miR-4735-3p inhibits cell migration and invasion of gastric cancer by downregulating NEDD9

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Abstract. Gastric cancer (GC) comprises the 3rd cause of cancer-related death worldwide. Increased expression of neural precursor cell expressed, developmentally downregulated 9 (NEDD9) is commonly observed in GC, however, its underlying molecular mechanism in GC remains unknown. The potential interaction between miR-4735-3p and NEDD9 was predicted by TargetScan 7.1. Expression profiles of miR-4735-3p and NEDD9 were examined between GC tissues and normal tissues by reverse transcription-quantitative (RT-q) PCR. The relationship between miR-4735-3p and NEDD9 was validated by RT-qPCR, western blotting, dual luciferase reporter assay and RNA immunoprecipitation assay. Biological relationship between miR-4735-3p and NEDD9 was evidenced by the cell invasion and cell migration assays. NEDD9 level was negatively associated with miR-4735-3p level in GC tissues. miR-4735-3p suppressed NEDD9 levels in GC cells. NEDD9 was revealed to be a target gene of miR-4735-3p. miR-4735-3p overexpression suppressed cell migration and invasion of GC cells, which were antagonized by overexpression of NEDD9. Moreover, miR-4735-3p mimic decreased the levels of matrix metalloproteinases 2/9, increased the level of E-cadherin, which were reversed by overexpression of NEDD9. Collectively, the present study provided a potential mechanism for the tumor suppressor role of miR-4735-3p in GC by targeting NEDD9.

Introduction

Gastric cancer (GC) ranks the 5th malignancy and the 3rd cause of cancer-related death according to the global cancer statistics in 2018 (1). GC progresses rapidly and numerous of the cancer cases are at a late stage at the time of diagnosis. The 5-year survival rate of patients with GC is less than 20% worldwide (2). The poor prognosis in patients with GC is mainly associated with the recurrence and metastasis of GC (3). Surgery and chemotherapy are conventional treatment approaches for patients with GC (4). Target therapies, such as anti-EGFR signaling agent, have been proven to improve treatment efficacy (5). However, the molecular mechanisms underlying GC progression and treatments remain largely unclear.

Neural precursor cell expressed, developmentally down-regulated 9 (NEDD9) is a well-characterized focal adhesion scaffold protein (6). High expression of NEDD9 is observed in the lung, kidney and fetal brain tissues (7). In cancer cells, overexpression of NEDD9 is also detected and related to elevated cell motility (8). Analysis of NEDD9 expression in gastric tumors suggested that NEDD9 overexpression was associated with metastasis and poor prognosis (9).

The translation or degradation of messenger RNAs (mRNAs) can be suppressed by microRNAs (miRNAs) (10), which are endogenous small non-coding RNA molecules of ~18-25 nucleotides in animals and plants, comprising one of the most abundant groups of gene regulatory molecules in multicellular organisms and affecting the output of numerous protein-coding genes (11). miRNAs are validated as oncogenes or tumor suppressors, and may predict patient outcome (12). Moreover, NEDD9 can be targeted by multiple miRNAs in various cancer types: For instance, miR-5195-3p inhibits cell proliferation by targeting NEDD9 in osteosarcoma (13), miR-145 suppresses cell growth and metastasis by targeting NEDD9 in renal cell carcinoma (14) and miR-1252-5p reduces cell invasion by targeting NEDD9 in pancreatic cancer (15). However, at present, there are no studies regarding the miRNAs which can target NEDD9 in GC. In the present study, it was investigated whether there are other novel miRNAs that can target NEDD9 and function in GC.

Materials and methods

Collection of specimens. In total, 25 GC tissues and the matched normal tissues were obtained from patients with GC from the General Hospital of Southern Theatre Command (Guangzhou, China) from June 2018 until September 2019. The clinical data from patients with GC are presented in Table I. Patients

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who received any chemotherapy or radiotherapy treatments were excluded from the study. Written informed consent was provided by all participants before the study. All experiments were approved by the Ethics Committee of General Hospital of Southern Theater Command (approval no. GHSTC-2018-06; Guangzhou, China). The tissues were immediately stored at -80˚C before subjected to the future experiments.

Cell culture. The GC cell line AGS was purchased from American Type Culture Collection. Cells were maintained in RPMI-1640 supplemented with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37˚C with 5% CO_2.

miR-4735-3p overexpression. miR-4735-3p mimic (50 nM; 5'-AAAGGUGCUCAAAUUAGACAU-3') and miR-NC (50 nM; negative control, 5'-UAGUCUCGGGAGACUCAC UACC-3') mimic were purchased from Guangzhou RiboBio Co., Ltd. For overexpression of miR-4735-3p, miR-4735-3p mimic was transfected into AGS cells ($2x10^5$ cells/well) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Then, cells were incubated in DMEM for 48 h at 37˚C. The protein and mRNA were extracted from cells at 48 h after transfection.

Wound healing assay. To assess cell migration ability, $1x10^6$ AGS cells were seeded in six-well plates and cultured in serum-free RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.). When the cell density reached 100% confluence, a wound was made at the centre of cell monolayer in each well with a 10-μl pipette tip. The culture medium was then replaced with serum free medium. At 0 and 24 h after transfection, images of wound area were captured with a light microscope. The relative area of wound was calculated using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.) to reflect cell migration ability.

Cell invasion assay. AGS cells resuspended in 200 μl serum-free RPMI-1640 (both from Gibco; Thermo Fisher Scientific, Inc.) were placed in the upper chamber of 24-well Transwell plates (8 μm-pore; Corning, Inc.), while RPMI-1640 containing 20% FBS was added to the lower chamber and acted as a chemoattractant. The Transwell membranes were precoated with Matrigel at 4˚C for 12 h. At 48 h, the non-invasive cells in the upper chamber were removed, while the invasive cells were fixed by 70% ethanol for 15 min at room temperature and stained by 0.1% crystal violet (Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature. Images were captured by an optical microscope.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated from cells and tissues with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription of RNA into cDNA was achieved using MMLV-reverse transcriptase (Promega Corporation) with M-MLV Reaction Buffer and dNTPs (dATP, dCTP, dGTP and dTTP), at 70˚C for 5 min and 37˚C for 1 h. qPCR was performed using the PrimeScript™ RT reagent kit according to the manufacturer's protocol (Takara, Bio, Inc.) on a CFX-96 system (Bio-Rad Laboratories, Inc.). The thermocycling conditions for qPCR were as follows: 95˚C for 30 sec followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The relative expression of genes was normalized to internal controls using the $2^{-\Delta\Delta Cq}$ method (16). β-actin and U6 were internal controls for mRNA and miRNA, respectively. The primers were designed using Primer Premier software (version 6; Primer Premier) and synthesized by GenScript. The primer sequences were as follows: miR-4735-3p forward, 5'-AAAGGTGCTCAAATTAGACAT-3' and reverse, 5'-ATG TCTAATTTGAGCACCTTT-3'; U6 forward, 5'-CTCGCT TCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATT TGCGT-3'; NEDD9 forward, 5'-ACCATGAATTACGAA GCACCTTA-3' and reverse, 5'-TAAGGTGCTTCGTAATTC ATGGT-3'; β-actin forward, 5'-AAATCTGGCACCACACCT TC-3'; and reverse, 5'-GGGGTGTTGAAGGTCTCAAA-3'.

Protein extraction and western blot analysis. Lysates were prepared in RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) following the manufacturer's protocol. β-actin antibody (1:10,000; cat. no. A1978) was purchased from Sigma-Aldrich; Merck KGaA. NEDD9 (1:1,000; cat. no. 4044), E-cadherin (1:1,000; cat. no. ab231303), matrix metalloproteinase (MMP)2 (1:1,000; cat. no. 40994) and MMP9 (1:1,000; cat. no. 13667) antibodies were obtained from Cell Signaling Technology, Inc. Secondary HRP-conjugated antibodies [rabbit anti-mouse IgG H&L (1:10,000; cat. no. ab6728) and goat anti-rabbit IgG H&L (1:10,000; cat. no. ab6721)] were purchased from Abcam. Protein concentration was determined by bicinchoninic acid assay. Briefly, 20 μg protein lysate was added into an SDS-PAGE gel (10%) and separated by electrophoresis. The proteins were transferred onto a PVDF membrane, then blocked in 5% non-fat milk at room temperature for 2 h. Subsequently, the membrane was incubated with the aforementioned primary antibodies at 4˚C overnight. Following the primary incubation, membranes were incubated with secondary antibodies at room temperature for 1 h sequentially. The membrane was washed between the incubation with the primary antibodies and secondary antibody by 0.2% TBST (2 ml Tween-20 in the 1 l TBST solution). The membrane was detected with ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.). ImageJ software 1.48u (National Institutes of Health) was used for densitometric analysis.

Plasmid construction. Full length of NEDD9 was amplified from AGS cDNA using PrimeSTAR® GXL DNA Polymerase (Takara Bio, Inc.). The fragment was annealed into pcDNA3.1 plasmid (1 μg) with T4 DNA ligase (Takara Bio, Inc.). For overexpression of NEDD9, pcDNA3.1-NEDD9 was transfected into AGS cells ($5x10^6$) with Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol for 48 h at 37˚C. At 48 h after transfection, cells were collected for the subsequent experiments.

Bioinformatic analysis and dual luciferase reporter assay. TargetScan 7.1 database (https://www.targetscan.org/vert_71/) was used to predict the potential miRNAs for NEDD9. The 3'-untranslated region (UTR) of NEDD9 was amplified from AGS cDNA using PrimeSTAR® GXL DNA Polymerase (Takara Bio, Inc.) and ligated into pGL3 basic with T4 DNA ligase (Takara Bio, Inc.). Two site mutations were introduced into

Table I. The basic characteristics of patients with gastric cancer.

Parameters	Number of patients	Expression level of microRNA-4735-3p		P-value
		Low, n	High, n	
Sex				0.69
Female	10	4	6	
Male	15	8	7	
Age, years				>0.99
≥55	20	10	10	
<55	5	2	3	
Histology grade				0.57
Well-moderately	11	5	6	
Poorly-signet	14	7	7	
Stage				0.23
I-II	10	3	7	
III-IV	15	9	6	
Size, cm				0.01
<3	11	2	9	
≥3	14	10	4	

pGL3-NEDD9 wild-type (WT) to construct pGL3-NEDD9 mutant (Mut) with QuickChange Site-Directed Mutagenesis kit (Stratagene; Agilent). For dual luciferase reporter assay, pGL3-NEDD9 WT or pGL3-NEDD9 Mut was co-transfected with miR-4735-3p mimic or miR-NC mimic into AGS cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, the firefly and *Renilla* luciferase activity of each group was detected by a Dual Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's protocol.

RNA immunoprecipitation (RIP). For RIP assay, anti-Ago2 (catalog no. ab186733, 1:50) and anti-IgG (catalog no. ab205718, 1:50) (both from Abcam) primary antibodies were used. Nuclei were isolated from cells by centrifugation for 10 min at 15,000 x g and 4˚C, then lysed with RNA lysis buffer (Promega Corporation) supplemented with protease and RNase inhibitors, and incubated with primary antibodies at 4˚C overnight. Next, RNA immunoprecipitated with RNA-binding proteins was isolated after addition of protein A agarose (catalog no. ab193255; 25 μl) and protein G agarose (catalog no. ab193258; 25 μl) (both from Abcam). Following washes, RNA was purified and reverse transcribed into cDNA. The expression of targets was determined by RT-qPCR.

Statistical analysis. All data were analyzed with GraphPad Prism 6.0 (GraphPad Software, Inc.) and presented as the mean ± SD. The differences of two groups were compared with Students' t test (unpaired Students' t-test for cells, paired Students' t-test for tissues). Pearson's correlation analysis was used to detect the correlation between miR-4735-3p and NEDD9. For three groups, the comparisons were conducted using one-way ANOVA followed by Newman Keuls analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-4735-3p is inversely related with NEDD9 in GC tissues. TargetScan 7.1 exhibited that there was a putative binding site between miR-4735-3p and NEDD9 3'UTR, and the context++ score percentile between miR-4735-3p and NEDD9 was as high as 99 (Fig. 1A). The putative binding site between miR-4735-3p and NEDD9 3'UTR was conserved in numerous species, including chimp, rhesus and squirrel (Fig. 1B). In GC tissues, an increase of NEDD9 mRNA level was observed (Fig. 1C) and a decrease of miR-4735-3p level (Fig. 1D) compared with normal tissues. It was observed that miR-4735-3p expression was negatively correlated with GC tumor size, but not sex, age, histology grade or stage (Table I). Notably, using Pearson's correlation analysis, a significant negative correlation was detected between miR-4735-3p and NEDD9 (Fig. 1E), suggesting that there may be a regulatory association between miR-4735-3p and NEDD9 in GC.

NEDD9 is negatively regulated by miR-4735-3p in GC cells. To investigate whether miR-4735-3p regulated NEDD9 expression in GC, miR-4735-3p mimic was used to overexpress miR-4735-3p in GC cell line AGS. As revealed in Fig. 2A, transfection of miR-4735-3p mimic elevated miR-4735-3p expression in AGS cells. RT-qPCR and western blotting showed that miR-4735-3p mimic decreased NEDD9 mRNA and protein levels (Fig. 2B-D). These data indicated that NEDD9 was negatively regulated by miR-4735-3p in GC cells.

miR-4735-3p directly binds to NEDD9 in GC cells. To further explore whether miR-4735-3p directly regulated NEDD9, dual luciferase assay was used. Mutations at two nucleotides were introduced into NEDD9 3'UTR (from CC to UG) (Fig. 3A). miR-4735-3p mimic significantly decreased the relative

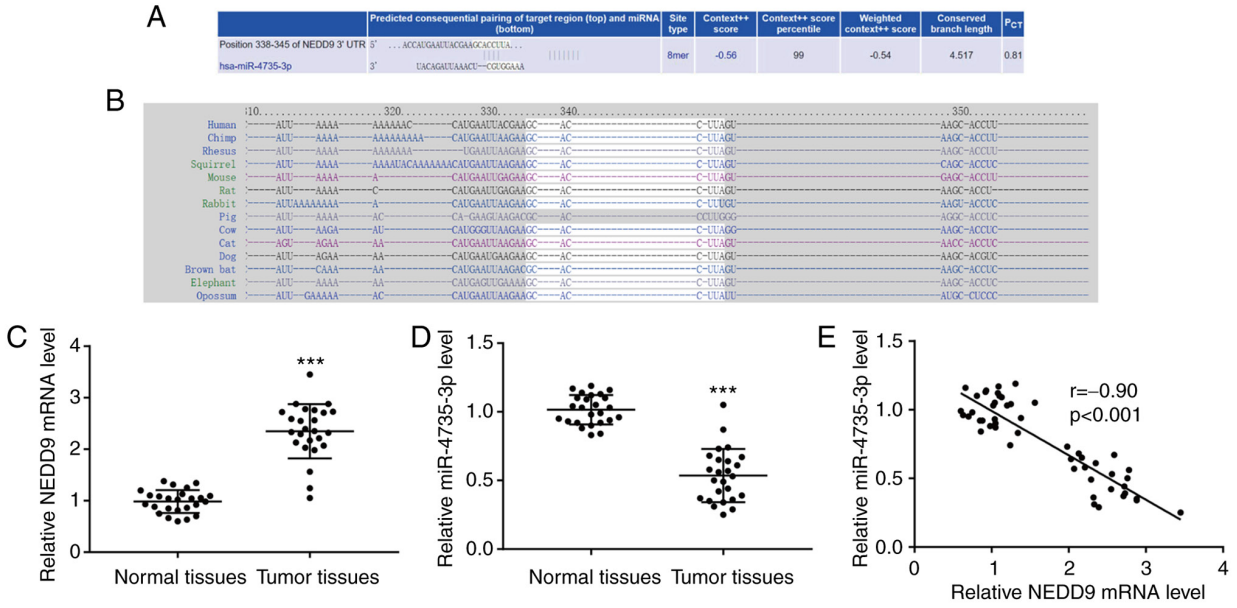

Figure 1. NEDD9 is inversely associated with miR-4735-3p in GC tissues. (A) The putative binding site between miR-4735-3p and NEDD9 was predicted by TargetScan 7.1. (B) The putative binding site was conserved in numerous species. (C and D) NEDD9 mRNA level was increased, and miR-4735-3p expression was decreased in GC tissues compared with the matched normal specimens. (E) Pearson's correlation analysis suggested that there was an inverse association between NEDD9 and miR-4735-3p in GC tissues. ***P<0.001 compared with normal tissues. NEDD9, neural precursor cell expressed, developmentally downregulated 9; miR, microRNA; GC, gastric cancer.

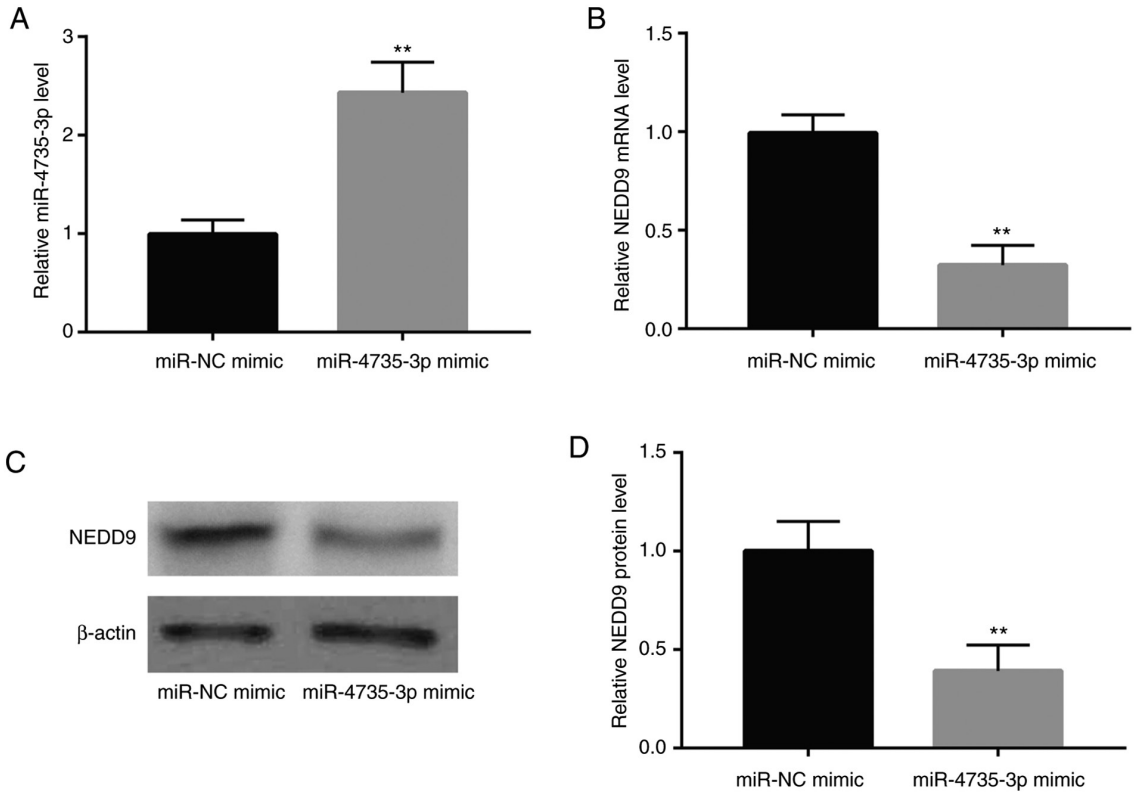

Figure 2. miR-4735-3p mimic decreases NEDD9 expression in gastric cancer cells. (A) In AGS cells, miR-4735-3p mimic significantly increased miR-4735-3p level. (B and C) In AGS cells, miR-4735-3p mimic decreased NEDD9 (B) mRNA and (C) protein levels. (D) Quantification of NEDD9 protein expression presented in panel C. **P<0.01 compared with miR-NC mimic. miR, microRNA; NEDD9, neural precursor cell expressed, developmentally downregulated 9; NC, negative control.

luciferase activity of AGS cells transfected with NEDD9 3'UTR-WT; while no effect was observed in the relative luciferase activity of AGS cells transfected with NEDD9 3'UTR-Mut (Fig. 3B). Furthermore, RIP assay demonstrated that miR-4735-3p and NEDD9 were highly enriched in the Ago2 group compared with the anti-IgG group (Fig. 3C). The

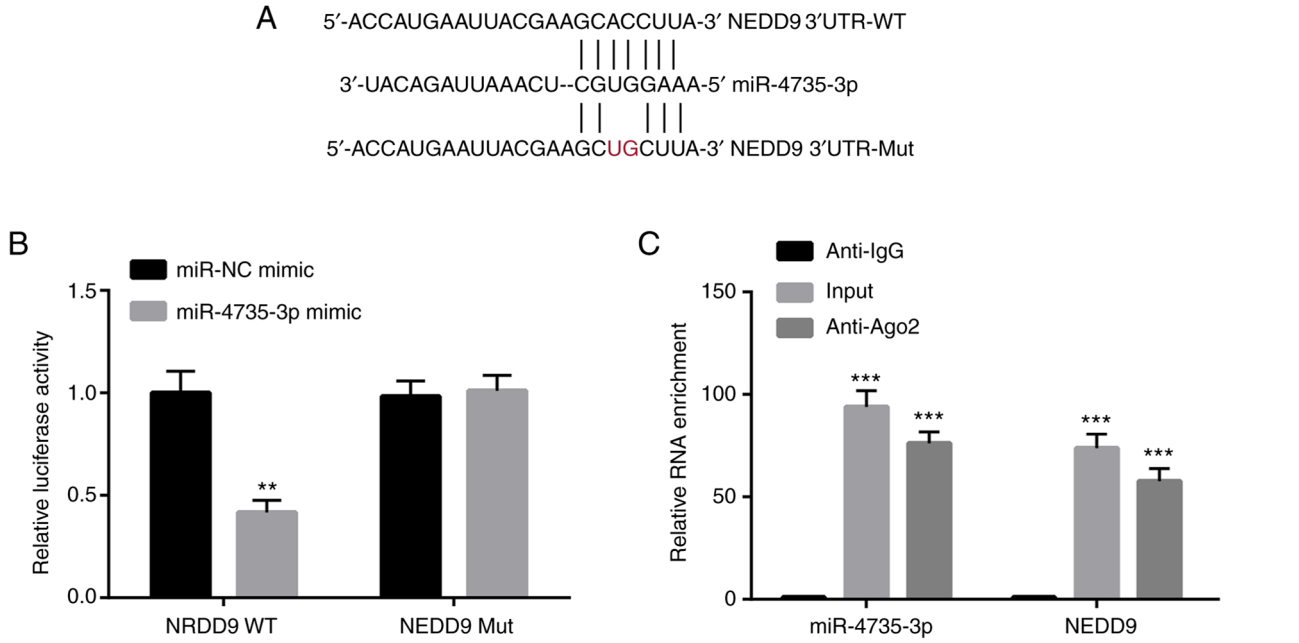

Figure 3. NEDD9 is regulated by miR-4735-3p in gastric cancer cells. (A) Mutations were introduced into the putative miRNA binding site in NEDD9 3'UTR to create NEDD9 3'UTR-Mut. (B and C) The dual luciferase reporter and RNA immunoprecipitation assays indicated that miR-4735-3p negatively regulated and targeted NEDD9 in AGS cells. **P<0.01 compared with miR-NC mimic. ***P<0.001 compared with anti-IgG. NEDD9, neural precursor cell expressed, developmentally downregulated 9; miR or miRNA, microRNA; UTR, untranslated region; Mut, mutant; WT, wild-type; NC, negative control.

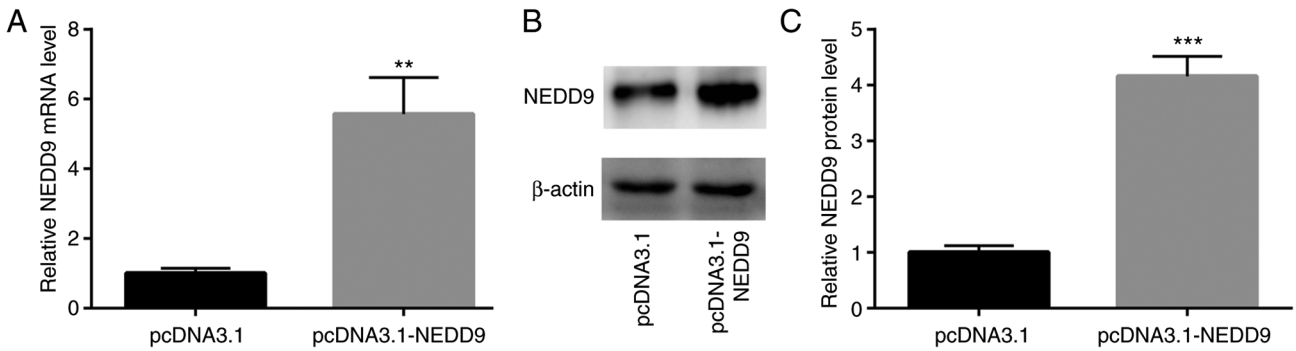

Figure 4. Overexpression of NEDD9 in gastric cancer cells. (A and B) In AGS cells, pcDNA3.1-NEDD9 increased NEDD9 (A) mRNA and (B) protein expression levels. (C) Quantification of protein expression levels presented in panel B. **P<0.01, ***P<0.001 compared with pcDNA3.1. NEDD9, neural precursor cell expressed, developmentally downregulated 9.

aforementioned results confirmed that miR-4735-3p directly regulated NEDD9 in GC cells.

miR-4735-3p overexpression suppresses GC cell migration and invasion by regulating NEDD9. Next, transfection of recombinant NEDD9 was used to study the function of NEDD9 in GC cells. As revealed in Fig. 4A-C, transfection of NEDD9 elevated NEDD9 mRNA and protein expression in AGS cells.

In a wound healing assay, miR-4735-3p mimic significantly inhibited migration of AGS cells; in addition, transfection of recombinant NEDD9 reversed miR-4735-3p-induced inhibition of AGS cell migration (Fig. 5A and B). In a Matrigel invasion assay, miR-4735-3p overexpression significantly inhibited invasion of AGS cells; in addition, transfection of recombinant NEDD9 reversed miR-4735-3p-induced inhibition of AGS cell invasion (Fig. 6A and B).

These data collectively showed that miR-4735-3p was a tumor suppressor in GC cells by regulation of NEDD9.

miR-4735-3p downregulates MMP2 and MMP9 in GC cells. In AGS cells, miR-4735-3p mimic decreased NEDD9 mRNA and protein levels accompanied with downregulation of MMP2 and MMP9, while increased E-cadherin mRNA and protein levels. Furthermore, transfection of recombinant NEDD9 led to an elevation of NEDD9, MMP2 and MMP9 proteins and a decrease of E-cadherin (Fig. 7A-C). These data suggested that miR-4735-3p could downregulate MMP2 and MMP9 and upregulate E-cadherin by suppression of NEDD9.

Discussion

Recently, the investigations on NEDD9 in GC have suggested that high NEDD9 expression was associated with poor differentiation,

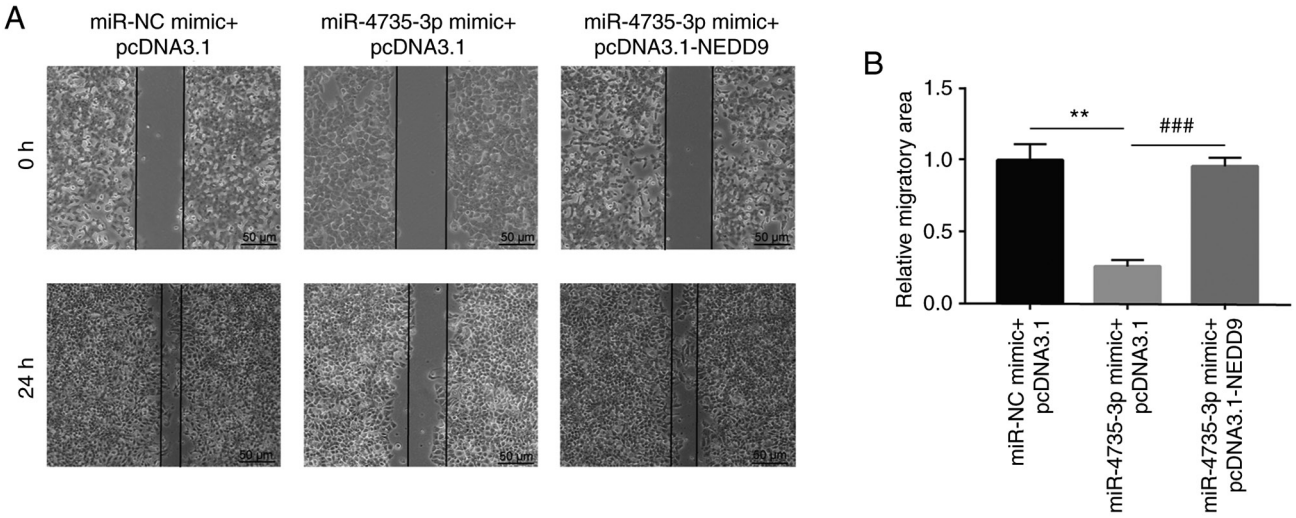

Figure 5. miR-4735-3p mimic inhibits gastric cancer cell migration by downregulation of NEDD9. (A) miR-4735-3p mimic reduced the wound closure area which was reversed by pcDNA3.1-NEDD9. (B) Quantification of migration area presented in panel A (scale bar, 50 μm). **P<0.01 compared with miR-NC mimic + pcDNA3.1. $^{###}$P<0.001 compared with miR-4735-3p mimic + pcDNA3.1. miR, microRNA; NEDD9, neural precursor cell expressed, developmentally downregulated 9; NC, negative control.

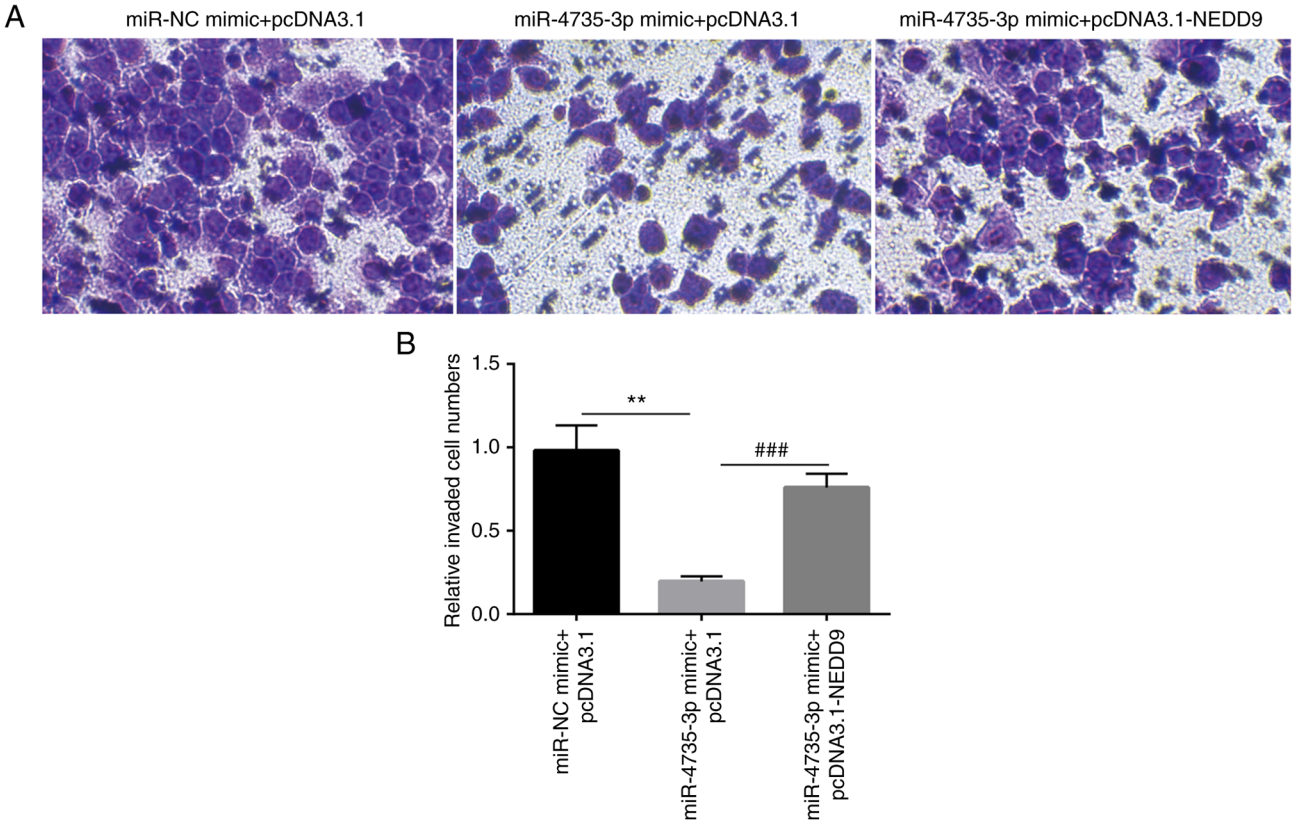

Figure 6. miR-4735-3p mimic inhibits gastric cancer cell invasion by downregulation of NEDD9. (A) miR-4735-3p mimic reduced the invasive ability of AGS cells, which was reversed by pcDNA3.1-NEDD9 (x200 magnification). (B) Quantification of migration area presented in panel A. **P<0.01 compared with miR-NC mimic + pcDNA3.1 and $^{###}$P<0.001 compared with miR-4735-3p mimic + pcDNA3.1. miR, microRNA; NEDD9, neural precursor cell expressed, developmentally downregulated 9; NC, negative control.

venous invasion, invasive depth, preset lymph node metastasis, distant metastasis and high clinical stage in patients with GC (17). Later, an *in vivo* study revealed that silencing of NEDD9 reduced the tumor volume in nude mice bearing gastric tumor (18); the *in vitro* assays indicated that decreased level of NEDD9 inhibited GC cell proliferation and migration (19,20). In the present study, it was also found that NEDD9 level was upregulated in tumor tissues of patients with GC, suggesting its oncogenic role in GC. Moreover, during the preparation of our article, several papers published to exhibit the oncogenic role of NEDD9 in GC; for

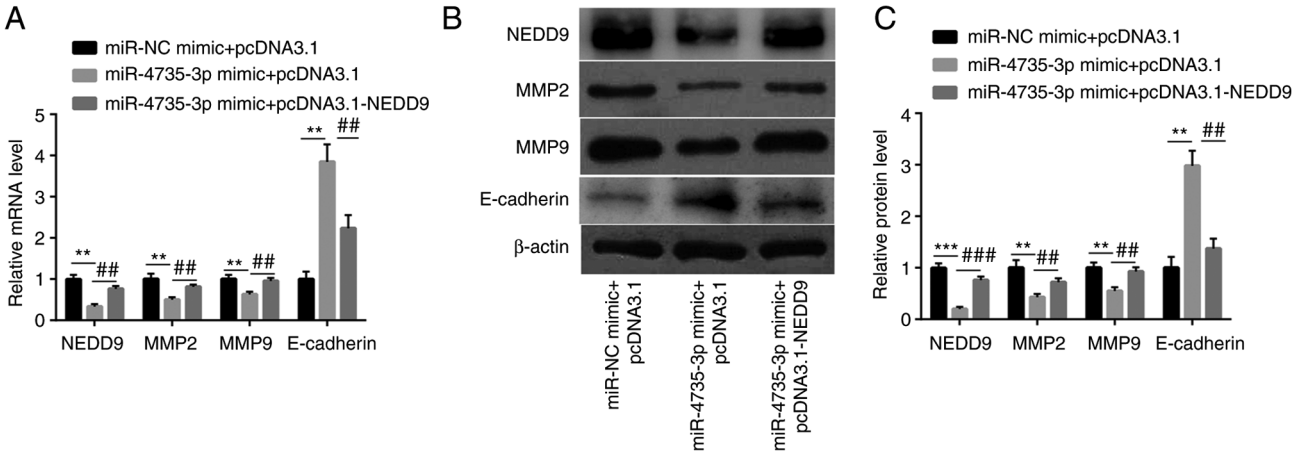

Figure 7. miR-4735-3p mimic suppresses MMP2 and MMP9 expression by regulation of NEDD9. (A and B) In AGS cells, miR-4735-3p mimic decreased NEDD9, MMP2 and MMP9 mRNA and protein levels and increased E-cadherin mRNA and protein levels. These effects were reversed by pcDNA3.1-NEDD9. (C) Quantification of protein expression levels presented in panel B. **P<0.01 and ***P<0.001 compared with miR-NC mimic + pcDNA3.1; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with miR-4735-3p mimic + pcDNA3.1. miR, microRNA; MMP, matrix metalloproteinase; NEDD9, neural precursor cell expressed, developmentally downregulated 9; NC, negative control.

instance, higher NEDD9 level was discovered in intestinal type GC compared with the adjacent non-neoplastic mucosa (21) and higher epithelial NEDD9 expression was related to higher mortality (22), which further proved the present findings.

miR-4735-3p acts as an oncogene or tumor suppressor in different cancer types. For example, miR-4735-3p acted as an oncogene in non-small cell lung cancer (NSCLC), as it was increased in NSCLC tissues and cell lines (23); however, it also served as a tumor suppressor in other types of cancer, as patients with low miR-4735-3p expression exerted an improved overall survival than those with high expression in prostate cancer (24). Long non-coding (lnc)RNA ARAP1-AS1 aggravated the development of bladder cancer by sponging miR-4735-3p/NOTCH2 axis (25). LncRNA ARAP1-AS1 promoted the progression of ovarian cancer by sponging miR-4735-3p/PLAGL2 axis (26). In consistency with previous studies (24-26), a decreased expression of miR-4735-3p was observed in GC tissues compared with matched normal tissues. In addition, using RT-qPCR, western blotting, dual luciferase reporter and RIP assay, it was found that NEDD9 was inversely associated with and targeted by miR-4735-3p in GC tissues.

Moreover, function assays indicated that miR-4735-3p regulated GC cell migration and invasion by suppression of NEDD9. However, the potential molecules related to metastasis of GC and NEDD9 are left to be explored.

As acknowledged, MMPs are endopeptidases involved in metastasis of cancer cells (27). Among 26 MMP family members, MMP2 and MMP9 were extensively studied due to their pivotal role in mediating cell invasion and migration in various cell types (28,29). E-cadherin acted as an invasion-suppressor molecule (30). It was reported that NEDD9 promoted metastasis of oral squamous cell carcinoma cells via upregulation of MMP9 (31), suggesting that NEDD9 regulated cell motility via MMPs. Furthermore, it was reported that downregulation of NEDD9 increased the expression levels of E-cadherin in colon cancer (32). Consistently, in the present study, it was identified that miR-4735-3p could regulate E-cadherin, MMP2 and MMP9 by suppression of NEDD9.

In conclusion, the present study demonstrated a tumor suppressor role of miR-4735-3p in GC by directly targeting NEDD9.

However, there remain 2 questions to be addressed: i) in the present study, the relationship between miRNA-4735-3p and NEDD9 was mainly investigated by examining the mRNA level. The protein level of NEDD9 in normal tissues and tumor tissues was not tested, which will be evaluated in future work; ii) in the present study, the function regulation of miRNA-4735-3p/NEDD9 was mainly evaluated by over-expression of miRNA-4735-3p and NEDD9; Cell lines with knock-out of NEDD9 will be examined in future studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YM conceptualized the study, performed the experiments and wrote the manuscript. YM confirms the authenticity of all the raw data. YM read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of General Hospital of Southern Theater Command (Guangzhou,

China). Written informed consent was provided by all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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