

## MicroRNA-133a Inhibits Proliferation of Gastric Cancer Cells by Downregulating ERBB2 Expression

Chang Li,\* Xiaoping Li,† Shuohui Gao,\* Chang Li,‡ and Lianjun Ma§

\*Department of Gastrointestinal Internal Medicine, China–Japan Union Hospital of Jilin University, Changchun, P.R. China

†Department of Pediatrics, The First Hospital of Jilin University, Changchun, P.R. China

‡Department of Cadre’s Ward, China–Japan Union Hospital of Jilin University, Changchun, P.R. China

§Endoscopy Center, China–Japan Union Hospital of Jilin University, Changchun, P.R. China

Gastric cancer is the fourth most common type of cancer and the second highest leading cause of cancer-related deaths worldwide. It has already been established that miR-133a is involved in gastric cancer. In this study, we investigated the molecular mechanisms by which miR-133a inhibits the proliferation of gastric cancer cells. We analyzed the proliferative capacity of human gastric cancer cells SNU-1 using an MTT assay. Cell apoptosis was determined using flow cytometry. The expression levels of ERBB2, p-ERK1/2, and p-AKT in SNU-1 cells were determined using Western blot analysis. To confirm that ERBB2 is a direct target of miR-133a, a luciferase reporter assay was performed. Results showed that miR-133a overexpression inhibited SNU-1 cell proliferation and increased apoptosis. ERBB2 was a direct target of miR-133a, and it was negatively regulated by miR-133a. Interestingly, ERBB2 silencing has a similar impact to miR-133a overexpression, in that it significantly induced apoptosis and inhibited ERK and AKT activation. Our study showed that miR-133a inhibits the proliferation of gastric cancer cells by downregulating the expression of ERBB2 and its downstream signaling molecules p-ERK1/2 and p-AKT. Therefore, miR-133a might be used as a therapeutic target for treating gastric cancer.

**Key words: MicroRNA-133a (miR-133a); Gastric cancer; ERBB2; p-ERK1/2; p-AKT**

### INTRODUCTION

Gastric cancer is the fourth most common type of cancer and the second highest leading cause of cancer-related deaths globally<sup>1</sup>. The common presenting symptoms of gastric cancer include weight loss, poor appetite, abdominal pain and discomfort, heartburn, nausea, vomiting, and pallor<sup>2</sup>. Generally, gastric cancer is diagnosed at an advanced stage, and the prognosis of patients with gastric cancer is often poor<sup>3</sup>. In addition, the molecular mechanisms involved in the development and progression of gastric cancer are not clear. It is very important to understand the different molecular pathways implicated in gastric cancer, as it would help in the identification of target biomarkers and therefore the development of novel drugs.

It has been shown that the deregulation of oncogenes or tumor suppressors, including microRNAs (miRNAs), leads to the development and progression of gastric cancer<sup>4,5</sup>. miRNAs are small short noncoding RNAs that are ~22 nucleotides in length<sup>6</sup>. miRNAs act at the posttranscriptional level and modulate various cellular

processes such as cell proliferation, invasion, and apoptosis<sup>7</sup>. Upregulation or downregulation of miRNAs affects the expression of various proteins and is therefore involved in tumorigenesis by facilitating proliferation and invasion<sup>8</sup>.

It has been found that aberrant expression of certain miRNAs, including miR-133a<sup>9</sup>, is associated with the development and progression of gastric cancer. In addition, the role of miR-133a as a tumor suppressor has been studied in other cancers including ovarian<sup>10</sup>, non-small cell lung<sup>11</sup>, osteosarcoma,<sup>12</sup> and esophageal squamous cell carcinoma<sup>12</sup>. Recently, Qiu et al.<sup>9</sup> reported that miR-133a inhibits cell proliferation, invasion, migration, and cell cycle progression via targeting transcription factors, especially protein 1, in gastric cancer. As miRNAs target a range of mRNAs, other oncogenes may also be involved in miR-133a-mediated cell proliferation and invasion in gastric cancer.

In the present study, we examined the role of miR-133a in gastric cancer cell proliferation and apoptosis, and the relationship between miR-133a and ERBB2 expressions

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Address correspondence to Chang Li, Department of Cadre’s Ward, China–Japan Union Hospital of Jilin University, No. 126, Xiantai Street, Changchun 130033, P.R. China. E-mail: [lichang23456@126.com](mailto:lichang23456@126.com) or Lianjun Ma, Endoscopy Center, China–Japan Union Hospital of Jilin University, No. 126, Xiantai Street, Changchun 130033, P.R. China. E-mail: [horsejlm@jlu.edu.cn](mailto:horsejlm@jlu.edu.cn)

in gastric cancer cells. ERBB2 (also known as HER2) is a transmembrane tyrosine kinase receptor and a member of the epidermal growth factor receptor (EGFR) family. ERBB2 is expressed in many tissues, including the stomach, breast, heart, and kidney. Overexpression of ERBB2 can promote cell proliferation and inhibit apoptosis, resulting in uncontrolled or excessive cell growth and tumorigenesis<sup>13</sup>. Thus, overexpression of ERBB2 plays an important role in the development and progression of human cancers.

## MATERIALS AND METHODS

### Cell Culture

Human gastric cancer cell line SNU-1 was grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/ml penicillin sodium, and 100 µg/ml streptomycin sulfate (Sigma-Aldrich, St. Louis, MO USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub><sup>14</sup>.

### Cell Proliferation Analysis

The proliferative capacity of SNU-1 cells was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. On each day of the assay, 100 µl of cells was placed into a 96-well plate, in triplicate, and incubated from day 1 to day 5 at 37°C. To assess cell proliferation, 50 µg of MTT (Sigma-Aldrich) was added to each well and then incubated for 4 h at 37°C. After incubation, 100 µl of 0.04 N HCl in 2-propanol was added and mixed thoroughly. The absorbance was detected at 570 nm using a Microplate Reader (Molecular Devices, Sunnyvale, CA, USA), with a subtraction of background reading at 650 nm<sup>15</sup>.

### Cell Transfection

For precursor miRNA transfection, hsa-miR-183 pre-miR miRNA precursor was obtained from Ambion (No. A25576; Austin, TX, USA). Precursor molecules were transfected with SNU-1 cells using siPORT Amine transfection reagent (Ambion) according to the manufacturer's instructions. Transfection effects were evaluated after 48 h. This experiment was performed in triplicate. Cells transfected with the Pre-miR<sup>TM</sup> miRNA precursor molecule (negative control) were used as a control in all transfection experiments.

For ERBB2 knockdown, siRNA-targeted ERBB2 (si-ERBB2) and siRNA negative control (si-NC) were purchased from Invitrogen. Cells were transfected with si-ERBB2 and si-NC using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

The efficiency of the overexpression of miR-133a and the silencing of ERBB2 were evaluated by quantitative RT-PCR (qRT-PCR).

### Western Blotting

SNU-1 cells were washed twice with phosphate-buffered saline (PBS) and lysed with 1× sodium dodecyl sulfate (SDS)-loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue). The protein samples [ERBB2, β-actin, p-AKT, and extracellular signal-regulated kinases 1 and 2 (ERK1/2)] were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting analysis was carried out with the respective primary antibodies: anti-ERBB2 and anti-β-actin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA); anti-p-AKT and anti-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA). The proteins were detected by enhanced chemiluminescence (ECL-plus; Amersham Pharmacia Biotech, Piscataway, NY, USA). In these analyses, β-actin served as a control for ERBB2, phosphorylated ERK1/2 (p-ERK1/2), and p-AKT<sup>16</sup>.

### Apoptosis Analysis

The Dead Cell Apoptosis Kit with Annexin-V FITC and PI (V13242; Invitrogen) was used to perform cell apoptosis analysis according to the manufacturer's instructions. Briefly, cells (3×10<sup>6</sup>) were stained using the apoptosis kit and suspended in annexin V-binding buffer (Invitrogen). The suspended cells were used to perform flow cytometry. Cell apoptosis was determined using the FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). In total, 10,000 cells were analyzed per measurement. Data were analyzed using the FlowJo 10.0.7 software (Treestar Inc., Ashland, OR, USA)<sup>17</sup>.

### qRT-PCR Analysis

Total RNA was extracted with TRIzol reagent (Invitrogen). RNA (500 ng) was polyadenylated and reverse transcribed to cDNA using an NCode miRNA First-Strand cDNA synthesis kit (Invitrogen). cDNA was used as a template for real-time PCR FastStart Universal SYBR green Master (Roche) with the universal reverse primer provided in the kit. RQ-PCR was performed on an Applied Biosystems real-time detection system (Applied Biosystems, Foster City, CA, USA), and the thermocycling parameters were 95°C for 3 min and 40 cycles of 95°C for 15 s followed by 60°C for 30 s. Each sample was run in triplicate and was normalized to U6 snRNA levels [U6 primers 5'-CTT CGG CAG CAC ATA TAC T-3' (forward) and 5'-AAA ATA TGG AAC GCT TCA CG-3' (reverse)]. Melting curve analysis was performed to confirm the specificity of the PCR products. The replicates were then averaged, and fold induction was determined by a ΔΔCT-based fold change calculation<sup>18</sup>.

### Luciferase Activity Assay

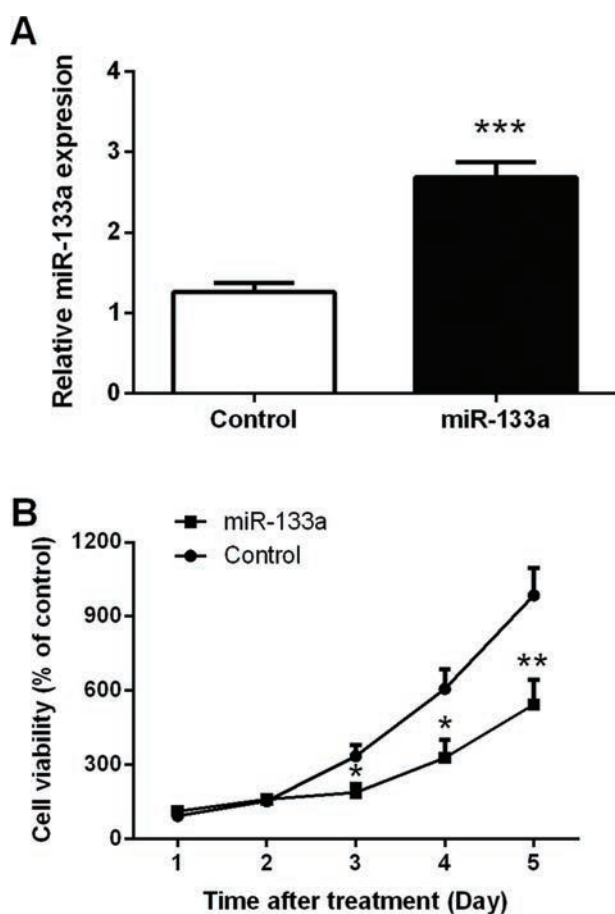
The 3'-UTR segment of the ERBB2 gene, containing the miR-133a binding site, was amplified through

PCR and inserted into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). SNU-1 cells were cotransfected with ERBB2 3'-UTR and miR-133a or control using Lipofectamine 2000 (Invitrogen). The luciferase activity was analyzed at 48 h posttransfection using the Dual-Luciferase Reporter Assay System (Promega). For each transfection, the luciferase activity was averaged from three replicates<sup>19</sup>.

## RESULTS

### *miR-133a Overexpression Inhibits Proliferation of Gastric Cancer Cells*

Human gastric cancer cells (SNU-1) were transfected with miR-133a or control, and the expression level of miR-133a was determined using qRT-PCR. The expression level of miR-133a was higher than that of the



**Figure 1.** miR-133a overexpression inhibits the proliferation of gastric cancer cells. (A) Quantitative RT-PCR (qRT-PCR) was performed to determine the relative expression of microRNA-133a (miR-133a) in SNU-1 cells transfected with miRNA-133a or control. (B) MTT assay was used to determine the proliferative capacity of SNU-1 cells, transfected with miR-133a or control, at day 1 to day 5. Data are presented as mean  $\pm$  standard error. Control: Pre-miR<sup>TM</sup> miRNA precursor molecule. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

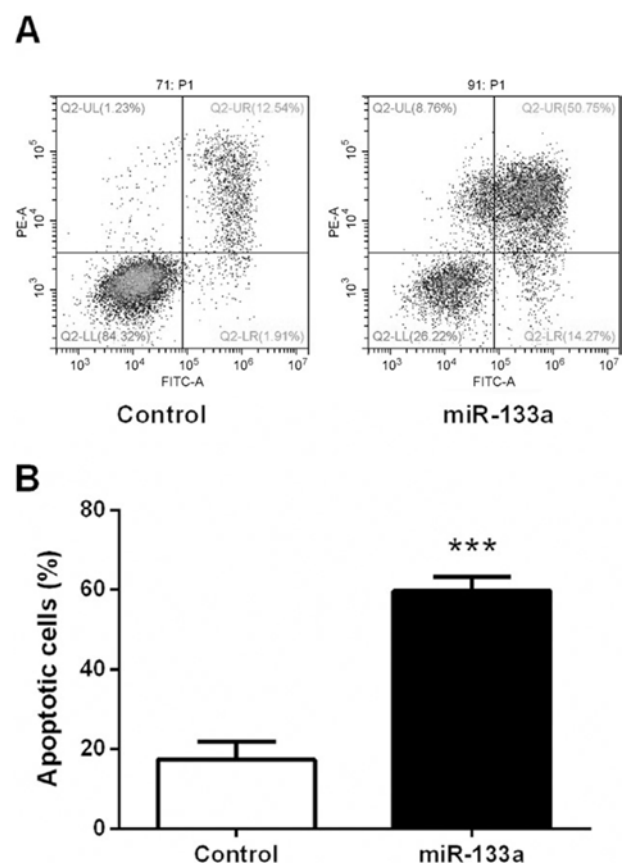
control ( $p < 0.001$ ) (Fig. 1A). We then used an MTT assay to determine the proliferative capacity of SNU-1 cells. Overexpression of miR-133a markedly inhibited the proliferation of SNU-1 cells compared to the control (Fig. 1B).

### *miR-133a Overexpression Promotes Apoptosis of Gastric Cancer Cells*

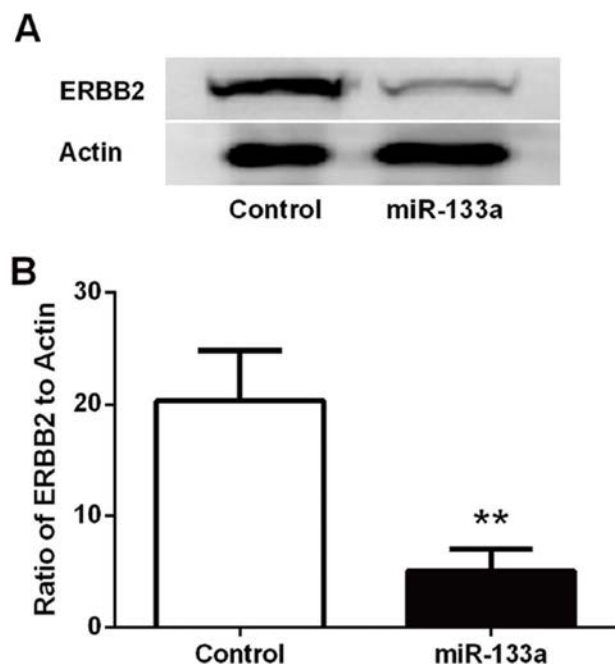
Flow cytometry was used to determine the apoptosis of SNU-1 cells that were transfected with miR-133a or control. The apoptotic cell rate was higher in the miR-133a-transfected group than those in the control group ( $p < 0.001$ ) (Fig. 2), indicating that overexpression of miR-133a promoted apoptosis of SNU-1 cells.

### *miR-133a Overexpression Downregulates the Expression of ERBB2 in Gastric Cancer Cells*

Western blot analysis was used to determine the expression levels of ERBB2 with  $\beta$ -actin expression as control in SNU-1 cells transfected with miR-133a or control. The expression of ERBB2 was fourfold lower in the



**Figure 2.** miR-133a overexpression promotes apoptosis of gastric cancer cells. A cell apoptosis assay was performed to examine the rate of apoptosis of SNU-1 cells transfected with miR-133a or control. (A) Flow cytometry analysis. (B) Data from the three samples were quantitated and expressed as mean  $\pm$  standard error. Control: Pre-miR<sup>TM</sup> miRNA precursor molecule. \*\*\* $p < 0.001$ .



**Figure 3.** miR-133a overexpression downregulates the expression of ERBB2 in gastric cancer cells. Western blotting was performed to examine the expression level of ERBB2 and  $\beta$ -actin in SNU-1 cells transfected with miR-133a or control.  $\beta$ -Actin served as control for ERBB2. (A) Western blot images. (B) Data from the three samples were quantitated and expressed as mean  $\pm$  standard error. Control: Pre-miR<sup>TM</sup> miRNA precursor molecule. \*\* $p < 0.01$ .

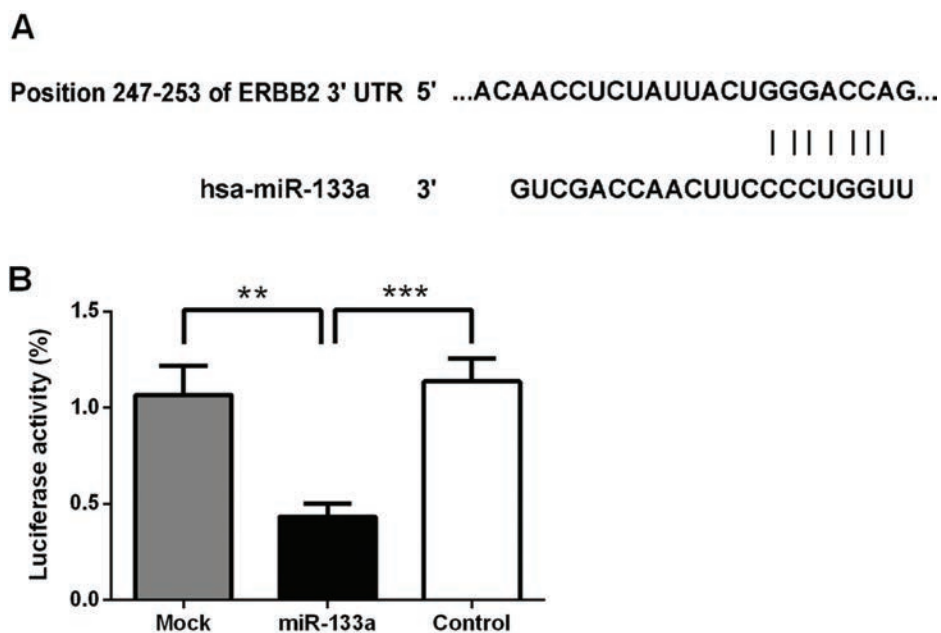
miR-133a-transfected cells compared to that in control-transfected cells ( $p < 0.01$ ) (Fig. 3). This indicates that overexpression of miR-133a reduced the expression level of ERBB2 in gastric cancer cells.

#### *ERBB2 Is a Direct Target of miR-133a in Gastric Cancer Cells*

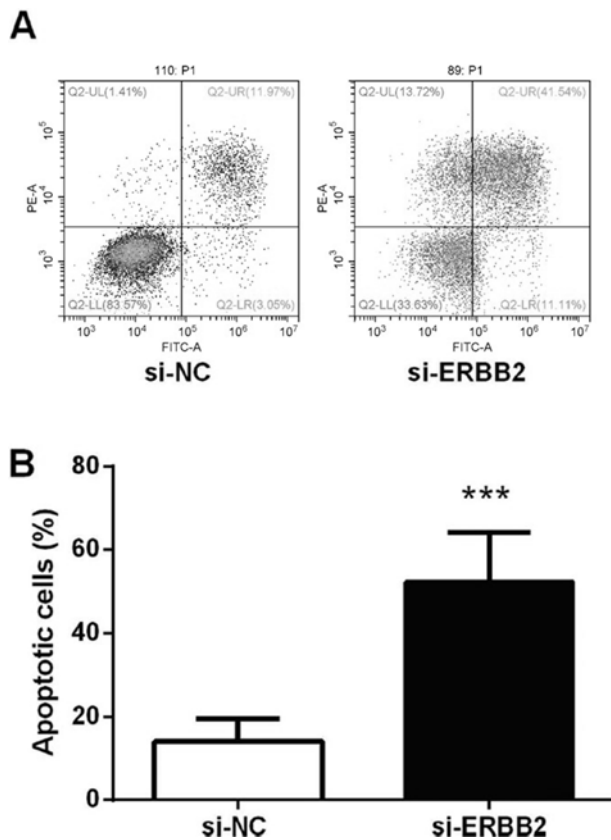
The putative seed sequence for miR-133a at the 3'-UTR of ERBB2 was indicated based on bioinformatics analysis (Fig. 4A). To further confirm that ERBB2 is a direct target of miR-133a, a luciferase reporter assay was performed using SNU-1 cells cotransfected with 3'-UTR of ERBB2 and miR-133a, control, or mock. Luciferase activity was markedly decreased in cells cotransfected with miR-133a and 3'-UTR of ERBB2 compared to the control ( $p < 0.001$ ) and mock samples ( $p < 0.01$ ) (Fig. 4B). This suggests that ERBB2 is a direct target of miR-133a in SNU-1 cells.

#### *ERBB2 Silencing Promotes Apoptosis of Gastric Cancer Cells*

Flow cytometry was performed again to assess the effects of ERBB2 silencing on SNU-1 cell apoptosis. ERBB2 silencing had a similar effect as miR-133a overexpression on SNU-1 cell apoptosis, in that it significantly enhanced apoptotic cell number ( $p < 0.001$ ) (Fig. 5). These results indicating miR-133a overexpression enhanced gastric cancer cells apoptosis possibly via targeting ERBB2.



**Figure 4.** ERBB2 is a direct target of miR-133a in gastric cancer cells. (A) Seed sequences of miR-133a in 3'-UTR of ERBB2 are indicated. (B) SNU-1 cells were cotransfected with 3'-UTR of ERBB2 and miR-133a, control, or mock, and then luciferase activities were measured. Data are presented as mean  $\pm$  standard error. Control: Pre-miR<sup>TM</sup> miRNA precursor molecule. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 5.** ERBB2 silencing promotes apoptosis of gastric cancer cells. A cell apoptosis assay was performed to examine the rate of apoptosis of SNU-1 cells transfected with ERBB2 siRNA or negative control. (A) Flow cytometry analysis. (B) Data from the three samples were quantitated and expressed as mean  $\pm$  standard error. \*\*\* $p < 0.001$ .

#### *miR-133a Overexpression Reduces the Expression of ERBB2 Downstream Signaling Molecules p-ERK and p-AKT*

Western blot analysis was used to determine the expression levels of p-ERK1/2, ERK1/2, p-AKT, and AKT in miR-133a-transfected cells and ERBB2-silenced cells. The expression levels of p-ERK1/2 and p-AKT were markedly lower in the miR-133a-transfected cells compared to those in the control-transfected cells ( $p < 0.05$  and  $p < 0.01$ ) (Fig. 6A and B). In addition, the expression levels of p-ERK1/2 and p-AKT were both lower in the ERBB2-silenced cells than those in si-NC cells ( $p < 0.01$ ) (Fig. 6C and D). This indicates that overexpression of miR-133a reduced the expression of ERBB2 and subsequently regulated p-ERK1/2 and p-AKT in SNU-1 cells.

### DISCUSSION

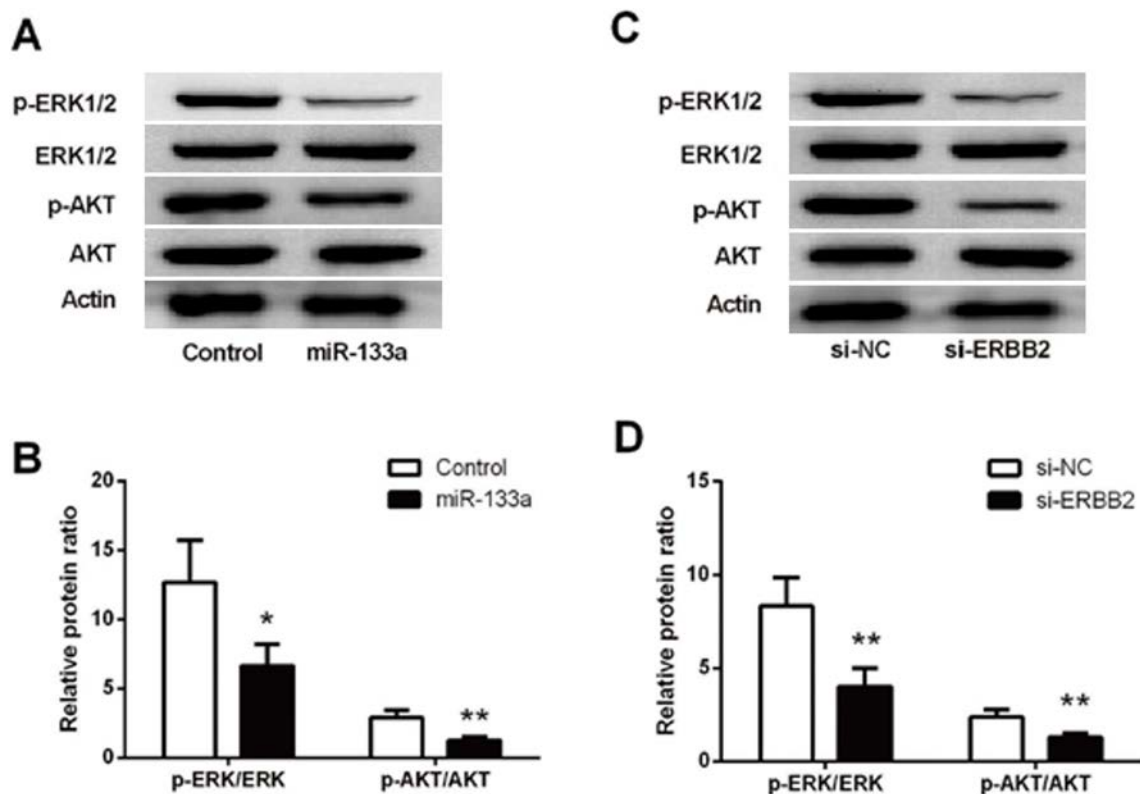
To our knowledge, studies evaluating the association of miR-133a and ERBB2 in gastric cancer have not been conducted or published. In the present study, we showed that miR-133a and ERBB2 play an important role in gastric

cancer. We demonstrated that miR-133a overexpression inhibits the proliferation of SNU-1 gastric cancer cells. Importantly, we identified that ERBB2 is a target of miR-133a in SNU-1 gastric cancer cells as overexpression of miR-133a downregulates the expression level of ERBB2 and its downstream signaling molecules p-ERK1/2 and p-AKT in gastric cancer cells. Considering these results, we strongly believe that the inhibitory effect of miR-133a on the proliferation of gastric cancer cells could be via the direct inhibition of ERBB2.

The effects of miR-133a on gastric cancer cell proliferation and apoptosis have been studied previously, albeit using different cell lines and biomarkers<sup>9,20</sup>. Qiu et al.<sup>9</sup> showed that miR-133a inhibits proliferation, migration, invasion, and cell cycle progression in gastric cancer cells (SGC7901, MKN45, and BGC823) via targeting transcription factor Sp1. A similar finding was reported by Lai et al.<sup>20</sup>, who demonstrated that miR-133a inhibits proliferation and invasion and induces apoptosis in gastric cancer cells (HGC27, GC7901, and AGS) via targeting fascin actin-bundling protein 1 (FSCN1). Our findings are consistent with theirs, as we have demonstrated that overexpression of miR-133a inhibits proliferation and promotes apoptosis of SNU-1 gastric cancer cells by direct inhibition of ERBB2.

It has been shown that ERBB2 is overexpressed in 7% to 34% of gastric cancers<sup>21-24</sup> and in many other cancers, including breast<sup>25</sup>, ovarian<sup>25</sup>, salivary<sup>26</sup>, bladder<sup>27</sup>, and lung cancers<sup>28</sup>. Furthermore, it has been reported that the expression level of ERBB2 is modulated by several miRNAs, including miR-205<sup>29</sup>, miR-125b<sup>30</sup>, and miR-4728<sup>31</sup>. The association between miR-133a and ERBB2 has been reported in many cancers, including breast<sup>32</sup>, prostate<sup>33</sup>, and cervical cancer<sup>34</sup>. In the present study, we demonstrated that miR-133a overexpression downregulates the expression of ERBB2 in gastric cancer cells. In addition, we identified that ERBB2 is a direct target of miR-133a in gastric cancer cells.

To further understand the mechanism by which miR-133a inhibits cancer cells by targeting ERBB2, we analyzed the expression levels of the downstream signaling molecules of ERBB2, namely, p-ERK1/2 and p-AKT. Dimerization of ERBB results in subsequent phosphorylation of some downstream effector proteins, including the ERK1/2 and AKT pathways<sup>35</sup>. It has been shown that inhibition of ERBB2 phosphorylation results in the inhibition of downstream proteins p-ERK1/2 and p-AKT<sup>36</sup>. ERK1/2 are members of the mitogen-activated protein kinase super family<sup>37</sup>. ERK1/2 is activated by the phosphorylation of ERK1/2 (p-ERK1/2), which generally promotes cell proliferation and inhibits apoptosis<sup>38</sup>. It has been shown that p-ERK1/2 is activated in many cancers, including non-small cell lung<sup>39</sup>, oral<sup>40</sup>, and pancreatic cancer<sup>41</sup>. Protein kinase B (AKT) is a signal transduction



**Figure 6.** miR-133a overexpression reduces the expression of ERBB2 downstream signaling molecules phosphorylated extracellular signal-regulated kinase (p-ERK) and p-AKT. (A) Western blotting was performed to examine the expression level of p-ERK1/2, ERK1/2, p-AKT, AKT, and  $\beta$ -actin in SNU-1 cells transfected with miR-133a or control (Pre-miR<sup>TM</sup> miRNA precursor molecule). (B) The data from three samples were quantitated for p-ERK1/2 and p-AKT and expressed as mean  $\pm$  standard error. (C) The expression of these proteins in SNU-1 cells transfected with ERBB2 siRNA or negative control was also detected. (D) Quantitative results from three samples were expressed as mean  $\pm$  standard error.  $\beta$ -Actin served as control for other proteins. \* $p < 0.05$ ; \*\* $p < 0.01$ .

protein involved in various cellular processes, including cell proliferation, invasion, and apoptosis. Several studies have revealed that AKT phosphorylation (p-AKT) plays a critical role in various cancers of various organs, including stomach, breast, prostate, and lung<sup>42</sup>. In the present study, we have demonstrated that miR-133a overexpression reduces the expression levels of p-ERK1/2 and p-AKT in gastric cancer cells. Similar findings have been reported by Zhang et al.<sup>43</sup>, who demonstrated that overexpression of miR-133a inhibits activation of the AKT and ERK signaling pathways, which contributes to the suppression of hepatocellular carcinoma cell growth. In addition, Wang et al.<sup>44</sup> reported that miR-133a suppressed tumor growth and metastasis in colorectal cancer by inhibiting p-ERK.

In conclusion, our data demonstrate that miR-133a inhibits proliferation and induces apoptosis of gastric cancer cells by downregulating the expression of ERBB2 and its downstream signaling molecules p-ERK1/2 and p-AKT. Our findings suggest that miR-133a may be considered to be a potential therapeutic target for the treatment of gastric cancer.

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