Death-associated protein kinase is essential for the survival of various types of uterine cancer cells

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Abstract. We recently showed that targeted knockdown of death-associated protein kinase (DAPK) expression induces apoptosis in the human endometrial adenocarcinoma cell line HHUA. To investigate the possibility that DAPK may represent a molecular target for anticancer therapies for advanced uterine cancers, we examined the effects of DAPK siRNA transfections on the viability of five different human uterine cancer cell lines. The five uterine cell lines comprised three differentiated endometrial adenocarcinomas, one leiomyosarcoma and one carcinosarcoma. Cell death assays showed that the DAPK siRNA transfection significantly increased the cell death in all five uterine cancer cells examined. Ribonuclease protection assays did not show any remarkable changes in the bcl-2 family gene expressions after the DAPK siRNA transfection in HHUA cells. Since DAPK-mutant mice were reported to be fertile and do not show lethality, DAPK may play a central role in the immortalization and carcinogenesis of uterine cancer cells, possibly without bcl-2 family-related apoptotic regulation. These results indicate that DAPK can be a convincing candidate for molecularly targeted anticancer therapies for patients with various types of advanced uterine cancers, including carcinosarcoma and leiomyosarcoma.

Introduction

Death-associated protein kinase (DAPK) is a Ca^{2+}/calmodulin-dependent serine/threonine kinase that functions as a positive mediator of apoptosis triggered by IFN-ß, TNF-α, anti-Fas antibodies, TGF-ß, c-myc and E2F oncogenes, ceramide and detachment from the extracellular matrix (1-8). Moreover, loss of DAPK expression has been implicated in tumorigenesis and metastasis (9,10), thereby suggesting a crucial role for DAPK in the apoptotic process under pathological conditions. On the other hand, several lines of evidence have indicated that DAPK may have an antiapoptotic function. Inhibition of DAPK expression in HeLa cells, 3T3 fibroblasts and primary human vascular smooth muscle cells with an antisense DAPK was found to increase apoptosis (11,12). These studies support the notion that DAPK plays cytoprotective roles under certain cell survival conditions.

In a previous study, we detected reduced DAPK protein expression in several ovarian and uterine carcinoma cell lines while a few uterine cancer cell lines showed high DAPK protein expression (13). We recently reported that targeted knockdown of DAPK expression in the HHUA cell line (14), a highly differentiated endometrial adenocarcinoma cell line, induced cell death by enhancing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in an autocrine/paracrine manner (15). These findings suggest that endogenous DAPK can be a potential candidate for molecularly targeted anticancer therapies for patients with endometrial adenocarcinoma. Therefore, we examined whether DAPK represents a candidate molecule for targeted anticancer therapies for various advanced uterine cancer patients. To achieve this, we utilized specific DAPK siRNAs to knockdown endogenous DAPK expression in five human uterine cancer cell lines to directly investigate the role of endogenous DAPK in uterine cancer cell survival.

Materials and methods

Cell lines and culture. The HEC-1 cell line (16) (moderately differentiated human endometrial endometrioid adenocarcinoma), Kuramochi cell line (17) (human ovarian undifferentiated carcinoma), MCAS cell line (18) (human ovarian mucinous cystadenocarcinoma) and HeLa cell line (19) (human cervical adenocarcinoma) were obtained from the Japan Resources Cell Bank (Tokyo, Japan). The HHUA cell line (14) (highly differentiated human endometrial endometrioid adenocarcinoma), SKN cell line (20) (human uterine leiomyosarcoma) and HTMMT cell line (21) (human uterine carcinosarcoma including a leiomyosarcoma component) were obtained from the Riken Cell Bank (Tsukuba, Japan). The Ishikawa cell line (22) (highly differentiated human endometrial endometrioid adenocarcinoma) was a kind gift from Dr M. Nishida (Kasumigaura Hospital, Ibaraki, Japan). All cell lines were cultured in OPTI-MEM (Invitrogen Corp.,

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Carlsbad, CA) supplemented with 5% FBS (Equitech Bio Inc., Ingram, TX), penicillin (100 U/ml), streptomycin (100 U/ml) and Fungizone (0.25 μg/ml; Invitrogen Corp.) in the presence of 5% CO_2 and 95% air at 37˚C.

Transfection of DAPK siRNAs. Two DAPK siRNA duplexes were designed and synthesized by iGENE Therapeutics Inc. (Tsukuba, Japan). The DAPK siRNA sequences were as follows: DAPK siRNA-1 duplex, 5'-CAACAUCAUGC AAAGUGAAACAGUU-AG-3'/3'-AU-GUUGUAG UACGUUUCACUUUGUCAA-5'; DAPK siRNA-2 duplex, 5'-AGCCAAGAAUUAAGCUCAAGCUGUU-AG-3'/3'-AU-UCGGUUCUUAAUUCGAGUUCGACAA-5'. A negative control siRNA was purchased from Ambion Inc. (Austin, TX). Lipofectamine 2000 (Invitrogen Corp.) was used as the transfection reagent according to the manufacturer's instructions. For experiments, cells were seeded in 6-well plates ($2.5x10^5$ cells/well) or 10-cm dishes ($2x10^6$ cells/dish), cultured for 24 h and then transfected with the DAPK siRNAs or control siRNA at a final concentration of 50 nM. Subsequently, the cells were cultured for 24-48 h for mRNA analyses and 48-72 h for protein assays before being harvested as indicated.

Western blot analysis. For Western blot analysis of siRNA-transfected cells, the cells were collected at 48-72 h after transfection with the DAPK siRNAs or control siRNA, and lysed in PBS containing 1% NP-40, 0.1% SDS, Complete protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN) and 1 mM PMSF. The protein concentrations of the cell lysates were quantified by Coomassie Plus Protein Assays (Pierce Biotechnology Inc., Rockford, IL). Equal amounts of total proteins were separated by SDS-PAGE using a 7.5% gel, and then transferred to a polyvinylidene fluoride membrane (ATTO Corp., Tokyo, Japan). After sequential incubations with primary and secondary antibodies, the immunocomplexes on the membranes were detected using ECL or ECL plus kits (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies used were purchased from the following sources: mouse monoclonal anti-DAPK antibody (BD Pharmingen, San Diego, CA); mouse monoclonal anti-ß-actin antibody (Sigma, St. Louis, MO).

Early cell death assay by fluorescence microscopy. HHUA cells were transfected with the control siRNA or DAPK siRNAs as described above. At 1 day after the transfections, the cells were stained with Alexa Fluor 488-conjugated annexin V (Molecular Probes, Eugene, OR) for 1 h at room temperature according to the manufacturer's protocol, washed with PBS and counterstained with 10 μg/ml propidium iodide (Sigma) to stain all the cell nuclei and allow determination of the total cell number. The fluorescently stained cells were washed with PBS and viewed under a fluorescence microscope equipped with fluorescein and rhodamine filters. Images of the cells were acquired with a digital camera (HV-C20S; Nikon, Tokyo, Japan) and the numbers of stained cells were counted. Early cell death ratios were determined as the percentages of Alexa Fluor 488-stained cells relative to the total numbers of propidium iodide-stained cells. The mean ± SD values of the early cell death ratios (n=9) were statistically compared between the control siRNA-transfected and DAPK siRNA-transfected cells.

Advanced cell death assay by counting non-adherent cell numbers. Since all the uterine cancer cells examined in this study proliferated in an adherent manner on the culture dishes, cells with advanced death became detached from the dishes. Therefore, the non-adherent cell number ratios represented the advanced cell death ratios in the cultures. The cancer cells were transfected with the control siRNA or DAPK siRNAs as described above. At 48 h after the transfections, adherent and non-adherent cells were collected and counted respectively. Advanced cell death ratios were determined as the percentages of non-adherent cells relative to the total cell numbers. The mean ± SD values of the advanced cell death ratios (n=4) were statistically compared between the control siRNA-transfected and DAPK siRNA-transfected cells.

RNase protection assay (RPA). A multiprobe RPA was performed using a RiboQuant™ hAPO-2C Multiprobe Template Set and an RPA Kit (BD Pharmingen) according to the manufacturer's protocol. Biotin-conjugated probes were prepared using a Non-Rad *In Vitro* Transcription Kit (BD Pharmingen). The probes were combined with 10 μg of total RNA isolated from DAPK siRNA-transfected or control siRNA-transfected HHUA cells. After denaturation at 95˚C for 3 min, the mixtures were allowed to hybridize at 56˚C for 12-16 h before RNase digestion. RNA hybrids were separated in 4.75% acrylamide/8 M urea denaturing gels, and the protected fragments were transferred to positively charged nylon membranes. The fragments on the membranes were detected using the RPA Kit and the protected fragments were quantified with a Luminocapture apparatus and lane analyzer software (ATTO Corp.). The relative expression of each mRNA species was calculated after normalization by the expression of the housekeeping gene L32.

Statistical analysis. The data were expressed as means ± SD. Comparisons between experimental groups were performed by analysis of variance (ANOVA). If the ANOVA was significant, *post hoc* comparisons were conducted using Scheffe's test. The level of statistical significance was set at $p<0.05$.

Results

Western blot analysis of the human ovarian and uterine cancer cell lines revealed that various types of cancer cells, except for MCAS cells, constitutively expressed DAPK protein (Fig. 1A), as reported previously (13). The endometrial adenocarcinoma cell lines HEC-1 and Ishikawa expressed slightly higher DAPK protein levels than HeLa cells, from which the DAPK cDNA cloning was performed (1). Moreover, both the endometrial adenocarcinoma cell line HHUA and uterine leiomyosarcoma cell line SKN expressed much higher DAPK protein levels than HeLa cells. As reported previously (15), transfection of the DAPK siRNAs strongly suppressed endogenous DAPK protein expression in HHUA cells, which expressed high DAPK protein levels (Fig. 1B).

Recently, it was reported that DAPK siRNA transfection into HHUA cells increased DNA fragmentation, as evaluated

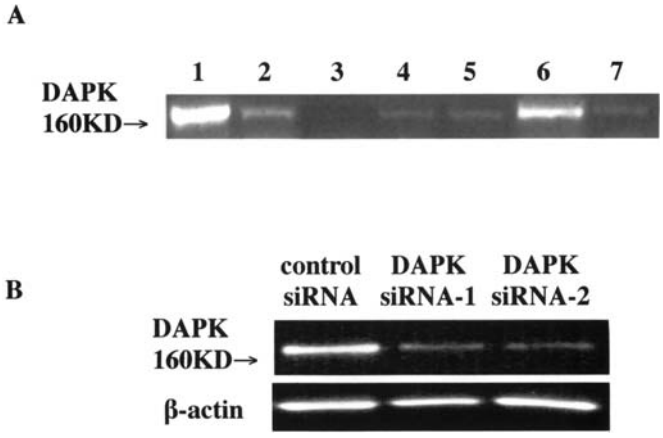

Figure 1. Western blot analyses of DAPK protein expression levels in the human cancer cell lines and the DAPK siRNA-transfected HHUA cells. (A) DAPK protein expression levels in the human cancer cell lines. 1, SKN; 2, Kuramochi; 3, MCAS; 4, Ishikawa; 5, HEC-1; 6, HHUA; 7, HeLa. (B) DAPK protein and ß-actin protein expression levels in the DAPK siRNA-transfected HHUA cells (DAPK siRNA-1 and DAPK siRNA-2) and control siRNA-transfected cells.

by flow cytometric DNA analyses and activated caspase assays (15). In the present study, apoptosis was measured by using Alexa 488-conjugated annexin V labeling. Annexin V binds to phosphatidylserine exposed on the surface of apoptotic cells, which is a reliable method for *in vitro* apoptosis research (23-25). As shown in Fig. 2, transfection of the DAPK siRNAs into HHUA cells increased the numbers of annexin V-labeled cells, representing early apoptotic cells. Next, we examined the effects of DAPK siRNA transfections into five different uterine cancer cell lines including HHUA cells. As shown in Fig. 3, microscopic findings revealed that the DAPK siRNA transfections into the uterine cancer cells apparently increased the numbers of non-adherent cells compared with the control siRNA-transfected cells. Since cells with advanced cell death became detached from the bottom of the culture dishes, the numbers of non-adherent cells relative to the total cell numbers were compared between the DAPK siRNA-transfected cells and the control siRNA-transfected cells (Figs. 3 and 4). In the five uterine cancer cell lines examined, the DAPK siRNA transfections significantly increased the ratios of non-adherent cell numbers, representing the advanced cell death ratios.

Recently, it was reported that the DAPK siRNA transfection-induced cell death in HHUA cells was partly caused by enhancement of TRAIL-mediated apoptosis (15). Therefore, in this study, RPAs were performed to examine whether the mRNA expressions of bcl-2 family genes were affected by the DAPK siRNA transfections. However, no remarkable changes in the bcl-2 family mRNA expression levels were observed in the DAPK siRNA-transfected cells (Fig. 5).

Discussion

This study is the first to demonstrate that DAPK regulates the survival of various kinds of human uterine cancer cell lines. Our recent study showed that targeted knockdown of endogenous DAPK protein expression by DAPK siRNA transfections can induce apoptosis in HHUA cells, which are highly differentiated endometrial endometrioid adenocarcinoma cells expressing estrogen receptors and progesterone receptors (15). In this study, we have shown that DAPK siRNA transfections can also induce cell death in two other differentiated

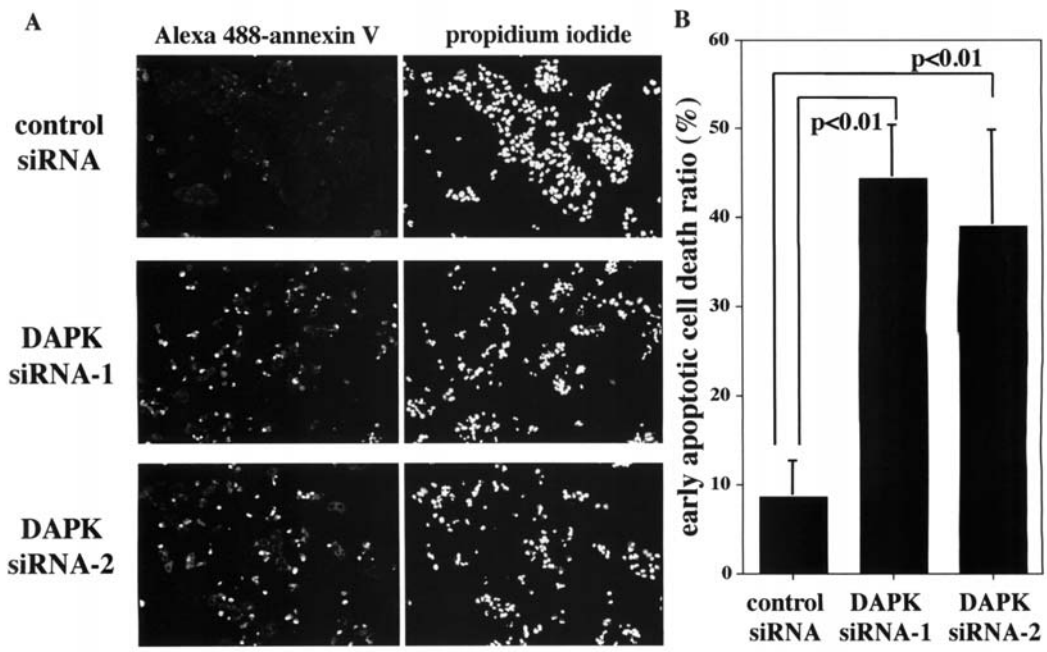

Figure 2. Fluorescence microscopic assays of the early cell death ratios in the DAPK siRNA-transfected and control siRNA-transfected HHUA cells. (A) Fluorescence microscopic findings of the siRNA-transfected HHUA cells. Alexa 488-annexin V staining (green fluorescence) indicates early apoptotic cells while propidium iodide staining (red fluorescence) shows the cell nuclei. (B) Early cell death ratios were calculated as the percentages of the Alexa 488-stained cell numbers relative to the propidium iodide-stained cell numbers (n=9). The DAPK siRNAs significantly increase the early cell death ratios in HHUA cells.

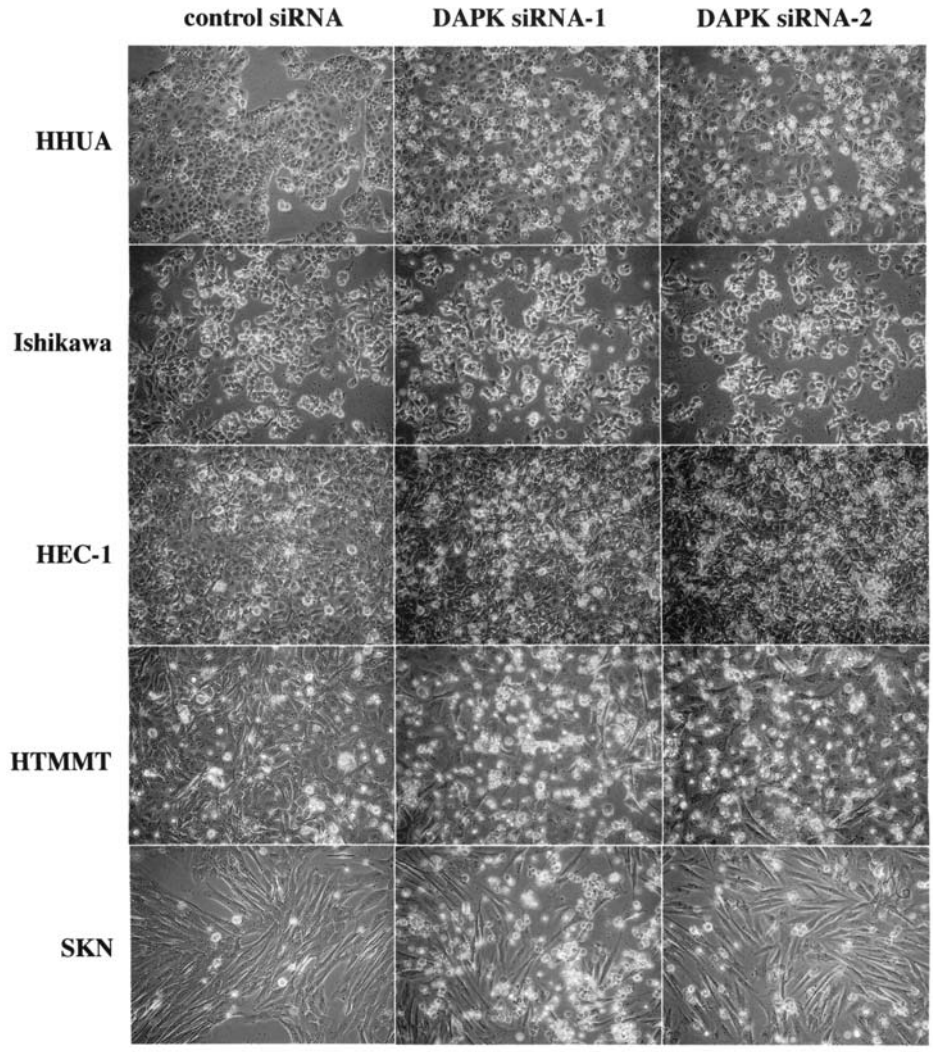

Figure 3. Phase-contrast microscopic findings for the DAPK siRNA-transfected and control siRNA-transfected uterine cancer cells. The images show the microscopic findings for five cancer cell lines at 42 h after the siRNA transfections. In the DAPK siRNA-transfected cells, increased cell detachment from the culture dishes is observed.

endometrial endometrioid adenocarcinoma cell lines, HEC-1 and Ishikawa. Moreover, DAPK siRNA transfections were also able to induce cell death in both the uterine leiomyosarcoma cell line SKN and uterine carcinosarcoma cell line HTMMT, which are clearly different uterine cancer cells from endometrial adenocarcinoma cells. Therefore, targeted knockdown of endogenous DAPK protein expression by DAPK siRNA transfections was able to induce cell death in all five human uterine cancer cell lines examined, which have different histotypes from one another. These results indicate that DAPK plays an important role in the survival of various uterine cancer cells.

Targeted knockdown of endogenous DAPK protein expression by DAPK siRNA transfections induced cell death in the uterine carcinosarcoma cell line HTMMT and uterine leiomyosarcoma cell line SKN in addition to the human endometrial adenocarcinoma cell lines HHUA, Ishikawa and HEC-1. These results suggest that DAPK may be essential for the survival of various types of human uterine cell lineages. However, we previously observed that DAPK-mutant mice showed similar survival rates to normal adult mice, and became pregnant and had offspring when they were bred for several years to investigate their nervous systems and ovarian functions (unpublished data). Taken together, these observations suggest that DAPK is neither a lethal molecule for an individual or an essential molecule for reproduction and that targeted deletion of endogenous DAPK functions does not induce cell death in normal murine uterine cells. Therefore, DAPK appears to regulate the survival of uterine cancer cells but not of normal uterine cells, indicating that it may play an important role in the immortalization and/or carcinogenesis of uterine cells.

It is notable that targeted knockdown of endogenous DAPK protein expression was able to induce cell death in carcinosarcoma cells and leiomyosarcoma cells. Most patients with uterine carcinosarcoma or uterine leiomyosarcoma clinically show a poor prognosis because these cancer cells are well known to be much more multidrug-resistant and radioresistant than other cancer cells. Therefore, DAPK may represent a convincing molecular target for the treatment of patients with various unresectable uterine cancers. The present study has shown that specific molecularly targeted therapies against DAPK can induce cell death in uterine cancer cells without any remarkable effects on normal uterine cells. Therefore, we are presently investigating the DAPK signaling cascades

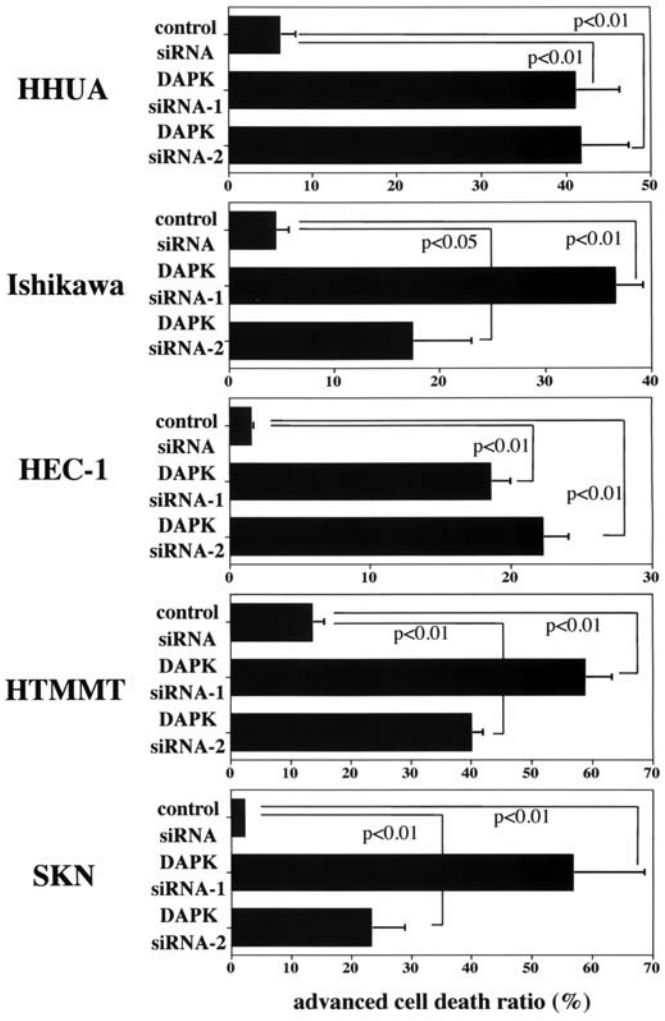

Figure 4. Advanced cell death ratios in the DAPK siRNA-transfected uterine cancer cell lines. Non-adherent cell numbers were counted at 48 h after the siRNA transfections. The DAPK siRNAs significantly increase cell detachment from the culture dishes and the advanced cell death ratios in all five uterine cancer cell lines.

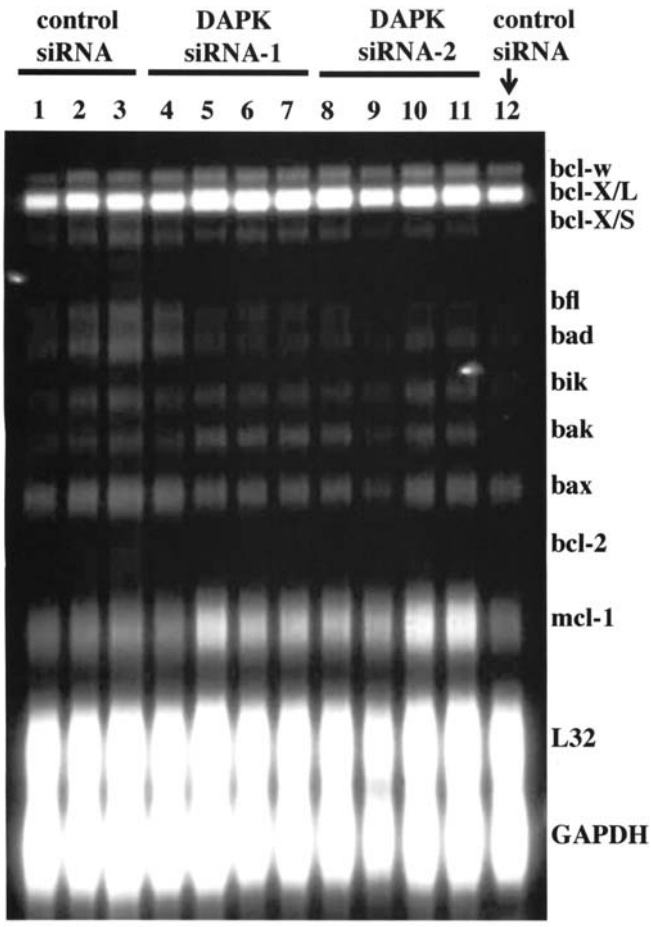

Figure 5. RPAs for the effects of the DAPK siRNA transfections on bcl-2 family gene expressions in HHUA cells. The DAPK siRNA-transfected cells show no apparent differences in the mRNA expression for bcl-2 family genes compared with the control siRNA-transfected cells. The panel shows a representative set of electrophoresis data for the DAPK siRNA-transfected and control siRNA-transfected cells. Lanes 1-3 and 12, control siRNA-transfected cells (n=4); lanes 4-7, DAPK siRNA-1-transfected cells (n=4); lanes 8-11, DAPK siRNA-2-transfected cells (n=4).

in human uterine cancer cells to identify DAPK-regulated molecules, which may be also potential candidates for molecularly targeted anticancer therapies.

There are many apoptosis-regulating molecules. Bcl-2 family molecules are well-known apoptosis-regulating molecules that can enhance or inhibit various types of apoptotic signals. In this study, we examined the effects of the DAPK siRNA transfections on the mRNA expressions of bcl-2 family genes in HHUA cells. However, we did not find any apparent common changes in the mRNA expressions of the bcl-2 family genes in the DAPK siRNA-transfected HHUA cells. These results suggest that the cell death induced in uterine cancer cells by DAPK siRNA transfections may not be regulated by bcl-2 family genes. The findings further suggest that bcl-2 family molecules cannot be effective molecular targets for anticancer targeted therapies for endometrial adenocarcinoma patients.

There are many reports showing that DAPK mRNA expression is mainly regulated by the methylation status of the DAPK gene promoter regions (26-32). In human uterine cancer cells and human ovarian cancer cells, DAPK mRNA expression is partly regulated by the methylation status of the DAPK gene promoter regions (14). The cisplatin sensitivity of human cervical cancer cells can also be affected by the methylation status of the DAPK gene promoter regions (33). Based on the present findings that DAPK siRNA transfections into various types of uterine cancer cells induced cell death, suppression of DAPK expression by methylation regulators or DAPK siRNA transfections may enhance the antitumor effects of conventional anticancer chemotherapies.

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