Hypermethylation of the Death-Associated Protein Kinase Promoter Attenuates the Sensitivity to TRAIL-Induced Apoptosis in Human Non–Small Cell Lung Cancer Cells

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Abstract

Death-associated protein (DAP) kinase plays an important role in IFN-γ, tumor necrosis factor (TNF)-α, or Fas ligand–induced apoptosis. TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF ligand family and can induce caspase-dependent apoptosis in cancer cells while sparing most of the normal cells. However, some of the cancer cell lines are insensitive to TRAIL, and such resistance cannot be explained by the dysfunction of TRAIL receptors or their known downstream targets. We reported previously that *DAP kinase* promoter is frequently methylated in non–small cell lung cancer (NSCLC), and such methylation is associated with a poor clinical outcome. To determine whether *DAP kinase* promoter methylation contributes to TRAIL resistance in NSCLC cells, we measured *DAP kinase* promoter methylation and its gene expression status in 11 NSCLC cell lines and correlated the methylation/expression status with the sensitivity of cells to TRAIL. Of the 11 cell lines, 1 had a completely methylated *DAP kinase* promoter and no detectable DAP kinase expression, 4 exhibited partial promoter methylation and substantially decreased gene expression, and the other 6 cell lines showed no methylation in the promoter and normal DAP kinase expression. Therefore, the amount of DAP kinase expression amount was negatively correlated to its promoter methylation ($r = -0.77$; $P = 0.003$). Interestingly, the cell lines without the DAP kinase promoter methylation underwent substantial apoptosis even in the low doses of TRAIL, whereas those with DAP kinase promoter methylation were resistant to the treatment. The resistance to TRAIL was reciprocally correlated to DAP kinase expression in 10 of the 11 cell lines at 10 ng/mL concentration ($r = 0.91$; $P = 0.001$). We treated cells resistant to TRAIL with 5-aza-2′-deoxycytidine, a demethylating reagent, and found that these cells expressed DAP kinase and became sensitive to TRAIL. These results suggest that DAP kinase is involved in TRAIL-mediated cell apoptosis and that a demethylating agent may have a role in enhancing TRAIL-mediated apoptosis in some NSCLC cells by reactivation of DAP kinase. (Mol Cancer Res 2004;2(12):685–91)

Introduction

Death-associated protein (DAP) kinase is a Ca^{2+}/calmodulin-regulated, 160-kDa serine/threonine, microfilament-bound kinase shown recently to be involved in IFN-γ, tumor necrosis factor-α, or Fas ligand–induced apoptosis (1-3). Aggressiveness of malignant tumors has been associated with methylation of the promoter region of the *DAP kinase* gene (4-8) and loss of DAP kinase expression (9).

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL, also known as Apo-2L), a member of the tumor necrosis factor ligand family, is a cytokine that can induce a rapid caspase-dependent and tumor-specific apoptosis (10-16) through its specific death receptors DR4 and DR5 (17). The activation of DR4 and DR5, like that of Fas/Apo, leads to the activation of the initiator caspase, caspase-8, and its downstream targets (18). TRAIL seems to exert selective toxicity toward neoplastic cells, whereas most normal cells are resistant to TRAIL (19). Multiple factors have been proposed for such resistance, including the presence of the decoy receptors DcR1 and DcR2, the downexpression of DR4 and DR5, the silencing of caspase-8, the inactivity of Akt, and the overexpression of cFLIP and cyclooxygenase-2 (20-27).

In this study, we investigated whether DAP kinase plays a role in determining the sensitivity of cells to TRAIL using non–small cell lung cancer (NSCLC) as a model. Our data suggest that the DAP kinase may be involved in TRAIL-induced apoptosis, and restoration of DAP kinase expression may overcome TRAIL resistance in certain NSCLCs.

Results

Establishment of a Multiplex Methylation-Specific PCR

Methylation-specific PCR (MSP; ref. 28) is the most extensively used method for detecting the methylation status

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of CpG islands in the promoter regions of genes. Combined bisulfite restriction analysis is more quantitative (29) but requires more strict experimental condition. In this study, we combined multiplex PCR and MSP to create multiplex MSP (MMSP). In this method, unmethylated and methylated DNA are amplified simultaneously with two primer sets specific for methylated and unmethylated CpG islands in the DAP kinase promoter. The intensities of PCR products between methylated and unmethylated DNA were used to determine their relative ratios. Our results indicate that MMSP is robust in quantifying methylated DAP kinase promoter (Fig. 1).

DAP Kinase Promoter Methylation in NSCLC Cell Lines

Using MMSP, we established the methylation status of the *DAP kinase* promoter in the 11 NSCLC cell lines. In 5 of the 11 (46%) cell lines, the CpG island in the promoter region was methylated (Fig. 2A). This percentage is close to the 44% that we found in early-stage NSCLC tissues in an earlier study (8). In the 5 promoter-methylated cell lines, the promoter in Calu-1 was completely methylated, whereas in H157, H460, H1792, and SK-MES-1 the promoter was partially methylated to different degrees.

To further determine the nature of mixed methylation status in some NSCLC cell lines, we isolated 98 individual clones of H460 cells and analyzed their methylation status. We found 77 clones that carried both methylated and unmethylated promoter alleles, 6 clones that contained a completely methylated promoter, and 15 clones that contained a completely unmethylated promoter. It suggests that H460 parental cell line is heterogeneous with respect to DAP kinase promoter methylation and its gene expression. It also suggests that the status of DAP kinase promoter methylation is also unstable in some of the subclones.

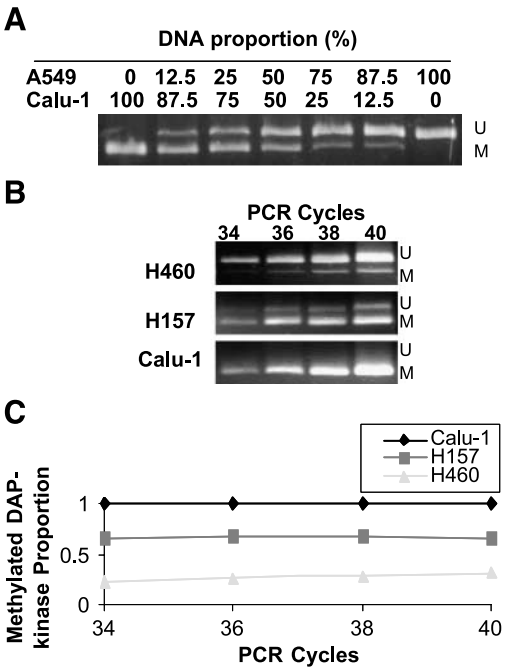

FIGURE 1. MMSP consistency for *DAP kinase* promoter methylation. **A.** MMSP was done using bisulfite-modified DNA from A549 cells (with unmethylated *DAP kinase* promoter) and Calu-1 cells (with methylated *DAP kinase* promoter), with ratios on *top of each lane*. **B.** MMSP using bisulfite-modified DNA isolated from NSCLC cell lines (H460, H157, and Calu-1). *U,* 279-bp unmethylated fragments; *M,* 218-bp methylated fragments. **C.** Scanning densitometry was used to measure and analyze the intensity of the fragment signal of **B**. Relative quantity of the methylation promoter was then calculated.

Expression of DAP Kinase in the NSCLC Cells Negatively Correlates with Its Promoter Methylation Status

Using multiplex reverse transcription-PCR, we examined DAP kinase expression in the 11 lung cancer cell lines. DAP kinase expression was completely or partially silenced in Calu-1, H1792, H157, H460, and SK-MES-1 cells (Fig. 2B), which have methylated CpG islands in their promoters, but not in A549, H226, H292, H522, H596, and H1944 cells, which have an unmethylated promoter. The extent of methylation detected in the *DAP kinase* promoter was negatively correlated with the DAP kinase mRNA level in the 11 cell lines ($r = -0.77$; $P = 0.003$ by linear correlation and regression analysis; Fig. 2C). Of the H460 subclones tested, H460-12 and H460-126, which were completely methylated, had no detectable *DAP kinase* gene and protein expression (example in Fig. 3C and D) but H460-14, H460-120, and H460 -124, which were unmethylated, had detectable DAP kinase expression (example in Fig. 3C and D).

TRAIL-Induced Apoptosis of NSCLC Cells Correlates with DAP Kinase Promoter Methylation and Its Gene Expression

The 11 NSCLC cell lines with different *DAP kinase* promoter methylation status were treated with TRAIL at different doses (10, 40, and 160 ng/mL) to determine their response to TRAIL. The results (Table 1) show that cell deaths induced by TRAIL correlated with DAP kinase expression. When analyzed as a whole, the degree of cell death (% of cells dying) induced by TRAIL at low doses (10 and 40 ng/mL) were positively correlated with DAP kinase mRNA expression (10 ng/mL, $r = 0.91$, $P < 0.001$; 40 ng/mL, $r = 0,80$; $P = 0.008$ by linear correlation and regression analysis) but not at 160 ng/mL ($r = 0.46$; $P = 0.187$; Table 1). Immunochemical double staining with DAP kinase antibody and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) showed that only the DAP kinase–expressing H460 cells underwent apoptosis after 12-hour incubation with TRAIL (10 ng/mL; Fig. 3A). To address the relationship of DAP kinase expression to TRAIL-induced apoptosis, we compared two H460 subclones (H460-12 and H460-124) in which the *DAP kinase* promoter was either completely methylated or unmethylated (Fig. 3B-D). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Fig. 3E) and DNA fragmentation (Fig. 3F) analyses showed that the H460-124 cells were sensitive, whereas the H460-12 cells were resistant to TRAIL-induced cell death.

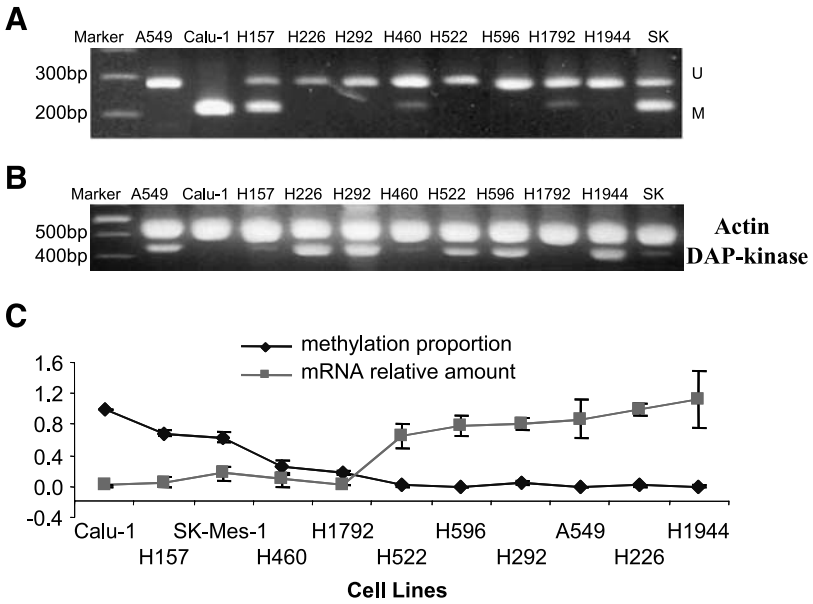

FIGURE 2. Correlation of *DAP kinase* promoter methylation status and expression of the gene in NSCLC cells. **A.** Methylation status of the *DAP kinase* promoter of the 11 NSCLC cell lines. *U,* 279-bp unmethylated PCR fragment; *M,* 218-bp methylated PCR fragment. **B.** DAP kinase mRNA expression in the 11 NSCLC cell lines. *Lane 1,* DNA size marker. **C.** Correlation of *DAP kinase* promoter methylation and expression of the gene based on data from **A** and **B**. *Points,* mean proportions of methylated *DAP kinase* promoter or relative gene expression level of three independent replicates; bars, unbiased SD.

The Restoration of DAP Kinase Expression and TRAIL Sensitivity by Promoter DNA Demethylation

To further establish that DAP kinase is involved in TRAIL-induced apoptosis in NSCLC cells, we pretreated Calu-1 and H460-12 cells, which contain a methylated *DAP kinase* promoter and lack *DAP kinase* gene expression, with 5-aza-2′-deoxycytidine (5ADC), a commonly used demethylation reagent, before TRAIL treatment. After pretreatment for 48 hours at 1 or 2 μmol/L, expression of DAP kinase was restored in both cell lines (Fig. 4A). The treatment substantially enhanced the levels of TRAIL-induced cell death/apoptosis in these cells as measured by MTT and DNA fragmentation assays (Fig. 4B and C).

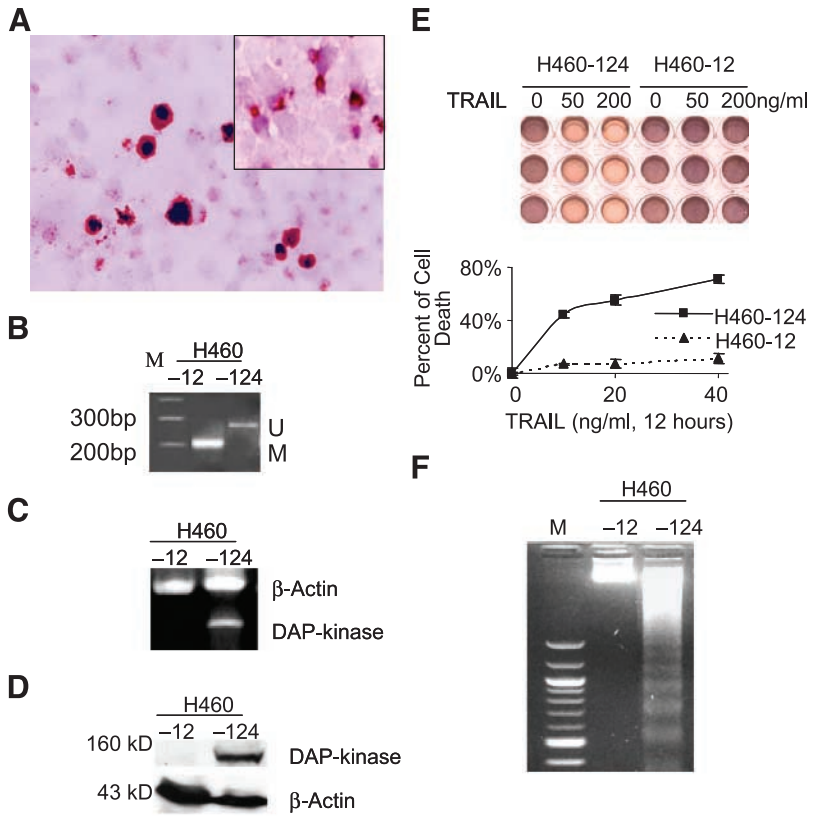

FIGURE 3. Correlation of DAP kinase expression with TRAIL-induced apoptosis in H460 cells. **A.** Double immunohistochemistry staining of H460 cells. *Black nuclear staining,* apoptosis; *red cytoplasmic staining,* expression of DAP kinase. *Top right box,* control panel without TRAIL treatment. **B.** Methylation status of two H460 subclones measured by MMSP. *U,* unmethylated fragment; *M,* methylated fragment. **C.** *DAP kinase* gene expression in H460-12 and H460-124. **D.** DAP kinase protein expression in H460-12 and H460-124. β-actin level was used as a loading control. **E.** TRAIL-induced cell death measured by MTT assay. *Top,* scanned image of the MTT results; *bottom,* measured rates of cell death. *Points,* mean cell death rates of three independent replicates; bars, SD. **F.** TRAIL-induced DNA fragmentation in the two subclones after TRAIL treatment (80 ng/mL for 12 hours). *M,* DNA size marker.

Discussion

In this study, we showed that *DAP kinase* promoter methylation and its gene expression correlate with TRAIL-induced apoptosis in NSCLC cells. We also showed that a demethylation agent activates *DAP kinase* gene expression and sensitizes the cells to TRAIL. We have shown previously that the promoter of the *DAP kinase* gene is methylated in 44% of primary NSCLC tumors (8) similar to the 46% rate we observed in NSCLC cell lines in present study. Methylation of the DAP kinase promoter in the primary tumors is associated with poor survival in patients with early-stage NSCLC (8). The methylation and lack of gene expression in hepatoma also correlated with a poor clinical outcome in patients (30). The inactivation of *DAP kinase* through its promoter methylation has been frequently detected in aggressive tumors of the brain (31), lymphoma (32), and colorectal cancer (33).

Of the 11 NSCLC cell lines studied, 5 contained a methylated *DAP kinase* promoter, including 4 that contained both methylated and unmethylated *DAP kinase* promoter, indicating that they are heterogeneous either due to differential methylation in one of the two *DAP kinase* alleles or the presence of subclones.

A highly negative correlation between *DAP kinase* methylation and gene expression was verified in the 11 NSCLC cell lines ($r = -0.77$; $P = 0.003$) and in the H460 subclones ($r = -0.97$; $P = 0.001$; data not shown). The fact that the promoter methylation status was consistent with *DAP kinase* gene expression indicates that the promoter methylation is the major mechanism to inactivate *DAP kinase*. Measurement of DAP kinase expression either by immunohistochemistry or *in situ* MSP would be helpful for predicting the functional status of the gene.

Previous studies have shown that *DAP kinase* is an important death messenger in IFN-γ, tumor necrosis factor-α, and Fas ligand–mediated apoptosis (3, 9), but *DAP kinase* involvement with TRAIL-induced cell apoptosis has not been reported. Consistent with this involvement is the observation that cells lacking DAP kinase expression were less sensitive to TRAIL-induced apoptosis than cells expressing DAP kinase. The fact that only cells expressing DAP kinase protein underwent apoptosis (Fig. 3) further supports the involvement of DAP kinase in TRAIL-induced apoptosis.

The expression of TRAIL decoy receptors DcR1 and DcR2 and death receptors DR4 and DR5 as messenger receivers directly influences TRAIL-induced apoptosis. In our previous study, we have shown DR5 was expressed, whereas decor receptors were not expressed in H157 cells (34), yet these cells were resistant to TRAIL (Table 1), suggesting the involvement of other mechanisms for this resistance. We have not observed a strong correlation between the expression of TRAIL receptors and their sensitivity to TRAIL in the 11 cell lines we studied.[2] It has been shown that the binding of death ligands to receptors might result in recruitment of the cofactor Fas-associated death domain–containing protein with formation of a death-inducing signaling complex, which results in activation of caspase-8 (18, 35). Recent studies reported that the gene for *caspase-8* is silenced preferentially by aberrant promoter methylation in

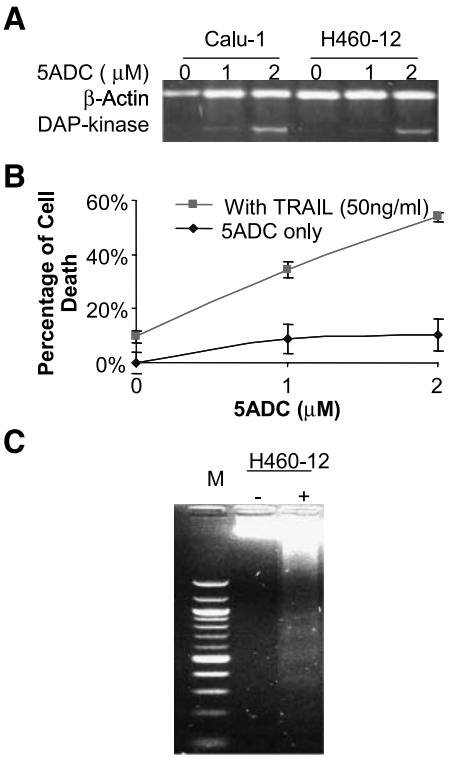

FIGURE 4. Restoration of DAP kinase expression and sensitivity to TRAIL treatment by promoter demethylation. **A.** DAP kinase expression was restored after treatment with 5ADC at the concentration of 1 or 2 μmol/L. **B.** MTT assay measuring cell death induced by TRAIL with or without 5ADC in H460-12 cells. Bars, unbiased SD of the triplicates. **C.** TRAIL-induced DNA fragmentation in H460-12 cells treated with TRAIL with (+) or without (–) 5ADC.

Table 1. Expression of DAP Kinase and TRAIL-Induced Cell Death in NSCLC Cell Lines

Cell Lines	DAP Kinase Expression*	TRAIL-Induced Cell Death (% of Control)		
		10 ng/mL†	40 ng/mL	160 ng/mL
Calu-1	0.000	0.00	6.79	7.22
H1792	0.022	0.00	10.79	24.87
H157	0.059	0.00	0.00	23.97
SK-MES-1	0.176	0.87	13.47	62.26
H522	0.644	9.48	17.95	35.81
H596	0.783	11.20	31.24	59.26
H292	0.812	12.47	13.97	43.22
A549	0.874	8.55	20.31	45.39
H226	0.999	12.61	19.13	29.22
H1944	1.120	25.85	30.05	45.70
r‡ (DAP Kinase Expression: TRAIL-Induced Cell Death)		0.91	0.80	0.46
P		0.001	0.008	0.187

*mRNA relative amount to β-actin detected with multiplex reverse transcription-PCR.
†TRAIL, incubated for 12 hours, MTT analysis.
‡Linear correlation and regression analysis.

[2] Unpublished data.

neuroblastomas, bronchial carcinoids, and small cell lung cancer but not in NSCLC (36, 37). Nevertheless, a potential relationship between TRAIL receptors and DAP kinase warrants further investigation.

When NSCLC cells containing a methylated DAP kinase promoter were treated with 5ADC (a demethylation agent), the cells restored expression of DAP kinase and TRAIL sensitivity (Fig. 4). These results support the involvement of DAP kinase in TRAIL-induced apoptosis and provide justification for combination of demethylation agent(s) with death ligand(s) to improve the therapeutic effects.

Although different cancer cell lines carry distinct patterns of methylated promoters and 5ADC treatment sensitized TRAIL response in multiple cell lines (Fig. 4),[3] it is still possible that activation of other genes by 5ADC treatment contributes to the sensitization. Additional experiments to specifically activate *DAP kinase* or specifically inhibit *DAP kinase* may provide more direct evidence to support the role of DAP kinase in rescuing TRAIL-induced apoptosis in NSCLC.

Materials and Methods

Cell Lines, Cell Culture, and Cell Subcloning

Human NSCLC cell lines A549, Calu-1, H157, H226, H292, H460, H522, H596, H1792, H1944, and SK-MES-1 were obtained from the American Type Cell Culture (Rockville, MD) and grown in DMEM (Life Technologies, Rockville, MD) containing 10% fetal bovine serum and antibiotics. The cells were maintained at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air in monolayers.

For each subcloning experiment, 30 to 50 H460 parental cells were seeded into a 100-mm dish and incubated for 4 hours; attached single cells were then identified by marking the back of the dish. After being cultured for 7 days, the individual clones originating from the marked single cells were transferred to 24-well plates for further culture before being transferred to 100-mm dishes for further expansion. All the subclones were harvested once on the same day to avoid biological variation in different generations.

cDNA Preparation and Multiplex Reverse Transcription-PCR

Total RNA was extracted and purified from cultured cells using RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA (1 μg) was used to synthesize cDNA with SuperScript II reverse transcriptase (Life Technologies) in 20 μL, and the final product was diluted to 100 μL. The cDNA was then used for the quantitative assay of DAP kinase expression by multiplex PCR. The multiplex reverse transcription-PCR was carried out under optimal conditions after which a linear correlation between cycle numbers and product amount of PCR was obtained (data not shown). PCR reaction mixture (10 μL) contained 1 μL diluted cDNA sample, 1 unit Hotstart polymerase (Qiagen), 0.1 mmol/L deoxynucleotide triphosphates (dATP + dTTP + dCTP + dGTP), 1 μL DMSO, and 50 ng DAP kinase primers (forward 5′-GACACCGGCGAGGAACTTGGC-3′ and reverse 5′-AAAGTCAATGATCTTGATCCGA-3′). The reaction was done according to the following program: at 94°C for 15 minutes for activating the polymerase; for 34 cycles at 94°C for 30 seconds, at 60°C for 1 minute, and at 72°C for 1 minute; and at 72°C for 7 minutes. At the 6th cycle of the 2nd step, β-actin primers (forward 5′-GTTGCTATCCAGG CTGTGC-3′ and reverse 5′-GCATCCTGTCGGCAATGC-3′) were added into the reaction mixture as an internal control. The PCR products were electrophoresed on 2.5% agarose gel. Then, the gel was stained with ethidium bromide and photographed under UV light. The absorbance of the DAP kinase–specific fragments was measured by scanning densitometry using the absorbance of the internal control (β-actin) as the standard. The relative amount of the DAP kinase mRNA was calculated as absorbance of *DAP kinase*/*actin.*

DNA Isolation and MMSP

DNA was extracted and purified from cultured cells. The cells were collected and digested in 200 μL of digestion buffer containing 50 mmol/L Tris-HCl (pH 8.0), 1% SDS, and 0.5 mg/mL proteinase K and incubated at 42°C for 36 hours. The digested products were purified with phenol-chloroform twice. DNA was then precipitated using the ethanol precipitation method and recovered in distilled DNase-free water. Bisulfite modification of DNA was done as described by Herman et al. (28). Briefly, 1 μg of the DNA from each sample was denatured using NaOH and then treated with sodium bisulfite (Sigma, St. Louis, MO) for 16 hours. After purification using the Wizard DNA purification kit (Promega, Madison, WI), the purified DNA was treated again with NaOH, precipitated with ethanol, and recovered in distilled water. The relative quantity of methylated and unmethylated DAP kinase promoters was determined by the MMSP. The primers specific for unmethylated CpG islands were (forward) 5′-TTGTGAGTTGTTGATTTTTTTTTGT-3′ and (reverse) 5′-ATACACAATAAAACACACCAACAAA-3′, which cover a 279-bp fragment; the primers specific for methylated CpG islands were (forward) 5′-CGAGTTGTC-GAGTTTTTTTCGC-3′ and (reverse) 5′-CCGCGC-AAAACCCGCAACG-3′, which cover a 218-bp fragment. The PCR products were separated in 2.5% of agarose gel, stained with ethidium bromide, and then photographed under UV light. The absorbances of the unmethylated fragments (U) at 279 bp and methylated fragments (M) at 218 bp were determined by scanning densitometry of the photographs. The proportion of methylation alleles was calculated as $M / U + M$.

Protein Extraction and Western Blot Analysis

Cellular proteins were collected in lysis buffer containing 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 μg/mL phenylmethylsulfonyl fluoride, 30 μg/mL aprotinin, and 50 mmol/L Tris-HCl (pH 8.0). The samples were then placed on ice for 60 minutes and then centrifuged at 14,000 × g at 4°C for 30 minutes. The protein concentration was measured using a protein assay kit (Bio-Rad, Hercules, CA). Each protein sample (30 μg) was subjected to SDS-PAGE

[3] Unpublished data.

using 8% denaturing polyacrylamide gel. The proteins were then transferred to Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The nitrocellulose membranes were incubated in a blocking solution containing 5% bovine skim milk in 10 mmol/L PBS containing 0.1% Tween 20 for 1 hour followed by incubation for 3 hours with monoclonal anti-DAP kinase antiserum (DAPK-55, Sigma) at a concentration of 1:1,000 or monoclonal anti-β-actin (AC15, Sigma) antibody at a concentration of 1:5,000. The membranes were washed with PBS and then incubated with the secondary anti-mouse antibody supplied in the enhanced chemiluminescence kit (Amersham Pharmacia Biotech) for 1 hour. After this incubation, the membranes were washed thrice in PBS, developed in enhanced chemiluminescence solution for 1 to 2 minutes, and exposed to X-ray film for chemiluminescence detection of the positive protein bands.

TUNEL and DAP Kinase Protein Detection by Immunohistochemistry Double Staining

H460 cells were seeded on two glass slides, incubated for 24 hours, and then treated with or without TRAIL (10 ng/mL, Calbiochem, San Diego, CA) for 12 hours. The slides were then fixed in 10% paraformalin [prepared with PBS (pH 7.4)] for 20 minutes and washed in PBS for 5 minutes. The TUNEL assay was done with TUNEL kit (Intergen, Burlington, MA) according to instructions provided by the manufacturer. 3,3′-Diaminobenzidine-conjugated nickel was used as a chromogen to stain the apoptotic cells. The slides were then subjected to double staining with immunohistochemistry to detect the expression of DAP kinase protein by the following procedures: the slides were treated thrice for 5 minutes in a microwave oven with 10 mmol/L citrate buffer (pH 6.0) to retrieve the antigenicity, immersed in methanol containing 0.3% hydrogen peroxidase for 20 minutes to block the endogenous peroxidase, and incubated in 2.5% blocking serum to reduce nonspecific binding. The slides were incubated overnight at 4°C with primary anti-DAP kinase monoclonal antiserum (DAPK-55, Sigma) at a dilution 1:160 and then processed using standard avidin-biotin immunohistochemistry according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Vector NevaRed (Vector Laboratories) was used as a chromogen to detect DAP kinase expression as red in the cytoplasm. Methyl green was used for nuclear counterstaining.

DNA Fragmentation Assay

After treatment with TRAIL, both floating and attached cells were collected, pelleted, and resuspended in Tris-EDTA buffer (pH 8.0). The plasma membrane of the cell was lysed on ice in a mixture of 10 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, and 0.5% Triton X-100 for 15 minutes. The lysate was centrifuged at 12,000 × *g* for 15 minutes to separate the soluble (fragmented) from pellet (intact genomic) DNA. The soluble DNA was treated with RNase A (50 μg/mL) at 37°C for 1 hour and proteinase K (100 μg/mL) in 0.5% SDS at 50°C for 2 hours, extracted with phenol-chloroform, precipitated in ethanol, electrophoresed on a 1.8% agarose gel, and stained with ethidium bromide. The gels were then photographed under UV illumination.

Demethylation

Cells were treated with 5ADC (Sigma) at 1 or 2 μmol/L concentrations for 48 hours before further treatment with TRAIL.

MTT Assay

About 30,000 cells of each line were seeded in 96-well plates in 0.1 mL DMEM in triplicate and incubated for 24 hours. The medium was then replaced with medium containing a designated concentration of TRAIL and incubated for another 12 hours. At the end of treatment, 10 μL (5 mg/mL) MTT (Sigma) were added and incubated for 3 hours. The medium containing MTT was absorbed off and washed with PBS carefully, and 0.1 mL DMSO was added to each well. Absorbances of controls (A_c) and experimental samples (A_t) at a wavelength of 540 nm with background subtraction at 620 nm were measured using the E_{max} precision microplate reader (Molecular Devices, Sunnyvale CA). Cell death (%) is calculated as $100 \times (1 - A_t / A_c)$.

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