Original Article
Down-regulation of microRNA-133b protects human esophageal squamous cells Het-1A from hydrochloric acid-induced apoptosis

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Abstract: Reflux esophagitis (RE) is an esophageal mucosal injury, the directly main cause of RE is excessive exposure of gastric contents. This study was aimed to investigate whether miR-133b was involved in hydrochloric acid (Hcl) induced RE. Human esophageal squamous cells Het-1A were employed and were exposed to Hcl, subsequently the expression of miR-133b was detected by quantitative PCR (qPCR). Cells were transfected with miR-133b mimic or inhibitor, and then cell viability, apoptosis and the expression levels of apoptosis related factors were respectively determined by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT), flow cytometry and Western blot. Further, cells were treated with Hcl or transfected with miR-133b mimic or/and inhibitor, Western blot was performed to explore the expression of extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase/serine/threonine kinase (PI3K/AKT) pathway proteins. Results showed that Hcl exposure significantly inhibited cell viability ($P < 0.05$) and up-regulated miR-133b ($P < 0.001$). Overexpression of miR-133b decreased cell viability ($P < 0.01$), induced apoptosis ($P < 0.001$), and up-regulated the levels of caspase-3, Fas and p53 ($P < 0.01$ or $P < 0.001$). Besides, Hcl exposure and miR-133b overexpression inhibited the phosphorylations of ERK1/2, AKT and PI3K ($P < 0.05$, $P < 0.01$ or $P < 0.001$), However, miR-133b suppression abolished the regulatory effects of miR-133b overexpression on these factors ($P < 0.01$ or $P < 0.001$). In conclusion, the expression of miR-133b might be involved in Hcl induced Het-1A cells injury via regulating ERK and PI3K/AKT pathways.

Keywords: Reflux esophagitis, miR-133b, hydrochloric acid, cell viability, apoptosis

Introduction

Reflux esophagitis (RE) is an esophageal mucosal injury that is characterized by esophageal erosions and can result in complications, such as esophageal stricture, Barrett's esophagus, and esophageal adenocarcinoma [1]. The pathogenesis of RE remains poorly understood, while age, unhealthy diet, *Helicobacter pylori* infection and central obesity have been identified as risk factors of RE [2, 3]. More important, excessive exposure to gastric contents is a directly main cause of RE, that hydrogen ion in gastric contents diffuses into the mucosa, and leads to tissue necrotic damage [4, 5]. Thus, pH control has been considered as important RE management, and alleviates the effects of hydrogen ion on mucosa can be a novel direction to prevent and treat this disease [6].

MicroRNAs (miRNAs), a class of short non-coding RNAs, have gained recognition as key post-transcriptional regulators in almost all biological processes [7]. In addition, miRNAs are implicated with a large number of diseases, including esophagitis. For instance, miR-143, miR-145 and miR-205 were expressed at higher levels in squamous mucosa of individuals with ulcerative esophagitis [8]. MiR-143, miR-145 and miR-194 expressions were increased in RE biopsies compared with normal squamous epithelium [9]. Besides, miRNAs have been found play a regulatory role in the pathogenesis of eosinophilic esophagitis by targeting immune-related genes [10]. These previous literatures lead us to hypothesize that miRNAs may associated with the occurrence and progression of RE.

MiR-133b has been reported as a tumor suppressor in numerous types of cancers, such as colorectal, gastric, bladder and lung cancer [11]. However, to the best of our knowledge, the role of miR-133b in RE has not yet clear. Thus, in this study, human esophageal squamous cell line Het-1A was employed and exposed to hydrochloric acid (Hcl; pH 4.0), then the expression of miR-133b was detected, to reveal the precise correlations between Hcl and miR-133b. Additionally, Het-1A cells were transfected with miR-133b mimic or inhibitor, and then the detailed functional effects of dysregulated miR-133b on cell were determined. Further, cells were treated with Hcl or transfected with miR-133b mimic or/and inhibitor, and then the protein expression changes of extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase/serine/threonine kinase (PI3K/AKT) pathways were monitored, to explore the possibly underlying mechanism of Hcl in Het-1A cells. This study might provide us a basic understanding of Hcl on the pathogenesis of RE.

Materials and methods

Cell culture

Human esophageal squamous cell line Het-1A was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in bronchial epithelial cell medium (BEGM; BulletKit, Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) [12]. Cells were incubated in a humidified 5% CO_2 incubator at 37°C.

Acid treatment and cell transfection

Cells were plated in 6-well plates at a density of 2×10^5 cells/well and incubated for 24 h. HCl was added to medium to bring the solution to pH 4.0, and then cells were cultured for 5, 15 and 30 min at 37°C. The cells in negative control group were treated with the same volume of phosphate-buffered saline (PBS). Finally, cells were washed three times with complete medium and cells were collected for the forthcoming analyses [13].

For cell transfection, cells were divided into four groups and respectively transfected with miR-133b mimic, miR-133b inhibitor or their corresponding controls (GenePharma, Shanghai, China). All transfections were performed by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. At 48 h after transfection, cells were collected and used in the following experiments.

Real-time quantitative PCR (qPCR)

Total RNA in cells was lysed in TRIzol (Invitrogen) for RNA isolation and DNaseI (Promega) was used for removing the DNA contamination. Afterward, 1 μg RNAs from each samples were applied to reverse transcription into cDNAs by using Transcriptor First Strand cDNA Synthesis Kit (Roche, USA), according to the manufacturer's instructions. qPCR was performed using FastSTART Universal SYBR Green Master (ROX; Roche, USA) and was conducted on the ABI PRISM 7500 real time PCR System (Applied Biosystems, Foster City, CA) [14]. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method, and were normalized to GAPDH or U6 snRNA expression. All primers were synthesized by GenePharma (Shanghai, China).
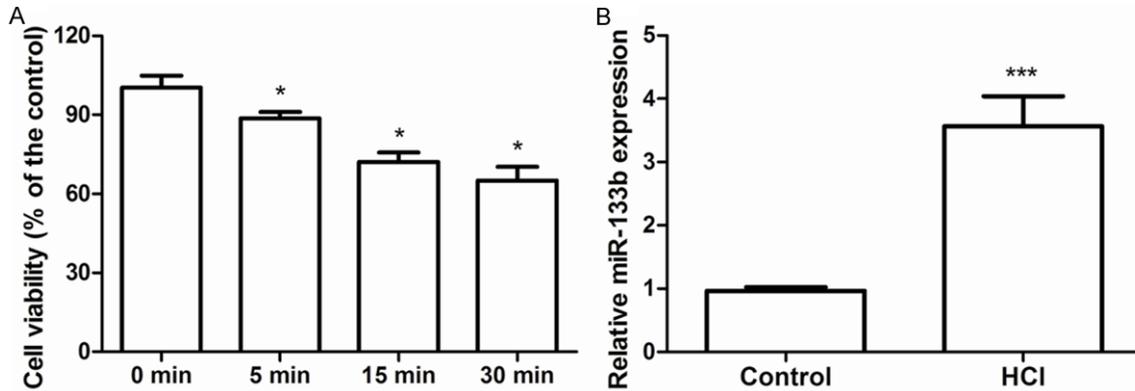
Cell viability assay

Cell viability was determined using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bro-mide (MTT) assay. Cells were treated with Hcl or transfected with miR-133b mimic or inhibitor, and then cells were collected and seeded into 96-well plates at a density of 2×10^3 cells/well. After 1-4 days' culture, 20 μL of MTT (5 mg/mL; Sigma, St. Louis, MO, USA) was added to each well and incubated for another 4 h. Dimethylsulfoxide (DMSO) of 150 μL was added into each well to dissolve the formazan crystals. The plates were shaken for 10 min gently and the absorbance was measured by a microplate reader (Bio Rad Laboratories, Hercules, CA, USA) at a wavelength of 490 nm [15].

Quantification of apoptotic cells

Cell apoptosis was performed by using the Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). After transfection, cells in each group were collected and resuspended in 200 μL binding buffer. Cells were then stained by 10 μL Annexin V-FITC and 5 μL PI for 30 min in the dark at room temperature. Finally, 300 μL phosphate buffer saline

Figure 1. Hcl inhibited Het-1A cells viability and up-regulated the expression of miR-133b. A: Het-1A cells were exposed to pH 4.0 for 0-30 min, and then cell viability was measured by MTT analysis. B: After 30 min of acid exposure, the mRNA level expression of miR-133b in cells was monitored by qPCR. Hcl, hydrochloric acid; miR-133b, microRNA-133b; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide; qPCR, quantitative PCR; *$P < 0.05$; ***$P < 0.001$.

(PBS) was added and 2×10^4 cells were analyzed on the flow cytometry (Becton Dickinson) [16].

Western blot

Cells were treated with Hcl or transfected with miR-133b mimic or/and inhibitor. Then, cells were collected and cellular proteins were extracted using the lysis buffer (Beyotime, Shanghai, China). Quantification of proteins was performed using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Equal amounts of proteins in each sample were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA) [17]. The blots were blocked within 5% skim milk for 1 h at room temperature, and then were incubated overnight at 4°C with primary antibodies. Active caspase-3 (ab2302), pro caspase-3 (ab32150), Fas (ab82419), p53 (ab1431), p-PI3K (ab182651), t-PI3K (ab86714) and GAPDH (ab9485) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); p-ERK1/2 (sc-136521), t-ERK1/2 (sc-292838), p-AKT (sc-33437) and t-AKT (sc-8312) were obtained from Abcam (Cambridge, MA). The blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were developed by chemiluminescence and autoradiography using X-ray film (Applygen Technologies, Beijing, China). The intensity of the bands was quantified using ImageJ 1.49

(National Institute of Health, Bethesda, MD) [18].

Statistical analysis

Data were expressed as mean ± standard deviations (SD) from three independent assays. Differences between the mean values of two groups were analyzed by student *t* tests. Statistical significance was considered at $P < 0.05$ with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Hcl inhibited Het-1A cells viability and up-regulated the expression of miR-133b

To verify the impacts of Hcl on human esophageal squamous cells, Het-1A cells were exposed to pH 4.0, and then cell viability was measured. As results showed in **Figure 1A**, cell viability was significantly decreased after cells were treated with Hcl for 5-30 min (all *P* < 0.05). Besides, it seemed that Hcl effectively inhibited cell viability in a time dependent manner. Subsequently, in order to explore the underlying mechanism in which Hcl affected Het-1A cells, the mRNA level expression of miR-133b in cells which were treated with Hcl for 30 min was detected by qPCR. We found that (**Figure 1B**), Hcl significantly increased the expression level of miR-133b. Thus, we temporarily speculated that *in vitro* exposure of Hcl caused significantly inhibition of cell viability, and miR-133b might be involved in the effects of Hcl in Het-1A cells.
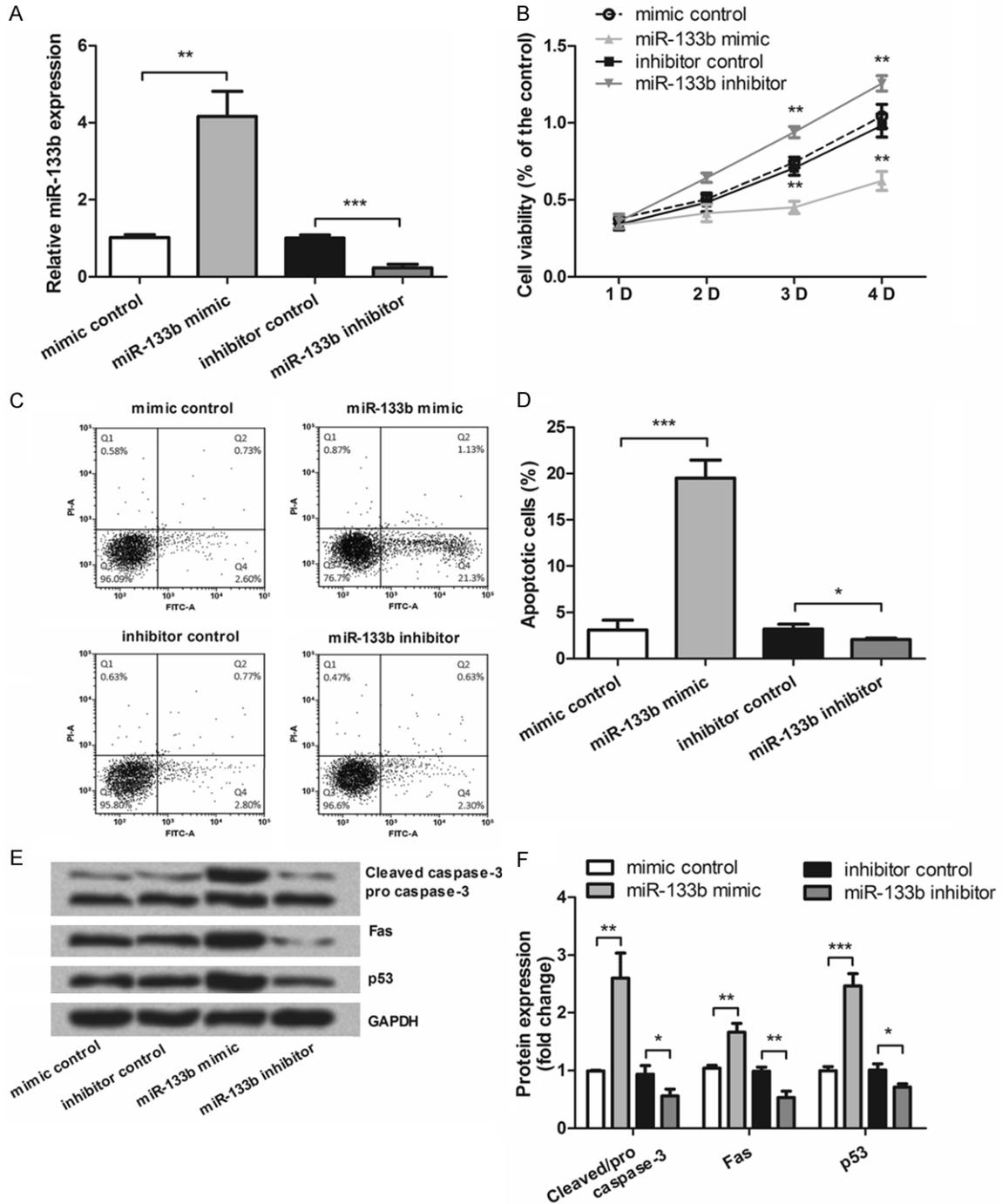
Figure 2. Overexpression of miR-133b decreased cell viability and induced apoptosis. A: Het-1A cells were transfected with miR-133b mimic, miR-133b inhibitor, or their corresponding controls, subsequently transfection efficiency was verified by qPCR. B: After transfection, cells were cultured for 1-4 days, and cell viability was measured by MTT. C and D: The transfected cells apoptosis was detected by flow cytometry. E and F: The expression levels of apoptosis related proteins in transfected cells were detected by Western blot. MiR-133b, microRNA-133b; qPCR, quantitative PCR; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Overexpression of miR-133b decreased cell viability and induced apoptosis

To explore the detailed functions of miR-133b on human esophageal squamous cells, Het-1A cells were transfected with miR-133b mimic, miR-133b inhibitor, or their corresponding controls. Transfection efficiency was verified by qPCR and results showed that, miR-133b overexpressed and suppressed cells were obtained
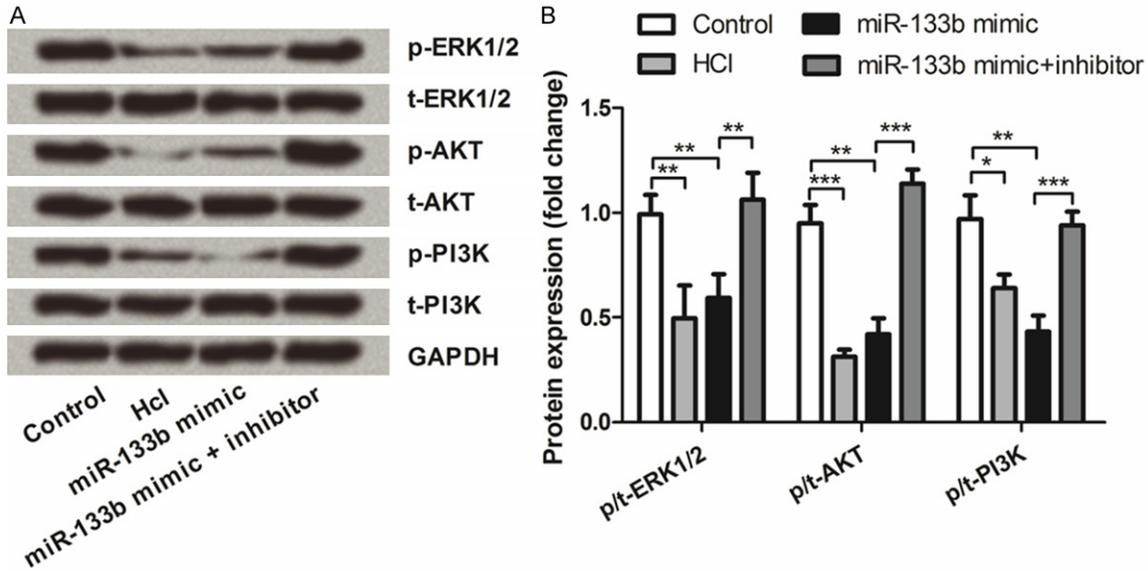
Figure 3. Down-regulation of miR-133b alleviated Hcl induced apoptosis via activation of ERK and PI3KAKT pathways. A and B: Het-1A cells were exposed to pH 4.0 for 30 min, or transfected with miR-133b mimic or/and miR-133b inhibitor. Afterward, the expression levels of ERK and PI3K/AKT pathway proteins were detected by Western blot. MiR-133b, microRNA-133b; Hcl, hydrochloric acid; ERK, extracellular signal-regulated kinase; PI3K/AKT, phosphatidylinositol-3-kinase/serine/threonine kinase; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

successfully (**Figure 2A**; $P < 0.01$ or $P < 0.001$). Afterward, cell viability and apoptosis were determined by MTT analysis and flow cytometry respectively. As results showed in **Figure 2B-D**, miR-133b overexpression significantly inhibited cell viability ($P < 0.01$), while induced apoptosis ($P < 0.001$). However, miR-133b suppression displayed the reversed impacts towards cell viability and apoptosis ($P < 0.05$ or $P < 0.01$). Furthermore, the expressions of apoptosis related factors in transfected cells were detected by Western blotting. Results showed that (**Figure 2E** and **2F**), the levels of caspase-3, Fas and p53 were significantly up-regulated by miR-133b overexpression ($P < 0.01$ or $P < 0.001$), whereas were down-regulated by miR-133b suppression ($P < 0.05$ or $P < 0.01$). Taken together, miR-133b might be a pivotal regulator in Het-1A cells viability and apoptosis.

Down-regulation of miR-133b alleviated Hcl induced apoptosis via regulating ERK and PI3K/ AKT pathways

To further investigated the connection between Hcl treatment and miR-133b expression, Het-1A cells were treated with Hcl for 30 min, or transfected with miR-133b mimic or/and miR-133b inhibitor. Afterward, the expression levels of ERK and PI3K/AKT pathway proteins were detected by Western blot. As results showed in **Figure 3A** and **3B**, down-regulations of p-ERK1/2, p-AKT and p-PI3K were found in cells which were treated with Hcl or were transfected with miR-133b mimic ($P < 0.05$, $P < 0.01$ or $P < 0.001$). Surprisingly, miR-133b inhibitor significantly recovered the regulatory effects of miR-133b mimic on these factors ($P < 0.01$ or $P < 0.001$). Therefore, we inferred that, down-regulation of miR-133b alleviated Hcl induced injury might be via regulating ERK and PI3K/ AKT pathways.

Discussion

In the present study, we found that Hcl inhibited Het-1A cells viability and up-regulated the expression of miR-133b. In addition, overexpression of miR-133b notably decreased cell viability, induced apoptosis and up-regulated the expressions of apoptosis related factors, *i.e.,* caspase-3, Fas and p53. Furthermore, miR-133b overexpression and Hcl have the similarly regulatory impacts on the ERK and PI3K/AKT pathways. However, miR-133b inhibitor significantly recovered these regulatory functions.

Acid-induced cell injury has been observed previously in humans and in experimental models

of esophagitis. Zhang *et al.* have shown that acid exposure in non-neoplastic Barrett's epithelial cells causes early anti-proliferative effects that are mediated by the activation of Checkpoint Kinase 2 (Chk2) [19]. Yamada *et al.* suggested that gastric acid, together with bile acid reflux, could affect the esophageal mucosa, even under reflux times of a few minutes [20]. Consistent with these previous studies, in this study, we found that Hcl could remarkably inhibit Het-1A cells viability and induce apoptosis. However, the underling mechanism of which Hcl impacts Het-1A cells is still a mystery.

An increasing number of literatures have reported that miR-133b serves as a pivotal regulator in multiple biological progresses, especially in cell proliferation and apoptosis. Chen *et al.* found that miR-133b could inhibit the proliferation and induce apoptosis in urothelial carcinoma of the bladder cells [21]. Using a model of prostate cancer, Patron *et al.* demonstrated that overexpression of miR-133b impaired proliferation and enhanced death receptor-induced apoptosis [22]. Nevertheless, our study provided the first evidence suggesting that miR-133b also play a regulatory role in Het-1A cells viability and apoptosis. Fas is widely known as a key modulator in apoptosis, and it triggers apoptosis via two distinct mechanisms dependent on cell types [23]. That is, Fas mediates apoptosis via activating effector caspase-3 and caspase-7, or via modulating mitochondrial pathway [24]. Cells lacking the p53 gene can also undergo apoptosis via the modulation of different proteins [25]. Moreover, previous evidences suggested an intriguing link between Fas and p53, that Fas death receptor gene contains a p53-responsive element, which is bound only by wild-type p53 [26]. In the present study, the protein expression levels of caspase-3, Fas and p53 were all up-regulated by miR-133b overexpression, proved that miR-133b served as an apoptosis inductor in Het-1A cells.

PI3K/AKT represents the main signaling pathway responsible for cell proliferation, survival, metabolism and motility regulation and apoptosis [27]. In some contexts, ERK acts as downstream target of PI3K [28]. Additionally, ERK pathway possesses anti-apoptotic functions, depending on cell type and stimuli [29]. Previous studies have linked PI3K/AKT and ERK2

signaling pathways in acid exposure induced esophagitis and Barrett's esophagus [30, 31]. Interestingly, our data showed that the phosphorylated forms of ERK1/2, AKT and PI3K were all down-regulated by Hcl, indicating that Hcl affected Het-1A cells via regulating these factors. Furthermore, miR-133b overexpression displayed the similar impacts as Hcl in these proteins, and miR-133b suppression abolished these regulatory impacts. Thus, we inferred that miR-133b might be a regulator in Hcl induced cell injury by regulating ERK and PI3K/AKT pathways.

In summary, Hcl exposure inhibited Het-1A cells viability in a time-dependent manner. MiR-133b was positively regulated by Hcl, and miR-133b might be involved in Hcl-induced cell injury via blockade of ERK and PI3K/AKT pathways. Nevertheless, more work still needed to strive to confirm these hypotheses.

Disclosure of conflict of interest

None.

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