MiR-128b is down-regulated in gastric cancer and negatively regulates tumour cell viability by targeting PDK1/Akt/NF-κB axis

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Gastric cancer (GC) is the fourth most prevalent type of cancer worldwide, which is usually caused by the interaction between environmental and genetic factors, or epigenetic aspects. Referring to the non-coding RNAs, miR-128b has been reported to be associated with many tumour cases, and exerts distinct functions in different types of cancers. However, the function of miR-128b in GC onset and progression largely remains unknown. In the present study, we found that miR-128b expression was down-regulated in tissues from 18 GC patients and 3 carcinoma cell lines. In turn, over-expression of miR-128b suppressed GC cell proliferation, invasion and promoted apoptosis. Moreover, miR-128b was predicted to bind the 3′UTR of *PDK1* gene using bioinformatic target-screening tools. Accordingly, ectopic expression of miR-128b inhibited the PDK1 expression at both transcriptional and post-transcriptional levels, and furthermore, the expression of gene tailed by the 3′UTR of *PDK1* gene was significantly decreased in a dual-luciferase reporter assay, suggesting that *PDK1* was a direct target of miR-128b in GC cells. In the conditon of miR-128b over-expression, we also observed spontaneous inactivation of the Akt/NF-κB signalling, implying PDK1 was a potential regulator of this pathway. In conclusion, our study shed some novel light on miR-128b-PDK1/Akt/NF-κB axis on GC progression.

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1. Introduction

Gastric cancer (GC) is one of the most prevalent cancers in China, with small improvement in long-term survival in the past years (Siegel *et al.* 2014). Gastric cancer is always diagnosed at advanced stages and has high rate of recurrence and metastasis after treatment. Thus, better understanding of the genetic and epigenetic alterations during the development and progression of GC is needed.

Much evidence has showed that there is a strong link between tumour progression and microRNA expression. MiRNAs are a class of endogenous, small, short single-stranded non-coding RNAs of 21–25 nucleotides in length that directly bind to the 3′-untranslated regions (3′UTRs) of target messenger RNAs (mRNAs), leading to mRNA degradation or translation suppression, and playing an important role in several cancers (Wu *et al.* 2010; Yates *et al.* 2013; Tong *et al.* 2014). Regarding GC, a group of miRNAs, such as miR-21 and miR-296-5p, were able to promote cell proliferation and invasion (Zhang *et al.* 2012; Li *et al.* 2014). However, other miRNAs, including miR-200 family, miR-124 and miR-375, showed inhibitory effect on cell proliferation and invasion in GC (Katada *et al.* 2009; Kurashige *et al.* 2012; Xia *et al.* 2012). Recent studies have indicated that the aberrant expression of miR-128b and miR-148a were associated with tumour classification and prognosis (Tchernitsa *et al.* 2010; Tsukamoto *et al.* 2010a, b; Zheng *et al.* 2011).

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MicroRNA-128 (miR-128) is brain-enriched, including miR-128a and miR-128b (Li *et al.* 2013). Aberrant expression of miR-128 is observed in many malignant tumours; however, the role of miR-128 in individual tumours is different. A study showed that miR-128 could suppress tumorigenesis of non-small-cell lung cancer (NSCLC) at least partly by targeting VEGF-C (Hu *et al.* 2014), while another study reported that miRNA-128 promoted cell proliferation in osteosarcoma cells by down-regulating PTEN (Shen *et al.* 2014). Katada detected the expression of miR-128 in the specimens of 42 undifferentiated gastric cancer tissues and the corresponding normal tissues and found that miR-128a was up-regulated and miR-128b was down-regulated (Tsukamoto *et al*. 2010a, b). In a study on GC, Tsukamoto found that miR-128b was up-regulated in the microarray data, although no detailed research was performed (Katada *et al.* 2009). However, the function of miR-128b in GC is not yet known. Online bioinformatic tools indicate that miR-128b has a conserved binding site in the PDK1 3′UTR, and therefore, we hypothesize that miR-128b may regulate cell progression by targeting PDK1 in GC.

2. Materials and methods

2.1 *Tissues and cells*

GC tissues were surgically resected from 18 patients at The First Affiliated Hospital of Nanchang University. Among the patients, 10 were males and 8 were females, with an average age of 59 years. All the patients were confirmed through pathological diagnosis by at least two specialists. Meanwhile, biopsy specimens from 10 volunteers with superficial gastritis were obtained as controls. Tissues were stored at −80°C. The use of the tissue samples for the experiment was approved by the patients and the ethics committee of The First Affiliated Hospital of Nanchang University. Human gastric adenocarcinoma cell line SGC-7901, normal gastric mucosal epithelial cell line GES1, gastric adenocarcinoma cell line BGC-823 and gastric carcinoma cell line HGC-27 were stored in our laboratory.

2.2 *Quantitative real-time PCR*

Total RNA was isolated from human GC specimens or cells using Trizol Reagent (Invitrogen, USA), and was transcribed to complementary DNA (cDNA) using a EasyScript First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotech, China) according to the manufacturer's instructions. Quantative real-time PCR (qPCR) was carried out in a ABI Step One Plus system (Applied Biosystems, USA) by using SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen, USA). Real-time PCR was run in triplicate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 65°C for 30 s. The primers used were as follows: miR-128b mimics: 5′-UCACAGUGAACCGGUCUCUUU-3′ (sense), 5′-AGAGACCGGUUCACGGUGAUU-3′ (anti-sense); scrambled miRNA (NC): 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense), 5′-ACGUGACACGUUCGGAGAATT-3′ (anti-sense). PDK1:5′-CTCAGGACACCATCCGTTCA-3′ (sense), 5′-ATCTTGCAGGCCATACAGCA-3′ (anti-sense). All the primers were synthesized by Genepharm Company (Shanghai, China). U6 or GAPDH was used as an reference for the expression of miR128b or PDK1, respectively. Relative expressions were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.3 *Cell culture and miRNA transfection*

The human gastric cell lines SGC-7901, BGC-823, HGC-27 and normal gastric mucosal epithelial cell line GES1 were cultured in PRIM 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, USA). The cells were maintained at 37°C in 5% CO_2, Human miR-128b mimics and the miRNA negative control mimics (NC) plasmids were purchased from Genepharm Company (Shanghai, China). According to the product specification, the plasmids were transfected into cells, reaching 60% convergence with Lipofectamine 2000 (Invitrogen, USA).

2.4 *Cell proliferation assay*

The methyl thiazolyl tetrazolium (MTT) (Sigma, St. Louis, MO, USA) colorimetric assay was used to detect cell proliferation ability. The cells were seeded in 96-well plates at a density of 1×10^3 cells/well and transfected with miR-128b mimics or NC control. The cells were trypsinized after 1–6 days of transfection, and suspended in phosphate buffer solution solution (PBS). Cell number was daily counted for 6 days by measuring the absorbance at 490 nm. The assay was performed in triplicate.

2.5 *Colony formation assay*

MGC-823 and HGC-27 cells (3×10^4/well) were seeded in a 24-well plate and transfected with miR-128b mimics or NC plasmid. After 24 h of transfection, the cells were collected and seeded (500/well) in a new 6 cm dish and cultured for 10 more days. Effective colonies (>50 cells per colony) were counted after fixed with methanol/acetone (1:1) and stained with 5% Gentian Violet (ICM Pharma, Singapore, Singapore). The experiment was repeated three times.

2.6 *Cell invasion assay*

Cell invasion ability was detected by matrigel invasion assay (BD Biosciences). Briefly, the MGC-823 and HGC-27 cells were transfected with miR-128b mimcs or NC plasmid and harvested 48 h post-transfection. Then, the cells were suspended in 500 μL serum-free medium (1×10^4 cells) and seeded in the upper chamber. The lower chamber contained 800 μL PRIM1640 medium with 25% FBS. After 48 h incubation, cells that had invaded through the matrigel membrane were stained with crystal violet and then counted (five high power fields, ×100 magnification). Three independent experiments were conducted.

2.7 *Cell cycle analysis and apoptosis assay*

1×10^6 cells were harvested and washed in ice-cold PBS, and then fixed in 75% ice-cold ethanol. Before staining, cells were collected and resuspended in cold PBS. Bovine pancreatic RNase (Sigma-Aldrich) was added to a final concentration of 2 μg/mL and incubated with cells at 37°C for 30 min, followed by incubation with 20 μg/mL propidium iodide (PI, Sigma-Aldrich) for 30 min at room temperature. The cell cycle profiles were analysed using FACS Calibur flow cytometer (BD, Bedford, MA).

Apoptosis was determined by dual staining with Annexin V-FITC and Propidium iodide (PI) (BD Biosciences, Erembodegem, Belgium). Cells were collected 48 h later after being transfected with miR-128 mimics or NC plasmid, and then stained with Annexin V-FITC and PI according to the manufacturer's instruction. The combination of Annexin V-FITC and PI staining distinguished early apoptotic cells (Annexin V-FITC+, PI−) and late apoptotic cells (Annexin V-FITC+, PI+). Three independent experiments were conducted.

2.8 *Western blotting*

Total protein was extracted by lysis buffer (50 mM Tris (pH 7.4),150 mM NaCl,1% Triton X-100,1% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktail. Primary antibodies of PDK1 (1:1000 dilute), Akt (1:1000 dilute), p-Akt (Thr308, Ser473) (1:200 dilute), NF-κB (1:1000 dilute) and Bcl-2 (1:500 dilute) from cell signalling technology (New England Biolabs) were used to recognize the target proteins. Subsequently, a secondary antibody conjuncted with horseradish peroxidase (Santa Cruz, USA) (1:5000 dilute), coupled with enhanced chemiluminescent substrate (Life technologies, USA), was employed to vasualize the protein expression. Meanwhile, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as the endogenous reference.

2.9 *Dual-luciferase reporter assay*

According to the binding of miR-128b on 3′UTR of *PDK1* gene, one wild-type (wt) luciferase reporter plasmid and a mutant plasmid (mut:UCA——GUGAACCGGUCUCUUU) were cloned and inserted into pGL3 vector (Life technologies, USA). 293T cells were seeded into 24-well plates at a density of 2×10^5 per well. PGL3-PDK1-3′UTR (wt/mut) was co-transfected into 293T cells with 100 nM miR-128b mimics or NC plasmid using Lipofectamine 2000. Renilla and firefly luciferase activities were measured by Dual-Luciferase Reporter Assay (Promega) 48 h after transfection.

2.10 *Statistical analysis*

All the data was analysed using the SPSS 18.0 software and presented as mean ± standard deviation (SD). Student's *t*-test was used for comparisons. $P<0.05$ was considered statistically significant, and '*'indicates $P<0.05$ and '**' indicates $P<0.01$.

3. Results

3.1 *miR-128b was down-regulated in gastric tissues and cells*

To investigate the association between miR-128b and tumour progression, the expression of miR-128b in both human GC tissue and cells was quantified by using qPCR. In the surgically resected carcinoma tissues from 18 GC patients, we observed a significant reduce of miR-128 expression than that in control tissues (figure 1A). Further, the expression of miR-128b in cultured GC cell lines SGC-7901, BGC-823 and HGC-27 was markedly decreased than that in normal gastric mucosal epithelial cell line GES-1 (figure 1B). Especially, BGC-823 and HGC27 exhibited the most decreased RNA abundance with 2.5- or 10-fold reduction. Therefore, these two cell lines were chosen for further functional analysis. The down-regulated expression of miR-128b in GC tissues and cells suggested that it might negatively modulate the carcinoma progression.

3.2 *miR-128b inhibited proliferation of GC cells*

To assess the potential function of miR-128b as a tumour suppressor in GC cells, we up-regulated the expression of miR-128b in two GC cell lines (BGC-823, HGC-27) by transfecting miR-128b mimics. MTT assay showed that over-expression of miR-128b decreased cell proliferation compared with NC group (figure 2A–C). In colony

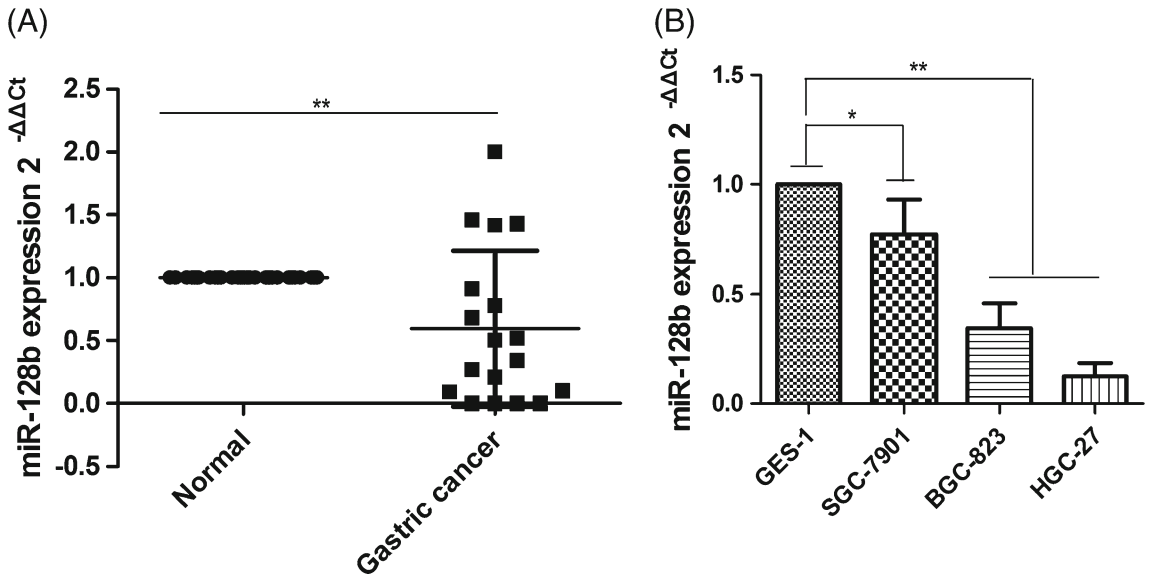


Figure 1. miR-128b expression in tissues from GC patients (**A**) and cell lines (**B**). GES1, human normal gastric mucosal epithelial cell line; SGC-7901, BGC-823 and HGC-27, human gastric adenocarcinoma cell lines. Data is displayed as mean ± standard deviation.

formation assay, fewer colonies formed in condition of miR-128b treatment (figure 2D).

3.3 *miR-128b suppressed cell cycle, invasion and induced apoptosis in GC cells*

To further explore how miR-128b inhibited GC cell viability, cell cycle analysis was performed. When BGC-823 and HGC-27 cells were challenged with miR-128b mimics, the proportion of cells in G0/G1 phase increased by about 10% ($P<0.05$) (figure 3A). In addition, cell number detected in the lower chamber reduced by 58–64% (figure 3B), and apoptosis rate increased by 6.5- to 8.8-fold (figure 3C) compared with the NC group. These results indicate that miR-128b inhibited cell viability by arresting them in G0/G1 phase, suppressing invasion and accelerating apoptosis.

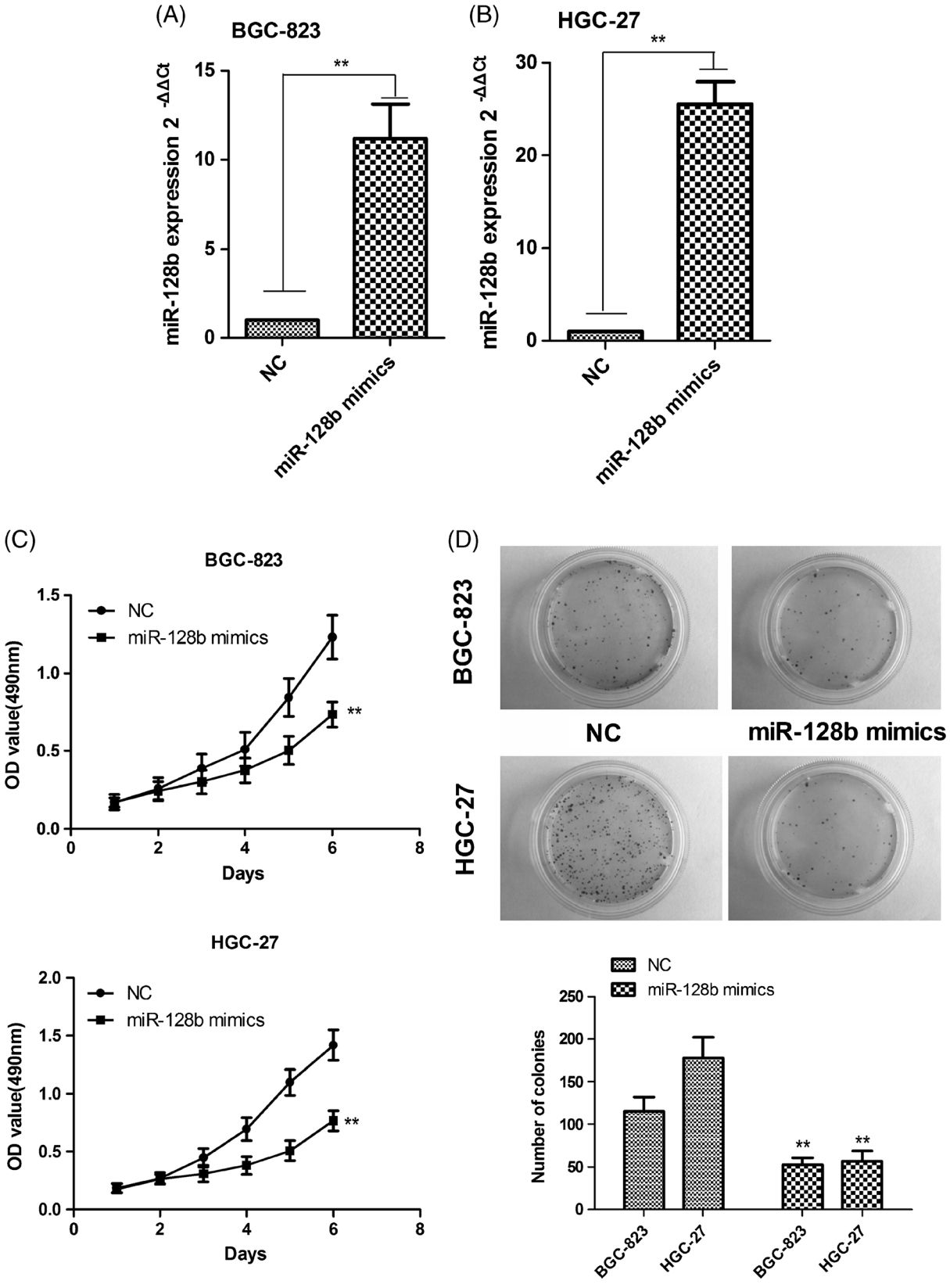
3.4 *PDK1 is a direct target of miR-128b*

MiRNAs usually exert functions by binding their target genes. To identify effectors of miR-128b, we used TargetScan and miRanda to predict the potential targets. Results from the two online tools based on different algorithms showed that miR-128b perfectly matched with the 3′UTR of *PDK1* gene and suggested that *PDK1* was likely to be a direct target of miR-128b (figure 4A). Therefore, the 3′UTR of *PDK1* gene was cloned and inserted into a luciferase reporter construct. Overexpression of miR-128b reduced luciferase activity when the reporter construct contained the wild-type *PDK1* 3′UTR (figure 4B). However, mutation of miR-128b potential binding site abolished the reduction of luciferase expression (fgure 4B). Moreover, transfection of miR-128b mimics in GC cells resulted in a reduction of *PDK1* mRNA and protein expression (figure 4C–D). These evidences demonstrated that *PDK1* was a target of miR-128b in GC cells.

3.5 *miR-128b affected the AKT-NF-κB axis partly via targeting PDK1*

In tumour cells, Akt/NF-κB axis was reported to induce cell cycle arrest and apoptosis (Belkhiri *et al.* 2008). Here we asked whether miR-128b suppressed GC cell growth and promoted apoptosis by modulating AKT-NF-κB signalling. Therefore, we detected the protein expression of the key memebers of this axis, including Akt, p-Akt, NF-κB and Bcl-2. Data showed that up-regulation of miR-128b led to significant decrease of the expression of p-Akt, NF-κB and

Figure 2. Cell proliferation in condition of miR-128b over-expression. (**A**) Over-expression of miR-128b in BGC-823 cell line. (**B**) Over-expression of miR-128b inHGC-27 cell line. (**C**) MTT assay in BGC-823 and HGC-27 cell lines. (**D**) Cell colony formation in cell plates and the corresponding colony count. ▶


(A)
BGC-823
miR-128b expression 2 -ΔΔCt
15
10
5
0
**
NC
miR-128b mimics
(B)
HGC-27
miR-128b expression 2 -ΔΔCt
30
20
10
0
**
NC
miR-128b mimics
(C)
BGC-823
OD value(490nm)
1.5
1.0
0.5
0.0
NC
miR-128b mimics
**
0
2
4
6
8
Days
HGC-27
OD value(490nm)
2.0
1.5
1.0
0.5
0.0
NC
miR-128b mimics
**
0
2
4
6
8
Days
(D)
BGC-823
HGC-27
NC
miR-128b mimics
NC
miR-128b mimics
Number of colonies
250
200
150
100
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BGC-823
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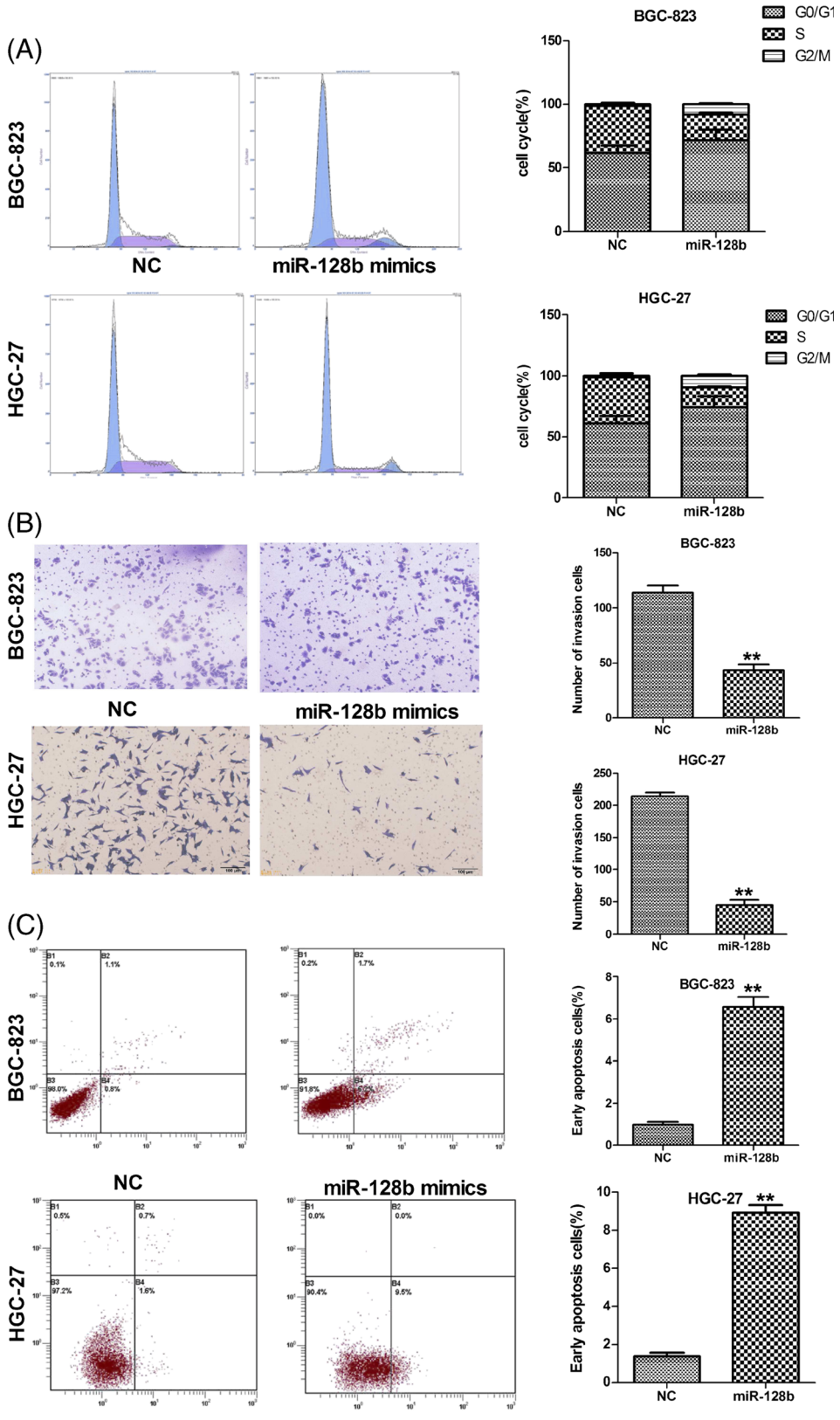

Figure 3. Viability of BGC-823 and HGC-27 cells depicted by cell cycle (**A**), cell invasion (**B**) and cell apoptosis (**C**).

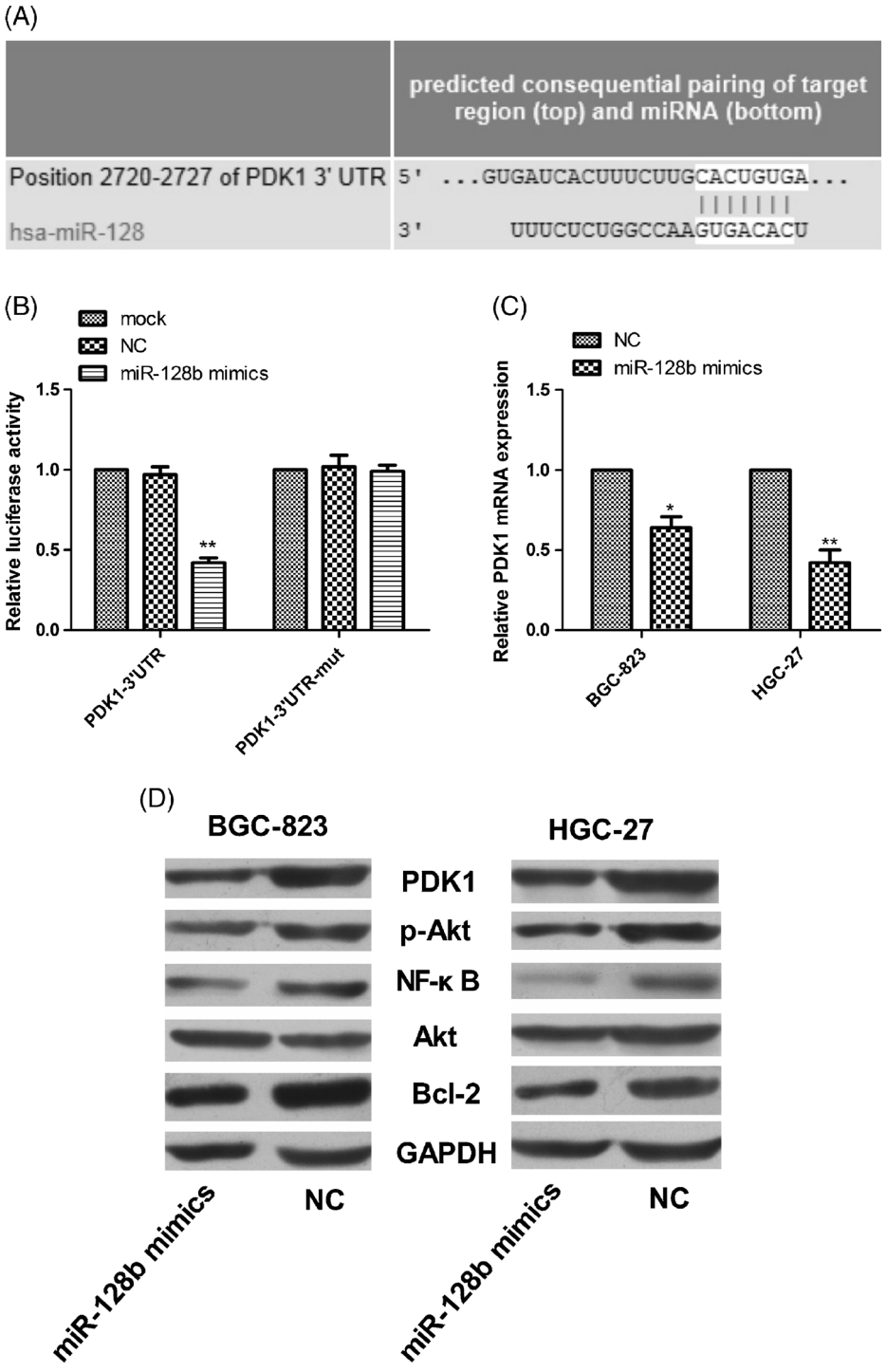

Figure 4. miR-128b targeting PDK1 and resulting in the inactivation of Akt/NF-κB axis. (**A**) miR-128b binding the 3′UTR of PDK1 predicted by the bioinformatic tool. The pairing region between miR-128b and PDK1 3′UTR is in the white box. (**B**) Luciferase activity of reporter gene tailed by wild-type or mutant PDK1 3′UTR. (**C**) mRNA expression of PDK1 response to miR-128b challenge. (**D**) Protein expression of the key components of the PDK1/Akt/NF-κB axis.

Bcl-2, but not the total Akt protein (figure 4D), representing the inactivation of this axis. As PDK1 can interact with and phosphorylate Akt1 (Bayascas 2008), it was rational to hypothesize that the decreased p-Akt expression was caused by the miR-128b/PDK1 cascade. In all, we concluded that miR-128b inactivated Akt/NF-κB axis through targeting PDK1 to inhibit GC cell viability and induce apoptosis.

4. Discussion

Previous studies have shown that miR-128b was down-regulated in undifferentiated GC tissues. However, the role of miR-128b in GC cells remains uninvestigated. In this study, we demonstrated that miR-128b expression was down-regulated in GC tissues and cell lines. Over-expression of miR-128b suppressed cell proliferation and invasion, and induced cell apoptosis through Akt-NF-κB axis by targeting PDK1. These findings suggested that miR-128b had an important role on inhibiting the development and progression of GC.

MiR-128 is a kind of intronic miRNAs and encoded by two distinct genes, *miR-128-1* and *miR-128-2*, which are located on human chromosome 2q21.3 and 3p22.3 respectively (Li *et al.* 2013). Volinia found that the expression of miR-128b was significantly up-regulated in tumour tissues of colon, lung and pancreas comparing with normal tissues (Volinia *et al.* 2006). In contrast, Katada showed that miR-128b was down-regulated in undifferentiated GC tissues (Li *et al.* 2013). These results revealed miR-128b might have different roles depending on the cancer type. Numerous researches have demonstrated that miR-128 regulated cell proliferation, differentiation and apoptosis in various cancers (Qian *et al.* 2012; Woo *et al.* 2012; Palumbo *et al.* 2013). For instance, miR-128 inhibited glioma cells proliferation by targeting transcription factor E2F3a (Zhang *et al.* 2009). Another study showed that up-regulation of miR-128 inhibited Reelin and DCX expression and reduced neuroblastoma cell motility and invasiveness (Evangelisti *et al.* 2009). In addition, reduction in miR-128 contributed to chemotherapeutic resistance by resisting of chemotherapy-induced apoptosis. Ectopic expression of miR-128 sensitizes breast tumour-initiating cells (BT-Ics) to the proapoptotic and DNA-damaging effects of doxorubicin via Bmi-1 and ABCC5 (Zhu *et al.* 2011). Consistent with these studies, here we showed that miR-128b inhibited cell proliferation and induced apoptosis in GC cells.

To investigate the mechanisms of miR-128b inhibiting cell growth and repressing cell invasion, an online bioinformatic tools was used, which indicated that miR-128b had a conserved binding site in the PDK1 3′UTR. In a previous study, researchers also demonstrated that miR-375 negatively regulated the Akt signalling by targeting PDK1 in pancreatic carcinoma tissues and cell lines (Zhou *et al.* 2014). Much evidence has revealed that PDK1 is an oncogenic gene in a variety of cancers, including GC (Tsukamoto *et al.* 2010a, b; Raimondi and Falasca 2011). PDK1 has been identified for its ability to phosphorylate and activate Akt (Dieterle *et al.* 2014), and aberrant activation of AKT is one of the most common change in GC (Almhanna *et al.* 2011). The activation of Akt plays a pivotal role in cell proliferation, invasion and apoptosis via regulating NF-κB, P21, Foxo1 and BCL-2 (Belkhiri *et al.* 2008; Yu *et al.* 2010; Xia *et al.* 2012). Studies have shown that the activation of the PDK1/Akt pathway plays an important role in cell proliferation and tumorigenesis (Bayascas 2008); therefore, the PDK1/Akt pathway is believed to be a critical therapy target for cancer intervention. In the present study, ectopic expression of miR-128b inhibited the expression of PDK1 at both transcriptional and post-transcriptional level. Furthermore, dual-luciferase reporter assay showed that PDK1 was a direct target of miR-128b. Based on the results that cell proliferation, invasion and apoptosis were regulated by miR-128b in GC, we investigated if the Akt/NF-κB axis participated in this process. Our results showed that up-regulation of miR-128b significantly decreased p-Akt and NF-κB, but did not influence the total Akt protein. This results showed that miR-128b promoted Akt/NF-κB axis activation partly through targeting PDK1.

In conclusion, we found that miR-128b expression was down-regulated in GC tissues and cell lines. More importantly, miR-128b over-expression in GC cells could inhibit cell proliferation, invasion and induce cell apoptosis. Our study further demonstrated that miR-128b over-expression inhibited GC cell progression by targeting PDK1 and inactivating Akt/NF-κB pathway. The miR-128b-PDK1/Akt/NF-κB axis may provide novel insights into the mechanisms of GC progression.

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