Melanoma differentiation-associated gene-7 suppresses human gastric cancer cell invasion and migration

LI XU[1], JINYAN CHEN[2], WEI LIN[1,3], JINKUN CHEN[3] and ZHIWEI CHEN[3]

[1]Department of Physiology, Basic Medical College of Putian University, Putian, Fujian 351100; [2]Institute for Immunology, Academy of Medical Sciences of Fujian, Fuzhou, Fujian 350001; [3]Department of General Surgery, Affiliated Hospital of Putian University, Putian, Fujian 351100, P.R. China

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Abstract. Gastric cancer is one of the most common types of cancer in the world. Patients with gastric cancer often respond poorly to conventional chemotherapies, therefore more comprehensive therapy is required. Melanoma differentiation-associated gene-7 (MDA-7), also termed interleukin-24, is a potent tumor suppressor gene. Numerous studies have demonstrated that MDA-7 suppresses the growth and induces the apoptosis of cancer cells. In the present study, the MDA-7 gene was transfected into human gastric cancer AGS cells using adenovirus. Transwell and wound healing assays were performed to evaluate AGS cell invasion and migration, respectively. Western blotting was used to detect the expression of epithelial (E)-cadherin, cluster of differentiation (CD)44 and matrix metalloproteinase (MMP)-2 and MMP-9 proteins. A recombinant virus package was successfully constructed, and it was verified using western blotting that exogenous MDA-7 was highly expressed in the AGS cells. MDA-7 overexpression inhibited invasion and migration, decreased CD44, MMP-2 and MMP-9 expression, and increased epithelial (E-)cadherin expression in the AGS cells. Results of the present study revealed that MDA-7 inhibits gastric cancer invasion and metastasis by inhibiting CD44, MMP-2 and MMP-9 expression and by promoting E-cadherin expression.

Introduction

Gastric cancer is the third most common type of cancer and one of the leading causes of cancer-associated mortality in China (1,2). Patients with gastric cancer often respond poorly to conventional chemotherapies, and, therefore, more comprehensive therapy is required (3). In general, gastric cancer remains difficult to cure, primarily due to gastric cancer cells possessing high invasion and metastasis capability. Melanoma differentiation-associated gene-7 (MDA-7), also termed interleukin (IL)-24, is a member of the IL-10 gene family, and *in vitro* and *in vivo* studies have indicated that MDA-7 overexpression suppresses tumor growth and causes tumor cell apoptosis in several types of human cancer, including mesothelioma, osteosarcoma, melanoma, lung cancer, breast cancer, pancreatic cancer, glioblastoma and prostate cancer (4-6). It is known that MDA-7, a cytokine-tumor suppressor gene and the only tumor suppressor gene in the IL-10 family, not only inhibits tumor growth but also stimulates the immune system and has an antitumor effect; these features render the MDA-7 gene a promising option for the treatment of cancer (7). Currently, basic studies regarding MDA-7/IL-24 in gastric cancer have been limited; to the best of our knowledge, whether MDA-7/IL-24 inhibits gastric cancer cell invasion and metastasis and the potential underlying mechanisms of action have not been reported.

The present study evaluated the effect of MDA-7/IL-24 inhibition on the invasive and metastatic capability of human gastric cancer AGS cells. Western blotting was used to detect the expression of epithelial (E)-cadherin, cluster of differentiation (CD)44, matrix metalloproteinase (MMP)-2 and MMP-9 proteins.

Materials and methods

Cell culture. AGS cells were purchased from the Chinese Academy of Sciences (Shanghai Institute for Biological Science, Shanghai, China) and were cultured in Ham's F12 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C and 5% CO_2.

Plasmid construction. Total RNA from AGS cells was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA concentration was quantified by assessment of the optical density; only RNA samples with an A260-A280 ratio between 1.8 and 2.0 were used to obtain complementary DNA (cDNA). A total of 2 μg of each RNA was reverse-transcribed into cDNA by using the first-strand cDNA synthesis kit (Promega

Correspondence to: Dr Wei Lin, Department of General Surgery, Affiliated Hospital of Putian University, 999 Dongzhen East Road, Licheng, Putian, Fujian 351100, P.R. China
E-mail: lwpt1971@163.com

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Corporation, Madison, WI, USA), and the complete MDA-7 sequence was amplified from the coding region of the gene (GenBank accession no. NM_001185156.1). The PCR cycling conditions were as follows: 94˚C for 2 min, 25 cycles of 94˚C for 30 sec, 72˚C for 30 sec and 56˚C for 1 min, followed by a final extension 72˚C for 7 min. The forward and reverse primers used were 5'-ACGCGTCGACGCATGAATTTT CAACAGAGGCTG-3' (*Sal*I restriction site underlined) and 5'-CCGCTCGAGGAGCTTGTAGAATTTCTGCATC-3' (*Xho*I restriction site underlined), respectively. The *Sal*I and *Xho*I sites were cloned into the vector pShuttle-IRES-hrGFP-1 (Stratagene; Agilent Technologies Inc., USA) and verified by DNA sequencing. Subsequently, they underwent homologous recombination with *Pme*I-linearized plasmid with the BJ5183 strain backbone carrier pAdEasy-1 (Stratagene, Agilent Technologies Inc.), and positive recombinants were identified using the *Pac*I restriction product, termed pAd-MDA-7.

Transfection of MDA-7/IL-24. Following *Pac*I linearization, the recombinant adenovirus vector pAd-MDA-7 and the empty vector were transfected with the aid of Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) into a 293A cell line (low passage; Chinese Academy of Sciences Cell Bank) and were transfected with the intracellular adenovirus packages Ad-MDA-7 or Ad-Control (empty negative control). The virus was repeatedly amplified in the 293A cells, and virus titer was determined by the green fluorescent protein counting method (8). AGS cells were subsequently infected with Ad-MDA-7 or Ad-Control using adenovirus (Stratagene; Agilent Technologies Inc.), and total cellular protein was collected from the lysed cells after 48 h. Western blotting was used to detect MDA-7 expression in 30 μg protein. The primary antibody used was a fusion protein tag antibody: Anti-FLAG M2 antibody (dilution, 1:500; cat. no. F3165; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and then incubated with anti-Mouse IgG for 1.5 h at room temperature (dilution, 1:3,000; cat. no. A9044; Sigma-Aldrich; Merck KGaA). The experiment was performed according to the Press Western breeze kit (Invitrogen; Thermo Fisher Scientific, Inc.) protocol.

Matrigel Transwell invasion assay. AGS cells were infected with Ad-MDA-7 or Ad-Control. After 6 h, the cells were harvested by centrifugation at 300 x g at room temperature for 5 min, and resuspended in serum-free F12 medium, and then transferred to the upper chambers of a Matrigel-coated Transwell system (25,000 cells/well); the bottom chambers contained F12 medium with 20% FBS. The cells were incubated for 24 and 48 h at 37˚C, and then invaded to the bottom surface of the membrane. The membranes were fixed in 4% paraformaldehyde at room temperature for 30 min, stained with hematoxylin at room temperature for 1 min and counted using a light microscope (magnification, x100); the relative cell number was calculated. The average number of cells in four random fields/membrane was used to calculate the relative cell number.

Wound healing assay. AGS cells were infected with Ad-MDA-7 or Ad-Control. After 24 h, a pipette tip was used to scratch a straight line down the middle of the cell monolayer. The cells were observed and images were captured under a microscope at 72 h using light microscope (magnification, x100).

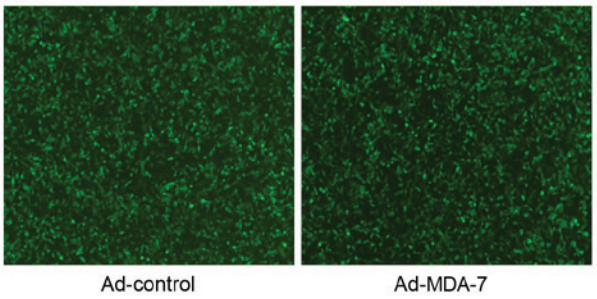

Figure 1. Fluorescence microscopy imaging of MDA-7 expression in AGS cells following infection with Ad-Control or Ad-MDA-7 (magnification, x100). MDA-7, melanoma differentiation-associated gene-7.

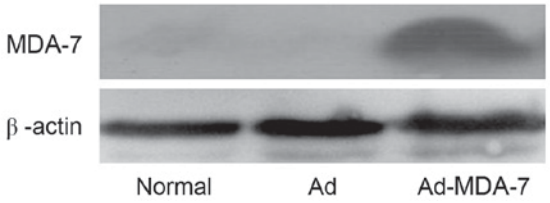

Figure 2. Western blot analysis detection of MDA-7 protein expression in AGS cells following infection with Ad-Control (Ad) or Ad-MDA-7. MDA-7, melanoma differentiation-associated gene-7.

Western blot analysis. AGS cells were infected with Ad-MDA-7 or Ad-Control over 48 h, harvested and lysed by adding ice-cold lysis buffer containing 1 mM phenylmethylsulphone fluoride to extract the total proteins. Protein concentrations were determined using the bicinchoninic acid method. For western blot analysis, 30 μg protein were separated by 12% SDS-PAGE (110 V, 1.5 h), and the membranes were blotted by wet transfer (110 V, 1.5 h, 4˚C) onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked in 5% non-fat milk solution in TBS for 1 h at room temperature, and then incubated overnight at 4˚C with primary antibodies against CD44 (cat. no. 3578), epithelial (E-) cadherin (cat. no. 5296), MMP-2 (cat. no. 4022) and MMP-9 (cat. no. 3852) (dilution for all, 1:1,000; all Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were washed with TBS-Tween (TBST), and then incubated for 1.5 h at room temperature with a goat anti-Mouse IgG horseradish peroxidase-conjugated secondary antibody (dilution, 1:1,000; cat. no. 62-6520; Invitrogen; Thermo Fisher Scientific, Inc.). Following washing with TBST, the membranes were exposed to X-ray film (1-15 min) to visualize the immunoreactive bands. Densitometric analysis of specific bands was performed using Image Lab software version 5.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The quantity of the target protein was calibrated with respect to β-actin, and the control value and relative intensities were obtained.

Statistical analysis. The results are presented as the mean ± standard error of the mean. The significance between experimental values was determined using Student's paired

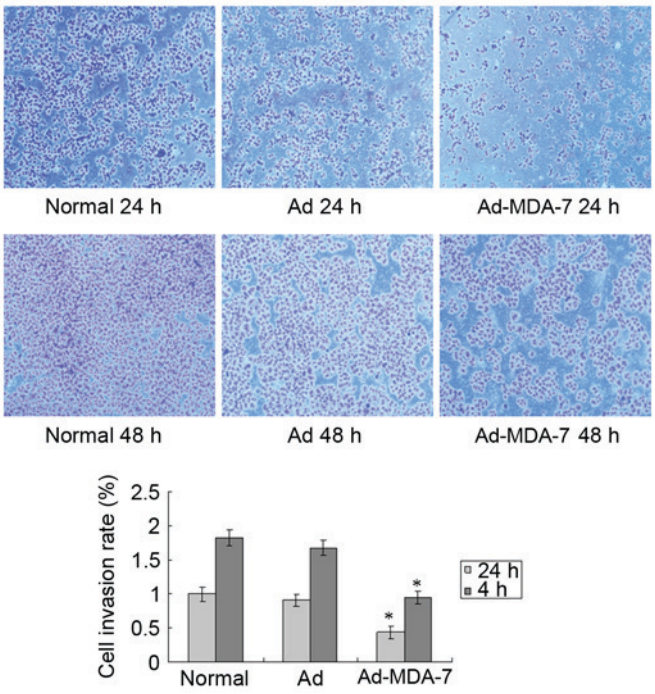

Figure 3. Invasion assay revealing the mean number of invaded cells following transfection (magnification, x100). MDA-7 inhibited tumor cell invasive potential in AGS cells as compared with the normal and Ad-Control groups. MDA-7, melanoma differentiation-associated gene-7.

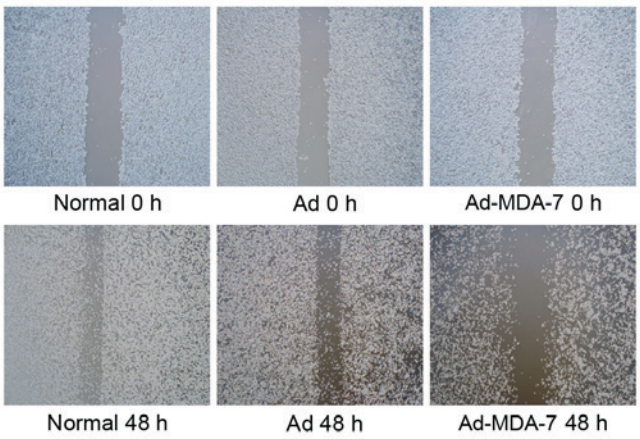

Figure 4. Wound healing assay of transfected AGS cells (magnification, x100). MDA-7 significantly prolonged the gap closure of AGS cells as compared with the normal and Ad-Control groups. MDA-7, melanoma differentiation-associated gene-7.

t-tests; one-way analysis of variance using the statistical software SPSS16.0 (SPSS, Inc., Chicago, IL, USA) was used to test differences in repeated measures across experiments. P<0.05 was considered to indicate a statistically significant difference. Values were analyzed using the statistical package SPSS (version 16.0; SPSS, Inc., Chicago, IL, USA).

Results

Ad-Control or Ad-MDA-7 infection of AGS cells. AGS cells infected with Ad-MDA7 or Ad-Control for 48 h were observed under a fluorescence microscope. In total >95% of cells emitted strong green fluorescence (Fig. 1). Western blotting using the anti-FLAG M2 antibody revealed that cells infected with Ad-MDA-7 expressed MDA-7 protein, whereas the cells infected with Ad-Control did not (Fig. 2).

Ad-MDA-7 inhibits AGS cell invasion and migration. The *in vitro* Transwell invasion assay demonstrated that, after 24 and 48 h, there was significantly lower invasion and metastasis by the AGS cells transfected with the MDA-7 gene in comparison with the normal cells and the Ad-Control group (P<0.05; Fig. 3).

In the wound healing assay, gap closure after 48 h by AGS cells transfected with the MDA-7 gene was markedly prolonged as compared with the normal and Ad-Control groups (Fig. 4).

Ad-MDA-7 decreases the expression of CD44 and E-cadherin proteins. To understand the underlying molecular mechanism of MDA-7 inhibition on cell invasion and metastasis, CD44 and E-cadherin expression was examined in the cells (Fig. 5). Compared with the normal and Ad-Control groups, MDA-7 gene transfection decreased CD44 protein expression (P<0.05) and increased E-cadherin protein expression after 48 h (P<0.05).

Ad-MDA-7 decreases the expression of MMP-2 and MMP-9 proteins. Compared with the normal and Ad-Control groups, MDA-7 gene transfection decreased MMP-2 and MMP-9 expression after 48 h (P<0.05; Fig. 6).

Discussion

Invasion and metastasis are the primary biological characteristics of malignant tumors and are the primary causes of surgical, radiotherapy and chemotherapy failure. Invasion and metastasis are the most important causes of mortality in patients with cancer (9). The incidence of gastric cancer is high (10) and the current means of treating gastric cancer in the clinic is surgery post-operatively; however, its effect on progressive patients is not ideal (11). Distal metastasis of gastric cancer cells is the primary cause of mortality in patients with gastric cancer; a biological characteristic of gastric cancer, it is a major obstacle to long-term survival and to improving prognosis (12).

Tumor metastasis involves multi-gene participation and is a complex multi-stage evolution of the biological processes. It was revealed that MDA-7 expression levels were negatively associated with tumor cell proliferation (13). Jiang *et al* (14) reported that MDA-7 consists of melanoma cells and megakaryocytes, and it was demonstrated that the gene sequence and protein structure of MDA-7 have IL-10 homology domains belonging to the IL-10 family (15). MDA-7 inhibits the growth of a variety of tumor cells and induces features of apoptosis, but does not affect normal cells. The present study suggests that, as a novel tumor suppressor gene, MDA-7 is capable of inhibiting tumor cell growth and angiogenesis, and inducing apoptosis, while stimulating immune cell cytokine expression (16-18). A number of studies have demonstrated MDA-7 expression *in vitro* and *in vivo* in liver cancer, lung cancer, pancreatic cancer, breast cancer and esophageal cancer, where it significantly inhibited tumor growth and induced apoptosis (19-22).

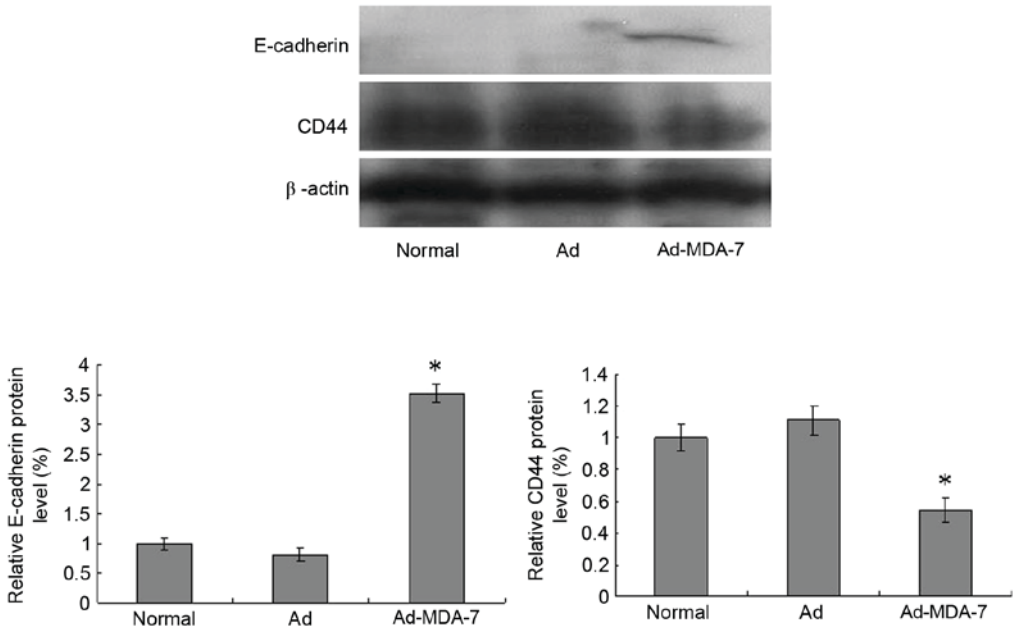

Figure 5. Ad-MDA-7 decreases CD44 protein expression and increases E-cadherin protein expression. *P<0.05. CD44, cluster of differentiation 44; E-cadherin, epithelial cadherin; MDA-7, melanoma differentiation-associated gene-7.

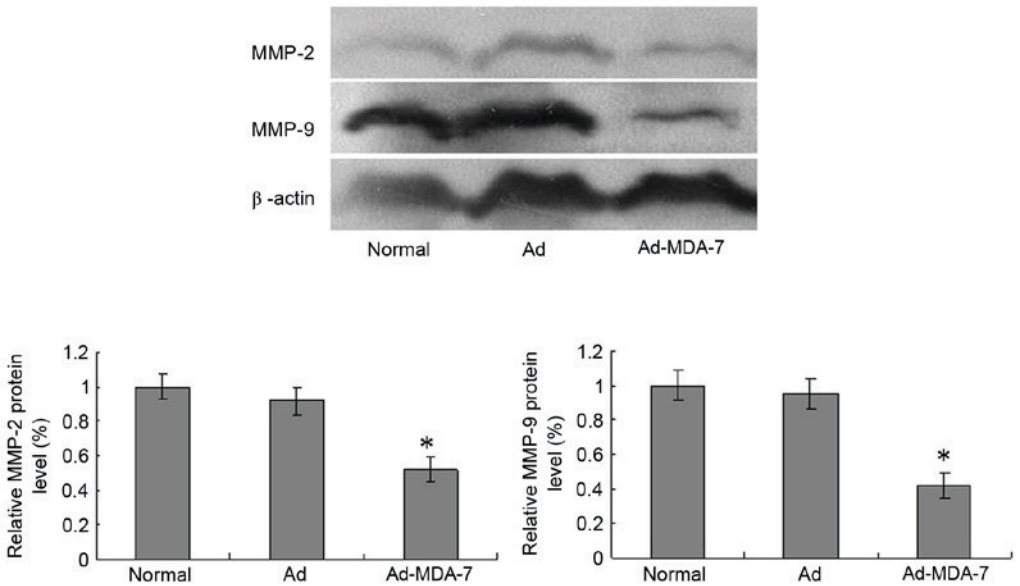

Figure 6. Ad-MDA-7 decreases MMP-2 and MMP-9 protein expression. *P<0.05. MMP, matrix metalloproteinase; MDA-7, melanoma differentiation-associated gene-7.

To study the effect of MDA-7 on invasion and metastasis in gastric cancer, the MDA-7 gene was inserted into an adenovirus vector-expressing recombinant adenovirus. The recombinant adenovirus was used to infect gastric cancer AGS cells to obtain increased expression of MDA-7. Matrigel, wound healing and Transwell assays were used to investigate gastric cancer cell adhesion, migration and invasion ability, respectively. The assays demonstrated that MDA-7 inhibits gastric cancer cell invasion and metastasis.

To investigate the underlying molecular mechanism, the expression of E-cadherin, CD44, MMP-2 and MMP-9 proteins was examined in gastric cancer cells transfected with MDA-7.

Tumor invasion and metastasis is a complex multi-step process involving cancer cells shed from the primary site and the degradation of basement membrane binding. Cancer cells invade the blood vessels and lymphatic vessels, eventually colonizing them, and the proliferation forms metastases. Tumor cells shedding from the primary site, which is associated with decreased cell adhesion, is the first step in metastasis. Therefore, mediating cell-cell adhesion of the calcium-dependent cell adhesion molecule E-cadherin serves an important role in tumor invasion and metastasis (23). A study on breast cancer, colorectal cancer, bladder cancer and other malignant tumors (24) revealed that E-cadherin is an inhibitory factor in tumor malignant transformation, invasion

and metastasis. E-cadherin downregulation or loss of function is significantly associated with tumor differentiation, invasive growth, metastasis and poor prognosis.

Another important step in tumor invasion and metastasis is the degradation and destruction of the extracellular matrix (ECM) and basement membrane structure barrier, which creates favorable conditions for tumor cell metastasis. MMPs are key enzymes of ECM catabolism, and MMP-2 and MMP-9 serve an important role in tumor invasion and metastasis (25). The actions of MMPs are facilitated by the degradation of ECM and basement membrane type IV collagen fibers, and they promote tumor angiogenesis and cancer cell invasion and metastasis (26,27).

Following infiltration from the blood vessels and lymph vessels by tumor cells and the development of metastases, cell adhesion is subsequently involved in the process and performs an important role. CD44 belongs to the adhesion molecule family and mediates ECM-cell adhesion; its expression is increased in a variety of tumor cells, promoting tumor cell invasion and metastasis, and performing an important role in tumor occurrence, development and metastasis (28,29).

In the present study, CD44, MMP-2 and MMP-9 protein expression was decreased and E-cadherin protein expression was increased in gastric cancer cells transfected with the MDA-7 gene. It was considered that MDA-7 inhibited CD44, MMP-2 and MMP-9 protein expression and promoted E-cadherin protein expression, consequently inhibiting the invasion and metastasis of gastric cancer cells. These features render the MDA-7 gene a promising cancer treatment approach. However, for MDA-7 gene therapy as well as the means of gene transfer to be effective, in-depth study of the antitumor capacity, efficiency and persistence of gene expression is required. The antitumor effect of MDA-7 in Stage I clinical trials has been demonstrated (30,31); in the future, the MDA-7 gene may be a novel tool in cancer gene therapy.

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