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miR-183-5p acts as a potential prognostic biomarker in gastric cancer and regulates cell functions by modulating EEF2

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ABSTRACT

Background: Gastric cancer (GC) is the fourth most prevalent malignant tumor and the second leading cause of cancer-related death around the world. Aberrant proliferation and metastasis are the mainspring of death in patients with GC. However, the specific mechanism of gastric cancer is far from being fully elucidated. Accumulating evidence revealed that miRNA played a significant role in the tumorigenesis and development.
Methods: The level of miR-183-5p was detected in 102 GC patients by using qRT-PCR. The prognostic value of miR-183-5p in GC was evaluated. Cell function assays (CCK-8 and transwell assays) were conducted to assess the role of miR-183-5p in proliferation and metastasis in GC. Dual luciferase report assay and western blot were performed to validate this potential target regulated by miR-183-5p in GC.
Results: miR-183-5p was down-regulated in GC tissues and cell lines. Remarkable pertinence was obtained between miR-183-5p level and TNM stage, tumor size, invasion depth, and lymph node metastasis. TNM stage, differentiation and miR-183-5p level were independent causes impacting on the overall survival in GC in multivariate analysis. GC individuals with high miR-183-5p level would experience a relatively better survival prognosis. Upregulation of miR-183-5p restrained GC cell proliferation and migration. EEF2 may be a potential target gene regulated by miR-183-5p in GC.
Conclusion: miR-183-5p acts as a potential prognostic biomarker in gastric cancer and regulates cell functions by modulating EEF2.

1. Background

Gastric cancer (GC) is the fourth most prevalent malignant tumor and the second leading cause of cancer-related death around the world with high morbidity and mortality [1–3]. One million cases initially diagnosed with GC and 0.74 million deaths from it are estimated to occur annually on a worldwide basis [4]. GC is also the third leading cause of cancer disability-adjusted life expectancy burden in men worldwide, accounting for 20 percent of the global burden [5]. Established risk factors for GC include *H. pylori* infection and Epstein-Barr virus [6,7]. Aberrant proliferation and metastasis are the mainspring of death in patients with GC. Patients with gastric cancer are often present with resistance to chemotherapeutics and irregular prognosis management, supporting the need for the identification of potential prognostic biomarkers and potential therapeutic targets.

As a series of endogenous non-coding RNA, microRNAs (miRNAs) can exert their gene regulatory functions at post-transcriptional levels, resulting in the degradation of target mRNA or inhibition of the mRNA translation [8]. miRNAs are involved in the proliferation, apoptosis and migration of tumor cells, thus affecting the tumorigenesis and development of tumors, including GC [9,10]. In addition, miRNAs have even been considered by many experts as markers to predict the prognosis of tumor patients [11,12]. It is believed that miRNAs are involved in gene regulation in over half of the protein-coding genes. Therefore, the study on the role of miRNAs in GC may be of landmark significance for the exploration of the specific mechanism of the tumorigenesis and development of GC and even the development of therapeutic targets for GC.

As a vital one of the miRNA family, miR-183-5p plays the character of oncogene or anti-oncogene in pancreatic cancer, breast cancer, glioblastoma, lung cancer, and cervical cancer [13–17]. Previous studies revealed that miR-183 was decreased in GC and inhibited cell functions, such as cell proliferation and invasion [18,19]. However, the

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mechanism and functions of miR-183 in GC have not been fully elucidated. In our study, the level, functions and mechanism of miR-183 GC would be explored. Moreover, the value of miR-183 in predicting the prognosis of GC patients had also been evaluated and verified.

2. Materials and methods

2.1. Specimen collection

A number of 102 GC tissues and para-carcinoma tissue were recruited from the patients who had their tumor removed at Tianjin Medical University General Hospital from June 2016 and July 2018. All the patients enrolled in our study didn't receive any therapy pre-operation. The normal gastric tissues were excised over 2 cm far from the visible mass. All collected tissues were processed within 30 min after removal. All tissues were classified and managed via hematoxylin and eosin staining under an experienced pathologist. Our research was supported and managed by the Ethics Committees of Tianjin Medical University General Hospital. Every patient was told the informed consent. Each patient included in our study did not receive any treatment before surgery. Those patients with other tumors or diseases would be excluded out of our study. The tissues were used in accordance with IRB approval. After the fresh tissues were collected, they were immersed in RNAlater reagent (Qiagen GmbH, Inc., Hilden, Germany) for 30 min. and kept in liquid nitrogen container.

2.2. Cell culture and transfection

The cell lines in our research included gastric epithelial cell line (GES-1; Cell Resource Center of the Chinese Academy of Sciences, Shanghai, China) and GC cell lines (BGC823 and MKN45; American Type Culture Collection, USA). Complete medium for cell culture was composed of Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, USA), fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific), antibiotics (100 μl/ml penicillin and 100 mg/ml streptomycin sulfates) and glutamine. All cells were incubated at 37 °C in cell incubator with 5% CO_2. For cell transfection, miR-183-5p mimic or negative control (NC) (GenePharma, Shanghai, China) were transfected in BGC823 and MKN45 cells with Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific). This was followed by Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for Verification of transfection efficiency or Further functional experiments.

2.3. RNA extraction and RT-qPCR

In our research, RNA extraction from tissues was conducted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA extraction from cell lines were conducted with the RNeasy Maxi Kit (Qiagen GmbH, Inc.). After that, the RNA samples were detected under a NanoDrop 2000c (Thermo Fisher Scientific, Inc.) and those RNA samples with the optical density ratio (260/280) of 1.8-2.0 were included in our further study. This was followed by cDNA synthesis with 1 ul RNAs and miScript II RT Kit (Qiagen GmbH). miR-183-5p level in tissues and cell lines was ascertained with RT-qPCR under the Roche LightCycler 480 RT-qPCR System. In this assay, miScript SYBR-Green PCR Kit from Qiagen GmbH was used and run program were: 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. The relative miR-183-5p level between groups was calculated with $2^{-\Delta\Delta Ct}$ method after normalization to U6.

2.4. Cell proliferation assay

Cell counting kit-8 (CCK-8, Everbright lnc) was used in this assay. In brief, BGC823 and MKN45 cells transfected with miR-183-5p mimic or NC were inoculated in 96-well cell culture plate with a density of 4×10^3 per well. 10 μl/well of CCK-8 was added at every 24 h for a total of 72 h. Incubate for another 90 min, the cells were detected with an ELISA microplate reader for obtaining the optical density value of each well.

2.5. Cell migration assay

Transwell assay was conducted to appraise GC cells migratory aptitude. In our study, transwell chamber inserts (BD Biosciences, New York, NJ, USA) were used. In brief, serum-free medium containing BGC823 and MKN45 cells transfected with miR-183-5p mimic or NC were added to the upper transwell chamber inserts with a density of 3×10^4 per well. After that, the lower 24-well plate was filled with 500 ul complete medium as a chemoattractant. Incubated for another 36 h, remanent BGC823 and MKN45 cells on the upper surface of the membrane were cleared, while the migration cells on the bottom surface of the membrane were fixed and stained with utilization of methanol and crystal violet, respectively. A microscope (Leica Microsystems GmbH) was utilized to count the cells number in three random fields.

2.6. Bioinformatics analysis

Survival data from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov) was obtained to clarify the prognostic value of miR-183-5p in GC. miR-183-5p was submitted to OncoLnc (http://www.oncolnc.org/) and Kaplan-Meier curve was plotted for prognostic analysis. TargetScan (http://www.targetscan.org/vert_72/), miRWalk (http://mirwalk.umm.uni-heidelberg.de) and starBase 3.0 (http://starbase.sysu.edu.cn) were used to predict target gene regulated by miR-183-5p in GC cells. In brief, StarBase 3.0 was firstly used to predict target gene of miR-183-5p on this arrangement of binding Sites of AGO protein on target. The top three target having the most binding sites of AGO protein with miR-183-5p were selected. After that, TargetScan and miRWalk were used to further verify these three target genes.

2.7. Dual luciferase reporter assay

The full-length EEF2-UTR containing the predicted binding site of miR-183-5p as well as its mutant fragment was cloned into the psiCHECK2 luciferase reporter vector (Syngen, Beijing, China). Co-transfection (Wild type, mutant type or NC with miR-183-5p mimic or NC) was performed with Lipofectamine 3000. This is followed by the detection of Renilla and firefly luciferase activities with dual luciferase reporter assay system (Promega Corporation, USA).

2.8. Western blot assay

BGC823 and MKN45 cells transfected with miR-183-5p mimic or NC were washed with PBS. Cell lysis was determined by ice-cold Radio Immunoprecipitation Assay (RIPA) buffer. We then performed homogenization centrifugation to collect the supernatant. The same amount of protein separation and the transfer was performed with 12% sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and polyvinylidene difluoride (PVDF) membranes, respectively. After saturated with 5% skim milk, the membranes were incubated with primary antibodies (rabbit polyclonal anti-EEF2 (1:1000, Proteintech, Wuhan, China) and GAPDH (1:2000, Proteintech, Wuhan,China)) at 4 °C for 24 h. This was followed by the membranes being incubated with HRP conjugated goat anti-rabbit antibody (1:1000, Sigma) for 2 h and exposure to enhanced chemiluminescence substrate (Millipore, Rockford, USA). At last, Bio-Rad Gel Doc XR + system (Bio-Rad, Hercules, CA, USA) was utilized to visualize the band.

2.9. Statistical analysis

All data were verified three times. All data was disposed with SPSS

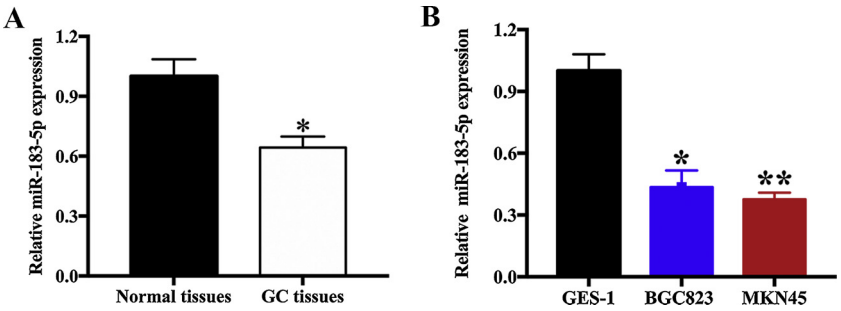

Fig. 1. miR-183-5p level in GC tissue and cell lines. (A) Relative level of miR-183-5p in GC tissues and normal tissues. (B) Relative level of miR-183-5p in cell lines. GC, gastric cancer. *P < 0.05, **P < 0.01.

21.0 or Graphpad prism 7 software. Between-group variance was determined by Student's t-test, chi-squared tests, one-way ANOVA, and Kaplan–Meier log-rank. The univariate and multivariate levels between miR-183-5p expression and clinicopathological variables or survival were determined using Cox proportional hazard regression analysis. Cut-off point was set as the median value of miR-183-5p expression for dividing the patients as High/low miR-183-5p group. P value less than 0.05 represented a statistically significant difference.

3. Results

3.1. miR-183-5p was down-regulated in GC tissues and cell lines

A total of 102 cases of GC and normal tissues was collected. The data of RT-qPCR revealed that miR-183-5p level was remarkably reduced in GC tissues in contrast to corresponding normal tissues (P < 0.05, Fig. 1A). We got a similar result in cell lines. The level of miR-183-5p was only 0.4 times and 0.46 times in BGC823 (P < 0.05) and MKN45 (P < 0.01) cell in contrast to GES-1 cell, respectively (Fig. 1B). Moreover, we then investigated the pertinence between miR-183-5p level and clinicopathological parameter of GC patients. Remarkable pertinence was got between miR-183-5p level and TNM stage (P = 0.001), tumor size (P = 0.01), invasion depth (P = 0.001), and lymph node metastasis (P = 0.001) (Table 1). We found that GC patients with low miR-183-5p level are more likely to suffer from advanced TNM stage, large tumor size, high invasion depth, and positive lymph node metastasis.

Table 1
Correlation of miR-183-5p expression and clinicopathological features in GC.

Variables	miR-183-5p expression			p value
	All cases (n = 102)	Low expression (n = 58)	High Expression (n = 44)	
Age (years)				0. 692
< 60	44	24	20	
≥60	58	34	24	
Gender				0.684
Male	62	34	28	
Female	40	24	16	
Tumor size (cm)				**0.01**
< 5	71	31	38	
≥5	31	27	6	
TNM stage				**0.001**
I & II	44	8	36	
III & IV	58	50	8	
Differentiation				0.318
Well & moderate	49	25	24	
Poor	53	33	20	
Invasion depth				**0.001**
T1 & T2	35	9	26	
T3 & T4	69	49	18	
Lymph node metastasis				**0.001**
Positive	27	25	2	
Negative	75	33	42	

3.2. The prognostic value of miR-183-5p in GC

Cox proportional hazard regression analysis and Kaplan-Meier survival curve were performed to evaluate the prognostic value of miR-183-5p in GC. Table 2 showed the results of the univariate and multivariate analysis. We found that TNM stage (HR = 4.933, 95% CI = 2.544–9.565, P = 0.001), differentiation (HR = 2.597, 95% CI = 1.487–4.563, P = 0.002), invasion depth (HR = 1.988, 95% CI = 1.084–3.644, P = 0.026), lymph node metastasis (HR = 3.017, 95% CI = 1.808–5.341, P = 0.001), and

miR-183-5p expression (HR = 0.210, 95% CI = 0.110–0.399, P = 0.001) were independent causes impacting on the overall survival in GC in univariate analysis. Furthermore, we found that TNM stage (HR = 3.005, 95% CI = 1.317–6.858, P = 0.009), differentiation (HR = 0.942, 95% CI = 1.562–5.542, P = 0.001) and miR-183-5p level (HR = 0.368, 95% CI = 0.167–0.809, P = 0.013) were independent causes impacting on the overall survival in GC in multivariate analysis. Moreover, the Kaplan-Meier survival curve was drawn and suggestted that the GC individuals with high miR-183-5p level would experience a relatively better survival prognosis (P = 0.001, Fig. 2A). Our result was verified by TCGA data, which revealed that GC individuals with high miR-183-5p level would experience a relatively better survival prognosis (P = 0.019, Fig. 2B). All the data above demonstrated miR-183-5p may act as a promising biomarker for the prognosis of GC.

3.3. Validation of cell transfection efficiency

The changes of miR-183-5p level of BGC823 and MKN45 cell transfected with miR-183-5p mimic or NC was confirmed by RT-qPCR. BGC823 cells transfected with miR-183-5p mimic presented significantly elevated miR-183-5p level (p < 0.001, Fig. 3A). Furthermore, MKN-45 cells transfected with miR-183-5p mimic presented significantly elevated miR-183-5p level (p < 0.001, Fig. 3B).

3.4. miR-183-5p inhibited cell proliferation and migration

Statistics of proliferation assay revealed that the proliferative capacity of BGC823 cells could be remarkably inhibited by upregulation of miR-183-5p level (Fig. 4A). The same result was got in MKN-45 cell (Fig. 4B). Statistics of migration assays revealed that the migrative capacity of BGC823 cells can be remarkably suppressed by upregulation of miR-183-5p level (p < 0.01, Fig. 4C). Similarly, the migrative capacity of MKN-45 cells could be remarkably suppressed by upregulation of miR-183-5p level (p < 0.05, Fig. 4D). Above all, miR-183-5p inhibited GC cell proliferation and migration.

Table 2
Univariate and multivariate cox proportional hazard regression analysis.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender (Male VS Female)	0.782	0.460–1.392	0.363	–	–	–
Age (years) (< 60 VS ≥ 60)	0.991	0.583–1.683	0.972	–	–	–
Tumor size (cm) (< 5 VS ≥ 5)	0.697	0.973–2.960	0.062	–	–	–
TNM stage (I & II VS III & IV)	4.933	2.544–9.565	**0.001**	3.005	1.317–6.858	**0.009**
Differentiation (Well & moderate VS Poor)	2.597	1.487–4.563	**0.002**	0.942	1.562–5.542	**0.001**
Invasion depth (T1 & T2 VS T3 & T4)	1.988	1.084–3.644	**0.026**	0.673	0.332–1.361	0.27
Lymph node metastasis (Positive VS Negative)	3.017	1.808–5.341	**0.001**	0.996	0.521–1.903	0.989
miR-183-5p expression[a] (High vs Low)	0.210	0.110–0.399	**0.001**	0.368	0.167–0.809	**0.013**

[a] Cut-off point: median value of miR-183-5p expression.

3.5. EEF2 may be a target gene regulated by miR-183-5p

In our study, StarBase 3.0 was firstly used to predict target gene of miR-183-5p on this arrangement of binding Sites of AGO protein on target. SOX4, EEF2 and RTN4 have the most binding sites of AGO protein with miR-183-5p. Besides, the results of TargetScan and miRWalk revealed binding sites between miR-183-5p and EEF2, but not SOX4 and RTN4. The binding sequence between miR-183-5p and EEF2 was shown in Fig. 5A. Moreover, RT-qPCR was performed and revealed that EEF2 was significantly increased in BGC823 ($P < 0.001$) and MKN45 ($P < 0.001$) cell compared with that in GES-1 cell (Fig. 5B). Dual luciferase report assay and western blot were conducted to validate this potential target. As we expected, the luciferase activity of the WT group in the -UTR of EEF2 in HEK293T cells could be remarkably inhibited by upregulation of miR-183-5p ($p < 0.05$) while the luciferase activity of the WT group could not be affected by miR-183-5p level (Fig. 5C). In western blot assay, the relative protein level of EEF2 in the miR-183-5p mimic group of BGC823 ($p < 0.05$, Fig. 5D) and MKN-45 ($p < 0.01$, Fig. 5E) was remarkably reduced in contrast to the NC group. Above all, EEF2 may be a direct target gene regulated by miR-183-5p in GC.

4. Discussion

In recent years, multidisciplinary synthetic therapy including surgery, radiotherapy, chemotherapy and Chinese medicine treatments was applied in GC, however, the prognosis of GC patients is still poor, especially the patients with advanced or metastatic GC, with the 5-year survival rates being only 5–20% [20,21]. One of main reason was the specific mechanism of the tumorigenesis and development of GC was a mystery. Though some targeted therapeutic drugs including Anti-vascular endothelial growth factor (VEGF) drug, C-met-target drugs, PI3K signaling pathway inhibitors and PARP inhibitors were used for the treatment of gastric cancer clinically, no significant change were made in overall survival of gastric cancer. The emergence of miRNA provides a new direction for the study of targeted therapeutic drugs and prognostic biomarkers for gastric cancer [22]. Numerous miRNAs have been suggested to result in GC oncogenesis and development, including miR-183-5p [18,23]. Thus, it is significant to investigate thoroughly promising miRNAs related to the tumorigenesis and development of GC, since they were likely to provide a biomarker for better diagnosis and prognosis as well as therapy.

Previous research revealed that miR-183 acted as a biomarker in various cancers. Li et al. [24] suggested that high level of miR-183-5p was related to lower overall survival in renal cell carcinoma, indicating high miR-183-5p level being a biomarker for predicting worse prognosis. miR-183 level not only was elevated in esophageal squamous cell carcinoma (ESCC) patients compared with healthy controls, but also associated with TNM stages. Moreover, the sensitivity and specificity of miR-183 in diagnosing ESCC was 0.789, 0.762, respectively, which suggested that miR-183 acted as a prognostic biomarker in ESCC [25]. In tongue carcinoma, miR-183 level was related to tumor size and

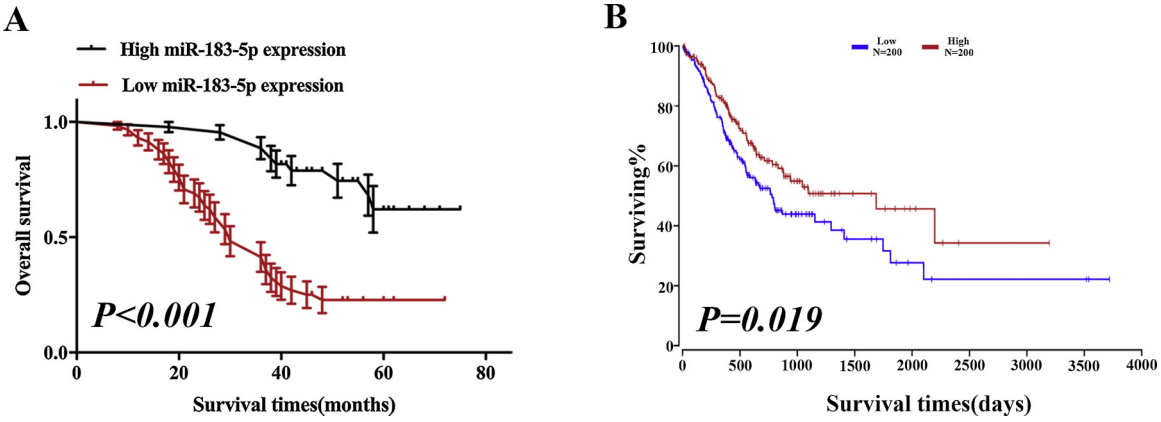

Fig. 2. Kaplan-Meier survival curves of miR-183-5p in the prognosis of GC. Kaplan-Meier curve of miR-183-5p obtained from GC tissues data (A) and TCGA database (B). GC, gastric cancer. Cut-off point: median value of miR-183-5p expression.

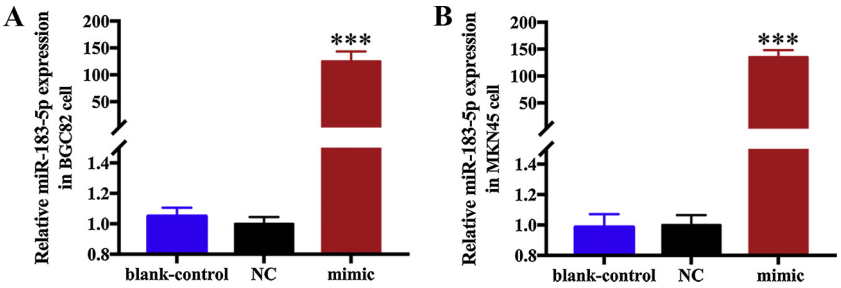

Fig. 3. Result of transfection efficacies. Transfection efficacies of miR-183-5p mimic in BGC823 (A) and MKN45 (B) cells. ***P < 0.001.

clinical stage and the patients with down-regulated miR-183 level had remarkably longer overall survival [26]. In colorectal cancer, miR-183 level was remarkably higher in plasma samples of CRC patients than healthy individual. High plasma miR-183 level was remarkably related to lymph node metastasis, advances pTNM stage and distant metastasis. Furthermore, the value of miR-183 level in ROC curve was higher than CEA and CA19-9 [27]. In our study, result of the Kaplan-Meier survival curve and Data of TCGA revealed that the GC individuals with high miR-183-5p level would experience a relatively better survival prognosis, demonstrating miR-183-5p may act as a promising biomarker for the prognosis of GC.

Previous research revealed that miR-183 played a vital regulatory role in various cancers. Zhou et al. revealed that miR-183 was increased in ovarian cancer tissues and decreased level of miR-183 significantly suppressed cell functions (proliferation, migration and invasion) via the TGF-β/Smad4 signaling pathway [28]. Studies about the functions of miR-183 in lung cancer were performed and demonstrated that upregulation of miR-183-5p could inhibit cell proliferation, invasion and induce cell cycle arrest, which was achieved by targeting PIK3CA [17]. Moreover, miR-183-5p expression was downregulated cervical cancer tissues and cell lines. Upregulation of miR-183-5p significantly suppressed cell migration and invasion by regulating the gene expressions of ITGB1 [16]. In bladder cancer, miR-183 level was increased in tissues and cell lines and upregulation of miR-183 promoted cell growth and

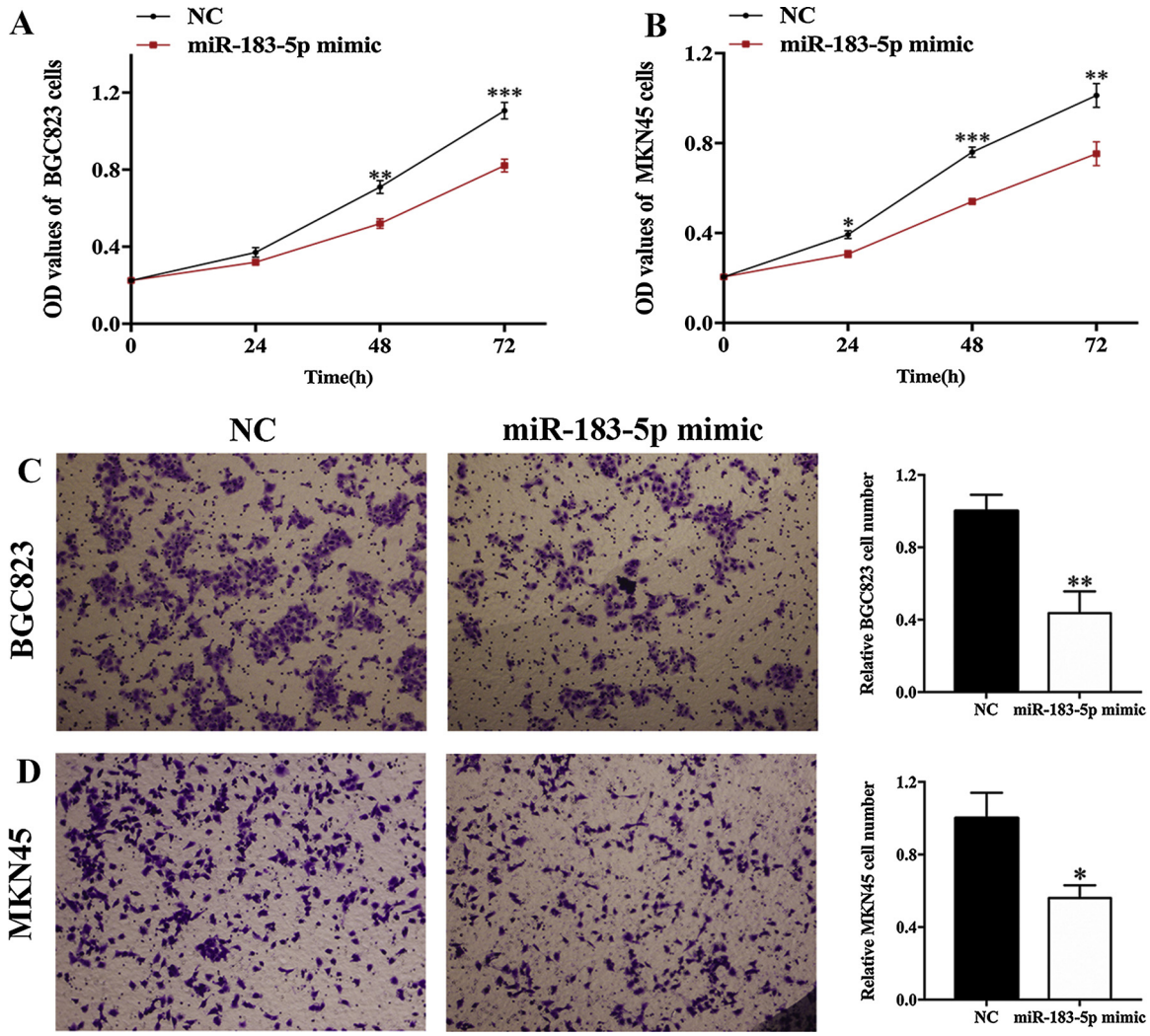

Fig. 4. The role of miR-183-5p in cell function. Cell proliferation in BGC823 (A) and MKN45 (B) cells after upregulation of miR-183-5p. Cell migration in BGC823 (A) and MKN45 (B) cells after upregulation of miR-183-5p. OD, optical density; *P < 0.05, **P < 0.01, ***P < 0.001.

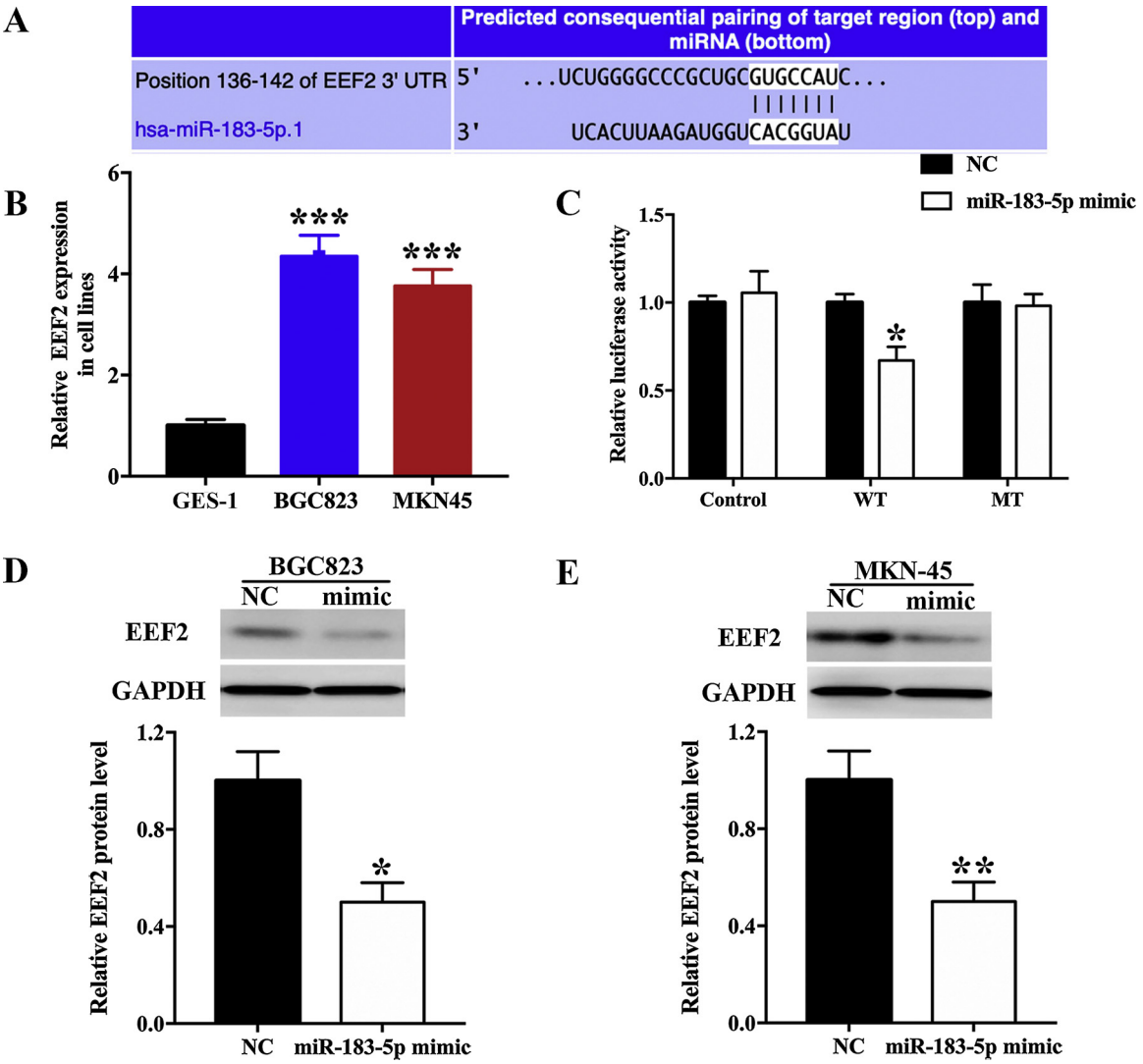

Fig. 5. Validation of EEF2 as a direct target of miR-183-5p. (A)Schematic diagram of the binding sites of miR-183-5p with the 3'-UTR of EEF2. (B) Relative expression of EEF2 in cell lines. (C)The luciferase activity in WT, MT and NC group treated with miR-183-5p mimic or NC. Protein expressions of miR-183-5p after over-expression of miR-183-5p in BGC823 (D) and MKN45 (E). *p < 0.05, **p < 0.01, ***p < 0.001.

inhibited apoptosis [29]. miR-183 also played a vital regulatory role in osteosarcoma, non-small cell lung cancer and endometrial cancer [30–32]. In our study, proliferative and migrative capacity of BGC823 and MKN45 cells can be remarkably suppressed by upregulation of miR-183-5p level.

Eukaryotic elongation factor 2 (EEF2), an important part of the translation machinery, exerts a significant funtions in the GTP-dependent translocation of the ribosome along mRNA [33,34]. Accumulating evidences demonstrated that EEF2 play a vital part in proliferation, migration and the survival of various cancers [33,35,36]. In our study, StarBase 3.0 was firstly used to predict target gene of miR-183-5p on this arrangement of binding Sites of AGO protein on target. SOX4, EEF2 and RTN4 have the most binding sites of AGO protein with miR-183-5p. Besides, the results of TargetScan and miRWalk revealed binding sites between miR-183-5p and EEF2, but not SOX4 and RTN4. Thus, EEF2 was selected as the target regulated by miR-183-5p in GC. After that, double luciferase assay and western blot was conducted and as we expected, the results revealed that EEF2 may be the direct target regulated by miR-183-5p in GC.

The limitation of this study is that the sample size in our analysis about miR-183-5p as a biomarker for prognosis of gastric cancer is limited and the same analysis in a large of sample size should be performed. Besides, specific molecular mechanism and signaling pathways of EEF2 in gastric cancer, and whether miR-183-5p can act as a diagnosis biomarker in gastric cancer hasn't been clarified. Rescue experiments and animal models should be performed in further study to clarify the function of EEF2 and the biological function of miR-183-5p.

In conclusion, miR-183-5p is downregulated and acts as a prognostic biomarker in gastric cancer. Up-regulation of miR-183-5p regulates cell proliferation and migration. EEF2 may be one of the potential targets of miR-183-5p.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Tianjin Medical University General Hospital.

Patient consent for publication

Written informed consent was obtained from all patients for publication of data.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgement

Not applicable.

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