Original Article
microRNA-433 inhibits gastric cancer cell proliferation and migration and promotes apoptosis by targeting JNK1

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Abstract: Gastric cancer (GC) is the most frequent gastrointestinal tumor in adults and is the most lethal form of human cancer. A number of microRNAs (miRNAs) have been implicated in cell cycle progression, growth, apoptosis, angiogenesis and metastasis in GC. To define novel modulators that regulate susceptibility to tumorgenesis, we focused on miRNA-433. Quantitative RT-PCR was employed to investigate the level of miRNA-433 in GC cell lines and normal human gastric mucosal epithelial cells (GES-1). Luciferase reporter gene assays were performed to identify the interaction between miR-433 and 3'UTR of Jun N-terminal kinase (JNK1) mRNA. In vitro cell proliferation, cell cycle, apoptosis assays, cell migration, and invasion assays were performed by CCK-8, flow cytometry and transwell assay, respectively, to elucidate biological effects of miRNA-433. The protein level of JNK1, Bax, Bcl-2, Caspase-3, PCNA, MMP-2 and MMP-9 was determined by Western blotting. We found that miR-433 was down-regulated in GC cell lines compared with normal gastric GES-1 cells. Restoring miR-433 expression in GC cells dramatically suppressed cells proliferation, invasion and migration. Importantly, our data showed that miR-433 down-regulated the expression of JNK1 through directly targeting its 3'UTR. In conclusion, miRNA-433 is potentially involved in GC progression and metastasis by targeting JNK1, which may provide a novel potential target for diagnostic and therapeutic applications in GC.

Keywords: Gastric cancer, miR-433, JNK1, MKN-45

Introduction

Cancer is a universal public health problem because of its high incidence and mortality rate. Currently, gastric cancer (GC) is the fourth most common human malignant disease and the second leading cause of cancer-related deaths worldwide [1], with an overall 5-year survival of approximately 20% in patients with late-stage GC [2]. Advances in treatment of this disease are likely to come from a fuller understanding of its biology and behavior. Eating habits and an increase in Helicobacter pyloriinfection are important causes for GC [3]. Data from several studies show that various genetic alterations affect the downstream signal transduction pathways involved in the control of cell growth and differentiation and confer a tremen-

dous survival and growth advantage to GC cells, thus cause tumorigenesis and progression of GC [4, 5]. However, markers for tumorigenesis and progression of gastric cancer have not yet been discovered and specific therapeutic targets have not been identified.

MicroRNAs (miRNA) are a class of small RNA molecules involved in regulating gene function through specifically binding to the 3'untranslated region (3'UTR) of target mRNAs for translation and degradation [6]. A growing body of evidence indicates that miRNAs are implicated in the pathogenesis of a variety of human diseases, notably cancers function as oncogenes or tumor suppressors [7, 8]. Several recent studies conducted in human specimens point to a physiological function of microRNA-433 (miR-

433) in human diseases. miR-433 was decreased in hepatocellular carcinoma and inhibited cell proliferation by targeting p21 activated kinase (PAK4) [9]. Decreased expression of miNA-433 is associated with the prognosis of ovarian cancer [10] and inhibits cell migration and invasion via targeting Notch1 [11]. miR-433 was down-regulated in GC patients [12] and low expression of miR-433 had poorer survival than did patients with high expression of miR-433 [13]. However, the role of miR-433 in GC remains largely unknown.

Protein kinases are the largest family of enzymes encoded by the human genome and have emerged as one of the most popular drug target class in the pharmaceutical industry [14]. Jun N-terminal kinase (JNK) is a major MAPK pathway, which is called stress activated protein kinase pathway and is often deregulated in cancers [15]. The JNK kinase family includes three proteins (JNK1, JNK2 and JNK3). The early findings that the transforming actions of several oncogenes could be JNKs dependent, suggested that the JNK signaling could contribute to the cellular transformation that supports cancer development [16, 17]. JNK1 is activated in primary hepatocellular carcinomas (HCC) and is associated with both a poor disease prognosis and expression of progenitor cell biomarkers [18], and absence of JNK1 impaired liver cell proliferation and tumor formation [19]. In sharp contrast to the tumor-promoting role of JNK1 described above, in breast cancer models, the JNK1 has been shown to suppress tumor development [20]. However, the role of JNK1 in GC tumorigenesis is still unknown.

In the present study, miR-433 expression was evaluated by qRT-PCR in 3 GC cell lines, which showed that miR-433 was downregulated in GC cell lines and inversely correlated with JNK1 expression. Our data further demonstrated that miR-433 suppressed GC cell proliferation, invasion and migration and induced cell cycle arrest and apoptosis. More importantly, we demonstrated that forced expression of JNK1 rescued the inhibitory effects of miRNA-433 on GC proliferation, apoptosis, invasion and migration. Therefore, our study suggests that miRNA-433 inhibits GC tumorigenesis by directly targeting JNK1.

Materials and methods

Cell culture and transfection

GES-1, BGC-823, MKN-28, and MKN-45 cell lines were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were grown routinely in RPMI-1640 medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA) plus 50 U/ml penicillin and streptomycin and cultured in a 37°C humidified atmosphere of 5% CO_2.

Lentiviral production and transduction

Transduction of cells was performed using lipofectamine 2000 (Invitrogen Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's protocol. Briefly, MKN-45 cells were seeded in 6-well plates at 30-40% confluence 24 h prior to transduction. The pre-miR-433 and JNK1 coding sequencewas cloned into the pLKO.1-C1 lentiviral vector. The constructs were then co-transducted into HEK 293T cells with lentiviral packaging vectors by using lipofectamine 2000. Viruses were collected 48 h after transduction and used to infect MKN-45 cells. Empty pLKO.1-C1 lentiviral vector was used as a negative control (Mock).

Luciferase reporter assays

The 3'UTR of human JNK1 gene that were predicted to interact with miR-433 were synthesized and inserted into downstream of the firefly luciferase gene in the pGL3 vector (Promega, Madison, WI, USA), yielding pGL3-JNK1. MKN-45 cells were seeded in 96-well plates, reporter plasmids were co-transfected with miR-433 expressing vector or negative control (Mock) using the Lipofectamine 2000 method. After 24 h, cells were lysed and activities of firefly luciferase and Renilla luciferase were examined using the Dual-Luciferase Reporter Assay System (Promega, Beijing, China). Firefly luciferase activity was normalized to Renilla luciferase activity.

CCK-8 assay

Cell viability was evaluated using a CCK-8 (Dojindo; Kumamoto, Japan) assay. The viability of MKN-45 cells was assessed at four time points (hour 0, 24, 48, and 72) after being seeded at 3×10^3 cells/well into 96-well culture
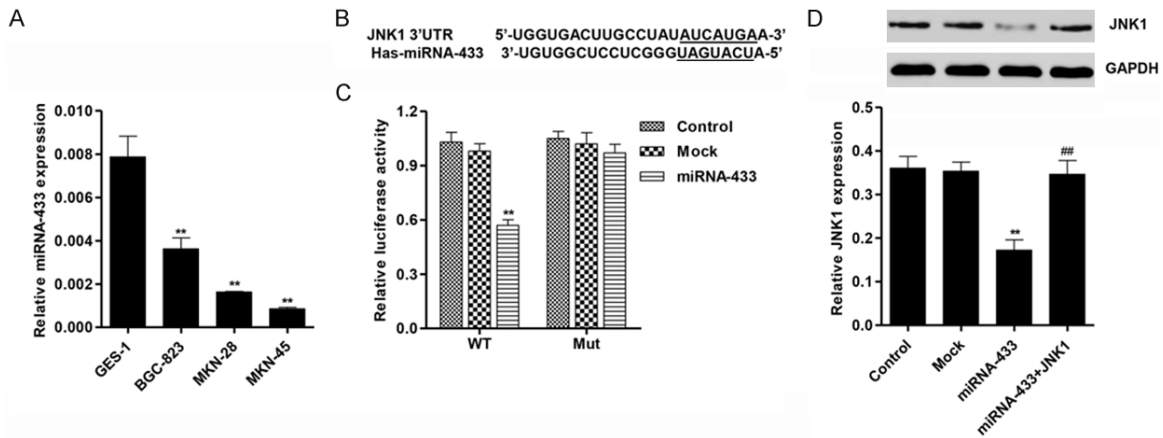
Figure 1. miRNA-433 directly targeted JNK1 in GC cells. A. JNK1 expression in GC cell lines and normal gastric GES-1 cells was measured by Real-time PCR. B. Base-pairing interaction between miRNA-433 seed sequences and JNK1, as predicted by TargetScan. C. Luciferase assay. MKN-45 cells were co-transfected with pGL3-JNK1 and miR-433 expressing vector or negative control (Mock). Firefly luciferase activity was normalized to Renilla luciferase activity. D. JNK1 protein levels were examined in MKN-45 cells transfected with miR-433 expressing vector or mock.**$P<0.01$ compared with GES-1 cells or Mock; ##$P<0.01$ compared with miRNA-433.

plates with serumfree medium for 24 h. Briefly, cells were incubated in 10% CCK-8 diluted in normal culture medium at 37°C until visual color conversion occurred. The absorbance of each well was measured with a microplate reader set at 450 nM.

Cell cycle and apoptosis assay

Cell cycle progression and apoptosis analysis was performed by flow cytometry. After being centrifuged, the cell pellets were resuspended in 500 μL propidium iodine (PI, 10 μg/mL) containing 300 μg/mL RNase (Sigma-Aldrich, USA) with subsequent incubation at 37°C for 30 min. Cell cycle distribution was calculated from $1×10^4$ cells for the FL-2 area using a flow cytometer (Beckman Coulter, Brea, CA). For apoptosis assay, $2×10^5$ cells were resuspended in 100 μL of 1×Annexin-binding buffer, 5 μl of Annexin V/FITC and 20 μl of PI solution (20 μg/mL). After incubation, the cells were left at room temperature in the dark for 15 min. Fluorescence was measured with a flow cytometer (Becton, Dickinson and Co., San Jose, CA), and the data were analyzed with Cell Quest software.

Cell migration and invasion assays

Cells were serum-starved for 24 h, and $5×10^4$ cells were seeded on Transwell (Corning, Inc., Corning, NY, USA) inserts. After 48 h, the non-migrated cells were removed from the insert with a cotton swab. The migrated cells were fixed for 10 min in 3.7% (v/v) formaldehyde in PBS before staining with 0.1% crystal violet for

15 min, followed by washing with PBS. Images were taken with a microscope (CX41RF; Olympus Corporation, Tokyo, Japan). The crystal violet-stained cells were counted. Cell invasion assays were performed as described for the migration assays, except that the Transwell was coated with Matrigel (BD Biosciences). Invading cells were fixed and stained. Images were taken, and cells were counted.

TaqMan RT-PCR for miRNA expression

Total RNA with miRNA was isolated from MKN-45 cells using TRIzol (Takara Inc., Dalian, P.R. China) according to the protocols supplied by the manufacturers. miR-433 was reverse transcripted by the looped primer, which binds to six nucleotides at the 3' portion of miR-433 molecules. Real-time PCR was performed using a TaqMan MicroRNA assay (Invitrogen, USA) on an ABI 7300 Real-Time PCR System (Applied Biosystems) according to the instructions of manufacturer. All mRNA quantification data was normalized to 5S according to the $2^{-\Delta\Delta Ct}$ method. The primer sequences were presented as follows: miR-433 5'-TGCGGTACGGTGAGCCT-GTC-3' (forward) and 5'-CCAGTGCAGGGTCCGA-GGT-3' (reverse), 5S 5'-CCATACCACCCTGGAAA-CGC-3' (forward) and 5'-TACTAACCGAGCCCGA-CC-CT-3' (reverse).

Western blot analysis

Western blot was performed to detect JNK1, Bax, Bcl-2, Caspase-3, PCNA, MMP-2, MMP-9 and GAPDH protein level in MKN-45 cells.
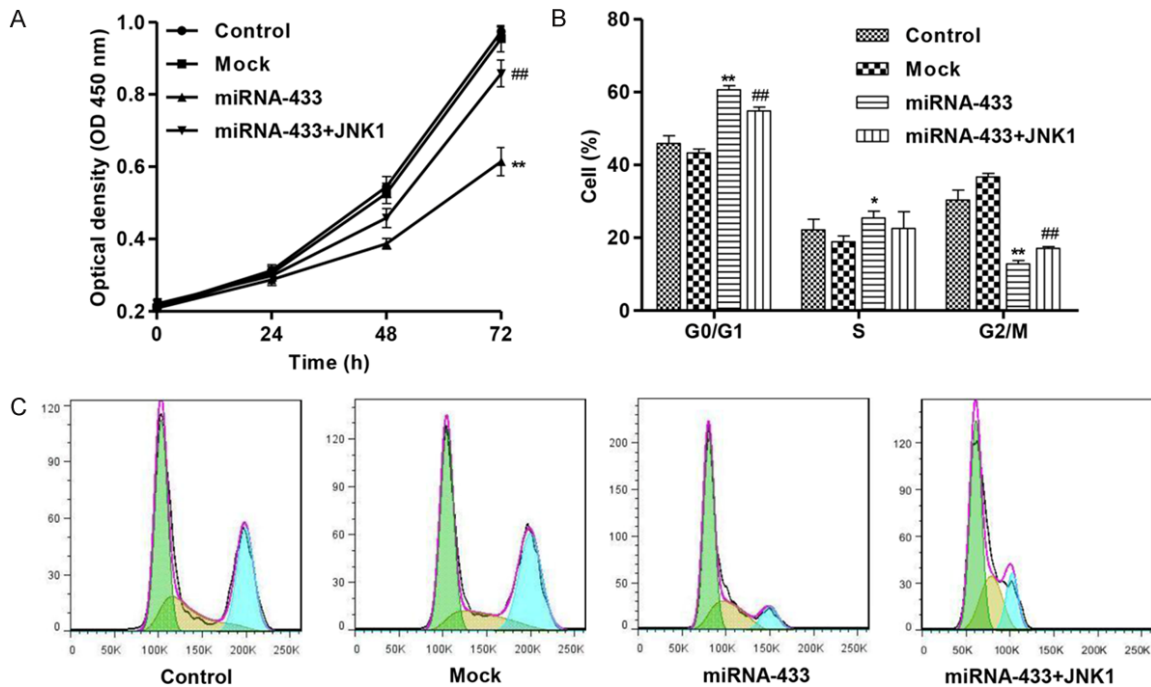
Figure 2. miRNA-433 inhibited MKN-45 cell growth and induced cell cycle arrest. A. Cell viability was determined in MKN-45 cells by CCK-8 assays. B, C. The proportion of cells in each phase of the cell cycle was determined in MKN-45 cells by flow cytometry assay. *$P<0.05$, **$P<0.01$ compared with Mock; ##$P<0.01$ compared with miRNA-433.

Cultured cells were lysed in RIPA buffer with 1% PMSF, and isolated protein was loaded onto a SDS-PAGE and transferred onto PVDF membrane. The membrane was then incubated with antibodies against JNK1, Bax, Bcl-2, Caspase-3, PCNA, MMP-2 and MMP-9 overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Signals were detected using ECL Substrates (Millipore, MA, USA). GAPDH was used as a loading control for normalization.

Statistical analysis

All the experiments were similarly done at least three times. All statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software, Inc., LaJolla, CA, USA). Statistical analyses were done by analysis of variance (ANOVA) or Student's t test and P<0.05 was considered statistically significant.

Results

miR-433 regulates JNK1 expression in MKN-45 cells

We first analyzed miR-433 expression in three GC cell lines, BGC-823, MKN-28 and MKN-45

cell lines and normal GES-1 cells by Real-time PCR (**Figure 1A**). miR-433 was expressed in lower level in BGC-823, MKN-28 and MKN-45 cell lines compared with GES-1 cells. To explore the regulation mechanism of miRNA-433, TargetScan bioinformatics analysis (www.targetscan.org) was employed. TargetScan identified that JNK1 mRNA contained potential binding sites of miRNA-433 (**Figure 1B**). To confirm JNK1 as a target and regulated by miR-433 in GC cells, JNK1 3'UTR was cloned and inserted into a luciferase reporter vector. The luciferase assay showed that miR-433 significantly suppressed luciferase activity containing the JNK1 3'UTR (**Figure 1C**). Western blot assay showed that, JNK1 was notably decreased after transfection of miR-433 into MKN-45 cells. Consistent with these results, a significant up-regulation of JNK1 was observed upon overexpression of JNK1 in miR-433-transfected MKN-45 cells (**Figure 1D**). These data suggested that JNK1 might be a direct target of miR-433 in GC cells.

miR-433 represses MKN-45 cell proliferation and induces cell cycle arrest

Then, miR-433 and miR-433+JNK1 expression vector were transfected into MKN-45 cells,
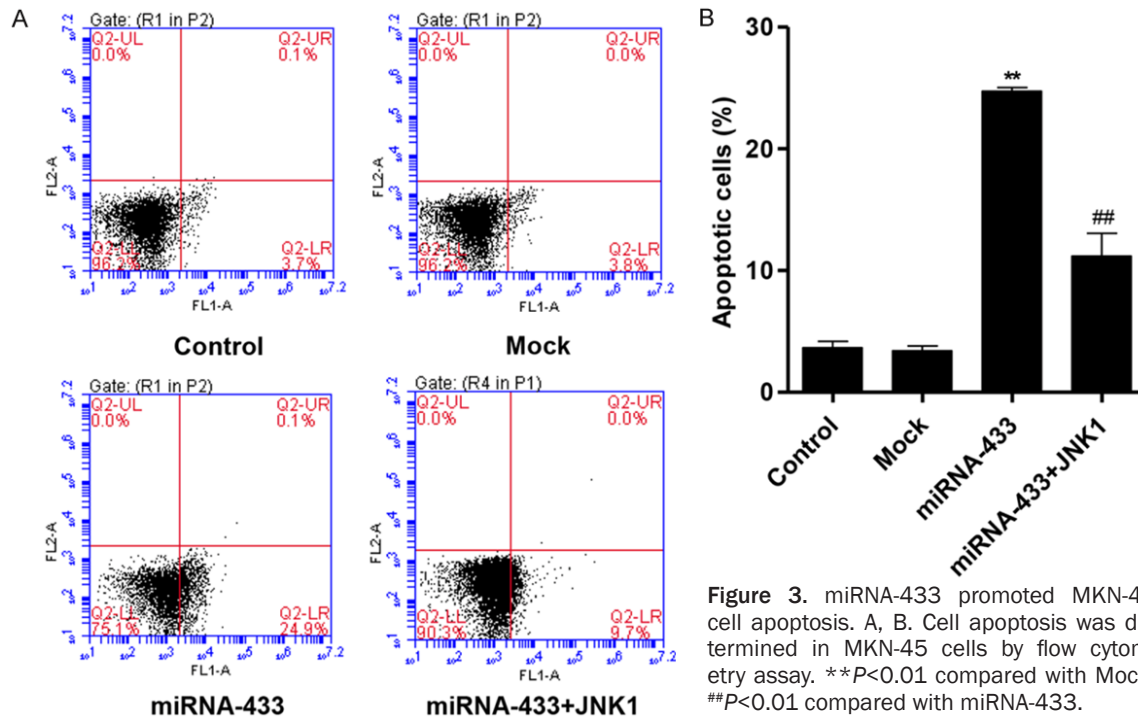
Figure 3. miRNA-433 promoted MKN-45 cell apoptosis. A, B. Cell apoptosis was determined in MKN-45 cells by flow cytometry assay. **$P<0.01$ compared with Mock; ##$P<0.01$ compared with miRNA-433.

respectively. 0, 24, 48 and 72 h after transfection, cell proliferation was analyzed using CCK-8 assay. As shown in **Figure 2A**, miR-433 induced great inhibition on cell proliferation compared with Mock sequence (Mock) in MKN-45 cells. To further validate the cell proliferation inhibition of miR-433, cell cycle was analyzed in MKN-45 cells. Cell cycle analysis showed that overexpression of miR-433 notably increased the rate of G0/G1 and S phase cells and reduced G2/M phase cell population in MKN-45 cells (**Figure 2B** and **2C**). On the contrary, overexpression of JNK1 slightly reduced G0/G1 and S phase population and increased the rate of G2/M phase cells in miR-433-transfected MKN-45 cells. These results indicated that miR-433 in GC cells may inhibited cell proliferation by arresting cell cycle progression in G0/G1 and S phases.

miR-433 induces MKN-45 cell apoptosis

Then, we evaluated the apoptotic function of miR-433 in MKN-45 cells by Annexin V-FITC/PI staining assay. As shown in **Figure 3A** and **3B**, flow cytometry analysis revealed that overexpression of miR-433 in MKN-45 cells significantly increased cell apoptosis by 6.27-fold compared to corresponding Mock. While, overexpression of JNK1 in MKN-45 cells reduced cell apoptosis by 54.7% compared with miR-433 overexpression.

miR-433 represses MKN-45 cell invasion and migration

As GC is a type of highly malignant tumor with a potent capacity to invade locally and distant metastasis, we next attempted to explore the effect of miR-433 restoration on GC cell invasion and migration. As shown in **Figure 4A** and **4B**, the transwell invasion assays showed that the number of cells that passed through Matrigel-coated membrane into the lower chamber was significantly reduced in the miR-433-transfected MKN-45 cells compared with the mock control-transfected cells (Mock). And ectopic expression of miR-433 using miR-433 expression plasmids significantly attenuated the migration potential of MKN-45 cells (**Figure 4C** and **4D**). However, overexpression of JNK1 in miR-433-transfected MKN-45 cells showed significantly increased invasion and migration potential (**Figure 4A-D**). These results suggest a role of miR-433 as a potential tumor suppressor.

Effects of miR-433 on apoptosis and metastasis-related protein expression

Moreover, four apoptotic factors Bcl-2, Bax, PCNA and Caspase-3 were evaluated by Western blot (**Figure 5A** and **5B**). In MKN-45 cells, overexpression of miR-433 induced down-regulation of PCNA and up-regualtion of

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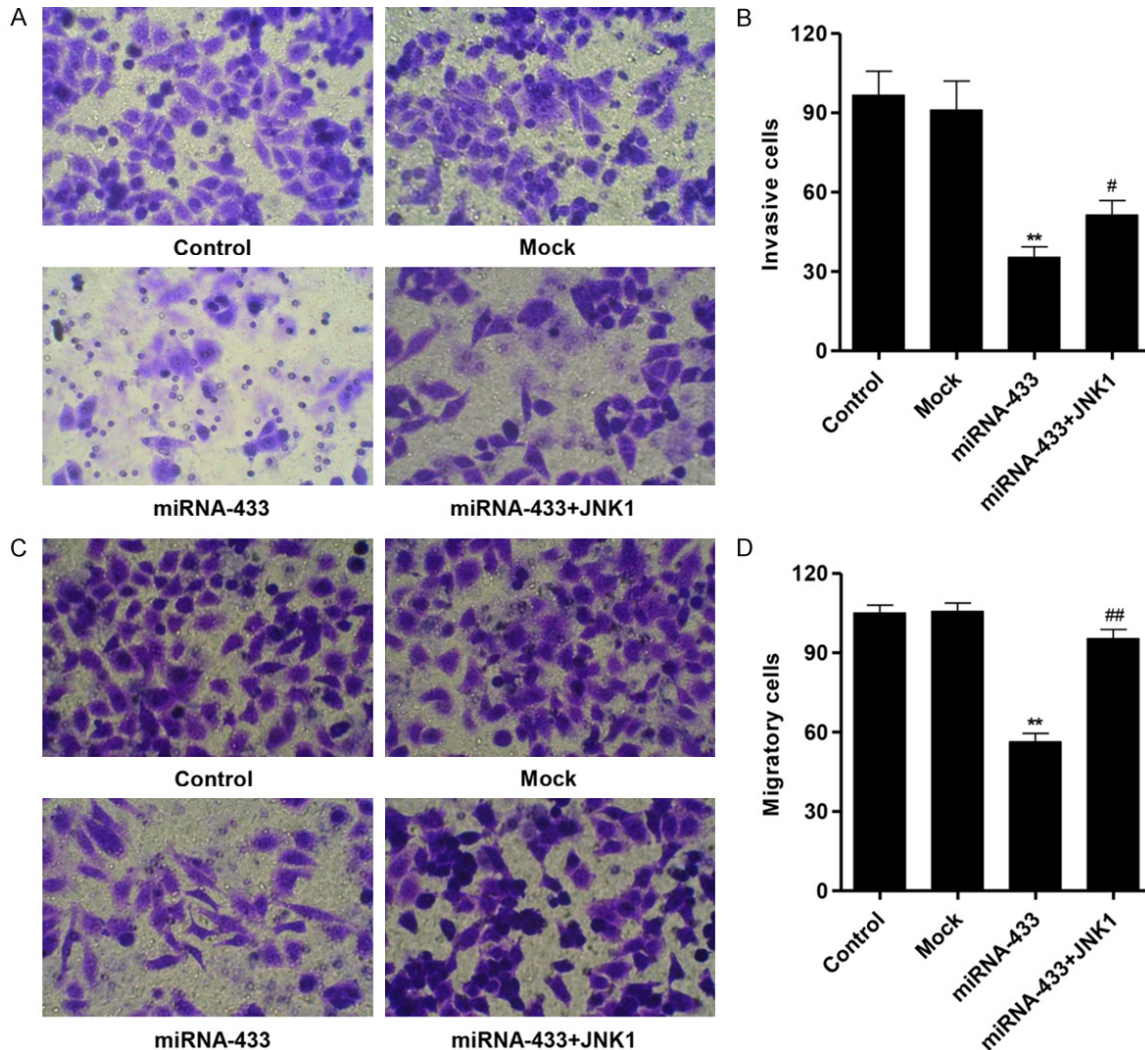
Figure 4. miRNA-433 inhibited MKN-45 cell invasion and migration. Cell invasion (A, B) and migration ability (C, D) was determined in MKN-45 cells by Transwell assay. **$P<0.01$ compared with Mock; ##$P<0.01$ compared with miRNA-433.

Bax/Bcl-2 ratio and Caspase-3. Since MMP-2 and MMP-9 play a vital role to facilitate the invasion and migration of GC cells, Western blot was performed to detect the two proteins expression. As shown in **Figure 5A** and **5C**, the expression of MMP-2 and MMP-9 was significantly reduced in miR-433-transfected MKN-45 cells compared with mock control cells. However, overexpression of JNK1 in MKN-45 cells significantly inhibited changes in protein expression induced by miR-433 (**Figure 5A-C**). Taken together, these results indicated that miR-433 induced cell apoptosis and inhibited migration in GC cells by targeting JNK1.

Discussion

In the present study, we found that JNK1 could promote GC cells proliferation, invasion and migration and inhibit apoptosis as an onco-gene, and miRNA-433 acts as a tumor suppressor that was inversely correlated with JNK1 expression in GC cells, and could inhibits proliferation, invasion and migration and increase apoptosis by regulation of JNK1.

In the last decade, miRNAs have emerged as critical regulators in regulation of cancer cell proliferation, invasion and migration, function as tumor suppressors or promoters of oncogenesis [21, 22]. However, to the best of our knowledge few previous reports have identified the miR-433 involvement in GC carcinogenesis. Low expression of miR-433 was associated with unfavourable outcome of GC patients in overall survival independent of clinical covariates, including depth of invasion, lymph-node
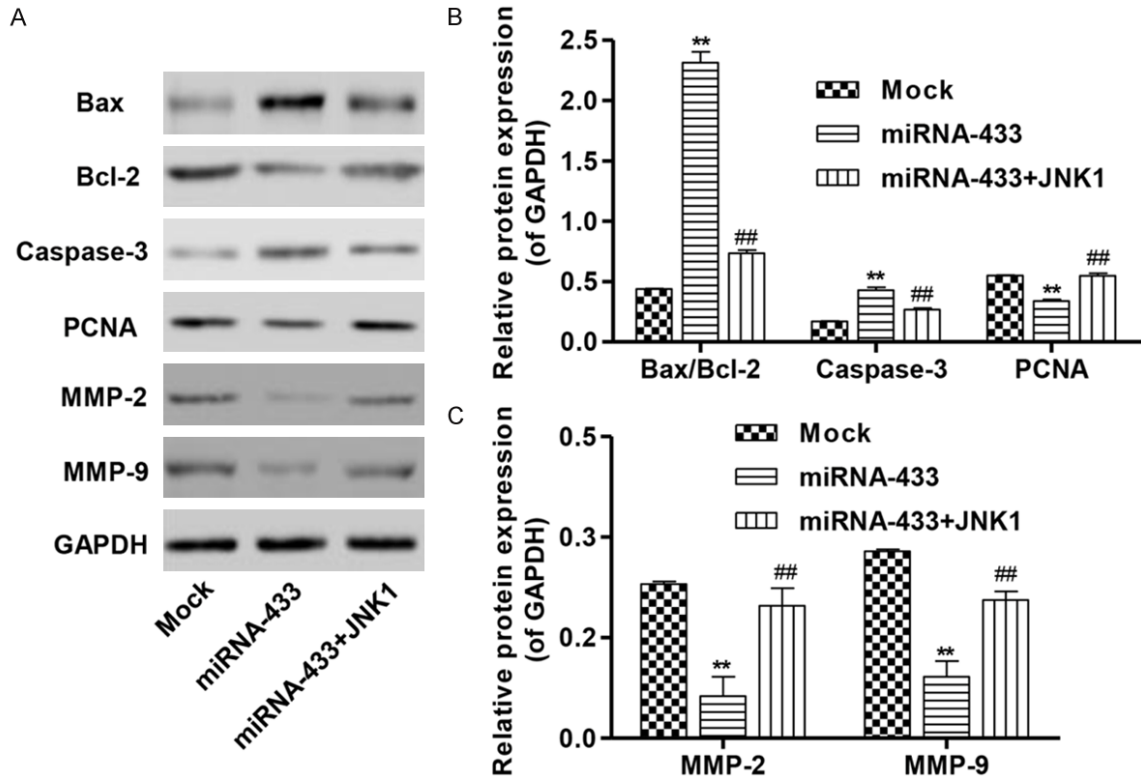
Figure 5. Protein expression in MKN-45 cells in response to miRNA-433 overexpression. A, B. The expression of apoptosis-related protein, including Bax, Bcl-2, Caspase-3 and PCNA, in MKN-45 cells was measured by Western blot assay. A, C. The expression of migration-related protein, including MMP-2 and MMP-9, in MKN-45 cells was measured by Western blot assay. $**P<0.01$ compared with Mock; $^{##}P<0.01$ compared with miRNA-433.

metastasis, and stage [13]. However, the mechanisms that dysregulated miRNA-433 have not been widely studied. In the present study, we attempt to investigate miR-433 expression in GC cell line and normal gastric GES-1 cells, and explore its biological function in GC carcinogenesis. Our data show that the relative expression of miR-433 in 3 GC cell lines (BGC-823, MKN-28 and MKN-45) was significantly down-regulated compared with GES-1 cells, suggesting that miR-433 is a candidate tumor suppressor in the pathogenesis of GC.

Further studies are required to clarify the function of miR-433 in the development and progression of GC cells. We thus test the putative function of miR-433 in GC cell lines, MKN-45. miR-433 overexpression in MKN-45 cells shows significant growth-suppressing effect by inhibiting cell proliferation and induced cell cycle arrest and apoptosis. Bcl-2 is a critical anti-apoptosis molecule, which has been shown to have inhibitory effects on the cell apoptosis, which in contrast to Bax [23]. The

Caspase-3 protein is a member of the cysteine-aspartic acid protease (caspase) family and activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [24]. PCNA was originally identified as an antigen that is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle and was a potential therapeutic target in cancer therapy [25]. In this study, we showed that miR-433 repressed the PCNA and promoted Caspase-3 protein and Bax/Bcl-2 ratio. Notably, we find that miR-433 inhibits GC cell invasion and migration in the present study. Previously we observed that the MMP-2 and MMP-9 belong to the MMP family and largely contribute to the growth, angiogenesis and progression of GC [26]. Our study verified that miR-433 significantly suppressed the protein expression of MMP-2 and MMP-9. These data suggest that miR-433 exhibits the growth, invasion and migration inhibitory ability in MKN-45 cells and acts as a potential tumor suppressor in GC pathogenesis.

Although the evidences have highlighted the importance of miR-433 as an onco suppressor in GC cells, the precise molecular mechanisms remain largely unclear. To better understand the tumor suppressive effect of miR-433 in GC tumorigenesis, bioinformatics analysis was used and identified JNK1 as a potential target of miR-433. With the 3'UTR luciferase reporter assay, JNK1 was identified as the direct target of miR-433. The increase in miR-433 expression is accompanied by down-regulation of JNK1 expression.

JNK1 has been proved to be activated in response to several stress signals, including tumor necrosis factor and hyperosmotic conditions, and is associated with induction of apoptosis [27] and acts as a crucial regulator of cell migration and epithelial morphogenesis [28]. Recent studies have demonstrated that JNK1 is a critical regulator of gastric tumorigenesis and frequent JNK1 activation in GC [15, 29]. Moreover, JNK activation has been detected in human GC samples and, consistent with this, JNK1 controls both tumor initiation and promotion by affecting cell proliferation and ROS production in a mouse model of GC [30]. In line with previous study, we found that overexpression of JNK1 in GC cells significantly enhanced cell proliferation, invasion and migration and inhibited cell apoptosis induced by miRNA-433.

In conclusion, the present study demonstrated that miRNA-433 could inhibit GC cell proliferation, invasion and migration via targeting JNK1. Further studies are required to fully understand the roles and mechanisms of miRNA-433 and JNK1 in GC, which will be beneficial for the development of therapeutic strategies for GC.

Disclosure of conflict of interest

None.

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