microRNA-145 inhibits cell proliferation, migration and invasion by targeting matrix metallopeptidase-11 in renal cell carcinoma

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Abstract. microRNA-145 (miR-145) has been reported to be frequently downregulated in various types of cancer, including renal, prostate, bladder, lung and colon cancer, as well as B-cell malignancies. The present study examined the effects of miR-145 on the cell proliferation, migration and invasion of renal cell carcinoma (RCC). Following transfection of miR-145, an MTT, cell migration, cell invasion and luciferase assays, and western blot analysis were conducted in RCC cell lines. The present study demonstrated that miR-145 inhibited cell proliferation, migration and invasion in 786-O and A498 cells. The present study also demonstrated for the first time, to the best of our knowledge, that miR-145 may directly target matrix metallopeptidase-11 (MMP-11) in RCC. miR-145 was demonstrated to suppress cell proliferation, migration and invasion by targeting MMP-11 in RCC cell lines. These results suggested that it may be investigated as a predictive marker for the early detection of tumor metastasis and for targeting therapeutic drugs to inhibit the invasion of RCC.

Introduction

Renal cell carcinoma (RCC) is a kidney cancer that originates from the proximal convoluted tubule. It represents the leading cause of mortality among urological malignancies (1,2). It is the 10th most common type of cancer in Europe (3). In the United States, there are ~65,000 novel cases and almost 14,000 mortalities from RCC annually (4). RCC includes several different histological subtypes that possess distinct biological behaviors and prognoses. The most common type of RCC is clear cell RCC (ccRCC), which originates in the lining of the proximal renal tubule, representing >75-80% of all cases of RCC (5). Approximately 30% of patients have metastatic disease when diagnosed with RCC and radical nephrectomy remains the main treatment for RCC patients due to resistance to radiation and chemotherapy (6). Although the overall survival rate is >60% over 5 years, ~30% of patients with a diagnosis of localized RCC develop metastatic recurrence (7,8). Patients with metastatic RCC face a poor prognosis and have limited therapeutic options. The median survival rate in a recent cohort was only 1.5 years with <10% of patients surviving to 5 years (9). The histological grade combined with clinical stage, which is considered to be the gold standard method for the prediction of patient prognosis, is not accurate when used alone (10). Thus, molecular markers and novel treatments are required in order to improve the prognosis for patients with RCC.

MicroRNAs (miRNAs) are a class of naturally occurring, endogenous small non-coding RNA, in the size range of 19-25 nt. miRNAs regulate gene expression at the post-transcriptional level by binding through partial sequence homology, to the 3' untranslated region (3'UTR) of mammalian target mRNAs and causing translational inhibition and/or mRNA degradation (11). Since they were initially described almost 20 years ago, there has been a steady increase in their identification and the latest Release 20 of miRBase has 24,521 entries of miRNAs from various species, including 734 mature miRNAs from *Gallus gallus* (12). It has been predicted that as many as 30% of protein-encoding genes may be regulated by miRNAs and they may function as oncogenes and tumor suppressors (13). Upregulated miRNAs in cancer may function as oncogenes by negatively regulating tumor suppressors. By contrast, downregulated miRNAs may normally function as tumor suppressor genes and inhibit cancer by regulating oncogenes (14,15). It has demonstrated the important roles of miRNAs in regulating various cellular functions, including cell apoptosis, cell proliferation, neural development and stem cell differentiation (16,17). Recently, miRNAs have also been revealed to be important in tumorigenesis, cancer invasion and metastasis (18).

miR-145 has been reported to be frequently downregulated in various types of cancer, including renal, prostate, bladder, lung and colon cancer, as well as B-cell malignancies. However, the functions of miR-145 are yet to be investigated

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in RCC. The present study focused on the functions and direct target of miR-145 in RCC.

Materials and methods

Cells and culture conditions. The human ccRCC-derived cell lines 786-O and A498 were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were incubated in RPMI-1640 (HyClone Laboratories, Inc., Logan, UT, USA) or Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 mg/l streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

Transfection of miR-145 mimics, scrambled control (NC) and luciferase reporter plasmid. Mature miR-145 mimics, the NC and the luciferase reporter plasmid were designed and synthesized by GenePharma (Shanghai, China). The sequence of miR-145 mimics was 5'-GUCCAGUUUUCC CAGGAAUCCCU-3'. The sequence of NC mimics was 5'-UUCUCCGAACGUGUCACGUTT-3'. The insertion fragment was confirmed by DNA sequencing. Cell transfection and cotransfection were performed using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions.

Cell viability assay. The cell proliferation was determined by the MTT assay. The cells transfected with miR-145 mimics or the NC were seeded in 96-well plates at a density of 3,000 cells per well. Cell proliferation was documented every 24 h for five days according to the manufacturer's instructions. Briefly, MTT solution was added into each well and incubated at 37˚C for 4 h. The plates were spun (200 x g for 10 min), and the purple colored precipitates of formazan were dissolved in 200 μl dimethylsulfoxide. Absorbance (optical density, OD) was measured at 490 nm using an automatic multi-well spectrophotometer (Bio-Rad, Richmond, CA, USA). There were six replicate wells for every time point in each group. The suppression rate was calculated using the formula: Suppression rate = (1 - $OD_{miR-145}$ / OD_{miR-NC}) x 100%. All the experiments were performed in triplicate.

Cell migration and invasion assay. Cell motility was measured using 8 μm-pore polycarbonate membrane Boyden chambers inserted in a transwell apparatus (Costar, Cambridge, MA, USA). The transfected cells (miR-145 mimics and NC) growing in the log phase were treated with trypsin/EDTA solution, washed once with serum-containing RPMI-1640 medium, centrifuged (200 x g for 10 mins), and re-suspended as single-cell solutions. A total of $1x10^5$ cells in 0.2 ml serum-free RPMI-1640 medium were seeded on a transwell apparatus (Costar). RPMI-1640 (600 μl) containing 20% fetal bovine serum was added to the lower chamber. An invasion assay was performed by the same procedure except that the filters of the transwell chambers were coated with 30 μg Matrigel (BD Biosciences, San Jose, CA, USA). After the cells were incubated for 12-24 h at 37˚C in a 5% CO_2 incubator, the cells on the top surface of the insert were removed by wiping with a cotton swab. The cells that migrated to the bottom surface of the insert were fixed in 100% methanol for 2 min, stained in 0.5% crystal violet for 2 min, rinsed in phosphate-buffered saline and then subjected to microscopic inspection (magnification, x200; BX51WI-DPMC; Olympus, Tokyo, Japan). The values for invasion and migration were obtained by counting five fields per membrane and represent the average of three independent experiments.

Western blot analysis. The primary antibodies used in the present study, including anti-MMP-11 and anti-β-actin were products of Bioworld Technology (St. Louis Park, MN, USA). The total protein of cells was prepared using RIPA lysis buffer. The protein concentration in the resulting lysate was determined using the bicinchoninic acid protein assay (Beyotime Institute of Biotechnology, Shanghai, China). Equal quantities of protein were loaded onto SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Following inhibition with 5% degreased milk in Tris-buffered saline with Tween-20 (TBST; containing 0.1% Tween-20), the membranes were incubated overnight with the appropriate primary antibody. The membranes were then washed and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit) at 1:1,000 dilution in TBST. The blot was developed with enhanced chemiluminescence solution (Pierce Biotechnology, Inc., Rockford, IL, USA) and images were captured by a FluorChem imaging system (Alpha Innotech, San Leandro, CA, USA). The intensity of each spot was read and analyzed using AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA). β-actin was used as a loading control.

Luciferase assay. The cells were plated in a 12-well plate at ~90% confluence and transfected with 0.5 μg reporter plasmid, 40 nmol miR-145 mimics or their negative control by Lipofectamine 2000. Each sample was also cotransfected with 0.05 μg pRL-CMV plasmid expressing *Renilla* luciferase (Promega Corporation, Madison, WI, USA) as an internal control for transfection efficiency. The cells were harvested with passive lysis buffer, a component of the Dual-Luciferase Reporter Assay system (Tecan, Theale, UK), 48 h after transfection according to the manufacturer's instructions. An appropriate volume of cell lysate was added to a well of the F96 MicroWell Plates, followed by 25 μl Luciferase Assay Reagent II (Tecan). Firefly luciferase activities and *Renilla* luciferase activities were measured using a luminometer (Tecan). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well. Each assay was replicated three times.

Statistical analysis. Data are presented as the mean ± standard deviation and compared using Student's t-test in Stata 10.0 (College Station, TX, USA). Double-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-145 suppresses cell proliferation in RCC cell lines. To measure the effect of miR-145 on cell proliferation, an MTT assay was used. As shown in Fig. 1, the upregulation of miR-145 significantly inhibited cell proliferation. MTT assays revealed

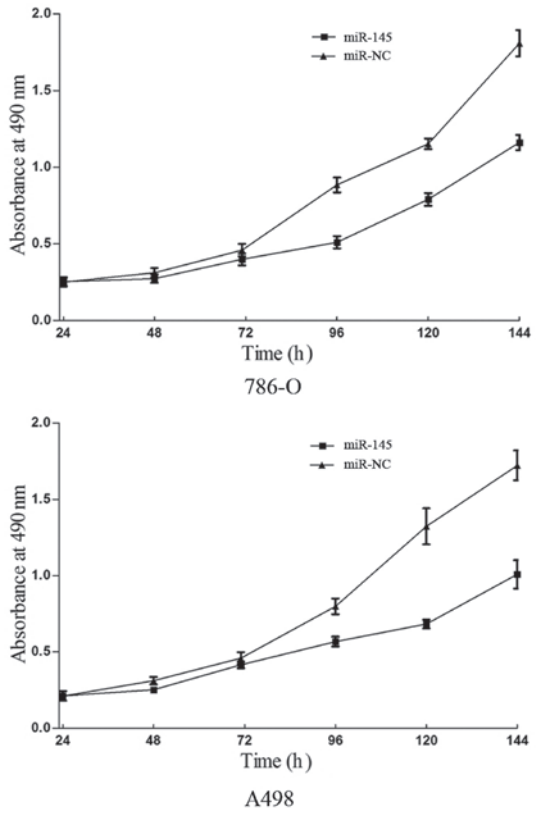

Figure 1. Viability of 786-O and A498 cells following transfection of miR-145. The cell proliferation was determined by an MTT assay. The results indicated that the upregulation of miR-145 significantly suppressed cell proliferation. miR-145, microRNA-145; NC, scrambled control.

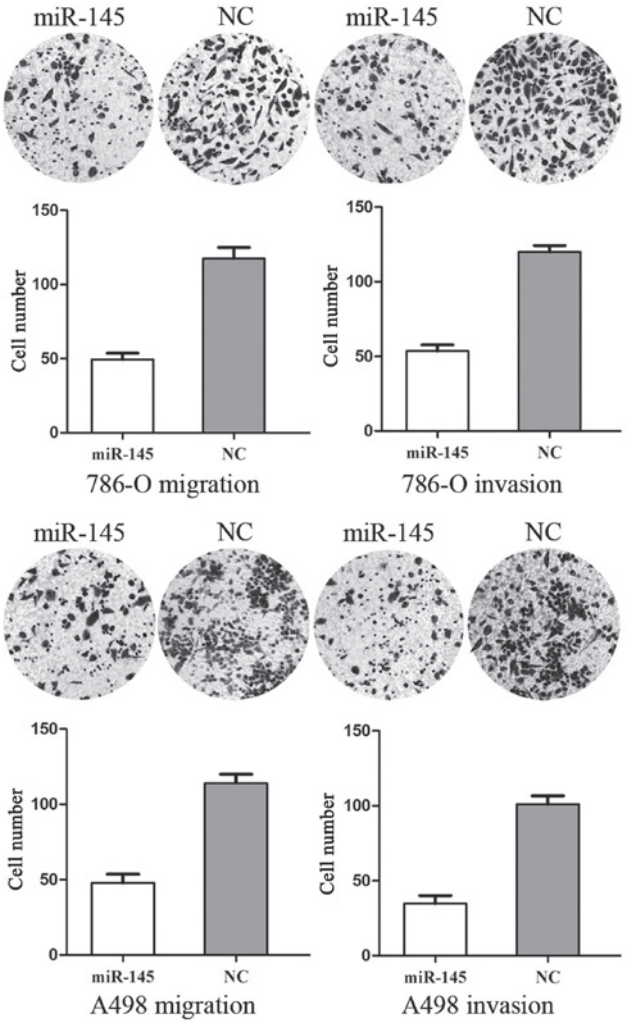

Figure 2. miR-145 inhibited cell migration and invasion in 786-O and A498 cells. Following 12 h incubation, cell migration was significantly decreased in the miR-145 groups compared with the control group. Following 24 h incubation, miR-145 transfected cells showed significantly decreased invasiveness compared with the control cells. miR-145, microRNA-145; NC, scrambled control.

that following 144 h of treatment, the suppression rate of miR-145 reached 35.81%±4.1% in 786-O cells and 41.15±3.5% in A498 cells. These results indicated that miR-145 may be important in 786-O and A498 cells.

miR-145 inhibits cell migration and invasion in RCC cell lines. To measure the effect of miR-145 on tumor cell migration and invasion, the transwell apparatus assay was used (Fig. 2). The transfected cells (miR-145 mimics and NC mimics) growing in the log phase were collected and cultured on transwell apparatus. Following 12 h incubation, cell migration was significantly decreased in the miR-145 groups compared with the control group ($P<0.05$). Using transwell apparatus pre-coated with Matrigel, the effects of miR-145 on cell invasiveness were examined. Following 24 h incubation, miR-145 transfected cells demonstrated significantly decreased invasiveness compared with the control cells ($P<0.05$). These results indicated that miR-145 inhibited cell migration and invasion in RCC cell lines.

miR-145 suppresses the expression of MMP-11 in RCC cell lines. Sachdeva *et al* revealed that miR-145 was able to target multiple metastasis related genes, including MMP-11 and ADAM-17 (19). Western blot analysis was performed to examine whether the protein level of MMP-11 was decreased following the transfection of miR-145. As shown in Fig. 3, MMP-11 was significantly decreased in 786-O and A498 cell lines 72 h after transfection of miR-145. Thus, miR-145 reduces the protein level of MMP-11 in RCC cells.

MMP-11 is a direct target of miR-145. Luciferase reporter assays were performed to evaluate whether the site was able to directly mediate the inhibition of expression. As shown in Fig. 4, the overexpression of miR-145 was able to suppress MMP-11 3'UTR-luciferase activity by 57% in 786-O cells and 45% in A498 cells ($P<0.05$). Therefore, MMP-11 may be a direct target of miR-145 *in vitro.*

Discussion

miR-145 was initially identified in mice from heart tissues using small RNA cloning techniques and then later reported in humans, revealing a unique seed sequence that is conserved in *Xenopus* and mammals (20). Human miR-145 (hsa-miR-145) is enriched in germline and mesoderm-derived tissues, including in the uterus, ovary, testis, prostate, spleen and heart (21). It is located on chromosome 5 (5q32-33) within a 4.09 kb region. The first study regarding the downregulation of miR-145 in various types of tumor was conducted by Michael *et al* (22). The authors demonstrated that the total number of clones

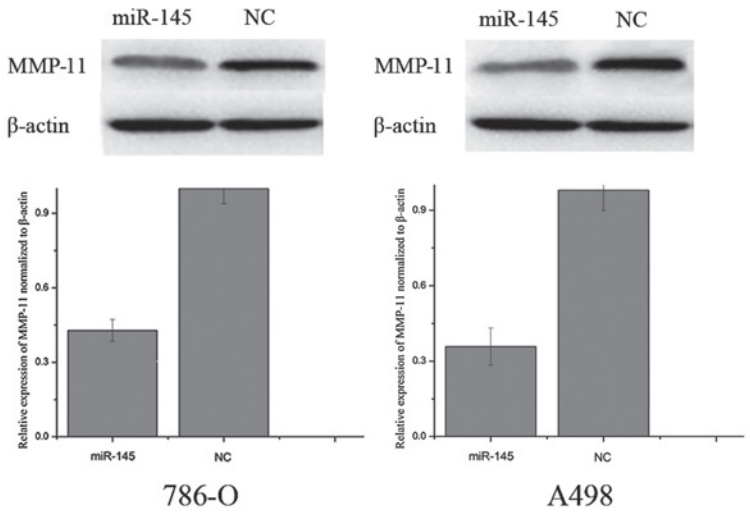

Figure 3. MMP-11 was significantly decreased in 786-O and A498 cells following the transfection of miR-145. MMP-11, matrix metallopeptidase-11; miR-145, microRNA-145; NC, scrambled control.

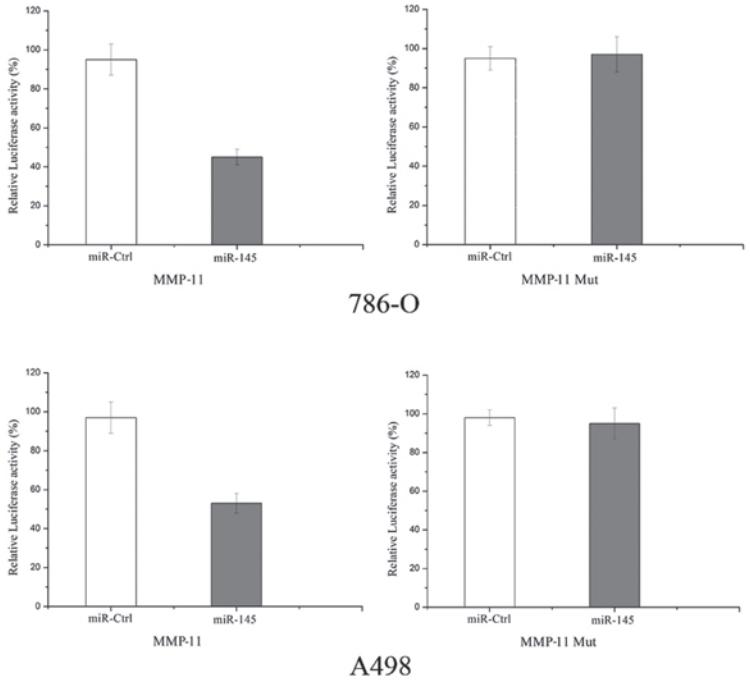

Figure 4. MMP-11 may be a direct target of miR-145 *in vitro*. Luciferase activity significantly decreased following cotransfection with miR-145 and the reporter plasmid in 786-O and A498 cells. Overexpression of miR-145 was able to suppress MMP-11 3' untranslated region-luciferase activity by 57% in 786-O cells and 45% in A498 cells. MMP-11, matrix metallopeptidase-11; miR-145, microRNA-145.

sequenced for miR-145 was two from patients with colon adenocarcinomas compared with eight from normal tissue using the small RNA cloning approach. The results were confirmed by northern blot analysis. Notably, the authors also found a decreased level of miR-145 in precancerous adenomatous polyps, suggesting a possible role in tumor initiation.

miR-145 has been reported to be frequently downregulated in various types of cancer, including renal, prostate, bladder, lung and colon cancer, as well as B-cell malignancies (23-27). The identification of miR-145 target genes is critical for understanding its role in tumorigenesis and is important for defining novel therapeutic targets. miR-145 suppresses tumor cell growth by targeting insulin receptor substrate-1 (28), c-Myc (29) and several other genes associated with carcinogenesis (19). miR-145 impacts migration, invasion and metastasis by targeting Fli 1 (30) and mucin 1 (31), respectively, and also affects p53-mediated cell cycle arrest by targeting p21 (32). In addition, the downregulation of miR-145 is associated with an aggressive phenotype and poor prognosis in prostate cancer (33,34). Therefore, upregulating miR-145 or providing analogous pharmaceutical compounds exogenously, may be effective cancer therapies for several types of tumor resulting from the activation or overexpression of these oncogenes. The present study revealed that miR-145 reduced cell migration and invasion by downregulating the expression of MMP-11. The results suggested that miR-145 may be used for

the development of novel molecular markers and therapeutic approaches to inhibit the metastasis of RCC.

Metastasis is a multi-step process that requires cancer cells to detach from the main tumor, migrate and invade through the stroma, intravasate, survive in the circulatory system, arrive at a secondary site and extravasate, invade and grow at the secondary site (35). MMPs have been previously shown to contribute to migration and invasion in various types of cancer. They are a family of zinc-dependent extracellular endoproteinases that are collectively capable of degrading essentially all components of the extracellular matrix and basement membrane (36). They regulate and shape the tumor microenvironment, and are synthesized and secreted by multiple cell types, including corneal epithelial cells and fibroblasts (37). To date, at least 24 different human MMPs have been identified and they can be classified into five groups on the basis of substrate specificity, including interstitial collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs (38,39).

MMPs are found in normal and pathological tissues in which matrix remodeling is involved, including embryonic development, wound healing, arthritis and angiogenesis, as well as tumor invasion and metastasis (40). Therefore, elevated levels of MMPs have been detected in the serum and urine of patients with several different types of cancer, including cancer of the bladder, breast, lung, colon, head and neck as well as melanoma (41). These MMPs are secreted as inactive zymogens (pro-MMPs) requiring extracellular activation and their activity is tightly regulated by specific tissue inhibitors (42). In view of the important role in tumor invasion and metastasis, inhibitors of MMP activity have been investigated as a method of preventing/decreasing tumor spread.

MMP-11, also termed stromelysin-3, is encoded by the MMP11 gene located on chromosome 22 q11.23 (43). It was originally identified by screening a breast cancer cDNA library for genes that were expressed at higher levels in invasive carcinomas compared with breast fibroadenomas (44). Additional investigation has demonstrated that MMP-11 is usually overexpressed in numerous types of human carcinoma, including breast, non-small cell lung and colorectal carcinomas, however, is rarely expressed in normal tissue, including the normal tissue surrounding the tumor. Notably, only adults have been shown to express MMP-11 in tumors and regenerating or healing tissues (45-47). The functions of MMP-11 in cancer progression have been demonstrated by several preclinical observations. The upregulated expression of MMP-11 enhances tumor incidence in mice (48), homing of malignant epithelial cells (49), cancer progression by remodeling extracellular matrix (50) and has antiapoptotic and antinecrotic effects on tumor cells (51,52). MMP-11 deficiency increases tumor-free survival rate and modulates local or distant invasion (53). The knockdown of MMP-11 mRNA in gastric cancer cells suppresses tumor growth *in vitro* and *in vivo* and inhibits the spread of murine hepatocarcinoma cells to lymph nodes (53). The levels of MMP-11 expression may serve as a marker for transformation and invasion in several types of cancer, otherwise, it may be a target for cancer therapy in order to inhibit metastasis. The results from the present study suggested that miR-145 suppressed the migration and invasion of RCC cells through the downregulation of MMP-11. It may be investigated as a predictive marker for the early detection of tumor metastasis and for targeting therapeutic drugs to inhibit the invasion of RCC.

In conclusion, to the best of our knowledge this is the first study to demonstrate that miR-145 inhibited RCC cell migration and invasion by downregulating the expression of MMP-11. These findings have therapeutic implications and may be exploited for further treatment of RCC.

Future investigation is required to address whether the potential of miR-145 may be fully realized in cancer treatment. If so, it may be beneficial for the treatment of RCC.

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