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Metadherin regulates radioresistance in cervical cancer cells

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Abstract. Metadherin (MTDH) promotes cancer metastasis, chemoresistance, invasion and angiogenesis. Upregulation of MTDH is correlated with both progression and poor clinical outcome of many types of cancers; however, there is currently no information regarding the role of MTDH in radiation sensitivity. Here, we investigated the effects of MTDH on the radiosensitivity of cervical cancer cells using the SiHa cell line. We discovered that cervical cancer cells in which MTDH was knocked down had significantly increased radiosensitivity as measured by a clonogenic assay. MTDH knockdown cells also had increased apoptosis and a decreased proportion of cells arrested in the G2 phase after radiation treatment. MTDH knockdown also weakened the repair of DNA double-strand breaks (DSBs) induced by radiation. These results indicate that MTDH affects the radiosensitivity of cervical cancer cells and that MTDH may be a novel target to improve cervical cancer radiation response.

Introduction

Cervical cancer is one of the most common cancers in women worldwide. There are approximately 500,000 new cervical cancer cases in the world each year, and more than 80% of these cases occur in developing countries (1,2). The current management for cervical cancer consists primarily of surgery, radiation and chemotherapy. The combination of external beam radiotherapy and intracavitary brachytherapy is regarded as the standard treatment for cervical cancer (3). Intracavitary brachytherapy allows for the delivery of higher doses of radiation to the tumor with less exposure to normal tissue (4). While definitive radiotherapy has high cure rates in early stage disease, the survival rates in locally advanced cervical cancer cases remains poor, with a 5-year overall survival of only 66% (5). Therefore, more effective strategies for the treatment of locally advanced cervical cancer need to be discovered.

Many genetic alterations, such as EGFR, c-erbB-2 and COX-2, have been discovered to be associated with survival and/or response to chemo- and radiotherapy (6). Previous studies have shown that EGFR expression decreased radiosensitivity of cervical cancer (7-9). Moreover, inhibition of EGFR with cetuximab plus radiotherapy increased overall survival when compared with the use of radiotherapy alone (10). Metadherin (MTDH, also known as AEG-1) was originally reported as a protein induced in primary human fetal astrocytes infected with HIV-1 or treated with either HIV gp120 or TNF-α (11,12). MTDH synergizes with oncogenic Ha-ras to enhance the soft-agar-colony-forming ability of non-tumorigenic immortalized melanocytes (13). Recently, clinical studies have revealed that MTDH is overexpressed in various malignancies, including breast, prostate, glioma, hepatocellular, and esophageal cancer and is associated with disease progression and poor clinical outcomes (14). Moreover, MTDH has been found to promote cancer metastasis, chemoresistance, invasion and angiogenesis (15-21), suggesting that it may function as an oncogene. Furthermore, MTDH knockdown has been found to sensitize breast cancer and neuroblastoma cells to cisplatin (15,17). Cisplatin is a platinol systemic agent that is widely used in the treatment of bladder, ovarian, cervical, testicular, and head and neck cancer (22). Cisplatin can cause cross-links in DNA (23). Therefore, based on the speculation that MTDH may similarly impact the radiosensitivity of cancer cells, we studied the role of MTDH in radioresistance.

In this study, we downregulated the expression of MTDH in a cervical cancer cell line (SiHa) and measured cell survival with a clonogenic assay. We discovered that knockdown of MTDH increased the radiosensitivity of SiHa to X-ray, and we further investigated the mechanism by which MTDH affects the radiosensitivity of cancer cells.

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Materials and methods

Cell culture. The human cervical squamous carcinoma cell line SiHa (24) [American Type Culture Collection (ATCC) Manassas, VA, USA] was routinely maintained in Minimum Essential Medium (MEM, Invitrogen, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (FBS, Haoyang Biological Manufacture Co., Ltd., Tianjin, China), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in an atmosphere of 5% CO_2.

Irradiation. The cells were irradiated at room temperature using a Faxitron Cabinet X-ray System (Faxitron, Wheeling, IL, USA) at a dose rate of 0.36 Gy/min. The X-rays were filtered through a 0.5-mm aluminum filter.

Construction of stably transfected cell lines. Endogenous MTDH was knocked down using the pSUPER.retro.puro vector containing short hairpin RNA (shRNA) against MTDH. The shRNA oligonucleotide sequence was ATGAACCAGAA TCAGTCAGC. The cells were transfected with either shRNA against MTDH or a control vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and mixed resistant clones were collected and cultured in medium containing puromycin (Invitrogen). Western blot analysis was used to detect the expression of MTDH in all of the cell lines described above.

Cell viability test. Cell viability was evaluated using the clonogenic assay. Briefly, exponentially growing cells were seeded into 6-well plates (400 cells/well) (Costar, Corning, NY, USA) and incubated for 12 h to allow the cells to attach. The cells were then irradiated with increasing fraction size (0, 1, 2, 3 and 4 Gy) and cultured for 12 days. The colonies were fixed with methanol for 30 min and stained with crystal violet. Colonies containing more than 50 cells were counted. The surviving fraction was calculated as follows: (number of colonies formed)/(number of cells plated x plating efficiency), and the plating efficiency was defined as: (number of colonies formed)/(number of cells plated for non-irradiated controls). Experiments were performed in triplicate and repeated three times.

Cell cycle analysis. To investigate cell cycle alterations after irradiation, cells (30-40x10^5) were plated on 60-mm culture dishes (BD Biosciences, San Jose, CA, USA) overnight and irradiated with the indicated doses. Cells were then harvested by digestion with 0.25% trypsin and washed twice with phosphate-buffered saline (PBS). Subsequently, 1x10^6 cells were resuspended in 1-ml staining solution (50 μg/ml PI, 20 μg/ml RNase A) and incubated for 30 min at room temperature protected from light and filtered using a 300-mesh nylon meshwork prior to detection. The DNA content was analyzed by flow cytometry with a FACScalibur (BD Biosciences). The results from 10,000 cells were analyzed with the ModFit software.

Apoptosis analysis. Cells (20-30x10^5) were plated on 60-mm culture dishes overnight and irradiated with the indicated doses. Proportions of apoptotic cells with/without irradiation were detected by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences). The experiment was performed according to the manufacturer's protocol. Floating cells were collected, and the attached cells were trypsinized and washed twice with ice-cold PBS at the indicated time. Then 1x10^5 cells were resuspended in 100 μl 1X binding buffer, and 5 μl of Annexin V-FITC and 5 μl of PI were added following incubation for 15 min at room temperature protected from light. Finally, 400 μl of 1X binding buffer was added to each sample. The samples were analyzed by flow cytometry within 1 h using the FACScalibur (BD Biosciences) with the CellQuest software. Cells that stained positive for FITC Annexin V and negative for PI were identified as apoptotic cells.

Immunofluorescence. Cells were irradiated with 0.5 Gy after attaching to chamber slides (Nest, Wuxi, China) in 12-well plates. At 0, 0.5, 2, 4 and 8 h post-irradiation, the cells were washed in PBS three times and fixed in 4% paraformaldehyde for 15 min at room temperature. The cells were then permeabilized in 0.2% Triton X-100 for 8 min after washing four times in PBS and blocked with 10% goat serum in PBS overnight at 4˚C, followed by incubation with mouse anti-human phospho-Histone H2AX monoclonal antibody (Millipore, Billerica, MA, USA) diluted 1:500 overnight at 4˚C. After being washed three times with PBS, the cells were incubated with Rhodamine-labeled secondary antibody (KPL, Gaithersburg, MD, USA) at room temperature for 1 h in the dark. The secondary antibody solution was removed, and the cells were washed with PBS three times before counterstaining with 1 μg/ml DAPI for 5 min in the dark. Finally, the slides were mounted with an antifading reagent and examined with a Leica fluorescence microscope.

Western blot assay. The cells were washed in PBS and lysed in RIPA buffer (Shennengbocai, Shanghai, China) (1% NP-40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate and 1 mM sodium vanadate) containing protease inhibitors (1 mM PMSF, 1 mM sodium fluoride). The samples were incubated on ice for 30 min and centrifuged at 12,000 x g for 15 min at 4˚C. The supernatants were collected, and the protein concentration was measured with the BCA Protein Assay kit (Merck, Darmstadt, Germany). An equal amount of total protein was separated by 10% sodium lauryl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a PVDF membrane (Millipore) using a semi-dry blotting apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% non-fat milk in TBST at room temperature for 1 h, incubated overnight with primary antibodies at 4˚C and then washed three times with TBST. This was followed by an additional 2 h of incubation with HRP-labeled secondary antibodies (KPL, Gaithersburg, MD, USA) in blocking buffer (1:5,000). Finally, the membranes were washed three times with TBST, and the protein bands were detected using an ECL system (Merck, Darmstadt, Germany) according to the manufacturer's instructions. The primary antibodies included MTDH (1:500, Abcam, Cambridge, MA, USA), Bcl-2 (1:200, Dako, Carpinteria, CA, USA), Ku70, Ku80, DNA-PKcs, Rad51 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA USA) and β-actin (1:5,000, Sigma-Aldrich, St. Louis, MO, USA).

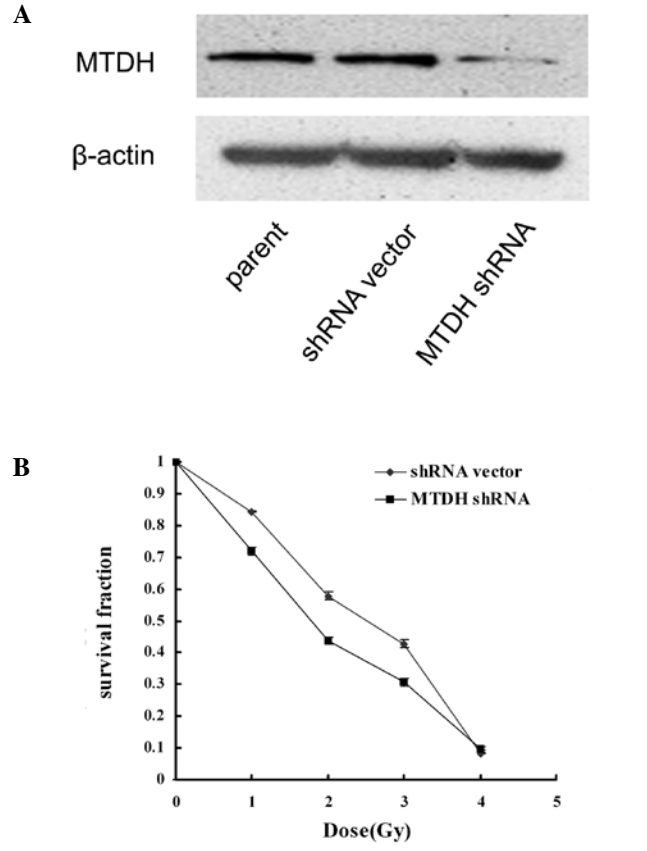

Figure 1. Knockdown of MTDH increases the X-ray radiosensitivity of SiHa cells. (A) The expression of MTDH in SiHa cells and their derivative cell lines was examined by western blot analysis. (B) Cell viability was evaluated using the clonogenic assay. A marked difference in the surviving fraction was observed between SiHa/shRNA vector and SiHa/MTDH shRNA cells (P<0.05). Data shown are the mean ± SD of three independent experiments.

Statistical analysis. The results were analyzed using SPSS 16.0 software (Chicago, IL, USA). Each experiment was performed three times, and the data were expressed as the mean ± SD. The two-tailed Student's t-test was used to analyze the differences between the means, and a P-value of <0.05 was considered significant.

Results

Knockdown of MTDH increases the radiosensitivity of SiHa cells. To knock down MTDH, we transfected SiHa cells with MTDH shRNA and obtained stable cell lines. The expression of MTDH in SiHa and its derivative cell lines was examined by western blot analysis. As shown in Fig. 1A, cells transfected with MTDH shRNA had significantly decreased levels of MTDH compared to the control groups.

A clonogenic assay was performed to determine the effect of MTDH on the radiosensitivity of cervical cancer cell lines. Radiation caused a dose-dependent decrease in the survival of all SiHa lines. As shown in Fig. 1B, knockdown of MTDH in SiHa markedly increased the cells' susceptibility to X-ray radiation. The surviving fractions of the SiHa/shRNA vector after 1, 2, 3 and 4 Gy of X-ray radiation were 84, 58, 43 and 8%, respectively, while the surviving fractions of SiHa/MTDH shRNA were 72% (P<0.05), 44% (P<0.01), 31% (P<0.01) and 10% after radiation treatment.

Knockdown of MTDH decreases G2 phase arrest induced by X-ray radiation. Cell cycle distribution after irradiation was assessed by PI staining. As shown in Fig. 2A, radiation induces G2 phase arrest of SiHa cells. At 10 h post-radiation, ~38% of SiHa/shRNA vector cells were arrested in the G2 phase when receiving 2 Gy of X-ray radiation, while only 33% (P<0.01) of SiHa/MTDH shRNA cells arrested in the G2 phase at the same time after being treated with an equivalent dose of X-rays. The proportion of SiHa/shRNA vector and SiHa/MTDH shRNA cells that arrested in G2 phase without radiation was equal (Fig. 2B).

Knockdown of MTDH increases radiation-induced apoptosis. We evaluated the frequency of apoptotic cells using Annexin V/PI staining at 24 h after X-ray radiation. The percentage of apoptotic cells was ~10 and 13% (P<0.05) in SiHa/shRNA vector and SiHa/MTDH shRNA cells, respectively, after exposure to 2 Gy of X-ray radiation (Table I). Knockdown of MTDH itself did not change the proportion of apoptotic cells in the SiHa cell line; the proportion of apoptotic cells was 3.51 and 4.75% (P>0.05) before and after silencing of MTDH, respectively, indicating that the increased radiosensitivity accompanying knockdown of MTDH was related to the increased apoptosis induced by radiation.

MTDH modulates the levels of Bcl-2 after radiation. To evaluate the mechanism underlying the radioprotection conferred by MTDH expression, the expression of Bcl-2 (an anti-apoptotic protein) in both SiHa/shRNA vector and SiHa/MTDH shRNA cells was determined in cells with or without radiation treatment. As shown in Fig. 3B, while there was no significant change in the basal levels of Bcl-2 in the two groups of cells (P>0.05), delivery of 2 Gy induced a significant upregulation in Bcl-2 expression in SiHa/shRNA vector cells

Table I. Percentage of early apoptotic cells in Annexin V/PI staining assay.

shRNA vector 0 Gy	MTDH shRNA 0 Gy	shRNA vector 2 Gy	MTDH shRNA 2 Gy
3.51±0.50%	4.75±0.25%	9.90±0.28%	12.59±0.52%

The apoptotic frequency of cells was detected 24 h after X-ray irradiation. The data presented are the mean ± SD of three independent experiments. Knockdown of MTDH in SiHa increased radiation-induced apoptosis (P<0.05). Knockdown of MTDH itself did not change the proportion of apoptosis in SiHa cells (P>0.05).

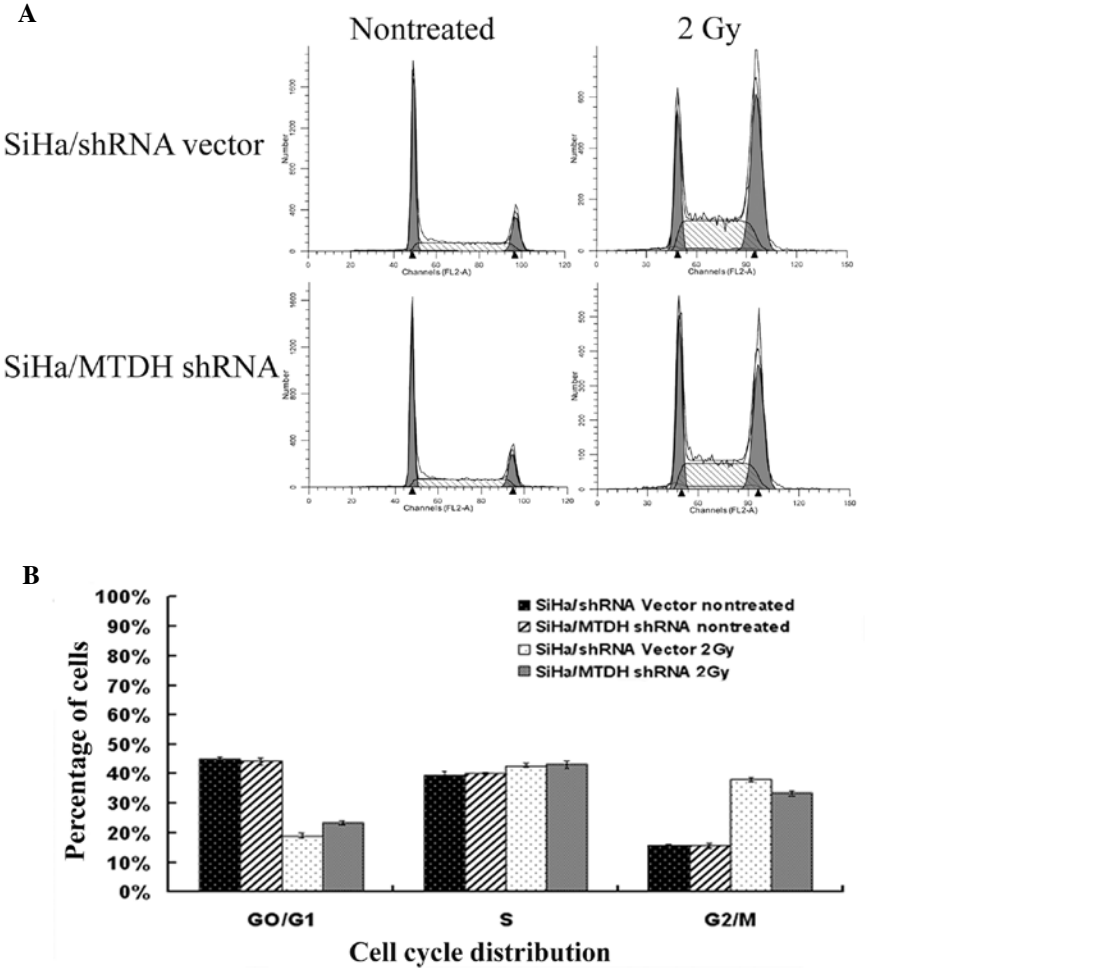

Figure 2. Cell cycle distribution by flow cytometry and the levels of Bcl-2 as measured by western blot analysis. (A) Knockdown of MTDH in SiHa cells decreased cell arrest in the G2 phase after radiation (P<0.01). The percent of SiHa/shRNA vector and SiHa/MTDH shRNA cells that arrested in G2 phase without radiation was equal.

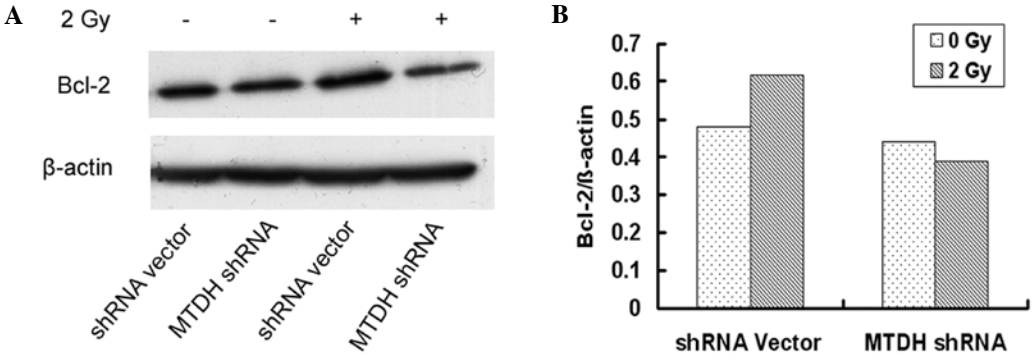

Figure 3. (A) Expression of Bcl-2 by western blot analysis. (B) The expression of Bcl-2 in SiHa/shRNA vector cells after 2 Gy of X-rays was upregulated compared with SiHa/MTDH shRNA cells exposed to an equivalent dose of radiation (P<0.01), while there was no significant change of the levels of Bcl-2 in the two cell lines without radiation (P>0.05).

(P<0.01). By contrast, radiation-induced upregulation of Bcl-2 was not observed in SiHa/MTDH shRNA cells.

MTDH enhanced the repair of DSBs after irradiation. A DNA double-strand break (DSB) is considered the most common and lethal type of radiation-induced damage (25). Phosphorylation of H2AX (γ-H2AX), which leads to the formation of foci at sites of DSBs, marks an early step in the cellular response to DSBs (26). The persistence of γ-H2AX foci after irradiation reflects an impaired cellular capacity to repair DSBs (27,28). We scored the γ-H2AX foci at different time points after the delivery of a single fraction of 0.5 Gy. While many foci emerged after radiation, the foci disappeared faster in the SiHa/shRNA vector cells, with the vast majority

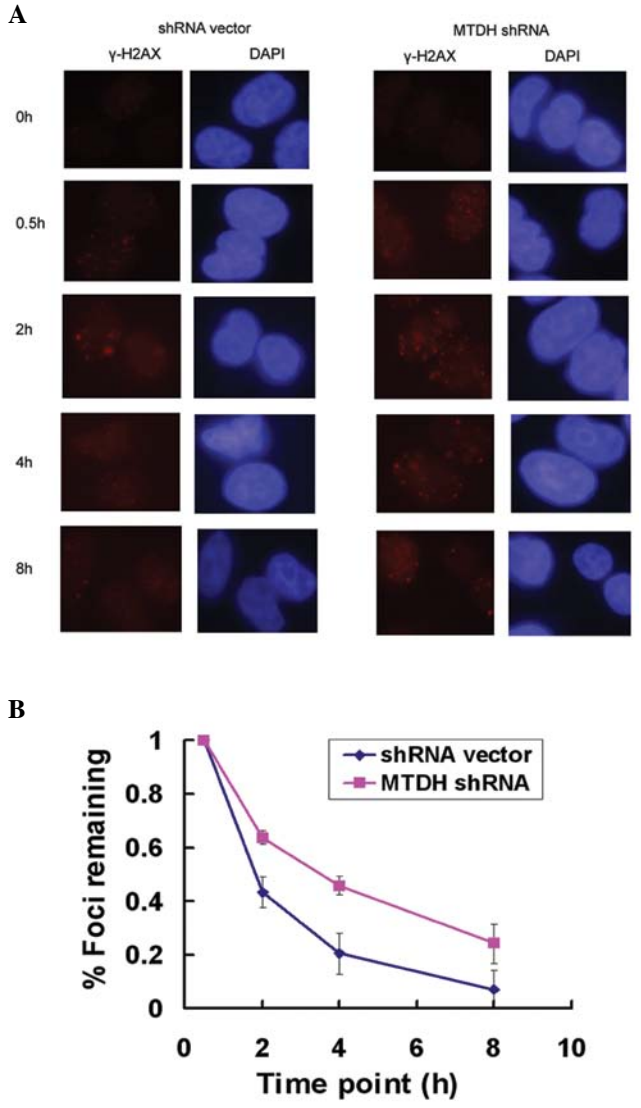

Figure 4. MTDH enhanced the capacity of cells to repair radiation-induced DNA DSBs. (A) Images of γ-H2AX foci at 0, 0.5, 2, 4 and 8 h after 0.5 Gy of X-rays using immunofluorescence. (B) The percent of foci remaining represents the number of foci at the indicated time compared with that at 0.5 h.

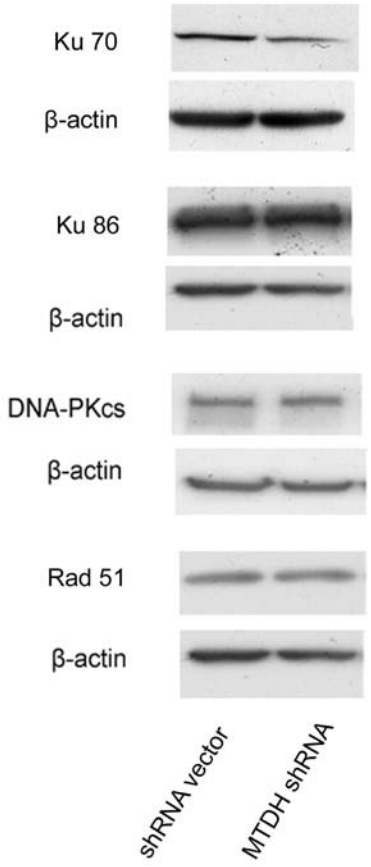

Figure 5. The proteins involved in the DSB repair pathway were examined by western blot analysis. Knockdown of MTDH resulted in downregulation of Ku70. No change was detected in the expression of other DSB repair proteins.

disappearing at 4 h post-radiation, in comparison to the SiHa/MTDH shRNA cells (Fig. 4), where the foci persisted for up to 8 h post-radiation. These results suggest that MTDH may play a role in enhancing the capacity of cells to repair radiation-induced DNA DSBs.

To investigate the mechanism by which MTDH promotes the repair of DSBs, we examined expression of DSB repair proteins Ku70, Ku80, DNA-PKcs and Rad51 by western blot analysis. As shown in Fig. 5, knockdown of MTDH resulted in a downregulation of Ku70 in SiHa cells, whereas no change was observed in the expression of the other repair proteins (Ku80, DNA-PKcs and Rad51). Therefore, MTDH likely promotes the repair of radiation-induced DSBs, mainly through the upregulation of Ku70.

Discussion

MTDH was initially identified as a human astrocyte gene that was elevated after either infection with HIV-1 or exposure to the viral glycoprotein gp120 (11). MTDH was found to be located at 8q22, a site of recurrent amplification in human gliomas and breast cancers; the full-length cDNA consists of 3611 bp encoding a 582 amino acid protein (13,15). In recent years, an increasing number of publications indicate that MTDH plays an important role in cancer progression and development. Our study is one of the first to evaluate the relationship of MTDH with radiation, and our findings demonstrate that cervical cancer cells are more sensitive to radiation following knockdown of MTDH. The increased radiosensitivity may be related to the disruption of cell cycle arrest, downregulation of anti-apoptotic genes and/or impairment of DNA DSB repair.

Irradiation results in a delay of progression through the G1, S and G2 phases of the cell cycle. The G2 arrest portion of the cell cycle is protective against radiation because the cells have more time to repair DNA damage and prevent cell death (29,30). In our study, the proportion of cells that were arrested in G2 phase after radiation was lower when MTDH was downregulated than in the control group.

Bcl-2 is an anti-apoptotic protein that plays an important role in radiation-induced apoptosis. Inhibition of Bcl-2 enhances radiation-induced apoptosis (31). Our results indicated that knockdown of MTDH generated more apoptotic cells after radiation and that Bcl-2 expression was lower in MTDH knockdown cells than in control cells, suggesting that the radioprotective effect of MTDH may be mediated via Bcl-2 modulation.

Radiation generates a variety of DNA lesions including base damages, single-strand breaks, DSBs and DNA-protein crosslinks. DSBs can cause genomic instability leading to either cell death or carcinogenesis (32). H2AX is a subset of the core histone H2A, and its phosphorylation (γ-H2AX) occurs rapidly after DSBs (33,34). The γ-H2AX foci caused by low-dose radiation in cells with MTDH knocked down were present longer than in the parental cells, indicating that MTDH may promote DSB repair as a potential mechanism of MTDH-associated radioresistance.

In mammalian cells, homologous recombination (HR) and DNA non-homologous end-joining (NHEJ) are two major DSB repair mechanisms (35,36). NHEJ is the main pathway to repair DSBs after radiation, while HR plays a minor role in repairing radiation-induced DSBs in adult cells (37,38). The proteins involved in NHEJ include Ku70, Ku80 and DNA-PKcs, which together compose the DNA-dependent protein kinase complex (DNA-PK) (38,39). In our study, knockdown of MTDH decreased the expression of Ku70, indicating that MTDH may enhance DSB repair and lead to radioresistance through the impairment of NHEJ.

In conclusion, we found that knockdown of MTDH increased the radiosensitivity of cervical cancer cells. MTDH levels may therefore be a useful marker for the predication of radiation response of cervical cancers and could be used as a novel target to increase the radiosensitivity of cervical cancer cells.

Acknowledgements

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