microRNA-145 modulates migration and invasion of bladder cancer cells by targeting N-cadherin

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Abstract. MicroRNA (miRNA)-145 has been demonstrated to serve a role in several types of tumors, however, the potential molecular mechanism of action of miRNA-145 in bladder cancer metastasis remains to be elucidated. This study aimed to investigate the potential modulation of miRNA-145 in bladder carcinoma and elucidate the underlying molecular mechanism. The expression of miRNA-145 in bladder adenocarcinoma tissues and bladder cancer cells was measured by reverse transcription-quantitative polymerase chain reaction. miRNA-145 mimics and inhibitor were transfected into bladder cancer (BC) cells to determine the role of miRNA-145 on cell motility and invasion measured by wound healing and transwell assays. Luciferase assay was performed to confirm whether N-cadherin was the direct target of miRNA-145. Subsequently, expression of N-cadherin and matrix metalloproteinase-9 (MMP9) in BC cells were detected by western blot analysis. miRNA-145 was significantly downregulated cells and tissues from patients with BC, compared with healthy controls. miRNA-145 markedly inhibited the ability of BC cells to migrate and invade. Furthermore, N-cadherin was identified as a target of miRNA-145 in BC cells. MMP9, acting downstream of N-cadherin, was downregulated in BC cells by miRNA-145. In the present study, miRNA-145 suppressed the migration and invasion of BC cells by regulating N-cadherin. The results of the present study indicated that miRNA-145 may function as a tumor suppressor and may have a potential to be a diagnostic and predictive biomarker, and a therapeutic target for treatment of BC.

Introduction

Bladder cancer (BC) is the most common malignant tumor of the urinary system in both sexes and occurs in the mucosa of the bladder, with 74,400 new cases and 29,400 new deaths occurring in 2013 in China as well as increasing incidence rate with age (1,2). In spite of advancements in bladder carcinoma therapeutic strategies, including radiotherapy, surgical resection and chemotherapy, the survival rate remains low due to recurrence and metastasis (3). The highly metastatic and invasive characteristics of BC reduce the effectiveness of treatments; therefore, these characteristics have become a focus of research (4,5). In addition, numerous clinicians emphasize that management, prevention and diagnosis are also of importance to effective control of BC (6-8). Therefore, it is necessary to elucidate the underlying molecular mechanisms of BC.

MicroRNAs (miRNAs) are non-coding small RNAs, encoded by genes of ~22 nucleotides in length, which regulate expression of other genes on post-transcriptional levels by promoting degradation of mRNAs or suppressing translation by complementary biding to the 3'untranslated region (UTR) of the target mRNA (9,10). Increasing amount of evidence indicates that miRNAs serve a role in regulating tumorigenesis and tumor development including growth, metastasis and apoptosis (11,12). miRNAs have been reported to modulate cancer progression and are considered as potential biomarkers for diagnosis, pathogenesis and progression prediction, as well as therapy in numerous cancers, including lung cancer, breast cancer, thyroid carcinoma and colorectal cancer (13-16). Particularly, miRNA-145 exerts a regulatory effect on multiple types of human tumors, including colorectal cancer, non-small cell lung cancer, pancreatic cancer and oral cancer (17-20). Nevertheless, the underlying mechanism of miRNA-145-mediated modulation of migration and invasion of BC cells remains to be elucidated.

In the present study, miRNA-145 was downregulated in BC tissues and cells. Furthermore, miRNA-145 inhibited migration/invasion of BC cells, which may be mediated by targeting N-cadherin and its downstream effector matrix metalloproteinase-9 (MMP9). Investigation of miRNA-145 and its targets may aid in identification of novel therapeutic targets and diagnostic biomarkers of BC.

Materials and methods

Reagents and antibodies. miRNA-145 mimics, inhibitor and negative controls (NCs) were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Lipofectamine®

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2000, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and a bicinchoninic acid (BCA) protein assay kit were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Transwell chamber was purchased from Corning Incorporated (Corning, NY, USA). RNAiso reagent, miRNA cDNA Synthesis kit and SYBR Premix Ex Taq™ kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Radioimmunoprecipitation assay (RIPA) lysis buffer was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Primary antibodies against N-cadherin, matrix metalloproteinase-9 (MMP9) and GAPDH, and anti-rabbit horseradish peroxidase (HRP) conjugated immunoglobulin G was purchased from Abcam (Cambridge, MA, USA).

Tissue specimens. Clinical tissue specimens were collected from patients with BC (n=10; all stage III; mean age, 61.8 years; 7 male and 3 female) who had undergone surgical resection of their bladder tumors and matched adjacent non-tumor bladder tissues (ANT) at Affiliated Hospital of BeiHua University from January 2014 to January 2015. All tissue specimens were snap frozen in liquid nitrogen and stored at -80˚C. Written informed consent was obtained from all patients and the present study was approved by the Ethics Committee of Affiliated Hospital of BeiHua University (Jilin City, China).

Cell culture. Human BC cell lines T24 and 5637 were obtained from American Type Culture Collection (Manassas, VA, USA). Normal bladder cells SV-HUC-1 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM medium supplemented with 10% (v/v) FBS, containing penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in a humidified incubator containing 5% CO_2. Cells in exponential growth phase (~1x10^6 cells/ml) were used for the subsequent experiments.

Transient transfection. T24 cells were seeded at a density of 1x10^5 cells/well into 6-well plates and cell confluence was 30% at the time of transfection. A total of 1 μg miRNA-145 mimics (forward 5'-TCGGGGGAGTCTCTTGACCTATT-3'; reverse 5'-TAGGTCAAGACTCCCCCGATT-3'), miRNA-145 inhibitor (5'-TAGGTCAAGACTCCCCCGA-3'), or negative control (NC: 5'-TAGGTCAAGAGACTCCCCCGA-3') were transfected into BC cells using Lipofectamine® 2000 following the manufacturer's protocol. Subsequent experiments were performed 48 h post-transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of tissues and cells were extracted with RNAiso reagent (Takara Biotechnology Co., Ltd.) following the manufacturer's protocol. RNA purity and concentration were examined spectrophotometrically and ribosomal ratios of 28S, 18S and 5S were detected by agarose gel electrophoresis. Reverse transcription was performed using miRNA cDNA Synthesis kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions with the following temperature protocol: 1 h at 37˚C and termination at 85˚C for 5 min. PCR was performed using the SYBR Premix Ex Taq™ kit (Takara Biotechnology Co., Ltd.) in accordance with manufacturer's protocols. The thermocycling conditions were as follows: 95˚C for 10 sec, followed by 95˚C for 5 sec and 60˚C for 20 sec for 40 cycles, 95˚C for 60 sec, 55˚C for 30 sec and 95˚C for 30 sec. U6 small nuclear RNA was used as internal control. Forward and reverse primers sequences for miRNA-145 were 5'-GTC CAGTTTTCCCAGGAATC-3' and 5'-AGAACAGTATTT CCAGGAAT-3', and primers for U6 included 5'CTCAACTGG TGTCGTGGAGTCGGCAATTGACAAGTTGAAATATG-3' and 5'-ACACTCCAAGGGCTGTAACGGGTGCCGGAA-3'. The qRT-PCR results of miRNA-145 expression levels were analyzed using the $2^{-\Delta\Delta Cq}$ calculation method (21) from three independent repeats.

Wound healing and transwell assays. To determine alterations in cell mobility following transfection with miRNA-145, wound healing and transwell assays were performed. A total of 1x10^5 cells were seeded into 6-well plates with complete culture medium (serum free DMEM supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin) and the confluent monolayer of cells was scratched using a 200 μl pipette tip. Subsequently, cell migration into the wound closure was visualized under a light microscope. For detection of cell migration by transwell assay, 100 μl cell suspension containing ~1x10^5 cells in serum-free medium was plated in the upper chamber while 500 μl DMEM containing 10% FBS was added into the lower chamber. Following incubation for 24 h, non-migrating cells on the top chamber were gently removed by a cotton-tipped swab. The cells that migrated through the membrane into the lower chamber were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 20 min at 37˚C. Subsequently, the migrated cells were captured under an inverted light microscope (Olympus Corporation, Tokyo, Japan) and counted. For detection of cell invasion, the experimental procedures were the same as for cell migration detection, but Matrigel was added into the bottom center of the upper chamber.

Western blot analysis. Cells were lysed with RIPA cell lysis buffer (Beyotime Institute of Biotechnology) and quantified using a BCA kit (Thermo Fisher Scientific, Inc.). Equal amounts (20 μg/lane) of protein were separated by 10% Tris-glycine gradient gels via SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Subsequently, the membranes were blocked with 5% skimmed milk at room temperature in TBST (containing 0.1% Tween-20) for 1.5 h and incubated with antibodies (1:1,000 dilution) against N-cadherin (cat. no. ab18203), MMP9 (cat. no. ab38898) and GAPDH (cat. no. ab8245) at 4˚C overnight, followed by incubation with anti-rabbit HRP-conjugated secondary antibody (1:5,000) for 2 h at room temperature. Western blot bands were visualized by Enhanced Chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.) and measured with Quantity One 4.6 version software (version 4.6; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Luciferase-reporter assay. To predict the potential target genes of miRNA-145, prediction software programs TargetScan 7.1 (http://www.targetscan.org/) and miRTarBase 7.0 (http://miRTarBase.mbc.nctu.edu.tw/) (22) were used. The

luciferase reporter assay was carried out to determine whether miRNA-145 directly targets N-cadherin. The wild-type (WT) and mutant-type (MUT) N-cadherin 3'-UTR were cloned into the pmirGLO-reporter vector (Promega Corporation, Madison, WI, USA). For the luciferase reporter assay, cells were seeded into 96-well plates for 24 h and co-transfected with luciferase reporter vectors with miRNA-145 mimic, miRNA-145 inhibitor or NC using Lipofectamine® 2000. Following transfection for 48 h, cells were collected. The relative luciferase activity was examined using a dual luciferase reporter assay system (Promega Corporation). Ratio of Firefly luciferase and *Renilla* luciferase activity was used for normalization. Each assay was performed in triplicate.

Statistical analysis. Statistical analysis was performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as the mean ± standard deviation of three independent experiments. One-way analysis of variance followed by Fisher's Least Significant Difference post-hoc test or two-tailed Student's t-test was used to determine the statistical significance of differences. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miRNA-145 is down-regulated in bladder cancer. In order to investigate the expression level of miRNA-145 in BC, miRNA-145 expression levels were determined by RT-qPCR in 10 cases of clinical BC tissues and corresponding ANT, as well as BC cell lines (T24 and 5637). Expression levels of miRNA-145 in tissues and cell lines were significantly reduced compared with paired normal bladder tissues and SV-HUC-1 cells, respectively ($P<0.01$; Fig. 1A and B). The expression level of miRNA-145 was the lowest in T24 cells, and, therefore, the subsequent experiments were performed using this cell line. Following transfection with miRNA-145 mimics, inhibitor or NC, expression of miRNA-145 was measured in T24 cells and the results indicated that miRNA-145 level in miRNA-145 mimics group was significantly higher compared with the other groups (both $P<0.01$; Fig. 1C) while miRNA-145 expression in miRNA-145 inhibitor group was markedly reduced compared with the miRNA-145 mimics group ($P<0.01$).

Inhibitory effect of miRNA-145 on migration and invasion of BC cells. To further measure the effect of miRNA-145 on cell migration and invasion, wound healing and transwell assays were performed. The wound healing assay demonstrated that transfection with miRNA-145 mimics significantly decreased wound-healing rate compared with the NC mimics group ($P<0.01$; Fig. 2A and B). Furthermore, the wound-healing rate of the miRNA-145 inhibitor group was significantly lower than the miRNA-145 mimic group ($P<0.01$). The results of transwell assay demonstrated that transfection with miRNA-145 mimics markedly reduced the migrated cell number and invaded cell number compared with cells transfected with NC mimics ($P<0.01$; Fig. 2C and D). Additionally, the migrated and invaded cell number in the miRNA-145 inhibitor group was significantly reduced compared with the miRNA-145 mimic group ($P<0.01$). These results indicate that miRNA-145 serves an inhibitory role in BC cell migration and invasion.

miRNA-145 regulates the expression of N-cadherin via binding to its 3'UTR. Using bioinformatics software, it was predicted that N-cadherin may be a potential target gene of miRNA-145. Further, in order to confirm this direct interaction between miRNA-145 and N-cadherin, a luciferase reporter assay was carried out. The results demonstrated that relative luciferase activity in the WT group co-transfected with N-cadherin 3'UTR and miRNA-145 mimics was lower compared with the miRNA-145 NC group ($P<0.01$; Fig. 3). Furthermore, the relative luciferase activity in the WT group co-transfected with miRNA-145 inhibitor was significantly higher than the miRNA-145 mimic group ($P<0.01$). Relative luciferase activity in the MUT group exhibited no significant difference between co-transfection with miRNA-145 mimics or miRNA-145 inhibitor and NC mimics. Furthermore, N-cadherin expression level was detected via western blot assay and it was determined that N-cadherin was significantly downregulated in the miRNA-145 mimics group compared with the miRNA-124 NC mimics group ($P<0.01$; Fig. 4A). The expression of N-cadherin was increased in the miRNA-145 inhibitor group compared with the miRNA-145 mimics group ($P<0.01$). Furthermore, since MMP9 was previously demonstrated to be one of N-cadherin downstream molecules (23), its expression level was determined by western blotting. The results were similar to that of N-cadherin described above ($P<0.01$; Fig. 4B).

Discussion

The results of the present study revealed the regulatory mechanism of miRNA-145 and N-cadherin in the migration and invasion of in BC cells. It was identified that miRNA-145 was downregulated in BC tissues and cell lines. Furthermore, miRNA-145 suppressed BC cell migration and invasion. miRNA-145 directly targeted N-cadherin and regulated its and MMP9 expression level, suggesting that miRNA-145 may exert a potentially inhibitory role on migration and invasion of BC cells by targeting N-cadherin and regulating MMP9. These results provide novel insights into molecular mechanism underlying BC metastasis and invasion and regulation of miRNA-145 could become a potential biomarker or a therapeutic target for treatment of BC.

Bioinformatics tools have predicted that high-energy bonding exists in the interactions between miRNA and target genes, and that miRNA overexpression results in target protein expression downregulation (9,24). It is well-known that numerous genes are responsible for carcinogenesis and cancer development. Therefore, miRNAs can act as tumor suppressors or oncogenes, by binding to regulators associated with cancer cell survival, proliferation, apoptosis and metastasis (25,26). A growing body of evidence has demonstrated that miRNA-145 serves a suppressive role in various cancers (27-30). It has been observed that miRNA-145 exerts tumor suppressive effects, by modulating cell proliferation, metastasis, invasion, apoptosis, chemo-resistance effect and epithelial-to-mesenchymal transition of multiple tumors (31-34). However, the effect and underlying

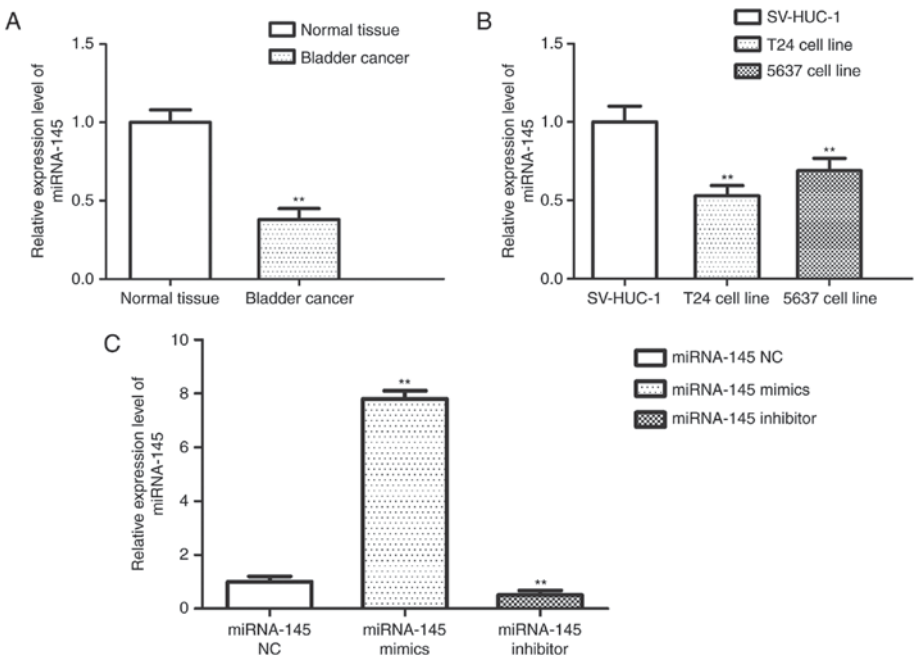

Figure 1. miRNA-145 expression was downregulated in BC cells and tissues compared with the controls. (A) miRNA-145 expression was measured in surgical specimens and corresponding non-cancerous tissues by RT-qPCR. (B) miRNA-145 expression was detected in BC cell lines and normal BC cells using RT-qPCR. (C) Change of miRNA-145 expression was determined after cell transfected with miRNA-145 mimics, miRNA-145 inhibitor or NC mimics by RT-qPCR. Data are presented as the mean ± standard deviation. **P<0.01 vs. NC, bladder non-cancerous tissues and normal BC cells (SV-HUC-1); $^{\#\#}$P<0.01 vs. miRNA-145 mimics group. miRNA, micro RNA; NC, negative control.

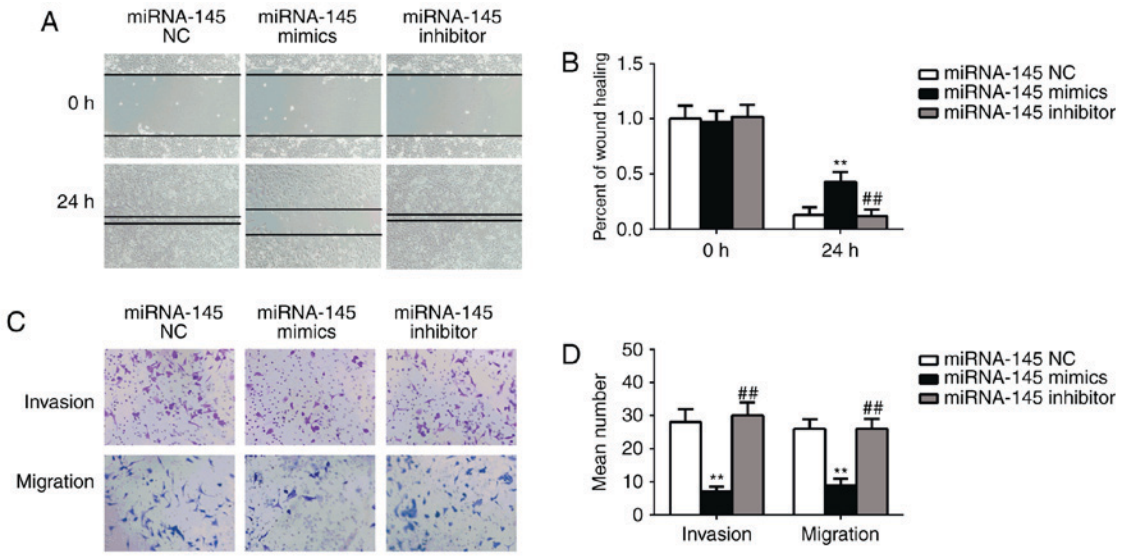

Figure 2. Cell migration and invasion were detected by wound healing and transwell assays. (A) Cell mobility was determined by wound healing assay and (B) the results were quantified. (C) The migrated and invaded cells were captured under an inverted light microscope and (D) the results were quantified. **P<0.01 vs. NC, $^{\#\#}$P<0.01 vs. miRNA-145 mimics group. Magnification, x200. miRNA, microRNA; NC, negative control.

mechanisms of miRNA-145 in BC remain to be elucidated. In line with aforementioned studies, the present study demonstrated that miRNA-145 acted as a tumor-suppressor and was downregulated in patients with BC. Furthermore, the present study revealed that miRNA-145 inhibited migration and invasion in BC cells.

Metastasis and invasion are the main characteristics of malignancy and are responsible for the majority of tumor-associated mortality (35,36). N-cadherin, a homophilic transmembrane adhesion glycoprotein, acts as a transmembrane signal transduction receptor influencing cell-cell adhesion and therefore serves a role in invasion and migration during cancer progression (37-39). Accumulating evidence suggests that N-cadherin is implicated in promoting cancer cell motility (40), thyroid tumorigenesis (41), migration and invasion (42). One report suggests that N-cadherin serves a prognostic role in patients with BC (43). Further, another research group has demonstrated that miRNA-145 can target N-cadherin in lung adenocarcinoma cells (44), whereas, to the best of the authors' knowledge, the modulation

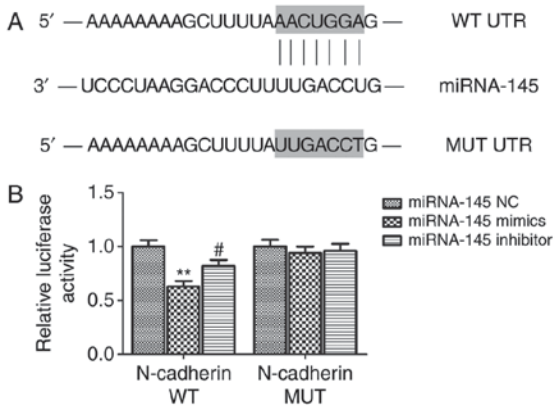

Figure 3. The direct binding between miRNA-145 and N-cadherin was determined by luciferase report assay. (A) Predicted miRNA-145 target sequences in the 3'UTR of N-cadherin. The gray rectangles represent the target sequences in WT N-cadherin 3'-UTR and the mutant sequences in MUT N-cadherin 3'-UTR. (B) Luciferase reporter assay data demonstrated that co-transfection of T24 cells with miRNA-145 and WT N-cadherin 3'-UTR results in a reduction in luciferase activity compared with the miRNA-145 NC group. Data are presented as the mean ± standard deviation. **P<0.01 vs. NC, $^{\#}$P<0.05 vs. miRNA-145 mimics group. miRNA, microRNA; NC, negative control; MUT, mutant-type; WT, wild-type; UTR, untranslated region.

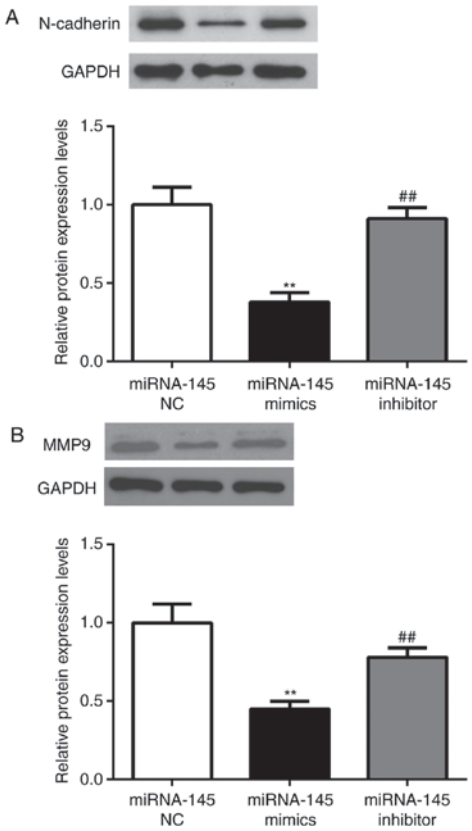

Figure 4. N-cadherin and MMP9 protein expression were detected by western blot analysis. (A) Expression level of N-cadherin was measured in T24 cells following transfection with miRNA-145 mimics, miRNA-145 inhibitor or NC. (B) Expression level of MMP9 was measured in T24 cells following transfection with miRNA-145 mimics, miRNA-145 inhibitor or NC. Data are presented as the mean ± standard deviation (SD). **P<0.01 vs. NC, $^{\#\#}$P<0.01 vs. miRNA-145 mimics group. MMP9, matrix metalloproteinase-9.

of cell metastasis via N-cadherin by miRNA-145 has not been previously studied in the context of BC. Based on the aforementioned studies, the authors of the present study hypothesize that N-cadherin may serve as an oncogene mediating the effect of miRNA-145 on BC metastasis. In the present study, luciferase-reporter assay and western blot analysis results suggested that miRNA-145 contributed to inhibition of BC cells invasion and migration by directly targeting N-cadherin. MMPs, known as proteolytic enzymes, were previously reported to be associated with tumor metastasis and invasion (45,46). Additionally, MMP9, an extracellular matrix-degrading enzyme, serves a role in metastasis and invasion of carcinoma cells (47,48). MMP9 was identified as a modulatory target of N-cadherin-dependent signaling (23). Furthermore, MMP9 expression level was upregulated in the presence of N-cadherin (49). Nonetheless, MMP9 is not a direct target of miRNA-145, as previously demonstrated by a luciferase assay, but its expression was decreased in miRNA-145-transfected gastric cell lines (50). In agreement with this evidence, the results of the present study indicated that levels of MMP9 were decreased in BC cells transfected with miRNA-145 mimics while these levels increased in BC cells transfected with miRNA-145 inhibitor. The above results indicate that miRNA-145 exerts an anti-invasive and anti-migratory roles in BC by directly targeting N-cadherin and regulating MMP9. However, the limitations of the present study included lack of verification of the results in an animal model of BC. Furthermore, the modulatory roles of miRNA-145 and N-cadherin in BC are likely through complex molecular mechanisms; thus, further investigation is required.

In conclusion, the present study demonstrated that miRNA-145 served as a tumor-inhibitor and was downregulated in BC tissues and cell lines. Furthermore, miRNA-145 directly regulated expression of N-cadherin in BC cells and its exogenous expression inhibited migration and invasion of BC cells. It can be hypothesized that miRNA-145 may inhibit migration and invasion of BC cells possibly by directly suppressing the protein expression of N-cadherin. Therefore, miRNA-145 and N-cadherin may be novel candidates for development of efficient therapeutic strategies for treatment of BC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XQZ designed the study and revised the manuscript. XFZ performed the experiments and wrote the manuscript. ZXC, CCW and HG performed the experiments and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients and the present study was approved by the Ethics Committee of Affiliated Hospital of BeiHua University (Jilin City, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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