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MiR-877-5p targets PDK-1 to promote aspirin-induced apoptosis in gastric mucosal cells

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This study aimed to investigate the role of miR-877-5p in aspirin-induced gastric mucosal injury. MiRNA microarray analysis was performed using paired gastric mucosal samples to find differentially expressed miRNAs. miR-877-5p was selected for subsequent analyses. Used as a model system, gastric epithelial cells (GES-1) were transfected with miR-877-5p mimic/inhibitor, then treated with aspirin. The expression of miR-877-5p in GES-1 cells was examined using quantitative real-time PCR (qRT-PCR). Flow cytometry analysis was used to detect cell apoptosis. Western blot assay was used to measure the protein levels of PDK1. The interaction between miR-877-5p and PDK1 was determined by luciferase reporter assay. The expression of miR-877-5p in gastric mucosal injury samples was higher than that in normal samples. Also, depletion of miR-877-5p reduced the apoptosis of GES-1 cells. Luciferase reporting assay confirmed that PDK1 was a target gene of miR-877-5p. PDK1 inhibited the apoptosis of GES-1 cells treated by aspirin. Moreover, this inhibitory effect was abrogated after PDK1 knockdown. Downregulation of miR-877-5p reduced the apoptosis by targeting PDK1 in GES-1 cells treated by aspirin, indicating that miR-877-5p may be a potential therapeutic target for gastric mucosal injury caused by aspirin.

1. Introduction

Aspirin is a common antiplatelet drug used to prevent and treat cardiovascular disease. However, the long-term use of aspirin can increase the risk of gastrointestinal symptoms, including ulcers and bleeding (ATT Collaboration et al. 2009). It is important to fully clarify the mechanism underlying aspirin-induced gastric bleeding and ulceration.

As an inhibitor of cyclooxygenase (COX), aspirin blocks the synthesis of prostaglandins to impair gastric mucosal defense (Fanaroff et al. 2016; Wallace 2008). Prostaglandin-independent mechanisms are involved in the attenuation of gastric mucosal surface hydrophobicity in a COX-1 knockout mouse model (Darling et al. 2004). Furthermore, aspirin can impair tight junctions (TJs) to increase gastric permeability (Oshima et al. 2008). Occludin and claudins, two main components of TJs, play an important role in the maintenance of paracellular permeability (Tsukita et al. 2000). The occludin expression is significantly decreased in gastric epithelial cells (GES-1) treated with aspirin, markedly reducing TJ integrity (Liu et al. 2018). Our team (Zhang et al. 2011) has found that aspirin can inhibit the growth of GES-1 and downregulate occludin expression, but the mechanism is still unclear.

MicroRNAs (miRNAs) are small (18-22 nt) endogenous, noncoding RNA molecules that suppress gene expression post-transcriptionally through base-pairing to complementary sequences in mRNAs (Bartel 2009; Lee et al. 1993). Previous studies have shown that miRNAs regulate various cellular processes, including cell differentiation, proliferation, and apoptosis (Brennecke et al. 2005; Lewis et al. 2005). miR-101 significantly promotes the apoptosis of gastric mucosal epithelial cells (Dong et al. 2019). Furthermore, *H. pylori* infection may disable the gastric epithelial barrier by increasing miR-100 levels (Hu et al. 2018). However, there is no report on the relationship between miRNAs and aspirin induced gastric mucosal injury.

To verify this relationship, the miRNA expression profile in aspirin-injured gastric mucosal samples was assessed using microarray analysis. Based on analytical validation and literature review, miR-877-5p was found differentially expressed using Real-time PCR. A dual-luciferase reporter assay was used to investigate the performance of PDK1, a potential target of miR-877-5p. Our findings may prop up a clearer molecular mechanism of miR-877-5p in aspirin induced gastric mucosal injury.

2. Investigations and results

2.1. miR-877-5p was upregulated in aspirin-injured gastric mucosal samples

Firstly, based on miRNA microarray analysis, the samples from ten patients with gastric mucosal injury caused by aspirin and paired healthy volunteers were compared to identify differential expressed miRNAs (≥ 2.0-fold changes). In total, 32 miRNAs were downregulated and 30 were upregulated (Fig. 1A). Subsequently, the five miRNAs with the greatest differences (>4.0-fold values), including miR-877-5p, miR-4440, miR-1469, miR-6782-5p and miR-122-5p, were selected to verify the results of the microarrays. Significant differences in miR-877-5p expression was noticed ($P<0.001$) (Fig. 1B). Ultimately, miR-877-5p was selected for further research.

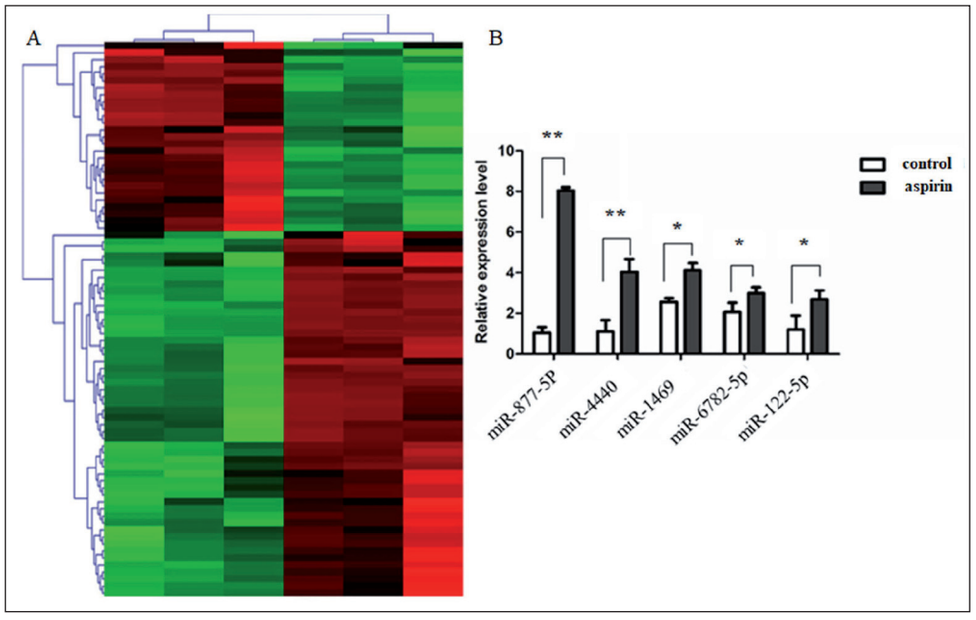

Fig. 1: miRNAs differentially expressed between aspirin-injured gastric mucosal samples and normal samples. (A) Microarray analysis of differentially expressed miRNAs from 10 patients with gastric mucosal injury caused by aspirin. Each row represents a miRNA and each column represents a sample pair. (B) Bar graphs present RT-qPCR results of miR-877-5p, miR-4440, miR-1469, miR-6782-5p and miR-122-5p in aspirin-injured gastric mucosal samples and normal samples. Each RT-qPCR was run in triplicate. The data are presented as relative expression following normalization. **, P <0.001; *, P <0.05 vs. control.

2.2. Lowly expressed miR-877-5p inhibited the apoptosis of GES-1 cells in vitro

To evaluate the role of miR-877-5p in gastric mucosal injury, GES-1 cells were transfected with in-miR-877-5p to downregulate the expression of miR-877-5p. The results of qRT-PCR revealed lower miR-877-5p expression than that in in-miR-NC group (Fig. 2A). Then, flow cytometry showed that the apoptosis of podocytes was evidently increased after aspirin treatment, but then abolished after transfection with in-miR-877-5p (Fig. 2B).

2.3. miR-877-5p directly targeted PDK1

We used TargetScan and miRanda to perform target prediction analysis, and found that PDK1 was a potential target of miR-877-5p (Fig. 3A). A dual luciferase reporter assay, in which the wild-type PDK1- 3'UTR was expressed with luciferase, revealed that miR-877-5p could significantly decrease the expression of luciferase (P<0.05) (Fig. 3B). However, no decrease was seen when luciferase was expressed with a PDK1-3'UTR mutant. This indicated a direct interaction between miR-877-5p and PDK1 mRNA.

2.4. PDK1 inhibited the apoptosis of GES-1 cells treated with aspirin

After GES-1 cells were cultured with aspirin for 24 h, then transfected with pcDNA, PDK1, si-NC or si-PDK1, respectively. Overexpression of PDK1 led to an apparent increase of PDK1 protein expression, and suppression of PDK1 led to an evident decrease of PDK1 protein expression in GES-1 cells (Fig. 4A). Then flow cytometry analysis exhibited that the apoptosis was remarkably reduced in the PDK1 group compared with the pcDNA group, and markedly enhanced in the si-PDK1 group over the si-NC group (Fig. 4B).

2.5. PDK1 knockdown abrogated the inhibitory effect of miR-877-5p inhibitor on the apoptosis of GES-1 cells

GES-1 cells were cultured with aspirin for 24 h, then transfected with in-miR-NC, in-miR-877-5p, in-miR-877-5p+si-NC or in-miR-877-5p+si-PDK1. The results revealed that inhibition of miR-877-5p resulted in an evident increase of PDK1 protein expression, while this effect was abolished after suppression of PDK1 (Fig. 5A). Additionally, knockdown of miR-877-5p conspicuously decreased the apoptosis of GES-1, whereas this effect was reverted by down-regulating PDK1 (Fig. 5B).

3. Discussion

Studies indicate that 2-4% of NSAID treated subjects suffer from serious gastric mucosal erosions (Goldstein et al. 1997) and long-term NSAIDs therapy may induce peptic ulcers (Naesdal et al. 2006). Reducing the complications of aspirin, while maximizing

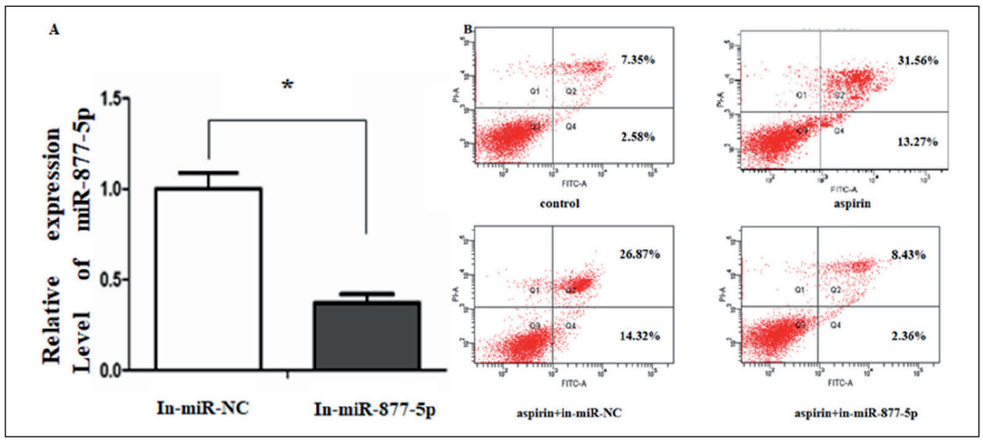

Fig. 2: Depletion of miR-877-5p alleviated aspirin-induced apoptosis in GES-1 GES-1 cells were transfected with in-miR-NC, in-miR-877-5p. (A) The expression level of miR-877-5p was detected by qRT-PCR analysis. (B) Cell apoptotic rate was evaluated by flow cytometry.

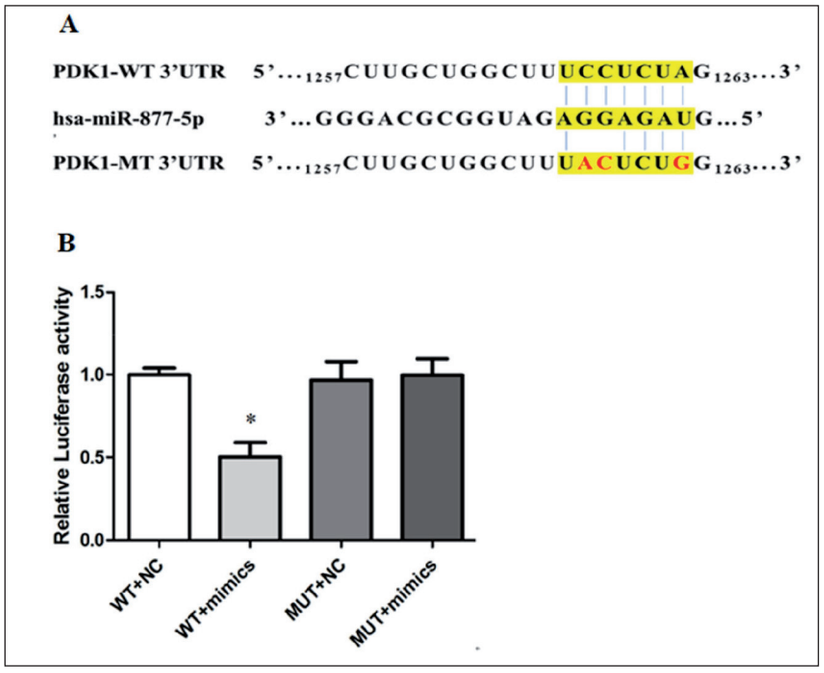

Fig.3: miR-877-5p directly targeted PDK1. A. Bioinformatics-based target prediction analysis showed that PDK1 was a potential target of miR-877-5p. B. A dual luciferase reporter assay revealed a direct interaction between miR-877-5p and PDK1. *$P < 0.05$ vs. control.

its benefits, is a major concern of clinical physicians (Mora et al. 2016; Bibbins-Domingo 2016). Our study (Zhang et al. 2011) has found that aspirin can inhibit the growth of GES-1 and downregulate occludin expression, but the underlying mechanism has not been completely understood.

MicroRNAs (miRNAs) are small conserved RNAs directly controlling temporal and spatial post-transcription (Friedman et al. 2019). In this study, miR-877-5p was selected as a miRNA of interest based on microarray and RT-qPCR results. MiR-877-5p expression was enhanced in gastric mucosal injury caused by aspirin, and silencing miR-877-5p inhibited aspirin-induced GES-1 cell apoptosis. MiR-877-5p can trigger apoptotic cell death or inhibit cell proliferation *via* different cellular signaling pathways, especially AKT (Mitsugi et al. 2016; Shi et al. 2016). A study has reported that miR-877-5p promotes the apoptosis of HepG2 cells by targeting FOXM1 (Huang et al. 2015). It was also demonstrated that miR-877-5p increases the levels of PEPCK in the onset of apoptosis in liver cells (Gershon et al. 2015). In addition, miR-877 inhibits renal cell carcinoma proliferation by modulating eEF2K/eEF2 signaling cascade (Shi et al. 2016).

The downstream targets of miR-877-5p that may also regulate the proliferation and apoptosis of GES-1 cells remain to be elucidated. Our data indicated that PDK1 gene may be targeted by miR-877-5p in aspirin induced cells injury. PDK1, composed by 556 amino acids (Gagliardi et al. 2018), serves as a constitutively active enzyme, due to its autophosphorylation on Ser-241 (Casamayor et al. 1999). The mechanism of phosphoinositide 3-kinase (PI3K)/PDK1/Akt pathway in cell survival and metabolism has been well elucidated (Franke et al. 1997).

In the present study, PDK1was overtly downregulated in GES-1 cells treated with aspirin. Additionally, PDK1 was negatively regulated by miR-877-5p. PDK1 restricted the apoptosis of GES-1 cells treated with aspirin. Meanwhile, rescue experiments exhibited that knockdown of PDK1 recuperated the effect of miR-877-5p on apoptosis.

In conclusion, miR-877-5p downregulation restrained aspirin-induced apoptosis of GES-1 cells *via* targeting PDK1, a molecular mechanism that may provide a new treatment option for gastric mucosal injury caused by aspirin. However, further in vivo experiments with mouse models are essential for confirming our conclusions.

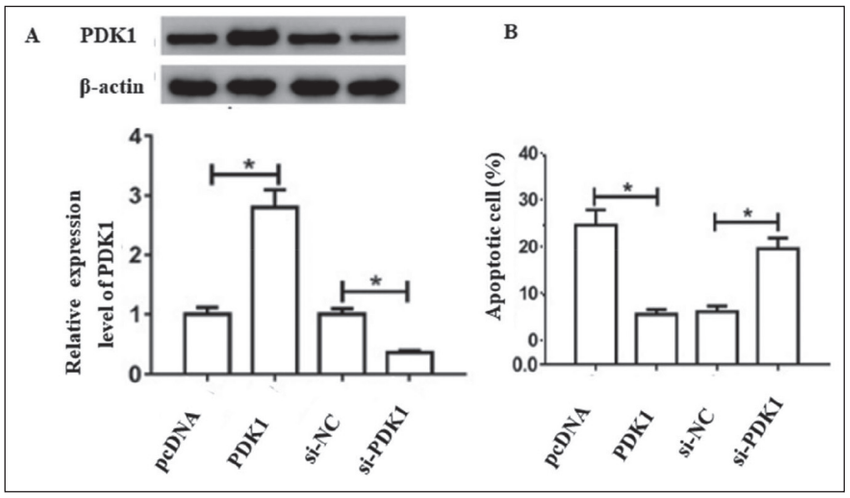

Fig. 4: PDK1 inhibited the apoptosis of GES-1 cells treated by aspirin. GES-1 cells were transfected with pcDNA, PDK1, si-NC or si-PDK1, respectively. (A) The protein level of PDK1 was examined using Western blot. (B) Cell apoptotic rate was estimated by flow cytometry. *, $P < 0.05$.

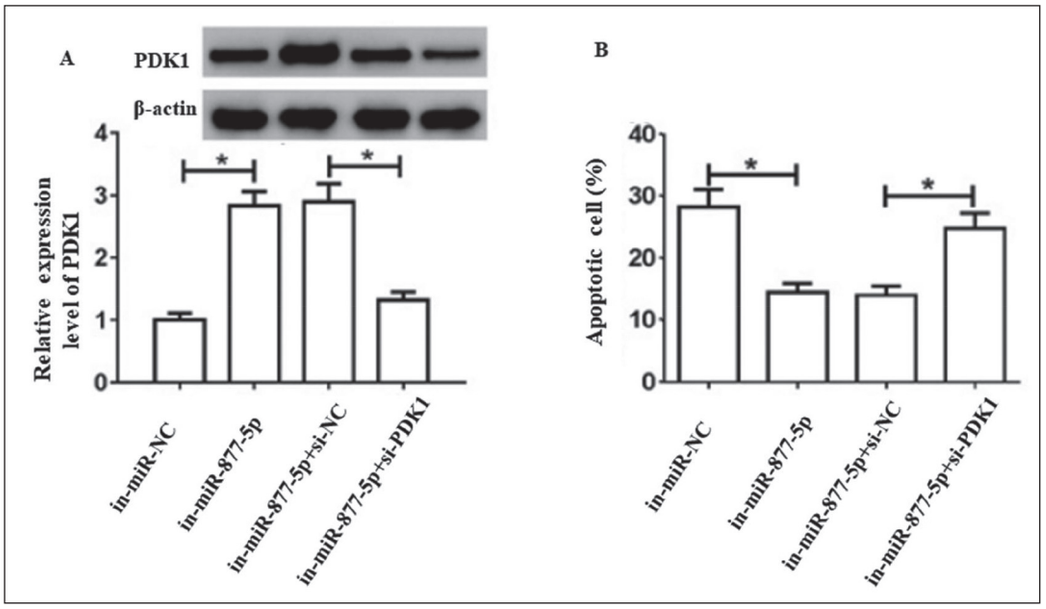

Fig. 5: PDK1 knockdown abrogated the inhibitory effects of miR-877-5p inhibitor on the apoptosis of GES-1 cells. (A) Western blot assay was used to detect the protein level of PDK1. (B) Flow cytometry was conducted to monitor cell apoptosis. *, P <0.05.

4. Experimental

4.1. Materials

Through endoscopic biopsy, gastric mucosal samples were obtained from ten patients taking aspirin and ten healthy volunteers in Nanjing First Hospital from 2018 to 2019, Exclusion criteria: serious disease history (chronic liver disease, kidney disease, blood system disease); malignant tumor disease; long-term use of antiplatelet drugs other than aspirin. Informed consent was obtained from all individuals who participated in this study. The study protocol was approved by the institutional review board of Nanjing Medical University, and all experiments were performed in accordance with related guidelines and regulations.

4.2. MiRNA microarray and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and QIAGEN miRNeasy mini kit (Qiagen GmbH, Hilden, Germany), quantified on the NanoDrop 2000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA), labeled using the FlashTag Biotin HSR RNA Labeling kit (Affymetrix, cat no. 901911; Thermo Fisher Scientific, Inc.) and hybridized onto the GeneChip2 Hybridization (Affymetrix, cat no. 902413; Thermo Fisher Scientific, Inc.). Having been washed, the slides were scanned using the GeneChip2 Scanner 3000 7G (Affymetrix; Thermo Fisher Scientific, Inc.). All data were normalized using the median normalization method. Differentially expressed miRNAs were identified through volcano plot filtering. Data were analyzed by GCBI online software (GCBI, R3.3.1, http://www. gcbi.com.cn; Genminix Informatics Co., Ltd., Shanghai, China). RT-qPCR analysis for miR-877-5p, miR-4440, miR-1469, miR-6782-5p and miR-122-5p was performed with a miDETECT A TRACK™ miRNA qRT-PCR Starter kit (RiboBio co., Ltd., Guangzhou, China). The miDETECT A TRACK™ Uni-RT primer was used for RT (Guangzhou RiboBio Co., Ltd., Guangzhou, China). The miDETECT A TRACK™ forward primers and uni-reverse primers were used for qPCR (Guangzhou RiboBio Co., Ltd.). The reactions were incubated in a 96-well plate at 95 °C for 10 min, followed by 40 cycles at 95 °C for 2 s, 60 °C for 20 s and 70 °C for 10 s. The miRNA primers used in RT-qPCR were synthesized by RiboBio, Co., Ltd., and the primer sequences were commercially restricted. The expression of miRNA was normalized to the small nuclear RNA U6 expression as an endogenous control. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

4.3. Cell culture

The normal human gastric epithelial cell line (or called GES-1) was obtained from the Division of Gastroenterology, Department of Medicine, People's Hospital of Jiangsu Province, China. GES-1 cells were cultured in DMEM (Gibco, America) supplemented with 10% FBS, 2.5 mg/ml amphotericin B, 50 U/ml penicillin, and 50 mg/ml streptomycin. The culture medium was changed every 48-72 h.

4.4. Cell transfection

When cell confluence reached ~70%, cell transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.). MiR-877-5p inhibitor (in- miR-877-5p), negative control inhibitor (in-miR-NC), miR-877-5p mimic (miR-877-5p), control mimic (miR-NC), PDK1 overexpression vector (PDK1), empty vector (pcDNA), small interfering RNA (siRNA) of PDK1 (si-PDK1) and control siRNA (si-NC) were synthesized by GenePharma (Shanghai, China).

4.5. qRT-PCR

Total RNA was extracted from GES-1 treated with aspirin with and without MiR-877-5p mimic or inhibitor using Trizol reagent (Invitrogen). The primer sequences used for amplification of genes were as follows: PDK1 F: 5'-AGGCAAAGGAAGTCCATCT-3', PDK1 R: 5'-CCCATGCATTGTGCTACC-3' GAPDH F: 5'-GUAUGACAACAGCCUCAAGTT-3' GAPDH R: 5'-CUUGAGGCUGUUGUCAUACTT-3' (Jima, Shanghai, China). qRT-PCR analysis was performed using a standard SYBR-Green PCR kit protocol on a Step One Plus system (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. The relative levels of miR-877-5p and PDK1 transcript were calculated and normalized to GAPDH, with at least three repeats per experimental group.

4.6. Flow cytometry analysis

Cell apoptotic rate was estimated using Annexin V, FITC Apoptosis Detection Kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Briefly, podocytes were seeded into six-well plates and cleansed with phosphate-buffered saline (PBS). Next, the cells were stained with annexin V-FITC and propidium iodide (PI) for 30 min. Data were analyzed by CellQuest v.5.1 software (BD Biosciences). The experiments were performed in triplicate.

4.7. Western Blotting

Total protein was extracted, boiled, and measured with a BCA kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). After separation with SDS-PAGE gels, protein was transferred onto the nitrocellulose membranes. The membranes were incubated with primary antibodies (PDK1 and β-actin at 1:1000 and 1:400 dilutions) at 4 °C overnight, then added with secondary antibody labeled HRP. Immunoblots were detected by densitometric analysis, and protein strips were analyzed using Image J software (Bethesda, MD, USA).

4.8. Luciferase reporter assay

The 3' UTR sequence of the PDK1 gene was cloned into the vector pGL3 containing the luciferase reporter gene, namely the Wt-3' UTR. Using the Site-Directed Mutagenesis kit (Thermo Fisher Scientific, Waltham, MA, USA), the core binding region of miRNA on the 3'UTR was mutated to an ineffective binding region to construct the control plasmid Mut-3' UTR. Full-length vectors and mutant vectors were transfected into cells by Lipofectamine 2000. Having been cultured for 36 h, the medium was discarded and the cells were rinsed for three times with PBS. Then the lysate was added and allowed to stand at room temperature for 10 min. A Dual Luciferase Reporter Assay System was subsequently used to detect fluorescence intensity.

4.9. Statistical methods

Data are presented as the means±SE. Statistical significance was determined by unpaired student's t-test or one-way analysis of variance (ANOVA), followed by the Bonferroni-Dunn post-hoc test. P? 0.05 was considered statistically significant. All

statistical analyses were performed using the Statistical Product and Services Solutions (SPSS) package (Version 20.0, SPSS, Science, Chicago, USA).

Author contributions: Yuyu Lu and Shikun Zhang performed the experiments; Shikun Zhang and Zhenyu Zhang analyzed the data; Zongdan Jiang and Weihao Sun designed the research; and Yuyu Lu and Shikun Zhang wrote the paper. All authors approved the final version of the manuscript.

Conflict of interest: All authors declare that they have no conflicts of interest.

References

Antithrombotic Trialists' (ATT) Collaboration, Baigent C., Blackwell L., et al (2009) Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Lancet 373: 1849–1860.

Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136: 215–233.

Bibbins-Domingo, K (2016) Aspirin Use for the Primary Prevention of Cardiovascular Disease and Colorectal Cancer: U.S. Preventive Services Task Force Recommendation Statement. Ann Int Med 164: 836–845.

Brennecke J, Stark A, Russell RB, Cohen SM (2005) Principles of microRNA-target recognition. PLoS Biol 3: e85.

Casamayor A, Morrice NA, Alessi DR (1999) Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. Biochem J 342: 287–292.

Darling RL, Romero JJ, Dial EJ, Akunda JK, Langenbach R, Lichtenberger LM (2004) The effects of aspirin on gastric mucosal integrity, surface hydrophobicity, and prostaglandin metabolism in cyclooxygenase knockout mice. Gastroenterology 127: 94–104.

Dong XQ, Zhang YH, Shang XQ, Zeng YJ (2019) Effects of miR-101 on the proliferation and apoptosis of gastric mucosal epithelial cells via Nrf2/ARE signaling pathway. Eur Rev Med Pharmacol Sci 23: 5187–5194.

Fanaroff AC, Roe MT (2016) Contemporary reflections on the safety of long-term sspirin treatment for the secondary prevention of cardiovascular disease. Drug Safety 39: 715–727.

Franke TF, Kaplan DR, Cantley LC (1997) PI3K: downstream AKTion blocks apoptosis. Cell 88: 435–437.

Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19: 92–105.

Gagliardi PA, Puliafito A, Primo L (2018) PDK1: At the crossroad of cancer signaling pathways. Seminars Cancer Biol 48: 27–35.

Gershon AA, Breuer J, Cohen JI, Cohrs RJ, Gershon MD, Gilden D, Grose C, Hambleton S, Kennedy PG, Oxman MN, Seward JF, Yamanishi K (2015) Varicella zoster virus infection. Nat Rev Dis Primers 1: 15016.

Goldstein JL, Larson LR, Yamashita BD, Boyd MS (1997) Management of NSAID-induced gastropathy: an economic decision analysis. Clin Ther 19: 1496–1509.

Hu G, Guo L, Ye G (2018) Helicobacter pylori infection impairs gastric epithelial barrier function via microRNA-100-mediated mTOR signaling inhibition, Mol Med Rep 18: 587–594.

Huang X, Qin J, Lu S (2015) Up-regulation of miR-877 induced by paclitaxel inhibits hepatocellular carcinoma cell proliferation though targeting FOXM1. Int J Clin Exp Pathol 8: 1515–1524.

Lee RC, Feinbaum RL, Ambros V (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin14. Cell 75: 843–854.

Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15–20.

Liu C, Duan Z, Guan Y, Wu H, Hu K, Gao X, Yuan F , Jiang Z , Fan Y, He B, Wang S , Zhang Z (2018) Increased expression of tight junction protein occludin is associated with the protective effect of mosapride against aspirin-induced gastric injury. Exp Ther Med 15: 1626–1632.

Mitsugi R, Itoh T, Fujiwara R (2016) MicroRNA-877-5p is involved in the trovafloxacin-induced liver injury. Toxicol Lett 263: 34–43.

Mora S, Manson JE (2016) Aspirin for primary prevention of atherosclerotic cardiovascular disease: advances in diagnosis and treatment. JAMA Int Med 176: 1195–1204.

Naesdal J, Brown K (2006) NSAID-associated adverse effects and acid control aids to prevent them: a review of current treatment options. Drug Safety 29: 119–132.

Oshima T, Miwa H, Joh T (2008) Aspirin induces gastric epithelial barrier dysfunction by activating p38 MAPK via claudin-7. Am J Physiol Cell Physiol 295: C800-806.

Shi Q, Xu X, Liu Q, Luo F, Shi J, He X (2016) MicroRNA-877 acts as a tumor suppressor by directly targeting eEF2K in renal cell carcinoma. Oncol Lett 11: 1474–1480.

Tsukita S, Furuse M (2000) Pores in the wall: claudins constitute tight junction strands containing aqueous pores. J Cell Biol 14: 13–16.

Wallace JL (2008). Prostaglandins, NSAIDs, and gastric mucosal protection: why doesn't the stomach digest itself? Physiol Rev 88: 1547–1565.

Zhang GY, Zhang ZY, Jiang ZD, He BS, Hu KW (2011) Effect and possible mechanism of aspirin on cell growth and occludin expression in Human gastric mucosal epithelial cells. Chin J Gastroenterol 16: 400-403.