MiR-877-5p down-regulation of PDK-1 involving in aspirin-induced gastric epithelial cells damage

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Abstract

It has been found that the expression of miR-877-5p is increased in serum of patients taking NSAIDs drugs. However, whether miR-877-5p play a role in aspirin-induced gastrointestinal mucosal erosion remains largely unknown. In this study, we investigated the effects of miR-877-5p on gastric epithelial cells (GES-1) proliferation and apoptosis in vitro. MiR-877-5p mimic/inhibitor and their oligonucleotides were transfected into GES-1 cells, then GES-1 cells were treated with different concentrations of aspirin (1.25, 2.5, 5 and 10 mmol/L). The bioinformatics software and dual-luciferase reporter assay were used to predict and verify the target gene of miR-877-5p. qRT-PCR and Western Blotting were employed to assess gene and protein expression, and CCK-8 assay and flow cytometry analysis were used to detect cell proliferation and apoptosis, respectively. qRT-PCR data showed that miR-877-5p level was significantly increased in aspirin incubated GES-1 cells. The proliferation of GES-1 cells were markedly inhibited and apoptosis was significantly induced in the miR-877-5p mimic groups compared to control groups. Using PITA, Targetscan and miRWalk database, the three databases indicated that PDK1 was a target gene of miR-miR-877-5p. Dual luciferase reporter assay confirmed that the existence of a direct interaction between miR-877-5p and PDK1 mRNA. Importantly, miR-877-5p knockdown resulted in a significant upregulation of PDK1 mRNA and its encoded protein in GES-1 cells. miR-877-5p plays a role in aspirin-induced gastrointestinal mucosal erosion, which may via down-regulation of targeting PDK1 gene.

Introduction

Aspirin, a member of NSAIDs, is the most prescribed agent for the prevention of atherothrombotic disease, beyond broad analgesic, antiinflammatory and antipyretic properties. The primary prevention trials demonstrated that aspirin administration achieved a 12% proportional reduction in serious vascular events, especially non-fatal myocardial infarction. Moreover, the secondary prevention trials showed that subject receiving aspirin therapy benefit more in reduction in serious vascular events compared to those control population without sexual differences[1]. However, gastrointestinal mucosal damages which range from small and asymptomatic gastrointestinal ulcer disease to risk of bleeding are well known adverse effects of aspirin[2].

Gastrointestinal mucosal erosion caused by aspirin is somewhat different to lesions caused by other NSAIDs[3]. As an inhibitor of cyclooxygenase (COX), aspirin impairs prostaglandins to gastric mucosal defense[4,5]. Using cyclooxygenase-1 knockout mice model, researchers found that prostaglandin-independent mechanisms are involved in attenuating gastric mucosal surface hydrophobicity[6]. Researchers also demonstrated that aspirin reduces the gastric mucosa autophagy, which is an important protective mechanism when the epithelium is injured[2]. Furthermore, study indicated that aspirin-induced impair of tight junctions (TJs) integrity results in the increase in gastric permeability[3]. Occludin and claudins are main components of TJs, which play an important role in maintain of paracellular permeability barrier[7]. Study further demonstrated that level of occluding expression, a transmembrane protein, was significantly decreased in Gastric epithelial cells (GES-1) treated with aspirin, resulted in a markedly reduced TJ integrity[8].

However, additional mechanisms may either contribution to the gastrointestinal toxicity induced by aspirin. Yu et al. observed that patients receiving acetaminophen, a number of NSAIDs, had a concentration dependent serum miR-877-5p level increase[9]. Studies indicated that miR-877-5p involved in the apoptosis cellular pathways[10,11], and possesses anti-carcinoma via suppressing the proliferation and migration of carcinoma cells[12]. So, we hypothesis that aspirin-induced gastrointestinal mucosal erosion may trigger the expression of miR-877-5p and the potential target genes.

In this study, we observed the effect of miR-877-5p on aspirin-induced GES-1 cell lesion using techniques such as gene transfection and dual-luciferase reporter assay in vitro.

Materials And Methods

Cell lines and culture conditions

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.

Different groups of aspirin induced GES-1 damage

To observe aspirin-induced cell lesion, GES-1 grown to near confluence were cultured in serum-free media containing 0.2% bovine serum albumin (BSA) (Invitrogen, Carlsbad, USA) overnight. Then GES-1 was treated with different concentrations of aspirin (1.25, 2.5, 5 and 10 mmol/L) for 24 h.

GES-1 cells transfection

To investigate the role of MiR-877-5p on GES-1 damage. MiR-877-5p mimic, inhibitor and their oligonucleotides were transfected into GES-1 cells. After cells reached near confluence, then cells were incubated with aspirin at a concentration of 2.30 mmo/L for 24 h. At the end of the experiment, the following assays were performed. All experiments were repeated 3–4 times.

qRT PCR

Total RNA of GES-1 treated with aspirin with and without MiR-877-5p mimic, inhibitor and their oligonucleotides were extracted using Trizol reagent (Invitrogen). The primer sequences used for amplification of genes were as follows: miR-877-5p: 5'-GUAGAGGAGAUGGCGCAGG-3'; U6 snRNA F: 5'-ATTGGAACGATACAGATACAGAGAAGATT-3', and U6 snRNA R: 5'-GGAACGCTTCAGAATTTG-3', 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 level

of MiR-877-5p and PDK1 transcript was calculated and normalized to GAPDH, with at least 3 repeats per experimental group.

CCK8 Assay

To evaluate aspirin-induced proliferation of GES-1 cells, a CCK8 assay was performed. Cells treated with different concentrations of aspirin (1.25, 2.5, 5 and 10 mmol/L) were seeded in 96well plates at a density of $5 \cdot 10^3$ cells/well in 200 μl DMEM and cultured at 37˚C for 12 h. Each well was added with 10 ∝l CCK8 reagent (YIFEIXUE Biotechnology, Nanjing, China) and cells were then cultured for another 1–2 h. The cells proliferation was determined by measuring the optical density absorbance at the wavelength of 450 nm.

Flow cytometry analysis

Apoptosis was determined by flow cytometry analysis. A FITC-labeled Annexin V/PI apoptosis assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was employed as previously described[8]. Cells were cultured at room temperature for 5 min using PI (50 μg/ml) solution and 5 μl fluorescein isothiocyanateconjugated AV (17.6 μg/ml), and then 400 μl of binding buffer was added to the cells before detection via flow cytometry assay (FACSCalibur; BD Biosciences, San Jose, CA, USA). Data were analyzed by CellQuest v.5.1 software (BD Biosciences). The experiments were performed in triplicate

GES-1 Transfection

Lipofectamine 2000 mixed with Opti-MEM for 5 min at room temperature, then added respectively pre-mixed miR-877-5p mimic, NC, inhibitor or INC and Opti-MEM for 20 min at room temperature. After GES-1 cells reached 60% confluence, cells were incubated with the mixed medium, medium was changed after 6 h. Total RNA and proteins were harvested at 48 and 72 h after transfection, respectively.

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. Then transferred membranes were incubated with primary antibodies (PDK-1 and β-actin at 1:1000 and 1:400 dilutions) at 4 °C overnight, and then secondary antibody labeled HRP was added. Immunoblots were detected by densitometric analysis, and protein strips were analyzed using Image J software (Bethesda, MD, USA).

Prediction and verification of target gene of miR-877-5p

The bioinformatics software was used to predict the target genes of miR-877-5p, and preliminary GO analysis and pathway analysis were performed on these screened target genes.

Statistics

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 to be statistically significant. All of the statistical analyses were performed using the Statistical Product and Services Solutions (SPSS) package (Version 20.0, SPSS, Science, Chicago, USA).

Results

Effect of aspirin on GES-1 cells

To investigate the effects of aspirin on the GES-1 cells, we tested the effects of four different concentrations of aspirin (1.25, 2.5, 5 and 10 mM) on the viability of the GES-1 cells using CCK8 assay. The cells were treated for 24 h, and their growth were inhibited by aspirin in a dose-dependent manner as compared with the control as shown in Fig. 1A. According to SPSS software analysis, the optimum concentration of aspirin IC10 was 2.30 mmol/L in this study. Using Flow cytometry analysis, our data demonstrated that GES-1 incubated with aspirin had a significantly increased cell apoptosis compared to control groups (Fig. 1B). In addition, we also observed an aspirin dose dependent of increased cell apoptosis ratio with the cell apoptosis was 4.7 ± 0.7%, 14.9 ± 2.1%, 27.4 ± 3.5%, and 34.5 ± 3.2%, respectively (all $P < 0.01$).

Aspirin induced miR-877-5p expression in GES-1 cells and effects of miR-877-5p mimic and inhibitor on proliferation and apoptosis of GES-1 cells

After GES-1 cells cultured with different aspirin concentrations for 24 h. As Fig. 2A showed that qRT-PCR data indicated that miR-877-5p mRNA expression levels were significantly increased in GES-1 cells treated with aspirin compared to control groups. In addition, a dose-dependent induced miR-877-5p expression levels induction was observed in this study (all $P < 0.05$).

A qRT-PCR assay was performed to access the efficacy of miR-877-5p mimic and miR-877-5p inhibitor after GES-1 transfection 24 h. Our data showed that miR-877-5p expression levels were 93.8 ± 7.7 and 0.54 ± 0.02 times in miR-877-5p mimic and miR-877-5p inhibitor transfection groups compared to those GES-1 cells in NC and INC groups ($P < 0.01$, respectively). Our data clearly showed the miR-877-5p mimic and miR-877-5p inhibitor transfection was successful. To observe the effect of miR-877-5p proliferation and apoptosis on GES-1 cells, we incubated miR-877-5p mimic/inhibitor transfected GES-1 cells with asprin (IC10, 2.3 mmol/L). Our CCK8 assay showed that proliferation rate of cell transfected with NC or INC was number but not statistically significantly increased compared to those treated with aspirin alone ($P > 0.05$). However, the proliferation rate of cells transfected with miR-877-5p mimic and miR-877-5p inhibitor achieved significantly decreased and increased proliferation rate compared to control groups, respectively (all $P < 0.05$) (Fig. 2B). In addition, flow cytometry analysis showed that the apoptosis rate in aspirin groups, NC + aspiprin groups, mimic + aspirin groups, INC + aspirin groups, and inhibitor + aspirin groups were 10.9, 12.3, 27.6, 14.2, and 6.6%, respectively. (Fig. 2C).

GO and pathway analysis then verify of interaction between miR-877-5p and PDK1

The potential miRNA target genes were identified based on the integrated gene and miRNA expression profile, using miRanda□Targetscan and miRWalk database. Among of the three databases, the candidate potential target genes were 1622, 2504, and 4320 in miRanda, Targetscan and miRWalk database, respectively, with 945 candidate genes located in the three databases. GO analysis showed that miR-877-5p metal-binding, transcription regulation and Intracellular Protein Modification ($P < 0.05$) (Fig. 3A a-c). KEGG signaling pathway analysis showed that among of the 65 enrichment pathways ($P < 0.05$) (Fig. 3Ad), PI3K-Akt, MAPK, AMP and Calcium signaling pathway were the more enriched pathways. MAPK, PI3K/AKT and AMPK signaling pathway, such as PDK1, SGK1, NEBL and UPRT, involved in the gastrointestinal mucosal erosion were well elucidated[2,13,14]. Given previous studies reporting that NSAIDs significantly inhibited expression of PDK1, we therefore hypothesis that PDK1 may be the potential miRNA target genes of miR-877-5p. Fortunately, Targetscan database clearly showed that miR-877-5p and PDK1 mRNA 3'-UTR had complementary sequence of base pairs(Fig. 3B).

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. 3C). However, no reduction was seen when luciferase was expressed with a PDK1-3'UTR mutant. This indicated the existence of a direct interaction between miR-877-5p and PDK1 mRNA.

Expression of PDK1 mRNA in aspirin-induced GES-1 cells and to observe the effect of miR-877-5p on PDK1 mRNA and protein profiles in GES-1 cells

qRT-PCR data showed that PDK1 mRNA expression levels were significantly decreased in GES-1 cells treated with aspirin compared to control groups. With the increase of aspirin concentration, the expression of PDK1 mRNA decreased. (all $P < 0.05$) (Fig. 4A).

To observe the effects of miR-877-5p on PDK1 in GES-1 cells, a qRT-PCR assay was performed to access the effect of miR-877-5p mimic and miR-877-5p inhibitor on PDK1 mRNA expression. Our data showed that PDK1 mRNA expression level was significantly decreased in miR-877-5p mimic transfected GES-1 cells groups compared to control groups ($P < 0.05$), and PDK1 mRNA expression level was significantly increased in miR-877-5p inhibitor transfected GES-1 cells groups compared to control groups ($P < 0.05$) (Fig. 4B). Furthermore, our Western Blotting data we also demonstrated a decrease and an increase in PDK1 protein expression in miR-877-5p mimic and miR-877-5p inhibitor transfected GES-1 cells groups, respectively ($P < 0.05$) (Fig. 4C).

Discussion

In this study, we observed that the expression of miR-877-5p was increased in aspirin treated GES-1 cells. We also observed that the overexpression of miR-877-5p inhibited GES-1cells proliferation and enhanced cells apoptosis. Importantly, our data indicated that PDK1 gene may be the targeting candidate gene of miR-877-5p involved in aspirin induced cells damage.

Evidence indicated that 2–4% of NSAIDs subjects suffered serious gastric mucosal erosions[15] and one fifth population receiving long-term NSAIDs therapy develop peptic ulcers[16]. So the major concern for clinicians balance of the benefits and bleeding risks when low-dose aspirin initiating as the primary prevention of CVD in order to maximize the benefits of aspirin[17,18].

MicroRNAs (miRNAs) are small conserved RNAs direct controlling temporal and spatial post-transcriptional[19]. We observed that the expression level of miR-877-5p was increased in aspirin treated GES-1 cells. miR-877-5p play an important role in triggering the onset of apoptotic cell death or inhibition cell proliferation via different cellular signaling pathways, especially AKT as a directly candidate target[10,12]. Previously study reporting that miR-877-5p promoted apoptosis in HepG2 cells by targeting FOXM1[11]. It was also demonstrated that miR-877-5p-induced the increase level of PEPCK responses to the onset of apoptosis in liver cells[20]. Studies also showed that miR-877 inhibits renal cell carcinoma proliferation by modulating the eEF2K/eEF2 signaling cascade[12]. In consistence with previous studies, our data also showed that overexpression of miR-877-5p inhibited GES-1 cells proliferation and enhanced cells apoptosis. However, the miR-877-5p downstream candidate target by which regulation of cells proliferation and apoptosis in GES-1 cells remained to be elucidated.

In this study, the potential miRNA target genes were identified based on the integrated gene and miRNA expression profile, using PITA, Targetscan and miRWalk database. The three databases indicated that PDK1 was a target of miR-miR-877-5p. Meanwhile, it was also found that the expression of miR-877-5p was increased and the level of PDK1 was reduced when GES-1 were cultured and exposed to aspirin. PDK1 is composed by 556 amino acids[21], which is considered as constitutively active enzyme, due to the autophosphorylation on Ser-241[22]. The cellular physiology of phosphoinositide 3-kinase (PI3K)/PDK1/Akt pathway on cell survival and metabolism was well elucidated, especially in oncology[23]. Evidence demonstrated that the aberrant activation of this pathway plays a pivotal role in human tumors initiation and development[24,25]. Extracellular stimulating signals activation of PDK1 via serine/threonine and/or tyrosine phosphorylation, subcellular localization, and binding to subunits and conformation of heterodimer complex, which activate PDK1 downstream targets gene responsible for the tumor cells apoptosis escape[26]. However, we had no data supporting that miR-877-5p/PDK1 triggered which cellular signaling pathway starts a cascade of GES-1 apoptosis. Further studies are needed to elucidate the whole mechanism of miR-877-5p/PDK1 induced apoptosis increase and proliferation decrease in GES-1 cells.

Conclusion

Our data showed that miR-877-5p/ PDK1 regulated inhibition of cell proliferation and enhancement to cell apoptosis in aspirin-induced cell damage.

Declarations

Funding

NO

Author contributions

Zongdan Jiang, Dan Ran and Chao Li performed the experiments; Zhibing Wang and Zhi Wang analyzed the data; Zhenyu Zhang and Weihao Sun designed the research; and Zongdan Jiang and Jianjun Zou wrote the paper. All authors approved the final version of the manuscript.

Declaration of competing interest

None.

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Figures

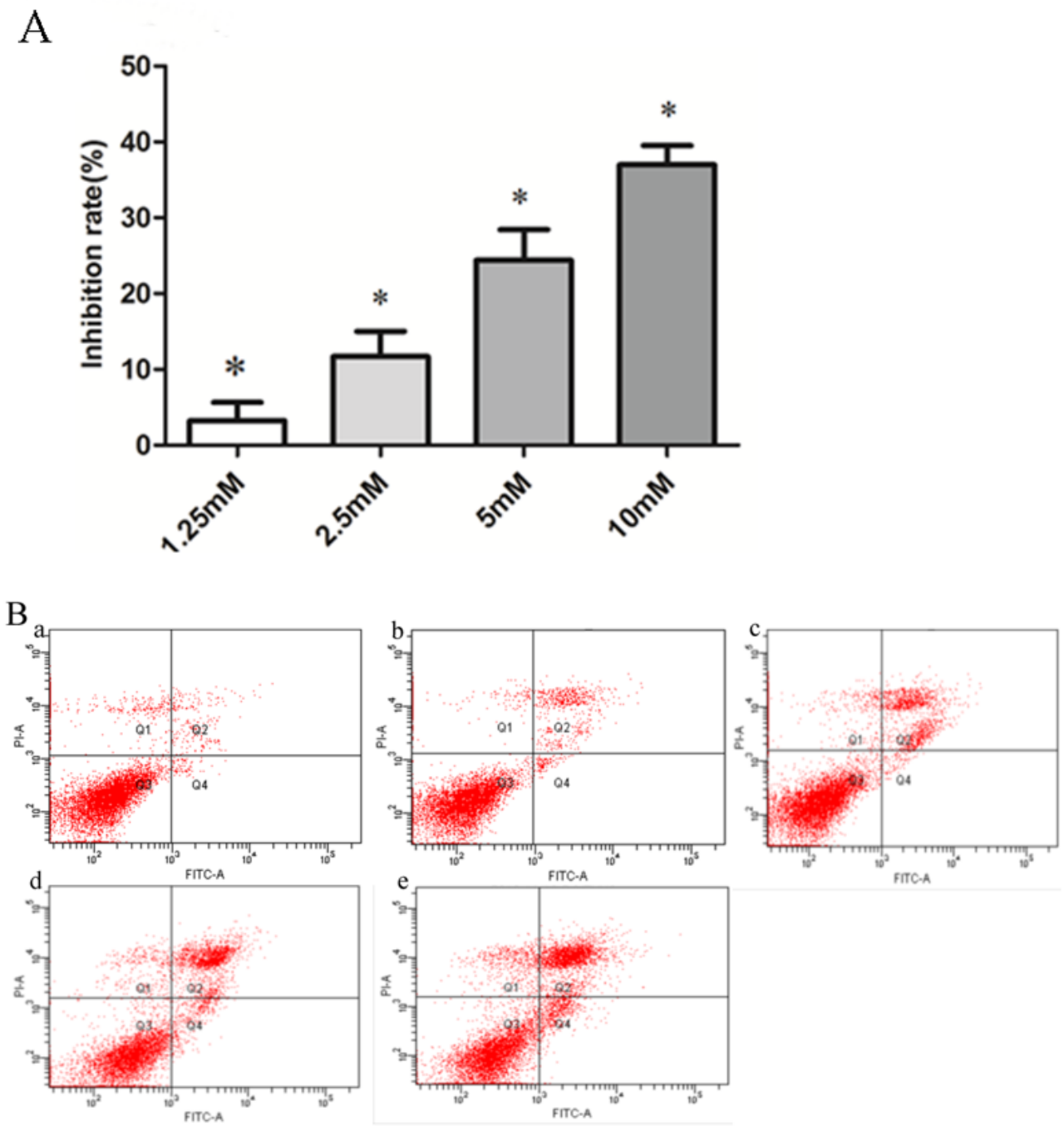

Figure 1

A. The effect of different aspirin concentrations on proliferation in GES-1 cells. Data were derived from three independent experiments. *P < 0.05 vs. control. B. GES-1cells were treated with different aspirin (a.control group b. aspirin 1.25 mM group c. Aspirin 2.5 mM group d. Aspirin 5 mM group E. Aspirin 10 mM group) for 24 h. The apoptosis was examined by AV/PI staining and flow cytometry analysis. Data were derived from three independent experiments. P < 0.01 vs. control.

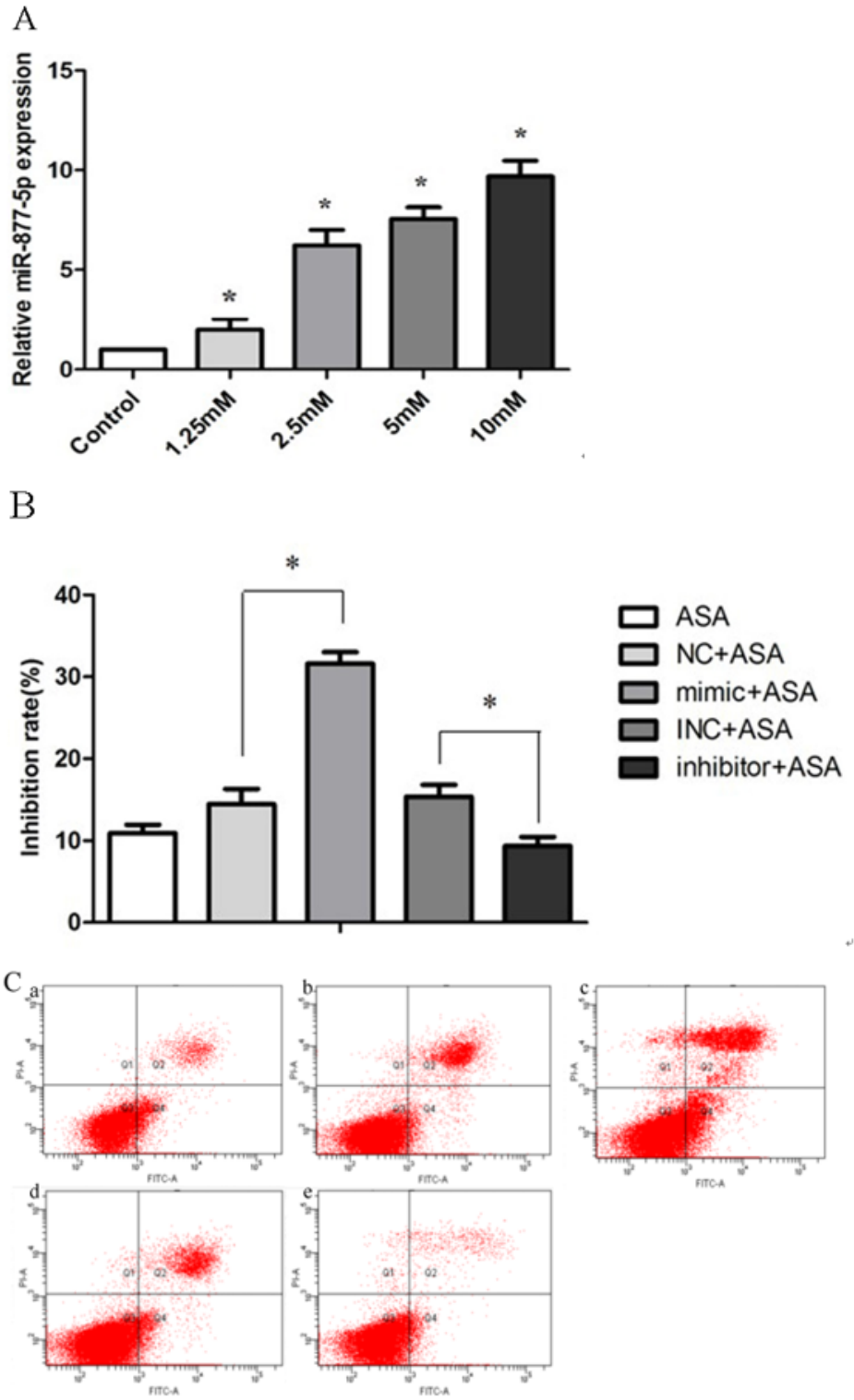

Figure 2

A. The effect of different aspirin concentrations on miR-877-5p mRNA expression in GES-1 cells. *P < 0.05 vs. control. B. The effect of aspirin on proliferation in miR-877-5p transfected GES-1 cells. *P < 0.05. C. The effect of aspirin on apoptosis in miR-877-5p transfected GES-1 cells (a, aspirin groups b, NC + aspiprin groups c, mimic + aspirin groups d, INC + aspirin groups e. inhibitor + aspirin groups). The

apoptosis rate of cells transfected with miR-877-5p mimic and miR-877-5p inhibitor achieved significantly decreased and increased proliferation rate compared to control groups, respectively (P<0.05)

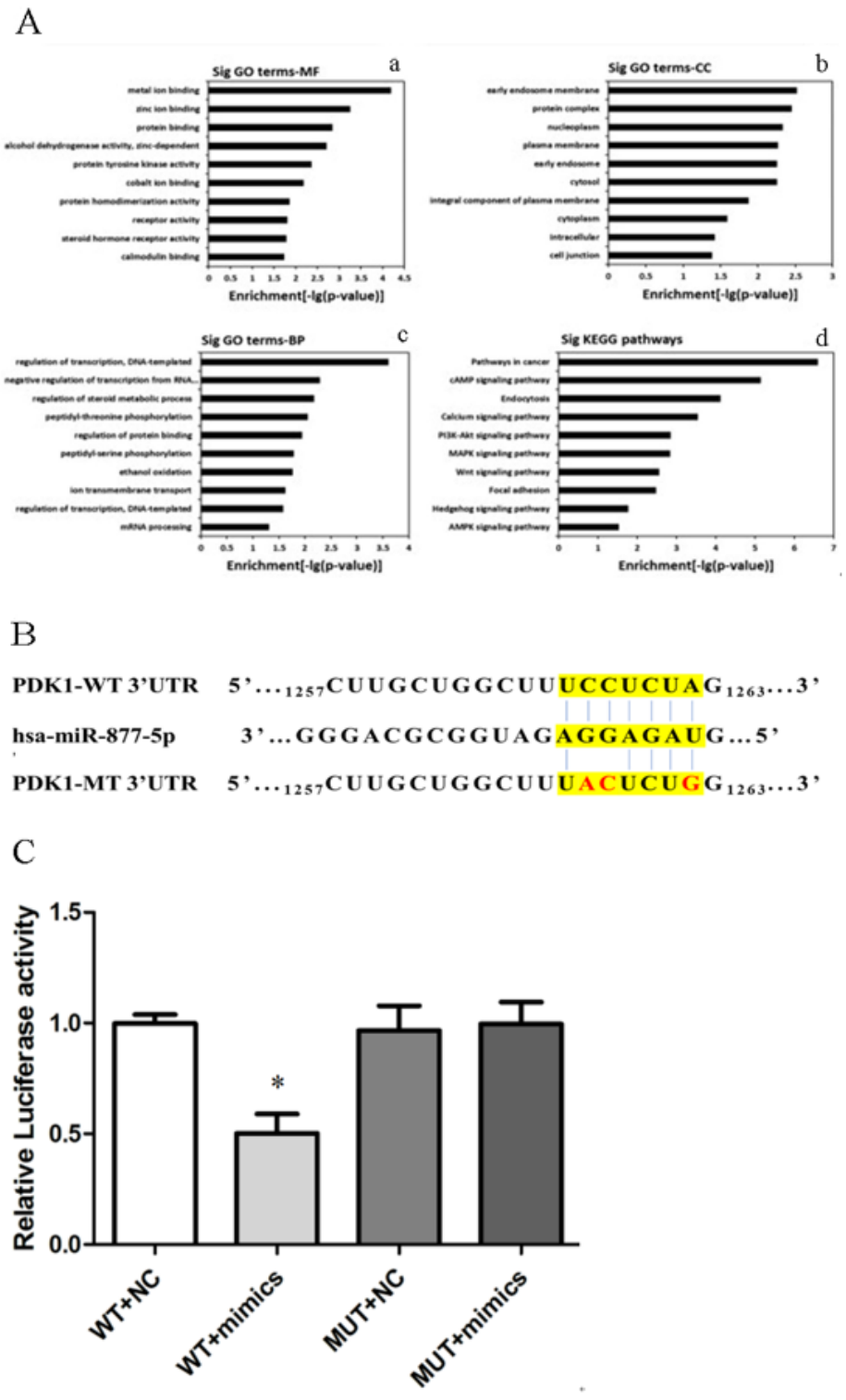

Figure 3

A. miR-877-5p gene GO and pathway analysis: (a) MF functional enrichment (b) GO functional enrichment; (c) GO functional enrichment; (d) KEGG pathway. B. miR-877-5p and PDK1 mRNA 3'-UTR had complementary sequence of base pairs. PDK1-3'UTR-wt/mut sequence of base pairs and the

complementary sequence of base pairs with miR-877-5p. The red highlight bases were the mutate sites. C. A dual luciferase reporter assay revealed that the existence of a direct interaction between miR-877-5p and PDK1. *P < 0.05 vs. control.

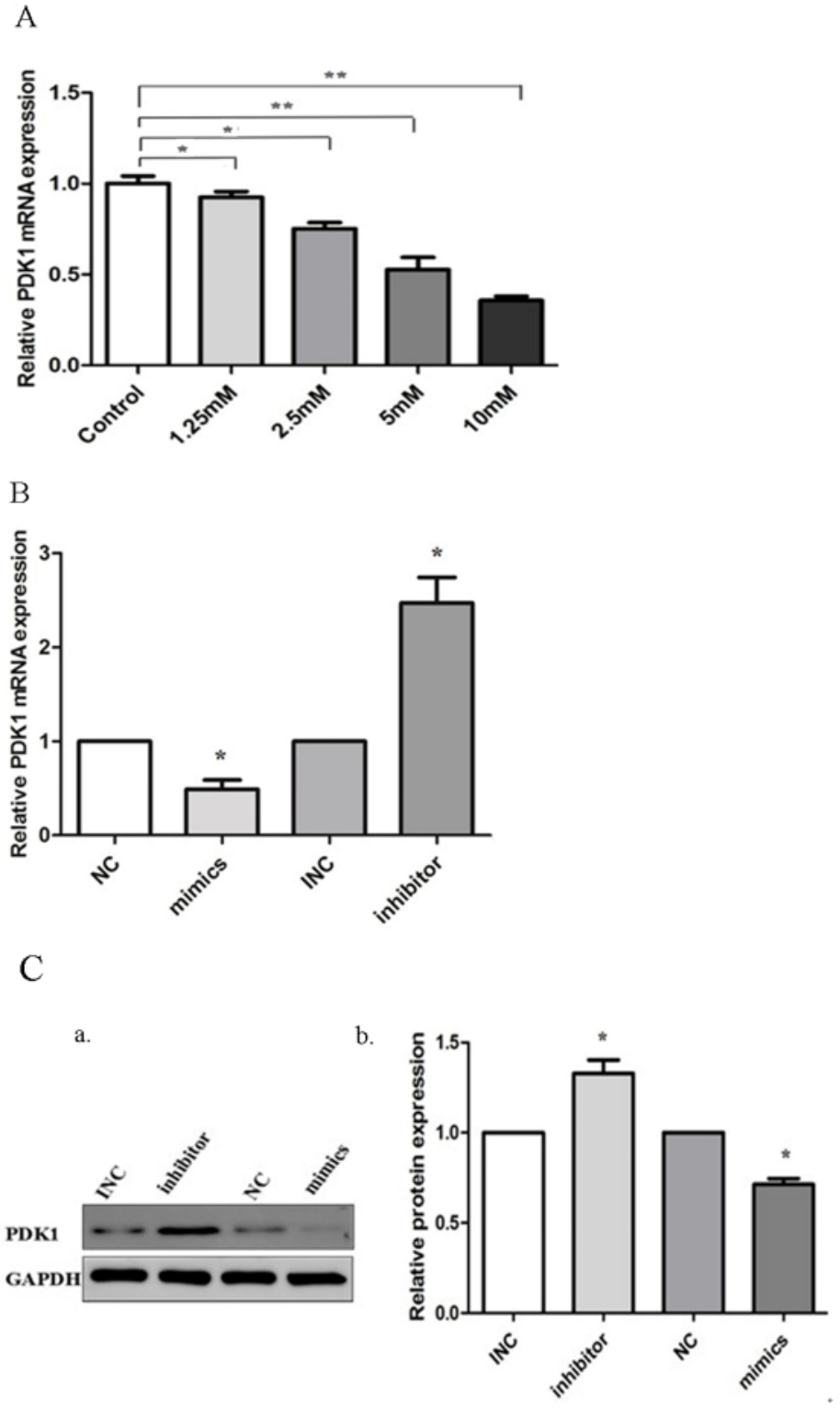

Figure 4

A. The effect of different aspirin concentrations on proliferation of PDK1 in GES-1 cells (compared to control groups, * P < 0.05, ** P < 0.001). B. The effect of miR-877-5p on PDK1 mRNA expression in GES-1

cells *P < 0.05 vs. control. C. The effect of miR-877-5p on PDK1 protein expression in GES-1 cells. *P < 0.05 vs. control.