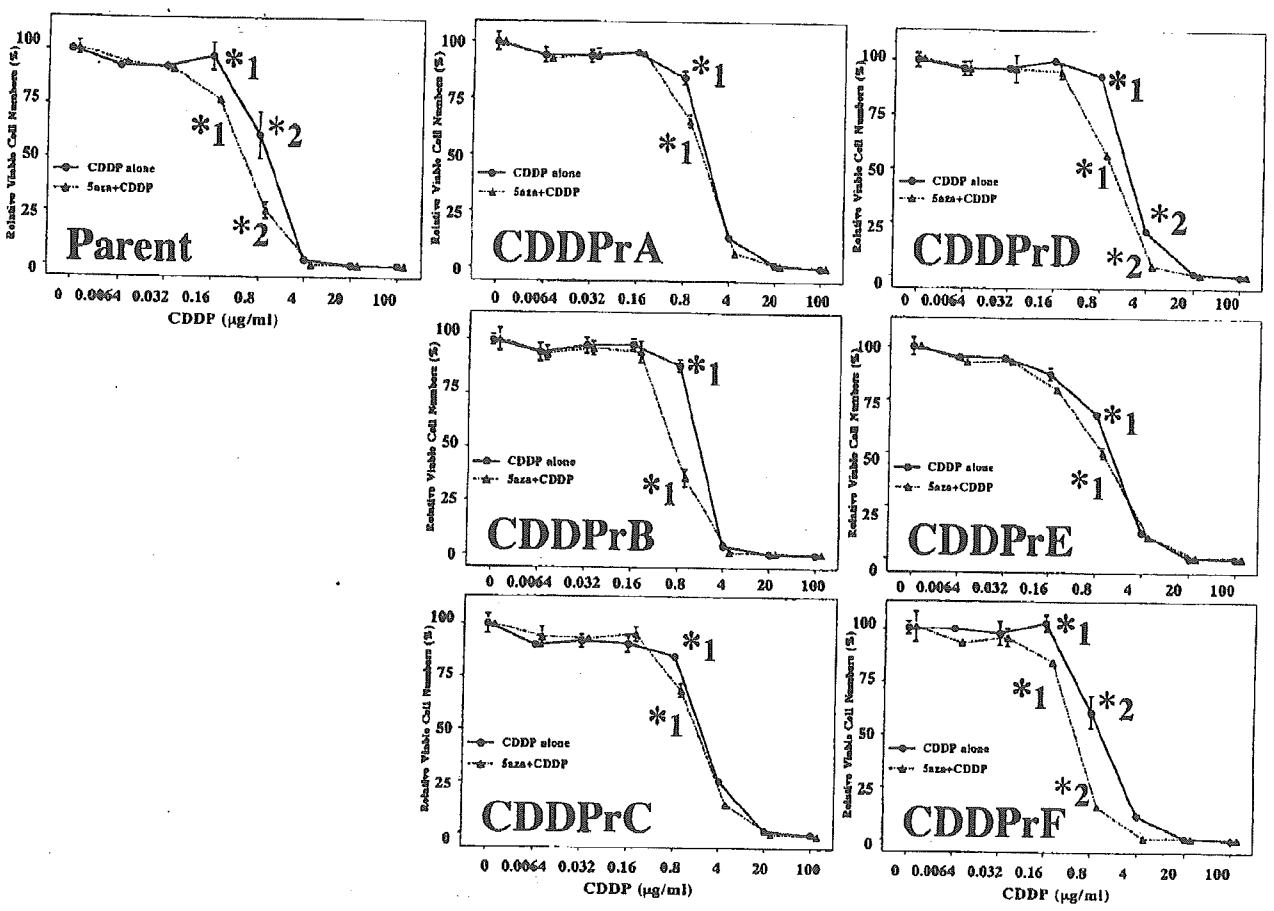

Figure 3. Effects of 5-aza-CdR treatment on the CDDP-sensitivities of the parent cells and CDDP-resistant subclones. The cells were concurrently treated with CDDP and 5-aza-CdR. The solid lines with closed circles are CDDP-sensitivity curves of cells without 5-aza-CdR treatment and the dotted lines with closed triangles are CDDP-sensitivity curves of cells treated with 5-aza-CdR. All the cells treated with 5-aza-CdR and CDDP show significantly higher CDDP-sensitivities than cells treated with CDDP alone. *$p<0.05$.

to CDDP in the parent cells and CDDP-resistant subclones for 24 h after 5-aza-CdR-pretreatment (Fig. 4). The results showed that the parent ME180 cells retained the CDDP-sensitivity restored by the 5-aza-CdR treatment after its removal, whereas all the CDDP-resistant subclones lost their restored CDDP-sensitivity.

Increased expression of DNMT genes in CDDP-resistant subclones. Since the CDDP-resistant subclones failed to maintain their restored CDDP-sensitivity, we speculated that the status of the DNMTs in the CDDP-resistant subclones may be altered during exposure to CDDP. To clarify whether DNMT expression was up-regulated in CDDP-resistant subclones compared with parent ME180 cells, we assessed the transcriptional expression of DNMTs by RT-PCR and quantitative real-time RT-PCR. As shown in Fig. 5A, RT-PCR readily detected the expression of DNMT 1, DNMT 3A and DNMT 3B in parent ME180 cells and all CDDP-resistant subclones. Moreover, quantitative real-time RT-PCR revealed that 3 CDDP-resistant subclones (A, C and E) showed significantly higher DNMT 3B mRNA expression and slightly higher DNMT 1 mRNA expression than the parent cells. As shown in Fig. 3, these 3 subclones showed smaller increases in their cellular CDDP-sensitivity than the other subclones upon co-treatment with 5-aza-CdR and CDDP, suggesting that the increased DNMT 3B and DNMT 1 levels could inhibit the effects of 5-aza-CdR in these 3 subclones.

The methylation status of the DAP kinase promoter in CDDP-resistant subclones. Several lines of evidence have shown that the hypermethylation of gene promoters is involved in drug-resistance (5,31,32). Previously, we reported that abnormal methylation of the DAP kinase gene promoter occurred and the level of DAP kinase protein was markedly diminished in the ME180 cell line (29). In view of the ability of DAP kinase to modulate apoptosis positively or negatively, depending on the cell type and apoptotic stimuli, we hypothesized that DAP kinase may be involved in anticancer drug-induced apoptosis and regulate the drug-sensitivity of malignant cells. Since the parent cells and CDDP-resistant subclones all showed increased CDDP-sensitivity upon co-treatment with CDDP and 5-aza-CdR, it is likely that the CpG island in the DAP kinase gene promoter is hypermethylated in CDDP-resistant subclones. To address this possibility, we examined the methylation status of the DAP kinase gene promoter using DAPK-MS-PCR (Fig. 6). In 2 of the 6 CDDP-resistant subclones (C and E), the band indicating hypermethylation of the DAP kinase gene promoter was not detected, while the others showed aberrant methylation.

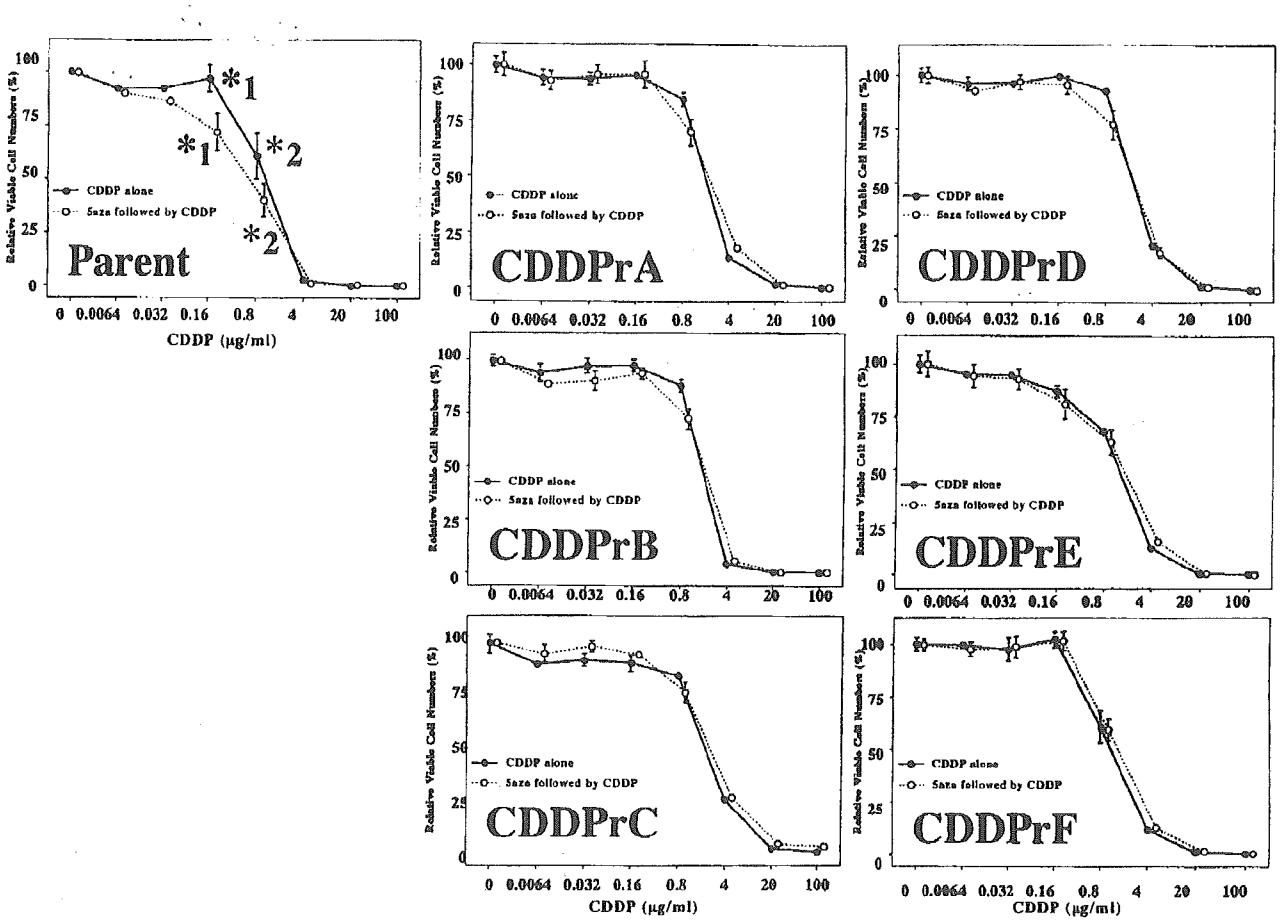

Figure 4. Effects of 5-aza-CdR removal on the CDDP-sensitivity of parent cells and CDDP-resistant subclones. The cells were stimulated with CDDP alone after pretreatment with 5-aza-CdR. The solid lines with closed circles are CDDP-sensitivity curves of cells treated with CDDP alone and the dotted lines with open circles are CDDP-sensitivity curves of cells treated with CDDP after 5-aza-CdR pretreatment. Although the parent cells exhibit a significantly higher CDDP-sensitivity after pretreatment with 5aza-CdR, all 6 CDDP-resistant subclones show no apparent changes in their CDDP-sensitivity with and without 5-aza-CdR pretreatment. *p<0.05.

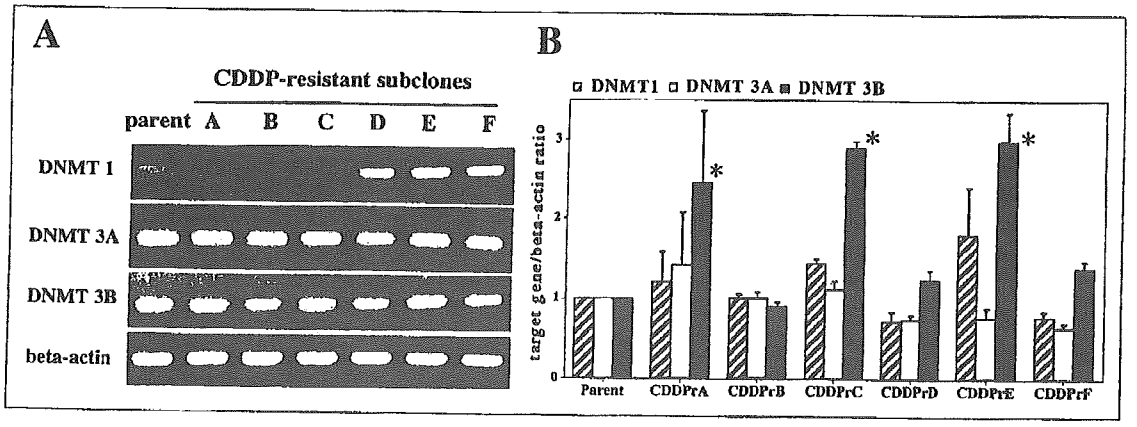

Figure 5. RT-PCR and quantitative real-time RT-PCR analyses of DNA methyltransferase mRNAs in CDDP-resistant subclones. A, RT-PCR analyses of DNA methyltransferase genes in CDDP-resistant subclones. Both the parent cells and CDDP-subclones express the 3 DNA methyltransferase mRNAs examined. B, Quantitative real-time RT-PCR analyses of 3 DNA methyltransferase genes: DNMT 1, DNMT 3A and DNMT 3B. *DNMT 3B mRNA expression is more than 2-fold higher in 3 CDDP-resistant subclones (CDDPrA, CDDPrC and CDDPrE) than in the parent cells. DNMT 1 mRNA expression is also slightly increased in these subclones, albeit to lesser extents.

The expression of DAP kinase protein in parent cells and CDDP-resistant subclones before and after treatment with 5-aza-CdR and/or TSA. Next, we examined the DAP kinase protein expression in parent cells and CDDP-resistant subclones by Western blot analysis. First, we investigated whether the DAP kinase protein expression in ME180 parent

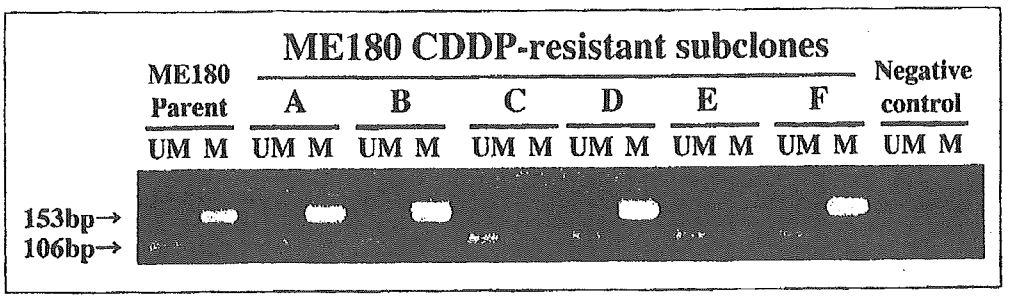

Figure 6. MS-DAPK-PCR analyses of CDDP-resistant subclones. Methylation-specific PCR of the DAP kinase gene was performed in ME180 parent cells and CDDP-resistant subclones. The parent cells have both the methylated and unmethylated bands. Two CDDP-resistant subclones (C and E) do not contain the methylated band. U, unmethylated; M, methylated.

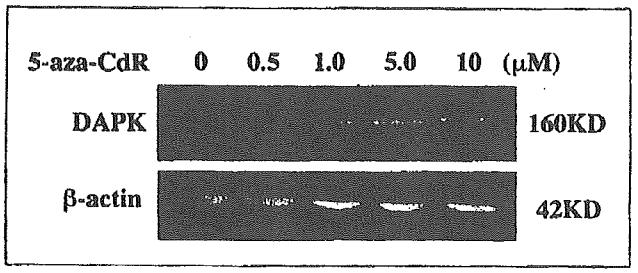

Figure 7. Effects of 5-aza-CdR treatment on DAP kinase protein expression in parent cells. The DAP kinase protein expression in parent ME180 cells is induced by 5-aza-CdR treatment in a dose-dependent manner.

cells was regulated by 5-aza-CdR treatment. In the parent cells, DAP kinase protein expression was remarkably suppressed. However, after treatment with various concentrations of 5-aza-CdR (0.5-10 μM) for 96 h, the parent cells showed increased DAP kinase protein expression in a dose-dependent manner (Fig. 7).

Secondly, we examined the DAP kinase protein expression in parent cells and CDDP-resistant subclones before and after treatment with 5-aza-CdR and/or TSA. Similar to the case for the parent ME180 cells, 5 of the 6 CDDP-resistant subclones (A, B, C, D and F) showed slightly detectable DAP kinase protein expression (Fig. 8). Next, we treated the parent cells and CDDP-resistant subclones with 1 μM 5-aza-CdR and/or 300 nM TSA, a histone deacetylase inhibitor (Fig. 8). In the parent cells, 5-aza-CdR treatment alone restored the expression of DAP kinase, and combined treatment of 5-aza-CdR and TSA caused a synergistic increase in DAP kinase protein expression. However, treatment of the parent cells with TSA alone induced little increase in the DAP kinase protein expression. In contrast to the parent cells, no distinguishable changes in DAP kinase protein expression were detected in any of the 6 CDDP-resistant subclones after treatment with 5-aza-CdR and/or TSA. These results demonstrated that the induction of DAP kinase protein expression was not responsible for the restoration of CDDP-sensitivity by 5-aza-CdR treatment shown in Figs. 3 and 4, since DAP kinase protein expression was not enhanced by 5-aza-CdR treatment in CDDP-resistant subclones whose CDDP-sensitivity was restored by 5-aza-CdR.

Transcription of the DAP kinase gene in parent cells and CDDP-resistant subclones. Although hypermethylation of the DAP kinase promoter was not detected in 2 of the 6 CDDP-resistant subclones by DAPK-MS-PCR, all 6 CDDP-resistant subclones failed to recover DAP kinase protein expression in response to demethylation after 5-aza-CdR treatment, suggesting that defective DAP kinase gene transcription may occur in CDDP-resistant subclones. Therefore, RT-PCR analysis was performed to estimate the DAP kinase gene transcription in parent cells and CDDP-resistant subclones. As shown in Fig. 9A, all of the cells tested showed readily detectable DAP kinase mRNA expression by RT-PCR. Next, quantitative real-time RT-PCR was carried out to investigate the mRNA expression levels in the parent cells and CDDP-resistant subclones. As shown in Fig. 9B, all 6 CDDP-resistant subclones expressed significantly higher levels of DAP kinase mRNA than the parent cells. These results indicated that impaired translation of the DAP kinase mRNA, rather than transcriptional repression of the DAP kinase gene, strongly reduced the DAP kinase protein expression in CDDP-resistant subclones.

Discussion

The epigenetic hypermethylation of gene promoters plays an important role in tumorigenesis as an alternative mechanism of genetic changes (36). The pattern of hypermethylation can be divided into age- or cancer-specific methylation (37,38). On the other hand, CDDP-resistance is a multifactorial condition, which involves decreased drug-uptake, increased drug-efflux and increased intracellular GSH (39) as well as genetic and epigenetic alterations, such as p53 mutations and hMLH1 hypermethylation (7). Furthermore, CDDP-resistance has also been reported to be associated with reciprocal EGF receptor and p21 expression in ME180 cells (40). Here, we focused on DAP kinase, an apoptosis modulator that has been reported to show abnormal methylation in many tumor types, including cervical cancer (25,41). We made several important observations regarding the relationship between DAP kinase expression and CDDP-resistance in the ME180 cervical squamous cell carcinoma cell line. To the best of our knowledge, this study represents the first report to investigate the relationships between CDDP-resistance and the regulatory system of DAP kinase expression. To evaluate the differing

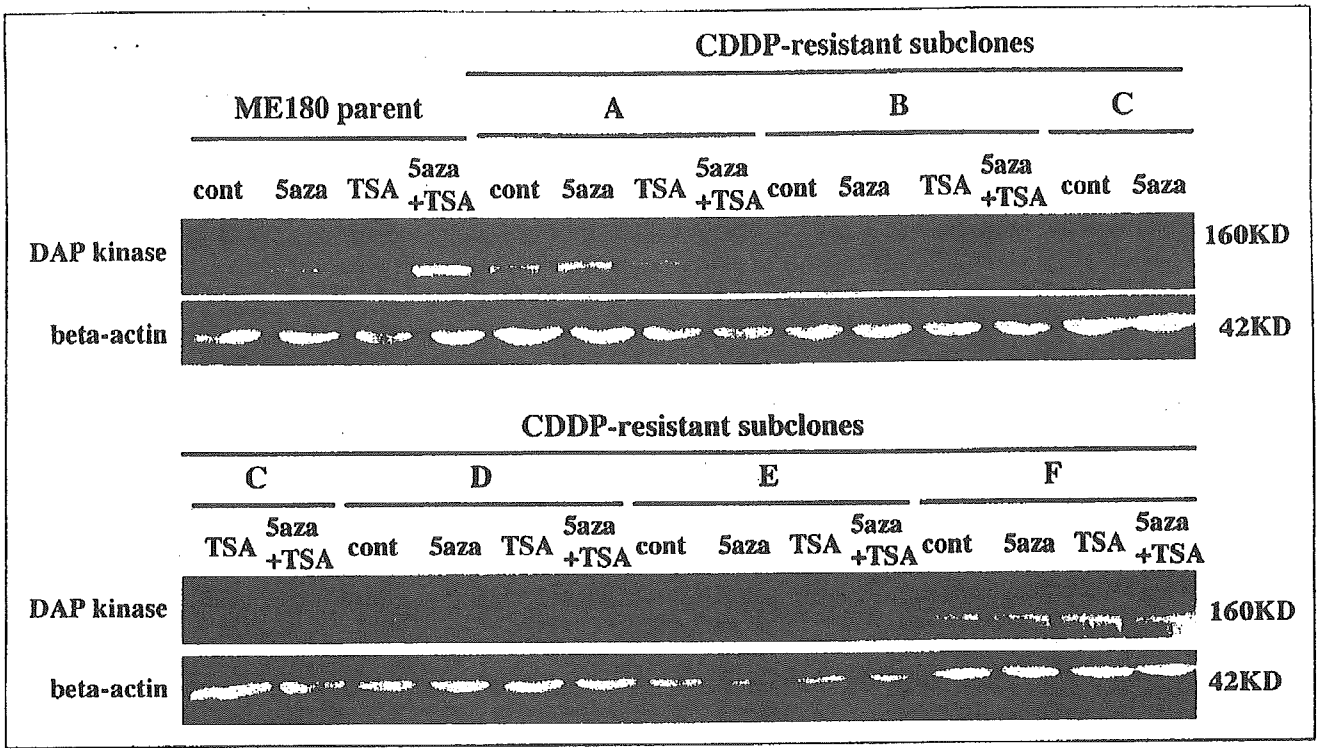

Figure 8. Effects of stimulation with 5-aza-CdR and/or TSA on DAP kinase protein expression in parent cells and CDDP-resistant subclones. Treatment with 5-aza-CdR clearly induces DAP kinase protein expression in parent cells. TSA synergistically enhances the DAP kinase protein expression induced by 5-aza-CdR in the parent cells. None of the CDDP-resistant subclones show induction of DAP kinase protein expression following treatment with 5-aza-CdR and/or TSA.

responsiveness to CDDP, we established 6 monoclonal CDDP-resistant subclones from ME180 cells, and then examined the effects of CDDP in combination with the demethylating agent, 5-aza-CdR, or the sequential treatment with 5-aza-CdR and CDDP of parent cells and CDDP-resistant subclones. The CDDP-sensitivity of the parent cells and CDDP-resistant subclones was significantly enhanced by 5-aza-CdR treatment, consistent with the previous finding that the cytotoxicity of a combination of CDDP and 5-aza-CdR against several tumor cell lines was synergistic (10). Based on the pattern of enhanced sensitivity to CDDP exposure after 5-aza-CdR treatment, the CDDP-resistant subclones were divided into two groups: one consisting of subclones B, D and F, which showed a relatively high responsiveness to 5-aza-CdR treatment; and the other consisting of subclones A, C and E, with a comparatively low responsiveness. We speculated that differences in the DNMT levels among these CDDP-resistant subclones may be responsible for the differing responsiveness to 5-aza-CdR between the 2 groups. DNMT 3A and DNMT 3B are thought to be the major *de novo* methylases that affect the methylation status of normally unmethylated CpG sites (42-44). Furthermore, *in vitro* methylation assays have shown that DNMT 3A and DNMT 3B cooperate with DNMT 1 to extend methylation within the Micrococcus luteus genome (45). Therefore, it seems likely that the lower CDDP-sensitivities of subclones A, C and E compared with the other subclones upon co-treatment with 5-aza-CdR and CDDP is partly attributable to the enhanced expression of DNMT 1 and DNMT 3B. Collectively, these observations suggest the

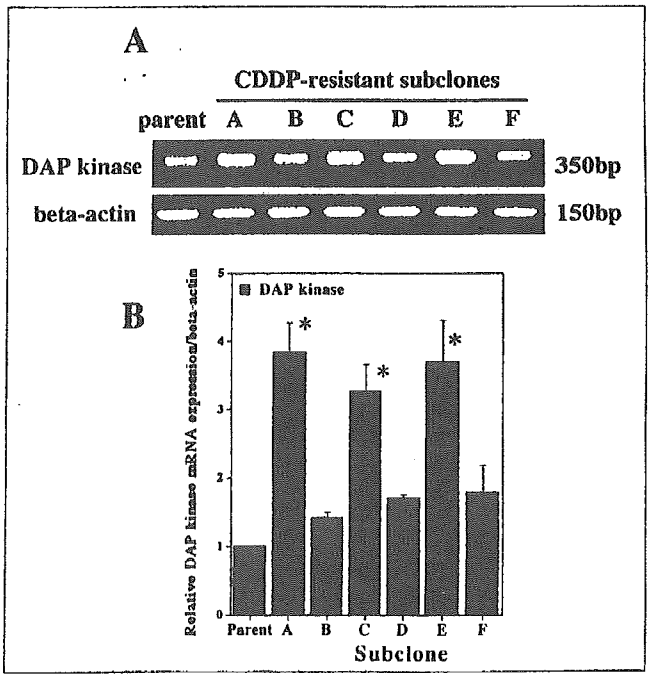

Figure 9. RT-PCR and quantitative real-time RT-PCR analyses of DAP kinase mRNA in CDDP-resistant subclones. A, RT-PCR analysis of CDDP-resistant subclones. B, Quantitative real-time RT-PCR analysis of DAP kinase mRNA. All of the CDDP-resistant subclones express higher DAP kinase mRNA levels than the parent cells. *DAP kinase mRNA expression is more than 2-fold higher in 3 CDDP-resistant subclones (CDDPrA, CDDPrC and CDDPrE) than in the parent cells.

possible application of a demethylating treatment, such as 5-aza-CdR, to cancer therapy, which could overcome acquired CDDP-resistance. In the present study, however, the CDDP-susceptibilities of the CDDP-resistant subclones rapidly returned to their original CDDP-sensitivity levels after the removal of 5-aza-CdR. Therefore, in order to overcome CDDP-resistance, long-acting demethylating drugs combined with CDDP may be essential for therapeutic use.

We also examined whether the CDDP-sensitivity of the parent cells and CDDP-resistant subclones correlated with the methylation status of the DAP kinase gene. Our previous study demonstrated that DAP kinase protein expression was strongly suppressed in ME180 cells, possibly due to aberrant methylation of the DAP kinase gene promoter (29). Thus, we examined the methylation status of the DAP kinase gene in CDDP-resistant subclones. We observed that the CDDP-resistant subclones showed little DAP kinase protein expression, similar to the ME180 parent cells. Interestingly, DAPK-MS-PCR was unable to detect a hypermethylated band in 2 of the 6 CDDP-resistant subclones (C and E), while hypermethylation of the DAP kinase promoter was easily detected in the other 4 CDDP-resistant subclones (A, B, D and F). A possible explanation is that our DAPK-MS-PCR is not sufficiently sensitive to identify the hypermethylation status of all of the targeted genes, and that undetected methylation sites are located in the DAP kinase promoter region in the 2 CDDP-resistant subclones (C and E). Further sequencing analyses of the full-length promoter region of the DAP kinase gene are necessary to verify this possibility. Alternatively, it is possible that these CDDP-resistant subclones isolated independently from the ME180 cell line may have diverged and acquired certain alterations during the selection cultures with CDDP. In addition, we noted that, even in the parent ME180 cells and 4 methylation-positive CDDP-resistant subclones, the methylated bands were always accompanied by unmethylated bands, suggesting that the 2 methylation-negative CDDP-resistant subclones expressing normal unmethylated DAP kinase promoters may originate from ME180 clones that possessed the unmethylated allele of the DAP kinase gene. Quantitative RT-PCR revealed that all of the CDDP-resistant subclones showed higher DAP kinase mRNA expression levels than the parent cells. In particular, much higher DAP kinase mRNA expression levels were found in the 3 subclones (A, C and E) whose CDDP-sensitivity was less affected by 5-aza-CdR than in the other 3 subclones (B, D and F). Since the 2 CDDP-resistant subclones without methylated bands in MS-DAPK-PCR (C and E) expressed much higher DAP kinase mRNA levels than the parent cells, DAP kinase mRNA transcription is partly regulated by methylation of the promoter region even in the CDDP-resistant subclones.

We then analyzed whether hypermethylation of the DAP kinase gene was sufficient to silence DAP kinase gene transcription and whether 5-aza-CdR-mediated demethylation affected the methylation status in the parent cells and CDDP-resistant subclones. Unexpectedly, we noted that the reduced DAP kinase protein expression in ME180 cells did not result from silenced transcription of the DAP kinase gene by hypermethylation of the DAP kinase gene promoter CpG island. DAP kinase mRNA expression was easily detected in the parent cells and all of the CDDP-resistant subclones. Thus, the post-transcriptional translation of DAP kinase mRNA was strongly suppressed in the ME180 parent cells and CDDP-resistant subclones. A similar discrepancy between the mRNA expression level and amount of protein expression was previously reported for the transcriptional regulatory factor, PAX6, in the SW837 human colorectal carcinoma cell line (40). However, the expression of DAP kinase protein was recovered to detectable levels in the ME180 parent cells upon 5-aza-CdR treatment in a dose-dependent manner, suggesting that the post-transcriptionally suppressed DAP kinase protein expression was at least partially attributable to aberrant methylation. Treatment with TSA alone had little effect on the DAP kinase protein re-expression in parent ME180 cells. Furthermore, combined treatment with 5-aza-CdR and TSA synergistically enhanced the DAP kinase protein expression in parent cells compared to treatment with 5-aza-CdR or TSA alone, consistent with a previous observation suggesting a more dominant role for methylation over histone deacetylase activity in mammals for the maintenance of gene silencing in association with CpG methylation (14). In contrast to the ME180 parent cells, 5 of the CDDP-resistant subclones (A, B, C, D and F) showed slightly detectable DAP kinase protein expression and all 6 retained DAP kinase mRNA expression. Moreover, quantitative RT-PCR analysis revealed that all 6 CDDP-resistant subclones had apparently higher DAP kinase mRNA expression levels than the parent cells. However, unlike the parent cells, all 6 CDDP-resistant subclones failed to show induction of DAP kinase protein expression upon treatment with 5-aza-CdR and/or TSA. Therefore, our results indicate that the acquired CDDP-resistance in the ME180-derived CDDP-resistant subclones is not caused directly by induction of DAP kinase protein expression. The impaired translation of DAP kinase mRNA after demethylation and/or inhibition of histone deacetylation was a common characteristic among all of the CDDP-resistant subclones. Thus, the acquisition of CDDP-resistance may be caused by gene mutations that strongly interrupt the post-transcriptional modulation of DAP kinase mRNA and simultaneously reduce the CDDP-sensitivity.

Our present results revealed that treatment with 5-aza-CdR could overcome CDDP-resistance in CDDP-resistant subclones established from the ME180 cervical carcinoma cell line, and that neither the methylation status of the DAP kinase promoter nor the DAP kinase protein expression level was directly involved in the acquisition of CDDP-resistance. CDDP-resistance may be caused by unknown molecular changes in the post-transcriptional mechanism that modulate the translation of DAP kinase mRNA. These currently unknown molecular changes in CDDP-resistant subclones are considered to be transiently restored by demethylation treatment. The present study is the first to demonstrate that the impaired translation mechanisms of DAP kinase mRNA are involved in the acquired CDDP-resistance of human cancer cells.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education

Science, Sports and Culture of Japan and a Grant-in-Aid for Scientific Research from the Ministry of Welfare and Labor of Japan.

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Glutathione-S-transferase and p53 polymorphisms in cervical carcinogenesis

Masatsugu Ueda[a,*], Yao-Ching Hung[b], Yoshito Terai[a], Junko Saito[c], Osamu Nunobiki[d], Sadamu Noda[d], Minoru Ueki[a]

[a]*Department of Obstetrics and Gynecology, Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan*
[b]*Department of Obstetrics and Gynecology, China Medical College, Taichung, Taiwan, ROC*
[c]*Department of Obstetrics and Gynecology, Kansai Medical College, Osaka, Japan*
[d]*Osaka Cancer Prevention Center, Osaka, Japan*

Received 29 July 2004
Available online 13 December 2004

Abstract

Objective. To investigate the clinical significance of glutathione-S-transferase GSTM1, GSTT1 and p53 codon 72 polymorphisms in cervical carcinogenesis.

Methods. GSTM1, GSTT1 and p53 codon 72 polymorphisms together with human papillomavirus (HPV) types were examined in a total of 198 cervical smear samples using multiplex polymerase chain reaction (PCR) and PCR restriction fragment length polymorphism (RFLP) techniques.

Results. Forty-two patients with high-grade squamous intraepithelial lesion (HSIL) had higher frequency of high-risk HPV and null GSTT1 genotype than 102 with low-grade SIL (LSIL) and 54 controls. Thirty-one patients with HSIL had also statistically higher frequency of null GSTT1 genotype than 28 with LSIL among 69 patients with high-risk HPV. There was no statistical difference in p53 Arg, Arg/Pro and Pro genotypes between SILs and controls with or without high-risk HPV.

Conclusion. GSTT1 null genotype in cervical cell samples may be associated with more severe precancerous lesions of the cervix in a Japanese population. The p53 codon 72 polymorphism is unlikely to be related to HPV status and the onset of cervical cancer.

Keywords: GST; p53; Polymorphism; SIL; Cervical carcinogenesis

Introduction

Cervical cancer is the second most common cancer in women worldwide, and is both a preventable and a curable disease especially if identified at an early stage. It is widely accepted that specific human papillomavirus (HPV) types are the central etiologic agent of cervical carcinogenesis. Recently, several candidate markers for cervical cancer risk, such as glutathione-S-transferase (GST) and p53, have been described [1–5]. Such markers could be used to direct high-risk women to more frequent cervical screening.

The genes of GST family encode enzymes that appear to be critical in cellular protection against the cytotoxic effects. GSTs play an important role in conjugating glutathione to the products of endogenous lipid peroxidation and inactivating organic hydroperoxides via selenium-independent glutathione peroxidase activity, thus protecting the cell from the deleterious effects of oxidative stress. GST classes mu (GSTM1) and theta (GSTT1) gene deletions may promote the development of cervical dysplasia by moderating the activation and detoxification of polycyclic hydrocarbons and other compounds that influence oxidative stress and DNA adduct formation [3]. A polymorphism at codon 72 of the p53 gene results in the substitution of arginine (Arg) for proline (Pro) in the gene product. It has been suggested that the homozygous Arg genotype increased the susceptibility

* Corresponding author. Fax: +81 72 681 3723.
E-mail address: gyn017@poh.osaka-med.ac.jp (M. Ueda).

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doi:10.1016/j.ygyno.2004.11.005

of p53 protein to degradation by E6 protein derived from oncogenic HPV [4].

Despite extensive studies on germline polymorphisms of GSTM1, GSTT1 and p53 genes in the patients with premalignant and malignant cervical lesions, no correlation has been reported so far between genetic polymorphisms of these genes and increased risk of cervical cancer [1–3,5–7]. In this study, we investigated GSTM1, GSTT1 and p53 codon 72 polymorphisms in exfoliated cervical cell samples from the patients with squamous intraepithelial lesion (SIL) of the cervix and evaluated the clinical significance of polymorphic frequency of these genes in cervical carcinogenesis.

Materials and methods

Cell sample

We conducted GST and p53 genotype analysis together with HPV typing in a total of 198 cervical smear samples obtained from the patients with consent who received cervical cancer screening. They consist of 54 normal, 102 low-grade SIL (LSIL) and 42 high-grade SIL (HSIL). All of 198 patients were Japanese women who visited Osaka Medical College, Kansai Medical College or Osaka Cancer Prevention Center in the past 5 years. Final histologic diagnosis was confirmed by colposcopy-directed biopsy for the patients with abnormal cytology.

DNA preparation

The exfoliated cervical cells were disrupted with lysis buffer [20 mM NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.5% SDS, 50 μg/ml proteinase K], and genomic DNA was extracted with phenol–chloroform and precipitated with ethanol using standard techniques. Purified DNA samples from the cells were stored at −20°C until use.

Genotyping of GSTM1, GSTT1 and p53 codon 72

The GSTM1 and GSTT1 genetic polymorphisms were evaluated using multiplex polymerase chain reaction (PCR) techniques according to the method reported by Chen et al. [8] with some modifications. For GSTM1, the primers 5′-GAACTCCCTGAAAAGCTAAAGC-3′ and 5′-GTTG-GGCTCAAATATACGGTGG-3′; for GSTT1, the primers 5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCAC-CGGATCATGGCCAGCA-3′; for β-globin as a positive control, the primers 5′-CAACTTCATCCACGTTCACC-3′ and 5′-GAAGAGCCAAGGACAGGTAC-3′ were used. One hundred nanograms of the DNA template from each cell sample was amplified by PCR in a final volume of 50μl reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM dNTP, 0.5μM each primer and 1.25 units Taq polymerase (Applied Biosystems, Branchburg, NJ) as previously described [9]. After an initial denaturation at 96°C for 3 min, 40 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 2 min) were carried out on a Perkin-Elmer GeneAmp PCR System 9700. The final extension was performed at 72°C for 10 min. After visualization of the PCR products by 2.0% agarose gel electrophoresis with ethidium bromide staining, gel images were obtained using the ATTO densitograph UV-image analyzer (ATTO Corp, Tokyo), and the presence or absence of the products was determined using ATTO's densitometry software version 2 (ATTO). The absence of amplified GSTM1 or GSTT1 product indicated the respective null genotype for each.

PCR restriction fragment length polymorphism (RFLP) analysis of codon 72 of the p53 gene, modified from a technique described by Ara et al. [10], was conducted to identify p53 genotypes with the primers 5′-TTGCC-GTCCCAAGCAATGGATGA-3′ and 5′-TCTGGGAAGG-GACAGAAGATGAC-3′. One hundred nanograms of the DNA template from each cell sample was amplified by PCR in a 50 μl reaction as described above with an annealing temperature at 60°C. After confirmation of an amplified fragment of the expected size (199 bp) on a 1.5% agarose gel, 17 μl of each PCR product was digested with 10 units of restriction enzyme *Bst*UI (New England Biolabs, ME) at 60°C for 3 h. DNA fragments were visualized on a 3.0% agarose gel with ethidium bromide as described above. The Arg allele is cleaved by *Bst*UI and yields two small fragments (113 and 86 bp). The Pro allele is not cleaved by *Bst*UI and has a single 199-bp band. The heterozygote contains three bands (199, 113 and 86 bp).

Sequence analysis

Polymorphisms in the p53 codon 72 were sequenced. Amplified DNA fragments were purified using the QIA-quick gel extraction kit (Qiagen, Valencia, CA) and directly sequenced using ABI PRISM 3100 sequencer (Applied Biosystems). Each single nucleotide polymorphism was verified in both the sense and antisense directions.

HPV typing

The presence of various HPV types was examined using L1-PCR according to the method reported by Nagano et al. [11]. Briefly, 100 ng of cellular DNA was subjected to PCR in the presence of published consensus primers (L1C1 and L1C2) [12]. Amplified HPV fragments were typed on the basis of the RFLP among HPVs. Initial typing of amplified HPV fragments was performed by digestions with *Rsa*I, *Dde*I, and then confirmed by digestions with several additional restriction enzymes as described previously [11,12]. HPV-negative or -positive cervical cancer cell line was used as a negative or positive control for HPV typing, respectively. L1-PCR can detect 22 registered low-risk (6, 11, 34, 40, 42, 43, 44) and high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 69) HPV types.

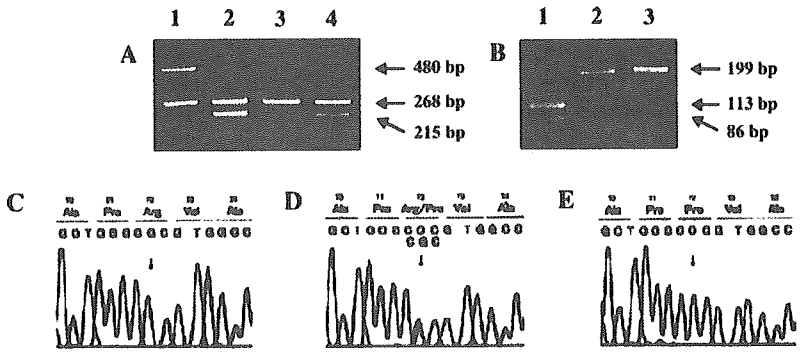

Fig. 1. Genotyping of GSTM1, GSTT1 and p53 codon 72 by multiplex PCR or PCR-RFLP and direct sequence. (A) Case 1, null GSTM1 genotype (absence of 215-bp fragment). Case 2, null GSTT1 genotype (absence of 480-bp fragment). Case 3, null GSTM1 and GSTT1 genotypes (absence of 215- and 480-bp fragments). Case 4, present GSTM1 and GSTT1 genotypes. β-globin as a positive control is detected as 268-bp fragment. (B) Case 1, Arg/Arg homozygotes. Case 2, Arg/Pro heterozygotes. Case 3, Pro/Pro homozygotes. The fragment of 199 bp is the nondigested PCR product from the Pro allele. Fragments of 113 and 86 bp result from *Bst*UI digestion of the Arg allele. The amplified fragments of the p53 codon 72 (cases 1–3 in B) were gel-purified and sequenced. Electropherograms of p53 codon 72 forward sequence indicate Arg (CGC) in case 1 (C), Arg/Pro (CGC/CCC) in case 2 (D), and Pro (CCC) in case 3 (E), respectively.

Statistical analysis

The HPV status and polymorphic features of GSTM1, GSTT1 and p53 genes in 198 cases were compared between normal, LSIL and HSIL, and checked by the Mann–Whitney and chi-square tests. A level of $P < 0.05$ was accepted as statistically significant.

Results

Fig. 1A shows an example for genotyping of GSTM1 and GSTT1. The polymorphic deletion of the GSTM1 and GSTT1 genes was determined by multiplex PCR. The absence of 215- or 480-bp fragment indicated null GSTM1 or GSTT1 genotype, respectively. The polymorphic site in exon 4 (codon 72) of the p53 gene was achieved by PCR-RFLP. As shown in Fig. 1B, the fragment of 199 bp indicated the nondigested PCR product from the Pro allele. Fragments of 113 and 86 bp resulted from *Bst*UI digestion of the Arg allele. The Arg/Pro heterozygote contained these three bands (199, 113 and 86 bp). Polymorphisms of p53 codon 72 detected by PCR-RFLP were also confirmed by sequence analyses. As can be seen in Figs. 1C–E, sequencing of the gel-purified PCR product indicated CGC for Arg, CGC/CCC for Arg/Pro, and CCC for Pro genotype at codon 72, respectively.

Table 1 shows HPV status and polymorphic frequency of GSTM1, GSTT1 and p53 codon 72 in 198 samples examined. The 42 patients with HSIL had significantly higher frequency of high-risk HPV than 102 with LSIL and 54 controls. There was no significant difference in the frequency of null GSTM1 genotype between SILs and controls, whereas the 42 patients with HSIL had statistically higher frequency of null GSTT1 genotype than 102 with LSIL and 54 controls. In contrast, the differences in the polymorphic frequency of p53 Arg, Arg/Pro and Pro genotypes between SILs and controls were statistically not significant.

As shown in Table 2, the 31 patients with HSIL had also statistically higher frequency of null GSTT1 genotype than 28 with LSIL among the 69 patients with high-risk HPV. When the Arg genotype was compared to the Arg/Pro + Pro genotypes, there was again no statistical difference in the

Table 1
Frequency of GSTM1, GSTT1 and p53 codon 72 polymorphisms in exfoliated cervical cell samples

Lesions	Number with high-risk HPV	GSTM1 null	GSTT1 null	Amino acid at p53 codon 72		
				Arg	Arg/Pro	Pro
Normal (n = 54)	10 (18.5%)[a,b]	28 (51.9%)	24 (44.4%)[c]	24 (44.4%)	23 (42.6%)	7 (13.0%)
LSIL (n = 102)	28 (27.5%)[d]	55 (53.9%)	40 (99.2%)[c]	38 (37.3%)	40 (39.2%)	24 (23.5%)
HSIL (n = 42)	31 (73.8%)[a,d]	20 (47.6%)	29 (69.0%)[c,e]	18 (42.9%)	16 (38.1%)	8 (19.0%)
All SIL (n = 144)	59 (41.0%)[b]	75 (52.1%)	69 (47.9%)	56 (38.9%)	56 (38.9%)	32 (22.2%)

[a] $P < 0.0001$, OR = 12.4 x^2 vs. normal.
[b] $P = 0.0031$, OR = 3.1 x^2 vs. normal.
[c] $P = 0.0162$, OR = 2.8 x^2 vs. normal.
[d] $P < 0.0001$, OR = 7.4 x^2 vs. LSIL.
[e] $P = 0.0011$, OR = 3.5 x^2 vs. LSIL.

Table 2
HPV status and frequency of GSTT1 and p53 codon 72 polymorphisms in exfoliated cervical cell samples

Study group	*n*	GSTT1 null	Amino acid at p53 codon 72	
			Arg	Arg/Pro + Pro
High-risk HPV−				
Normal	44	20 (45.5%)	20 (45.5%)	24 (54.5%)
LSIL	74	31 (41.9%)	26 (35.1%)	48 (64.9%)
HSIL	11	8 (72.7%)	4 (36.4%)	7 (63.6%)
All SIL	85	39 (45.9%)	30 (35.3%)	55 (64.7%)
High-risk HPV+				
Normal	10	4 (40.0%)	4 (40.0%)	6 (60.0%)
LSIL	28	9 (32.1%)[a]	12 (42.9%)	16 (57.1%)
HSIL	31	21 (67.7%)[a]	14 (45.2%)	17 (54.8%)
All SIL	59	30 (50.8%)	26 (44.1%)	33 (55.9%)

[a] $P = 0.0063$, OR = 4.4 x^2 vs. LSIL.

genotype prevalence between SILs and controls among the 129 and 69 patients without and with high-risk HPV, respectively.

Discussion

There is an expanding body of literature suggesting that host factors, including genetic polymorphisms, may explain some of the individual differences in cancer occurrence. A large number of previous studies have been conducted on the correlation between germline polymorphisms of cancer susceptibility genes and the higher risk of human malignant tumors.

The GSTM1 and GSTT1 gene products are thought to protect against somatic mutation in DNA by facilitating the conjugation and elimination of a variety of electrophilic species [13]. Previous epidemiological studies of GST and cervical neoplasia found no significant differences in the frequency of GSTM1 or GSTT1 in women with cervical SIL or cancer compared to controls with normal cervical pathology [1–3]. In our investigation using exfoliated cervical cell samples from a Japanese population, the GSTT1 null genotype was more common among HSIL cases than LSIL cases and controls. Moreover, the patients with HSIL also had higher frequency of null GSTT1 genotype than those with LSIL among high-risk HPV group. GSTT1 differs from other classes of GSTs in its lack of activity towards the GST model substrate 1-chloro-2, 4-dinitrobenzene and its failure to bind to S-hexyl-glutathione affinity matrices [14]. The gene defect of GSTT1 was reported to be associated with an increased risk of myelodysplastic syndromes [15], astrocytoma and meningioma [16]. However, there have been no other reports on the correlation between GSTT1 gene defects and cervical carcinogenesis. Recently, we have examined GSTM1 and GSTT1 genotypes in 104 cell lines originating from a variety of human malignant tumors and found that GSTT1 null genotype was more common in cervical cancer cells [17]. Further studies on the differential gene expression profiles between normal cervical keratinocytes and cervical cancer cell lines with or without GSTT1 deletion may provide the better understanding for the effect of this abnormal genotype in the sequence of cervical carcinogenesis. Moreover, it might be of interest to further examine the difference in the polymorphic frequency of the null GSTT1 genotype between SILs and invasive cervical cancer to clarify whether this genotype alteration occurs prior to the development of malignant phenotype cells or late in the development of neoplastic cells.

Tobacco smoking has been associated with the risk of cervical malignancy and SIL [18]. DNA adducts of bulky aromatic compounds have been found with increased frequency in the cervical epithelium of smokers compared to nonsmokers [19], providing biochemical evidence that smoking may act as a confounder in the etiology of cervical cancer. Molecular studies have identified polymorphic gene products that are associated with the metabolism of tobacco smoke procarcinogens and possibly with susceptibility to cancer. Lack of GST activity, caused by an inherited deletion of the GST gene, has also been reported to increase the risk of lung and other tobacco-related cancers [13]. It would be of interest to further examine the relationship of the development of SIL, smoking and GSTT1 null genotype in the group of patients we examined.

Initially, we evaluated p53 genotypes using the technique reported by Ara et al. [10]. Because these results may be affected by incomplete *Bst*UI digestion, we further confirmed p53 genotypes by sequence analyses. We found that incubation of PCR products with 10 units of *Bst*UI at 60°C for 3 h resulted in complete digestion and PCR-RFLP profiles exactly matched sequence data. Our present results revealed that the differences in the polymorphic frequency of p53 Arg, Arg/Pro and Pro genotypes between SILs and controls were statistically not significant. Moreover, neither Arg nor Pro allele affected the increased risk of SILs with or without high-risk HPVs compared to controls. Some previous studies have reported no correlation between germline polymorphisms of the p53 codon 72 and increased risk of cervical cancer [5–7]. The recent study reported by Nishikawa et al. [20] using cervical condyloma, dysplasia and cancer tissue samples also demonstrated that no statistically significant differences in the distribution of p53 genotypes were found among the patients with these diseases, regardless of HPV status. The other two reports examining for Japanese population supported their results [21,22]. These data suggest that p53 codon 72 polymorphism does not correlate with the development of HPV-associated cervical neoplasms.

To the best of our knowledge, this is the first study to examine the role of GST and p53 codon 72 polymorphisms in cytologic materials from women with premalignant cervical disease. GSTT1 null genotype in cervical cell samples may be associated with more severe precancerous lesions of the cervix in a Japanese population. However, the

p53 codon 72 polymorphism is unlikely to be associated with HPV status and the onset of cervical cancer. These observations are potentially important in managing SIL patients by cervical screening and in understanding the pathogenesis of cervical cancer.

Acknowledgments

We are grateful to Dr. Ken Ueki, Department of Obstetrics and Gynecology, Osaka Medical College, for collecting clinical materials. We also thank Kumiko Sato for her technical assistance. This work was supported in part by High-Tech Research Program of Osaka Medical College.

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Gynecologic Oncology 98 (2005) 129–133

Gynecologic Oncology

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Fas gene promoter −670 polymorphism (A/G) is associated with cervical carcinogenesis

Masatsugu Ueda[a,*], Yao-Ching Hung[b], Yoshito Terai[a], Hiroyuki Yamaguchi[a], Junko Saito[c], Osamu Nunobiki[d], Sadamu Noda[d], Minoru Ueki[a]

[a]*Department of Obstetrics and Gynecology, Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan*
[b]*Department of Obstetrics and Gynecology, China Medical College, Taichung, Taiwan, R.O.C.*
[c]*Department of Obstetrics and Gynecology, Kansai Medical College, Osaka, Japan*
[d]*Osaka Cancer Prevention Center, Osaka, Japan*

Received 22 February 2005
Available online 13 May 2005

Abstract

Objective. To investigate the biological significance of single nucleotide polymorphism (SNP) at Fas gene promoter in cervical carcinogenesis.

Methods. SNP at −670 of Fas gene promoter (A/G) together with human papillomavirus (HPV) types were examined in a total of 279 cervical smear samples and 8 human cervical squamous carcinoma cell lines using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) techniques.

Results. 49 patients with high-grade squamous intraepithelial lesion (HSIL) had higher frequency of high-risk HPV and GA + GG genotype than 167 with low-grade SIL (LSIL) and 63 controls. G allele frequency was also higher in HSIL than in LSIL and controls. There was an increased OR (6.00; CI, 1.32–27.37; $P = 0.021$) for GA + GG genotype in HSIL cases compared to controls among 96 patients with high-risk HPV. 7 of 8 cervical carcinoma cell lines also showed GA or GG genotype.

Conclusion. Fas gene promoter −670 polymorphism (A/G) may be closely associated with cervical carcinogenesis in a Japanese population.

Keywords: Fas; Polymorphism; SIL; Cervical carcinogenesis

Introduction

Cervical cancer is the second most common cancer in women worldwide, and is both a preventable and a curable disease especially if identified at an early stage. It is widely accepted that specific human papillomavirus (HPV) types are the central etiologic agent of cervical carcinogenesis. Other environmental and host factors also play decisive roles in the persistence of HPV infection and further malignant conversion of cervical epithelium [1]. Although many previous reports have focused on HPV and environmental factors, the role of host susceptibility to cervical carcinogenesis is largely unknown.

Apoptosis is a physiological process that regulates normal homeostasis and alterations of apoptosis-related genes are likely to contribute to the pathogenesis of autoimmune diseases [2] and malignant tumors [3]. Among various cell surface death receptors, Fas/CD95, a transmembrane receptor, is known as a member of tumor necrosis factor (TNF) receptors superfamily [4]. Downregulation of Fas with resultant resistance to death signals has been reported in many cancers [5–7]. The transcriptional expression of Fas gene is regulated by a number of genetic elements located in the 5′ upstream region of the gene. The promoter region of Fas gene consists of basal promoter, enhancer, and silencer regions [8]. Single nucleotide poly-

* Corresponding author. Fax: +81 72 681 3723.
E-mail address: gyn017@poh.osaka-med.ac.jp (M. Ueda).

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doi:10.1016/j.ygyno.2005.04.001

morphism (SNP) at −670 in the enhancer region (A/G) situates at a binding element of gamma interferon activation signal (GAS). G allele results in an abolishment of the GAS element and a significant decrease in Fas gene expression in response to interferon (INF)-γ stimuli [9,10]. Recent studies have demonstrated that the A/G SNP at −670 of Fas gene promoter is closely associated with the pathogenesis of autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus [10,11]. However, there have been very few reports on the correlation between SNP of Fas gene and cancer susceptibility [12,13]. In this study, we investigated Fas gene promoter −670 polymorphism together with HPV types in exfoliated cervical cell samples from the patients with squamous intraepithelial lesion (SIL) of the cervix or human cervical squamous carcinoma cell lines, and evaluated the biological significance of this genotype in cervical carcinogenesis.

Materials and methods

Cell sample

We conducted genotype analysis of Fas gene promoter −670 together with HPV typing in a total of 279 cervical smear samples obtained from the patients with consent who received cervical cancer screening. They consist of 63 normal, 167 low-grade SIL (LSIL), and 49 high-grade SIL (HSIL). All of 279 patients were Japanese women who visited Osaka Medical College, Kansai Medical College or Osaka Cancer Prevention Center in the past 5 years. Cervical cell samples from these patients were collected from the uterine ectocervix and the endocervical canal by cotton swabs, placed in phosphate-buffered saline, and stored at −20°C until use. Final histologic diagnosis was confirmed by colposcopy-directed biopsy for the patients with abnormal cytology.

Cell line

Eight human cervical squamous carcinoma cell lines (SKG-I, SKG-II, SKG-IIIa, SKG-IIIb, OMC-1, YUMOTO, QG-U, and QG-H) were also used for genotype analysis of Fas gene promoter −670 together with HPV typing. All cell lines were originating from Japanese women. The OMC-1 cell line [14] was established in our laboratory. The SKG-I [15], SKG-II [16], SKG-IIIa, and SKG-IIIb [17] cell lines were kindly provided by Dr. Shiro Nozawa, Keio University, Tokyo. The YUMOTO [18], QG-U, and QG-H [19] cell lines were kindly provided by Dr. Naotake Tanaka, Chiba University, Chiba. The SKG-I, SKG-II, SKG-IIIa, SKG-IIIb, and OMC-1 cell lines were maintained as monolayer cultures in Ham's F-12 medium (Flow Laboratories Inc., Irvine, Scotland) supplemented with 10% fetal bovine serum (Mitsubishi Chemical Co., Tokyo) at 37°C in a humidified incubator with 5% CO_2 in air. The YUMOTO, QG-U, and QG-H cell lines were cultured in RPMI-1640 medium (GIBCO BRL, Bethesda, MD) supplemented with 10% fetal bovine serum. The cells were grown in 75-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) and cell viability was determined by trypan blue dye exclusion prior to use.

DNA preparation

The exfoliated cervical cells or cell lines were disrupted with lysis buffer [20 mM NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.5% SDS, 50 μg/ml proteinase K], and genomic DNA was extracted with phenol-chloroform and precipitated with ethanol using standard techniques.

Genotyping of Fas gene promoter −670

Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis of the Fas gene promoter −670, modified from a technique described by Lee et al. [11], was conducted with the primers, 5′-CTACCTAAGAGCTATCTACCGTTC-3′ and 5′-GGCTGTCCATGTTGTGGCTGC-3′. 100 ng of the DNA template from each cell sample or cell line was amplified by PCR in a final volume of 50 μl reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM dNTP, 0.5 μM each primer, and 1.25 units Taq polymerase (Applied Biosystems, Branchburg, NJ) as previously described [20]. After an initial denaturation at 96°C for 3 min, 40 cycles of denaturation (94°C for 1 min), annealing (58°C for 1 min) and extension (72°C for 2 min) were carried out on a Perkin-Elmer GeneAmp PCR System 9700. The final extension was performed at 72°C for 10 min. After digestion of PCR products with restriction enzyme *Mva*I (Roche Applied Science, Penzberg, Germany) under recommended conditions, DNA fragments were visualized on a 3.0% agarose gel electrophoresis with ethidium bromide staining and gel images were obtained using the ATTO densitograph UV-image analyzer (ATTO Corp, Tokyo). The genotype was determined with A allele fragment length of 232 bp and G allele of 188 bp.

HPV typing

The presence of various HPV types was examined using L1-PCR according to the method reported by Nagano et al. [21]. Briefly, 100 ng of cellular DNA was subjected to PCR in the presence of published consensus primers (L1C1 and L1C2) [22]. Amplified HPV fragments were typed on the basis of the RFLP among HPVs. Initial typing of amplified HPV fragments was performed by digestions with *Rsa*I, *Dde*I, and then confirmed by digestions with several additional restriction enzymes as described previously [21,22]. L1-PCR can detect 22 registered low-risk (6, 11,

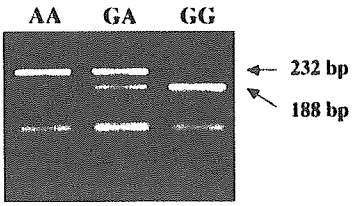

Fig. 1. Genotyping of Fas gene promoter −670 in exfoliated cervical cell samples by PCR-RFLP. The genotypes AA (232 bp), GA (188, 232 bp), and GG (188 bp) are shown.

34, 40, 42, 43, 44) and high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 69) HPV types.

Statistical analysis

To compare the HPV status and polymorphic features of Fas gene promoter −670 between normal, LSIL, and HSIL groups, chi-square test and Fisher's exact test were used. A level of $P < 0.05$ was accepted as statistically significant.

Results

Fig. 1 shows an example for genotyping of Fas gene promoter −670 in exfoliated cervical cell samples. The fragments of 232 and 188 bp indicated the AA and GG genotypes, respectively. The GA genotype contained these two bands.

Table 1 shows the frequency of high-risk HPV and Fas promoter −670 polymorphism in 279 samples examined. When AA genotype was compared to GA + GG genotype, 49 patients with HSIL had significantly higher frequency of high-risk HPV and GA + GG genotype than 167 with LSIL and 63 controls. G allele frequency was also higher in HSIL than in LSIL and controls. There was no statistical difference in the GA + GG genotype prevalence between SILs and controls among 183 patients without high-risk HPV as shown in Table 2. However, there was an increased OR (6.00; CI, 1.32–27.37; $P = 0.021$) for GA + GG genotype in HSIL cases compared to controls among 96 patients with high-risk HPV. There also appeared to be a trend toward decreased AA genotype from LSIL to HSIL in both groups (P test for trend <0.05).

Table 2
HPV status and frequency of Fas promoter −670 polymorphism in exfoliated cervical cell samples

Study group	*n*	Genotype at Fas promoter −670		OR	95% CI	*P* value
		AA	GA + GG			
High-risk HPV−						
Normal	53	15 (28.3%)	38 (71.7%)	1		
LSIL	121	36 (29.8%)	85 (70.2%)	0.93	0.44–1.95	0.847
HSIL	9	1 (11.1%)	8 (88.9%)	3.16	0.40–25.04	0.276
High-risk HPV+						
Normal	10	4 (40.0%)	6 (60.0%)	1		
LSIL	46	15 (32.6%)	31 (67.4%)	1.38	0.34–5.66	0.655
HSIL	40	4 (10.0%)	36 (90.0%)	6.00	1.32–27.37	0.021

As shown in Fig. 2, genotyping of Fas gene promoter −670 in 8 cervical squamous carcinoma cell lines revealed that AA genotype was detected only in the QG-U cell line, whereas the other 7 of 8 (87.5%) cell lines had GA or GG genotype. In addition, 7 of 8 cell lines except for YUMOTO were positive for high-risk HPV.

Discussion

There is an expanding body of literature suggesting that host factors, including genetic polymorphisms, may explain some of the individual differences in cancer occurrence. A large number of previous studies have been conducted on the correlation between germline polymorphisms of cancer susceptibility genes and the higher risk of human malignant tumors.

Polymorphisms in the promoter region or 5′ flanking region of genes can lead to different levels of gene expression and have been also implicated in a number of diseases. SNP at −670 of Fas gene promoter (A/G) has been found with potentially different transcriptional efficiency [9,23]. Several studies addressed the association of this SNP with autoimmune diseases [9–11,23]. Recently, Lai et al. [12] conducted Fas promoter −670 polymorphism analysis using surgical and biopsy tissue specimens of cervical

Table 1
Frequency of high-risk HPV and Fas promoter −670 polymorphism in exfoliated cervical cell samples

Lesions	Number with high-risk HPV	Genotype frequency		Allele frequency	
		AA	GA + GG	A	G
Normal ($n = 63$)	10 (15.9%)[a]	19 (30.2%)	44 (69.8%)[b]	67 (53.2%)	59 (46.8%)[e]
LSIL ($n = 167$)	46 (27.5%)[d]	51 (30.5%)	116 (69.5%)[c]	165 (49.4%)	169 (50.6%)[f]
HSIL ($n = 49$)	40 (81.6%)[a,d]	5 (10.2%)	44 (89.8%)[b,c]	37 (37.8%)	61 (62.2%)[e,f]

[a] $P < 0.0001$ χ^2 vs. normal.
[b] $P = 0.0107$ χ^2 vs. normal.
[c] $P = 0.0217$ χ^2 vs. normal.
[d] $P < 0.0001$ χ^2 vs. LSIL.
[e] $P = 0.0043$ χ^2 vs. LSIL.
[f] $P = 0.0422$ χ^2 vs. LSIL.