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Downregulation of microRNA-128-3p protects human esophageal squamous Het-1A cells from hydrochloric acid-induced cell injury by targeting E2F3

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This study aimed to investigate the role of microRNA-128-3p in regulating hydrochloric acid (HCl)-induced cell injury in human esophageal squamous Het-1A cells. Het-1A cells were treated with HCl (pH 4.0) to induce esophageal injury like this caused by reflux. Whether HCl induced cell injury was then assessed by detecting cell proliferation and apoptosis. The expression of miR-128-3p in HCl-treated Het-1A cells was investigated, followed by investigating the effects of miR-128-3p overexpression and suppression on HCl-induced Het-1A cell injury. In addition, the target of miR-128-3p was identified. Besides, the association between miR-128-3p and ERK and PI3K/AKT pathways was further explored. HCl inhibited the proliferation and increased apoptosis of Het-1A cells. miR-128-3p was upregulated in Het-1A cells after HCl treatment. Inhibition of miR-128-3p alleviated HCl-induced Het-1A cell injury, whereas miR-128-3p overexpression further aggravated this injury. Moreover, E2F3 was confirmed as a target of miR-128-3p, which could be negatively regulated by miR-128-3p. Besides, miR-128-3p inhibition remarkably alleviated the inhibitory effects of HCl on the activation of ERK and PI3K/AKT pathways, which were further reversed after inhibition of miR-128-3p and E2F3 at the same time. Alltogether, our findings indicate that downregulation of microRNA-128-3p may protect human esophageal squamous cells Het-1A from HCl-induced cell injury *via* targeting E2F3 and inhibiting the activation of ERK and PI3K/AKT pathways. These findings provide the experimental basis for targeted treatment of reflux esophagitis.

1. Introduction

Gastroesophageal reflux is characterized by the reflux of gastric contents into the esophagus, thus causing a variety of troublesome symptoms and/or complications (Liu et al. 2016). Reflux esophagitis is a common consequence of esophageal injury resulting from reflux-caused excessive esophageal acid exposure (Iwakiri 2017; Iwakiri et al. 2017). Patients with reflux esophagitis have a high rate of relapse after treatment withdrawal (Colombo 2000). Moreover, reflux esophagitis can result in Barrett's metaplasia, which is an abnormal esophageal mucosa predisposed to adenocarcinoma (Souza 2016), implying the potential risk from reflux esophagitis to esophageal adenocarcinoma. Therefore, it is imperative to further elucidate the key mechanism involved in reflux esophagitis and develop the effective treatment strategies for this disease.

MicroRNAs (miRNAs) are a class of small, non-coding RNAs that are able to control gene expression by regulating the degradation or translational repression of their target mRNAs (Caporali and Emanueli 2011; Catalanotto et al. 2016). miRNAs have been identified as key players in multiple cellular functions and events, such as cell proliferation, metabolism, and tumorigenesis (Schickel et al. 2008). Although many miRNAs are involved in esophageal adenocarcinoma development (Hemmatzadeh et al. 2016; Hu et al. 2017; Lynamlennon et al. 2016; Wang et al. 2018), there are few studies on exploration the key miRNAs involved in reflux esophagitis.

There is evidence that miR-128-3p plays a key role in the development of several human cancers (Cai et al. 2017; Mets et al. 2014; Yu et al. 2015), as well as protection against myocardial or cerebral ischemia/reperfusion injury (Chen et al. 2017; Shi et al. 2016). A recent study showed that microarray analysis reveals an upregulation of serum miR-128-3p on 21 days (chronic phase) after induction of reflux esophagitis in rats, however, real-time quantitative PCR (qPCR) do not observe the significantly expression change of miR-128-3p at this time point (Uemura et al. 2017). Whether miR-128-3 contributes to the development of reflux esophagitis is largely unknown, let alone its regulatory mechanism.

In this present study, Het-1A cells were treated with hydrochloric acid (HCl, pH 4.0) to induce esophageal injury caused by reflux similarly. Upregulation of miR-128-3p was observed in HCl-treated HET-1A cells, imply the potential role of miR-128-3p in acid-stimulated HET-1A cells. Our finding will provide a new insight for better understanding the molecular mechanism underlying reflux esophagitis.

2. Investigations and results

2.1. HCl inhibited proliferation and increased apoptosis of Het-1A cells

To detect whether HCl could induce Het-1A cell injury, the effects of HCl on Het-1A cell proliferation and apoptosis were investigated. The results of MTT assay showed that HCl inhibited Het-1A cell viability significantly in a time-dependent manner ($P < 0.05$, Fig. 1A). Consistent results were also obtained by BrdU labeling assay that HCl remarkably decreased the percentage of Brdu-positive cells ($P < 0.05$, Fig. 1B). Moreover, the expression levels of cell cycle-related proteins, including CDK6, CDK4, cyclin D1, CDK2 and cyclin E1 were gradually decreased with the increase of treated times of HCl (Fig. 1C). These data indicated that HCl inhibited Het-1A cell proliferation. Furthermore, the results of flow cytometry showed that HCl significantly

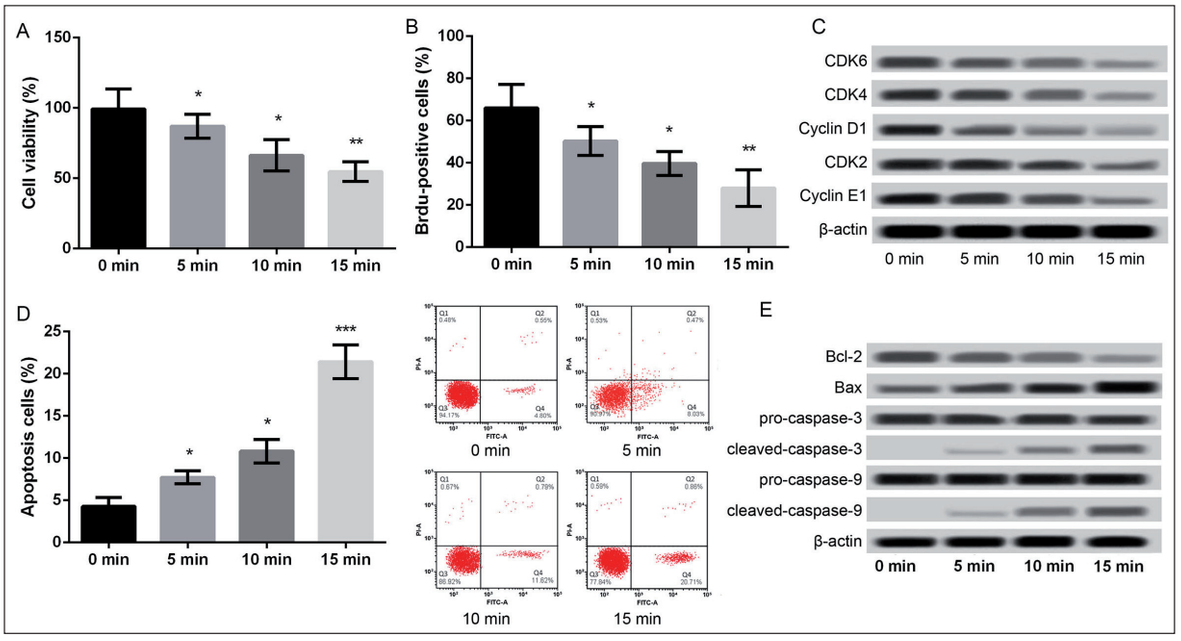

Fig. 1: Hydrochloric acid (HCl) inhibited proliferation and increased apoptosis of Het-1A cells. A: MTT assay showed that Het-1A cell viability after 5, 10, and 15 min of HCl treatment. B: BrdU labeling assay showed the percentage of Brdu-positive cells after 5, 10, and 15 min of HCl treatment. C: The expression levels of cell cycle-related proteins, including CDK6, CDK4, cyclin D1, CDK2 and cyclin E1 in Het-1A cells after 5, 10, and 15 min of HCl treatment. D: Flow cytometry showed Het-1A cell apoptosis after 5, 10, and 15 min of HCl treatment. E: The expression levels of apoptosis-related proteins in Het-1A cells after 5, 10, and 15 min of HCl treatment. Data are expressed as mean±standard deviations (SD) from three independent assays in each experiment. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with control.

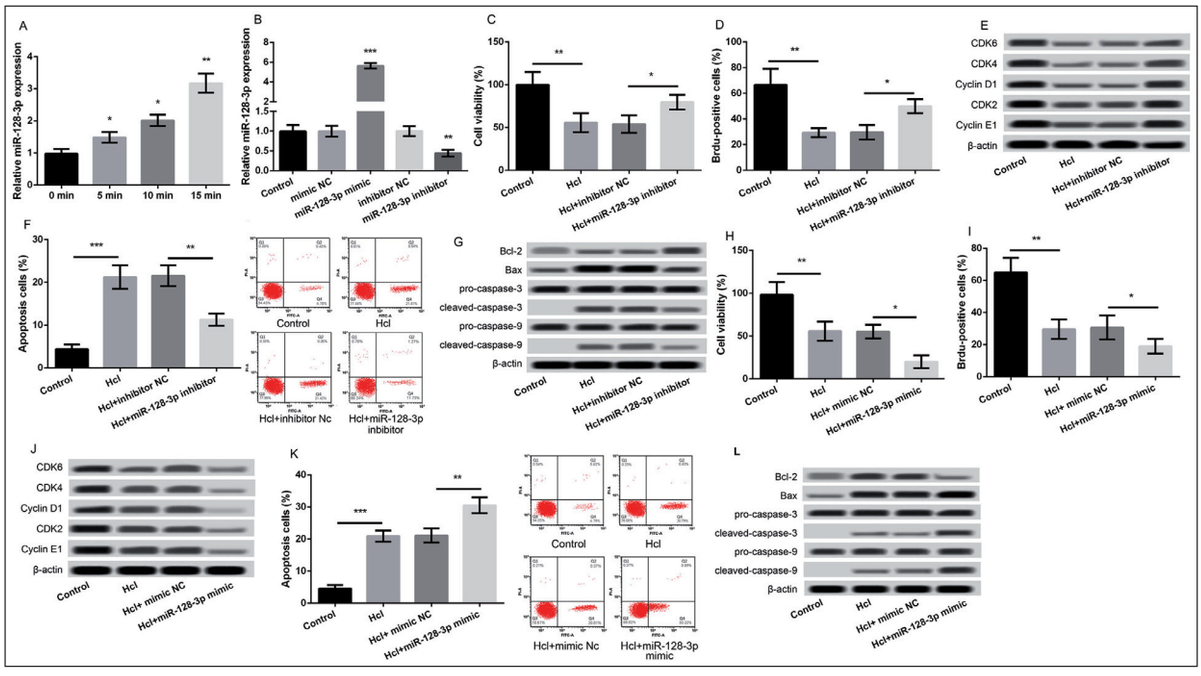

Fig. 2: HCl-induced cell injury was alleviated after miR-128-3p inhibition, but aggravated after miR-128-3p overexpression. A: HCl upregulated the expression of miR-128-3p. B: Real-time qPCR showed the expression of miR-128-3p after different transfections. C: MTT assay showed that Het-1A cell viability after HCl treatment and miR-128-3p inhibitor transfection. D: BrdU labeling assay showed the percentage of Brdu-positive cells after HCl treatment and miR-128-3p inhibitor transfection. E: The expression levels of cell cycle-related proteins, including CDK6, CDK4, cyclin D1, CDK2 and cyclin E1 in Het-1A cells after HCl treatment and miR-128-3p inhibitor transfection. F: Flow cytometry showed Het-1A cell apoptosis after HCl treatment and miR-128-3p inhibitor transfection. G: The expression levels of apoptosis-related proteins in Het-1A cells after HCl treatment and miR-128-3p inhibitor transfection. H: MTT assay showed that Het-1A cell viability after HCl treatment and miR-128-3p mimic transfection. I: BrdU labeling assay showed the percentage of Brdu-positive cells after HCl treatment and miR-128-3p mimic transfection. J: The expression levels of cell cycle-related proteins, including CDK6, CDK4, cyclin D1, CDK2 and cyclin E1 in Het-1A cells after HCl treatment and miR-128-3p mimic transfection. K: Flow cytometry showed Het-1A cell apoptosis after HCl treatment and miR-128-3p mimic transfection. L: The expression levels of apoptosis-related proteins in Het-1A cells after HCl treatment and miR-128-3p mimic transfection. Data are expressed as mean±SD from three independent assays in each experiment. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

induced Het-1A cell apoptosis in a time-dependent manner ($P < 0.05$, Fig. 1D). The expression levels of apoptosis-related proteins exhibited consistent results that the expression levels of Bax/Bcl-2, cleaved/pro-caspase-3 and cleaved/pro-caspase-9 were markedly increased with the increase of treated times of HCl (Fig. 1E).

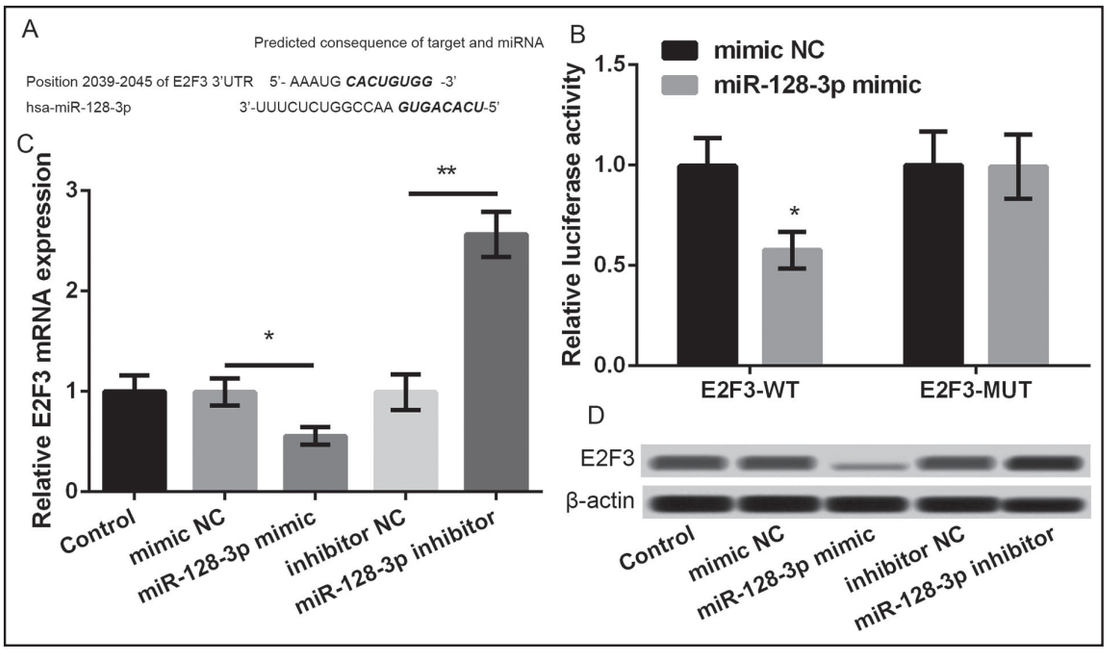

Fig. 3: E2F3 was verified as a target gene of miR-128-3p. A: E2F3 was predicted as a potential target of miR-128-3p by Targetscan software. B: Luciferase reporter assay confirmed the target regulatory relationship between E2F3 and miR-128-3p. C: Real-time q-PCR showed the expression of E2F3 mRNA after different transfections. D: Western blot showed the expression of E2F3 protein after different transfections. Data are expressed as mean±SD from three independent assays in each experiment. * $P < 0.05$, and ** $P < 0.01$.

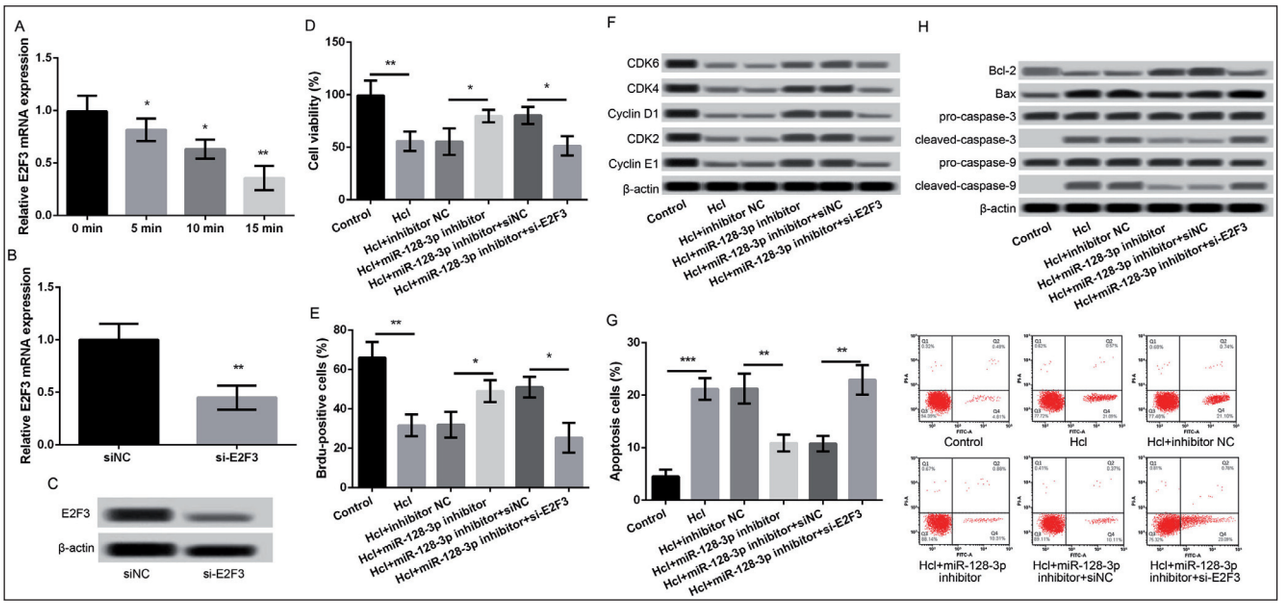

Fig. 4: miR-128-3p regulated Het-1A cell proliferation and apoptosis through E2F3. A: Real-time q-PCR showed the expression of E2F3 mRNA after 5, 10, and 15 min of HCl treatment. B: Real-time q-PCR showed the expression of E2F3 mRNA after transfection with si-E2F3. C: Western blot showed the expression of E2F3 protein after transfection with si-E2F3. D: MTT assay showed that Het-1A cell viability after HCl treatment and different transfections. E: BrdU labeling assay showed the percentage of Brdu-positive cells after HCl treatment and different transfections. F: The expression levels of cell cycle-related proteins, including CDK6, CDK4, cyclin D1, CDK2 and cyclin E1 in Het-1A cells after HCl treatment and different transfections. G: Flow cytometry showed Het-1A cell apoptosis after HCl treatment and different transfections. H: The expression levels of apoptosis-related proteins in Het-1A cells after HCl treatment and different transfections. Data are expressed as mean±SD from three independent assays in each experiment. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

2.2. HCl upregulated the expression of miR-128-3p

A previous study has reported that miR-128-3p is upregulated in rat reflux esophagitis (Uemura et al. 2017), however, the regulatory mechanism of miR-128-3p in this disease is largely unknown. Thus, we determined the expression levels of miR-128-3p in HCl-treated Het-1A cells. Expected results were got that HCl upregulated the expression of miR-128-3p in Het-1A cells in a time-dependent manner ($P < 0.05$, Fig. 2A).

2.3. HCl-induced cell injury was alleviated after miR-128-3p inhibition, but aggravated after miR-128-3p overexpression

To investigate the role of miR-128-3p in esophagitis, miR-128-3p was further overexpressed and inhibited in HCl-treated Het-1A cells by transfection with miR-128-3p mimic and miR-128-3p inhibitor, respectively. The high transfection efficiency was confirmed by qPCR ($P < 0.01$, Fig. 2B). Moreover, the results showed that

miR-128-3p inhibition significantly alleviated HCl-induced decreased Het-1A cell viability and the percentage of Brdu-positive cells ($P < 0.05$, Fig. 2C and 2D). Moreover, the decreased expression levels of cell cycle-related proteins, including CDK6, CDK4, cyclin D1, CDK2 and cyclin E1 were markedly reversed after miR-128-3p inhibition in HCl-treated Het-1A cells (Fig. 2E). Furthermore, miR-128-3p inhibition also reversed HCl-induced Het-1A cell apoptosis ($P < 0.01$, Fig. 2F) and the expression levels of apoptosis-related proteins (Fig. 2G). However, miR-128-3p overexpression significantly aggravated HCl-induced Het-1A cell injury by further decreasing cell viability (Fig. 2H), the percentage of Brdu-positive cells (Fig. 2I) and the expression levels of cell cycle-related proteins (Fig. 2J), and increasing cell apoptosis (Fig. 2K) and the expression levels of Bax/Bcl-2, cleaved/pro-caspase-3 and cleaved/pro-caspase-9 (Fig. 2L) (all $P < 0.05$).

2.4. E2F3 was verified as a target gene of miR-128-3p

The potential targets of miR-128-3p were further explored. As shown in Fig. 3A, E2F3 was a potential target of miR-128-3p according to the Targetscan software (http://www.targetscan.org/cgi-bin/targetscan/vert_71/view_gene.cgi?rs=ENST00000346618.3&taxid=9606&members=miR-128-3p&showcnc=0&shownc=0&shownc_nc=&showncf1=&showncf2=&subset=1). Further luciferase reporter assay confirmed the target regulatory relationship between E2F3 and miR-128-3p (Fig. 3B). Moreover, miR-128-3p mimic significantly downregulated the expression of E2F3 in Het-1A cells, but miR-128-3p upregulated (Fig. 3C and 3D), supporting the negative regulation between miR-128-3p expression and E2F3 expression.

2.5. miR-128-3p regulated Het-1A cell proliferation and apoptosis through E2F3

To further verify the roles of miR-128-3p in Het-1A cells through targeting E2F3, we firstly detected the expression of E2F3 after HCl treatment. The results showed that HCl significantly decreased E2F3 expression in a time-dependent manner ($P < 0.05$, Fig. 4A). Subsequently, E2F3 was successfully knocked down by transfection with si-E2F3 (Fig. 4B and 4C), followed by detecting the effects of cotransfection of miR-128-3p inhibitor and si-E2F3 on the proliferation and apoptosis of HCl-treated Het-1A cells. The results showed that inhibition of miR-128-3p and E2F3 at the same time in HCl-treated Het-1A cells significantly reversed the effects of miR-128-3p inhibition on cell viability (Fig. 4D), the percentage of Brdu-positive cells (Fig. 4E) and the expression levels of cell cycle-related proteins (Fig. 4F), and cell apoptosis (Fig. 4G) and the expression levels of Bax/Bcl-2, cleaved/pro-caspase-3 and cleaved/pro-caspase-9 (Fig. 4H) (all $P < 0.05$).

2.6. Effects of miR-128-3p on Het-1A cells through regulation of ERK and PI3K/AKT pathways

The association between miR-128-3p/E2F3 and ERK and PI3K/AKT pathways in Het-1A cells was further explored (Fig. 5). The results showed that HCl decreased the expression levels of p-ERK1/2, p-AKT and p-PI3K, indicating that HCl inhibited the activation of ERK and PI3K/AKT pathways. Furthermore, miR-128-3p inhibition remarkably alleviated HCl-induced the decreased expression of p-ERK1/2, p-AKT and p-PI3K, which were further reversed after inhibition of miR-128-3p and E2F3 at the same time.

3. Discussion

This study investigated the role of miR-128-3p in HCl-treated Het-1A cells. Interestingly, miR-128-3p was upregulated in Het-1A cells after HCl treatment. Inhibition of miR-128-3p alleviated HCl-induced Het-1A cell injury, whereas miR-128-3p overexpression further aggravated this injury. Moreover, E2F3 was confirmed as a target of miR-128-3p, which could be negatively regulated by miR-128-3p. Besides, miR-128-3p inhibition remarkably alleviated the inhibitory effects of HCl on the activation of ERK and PI3K/AKT pathways, which were further reversed after inhibition of miR-128-3p and E2F3 at the same time, supporting that effects of miR-128-3p on Het-1A cells were possible through regulation of ERK and PI3K/AKT pathways.

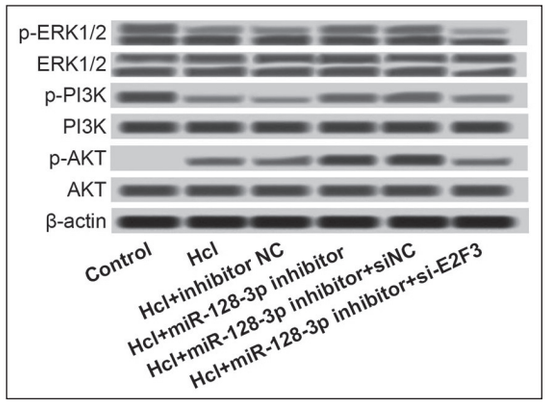

Fig. 5: Expression levels of ERK and PI3K/AKT pathways-related proteins after HCl treatment and different transfections.

A previous study has reported that miR-128-3p was upregulated in a rat model of acid reflux esophagitis by microarray analysis (Uemura et al. 2017). Moreover, miR-128-3p is reported to play a crucial role in regulating tumor metastasis in esophageal squamous-cell cancer (Zhao et al. 2018). These findings imply that miR-128-3p may be a key mediator to mediate the pathological development of esophageal diseases. In this study, we found that miR-128-3p was upregulated in Het-1A cells after HCl treatment. Inhibition of miR-128-3p alleviated HCl-induced Het-1A cell injury, whereas miR-128-3p overexpression further aggravated this injury. Therefore, we hypothesize that downregulation of microRNA-128-3p may protect human esophageal squamous cells Het-1A from HCl-induced cell injury.

Furthermore, E2F3 was confirmed as a target of miR-128-3p in our study. E2F3 belongs to the E2F family which can regulate both cellular proliferation and differentiation (Humbert et al. 2000). E2F3 is found to modulate cellular proliferation rate in human bladder and prostate cancer cells (Olsson et al. 2007). Overexpression of E2F3 is also shown to be associated with tumor cell proliferation and invasive tumor growth in urinary bladder cancer (Oeggerli et al. 2004). Moreover, miR-449a suppresses cell proliferation and promotes apoptosis in gastric cancer by directly repressing E2F3 (Li et al. 2015). Given the key role of E2F3 in regulating cellular proliferation, although there was no study reporting the role of E2F3 in acid reflux esophagitis, our results prompt us to infer that miR-128-3p may target E2F3 expression to further regulate the proliferation of HCl-treated Het-1A cells.

In addition, the association between miR-128-3p/E2F3 and ERK and PI3K/AKT pathways in Het-1A cells was further explored. PI3K/Akt signaling plays an important role in regulating cell survival and apoptosis (Fresno Vara et al. 2004). Previous studies have reported that AKT is associated with esophageal lesions (Fassan et al. 2012; Wang et al. 2015). Moreover, Zhuang et al. (2017) demonstrated that the activation of PI3K/Akt-NF-κB signaling pathway played a key role in the occurrence and development of reflux esophagitis. Rafiee et al. (2016) revealed that PI3K/AKT and p38 MAPK-regulated induction of Hsp70 and Hsp27 is key mechanisms to regulate the response of human esophageal microvascular endothelial cells to acidic pH stress. Furthermore, in esophageal epithelia in reflux esophagitis, ERK1/2 is found involved in regulating the tight junction proteins that are sensitive markers of reflux esophagitis (Tan et al. 2014). Based on our results, we assume that activation of ERK and PI3K/AKT pathways may be downstream mechanisms to mediate the role of miR-128-3p in regulating HCl-induced Het-1A cell injury. Alltogether, our findings indicate that downregulation of microRNA-128-3p may protect human esophageal squamous cells Het-1A

from HCl-induced cell injury *via* targeting E2F3 and inhibiting the activation of ERK and PI3K/AKT pathways. These findings provide the experimental basis for targeted treatment of reflux esophagitis. Further studies are required to confirm our observations.

4. Experimental

4.1. Cell culture

Human esophageal squamous cell line Het-1A (American Type Culture Collection, Manassas, VA, USA) was obtained and then cultured in bronchial epithelial cell medium (BulletKit, Lonza, Walkersville, MD) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), followed by maintaining in a humidified incubator with 5 % CO_2 at 37 °C.

4.2. Cell treatment

Het-1A cells (2×10^5) were plated in each well of 6-well plates and then incubated for 24 h. HCl was added to the medium until pH 4.0, which was then used to incubate cells for 5, 15 and 30 min at 37 °C. The same volume of phosphate-buffered saline (PBS) was used as negative control. After treatment, cells were washed with complete medium and collected for subsequent analyses.

4.3. Cell transfection

For cell transfection, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions. Control and HCl-treated Het-1A cells were transfected with miR-128-3p mimic, miR-128-3p inhibitor, siRNAs specially targeting E2F3 (si-E2F3) or their corresponding controls (GenePharma, Shanghai, China). At 48 h after transfection, cells were collected for the following experiments.

4.4. Real-time qPCR

TRIzol (Invitrogen) was used for RNA isolation from different treated Het-1A cells and DNaseI (Promega, Madison, WI, USA) was added for removing DNA contamination. Subsequently, 1 μg RNAs from each samples were reverse transcribed into cDNAs using Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). Using FastSTART Universal SYBR Green Master (ROX; Roche), real-time qPCR was completed on the ABI PRISM 7500 real time PCR System (Applied Biosystems, Foster City, CA). The primers were synthesized by GenePharma. Finally, the expression levels of miR-128-3p and E2F3 were normalized to GAPDH or U6 snRNA expression Data and then analyzed according to the $2^{-\Delta\Delta Ct}$ method.

4.5. Cell viability assay

Using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA), cell viability under different treatments was evaluated. Briefly, Het-1A cells were collected after different treatments and then seeded into 96-well plates (2×10^3 cells/well). After culture for 1-4 days, 20 μL of MTT (5 mg/mL) was added to each well for another 4 h of incubation. Followed by dissolving the formazan crystals with150 μL of dimethylsulfoxide for 10 min, the absorbance of each well at a wavelength of 490 nm was measured by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

4.6. BrdU labeling

Cells were plated on coverslips in 6-well plates, incubated in Dulbecco's modified eagle medium (DMEM) containing 0.1% FBS for 72 h and then fed with DMEM containing 10% FBS. At different treatments, Het-1A cells were incubated with DMEM containing BrdU solution for 2 h at 37 °C, followed by fixation and permeabilization. After denaturing DNA with HCl, Het-1A cells were incubated with mouse anti-BrdU antibody (G3G4, 1:20, Hybridoma Bank, Iowa City, IA) and detected by FITC antimouse secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA, USA).

4.7. Apoptosis analysis

After different treatments, Het-1A cells were collected and resuspended in 200 μL binding buffer. Using the Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China), apoptotic cells were detected on a flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA) after staining by 10 μL Annexin V-FITC and 5 μL propidium iodide (PI) for 30 min in the dark at 37 °C. The data was then analyzed by Cell Quest software (Becton, Franklin Lakes, NJ, USA).

4.8. Dual-luciferase reporter assay

The 3'-UTRs of E2F3 containing miR-128-3p binding sites were amplified and cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) to construct the luciferase vectors pmirGLO-E2F3-WT. Using the fusion PCR method, the E2F3 3'UTR carrying the mutated sequence in the complementary site for miR-128-3p was generated, which was also inserted into pmirGLO vector to construct the luciferase vectors pmirGLO-E2F3-MUT. For dual-luciferase reporter assay, Het-1A cells were cotransfected with the luciferase vectors with miR-128-3p mimic or mimic NC for 48 h. Luciferase activity was then detected by the Dual-Glo luciferase assay kit (Promega).

4.9. Western blot

After different treatments, cells were collected and cellular proteins in each group were extracted using the lysis buffer (Beyotime, Shanghai, China). After quantification of protein extracts using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA), proteins in each sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following transferring onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad), the protein blots were incubated with primary antibodies (1:1000) overnight at 4 °C. Anti-Bcl-2, anti-Bax , anti-pro-caspase-3, anti-cleaved-caspase-3, anti-pro-caspase-9, anti-cleaved-caspase-9, anti-CDK2, anti-CDK4, anti-clyclin D1; anti-CDK6, anti-clyclin E1, anti-E2F3, anti-ERK1/2, anti-p-ERK1/2, anti-p-PI3K, anti-PI3K, anti-p-AKT, anti- AKT and anti-β actin were purchased from Abcam (Cambridge, UK). Followed by further incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000, Abcam) for 1 h at 37 °C, the protein bands were developed by chemiluminescence and revealed by autoradiography (Applygen Technologies, Beijing, China).

4.10. Statistical analysis

Data were expressed as mean±standard deviations (SD) from three independent assays in each experiment. Differences between two groups were compared by student t tests or one-way ANOVA using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was obtained when $P < 0.05$.

Conflicts of interest: None declared.

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