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RESEARCH

MicroRNA-181a Functions as an Oncomir in Gastric Cancer by Targeting the Tumour Suppressor Gene ATM

Xiangyang Zhang · Yuqiang Nie · Xiaorong Li · Guifu Wu · Qun Huang · Jie Cao · Yanlei Du · Junda Li · Ruoyu Deng · Dongshen Huang · Baozhi Chen · Shang Li · Baojun Wei

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Abstract Based on our previous experiments, this study is to further investigate the functional significance of miR-181a and its target gene in gastric cancer. Expression of miR-181a was detected by qRT-PCR in three normal gastric tissues and three human gastric cancer cell lines (SGC-7901, MGC-803, and BGC-823 cells). After transfection with miR-181a inhibitor, proliferation, apoptosis, migration, and invasion of the SGC-7901 cells were evaluated. Ataxia-telangiectasia mutation (ATM) was predicted as a target gene of miR-181a with bioinformatics analysis, and was verified by lucifersae reporter assay. Expression of ATM protein in HEK293T cells and tissues was measured by Western Blot. Expression of ATM mRNA in HEK293T cells was measured by RT-PCR. Compared with three non-tumour tissues, the expression of miR-181a in three gastric cancer cells was significantly increased by 26.68, 14.83 and 14.96 folds; Compared with Negative Control(NC) and blank groups, transfection of miR-181a inhibitor led to inhibition of SGC7901 cell proliferation, invasion, and migration as well as promotion of apoptosis. A luciferase reporter assay demonstrated that ATM was a direct target of miR-181a, miR-181a mimics transfection down regulated ATM mRNA and protein expression. There was

X. Zhang
Department of Gastroenterology, Shenzhen Futian Hospital of TCM, No. 6001 North Central Avenue of Futian, Shenzhen, China 518034
e-mail: xiangyangzhang@163.com

Y. Nie (✉) · J. Cao · Y. Du
Department of Gastroenterology, Guangzhou Key Laboratory of Digestive Disease, Guangzhou First Municipal People's Hospital, Guangzhou Medical University, No.1 Panfu Road, Guangzhou, China 510180
e-mail: yuqiangnie@126.com

J. Cao
e-mail: xyz_701@163.com

Y. Du
e-mail: 136225125@qq.com

X. Li · G. Wu · Q. Huang
Department of Gastroenterology, The Fourth People's Hospital of Shenzhen City (Futian Hospital), No. 3025 Middle Shennan Road, Shenzhen, China 518033

X. Li
e-mail: 18823300801@126.com

G. Wu
e-mail: 1193277911@qq.com

Q. Huang
e-mail: 1779959514@qq.com

J. Li · R. Deng
Department of Gastroenterology, People's Hospital Of New District Longhua Shenzhen, Jianshe East Road of Longhua New City, Shenzheng, China 518019

J. Li
e-mail: 1026003538@qq.com

R. Deng
e-mail: 380630870@qq.com

D. Huang
Guangzhou Chest Hospital, Guangzhou, China 510180
e-mail: dr_dhs@163.com

B. Chen
Second People's Hospital of Lanzhou City, Lanzhou, China 730046
e-mail: 15811868828@163.com

S. Li
Jiuquan City Health School, Lanzhou, China 735000
e-mail: 312292027@qq.com

B. Wei
Lanzhou Xigu People's Hospital, Lanzhou, China 730060
e-mail: 357291669@qq.com

inverse correlation between miR-181a and ATM protein expression in gastric cancer and normal gastric tissues. Our study demonstrates that over-expression of miR-181a might be involved in development of gastric cancer by promoting proliferation and inhibiting apoptosis probably through directly targeting ATM. miR-181a modulation may be a potential strategy for the development of miRNA-based therapy of gastric cancer.

Keywords miR-181a · micro-RNA · Gastric cancer · Target gene · ATM

Abbreviations

miRNA	microRNA
BCL-2	B cell leukemia-2
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptional PCR
CLL	Chronic lymphatic leukemia
RISC	RNA-induced silencing complex
PBS	Phosphate balanced solution
NC	Negative control
ATM	Ataxia-telangiectasia mutated gene
rpm	Revolutions per minute
bp	Base pair
OD	Optical density
ddH_2O	Double distilled water
cDNA	Complementary deoxyribonucleic acid
pre-miRNA	Precursor of miRNA
pri-miRNA	Precursor of pre- miRNA
3′UTR	3′-untranslated region
DEPC	Dithylpyrocarbonate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
LNA	Locked nucleic acid
DMSO	Dimethyl sulphoxide
PLB	Passive lysis buffer
BCA	Bicinchoninic acid
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
TBST	Triethanolamine buffere saline solution with Tween-20
EDTA	Ethylene diamine tetraacetic acid
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PI	Propidium iodide

Introduction

Gastric cancer is the fourth most common cancer worldwide, and it is estimated that there are 603,003 new cases per year among men and 330,290 new cases per year among women [1]. Many well characterized tumour suppressors and oncogenes have been analyzed to define their respective roles in development and progression of gastric cancer, but only a few consistent and frequent genetic alterations have been identified [2]. MicroRNAs (miRNAs) post-transcriptionally regulate the expression of several hundreds of genes mainly by targeting their mRNAs [3]. MiRNAs play crucial roles in various cellular processes such as proliferation, apoptosis, development, differentiation, and metabolism [3]. Accumulating evidence suggests some miRNAs are up-regulated or down-regulated in a variety of cancer [4–6], including gastric cancer [7], which act as oncogenes or tumour suppressor genes [8–10] and are involved in cancer initiation, progression, and metastasis.

Human microRNA-181a (miR-181a), a member of the miR-181 family, plays a crucial role in development and differentiation of lymphocyte [10–12], blood vascular endothelial cell [13], and skeletal-muscle [14], and is associated with various human malignancies including leukemia [15, 16], multiple myeloma [17], breast cancer [18], and hepatocellular carcinoma [19, 20]. Using Illumina miRNAs microarray and real-time reverse transcription–quantitative PCR (qRT-PCR), our previous work has shown a 5.45-fold higher level of miR-181a in human gastric cancer tissues as compared with adjacent non- cancerous tissues. Among 60 patients studied, the over-expression of miR-181a was seen in 52 cases (86.67 %). The up-regulation of miR-181a in gastric cancer tissues is also observed in the studies conducted by Ueda, et al. [9] and Yao, et al. [21] respectively.

ATM, the gene defective in a autosomal recessive disorder ataxia telangiectasia (A-T), is localized to human chromosome 11q22–23. It encodes a serine/threonine protein kinase member of the phosphatidylinositol-3 kinase-related protein kinase (PIKK) family, and regulates cell cycle checkpoint, DNA repair, telomere maintenance, and apoptosis in response to DNA double-strand breaks by phosphorylating numerous substrates including p53 and BRCA1 [22, 23]. ATM is regarded as a tumour suppressor. Individuals with ATM mutations have an increased risk of breast cancer and perhaps gastric cancer [24], and ATM protein inhibits malignant transformation of human mammary gland epithelial cells [25]. The expression of total and phosphorylated ATM (active form) is decreased in gastric cancer tissues [26], which suggests that ATM might participate in development gastric cancer.

Materials and Methods

Cell Culture and Tissue Preparation

SGC-7901, MGC-803, BGC-823 and HEK 293T cell were purchased from the cell bank of Chinese academy of sciences.

Nine Gastric cancer specimens and their matching non-tumour tissues specimens were collected at the time of surgery from patients in First Municipal People's Hospital of Guangzhou. Samples were snap frozen immediately and stored at −80 °C. All tissue samples were obtained with informed consent and the study protocol was approved by the Medical Ethics Committee of First Municipal People's Hospital of Guangzhou.

RNA Isolation and Real-Time Reverse Transcription-Quantitative PCR (qRT-PCR)

Total RNA was extracted from cell lines and tissue samples with Trizol reagent (Invitrogen, Wisconsin, USA). The quality and quantity of the RNA samples were assessed by standard electrophoresis and spectrophotometric assay. Qualifying conditions is OD260/OD280=1.9~2.1. The RNA was reversely transcribed into complementary DNA (cDNA) with miRCURY LNA™ Universal cDNA Synthesis Kit (EXIQON, Vedbaek, Denmark) according to the manufacturer's instructions. Real-time PCR was performed using miRCURY LNA™ SYBR® Green master mix, Universal RT (EXIQON, Vedbaek, Denmark) on DNA Engine Opticon 2 Sequence Detection (MJ Research, Massachusetts, USA). The quantifications were normalized with U6 small nuclear RNA (RNU6B). The primer used in this study for detection of miR-181a was LNA™ PCR primer set provided by the EXIQON Company. ATM mRNA forward primer:5′*AGAGACAGGGTT GCCATTG3′*, reverse primer: 5′*TGCAGAAAGAGTTCCAGC TT*3′. The relative expression of miR-181a was quantified by the $2^{-\Delta\Delta CT}$ method [27].

miR-181a Targets Prediction

The miR-181a sequence was obtained from miRBase (http://www.microrna.sanger.ac.uk). The target genes were predicted using three algorithms including TargetScan [28], PicTar [29], and Miranda [30].

Transfection of Gastric Cancer Cells

SGC7901 cells were tansfected with plasmids pSIREN-RetroQZsGreen (Promega, WI, USA) connected with the miR-181a inhibitor sequence, The miR-181a inhibitor sequence was 5′-*ACTCACCGACAGCGTTGAATGTTTTC GACTCACCGACAGCGTTGAATGTT TTCGACTCACCG ACAGCGTTGAATGTT*-3′ (Landbiology, Guangzhou, China), and the plasmids containing a scrambled sequence (promega, WI, USA) were used as Negative Control (NC). Transfection efficiency was detected under a fluorescence microscope using FAM labeled NC by observing the cytoplasmic expression of enhanced green fluorescent protein. The experiments were repeated three times.

Cell Proliferation Assay

For MTS assay, at four different time points (24 h, 48 h, 72 h and 96 h) MTS (cellTiter96AQ, Promega, USA) solution (10 μl) was added to each well, followed by incubation for 4 h at 37 °C and the absorbance read at 490 nm; For viable cell count, the cells were harvested at five different time points (24 h, 48 h, 72 h, 96 h, and 120 h), and counted using haemocytometer (siakiayo, Shanghai, China) after stained with 0.1 % trypan blue. The experiments were repeated 3 times.

Colony Formation Assay

When single-cells grew into group-like colonies, colonies were washed once with PBS and fixed with 4 % paraformaldehyde for 20 min, then stained with crystal violet for 15 min. The excess crystal violet dye was siphoned off and washed with PBS. The automated enzyme-linked spot image analyzer Elisporeader 400 PRO-X (BIOSYS, Kiefernweg, Germany) was used to read the plates. The experiments were repeated three times.

In Vitro Tumour Cell Migration and Invasion Assays

Transwell cell culture plate (Becton Dickinson, New Jersey, USA) was used to measure cells' migratory and invasive ability. In both assays, six hundred microliter of 10 % FBS-containing medium was added to the lower chamber as a chemoattractant. Cells were incubated for another 24 h at 37 °C, and then non-migrating cells or non-invading cells on the upper surface of the chambers were scraped off with cotton swabs. Cells on the underside of the membrane were fixed and stained with Kristallviolet for 10 min. Stained cells were visualized under a microscope. The experiments were repeated 3 times.

Apoptosis Assay

Twenty-four hours after transfection, cells were labeled with annexin V-FITC and propidium iodide (PI) (keygen Biotech. Nanjin. China) according to the manufacturer's instructions. Cells were analyzed by flow cytometry (Becton Dickinson, New Jersey, USA). The experiments were repeated three times.

Western Blot

Expression of ATM protein was determined by Western Blot. Protein concentration was measured by BCA method [31]. Proteins were separated by 10 % SDS gel and blotted to Immobilon-P Transfer Membrane (MILLIPORE, Missouri, USA). Then the membranes were blocked with 5 % skim milk at room temperature for 2 h, followed by incubation with primary antibodies anti-ATM (Abnova, Taipei, Taiwan) of anti-GAPDH body (KangCheng, Shanghai, China) at 4 °C overnight. After incubation with the secondary antibody rabbit

anti-mouse IgG (H+L) (Southern biotech, Alabama, USA) for 1 h at room temperature, proteins were visualized and quantified by enhanced chemiluminescence (MILLIPORE, Missouri, USA). The experiments were repeated three times.

DNA Amplification and Cloning

The 3′UTR of the human ATM gene, which contained two predicted miR-181a binding sites at the positions 123nt and 3525nt was amplified with PCR from whole blood genomic DNA. The ATM primers included the forward: 5′-*TACGCGTC GACTCTT CAGTATATGAATTACCCTTTCATTC*-3′ and the reverse: 5′-*ATAAGAATGCGGCCGCGCTTTT AGAATTATTTAT TCAAAAACACT*-3′ (Invitrogen Biotechnology, Guangzhou. China). The PCR products were reclaimed from agarose gel electrophoresis, and cloned into the luciferase reporter vector PsiCHECK™-2 (Promega, WI, USA), yielding PsiCHECK™-wt-ATM. Site-directed mutagenesis of the miR-181a target-site in the ATM 3′UTR was carried out using TaKaRa MutanBEST Kit (U-Me Biotech, WuHan, China). Mut1-ATM is mutation occured at 123nt position of ATM 3′UTR (T→C), while mut2-ATM is mutation occured at 3525nt position (T→C); Mutation occured at these both sites is mut3-ATM. Primers for 123nt were forward 1 (5′-*GTGAACTATTGTGGGTTTTTTATCGATCTGGT TTTAATACT TGATTTAA*-3′) and reverse 1 (5′-*TTAAATCAAGTA TTAAAACCAGATCGATAAAAAACCCACAATAGTTCAC*-3′); the primers for 3525 nt position were forward 2 (5′-*TTCTATAG ATTTTAGTACTATATCGATCATTACTTT ACTGTTACC*-3′) and reverse 2 (5′-*GGTAACAGTAAAGTAATGATCGATATAGTACT AAAATCTA TAGAA*-3′); All constructs were verified by sequencing. Binding sites for the used oligos (PCR primers, siRNA oligos) were verified using blast.

Luciferase Assay

HEK 293T cells were transiently cotransfected with 0.5 μg PsiCHECK™-wt/mut plus 1 μl miR-181a mimics (25 nM) or NC, using lipofectamine™2000 (Invitrogen, Wisconsin, USA). Firefly and Renilla luciferase activities were measured consecutively by using Dual-Luciferase Reporter Assay System (Promega, Massachusetts, USA) according to the manufacturer's instructions, and normalized for transfection efficiency by the control vector pRL-TK (Promega, Massachusetts, USA) that contained Renilla luciferase. The experiments were repeated three times.

Statistical Analysis

Measurement data were presented as the mean ± standard deviation (SD), and enumeration data were presented as frequency (%). Measurement data and enumeration data were compared with Student's *t* test and Chi-square test, respectively. A 2-tailed P value of less than 0.05 was considered statistically significant.

Results

miR-181a Was Up-Regulated in Gastric Cancer Cells

Expression of miR-181a was analyzed by qRT-PCR in three different gastric cancer cell lines and three normal gastric mucosal tissues. Randomly select three Normal Tissues, use the average expression of miR-181a as control [32]. As shown in Fig. 1a, expressions of miR-181a in all of three gastric cancer cells were remarkably higher than in normal gastric tissues by 14.83 to 26.68 folds ($p < 0.05$). We chose SGC-7901 cells to further study.

miR-181a Inhibitor Decreased the Growth and Increased the Apoptosis of SGC-7901 Cells

According to the viable cell count and MTS assay, miR-181a inhibitor significantly decreased SGC-7901 cell growth 96–120 h after transfection compared with NC group (Fig. 1b and c, $*p < 0.05$). The proliferation-inhibiting effect of miR-181a inhibitor on gastric cancer cell growth was further confirmed by clony formation assay (Fig. 1e). 120 h later the clonies of miR-181a inhibitor group were significantly decreased than NC group (Fig. 1d, $*p < 0.05$).

The effects of miR-181a inhibitor on apoptosis of SGC-7901 cells were investigated by flow cytometry (Fig. 1j)

Twenty-four hours after transfection with miR-181a inhibitor, the apoptosis rate of SGC-7901 cells was significantly higher than that of cells transfected with NC (Fig. 1k, $*P < 0.05$). It means that miR-181a inhibitor could enhance the apoptosis of SGC-7901 cells compard to NC.

miR-181a Inhibitor Inhibited Migration and Invasion of SGC-7901 Cells

Impact of miR-181a inhibitor on migration (Fig. 1f) and invasion (Fig. 1g) of SGC-7901 cells were evaluated in vitro. We found that SGC-7901 cells transfected with miR-181a inhibitor migrated slower than those transfected with NC (Fig. 1h, $*p < 0.05$). It was also shown that miR-181a inhibitor significantly inhibit the invasive capability of SGC7901 compared with the NC (Fig. 1i, $*p < 0.05$).

miR-181a Directly Inhibited the Expression of ATM Through ATM 3′UTR

As shown in Fig. 2a, two potential miR-181a targeting sites were found at the positions 123nt and 3525nt in ATM 3′UTR. Comparative sequence analysis showed that the target sequences were evolutionarily conserved in different species (Fig. 2b). To confirm the influence of miR-181a on ATM

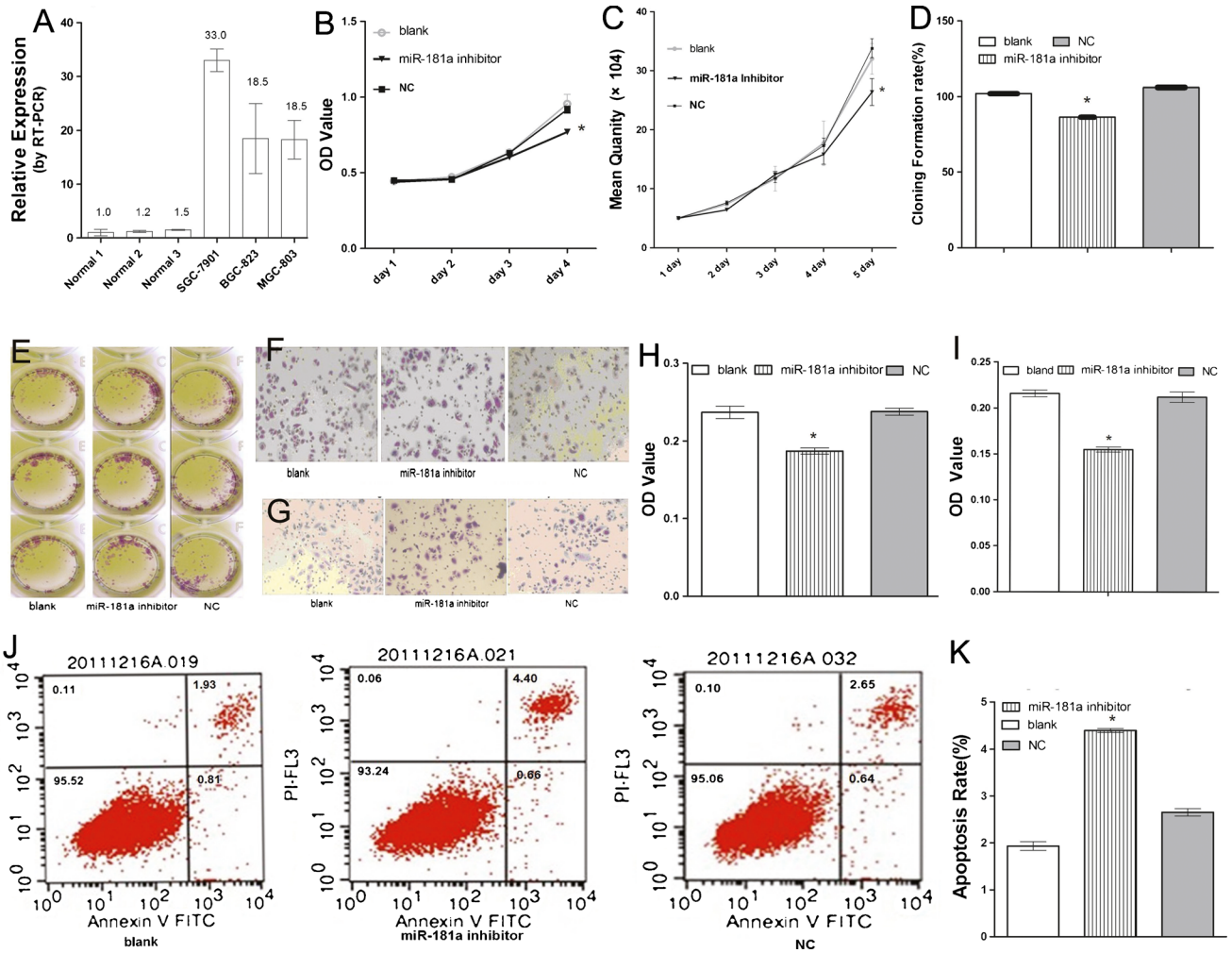

Fig. 1 **a**: The expression levels of miR-181a was found in three gastric cancer cell lines and three non-tumour mucosal tissues by qRT-PCR using specific primers. **b**: MTS assay. miR-181a inhibitor inhibited cell growth. (miR-181a inhibitor inhibited cell growth compared with NC by MTS assay analysis. *$p < 0.05$). **c**: Cell growth curve assay.(miR-181a inhibitor was found to down-regulate miR-181a expression compared with NC and blank control group. *$p < 0.05$). **d**: The graph shows the clony forming efficiency. Cell clonogenicity was strongly decreased by transfection of miR-181a inhibitor compared with NC and blank control group (*$p < 0.05$). **e**: miR-181a inhibitor inhibited clonogenicity of SGC-7901 cells in vitro. (100 cells per well in 96-plate, observed at the fifth days). **f**: miR-181a inhibitor decreases SGC-7901 cells migratory potentials. Transwell cell migration assay were performed after SGC-7901 cells transfected with miR-181a inhibitor or NC. The photograph is a representative example. SGC-7901 cells had significant difference migration between NC group and miR-181a inhibitor group. **g**. Effect of miR-181a inhibitor on invasion ability of SGC-7901 cells. Transwell cell invasion assay was performed after transfected with miR-181a inhibitor into SGC-7901 cells. The photograph is a representative example. SGC-7901 cells had significant difference invasive behaviors between miR-181a inhibitor and NC and blank control. **h** After transfected with miR-181a inhibitor, the cells ability of migration are decreased compared with cells transfected with NC and blank control (*$P < 0.05$). **i** The graph shows that after transfected with miR-181a inhibitor, the cells ability of invasion are decreased compared with cells transfected with NC and blank control (*$P < 0.05$). **j**: miR-181a inhibitor promoted the apoptosis of SGC-7901 gastric cancer cells. Detection of apoptosis by flow cytometry. SGC-7901 cells were transfected with miR-181a inhibitor or NC as described in the Materials and Methods. **k** shows miR-181a inhibitor promoted apoptosis as analyzed using flow cytometry during different time point (48 h) (*$P < 0.05$)

expression, we compared the ATM protein and mRNA levels in HEK 293T cells transfected with the miR-181a mimics or NC. As shown in Fig. 2c, e and f, miR-181a mimics transfection led to a significant decrease in ATM mRNA and protein level compared with NC groups (Fig. 2e, f, **$p < 0.01$). Expression of ATM and miR-181a were quantified in nine pairs of GC and Normal Tissues with western blot and qRT-PCR, respectively (Fig. 2d, g), we found that miR-181a expression was inversely correlated with ATM (correlation coefficient is −0.539, Fig. 2h). The findings above-mentioned suggest an interaction between miR-181a and ATM.

To show a direct interaction between miR-181a and the ATM 3′UTR, we then assessed luciferase activity in HEK 293T cells by co-transfecting the luciferase reporter vector bearing the wild-type or mutant 3′UTR of ATM with the miR-181a mimics or NC. Luciferase activity was markedly diminished in cells co-transfected with miR-181a mimics and luciferase reporter containing wild-type 3′UTR, compared

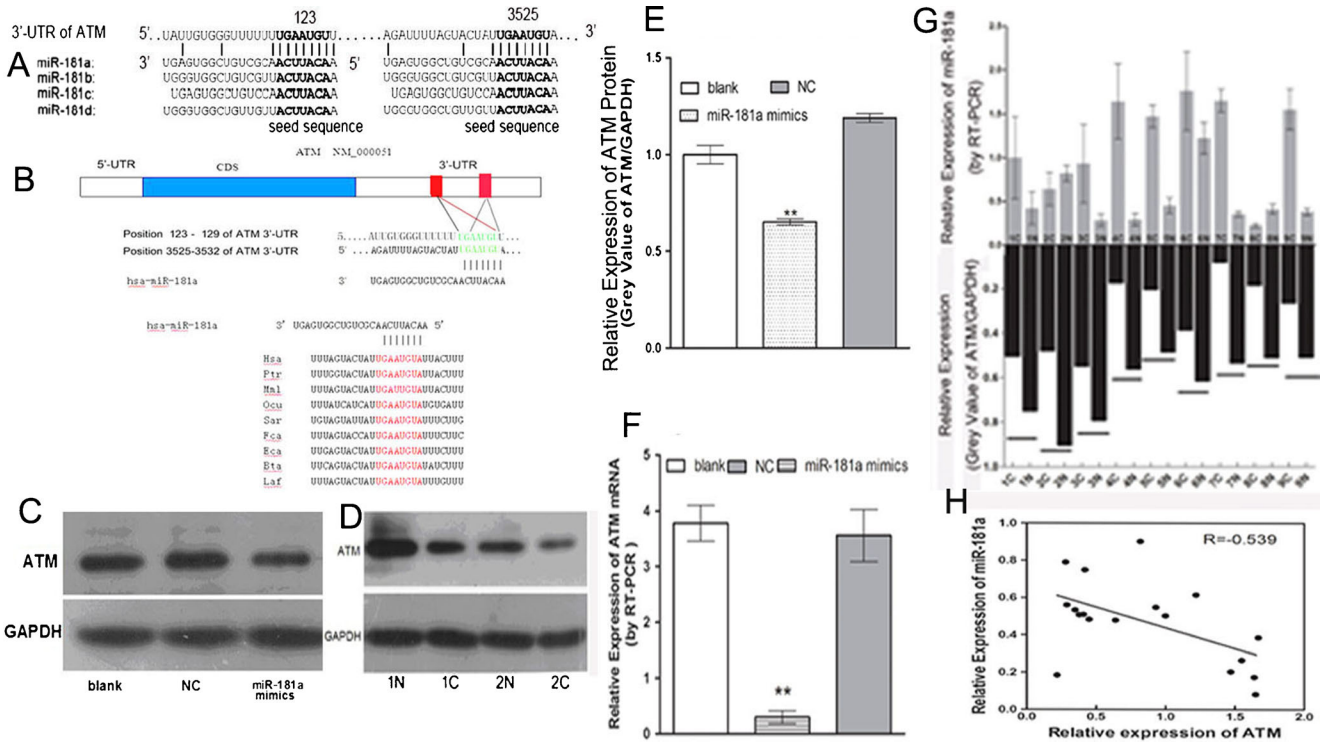

Fig. 2 **a** 3′UTR of ATM possesses two potential binding sites of miR-181a. It representative nucleotide sequence matches between possible target genes and miR-181a. Only matched nucleotides with miRNA seed sequences are indicated with vertical lines. **b** The genome position of putative two binding site of miR-181a in ATM 3′UTR region. The sequence conservation of around two putative binding sites was seen in 3′UTR of different species. The red letters specify the binding sites sequences. **c**: Effect of miR-181a mimics on ATM protein level. HEK 293T cells were transfected with 20 nM of miR-181a mimics or NC. Whole-cell lysates were prepared 72 h post-transfection and subjected to Western blot analysis using antibodies against ATM, and GAPDH. **d** Western blot Assay (gastric tissue); **e** The graph shows the relative expression of ATM as detected by Western blot. The expression levels of ATM were measured in triplicate, shown as mean ± SD. **f** Effect of miR-181a mimics on ATM mRNA level. HEK 293T cells were transfected with 20 nM of miR-181a mimics or NC. The graph shows the relative expression of ATM mRNA detected by RT-PCR. The expression levels were measured in triplicate, shown as mean ± SD. **g** Expression of miR-181a was quantitate by RT-PCR and expression of ATM was detected using Western blot. **h**. The lower expression of ATM inversely correlation with the over-expression of miR-181a in nine pairs gastric cancer and no-tumour tissues

with that in cells co-transfected with miR-181a mimics and mutant 3′UTR reporter plasmids containing one of the two mutated binding positions (Fig. 3b, $^{**}p < 0.01$,$^{*}p < 0.05$). Taken together, these data imply that miR-181a may attenuate the expression of ATM by targeting the ATM 3′UTR directly.

Discussion

In recent years, many studies have shown that aberrant expression of miRNAs emerges in human malignancies [33]. Tchernitsa et al. [34] reported that gastric cancer shows a intricate derangement of the miRNA-ome, including Drosha and Dicer. These changes may lead to impact on prognosis and local tumour growth and nodal spread of patients. However, knowledge of the aberrant expression and potential role of miRNAs in gastric cancer remains largely unknown. Identification of cancer-specific miRNAs as well as their targets is critical for understanding the roles of specific miRNAs in tumorigenesis and may be important for defining novel therapeutic targets. Using miRNA microarray screen, we have identified many dysregulated miRNAs in gastric cancer tissue, including the up-regulation of miR-181a. In this study, we focused on the potential oncomir miR-181a. MiR-181 family members include miR-181a-1, miR-181a-2, miR-181b-1, miR-181b-2, miR-181c and miR-181d. Their genomic organization appears to be evolutionarily conserved from bony fishes to mammals, and is involved in cell differentiation regulation [19]. The over-expression of miR-181a in gastric cancer tissues has been previously observed in two studies [9, 21]. However, the role of miR-181a in GC remains to be determined. Down-regulation of miR-181a has been observed in human primary glioblastoma [35], acute myeloid leukemia [15], chronic lymphocytic leukemia [16], non small cell lung cancer [36] and oral squamous cell carcinoma [37], and reported to act as tumour suppressor by targeting K-ras [37], Bcl-2 [12], PLAG1 [15]. However, miR-181a has also been found elevated in breast cancer [18], hepatocellular carcinoma [19], multiple myeloma [17], papillary thyroid carcinoma [38], functions as an oncomir by direct targeting tumour suppressive genes such as osteopontin [20], CDX2, GATA6, NLK [19], RASSF1A, PCAF [17],

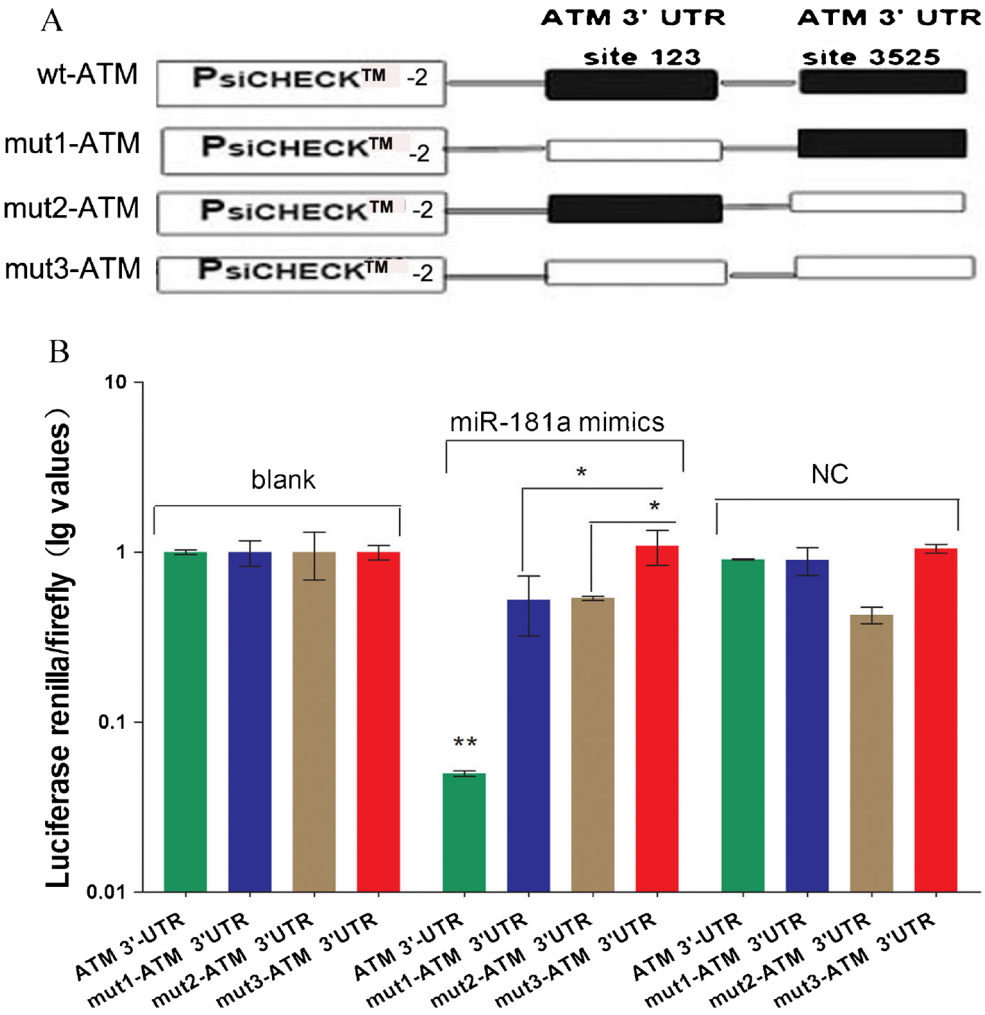

Fig. 3 miR-181a binds to the 3′UTR of ATM to post-transcriptionally repress protein expression. A total of 2×10^4 HEK 293T cells were transfected with 0.5 μg of wild-type-3′UTR-reporter or mutant-3′UTR-reporter constructs together with 20 nM of miR-181a mimics or NC. Forty-eight after transfection, firefly and renilla luciferase activities were measured using Dual-Luciferase Reporter Assay. Each bar represents mean values ± SD from three independent experiments (**$p<0.01$;*$p<0.05$). **a** pasimad model of wild and mutated of ATM; **b** Luciferase activity was markedly diminished in cells co-transfected with miR-181a mimics and luciferase reporter containing wild-type 3′UTR, compared with that in cells co-transfected with miR-181a mimics and mutant 3′ UTR reporter plasmids

THRB [38], uPA [39]. The apparent opposing roles of miR-181a in cancer development and progression may be that its expression and the relevant signaling pathway activation by that expression are cell/tissues type and microenvironment dependent [19]. miR-181 plays a critical role in cellular differentiation, and is upregulated in myoblast and regenerating muscle cells [14], embryonic blood vascular endothelial cells [13], early T progenitor DN (CD4 and CD8 double-negative) [11] and DP cells (CD4 and CD8 double-positive) of thymocyte [12]. Up-regulation of miR-181a by TGFβ induce mammosphere formation in breast cancer cells [18].

We used miR-181a inhibitor plasmid and NC plasmid to perform miR-181a down-regulation studies in SGC-7901 cells. The cell proliferation, apoptosis, colony formation, migration and invasion assays showed that down-regulation miR-181a in GC cell decreases the proliferation, enhance the apoptosis of gastric cancer cell, and also decreases GC cell migration and invasion capacity.

The current model of gastric carcinogenesis is based on the interaction of multiple risk factors including H. pylori infection, environmental factors and genetic susceptibility. H. pylori infection [40] and environmental carcinogen such as MNNG (N-methyl-N′- nitro-nitrosoguanidine) [41] can directly induce gastric mucosal cellular DNA damage, which involves the ATM cascade. DNA double-strand breaks are induced in primary and transformed murine and human epithelial and mesenchymal cells when cells directly contact with live bacteria. DNA damage trigger a damage-signaling and repair response involving the sequential ATM-dependent recruitment of p53-binding protein (53BP1), mediator of DNA damage checkpoint protein 1(MDC1) and histone H2A variant X(H2AX) phosphorylation [40]. It is suggested that DNA damage followed by potential imprecise repair due to prolonging infection or long-term carcinogen exposal may contribute to the genetic instability and frequent chromosomal aberrations that are hallmark of gastric cancer [40]. Because miR-181a promotes proliferation and suppresses apoptosis in gastric cell, it is reasonable to expect that miR-181a target genes may have tumour suppressor characteristics. Among the putative targets for miR-181a is ATM, which was regarded as tumour suppress gene that the patients with ATM mutation have an increased risk of cancer, primary for breast cancer and perhaps gastric cancer [22–24],

and ATM protein inhibits transformation of human mammary gland epithelial cells [25]. In response to DNA damage, this 350-kDa protein kinase appears to be required for checkpoints in G1, S, and G2 phases [23, 42]. Deficiency of ATM function would impair its maintenance of DNA stability and results in carcinogenesis, which could be caused by ATM gene mutation, promoter hypermethylation, inactivation of its kinase activity [24–26]. Expression and phosphorylation of ATM (active form) is down-regulated in gastric cancer tissues [26], while most human normal tissues including stomach contain the nonphosphorylated form of ATM [43]. But, the underlying mechanisms of down-regulation of ATM in gastric cancer tissues are unclear.

The bioinformatics analysis revealed that the conserved binding sites on ATM that can be recognized by miR-181a are located in the 3′UTR, which is suggestion that the expression of ATM may be post-transcriptionally modulated by miRNAs. To test this assumption, we investigated whether miR-181a affects ATM mRNA and protein levels. Our results show the ATM is indeed a direct target of miR-181a. First, we found that over-expressing miR-181a leads to a significant decrease in ATM mRNA and protein levels, suggesting that ATM is a functional target of miR-181a. Second, in human gastric cancer tissues, expression of ATM was inversely corelated with expression of miR-181a. Third, results from our dual-luciferase reporter assays suggest that ATM is one of the functional downstream targets of miR-181a. Lastly during our preparing this manuscript, a paper by Wang Y. et al. show that ATM is target gene of miR-181a in breast cancer cells [18]. Stable ATM silencing by RNA interference result in the cell proliferation increasing, cellular transformation, genomic instability, and formation of dysplastic in both MCF-10A and MCF-12A human mammary gland epithelial cell lines, or in primary human mammary gland epithelial cells [25]. Increased cell proliferation correlate with a reduction of $p21^{WAF1/CIP1}$ and $p27^{KIP125}$. Therefore, ATM directly inhibits the proliferation of normal mammary epithelial cells [25], which is in agreement with the results that overexpression of miR-181a/b, or depletion of ATM or its substate CHK2 are sufficient to induce mammosphere formation in breast cancer cells [18]. To nowadays what and how ATM affect gastric cancer cells are not know. In addition, ATM may not be the only target of miR-181a in gastric cancer, previous studies has shown that osteopontin, CDX2, GATA6, NLK, RASSF1A [19, 20] are the target genes of miR-181 in hepatocellular carcinoma cells. Of note, Liver and stomach are derived from endoderm during embryonic development, where miRNAs came from colon, pancreas, liver and stomach all can cluster together using hierarchical clustering program. Some validated target genes of miR-181a such as K-ras [37], Bcl-2 [12] have been known for many years to associated with gastric cancer. From in silico analysis, miR-181a is suspected to have a number of targets in addition to ATM, such as MTMR3 which mutations could be detected in GC with high microsatellite instability [44]. Moreover, miR-181a has shown to be an important modulator of immune cell differentiation, maturation and function [10–12], while in mice model gastric cancer precursor lesion induced by H.pylori infection arise as a consequence of a T-helper type 1 cell-driven immunopathological response to the infection [40]. Therefore, the reciprocal effect and underlying mechanisms among miR-181a, ATM and GC need further investigation.

In conclusion, we show that miR-181a is up-regulated in gastric cancer, and affects the proliferation and metastatic potential of gastric cancer cells, partly through regulating ATM. Thus, miR-181a functions as an oncomir in gastric cancer. The identification of miR-181a and its target gene, ATM, in gastric cancer may help in understanding the potential molecular mechanisms of gastric cancer development and may have therapeutic value in the future.

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