Original Article
microRNA-32-5p targets KLF2 to promote gastric cancer by activating PI3K/AKT signaling pathway

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Abstract: Krüppel-like factor 2 (KLF2) is a member of the zinc finger family, which is considered a potential tumor suppressor gene due to its reduced expression in gastric cancer. MicroRNAs (miRNAs) are a class of short non-coding single-stranded RNAs that are closely related to the development of gastric cancer. The purpose of this study was to investigate the miRNAs that regulate KLF2 and explore its specific mechanism in gastric cancer. Bioinformatics software Targetscan identified that microRNA-32-5p (miRNA-32-5p) may bind to KLF2 mRNA to regulate its expression. In order to verify the regulatory effect of miRNA-32-5p on KLF2, the proliferation and migration assays were performed in both KLF2 overexpression and KLF2 knockdown gastric cancer cells. Dual-Luciferase reporter assay proved that KLF2 could bind to the PTEN promoter to induce its expression. Moreover, research on molecular mechanisms indicated that both miRNA-32-5p and shKLF2 downregulated the expression of PTEN and activated the PI3K/AKT signaling to promote the development of gastric cancer. Targeting miRNA-32-5p and KLF2 is expected to provide new sign and target for gastric cancer treatment.

Keywords: KLF2, microRNA-32-5p, gastric cancer, PI3K/AKT signaling

Introduction

Gastric cancer is one of the most common malignant cancers of the digestive tract in the world with high morbidity and mortality. There are 951,594 new cases of gastric cancer, and 723,072 patients died from this disease worldwide in 2012, of which China accounted for 42.6% and 45.0% respectively [1]. Due to the early concealment of symptoms and lack of universal screening methods, gastric cancer is often found in the middle or late stages, which makes the treatment effect and prognosis very poor [2]. Surgery is still the main treatment strategy to cure gastric cancer. However, the curative effect on patients with advanced disease is not ideal. The commonly used chemotherapy drugs such as fluorouracil and cisplatin

for postoperative adjuvant chemotherapy are very limited in prolonging the survival of patients, and the 5-year survival rate of patients with advanced gastric cancer is less than 20% [3]. HER2 targeted drug, trastuzumab, has a relatively good clinical outcome in the treatment of advanced gastric cancer. While trastuzumab has little effect on HER2-negative patients, and its safety remains to be confirmed [4, 5]. In addition, there are no clinically specific tumor markers for the diagnosis of early gastric cancer. To a large extent, the number of clinical cures is insufficient, and exploiting novel effective targets and drugs is pressingly needed. Therefore, it is urgent to perform more in-depth researches on the biological mechanism of gastric cancer and find effective gastric cancer tumor markers and potential therapeutic tar-

gets. This is of great significance for the early detection, prevention and treatment of gastric cancer.

A prominent feature of the transcription factor Krüppel-like factor (KLF) family is three highly conserved classical Cys2/His2 zinc finger structures at the carboxyl terminus of the protein, which enable KLFs bind to the GC- and CACCC-boxes of DNA [6]. To date, 17 kinds of KLFs (KLF1-KLF17) have been found in humans, which are involved in many biological processes, including cell proliferation, differentiation, apoptosis, ontogeny, tumor cell migration and differentiation. Many studies revealed that different KLFs exert different roles in tumors. For example, KLF4 inhibits the invasion and metastasis of pancreatic cancer and lung cancer [7-9], while KLF7 and KLF8 promote the invasion and metastasis processes in colorectal cancer [10], breast cancer [11], lung cancer [12] and pancreatic cancer [13], etc. As an important member of KLFs family, KLF2 has a wide range of functions. Studies have demonstrated that KLF2 plays important roles in neovascularization [14, 15], endothelial cell proliferation and function [16-19], maintenance of B cell homeostasis and plasma cell homing [19, 20], mediation the granulocyte inflammatory response [21], immune cell function [19], and promotion of inflammatory T cells [19, 22, 23]. In addition, KLF2 is expressed in a variety of cancers, such as non-small cell lung cancer [19, 24], pancreatic ductal adenocarcinoma [25, 26], thyroid cancer [27], colorectal cancer [28, 29], ovarian cancer [30] and prostate cancer [31]. All of these studies have indicated that KLF2 overexpression inhibits tumor cell viability and promotes cell apoptosis. Furthermore, KLF2 possesses tumor suppressor functions in non-small cell lung cancer, which is significantly associated with lymph node metastasis and advanced TNM staging [24]. Therefore, KLF2 is considered as a potential tumor suppressor gene.

The expression of KLF2 in tumors is regulated by a variety of factors. Among them, microRNAs (miRNAs), a small endogenous non-coding single-stranded RNA containing 19-22 base, is one of the most important regulation mechanisms of tumor associated genes, which is also a research hotspot of life sciences in recent years. miRNAs target the 3'-UTR or 5'-UTR of mRNAs to suppress translation or degrade tar-

get mRNA, thereby affecting cell growth, proliferation, differentiation, metabolism and neuromodulation [32, 33], which is closely related to the occurrence and development of human diseases. For example, miRNA-223 targets the tumor suppressor EPB41L3 to promote gastric cancer invasion and metastasis [34]. miRNA-19 promotes osteosarcoma progression by targeting SOCS6 and Pax6 [35, 36]. A large number of researches have shown that the expression levels of miRNAs are closely related to various tumors and mediate many biological behaviors, such as tumor proliferation, apoptosis, metastasis and drug resistance. Given the importance of miRNAs in regulating cell growth and viability, miRNAs dysregulation is thought to be closely related to the development and progression of gastric cancer [37]. Therefore, in the near future, targeting miRNAs will become a new technology for clinical diagnosis, treatment and prediction of cancer patient prognosis. Since KLF2 significantly inhibits the development of gastric cancer, it is important to explore novel miRNAs targeting KLF2 for the development of gastric cancer therapeutic strategies.

In our previous researches, we have explored the role of KLF2 in liver cancer and hepatic steatosis [38, 39]. And we have revealed that KLF2 overexpression inhibits the proliferation and migration of gastric cancer cells by inducing PTEN expression to inhibit PI3K/AKT pathway [40]. However, the specific molecular mechanism of KLF2 acting on PTEN is not yet clear and the researches on upstream regulatory miRNAs of KLF2 are insufficient in gastric cancer.

Based on this, we screened that micoRNA-32-5p (miRNA-32-5p) targets KLF2 and identified the possible binding regions of KLF2 on PTEN promoters by bioinformatics software Targetscan, then explored their molecular mechanism relationship in gastric cancer. Finally, we expect our experimental results provide theoretical support for the discovery of new markers and targets in the diagnosis and treatment of gastric cancer.

Materials and methods

Cell lines and reagents

Human gastric cancer cell lines: MKN45, BGC-823 and MGC803 were obtained from Ameri-

can Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were maintained in a humidified incubator at 5% CO_2 at 37°C. The primary antibodies used in the study include the following: KLF2 (Abcam, ab194468, 1:1000), PTEN (CST, 1:1000, 9188T), AKT (CST, 4685, 1:1000), p-AKT (CST, 4060, 1:1000), mTOR (CST, 2972, 1:1000), p-mTOR (CST, 2971, 1:1000), p70S6K (CST, 9202, 1:1000), p-p70S6K (CST, 9208, 1:1000) and β-actin (sigma, A5441, 1:10000). Lipofectamine 2000 Transfection Reagent was purchased from Thermo Fisher Scientific. CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (MTS; Promega, USA). Dual-LuciferaseReporter Assay System (Promega, Madison, WI).

MTS assay

Cell proliferation was analyzed by the MTS assay according to the manufacturer's instructions [41]. Briefly, cells (1×10^4/well in a 96-well plate) were seeded in 96 well plates. After 1, 2 and 3 days of incubation, wild-type or transfected gastric cancer cells were incubated with 20 μL MTS test buffer per well. The formazan product was quantified by absorbance at 490 nm in a microplate reader (SpectraMax 190; Molecular Devices, USA). The number of viable cells was presented relative to untreated control. This assay was repeated three times independently. One-way ANOVA was used for the comparison among multiple groups, followed by the LSD multiple t-test.

Colony formation assay

The colony formation assay was performed as described [42]. Wild-type or transfected gastric cancer cells were trypsinized and seeded 2000 per well into a 6-well plate and cultured for 10 days. Then the cells were fixed with 4% paraformaldehyde for 20 min, and stained with 0.5% crystal violet for 15 min. The number of cell colonies was calculated and analyzed as the ratio of the number of treated samples to untreated samples. One-way ANOVA was used for the comparison among multiple groups, followed by the LSD multiple t-test.

Transwell assays

Cell migration was examined using the transwell assay as previously described. 300 μL of

5×10^4 wild-type or transfected gastric cancer cells were suspended in serum-free medium in the top of transwell chambers, 600 μL medium containing 10% FBS was added to the lower chambers. After 18 h of incubation at 37°C, cells on the upper surface were removed using a swab and cells on the lower surface of the membrane were fixed in 4% paraformaldehyde for 20 minutes, then dyed with 0.5% crystal violet for counting. Images were photographed using a digital camera attached to OLYMPUS inverted microscope, and invaded cells were quantified by manual counting. Three independent experiments were performed. One-way ANOVA was used for the comparison among multiple groups, followed by the LSD multiple t-test.

Plasmid construction and transfection

According to the expression of KLF2 in gastric cells, MGC803 and MKN45 cell lines were respectively selected to construct knockdown and overexpression cell lines. Three KLF2 shRNAs oligos were inserted into pLKO.1 vector. The sequences of KLF2-shRNA are as follows: KLF2-shRNA 1# sequence: CGGCACCGACGACGACCTCAA, KLF2-shRNA 2# sequence: GCCAAGAGTTCGCATCTG, KLF2-shRNA 3# sequence: CCTTTCGGTGGCCCTGGTT. 293T cells were transiently transfected with the following plasmids: lentiviral packaging plasmid pMD2.G, psPAX2, and lentiviral expression plasmid. Forty eight hours post-transfection, the supernatant was harvested for knockdown of KLF2. The full length human KLF2 cDNA was obtained from PCR. The cDNA of KLF2 were subcloned into pcDNA3.1 vector, and lipofectamine-mediated transfection of overexpression vector of KLF2 was performed. Both knockdown and overexpression cell lines were obtained by resistance screening.

Protein extraction and western blotting

Briefly, cells were washed twice with PBS and then harvested and lysed on ice for 30 min in lysis buffer containing PMSF, protease inhibitors and Phosphatase inhibitor. Protein concentrations were determined using BCA protein assay kit (Thermo Scientific, USA) and equalized before loading. Subsequently, proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, USA). The nitrocellulose membranes were then blocked with 5% BSA for 1 h, and incubated

with antibodies at 4°C overnight, washed rapidly 3 times with TBST and then followed by the fluorescence conjugated secondary antibodies for 1 h. The washing was again performed 3 times with TBST. Finally the proteins were detected by the infrared dichroic laser scanning imaging system (Li-Cor, Odyssey). Three independent experiments were performed.

Dual-luciferase reporter assay

For dual-luciferase screening assay, according to specific experimental needs, gastric cancer cells were transfected with wild-type or mutant MMTV-luc, Renilla-luc, miRNA-32-5p mimics, miRNA mimics NC AR or shNC, shKLF2 plasmids using lipofectamine 2000 according to the manufacturer's instructions. After transfection for 48 h, the renilla and firefly activities were then determined by luminometry using the Dual-Luciferase Reporter Assay System and the ratio was calculated. Results were expressed as the ratio of firefly to Renilla luciferase activity. The experiment was repeated three times independently. One-way ANOVA was used for the comparison among multiple groups, followed by the LSD multiple t-test.

Introduction of miRNA mimics

Mimics of miRNA-32-5p and miRNA mimics NC were synthesized by the GENEWIZ Company (Shanghai, China). For each transfection in a six-well plate, 100 nM miRNA mimics, mimic NC were used. The transfection of gastric cancer cells by lipofectamine 2000 transfection reagent was performed according to the manufacturer's instructions.

Statistical analysis

Data are presented as the means ± standard deviation. Statistical analyses were performed with SPSS 16.0 (SSPS, Inc., Chicago, IL, USA). Student's t-test was used for the comparison between two groups. One-way ANOVA was used for the comparison among multiple groups, followed by the LSD multiple t-test. A *p*-value of < 0.05 was considered statistically significant.

Results

miR-32-5p regulates KLF2 expression by binding 3'-UTR region of KLF2 mRNA

miRNAs are important regulators that mediate tumor proliferation, apoptosis, metastasis and drug resistance. In gastric cancer, miRNAs dysregulation plays an important role [37]. In addition, our previous research have found that the transcription factor KLF2 is closely related to the development of gastric cancer [40]. To explore how miRNAs regulated KLF2, bioinformatics software Targetscan was used and predicted that miR-32-5p may bind to the 3'-UTR (242-249 nucleotide) of human KLF2 mRNA (**Figure 1A** and **1B**). Besides that, the data of miR-32-5p expression in The Cancer Genome Atlas (TCGA) database show that miR-32-5p expression is much higher in gastric tumor samples (n = 387) compared with normal samples (n = 41) (P = 0.002165) (**Figure 1C**). Moreover, the correlation analysis of miRNA-32-5p and KLF2 in TCGA database shows that they are negatively correlated in gastric cancer tissues, and the correlation coefficient is r = -0.1258471 (P = 0.01571) (Data not shown). Therefore, we speculate that miR-32-5p may regulate KLF2 expression in gastric cancer cells. To verify this assumption, the dual-luciferase reporter assay was carried out. Wild-type (GUGCAAUA) and mutant (ACTTCCGC) KLF2 3'-UTR binding sites of miR-32-5p were cloned into luciferase reporter vectors respectively. miR-32-5p mimic suppressed the luciferase intensity in gastric cancer cells compared with miRNA-NC (negative control) in wild-type group. However, we did not notice a significant change in mutation group (**Figure 1D**). These results demonstrate that miR-32-5p directly binds to the 3'-UTR region of KLF2 mRNA to inhibit its expression in gastric cancer cells.

Effects of miRNA-32-5p and KLF2 on proliferation of gastric cancer cells

A literature survey indicates that KLF2 inhibits the development of gastric cancer [38], while very few researches focus on miRNA-32-5p in gastric cancer. Thus, the present study further revealed the biological function of miRNA-32-5p and explored its interaction with KLF2 in gastric cancer. Firstly, the expression of KLF2 in MGC803, MKN45 and BGC823 gastric cancer cell lines were analyzed (**Figure 2A**), and MKN45 and MGC803 cell lines were respectively selected to construct knockout and overexpression cells stably transfected strains for further research. The expression of KLF2 in MKN45 cell line infected with shKLF2 lentivirus are decreased significantly compared with control and shNC (negative control) (**Figure 2B** and **2C**). The shKLF 2# was selected for subsequent

A

miRNA	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type
Position 242-249 of KLF2 3' UTR hsa-miR-32-5p	5' UUGUUUUUCAAAAUGGUGCAAUA | | | | | | | | 3' ACGUUGAAUCAUUACACGUUAU	8 mer

Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	Pct
-0.51	99	-0.51	4.799	0.92

B

C

D
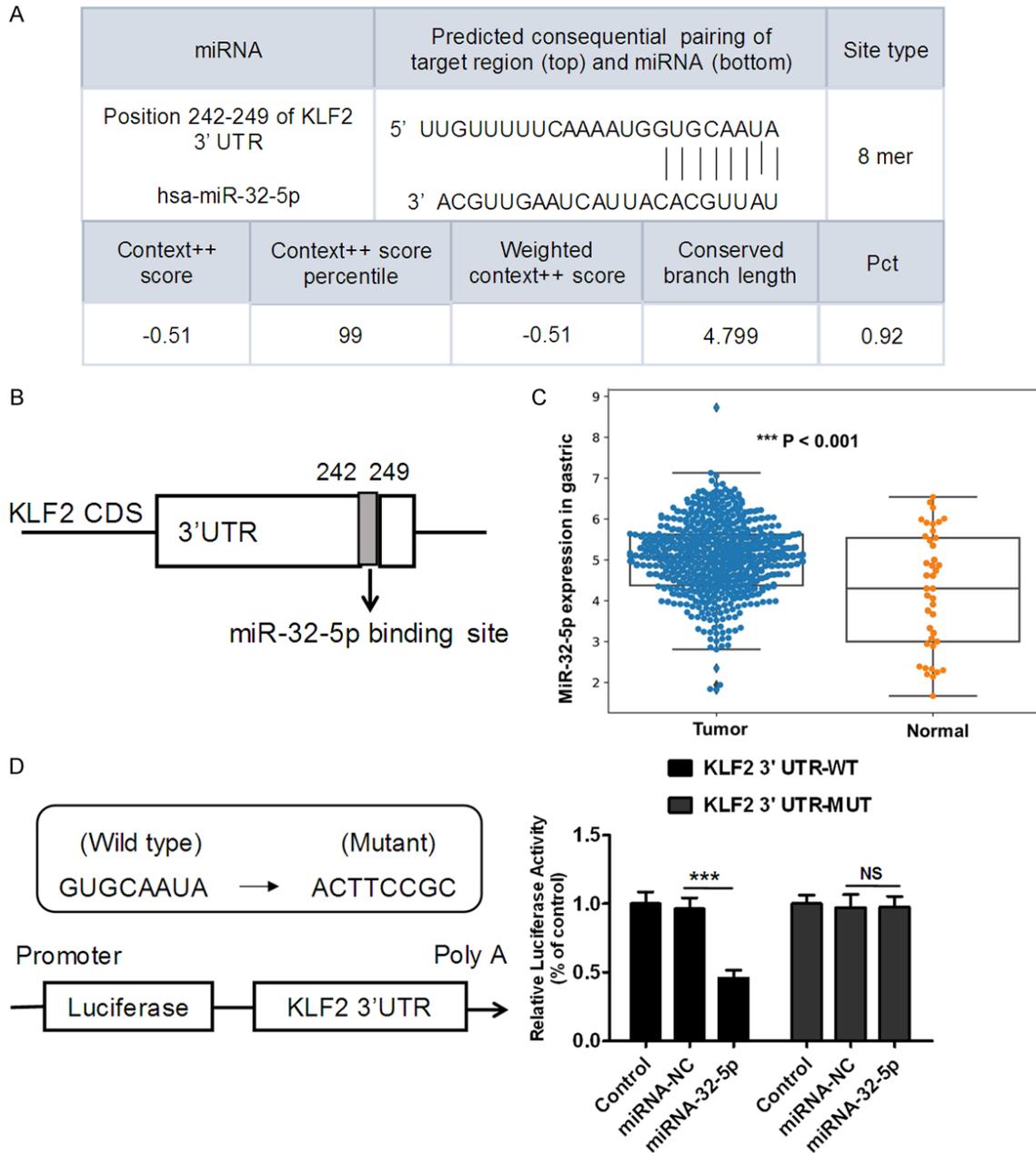
Figure 1. miR-32-5p inhibits KLF2 expression by binding KLF2 3'-UTR. A and B. Prediction of miR-32-5p binding sites in the 3'-UTRs of KLF2 gene by bioinformatics software Targetscan. C. Data from TCGA database showed average expression level of miR-32-5p is higher in gastric cancer specimens (n = 387) compared with normal samples (n = 41). D. The dual-luciferase reporter gene system shows that miR32-5p binds to the 3'-UTR of KLF2 and inhibits its expression. NS stands for non-significant (P > 0.05), ***P < 0.001.

experiments according to its best knockout efficiency. Our previously published article have revealed that KLF2 acted as a tumor suppressor by inhibiting the proliferation, migration and invasion of gastric cancer cells [40]. In this study, the MTS assay and colony formation assay were used to determine the effects of miRNA-32-5p and KLF2 on gastric cancer cells proliferation. MTS assay demonstrated that gastric cancer cells proliferation was increased upon transfection with miRNA-32-5p and shKLF2 compared with miRNA-NC and shNC control respectively. And co-transfection of miRNA-32-5p and shKLF2 resulted in faster
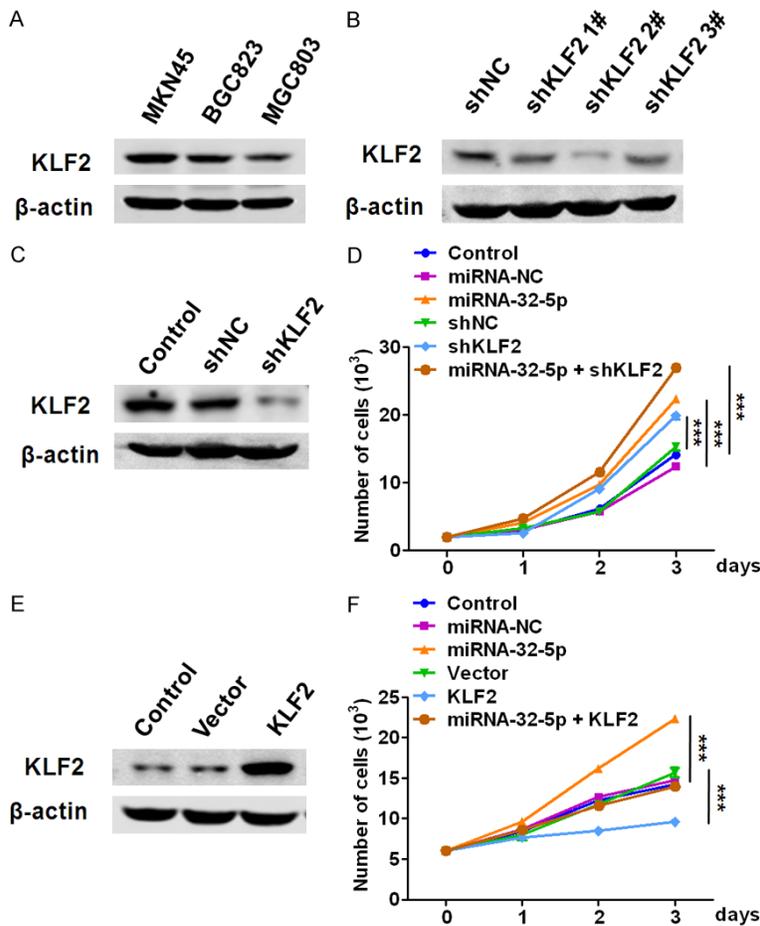
Figure 2. miR-32-5p and KLF2 knockdown synergistically promote gastric cancer cell growth. A. The expression of KLF2 in three gastric cancer cells was detected. MKN45 and MGC803 cell lines were respectively selected to construct knockout and overexpression cell lines. B. Western blot verified the knockout efficiency of KLF2 in the MKN45 knockdown cell line and shKLF2 2# was choosed for subsequent experiments. C. The knockout efficiency of KLF2 in shKLF2 2# cells was further confirmed. D. MTS cell proliferation assay revealed miR-32-5p and KLF2 knockdown synergistically promoted MKN45 cancer cells growth. E. The level of KLF2 in MKN45 cells infected with KLF2-overexpressing plasmid was verified by western blot. F. MTS cell proliferation assay showed KLF2 overexpression attenuated the proliferative effect of miR-32-5p. ***P < 0.001.

proliferation of gastric cancer cells (**Figure 2D**). The result of transfect with KLF2 overexpression plasmid was contrary. Overexpression of KLF2 restrains the proliferation of gastric cancer cells, while co-transfection with miRNA-32-5p abate the inhibition of KLF2 on cell proliferation (**Figure 2E** and **2F**). Then colony formation assays further validated the above results (**Figure 3**). These findings indicate that miR-32-5p has synergistic effect with KLF2 knockdown on proliferation of gastric cancer cells, which confirms the hypothesis that miR-

NA-32-5p induces the growth of gastric cancer by inhibiting the expression of KLF2.

Effects of miRNA-32-5p and KLF2 on migration of gastric cancer cells

Like the rapid proliferation of cancer cells, strong cell migration ability is also one of the most important biological characteristics of malignant tumors. We then evaluated the effect of miRNA-32-5p and KLF2 on cell migration of gastric cancer. The transwell assay showed that the invasive activity of MGC803 cells was increased following transfection with miRNA-32-5p and shKLF2 compared with miRNA-NC and shNC control respectively. And the number of cancer cells passing through the membrane in co-transfection of miRNA-32-5p and shKLF2 group was significantly higher than that of single transfected group (**Figure 4A**). By contrast, the KLF2 overexpression reduced invasive activity of MGC803 cells. And the miRNA-32-5p abate the inhibition of KLF2 on cell migration (**Figure 4B**). Then we explored the effect of miR-32-5p and shKLF2 on EMT in gastric cancer. Western blot showed that the expression of epithelial cell marker E-cadherin was drastically reduced. Conversely, mesenchymal cell markers fibronectin and vimentin were significantly upregulated, indicating that both miR-32-5p and shKLF2 promoted EMT with synergy effect (**Figure 4C**). Combined the results of proliferation experiment, it is concluded that KLF2 suppresses gastric cancer cells proliferation and migration capability. While miRNA-32-5p has the opposite effects, and it antagonizes the inhibition of KLF2 on gastric cancer cells proliferation and migration. Besides that, these results corroborate that
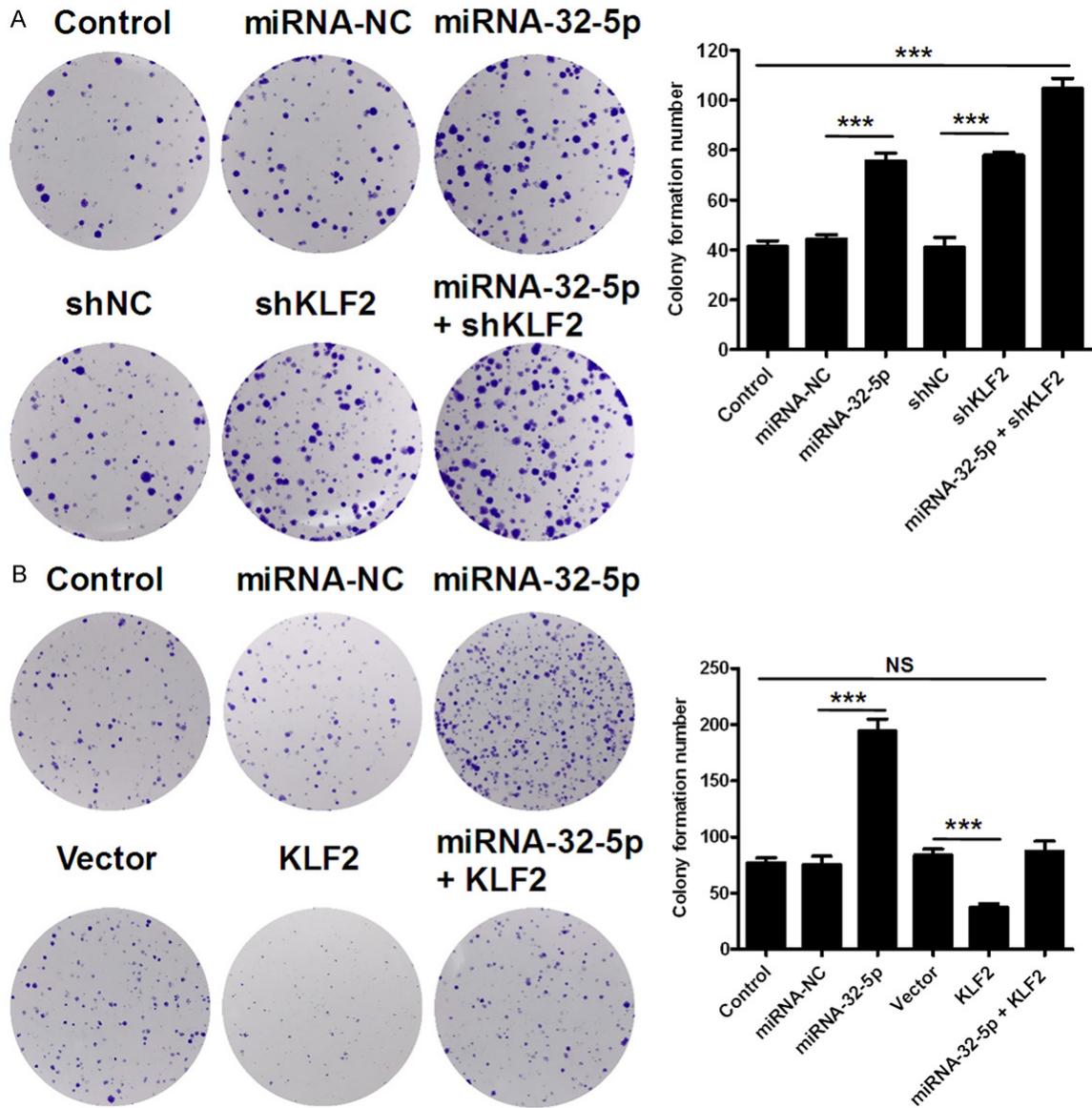
Figure 3. miR-32-5p and KLF2 knockdown synergistically promote gastric cancer cell colony formation. A. Both KLF2 knockdown and miRNA-32-5p mimic increased colony number of MGC803 cells and KLF2 knockdown and miRNA-32-5p mimic have a synergistic effect. B. KLF2 overexpression decrease colony number of MKN45 cells, while the inhibition effect was antagonized by miRNA-32-5p mimic. NS stands for non-significant (P > 0.05), ***P < 0.001.

miRNA-32-5p is negatively correlated with KLF2 expression.

KLF2 knockdown reduces the expression of PTEN

Our previous research has revealed that KLF2 inhibits PI3K/AKT signaling by inducing expression of PTEN protein, thereby suppressing the development of gastric cancer [40]. However, whether KLF2 directly targets PTEN has not

been verified. To further investigate the regulation of KLF2 on PTEN, three binding sites of KLF2 on PTEN promoter were predicted by bioinformatics software Targetscan. And then three wild-type binding sites and three mutant binding sites of KLF2 on PTEN promoter were cloned into luciferase reporter vectors respectively (**Figure 5A-C**). MKN45 cells were cotransfected wild-type or mutant luciferase reporter vector together with shKLF2 or shNC respectively. Luciferase reporter assay reveal-
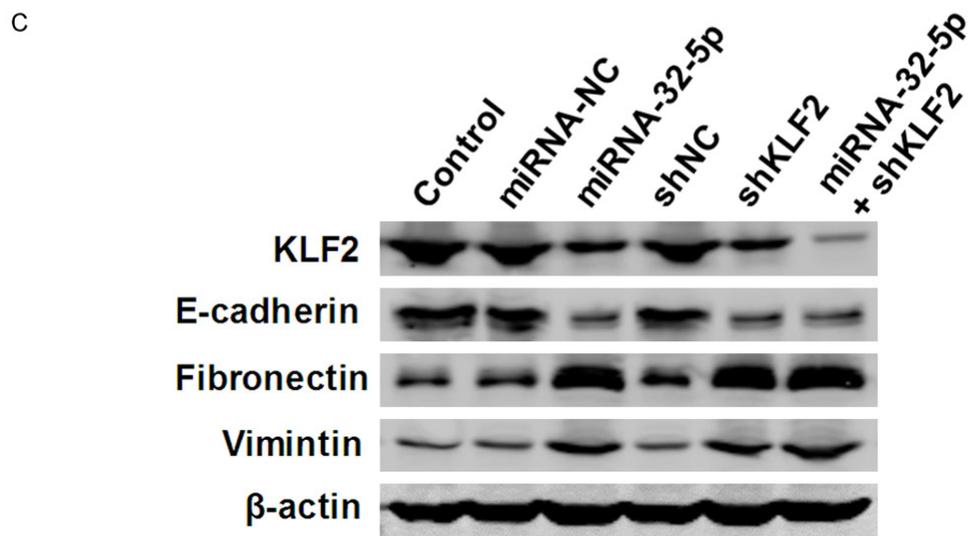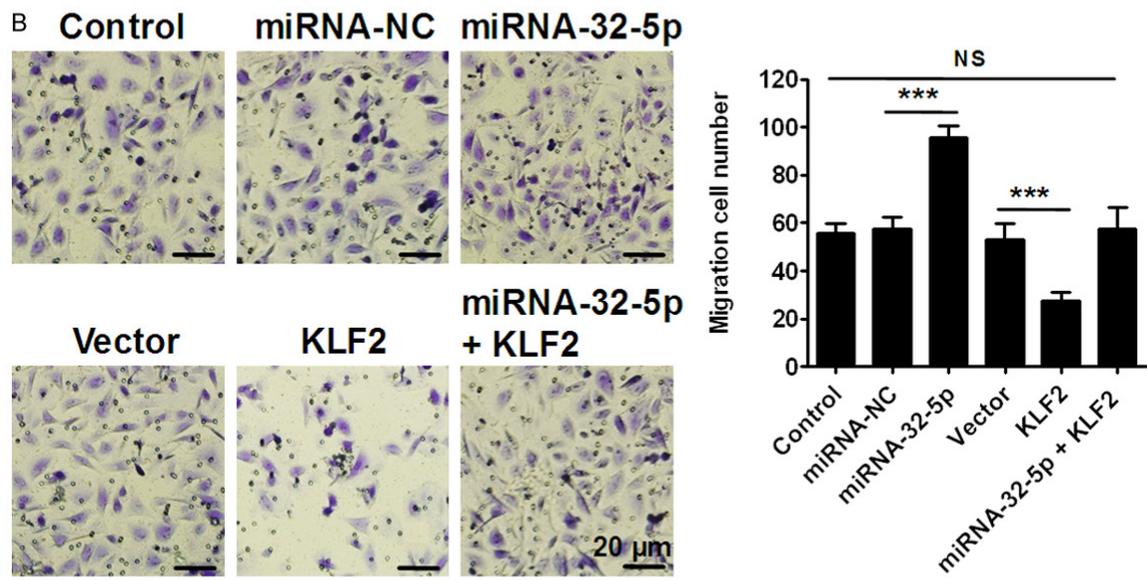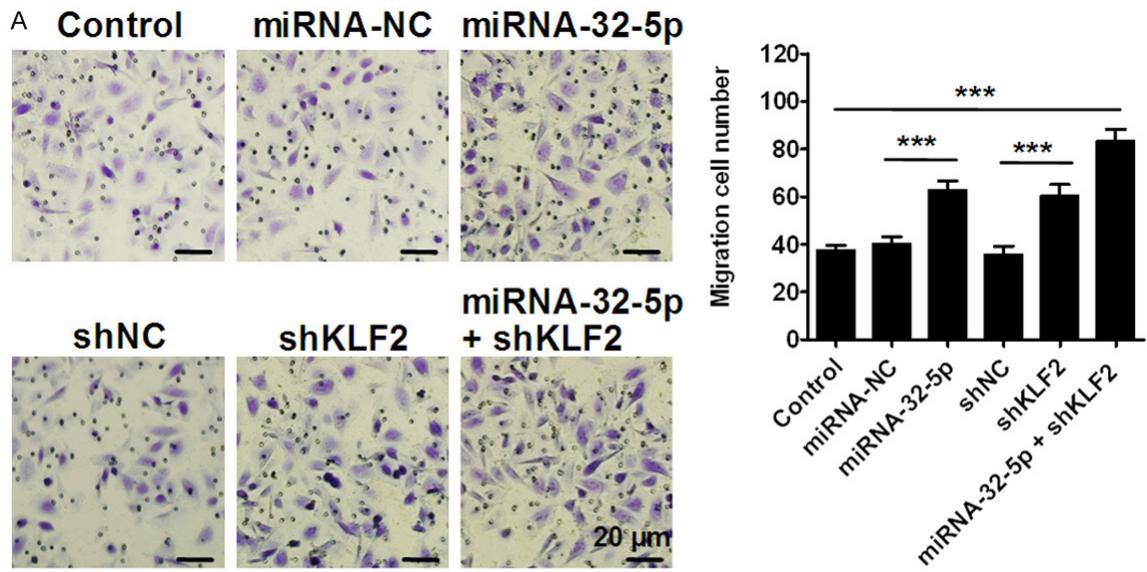
Figure 4. miR-32-5p and KLF2 knockdown synergistically promote gastric cancer cell migration. A. Transwell assay revealed that invasive cell number of KLF2 knockdown and miR-32-5p mimic group was higher than that in control group. B. Overexpression of KLF2 impaired the migration of gastric cancer, and miR-32-5p antagonized the inhibition effect. C. Western blot tested the level of E-cadherin, fibronectin and vimentin in gastric cancer cells transfected with miR-32-5p mimics and shKLF2. NS stands for non-significant (P > 0.05), ***P < 0.001.
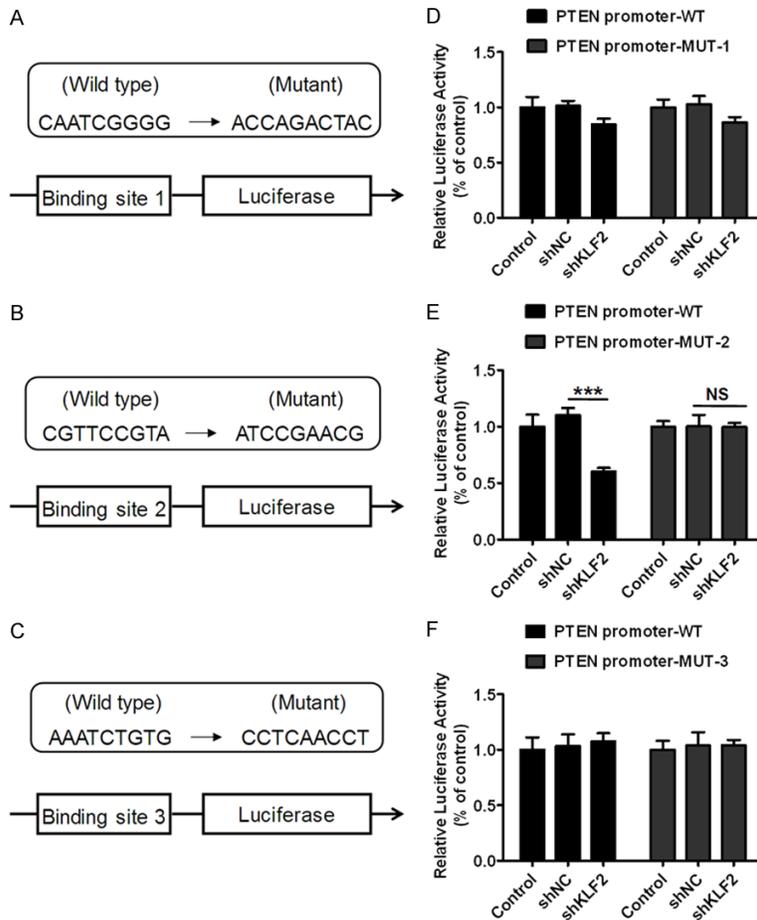
Figure 5. KLF2 induces PTEN expression by binding to its promoter. A-C. Three KLF2 binding sites in the PTEN promoter were predicted by bioinformatics software Targetscan and their mutant-type were constructed. D-F. Dual-Luciferase reporter showed that relative luciferase activity in KLF2 knockdown MGC803 cells transfected with wild-type PTEN promoter was lower than that of mutant PTEN promoter in binding site 2 group. However, there is no similar phenomenon in other binding site groups. NS stands for non-significant (P > 0.05), ***P < 0.001.

ed that shKLF2 suppressed the luciferase reporter intensity in gastric cancer cells compared with control and shNC (negative control) in PTEN promoter wild-type group respectively (**Figure 5E**, left panel). However, there was no significant change in mutation group (**Figure 5E**, right panel) and the other two binding site groups (**Figure 5D** and **5F**). These results suggest that KLF2 directly binds to the promoter of PTEN to induce its transcription.

miRNA-32-5p and shKLF2 activate PI3K/AKT signaling pathway

In the related literature, miRNA-32 inhibits PTEN expression in colorectal carcinoma cells at the protein level [43]. In order to further confirm the result in gastric cancer and explore the relationship between microRNAs and KLF2 in PI3K/AKT signaling pathway, we detected the effect of the signaling pathway in gastric cancer cells transfected with miRNA-32-5p mimic, shKLF2 and the co-transfection respectively (**Figure 6A**). The results indicated that both microRNA32-5p and sh-KLF2 inhibit the expression of PTEN and activate PI3K/AKT signaling pathway with synergistic effects, thus promoting the growth and metastasis of gastric cancer. The schematic diagram illustrated in **Figure 6B** summarized our current view of the study.

Discussion

KLF2 is a zinc finger structure-like transcription factor, which is closely related to the proliferation and differentiation of endothelial cells. Recent studies demonstrate that the overexpression of KLF2 suppresses tumor development by inhibiting cell proliferation, migration and promoting tumor cell apoptosis [19, 24-27]. Our previous study has revealed that the expression of KLF2 is decreased in gastric cancer compared with normal tissues and the overexpression of KLF2 significantly inhibits the proliferation and migration of gastric cancer cells [40]. In addition, previous research has demonstrated that KLF2 inhibits the PI3K/AKT

A

B
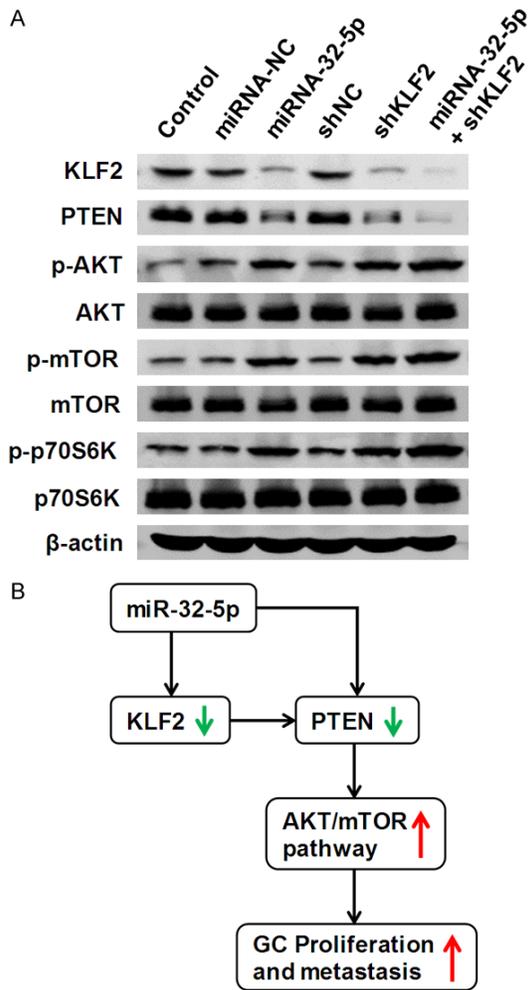
Figure 6. miRNA-32-5p and KLF2 knockdown synergistically activate PI3K/AKT signaling pathway. A. miRNA-32-5p mimic and KLF2 knockdown synergistically inhibited the expression of PTEN and increased the phosphorylation level of AKT, mTOR and p70S6K in MGC803 cells, thereby activating the PI3K/AKT signaling pathway. B. The schematic diagram of the mechanism of action in this study.

signaling pathway by inducing PTEN expression [40]. However, it is not clear whether KLF2 directly acts on PTEN and the specific mechanism of action needs to be further explored. In this research, we constructed KLF2 overexpression and knockdown stable transgenic strains, and confirmed its anticancer effects in colony formation and cell migration assays (**Figures 2-4**). In order to explore the specific mechanism, bioinformatics software Targetscan was used to predicted that whether KLF2 bound to the promoter of PTEN gene thus regulating its transcription. And we verified this hypothesis by a dual-luciferase reporter gene

system with binding site mutation (**Figure 5**). In addition, the expression of PTEN was further confirmed by western blot (**Figure 6A**). The results above indicated that KLF2 could directly regulate the PTEN promoter region and induce its expression, which in turn affected the proliferation and migration of gastric cancer cells (**Figure 6B**). These results provide the potential of KLF2 to act as a tumor suppressor in gastric tumorigenesis and a potential therapeutic target.

Since KLF2 plays important roles in the development of gastric cancer, we further explored what affected the expression of KLF2 in gastric cancer. There are many kinds of factors involved in gastric cancer development, among them miRNA is one of the most important regulators. miRNAs are a small class of endogenous, noncoding single-stranded RNAs that are processed from the stem-loop region of longer RNA transcripts [32, 33]. Many studies have found that miRNAs play important roles in the development of gastric cancer [34, 37, 44, 45]. In addition, several reports show that miRNAs regulate the expression of KLF2. For example, in acute hypoxia, miRNA-200b directly binds to KLF2 mRNA in endothelial cells and downregulates its expression to promote neovascularization [46]. Therefore, we envisaged that new miRNA targeted KLF2 to participate in the development of gastric cancer. Based on this, we used bioinformatics software Targetscan to predict and screen miRNAs that miRNA-32-5p may bind to KLF2 mRNA (**Figure 1**). Besides that, TCGA database show that miR-32-5p expression is higher in gastric tumor samples (n = 387) compared with normal samples (n = 41) (P = 0.002165). Moreover, TCGA dataset displays the negative correlation between miRNA-32-5p and KLF2 expression in gastric cancer (Data not shown). miRNA-32 is a widely studied cancer-promoting factor, which promotes cancer cell proliferation, migration and inhibition of apoptosis in many tumor studies, such as prostate cancer [47], colorectal cancer [43, 48], pancreatic cancer [49] and breast cancer [50, 51]. In addition, studies have found that miRNA-32 can reduce PTEN expression in tumor cells at both mRNA and protein levels by binding to the PTEN 3'-UTR region [43, 52, 53]. While the role of miRNA-32 in gastric cancer is controversial. Previous studies have identified that miRNA-32 promotes the development of gas-

tric cancer by targeting KLF4 [54, 55]. However another study indicates that miRNA-32 inhibits the proliferation and invasion of gastric cancer cells [56]. miRNA-32-5p is one of the effective fragments of miRNA-32. However, there is no research on miRNA-32-5p in gastric cancer. Therefore, in this study we focused on the effect of miRNA-32-5p and revealed that miRNA-32-5p significantly promote gastric cancer cell proliferation, migration and EMT. Study has shown that miR-32-5p also inhibits PTEN expression at mRNA and protein levels [57]. The data in our research showed that transfection of miR-32-5p mimics resulted in the downregulation of KLF2 transcription and decreased PTEN protein expression. Therefore, we conclude that miR-32-5p and KLF2 have antagonistic effects on the regulation of PTEN. Gene therapy generally refers to the transfer of specific genetic material into the patient's pathogenic target cells, which inhibits abnormal gene expression, corrects the expression of defective genes, and achieves the purpose of treating tumors with high specificity and efficiency. The intensive investigation of miRNA-32-5p also provides a new direction for gastric cancer genes therapy.

Taken together, the results of various cell experiments in this study indicate that KLF2 directly regulates the PTEN promoter and affects PTEN expression at the transcriptional level, and we have firstly identified the binding site of KLF2 on PTEN promoter. In addition, for the first time, we have discovered that miR-32-5p participates in the development of gastric cancer by targeting KLF2 and PTEN. And miR-32-5p and shKLF2 synergistically activate PI3K/AKT signaling pathway. The result enriches our understanding about the molecular mechanisms of gastric carcinogenesis and metastasis, and these basic works will be of great significance for the clinical diagnosis and treatment of gastric cancer. In gastric cancer, studies have also shown that besides miRNA-32-5p, other miRNAs are also overexpressed. This phenomenon suggests that miRNA inhibitors may combine with targeted drugs to cure gastric cancer.

Acknowledgements

Disclosure of conflict of interest

None.

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