



MicroRNA-301b-3p accelerates the growth of gastric cancer cells by targeting zinc finger and BTB domain containing 4

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ABSTRACT

MicroRNAs (miRNAs) have been found to be aberrantly expressed and exert essential roles in the tumorigenesis and progression of gastric cancer (GC). miR-301b-3p has been recognized as a cancer-related miRNA in lung cancer, bladder cancer and hepatocellular carcinoma. However, the function of miR-301b-3p in GC progression and its underlying mechanism have not been studied yet. In this study, we found that miR-301b-3p expression was up-regulated in GC tissues compared to adjacent noncancerous tissues. Furthermore, the elevated levels of miR-301b-3p were detected in GC cell lines (SGC-7901, AGS, MKN-45 and MGC-803) as compared with GES-1 cells. Interestingly, GC tissues from patients with tumor size ≥ 5 cm and advanced tumor stages showed obvious higher levels of miR-301b-3p compared to matched controls. Functionally, miR-301b-3p knockdown prominently inhibited cell proliferation, and induced cell cycle arrest at G1 phase and apoptosis in MGC-803 cells. Meanwhile, ectopic expression of miR-301b-3p conversely regulated these biological behaviors of MKN-45 cells. Next, we found that miR-301b-3p knockdown increased, whereas miR-301b-3p overexpression reduced the expression of zinc finger and BTB domain containing 4 (ZBTB4) in GC cells. Accordingly, luciferase reporter assay identified ZBTB4 as a direct target of miR-301b-3p. ZBTB4 overexpression markedly restrained the growth of MGC-803 cells. More importantly, ZBTB4 silencing partially reversed miR-301b-3p knockdown-induced tumor suppressive effects on MGC-803 cells. In conclusion, we firstly revealed that miR-301b-3p was highly expressed in GC and contributed to tumor progression via attenuating ZBTB4, which might provide a novel molecular-targeted strategy for GC treatment.

1. Introduction

Gastric cancer (GC), with around 1,000,000 newly diagnosed cases annually, is the third leading cause of cancer-associated mortality across the world [1]. Notably, Eastern Asia, especially, Korea, Japan, and China contribute to nearly two-thirds of cases [1]. The overall prognosis of GC patients is dismal, especially for those in advanced stage of GC [2]. Accelerated growth, occurrence of systemic metastasis and recurrence after surgical resection are main causes for the poor prognosis of GC [3]. Thus, it is urgent to discover the molecular mechanisms underlying the growth and metastasis of GC cells.

MicroRNAs (miRNAs) are known as single-strand, short (about 22

bp) RNAs, post-transcriptionally regulating gene expression via targeting 3' untranslated region (3'UTR) of mRNAs and resulting in the degradation of mRNA or suppressing the translation of functional proteins [4,5]. Large amount of studies confirm that miRNAs play essential roles in diagnosis, prognosis, development and progression of human cancer including GC [6–11]. Our previous study reports that miR-340 expression is strongly elevated in GC and contributes to tumor growth via attenuating cyclin G2 (CCNG2) [12]. Furthermore, we reveal that miR-125b functions as a promoting factor in GC metastasis by inhibiting StAR related lipid transfer domain containing 13 (STARD13) and neuraminidase 1 (NEU1) [13]. The aberrant expression of miR-301b-3p is widely determined in human cancers including

Abbreviations: GC, gastric cancer; miRNAs, microRNAs; 3'UTR, 3' untranslated region; CCNG2, cyclin G2; STARD13, StAR related lipid transfer domain containing 13; NEU1, neuraminidase 1; HCC, hepatocellular carcinoma; NSCLC, non-small cell lung cancer; VGLL4, vestigial like family member 4; CYLD, CYLD lysine 63 deubiquitinase; TNBC, triple-negative breast cancer; lncRNA, long noncoding RNA; MBNL1-AS1, MBNL1 antisense RNA 1; CSC, cancer stem cell; ZBTB4, zinc finger and BTB domain containing 4; BMP2, bone morphogenetic protein 2; TGF- β 2, transforming growth factor beta 2; NF- κ B, nuclear factor kappa B

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hepatocellular carcinoma (HCC) [14,15], bladder cancer [16], non-small cell lung cancer (NSCLC) [17], ovarian cancer [18] and prostate cancer [19]. miR-301b-3p expression is obviously up-regulated in HCC and contributes to the proliferation, cell cycle progression and apoptosis resistance by attenuating vestigial like family member 4 (VGLL4) [14]. Furthermore, miR-301b-3p functions as an oncogenic factor by targeting CYLD lysine 63 deubiquitinase (CYLD) in HCC [15] and triple-negative breast cancer (TNBC) [20]. miR-301b-3p is frequently over-expressed in lung cancer, and facilitates the growth and chemoresistance of cancer cells by reducing Bim-induced apoptosis [21]. Additionally, the high expression of miR-301b-3p is confirmed in NSCLC, and it mediates the inhibitory effects of long noncoding RNA MBNL1 antisense RNA 1 (lncRNA MBNL1-AS1) on the proliferation, mobility, drug resistance and sphere formation of cancer stem cell (CSC) [17]. But, the expression trend of miR-301b-3p and its biological function in GC are not reported yet.

This study investigated the aberrant expression of miR-301b-3p in GC. Furthermore, gain-of and loss-of-function experiments were performed to determine the biological functions of miR-301b-3p in GC cells. Additionally, the related mechanism underlying the role of miR-301b-3p in GC cells were discovered.

2. Materials and methods

2.1. Clinical samples

GC tissues and adjacent noncancerous tissues were obtained from 73 patients who underwent surgical resection in the First Affiliated Hospital of Xi'an Jiaotong University. The patients did not receive local or systemic therapy before surgery. All specimens were pathologically confirmed by pathologists. Samples were quickly frozen in liquid nitrogen and then maintained at -80 °C until being used. Written informed consent were obtained from patients. The protocols involved in human samples were reviewed and approved by the Ethnic Committee of the First Affiliated Hospital of Xi'an Jiaotong University.

2.2. Cell culture and transfection

The normal gastric epithelium cell line (GES-1) and GC cell lines (SGC7901, AGS, MKN-45, MGC-803) were maintained in our laboratory and cultured under standard condition as previously mentioned [12]. miR-301b-3p mimics, miR-301b-3p inhibitors and matched scrambled negative controls were obtained from RiboBio (Guangzhou, China). Zinc finger and BTB domain containing 4 (ZBTB4) expression plasmid was generated by inserting ZBTB4 cDNA into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). ZBTB4 siRNA (sc-93593) and scrambled siRNA (sc-37007) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) [22]. The above-mentioned plasmids or oligos were transfected into GC cells by using lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Quantitative real-time PCR (qRT-PCR) assay

Total RNAs in tissues and cells were isolated by using Trizol (Thermo Fisher Scientific). The expression of miR-301b-3p was detected by using the TaqMan Human MiRNA Assay kit (Applied Biosystems, Foster City, CA, USA). The expression of ZBTB4 mRNA was determined using a SYBR® Premix Ex Taq™ II kit (Takara, Shiga, Japan) in a CFX96 Touch™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) following the instructions [12]. The level of miR-301b-3p was normalized to that of U6 employing the $2^{-\Delta\Delta Ct}$ method. miR-301b-3p: RT primer, 5'GTCGTATCCAGTGCAGG GTCCGAGGTATTCCGACTGGATACGACGTCACGT3'; forward, 5'TCCG ACGAAACTGTTATAGTA 3'; reverse, 5'GTGCAGGGTCCGAGGT 3'. U6: RT primer, 5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCCGACTGGAT ACGACAAAATATGGAAGTGC3'; forward, 5'CTCGCTTCGCGACGACA

3'; reverse, 5'GTGCAGGGTCCGAGGT 3'. ZBTB4: forward 5'-AGGAAG TACCCCTGCCGCTA-3'; reverse 5'-TTGTAGCCTCCATTGGGTGT-3'. GAPDH: forward, 5'-TCAGTGGTGGACCTGACCTG-3'; reverse, 5'-TGCT GTAGCCAAATTCGTTG-3'.

2.4. Cell proliferation assay

The viability of GC cells was determined by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Dojindo, Japan) assay. Transfected GC cells were seeded into a 96-well plate (2×10^3 cells per well). At different time point, 10 μ L of CCK-8 solution was added into each well. After 4 h incubation, the absorbance values at 450 nm were measured by a microplate reader (Thermo Scientific). For EdU assay, transfected GC cells were seeded into a 6-well plate (1×10^4 cells per well). Following incubation with EdU solution (RiboBio) for 4 h, and the nuclei were stained with DAPI. The images were captured using a microscope (Leica, Wetzlar, Germany).

2.5. Flow cytometric assay

The PE Annexin V Apoptosis Detection Kit I (BD Bioscience; San Jose, CA, USA) was utilized for apoptosis analysis according to the manufacturer's directions. GC cells (1×10^5 cells per well) were plated at in a 6-well plate, cultivated for one night, and further suffered different transfections. Harvested cells were combined with a binding buffer comprising 5 μ L of PE Annexin V and 5 μ L of 7-AAD solution for 15 min of incubation in the dark place, followed by flow cytometry analysis via a FACSCalibur flow cytometer (BD Biosciences). The cell cycle distribution of GC cells was determined by using PI/RNase Staining Buffer (BD biosciences) in a FACSCalibur flow cytometer (BD Biosciences) as previously described [12].

2.6. Western blotting

Total proteins were extracted from GC cells using RIPA buffer (Beyotime, Shanghai, China) and protein concentration was estimated by a Bradford protein assay kit (Beyotime). Approximate 15 μ g of protein was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% skimmed milk for 1.5 h, the membranes were incubated with the primary antibodies against ZBTB4 (sc-514883; Santa Cruz Biotechnology) and GAPDH (sc-47724; Santa Cruz Biotechnology) at 4 °C overnight. After incubation, the membranes were washed by TBST and then incubated with secondary IgG-HRP (Santa Cruz Biotechnology) for 2 h at room temperature. ECL reagent (Millipore) was used to visualize the protein bands.

2.7. Luciferase reporter assay

The 3'UTR of ZBTB4 harboring the wild-type (wt) or mutant (mt) miR-301b-3p interacting sites was inserted into the reporter vector (pMirGLO; Promega, Madison, WI, USA). MGC-803 cells were cotransfected with Renilla plasmid, reporter plasmids containing wt or mt 3'UTR of ZBTB4 and miR-301b-3p mimics or inhibitors. The final luciferase activity was evaluated using the Dual-Luciferase reporter assay system (Promega) after transfection for 48 h.

2.8. Statistical analysis

The quantitative data were showed as mean \pm standard deviation (SD) and analyzed by student's *t*-test or analysis of variance (ANOVA) using GraphPad Prism 8.0 (GraphPad Inc., San Diego, CA, USA). A *P* value less than 0.05 was statistically significant.

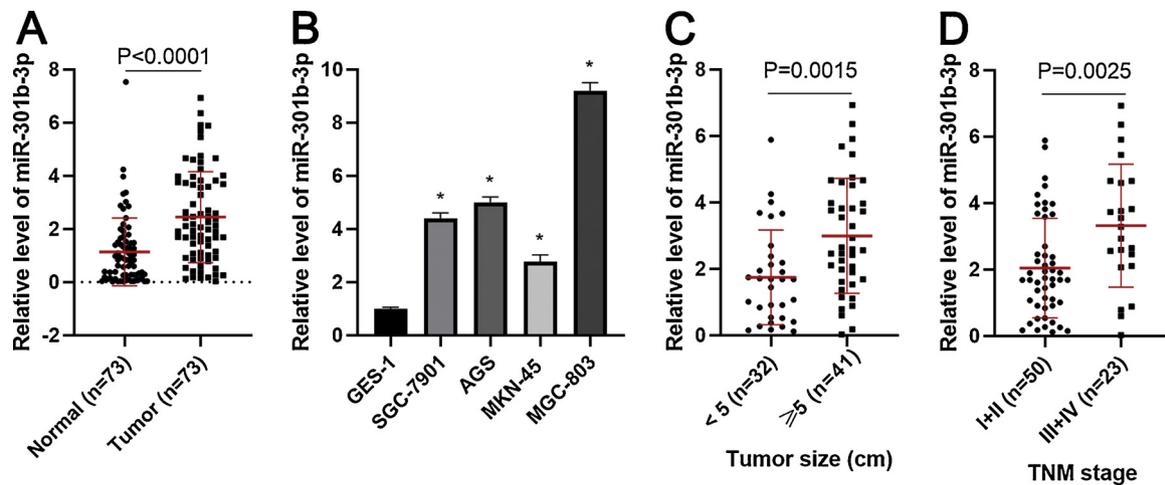


Fig. 1. miR-301b-3p is overexpressed in GC and correlates with clinical features. (A) The expressions of miR-301b-3p in GC ($n = 73$) and matched noncancerous tissues ($n = 73$) were detected by qRT-PCR. (B) qRT-PCR was performed to measure the levels of miR-301b-3p in the normal gastric epithelium cell line GES-1 and four GC cell lines. $n =$ three independent repeats. (C) The levels of miR-301b-3p were compared in GC tissues with tumor size < 5 cm ($n = 32$) or ≥ 5 cm ($n = 41$). (D) The levels of miR-301b-3p were compared in GC tissues with early tumor stages (I + II, $n = 50$) or advanced tumor stages (III + IV, $n = 23$). * $P < 0.05$.

3. Results

3.1. miR-301b-3p is highly expressed in GC

The levels of miR-301b-3p in 73 pairs of GC tissues and adjacent nontumor tissues were determined by qRT-PCR. Our data indicated that miR-301b-3p expression in GC tissues was obviously higher than that in adjacent noncancerous tissues ($P < 0.0001$, Fig. 1A). The Cancer Genome Atlas Stomach Adenocarcinoma (TCGA-STAD) data also indicated that the expression of miR-301b-3p was highly expressed in GC tissues ($P < 0.0001$, Supplementary Fig. 1A). Furthermore, the expressions of miR-301b-3p in GES-1, SGC-7901, AGS, MKN-45 and MGC-803 cells were further detected. As shown in Fig. 1B, the levels of miR-301b-3p were markedly higher in GC cell lines compared to GES-1 cells ($P < 0.05$). Interestingly, GC tissues from patients with tumor size ≥ 5 cm and advanced tumor stages showed significant higher levels of miR-301b-3p as compared with matched controls ($P < 0.01$, Fig. 1C and D). Thus, our findings indicated that the up-regulated expression of miR-301b-3p might contribute to GC progression.

3.2. miR-301b-3p facilitates the growth of GC cells

To further confirm the biological function of miR-301b-3p in GC cells, miR-301b-3p expression was knocked down by transfecting inhibitors in MGC-803 cells ($P < 0.05$, Fig. 2A). CCK-8 assay revealed that knockdown of miR-301b-3p prominently inhibited the viability of MGC-803 cells ($P < 0.05$, Fig. 2B). EdU analysis indicated that miR-301b-3p silencing obviously suppressed the proliferation of MGC-803 cells ($P < 0.05$, Fig. 2C). Moreover, flow cytometric assay demonstrated that miR-301b-3p knockdown significantly induced apoptosis and cell cycle arrest at G1 phase in MGC-803 cells ($P < 0.05$, Fig. 2D and E). Next, ectopic expression of miR-301b-3p was performed in MKN-45 cells ($P < 0.05$, Fig. 3A). Conversely, miR-301b-3p overexpression promoted cell proliferation, cell cycle progression, and repressed apoptosis in MKN-45 cells ($P < 0.05$, Fig. 3B-E). Collectively, miR-301b-3p contributed to the malignant phenotype of GC cells.

3.3. ZBTB4 is a direct target of miR-301b-3p

To discover the molecular mechanism related to the tumor promoting role of miR-301b-3p in GC cells, the predicted targets of miR-301b-3p were screened using starBase platform [23,24]. Accordingly, tumor suppressor ZBTB4 was recognized as a candidate target of miR-

301b-3p. As expected, miR-301b-3p knockdown markedly increased the level of ZBTB4 protein in MGC-803 cells ($P < 0.05$, Fig. 4A). While, miR-301b-3p overexpression remarkably reduced the expression of ZBTB4 protein in MKN-45 cells ($P < 0.05$, Fig. 4B). Additionally, our data and TCGA-STAD data consistently revealed that the expression of ZBTB4 was down-regulated in GC tissues ($P < 0.01$, Supplementary Fig. 1B and C). An inverse correlation between miR-301b-3p and ZBTB4 was observed in GC tissues from TCGA database ($r = -0.381$, $P < 0.0001$, Supplementary Fig. 1D). Importantly, overexpression of miR-301b-3p significantly reduced, but miR-301b-3p silencing obviously enhanced the luciferase activity of vector containing wt 3'UTR of ZBTB4 rather than mt 3'UTR of ZBTB4 ($P < 0.05$, Fig. 4C) in MGC-803 cells. Herein, ZBTB4 was a direct target of miR-301b-3p.

3.4. ZBTB4 partially mediates the tumor promoting role of miR-301b-3p

Next, we aimed to demonstrate whether miR-301b-3p promoted GC cell growth by targeting ZBTB4. ZBTB4 was overexpressed in MGC-803 cells by plasmid transfection ($P < 0.05$, Fig. 5A). The results verified that ZBTB4 overexpression repressed cell proliferation, and resulted in apoptosis and G1 phase arrest in MGC-803 cells ($P < 0.05$, Fig. 5B-5E and Supplementary Fig. 2). Subsequently, MGC-803 cells with miR-301b-3p knockdown were transfected with ZBTB4 siRNA ($P < 0.05$, Fig. 6A). Interestingly, ZBTB4 silencing partially reversed miR-301b-3p knockdown-induced growth arrest of MGC-803 cells ($P < 0.05$, Fig. 6B-E and Supplementary Fig. 3). Altogether, our data suggested that miR-301b-3p exerted a tumor promoting role in GC possibly by repressing ZBTB4.

4. Discussion

The aberrant expression and dysfunction of miRNAs have been confirmed in human GC [25–27]. Several studies have reported that miR-301b-3p is aberrantly expressed in human cancers including HCC, NSCLC and breast cancer [14,17,20]. However, the expression status of miR-301b-3p is unclear in GC. Our current study found that miR-301b-3p expression was obviously up-regulated in GC tissues compared to noncancerous tissues. GC cell lines also highly expressed miR-301b-3p compared to the normal gastric epithelium cell line. Notably, the elevated expression of miR-301b-3p was closely correlated with poor prognostic features including tumor size ≥ 5 cm and advanced tumor stages. Previous studies have revealed several mechanisms underlying the aberrant expression of miR-301b-3p in HCC, lung cancer and

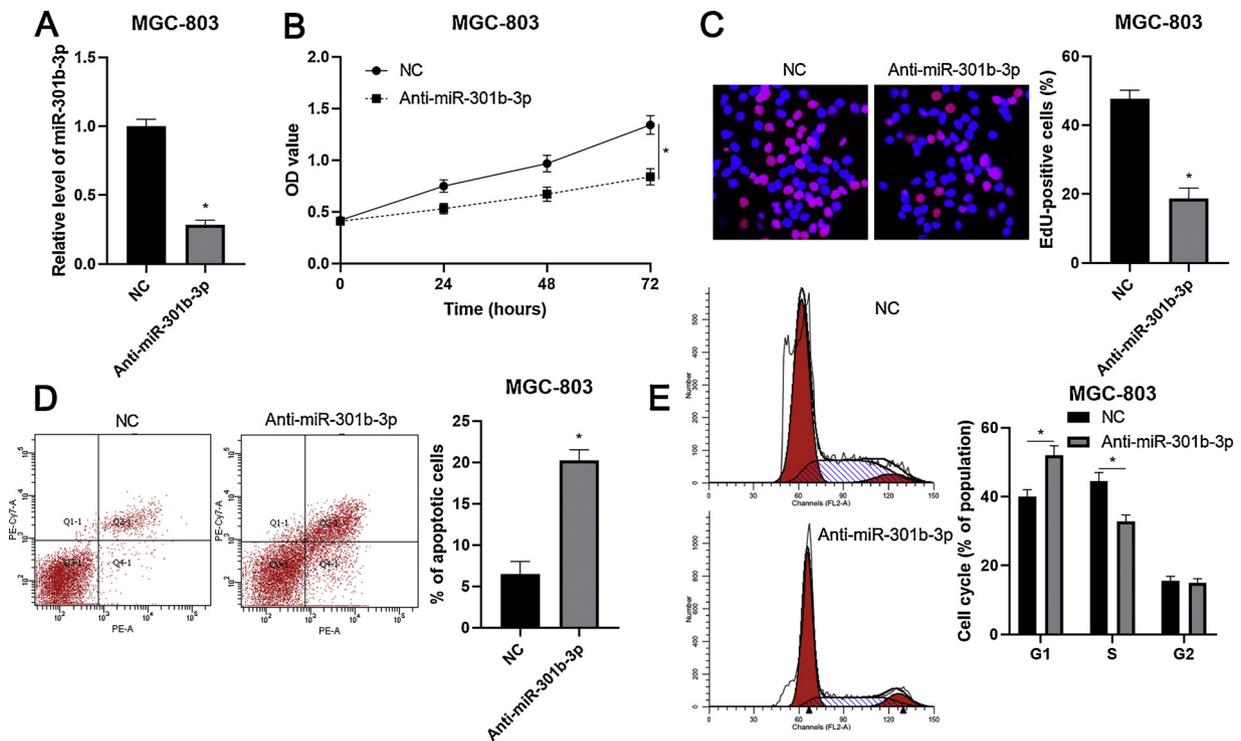


Fig. 2. miR-301b-3p knockdown suppresses the growth of MGC-803 cells. (A) miR-301b-3p inhibitors (anti-miR-301b-3p) and negative control (NC) were respectively transfected into MGC-803 cells. (B) The viability of MGC-803 cells was repressed by miR-301b-3p knockdown as detected by CCK-8 assay. (C) miR-301b-3p silencing reduced the proliferation of MGC-803 cells. (D) The percentage of apoptotic MGC-803 cells was increased by miR-301b-3p knockdown. (E) miR-301b-3p knockdown induced cell cycle arrest at G1 phase in MGC-803 cells. n = three independent repeats. *P < 0.05.

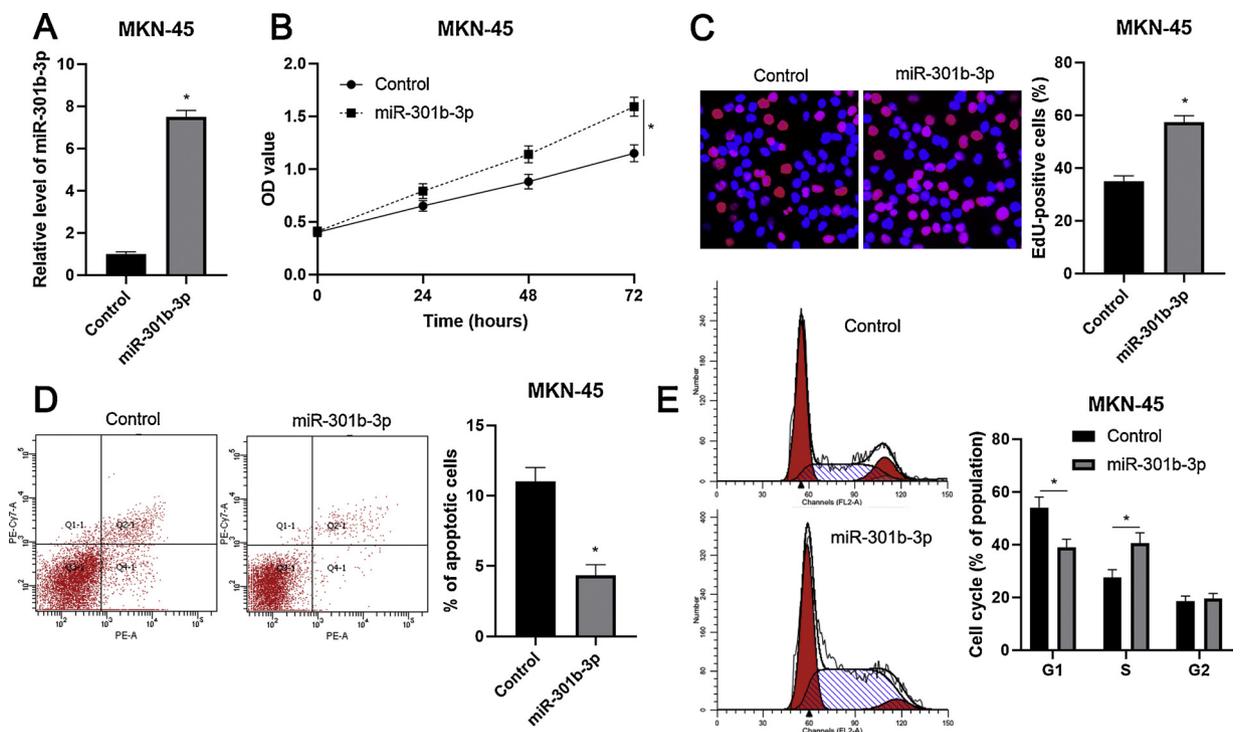


Fig. 3. miR-301b-3p overexpression promotes the growth of MKN-45 cells. (A) miR-301b-3p mimics or scrambled control was transfected into MKN-45 cells. (B) The viability of MKN-45 cells was promoted by miR-301b-3p overexpression as detected by CCK-8 assay. (C) Ectopic expression of miR-301b-3p enhanced the proliferation of MKN-45 cells. (D) The percentage of apoptotic MKN-45 cells was decreased by miR-301b-3p overexpression. (E) miR-301b-3p overexpression facilitated cell cycle progression in MKN-45 cells. n = three independent repeats. *P < 0.05.

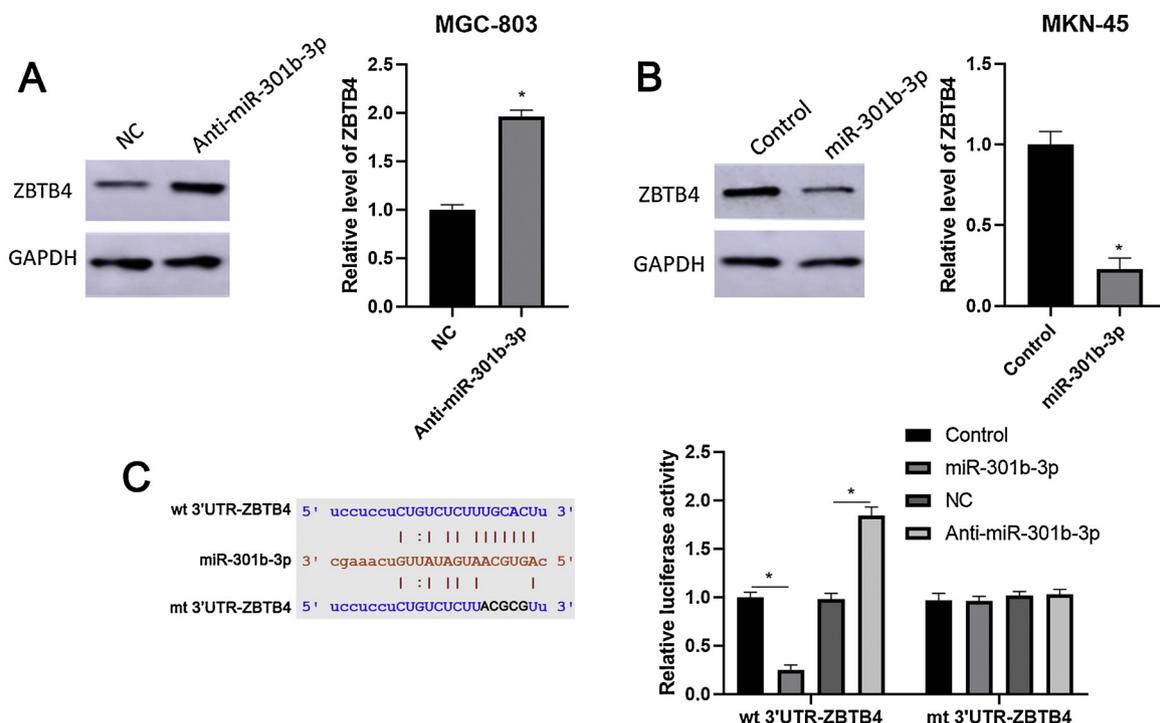


Fig. 4. ZBTB4 is a direct target of miR-301b-3p. (A) miR-301b-3p knockdown increased the expression of ZBTB4 protein in MGC-803 cells. (B) Ectopic expression of miR-301b-3p reduced the level of ZBTB4 protein in MKN-45 cells. (C) The reporter vectors carrying wild type (wt) or mutated (mt) 3'UTR of ZBTB4 and miR-301b-3p mimics or inhibitors were co-transfected into MGC-803 cells and the fluorescence intensity was detected. n = three independent repeats. *P < 0.05.

bladder cancer [15,16,21]. Bone morphogenetic protein 2 (BMP2) and transforming growth factor beta 2 (TGF-β2), which are secreted by tumor-associated neutrophils (TANs), induce miR-301b-3p expression in HCC cells [15]. Nuclear factor kappa B (NF-κB) is recognized as a positive regulator to transcriptionally active miR-301b-3p expression in bladder cancer [16]. Moreover, hypoxia is a strong inducer for miR-301b-3p overexpression in lung cancer [21]. Thus, it will be interesting to further investigate which mechanism involved in miR-301b-3p up-regulation in GC.

Our previous studies have demonstrated that miR-340 and miR-125b are critical oncogenic factors in GC progression [12,13]. Furthermore, miR-301b-3p is recognized as a tumor promoting miRNA in

human cancers [14,17,28]. However, it is unknown whether miR-301b-3p participates in the progression of GC. In this study, we revealed that miR-301b-3p knockdown significantly suppressed cell proliferation and led to G1 phase arrest and apoptosis in MGC-803 cells. Conversely, miR-301b-3p overexpression facilitated the growth of MKN-45 cells. Thus, our data suggested that miR-301b-3p acted as an oncogenic factor in human GC. It is well-known that the function of miRNA is determined by downstream regulatory targets. Thus, it is necessary to discover the target involved in the oncogenic role of miR-301b-3p in GC. Here, we predicted the potential targets for miR-301b-3p using starBase platform. The tumor suppressor ZBTB4 caught our attention. Further investigation found that miR-301b-3p inversely regulated the expression

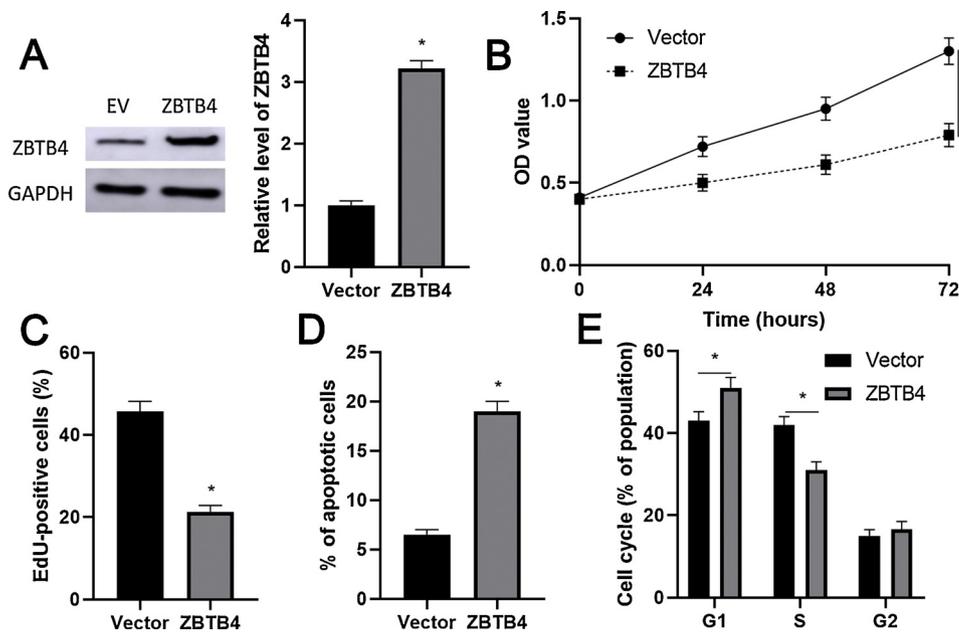


Fig. 5. ZBTB4 overexpression suppresses MGC-803 cell growth. (A) MGC-803 cells were transfected with empty vector or pcDNA3.1-ZBTB4, and detected by western blotting for ZBTB4 expression. (B) The viability of MGC-803 cells was inhibited by ZBTB4 overexpression. (C) ZBTB4 overexpression repressed the proliferation of MGC-803 cells. (D) ZBTB4 overexpression induced the apoptosis of MGC-803 cells. (E) Ectopic expression of ZBTB4 suppressed cell cycle progression in MGC-803 cells. n = three independent repeats. *P < 0.05.

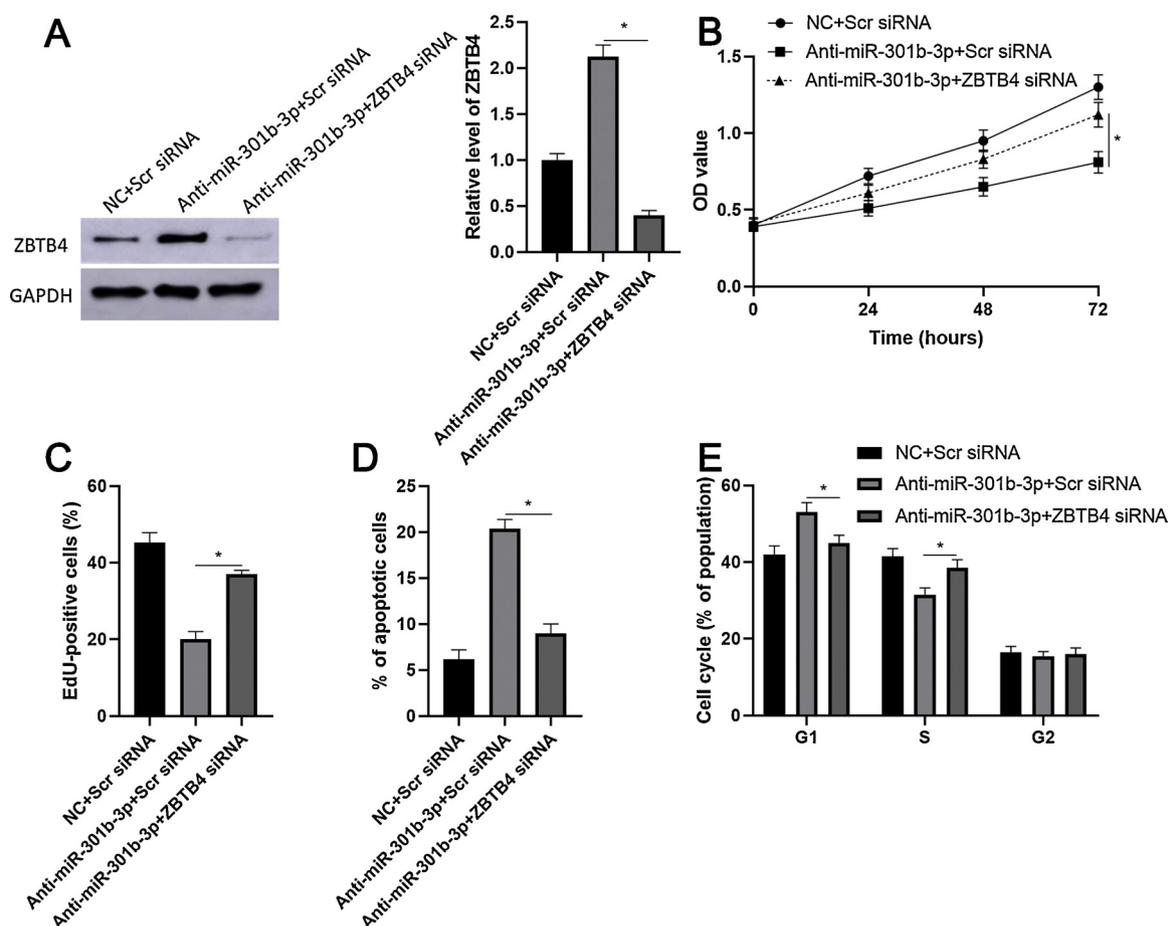


Fig. 6. ZBTB4 silencing reverses miR-301b-3p knockdown-induced the growth arrest of MGC-803 cells. (A) ZBTB4 expression was knocked down by transfecting ZBTB4 siRNA in MGC-803 cells with miR-301b-3p silencing. (B) CCK-8, (C) EdU, (D and E) flow cytometric analysis were performed to detect the proliferation, cell cycle distribution and apoptosis of MGC-803 cells with different transfections. $n =$ three independent repeats. * $P < 0.05$.

of ZBTB4 protein in GC cells. ZBTB4 expression was down-regulated in GC tissues and negatively correlated with miR-301-3p. Luciferase reporter assay demonstrated that miR-301b-3p directly regulated ZBTB4 expression via binding to 3'UTR. ZBTB4 functions as a tumor suppressor via regulating cell proliferation, cell cycle progression and apoptosis in Ewing sarcoma [22]. Ectopic expression of ZBTB4 suppresses breast cancer cell growth and invasion [29]. Loss of ZBTB4 breaks genomic stability to promote tumorigenesis [30]. Currently, the biological role of ZBTB4 in GC is still unexplored. In our study, we demonstrated that ZBTB4 functioned as a novel tumor-suppressor gene in GC cells. More importantly, ZBTB4 silencing partially abolished miR-301b-3p knock-down-induced inhibitory effects on GC cell growth. Altogether, our study suggested that miR-301b-3p contributed to GC progression possibly by attenuating ZBTB4.

In summary, we found that miR-301b-3p level was increased in GC and the elevated expression of miR-301b-3p correlated with poor prognostic features. ZBTB4 was identified as a direct target and functional effector of miR-301b-3p. miR-301b-3p facilitated the growth of GC cells possibly by targeting ZBTB4, indicating miR-301b-3p/ZBTB4 axis as a potential novel target to improve the molecular therapy for GC.

5. Conclusions

To conclude, we demonstrate that miR-301b-3p level is strongly elevated in GC tissues, and positively correlated with poor prognostic features. Gain-of and loss-of-function experiments reveal that miR-301b-3p promotes the growth of GC cells via regulating cell cycle

progression and apoptosis. Interestingly, ZBTB4 is identified as a novel tumor suppressor gene and partially mediates the oncogenic role of miR-301b-3p in GC. These data provide a new insight into the therapy of GC patients.

Declaration of Competing Interest

All authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.prp.2019.152667>.

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