Melanoma differentiation-associated gene-7/interleukin 24 inhibits invasion and migration of human cervical cancer cells in vitro

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ABSTRACT

Objectives: In this study, we used an adenoviral vector - melanoma differentiation-associated gene-7 (Ad-mda7) to examine the effect of the ectopic production of MDA-7/IL-24 on cell migration and invasion by human cervical cancer cells.

Methods: The study took place in the Department of Biochemistry and Molecular Biology, Chongqing Medical University, Chongqing, China, between April 2006 and November 2006. The change of metastasis of cervical cancer cells (CaSki) cells were detected by Cell Migration Assay and Cell Invasion Assay after treated with Ad-mda7. The production of proteins associated with cell migration and invasion were detected by western blot.

Results: Cervical cancer cells treated in vitro with Ad-mda7 migrated and invaded less than cells treated with phosphate-buffered saline (PBS) or Ad-Luc (vector control). Melanoma differentiation-associated gene-7 /IL-24 inhibited migration and invasion by down-regulating the production of matrix metalloproteinase-2 (MMP-2) and by up-regulating the production of p38 mitogen-activated protein kinase. relative to PBS and Ad-Luc.

Conclusion: These results show that MDA-7/IL-24 inhibits invasion and migration by cervical cancer cells by down- or up-regulating proteins associated with these processes, resulting in reduced metastasis. Thus, Ad-mda7 should be considered a therapeutic agent that can inhibit primary tumor growth and prevent metastasis.

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Cervical cancer remains as one of the biggest killers of women worldwide.[1,2] Despite significant achievements in the treatment of cervical cancer, it is still a deadly disease that readily metastasize in the earlier period;[3] hence, newer therapeutical modalities are needed. The melanoma differentiation-associated gene-7 (MDA-7); approved gene symbol (IL24) was reported to be a novel tumor-suppressor gene whose expression was lost during tumorigenesis.[4] On the basis of these observations, it was speculated that the MDA-7 protein plays a role in maintaining the normal physiology of melanocytes and that the inhibition of its production results in transformation and progression from a local, non-metastatic primary tumor to highly metastatic cancer. To support this concept, Ellerhorst et al[5] found that the MDA-7 protein is produced in human melanocytes and in primary melanomas, however, its production is progressively lost in metastatic melanomas. Strikingly, the authors of this study noted a significant correlation between loss of MDA-7 production and melanoma tumor invasion. They identified specimens in which the superficial layer of tumor was MDA-7 positive, yet MDA-7 production was lost as the tumor invaded the surrounding tissue. Thus, the restoration of MDA-7 production in tumor cells should inhibit tumor growth and tumor invasion and metastasis. However, no additional preclinical or clinical data exist showing a role for MDA-7/IL-24 in regulating cervical cancer metastasis. We therefore investigated the effects of the ectopic production of MDA-7/IL-24 on migration and invasion by human cervical cancer cells in vitro and attempted to identify the underlying mechanisms of metastasis suppression. In this study, we show for the first time that MDA-7 inhibits the migration and invasion of human cervical cancer cells in vitro by down-regulating the protein production of Matrix Metalloproteinase-

2 (MMP-2) and by up-regulating the production of p38 mitogen-activated protein kinase (MAPK). Thus, MDA-7/IL-24 may provide multiple benefits as an anti-cancer strategy since it can inhibit primary tumor growth and prevent metastatic spread.

Methods. *Cell culture*. This study was carried out in the Department of Biochemistry and Molecular Biology, Chongqing Medical University, Chongqing, China, from April 2006 to November 2006. Human cervical cell line (CaSki) (epidermoid carcinoma) was purchased from the American Type Culture Collection (Rockville, MD, USA). Tumor cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA), antibiotics (GIBCO), and L-glutamine. Before the experiments started, the absence of mycoplasma from the cells was verified. Cells were used in the log phase of growth.

Recombinant adenoviral vector. The replication-deficient human type 5 adenoviral vector carrying the MDA-7 gene (Ad-mda7) has been constructed. Viruses were propagated in human embryonic kidney 293 cells and purified by chromatography.

Cell migration assay. Tumor cells (CaSki) were seeded at a density of 5×10^5 cells/well in 6-well tissue culture plates. The next day, cells were infected with Ad-mda7 or adenoviral vector-luciferase (Ad-Luc) at a multiplicity of infection (moi) of 2500 viral particles (vp)/cell. At 6-hours after infection, the cells were trypsinized, washed in phosphate-buffered saline (PBS), and resuspended in serum-free RPMI 1640 medium. A cell migration assay was performed in a 24-well Transwell unit (Millipore, Cambridge, MA, USA), as described previously.[6] Briefly, polycarbonate filters with 12-μm pores were used. The lower chambers of the Transwell units were filled with serum-free medium, and the upper chambers were seeded with 1×10^4 cells from each treatment group in triplicate wells. After the 24- and 48-hour incubations, the cells that had passed through the filter into the lower wells were counted, and the number was expressed as a percentage of the sum of the cells in the upper and lower wells. The experiments were performed 4 times, and the results were recorded as the mean. In a parallel set of experiments, tumor cells subjected to various treatments as described above were subjected to cell viability assays at 24 and 48 hours, as described previously.[7,8] These experiments were performed to exclude the possibility that the inhibition of cell migration by MDA-7/IL-24 was a result of cytotoxicity.

Cell invasion assay. Tumor cells (CaSki) were seeded at 5×10^5 cells/well in 6-well tissue culture plates. The next day, cells were infected with Ad-mda7 or Ad-Luc at an moi of 2500 vp/cell. After transfection, cultures were replenished with complete medium. At 6 hour after treatment, cells were trypsinized, washed in PBS, and resuspended in serum-free RPMI 1640 medium. A cell invasion assay was performed in a 24-well Transwell unit coated with Matrigel (Becton, Dickinson, Franklin Lakes, NJ, USA), as described previously.[9] Briefly, the lower chambers of the Matrigel-coated Transwell units were filled with serum-free medium, and the upper chambers were seeded with 1×10^4 cells from each treatment in triplicate wells. After 24- and 48-hour incubations, the cells that had passed through the Matrigel-coated filter membrane into the lower well were counted as a measure of invasion. The invading cells were counted for each treatment and expressed as a percentage of the sum of the cells in the upper and lower wells. Experiments were performed at least three times, and the results were recorded as the mean of these experiments.

Immunoblotting. Immunoblotting using various antibodies was performed as described previously.[7] Briefly, cells were harvested by trypsinization and resuspended in lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, and 4 M urea). Protein samples (50 μg each) were diluted into a 20-μl solution of lysis buffer and 5% 2-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA, USA) and heated in a water bath at 95^0C for 5 minutes.Then, protein extracts were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a vertical-slab gel electrophoresis cell (Bio-Rad). Next, the separated proteins were transferred from the gel to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech) and then blocked in a blocking solution (5% dry milk and 0.3% Tween 20 in PBS) for one hour. Then, the membranes were incubated with the primary antibodies for MMP-2, p38MAPK(Santa Cruz Biotechnology), MDA-7 (Introgen Therapeutics, Inc., Houston, TX, USA), and ß-actin (Sigma Chemicals). The membranes were then incubated with horseradish peroxidase-labeled secondary antibodies (Amersham). Finally, the proteins were visualized on enhanced chemiluminescence film (Hyperfilm; Amersham) using Amersham's Enhanced Chemiluminescence Western Blotting Detection System. The relative changes in the protein expression levels after various treatments were quantified using ImageQuant software (Amersham Pharmacia Biotech) and expressed as a ratio with one being the value for PBS-treated cells.

Statistical analysis. The statistical significance of the experimental results was calculated using Analysis of Variance and the Mann-Whitney rank-sum test. The differences among groups were interpreted as statistically significant if the probability values are less than 0.05.

Results. In the present study, we tested the ability of Ad-mda7 to inhibit cell migration on CaSki. Tumor

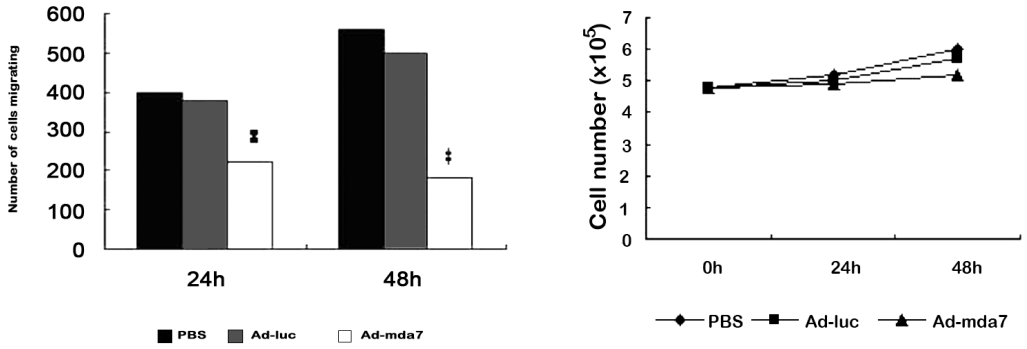

Figure 1 - Melanoma differentiation-associated gene-7/Interleukin 24 inhibits tumor cell migration. **a)** Caski cells were treated with phosphate-buffered saline (PBS), adenoviral vector-luciferase (Ad-Luc), or adenoviral vector - melanoma differentiation-associated gene-7 (Ad-mda7) and analyzed for cell migration. Cells treated with Ad-mda7 were significantly (p=0.002) less able to migrate than cells treated with PBS or Ad-Luc. **b)** Caski cells were treated with PBS, Ad-Luc, or Ad-mda7 and analyzed for cell viability at 24 hours and 48 hours after treatment.

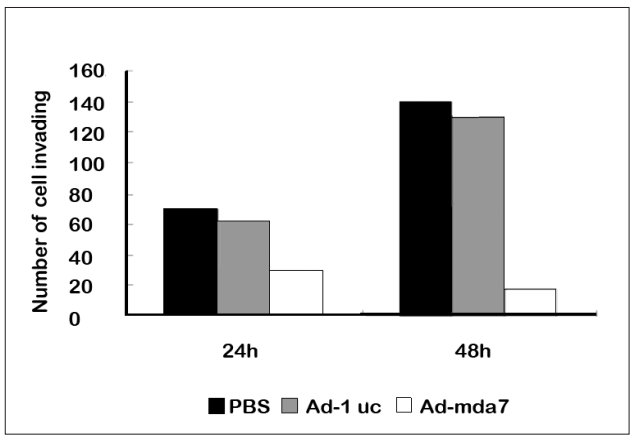

Figure 2 - Melanoma differentiation-associated gene-7/Interleukin 24 inhibits tumor cell invasion. CaSki tumor cells were treated with phosphate-buffered saline (PBS), adenoviral vector-luciferase (Ad-Luc), or adenoviral vector-melanoma differentiation-associated gene-7 (Ad-mda7). After 6 hours, cells were harvested, counted, and added into the upper wells of Matrigel-coated wells.

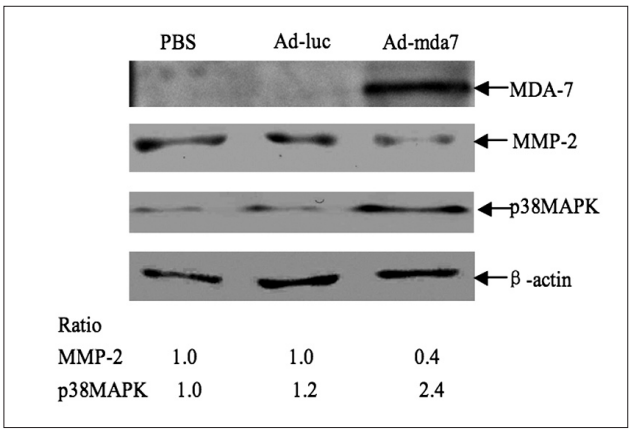

Figure 3 - Melanoma differentiation-associated gene-7 (mda-7)/ Interleukin 24 regulates production of proteins associated with migration and invasion. Tumor cells (CaSki) were treated with phosphate-buffered saline (PBS), Ad-Luc, or adenoviral vector-melanoma differentiation-associated gene-7 (Ad-mda7). MAPK - mitogen-activated protein kinase, MMP-2 - Matrix Metalloproteinase-2, Ad-luc - Adenoviral vector- luciferase

cells treated with Ad-mda7 were significantly (p=0.002) less able to migrate than cells treated with Ad-Luc or PBS (**Figure 1a**). The inhibitory effect on cell migration occurred as early as 24-hours (**Figure 1a**). To show that the inhibition of cell migration was not due to MDA-7/ IL-24- mediated cell death, in a separate but parallel set of experiments, we subjected cells treated with PBS, Ad-Luc, and Ad-Mda7 to a cell viability assay at 24 hours and 48 hours after infection. We observed no significant difference in cell viability indicating that the inhibition of migration by MDA-7/IL-24 was not due to cell death (**Figure 1b**). Note that at 24 hour post-transduction, all 3 experimental groups are superimposable, indicating no significant cell death. By 48 hours, some cell death is occurring; however, with this vector dose, significant Ad-mda7-mediated death is observed only at 72 and 96 hours posttransduction. These results show that MDA-7/IL-24 inhibits tumor cell migration and that the inhibitory effect is not due to cytotoxicity.

Figure 1 shows the MDA-7/IL-24 inhibits tumor cell migration.[1] Caski cells were treated with PBS, Ad-Luc, or Ad-mda7 and analyzed for cell migration. Cells treated with Ad-mda7 were significantly (p=0.002) less able to migrate than cells treated with PBS or Ad-Luc.[2] Caski cells were treated with PBS, Ad-Luc, or Ad-mda7 and analyzed for cell viability at 24- and 48- hours after treatment. That inhibition of cell migration was not due

to cytotoxicity was determined by cell viability assay. No significant inhibition of tumor cell proliferation was observed in Ad-mda7-treated cells compared to PBS- and Ad-Luc-treated cells. Bars denote standard error.

Tumor cells treated with Ad-mda7 were much less invasive, as indicated by the small number of cells on the outer membrane of the Matrigel invasion assay filter, than cells treated with PBS or Ad-Luc (**Figure 2**). The number of invading cells was significantly less after treatment with Ad-mda7 (p=0.001) than with PBS or Ad-Luc. We observed the inhibitory effect exerted by MDA-7/IL-24 as early as 24 hour after Ad-mda7 treatment (**Figure 2**). A cell viability assay showed that the inhibition was not a result of MDA-7/IL-24-mediated cell death (data not shown). These results show that MDA-7/IL-24 effectively inhibits cell invasion.

Melanoma differentiation-associated gene-7/IL-24 regulates production of proteins associated with migration and invasion (**Figure 3**). Tumor cells (CaSki) were treated with PBS, Ad-Luc, or Ad-mda7. Cells were harvested at 48 hours, total cell lysates were prepared, and proteins were separated by SDS-PAGE. Proteins were blotted using antibodies against MDA-7, MMP-2, p38MAPK. Beta-actin was used as a loading control. The relative change in the protein expression was expressed as a ratio with the value for PBS-treated cells arbitrarily set to one.

Discussion. In this study, we show for the first time that MDA-7 can inhibit the migration and invasion of human CaSki cancer cells in vitro. Tumor cells treated with Ad-mda7 were significantly inhibited from growth factor-induced migration. Protein production analysis showed that the inhibition of cell migration was due to MDA-7. It could be argued that the observed inhibition of cell migration was due to the cytotoxicity of Ad-mda7 as opposed to recombinant MDA-7 (rMDA-7) protein; however, that was ruled out by a cell proliferation assay. Note that at the doses and time frames employed in this study, more than 90% of the cells were viable. These results show that Ad-mda7 can inhibit tumor cell migration in vitro. Cell migration is an important element in the physiology of a normal cell and of a cell in pathologic states. For example, the migration of inflammatory cells to a site of injury is a requisite for wound healing.[10,11] Similarly, the migration of tumor cells to distant organ sites plays a pivotal role in metastasis.[12,13] Cell migration has previously been shown to be regulated by numerous molecules, including p38MAPK, pJNK, and et al.[14-18] In our study, we found that MDA-7 inhibited cell migration by up-regulating the production of the p38MAPK protein. In addition to migration, tumor cells need to invade to establish metastasis successfully at a distant site. Tumor cell invasion involves the degradation of the extracellular matrix, and this destruction has been attributed to the activity of proteolytic enzymes. Among the proteases implicated in tumor cell dissemination are MMPs,[19] which play an important role in tissue remodeling in normal cells. However, the production of MMPs has been observed in many invasive tumor cell lines and during tumor growth.[20] In fact, the MMP levels appear to be prognostically significant during tumor progression in the breast, lung, and colon.[21-23] Evidence that MMPs are involved in invasion and angiogenesis in cervical cancer comes from the observation that MMP-2 have been found in several cervical cancer cell lines and surgical specimens, that MMP-2 proteins are localized to the tumor neovasculature, and that the extent of MMP overproduction correlates with prognosis. On the basis of these reports, we next investigated the effect of Ad-mda7 on tumor cell invasion. In this study, we found that MDA-7-mediated inhibition of invasion occurred via the down-regulation of MMP-2. However, the exact mechanism by which MDA-7 inhibits MMP-2 is not clear. Thus, MDA-7 production may activate a signaling cascade resulting in regulation of transcription of MMPs. Whatever the underlying mechanism, the ability of Ad-mda7 to inhibit tumor cell invasion has been clearly shown in this study.

In conclusion, we have shown for the first time that MDA-7 inhibits cell migration and invasion by human cervical tumor cells in vitro. Thus, MDA-7 gene-based drugs may provide a novel therapeutic strategy that can inhibit tumor growth directly via induction of apoptosis and also prevent tumor invasion and ultimately reduce metastasis.

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Ethical Consent

All manuscripts reporting the results of experimental investigations involving human subjects should include a statement confirming that informed consent was obtained from each subject or subject's guardian, after receiving approval of the experimental protocol by a local human ethics committee, or institutional review board. When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.