

Original Article

Decreased expression of miR-193b by DNMT1 affects gastric cancer cell proliferation and migration

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Abstract: MicroRNA (miR)-193b has been implicated in numerous types of cancer, however, the mechanism underlying the effects of miR-193b in gastric cancer (GC) have remained to be elucidated. In this study, we aimed to identify the mechanisms by which miR-193b is regulated as well as the functional role of miR-193b in GC. Decreased expression levels of miR-193b was detected in GC samples and cell lines by reverse transcription quantitative polymerase chain reaction, the expression level of miR-193b correlated with lymph node metastasis in patients with GC ($P < 0.05$). 5-Aza-2'-deoxycytidine (5'-Aza) treatment and DNA methyltransferase 1 (DNMT1) knockdown restored the level of miR-193b in GC cells. Functional studies demonstrated that elevated expression of miR-193b by transient transfection was able to inhibit the proliferation and migration of GC cells. Furthermore, myeloid cell leukemia (MCL) 1 was validated as the target of miR-193b by luciferase assay, and overexpression of miR-193b suppressed the expression of MCL1. The results of the present study suggested miR-193b acts as a tumor suppressor in GC cells, and the reduced expression of miR-193b in GC cells may be in part due to epigenetic regulation via DNMT1.

Keywords: miR-193b, DNA Methylation, DNMT1, proliferation, migration, gastric cancer

Introduction

Gastric cancer (GC) is the third leading cause of cancer-associated mortality worldwide. According to data from GLOBOCAN, there were almost one million new cases of stomach cancer occurred in 2012, and nearly half of the total cases happened in China [1]. The pre-disposing factors of gastric cancer include family history, lifestyle, dietary habits, *Helicobacter pylori* infection [2]. Due to the absence of early detection, the majority of patients are diagnosed at an advanced stage, when treatments and prognosis may be unfavorable [3]. Therefore, an improved understanding regarding the molecular pathogenesis in GC is vital for developing novel molecular methods of diagnosis and therapy.

MicroRNAs (miRNAs) are a class of small single-stranded RNA, which belongs to the non-coding RNA species. miRNAs regulate gene expression by binding to the 3' untranslated region (UTR) of target mRNAs, following which they either block translation or degrade the

mRNA. miRNAs participate in numerous biological processes, including embryonic development, cell differentiation and apoptosis [4]. Dysregulation of miRNAs has been detected in numerous pathological processes, particularly in cancer. During carcinogenesis, miRNAs may act as oncogenes or tumor suppressors [5]. Emerging evidences has demonstrated that modification of miRNAs may result in their aberrant expression in cancer [6]. DNA methylation is a major type of epigenetic modification that regulates gene expression [7], and DNA methylation associated silencing of miRNAs has recently emerged as a hallmark of cancer [6].

miR-193b is located on 16p13.12 and belongs to the miR-193b-365 cluster. Previous studies have demonstrated miR-193b may participate in the tumorigenesis of numerous types of cancer, including gastric cancer [8], epithelial ovarian cancer [9], glioma [10], hepatocellular carcinoma [11], non-small cell lung cancer (NSCLC) [12], breast cancer [13] and melanoma [14]. However, the expression pattern and functional role of miR-193b in GC have yet to be elucidated.

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In this study, we showed the expression of miR-193b was decreased in GC tissues and cell lines, and the decreased expression of miR-193b may partly attribute to the CpG island methylation by DNMT1. Ectopic overexpression of miR-193b inhibited the cell proliferation and migration. We also confirmed that MCL1 is a direct target of miR-193b.

Materials and methods

Tissues samples and cell culture

Tissues samples from 35 patients were collected from Linyi People's Hospital. Tumor as well as adjacent non tumor samples was confirmed by two senior pathologists. Written informed consent was obtained from all patients. The present study was approved by the Ethical Committee of Linyi People's Hospital. The human GC Cell lines (GES-1, BGC-823, AGS, MGC-803 and SGC-7901) and human embryonic kidney (HEK) 293T cells were purchased from the Cell Resource Center of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Invitrogen Life Technologies) in a humidified atmosphere containing 5% CO₂ at 37°C.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues and cell lines with TRIzol reagent (Invitrogen Life Technologies) and homogenized using the TL2020 Cracker (DHS Life Science & Technology Ltd., Beijing, China), according to the manufacturer's instructions. Reverse transcription was performed using a reverse transcription kit (Takara Biotechnology, Co., Ltd., Otsu, Japan). qRT-PCR was performed using SYBR Premix Ex Taq Kit (Biotechnology Co., Ltd.) on ABI 7500 (Applied Biosystems, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). U6 was selected as the internal control. The sequences of the primers (GenePharma Co., Ltd, Shanghai, China) were as follows: miR-193b forward, 5'-TTGACCGGGAGTTTCAGG-3' and reverse, 5'-GTG-CAGGGTCCGAGGT-3', U6 forward, 5'-AAAGACC-TGTACGCCAACAC-3' and reverse, 5'-GTCATAC-TCTGCTTGCTGAT-3'. The PCR cycling condi-

tions consisted of an initial denaturation step at 95°C for 30 sec, followed by 40 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Relative mRNA levels were analyzed using 2^{-ΔΔCt} method [15].

Cell transfection

The small interfering (si)RNA sequences of DNMT1 was 5'-GGAUGAGAAGAGACGUAGATT-3' (sense) and negative control was 5'-AAU UCU CCG AAC GUG UCA CTT-3' (sense). The miR-193b mimics sequence was 5'-AACUGCCCC-UCAAAGUCCCGCU-3' (sense) and negative control was 5'-UUCUCCGAACGUGUCACGU-3' (sense). All of the oligonucleotides were synthesized by Shanghai GenePharma Co., Ltd. Briefly, 2×10⁵ cells were seeded into 6-well plate. After 24 h, once the cells had reached 70-90% confluence, the cells were transfected using Lipofectamine 2000 (Invitrogen) with a final siRNA concentration of 20 nM, and a final miRNA concentration of 100 nM, respectively. AGS cells transfected with DNMT1 siRNA or negative control were named AGS-SiDNMT1 or AGS-CDNMT1, respectively. SGC-7901 cells transfected with DNMT1 siRNA or negative control were labeled as 7901-SiDNMT1 or 7901-CDNMT1, respectively. AGS cells transfected with miR-193b mimics or negative control were named AGS-OV193b or AGS-C193b, respectively. SGC-7901 cells transfected with miR-193b mimics or negative control were labeled as 7901-OV193b or 7901-C193b, respectively.

5-aza-2'-deoxycytidine (5'-Aza) treatment

The AGS and SGC-7901 cells were planted and cultured with RPMI 1640 medium containing 0, 10, or 50 μM 5'-Aza (Sigma-Aldrich, St. Louis, MO, USA). Following a 72-h incubation, the cells were harvested and total RNA was extracted.

Cell counting kit-8 (CCK-8) assay

Cell proliferation was measured using CCK-8. AGS or SGC-1901 cells were seeded in 96-well plates at a density of 1×10³ cells/well and transfected with miR-193b mimics or negative control as described above. At various time-points (1, 2, 3, 4 and 5 days) post-transfection, the medium was replaced with 110 μl fresh medium containing 10 μl CCK-8 (Beyotime Institute of Biotechnology, Shanghai, China). The supernatants were removed following a 2-h

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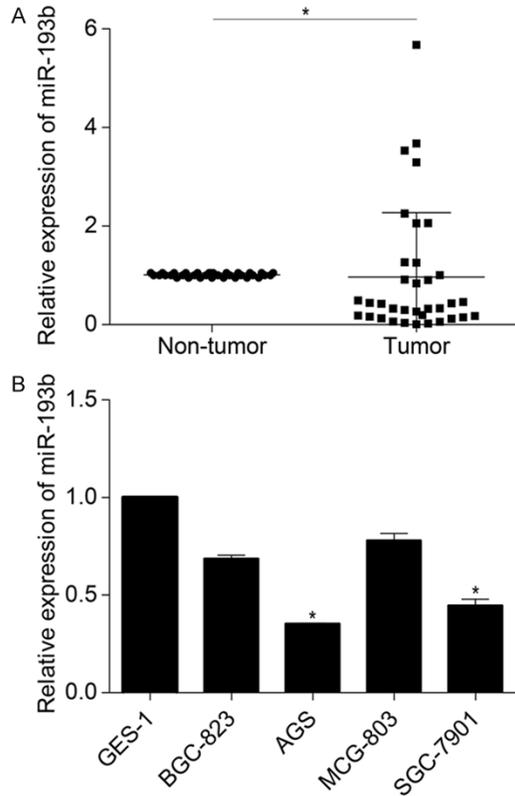


Figure 1. miR-193b is downregulated in GC tissues and cell lines. A. Expression levels of miR-193b in GC tissues were measured by reverse transcription-quantitative polymerase chain reaction. Each datapoint represents the tumor of one patient. Horizontal lines represent the mean value and error bars represent the standard deviation. B. Expression levels of miR-193b were detected in GC cell lines BGC-823, AGS, MGC-823 and SGC-7901, and the gastric mucosa cell line GES-1. Values are expressed as the mean \pm standard deviation from at least three independent experiments. ** $P < 0.01$. * $P < 0.05$, vs. GES-1. GC, gastric cancer; miR, microRNA.

incubation and the absorbance was measured at 450 nm using a microplate reader (Bio-Rad, CA, USA).

Wound healing assay

Cell migration was detected by using a scratch test. At 24 h post-transfection with miR-193b mimics or negative control, the cell layers were scratched using a 20 μ l Eppendorf tip and washed with phosphate-buffered saline in order to remove the non-adherent cells. Images of the cells were captured under a microscope (Nikon, Tokyo, Japan) 0 h and 48 h following generation of the wound.

Western blot analysis

The protein expression levels of E-cadherin, vimentin and MCL1 were detected by Western blotting. At 48 h post-transfection, the cells were lysed in RIPA lysis buffer (Beyotime) at 4°C for 30 min and then centrifuged at 12,000 g for 5 min at 4°C. Total extracted proteins in the supernatant were quantified using a BCA Protein Assay Kit (Beyotime). Equal amounts of protein (30 μ g) were separated by 10% SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were then incubated with the following primary antibodies: DNMT1 (1:1000, monoclonal mouse anti-human, Abcam, CA, UK), E-cadherin (1:500, monoclonal mouse anti-human, Cell Signaling Technology, Inc., Beverly, MA, USA), vimentin (1:500, monoclonal mouse anti-human, Cell Signaling Technology), MCL1 (1:2000, monoclonal mouse anti-human, Abcam), β -actin (1:8000, monoclonal mouse anti-human, Abcam), followed by an incubation with the secondary antibody (1:5000, goat anti-mouse, Boster Biological Technology, Wuhan, China). Protein bands were visualized using a BeyoECL Plus kit for enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology). B-actin served as an internal reference.

Dual luciferase assay

The wild type (WT) and mutant (MUT) 3'-UTR of human MCL1 were synthesized and cloned into the XbaI site of luciferase reporter vector pGL3 (Corporation, Madison, WI, USA) [16]. HEK293T cells were plated into 24-well plates, 100 ng of pGL3-MCL1-3'UTR (WT or MUT), 1 ng of pRL-TK, and 30 nM of miR-193b mimics or scramble oligonucleotides were transfected into cells using Lipofectamine 2000 (Invitrogen). After 48 h, cells were collected and the luciferase activities were analyzed using the Dual-Luciferase Reporter Assay System (Promega) with the TD20/20 Luminometer (Turner BioSystems, Sunnyvale, CA, USA). Experiments were performed three times independently.

Statistical analysis

All statistical analyses were performed using the SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Values are expressed as mean \pm SD at least three separate experiments. Statisti-

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Table 1. Association between miR-193b expression and clinicopathological characteristics of patients with gastric cancer

Feature	Relative miR-193b expression		P value
	Low	High	
Gender			
Male	12	8	0.686
Female	10	5	
Age			
<60	9	6	0.762
≥60	13	7	
Differentiation			
Poorly	8	3	0.659
Moderately/Well	14	10	
Local invasion			
Yes	15	4	0.073
No	7	9	
Lymph node metastasis			
Yes	14	3	0.049*
No	8	10	
Distant metastasis			
Yes	13	5	0.238
No	9	8	

*P<0.05. miR, microRNA.

cal comparisons were made with two-sided Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-193b is downregulated in gastric cancer tissues and cell lines

The expression level of miR-193b was detected in GC tissues and cell lines (GES-1, BGC-823, AGS, MGC-803, SGC-7901) by qRT-PCR. Results showed that miR-193b was downregulated in GC tissues, as compared with that in non-tumor tissues (22/35) (**Figure 1A**). In addition, the expression levels of miR-193b were decreased in the four GC cell lines, as compared with those in the immortalized gastric mucosa cell line GES-1 (**Figure 1B**).

To investigate the potential role of miR-193b in GC, the expression pattern of miR-193b and the clinicopathological features were analyzed. The 35 GC patients were categorized into two groups according to the difference in expression levels (<1.5-fold). The data revealed that

expression of miR-193b was negatively correlated with lymph node metastasis (P=0.049) of GC (**Table 1**). These results indicated miR-193b may act as a tumor inhibitor in GC progression.

5'-Aza treatment and DNMT1 knockdown restore levels of miR-193b

It has previously been reported that miR-193b is transcribed from a CpG island containing promoter [17], and the results of the present study indicated that miR-193b is downregulated in GC tissues. To investigate whether the decreased expression of miR-193 is due to the promoter hypermethylation, AGS and SGC-7901 cell lines were treated with DNA methylation inhibitor 5'-Aza. 72 h after treatment, RNA was extracted and qRT-PCR was performed. Results revealed that the expression levels of miR-193b was restored following treatment with 5'-Aza, and the levels of miR-193b increased in an 5'-Aza dose-dependent manner (**Figure 2A**).

5'-Aza may induce selective degradation of DNMT1. To provide further evidence regarding the effects of methylation on GC cells, DNMT1 expression was knocked-down in AGS and SGC-7901 cells (**Figure 2B**). The data indicated that Knockdown of DNMT1 increased the expression levels of miR-193b in two GC cell lines (**Figure 2C**). These results suggested that epigenetic silencing may partly contribute to the downregulation of miR-193b in GC cells.

miR-193b decreases cell proliferation, migration and epithelial-to-mesenchymal transition (EMT)

To evaluate the biology function of miR-193b, AGS and SGC-7901 cell lines were transfected with miR-193b mimics and negative control. The expression of miR-193b in AGS and SGC-7901 cells was significantly increased following transfection with the miR-193b mimics (**Figure 3A**). A CCK-8 assay was performed to detect the proliferative ability of cells overexpressing miR-193b. As shown in **Figure 3B**, cell proliferation was decreased following transfection with miR-193b, as compared with that in the in AGS and SGC-7901 cells transfected with negative control. In addition, the migration of the AGS-OV193b and 7901-OV193b cells was decreased, as compared with that of the AGS-C193b and 7901-C193b cells, as determined by wound healing assay (**Figure 3C**). The pres-

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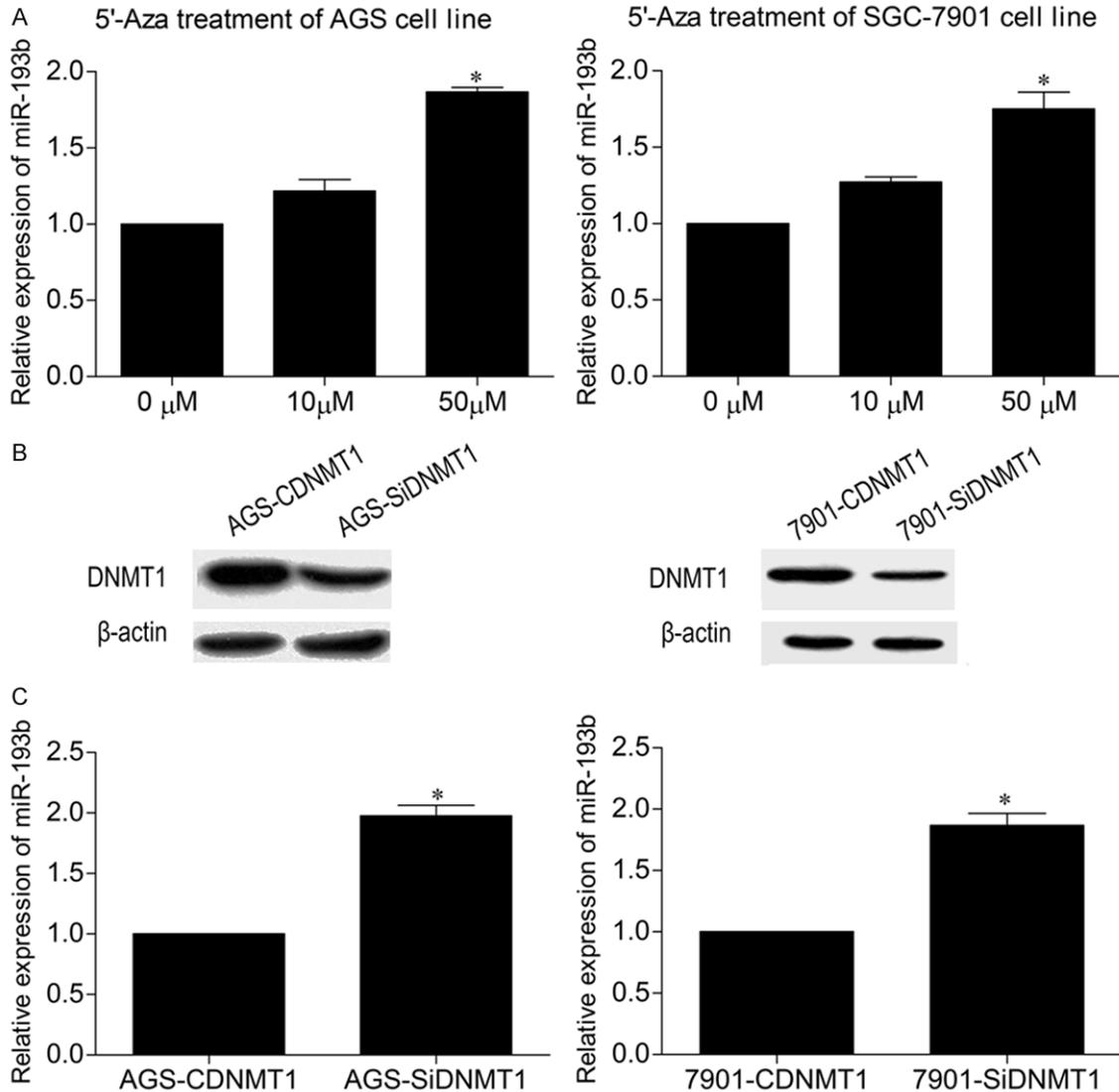


Figure 2. Expression levels of miR-193b following 5'-Aza treatment and DNMT1 knockdown. A. miR-193b expression was restored following 5'-Aza treatment. B. Expression of DNMT1 following transfection with siDNMT1. C. Knockdown of DNMT1 increased the expression levels of miR-193b. Values are expressed as the mean \pm standard deviation from at least three independent experiments * $P < 0.05$ vs. control. 5'Aza, 5-aza-2'-deoxycytidine; DNMT1, DNA methyltransferase 1; miR, microRNA; C/SiDNMT1, transfected with control/small interfering RNA specific for DNMT1.

ent study also characterized the expression of common EMT markers, results showed that the epithelial cell marker E-cadherin were upregulated and the mesenchymal cell marker vimentin was downregulated in miR-193b mimics-transfected GC cells, as compared with that in the negative control-transfected cells (**Figure 3D**).

MCL1 was a direct target of miR-193b

According to the results of three prediction software: TargetScan (www.targetscan.org), pic-

tar (pictar.mdc-berlin.de/) and mirbase (www.mirna.org), myeloid cell leukemia factor 1 (MCL1) was identified as a target of miR-193b. Luciferase reporter assay was used to validate the prediction. WT and Mut miR-193b binding site in 3'UTR region of MCL1 were inserted into pGL3 vector (**Figure 4A**). Following co-transfection of the HEK293T cells with pRL-TK and miR-193b mimics, we found the luciferase activity of the reporter gene containing WT MCL1 3'UTR, but not MUT MCL1 3'UTR, was significantly reduced (**Figure 4B**). Furthermore, the

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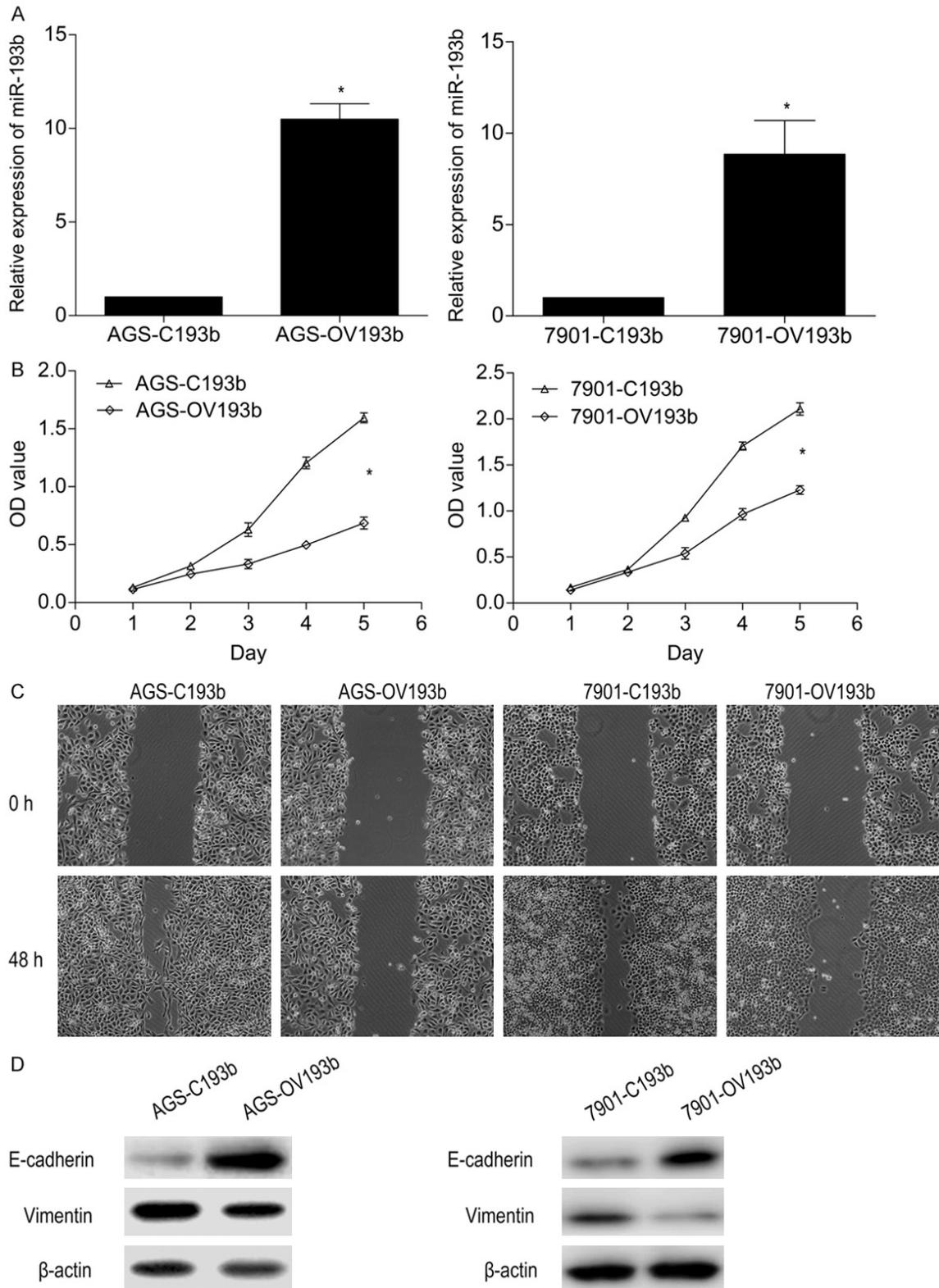


Figure 3. Ectopic expression of miR-193b inhibits the proliferation and migration of gastric cancer cells. A. Transfection of miR-193b mimics effectively elevated the expression of miR-193b in AGS and SGC-1901 cells. B. Cell proliferation was suppressed following transfection with miR-193b mimics, as determined by Cell Counting kit-8 assay. C. Cell migration was inhibited after transfection with miR-193b mimics, as determined by wound healing

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assay (magnification, $\times 200$). D. E-cadherin was upregulated and vimentin was downregulated in miR-193b mimics-transfected AGS and SGC-7901 cells. Values are expressed as the mean \pm standard deviation from at least three independent experiments. * $P < 0.05$ vs. control. DNMT1, DNA methyltransferase 1; miR, microRNA; OD, optical density; C/OV193b, cells transfected with control/miR-193b mimics.

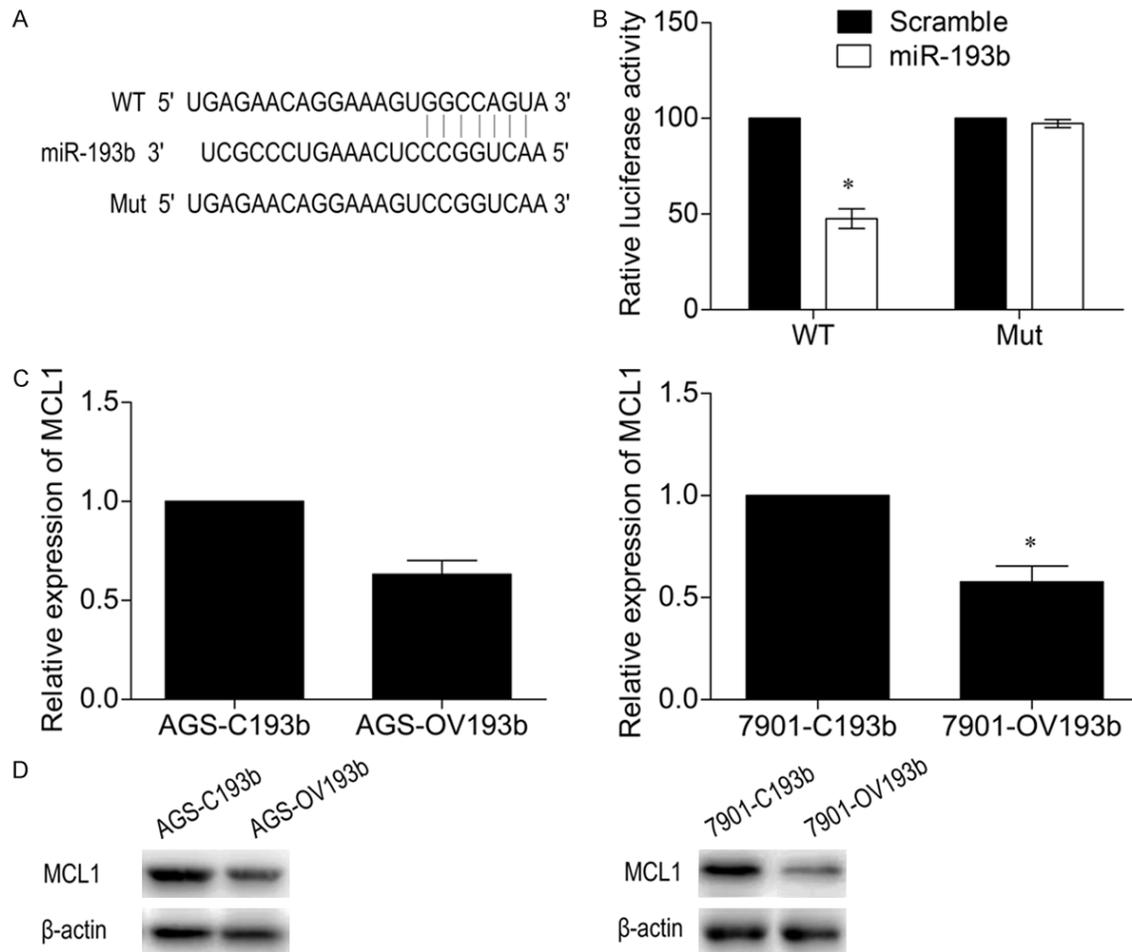


Figure 4. miR-193b targets MCL1. (A) The 3'-UTR of MCL1 aligns with miR-193b. (B) Transfection with miR-193b mimics reduced the luciferase activity of the reporter gene containing the wild-type MCL1 3'UTR in human embryonic kidney 293T cells, as determined by luciferase reporter assay. (C) Reverse transcription-quantitative polymerase chain reaction and (D) western blot analysis showed that the mRNA and protein expression levels of MCL1 were downregulated in the miR-193b mimics-transfected AGS and SGC-7901 cells. Values are expressed as the mean \pm standard deviation from at least three independent experiments. * $P < 0.05$ vs. scramble group. WT, wild-type; Mut, mutant; miR, microRNA; UTR, untranslated region; MCL1, myeloid cell leukemia; C/OV193b, cells transfected with control/miR-193b mimics.

expression levels of MCL1 were detected in the miR-193b-transfected GC cells; the ectopic overexpression of miR-193b reduced the mRNA and protein expression levels of MCL1 in the AGS and SGC-7901 cell lines (Figure 4C and 4D).

Discussion

Numerous miRNAs have been demonstrated to have significant roles in drug resistance, tumor

metastasis and prognosis, thus suggesting they have potential as a novel class of cancer biomarkers.

Previous studies have indicated downregulation of miR-193b is implicated in numerous types of cancers. Hu et al demonstrated that miR-193b was markedly downregulated in NSCLC cancer tissues, and inhibition of miR-193b increased the proliferation, migration and invasion of A549 cells [12]. In breast can-

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cer, downregulation of miR-193b contributed to enhanced urokinase-type plasminogen activator expression and tumor progression [13]. In melanoma, miR-193b repressed cell proliferation and was shown to regulate cyclin D1 [14]. Ziliak et al demonstrated miR-193b inhibited the expression of platinum-associated genes (CRIM1 and IFIT2) and increased miR-193b expression conferred greater platinum resistance in a randomly selected HapMap cell line [18]. Conversely, in glioma [10], head and neck squamous cell carcinomas [19], uveal melanomas [20], and multiple myeloma [21], miR-193b was upregulated and served as an oncogene.

The results of the present study demonstrated that miR-193b expression was markedly decreased in 62.86% of GC tissues. In addition, the expression levels of miR-193b were lower in the four GC cell lines, as compared with those in the immortalized gastric mucosa cell line GES-1. In a correlation analysis, the low expression level of miR-193b in GC patients was shown to be associated with tumor invasion and lymph node metastasis. Gain-of-function analyses indicated miR-193b was able to inhibit cell proliferation and migration. In addition, upregulation of miR-193b increased the expression of E-cadherin and decreased the expression of vimentin, suggesting that miR-193b may be associated with the regulation of EMT. These data indicated that miR-193b may function as a tumor suppressor and have an important role in GC metastasis.

miRNAs exert their effects by regulating target mRNAs, the present study identified MCL1 as a target gene of miR-193b. The results demonstrated that the mRNA as well as protein levels of MCL1 were downregulated in the miR-193b-transfected GC cells. MCL1 is a member of the B-cell lymphoma 2 pro-survival protein family [22], which is highly expressed in numerous cancer sub-types [23-25]. MCL1 regulates cell proliferation, differentiation and tumorigenesis by influencing cell viability [26]. The results of the present study indicated that miR-193b may modulate cell proliferation and migration by targeting MCL1.

miRNAs have an important role in numerous processes, however, little is currently known regarding the mechanism of their dysregulation in cancer. Previous studies have reported that epigenetic modification, such as DNA methylation, is associated with the aberrant expression

of miRNAs [27-29]. DNA methylation is an essential modification that regulates gene expression in mammalian cells. Aberrant alterations of methylation patterns may lead to oncogene activation and tumor suppressor gene inactivation [30]. Hypermethylation of the CpG islands may silence tumor suppressor miRNA expression in cancers. DNMT1 has been identified as the maintenance methyltransferase, which methylates hemi-methylated duplexes during DNA replication contributed to aberrant CpG-island methylation in human cancer cells [31].

Rauhala et al demonstrated that miR-193b was upregulated in six prostate cancer cell lines following treatment with 5'-Aza, and a methylation analysis indicated miR-193b was methylated at a CpG island ~1 kb upstream of the miRNA locus [17]. Conversely, Du et al indicated that miR-193b is a GC-associated gene that enriches CpG islands [32]. The results of the present study demonstrated that following 5'-Aza treatments, the expression level of miR-193b was restored in AGS and SGC-7901 cell lines. 5'-Aza is a DNMT1 inhibitor, which is widely used for cancer therapy [33]. A further experiment demonstrated that knockdown of DNMT1 increased the expression levels of miR-193b, suggesting that the low expression levels of miR-193b in GC tissues and cell lines may be partly caused by the promoter hypermethylation. However, the methylation status of the miR-193b promoter still requires verification by bisulfite genome sequencing.

In conclusion, the present study demonstrated that miR-193b was downregulated in GC tissues and was negatively correlated with lymph node metastases. The decreased expression of miR-193b may be partly due to the promoter methylation by DNMT1. Ectopic expression of miR-193b decreased the proliferation and migration of GC cell lines as well as the EMT, providing further evidence for the tumor-suppressive and anti-metastatic role of miR-193b in GC cells. Furthermore, MCL1 was proven to be a direct target gene of miR-193b. miR-193b may therefore represent a negative diagnostic factor or a therapeutic agent for the treatment of GC.

Disclosure of conflict of interest

None.

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