

# MiR-144 functions as tumor suppressor by targeting PIM1 in gastric cancer

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**Abstract. – OBJECTIVE:** Gastric cancer (GC) is one of the most prevalent types of malignant disease Worldwide. Mounting evidence has demonstrated the involvement of miRNAs in the development of GC. One of these miRNAs, miR-144 has been found aberrantly expressed in a variety of human malignancies.

**PATIENTS AND METHODS:** GC tissues were collected from patients, and the level of miR-144 was determined by qRT-PCR. GC cell lines SGC7901 and AGS were used as model cell lines and the anti-tumor effect of miR-144 in both cells were examined. The level of miR-144 was restored in GC cells using miR-144 mimic. Moreover, the target gene of miR-144 was identified.

**RESULTS:** In this study, our results showed that low miR-144 level significantly correlated with lymph node metastasis stage, TNM stage and differentiation degree. In addition, we found that miR-144 acted as a tumor suppressor in GC. Moreover, our findings showed that miR-144 exerted an anti-tumor effect by directly targeting RLIP76.

**CONCLUSIONS:** miR-144 acts as a tumor suppressor in GC and it is a potential therapeutic target for GC treatment.

Key Words:

Gastric cancer, miR-144, RLIP76.

## Introduction

Gastric cancer (GC) is one of the most prevalent malignant diseases in the world. As the fourth most prevalent human tumor and one of the leading causes of cancer death, GC accounts for 738,000 deaths<sup>1</sup>. Although advanced surgical and adjuvant therapeutic regimen have been employed, the prognosis of patients with GC still remains poor with a 5-year overall survival less

than 25%<sup>2</sup>. Therefore, the discovery of novel therapeutic targets for GC may provide insights into the development of chemotherapeutic agents.

MicroRNAs (miRNAs), a family of small non-coding RNAs, function as either oncogenes or tumor suppressors and regulate a variety of biological activities, such as cell differentiation, proliferation, apoptosis, migration and invasion through repressing the expression of target genes<sup>3</sup>. Recently, a meta-analysis of 28 studies on the microRNA profiling of 4000 cancer and adjacent normal tissues (including 33 controls) reported that miR-144 was tightly correlated with liver, lung and prostate cancers<sup>4</sup>. Accumulating evidence has showed that miR-144 can function as either oncogene or tumor suppressor. Among the cervical cancer cells, miR-144 exerted a pro-proliferative effect<sup>5</sup>. In addition, miR-144 has been reported to be aberrantly up-regulated in nasopharyngeal cancer and to promote cell proliferation and invasion<sup>6</sup>. In contrast, miR-144 is down-regulated in the colorectal cancer<sup>7</sup> and the follicular thyroid carcinoma<sup>8</sup>, suggesting the anti-tumor role of miR-144 in these malignancies. Another recent study carried out by Zhang et al<sup>9</sup> also found that miR-144 suppressed the invasion and metastasis of the laryngeal squamous cell carcinoma. In terms of GC, aberrantly down-regulation of miR-144 correlated with poor outcomes<sup>10</sup>. Lately, Liu et al<sup>11</sup> showed that reintroduction of miR-144 expression in GC cells significantly suppressed the metastasis of gastric cancer; however, the role of miR-144 in GC has not been fully appreciated.

RLIP76 (DNP-SG ATPase) is a stress-inducible non-ABC transporter mediating the transmembrane movement of a number of substances including the glutathione conjugates and the chemotherapy drugs in an ATP-hydrolysis

dependent manner<sup>12-14</sup>. Although the majority of early studies<sup>15-17</sup> have focused on the transporter activity of RLIP76, mounting evidence has revealed that RLIP76 is implicated in various physiological processes including cell proliferation, metastasis and ligand-dependent receptor endocytosis. Moreover, RLIP76 was overexpressed in most cancer cell lines and many human cancers<sup>18</sup> and RLIP76 targeted therapy by using antibody, shRNA or antisense RNA led to durable and complete remission in xenograft of human lung and colon cancer<sup>19</sup> as well prostate cancer and kidney cancer<sup>20,21</sup>. In this work, we found that the low level of miR-144 correlated with the poor prognosis and miR-144 level can be considered as an independent prognostic marker in clinical evaluations. Moreover, our results showed that ectopic over-expression of miR-144 in GC cell lines would lead to the suppression of proliferation and inducing apoptosis by directly targeting RLIP76.

## Patients and Methods

### *Patients and Tissues Samples*

Between June 2013 and May 2016, GC tissue samples and the matched normal tissues were obtained from a total of 67 patients without peritoneal dissemination, liver metastasis and distant metastasis at the First Affiliated Hospital of Zhengzhou University, Zhengzhou City, Henan Province, China. The present study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University, Zhengzhou City, Henan Province, China and written consent forms were obtained from all patients. The tissues specimen was snapped frozen and kept in liquid (-70 °C) until analysis. All samples were blindly examined by two senior pathologists. The clinical and pathological data for the patients are provided in Table I.

### *Cell Lines and Cultures*

Human GC cells SGC7901 and AGS were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) containing 4 mM/L glutamine, 3.7 g/L sodium bicarbonate, 4.5 g/L glucose and 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). Cells were maintained in a 5% CO<sub>2</sub> humidified incubator at 37 °C.

### *Quantitative Real-Time PCR (qRT-PCR)*

TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to extract total RNA from the cultured cells or human tissues. MiR-144 expression was quantified by real time PCR with a TaqMan Probe (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, cDNA was obtained by High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) and qRT-PCR was performed by using a TaqMan PCR kit and the ABI 7500 System (Thermo Fisher Scientific, Waltham, MA, USA). The relative expression of miR-144 in cells and tissues were normalized to that of U6. For RLIP76 mRNA expression, the primer was synthesized based on published sequence<sup>22</sup>. The first-strand cDNA was reversely transcribed from µg total RNA by using the Super M-MLV Reverse transcriptase (BioTeke Co., Beijing, China). PCR reaction solution included a master mix including SYBR GREEN mastermix (Solarbio Co., Beijing, China), forward primer, reverse primer and 10 ng template cDNA. GAPDH was used as internal control to normalize PCR results. PCR results were analyzed by using the comparative ΔCt method (ABPrism software, Applied Biosystems, Foster City, CA, USA).

### *Western Blotting Analysis*

Proteins were isolated from tissues by lysing frozen tissues in Radio ImmunoPrecipitation Assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA). Proteins were extracted from cells after cells were lysed by lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors (Sigma, St. Louis, MO, USA). The protein concentration was measured by the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Extracted proteins were separated on SDS-PAGE and then transferred electro-phoretically onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Proteins were probed into with specific antibodies in adherence to standard protocol. Specific primary antibodies against RLIP76 and β-actin were purchased from Abcam (Shanghai, China). The second antibodies used in this study included the goat anti-rabbit IgG-HRP, the goat anti-mouse IgG-HRP and the donkey anti-goat IgG-HRP (Beyotime Institute of Biotechnology, Shanghai, China). Signals were detected by using chemiluminescent substrate (KPL, Guildford, UK) and the blot intensity was quantified by using BandScan software (Glyko, Novato, CA, USA).

**Table 1.** Correlation between miR-144 level and clinicopathological features of HCC patients.

Variables	Patients (No.)	miR-144 expression		<i>p</i> -value
		Low expression	High expression	
Sex				0.6205
Female	27	15	12	
Male	40	19	21	
Age				0.2242
<60	31	13	18	
≥60	36	21	15	
Differentiation degree				0.0125
Well/moderate	40	15	25	
Poorly	27	19	8	
Lymph node metastasis				0.0136
N0/N1	38	14	24	
N2/N3	29	20	9	
Lymphatic invasion				0.3319
Absent	35	20	15	
Present	32	14	18	
Venous invasion				0.3268
Absent	38	17	21	
Present	29	17	12	
TNM stage				0.0005
I+II	38	12	26	
III+IV	29	22	7	

### Construction of Reporter Plasmids and Luciferase Assay

The reporter plasmid was constructed as previously described<sup>23</sup>. A fragment containing RLIP76 3'UTR was amplified by PCR from human genomic DNA, utilizing specific primers, and was inserted into a pGL3 vector (Promega, Madison, WI, USA) downstream, the stop codon of firefly-luciferase reporter gene, thus resulting in the pGL3-3'UTR/RLIP76. For luciferase assay 293T recipient cells were transiently co-transfected with 0.2 µg of pGL3-3'UTR/RLIP76 constructs, 0.02 µg of pRL-TK-Renilla luciferase reporter plasmids (Promega, Madison, WI, USA) containing the Renilla-luciferase for normalization, and with 5 pmol of miR-124 overexpression construct or control. 24 hours after transfection, the cells were lysed and the luciferase activity was measured with a luminometer by using the dual-luciferase reporter assay system according to the manufacturer's instructions.

### MiR-144 Knockdown or Overexpression

The lentiviral constructs of miR-144 mimics and miR-144 inhibitor (anti-miR-144) were synthesized by Genepharma (Shanghai, China). Cells were seeded into each well of a 96-well plate and incubated overnight, and then transfected with miR-370 mimic and miR-con or miR-370 inhibitor according to the manufacturer's instructions.

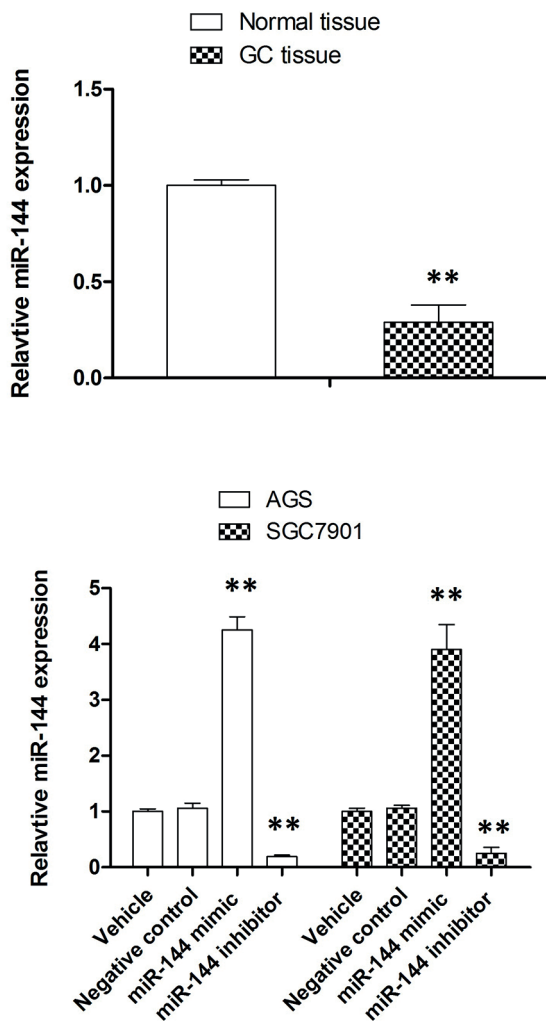
The transfection efficiency was confirmed by qPCR analysis.

### RLIP76 Overexpression

RLIP76 were overexpressed through transfection with the expressing construct using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RLIP76 overexpressing vector was constructed as previously described by using a plasmid vector pGCsi-H1<sup>18</sup>. The cells transfected with empty vector were used as controls. 48 hours after transfection, the cells were rinsed before being re-suspended in fresh culture media and the overexpression was verified by the Western blot analysis.

### Cell Viability Test

CCK-8 test was performed to determine the cell viability (WST-8 Cell Counting Kit-8, Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, the cells at a density of  $1 \times 10^5$  were seeded in the culture plates and maintained for 24 or 48 hours. Next, an aliquot of CCK-8 solution was added into the culture medium and the cells were incubated for another 1 hour before the viable cells were examined by measuring absorbance at 450 nm (Tecan Group Ltd, Männedorf, Switzerland).



**Figure 1.** MiR-144 is downregulated in GC tissues. \*\* $p < 0.01$  vs. vehicle.

**Colony Formation**

The cells suspended in RPMI-1640 agarose medium were seeded in each well of a 6-well plate over a bottom layer of solidified RPMI-1640 agarose medium. Cultures were maintained for 14 days without fresh medium feeding at 37 °C in a humidified atmosphere of 95% air and 5.0% CO<sub>2</sub>. Then the cell colonies with over 50 cells were enumerated and stained with violet crystal before being photographed by using a digital camera (Nikon DXM1200, Tokyo, Japan).

**Flow Cytometry**

FITC Annexin V apoptosis kit (BD Pharmingen, Franklin Lakes, NJ, USA) was used in the flow cytometry analysis. Briefly, the cells were adjusted to a final density of 5×10<sup>5</sup> cells/ml before

being stained with the annexin V-FITC and propidium iodide (PI) for 15 minutes in dark. Then the apoptotic cells were determined with a flow cytometer (Beckman Coulter Inc., Miami, FL, USA).

**Statistical Analysis**

All statistical analysis was performed by using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Values were presented as the mean ± SD. The comparison of miR-144 levels in tumor and normal tissue were performed by using the Student’s *t*-test. Statistical comparisons between cell lines were performed by one-way ANOVA followed by Dunnett’s *t*-test. The difference with a *p*-value less than 0.05 was defined as statistically significant.

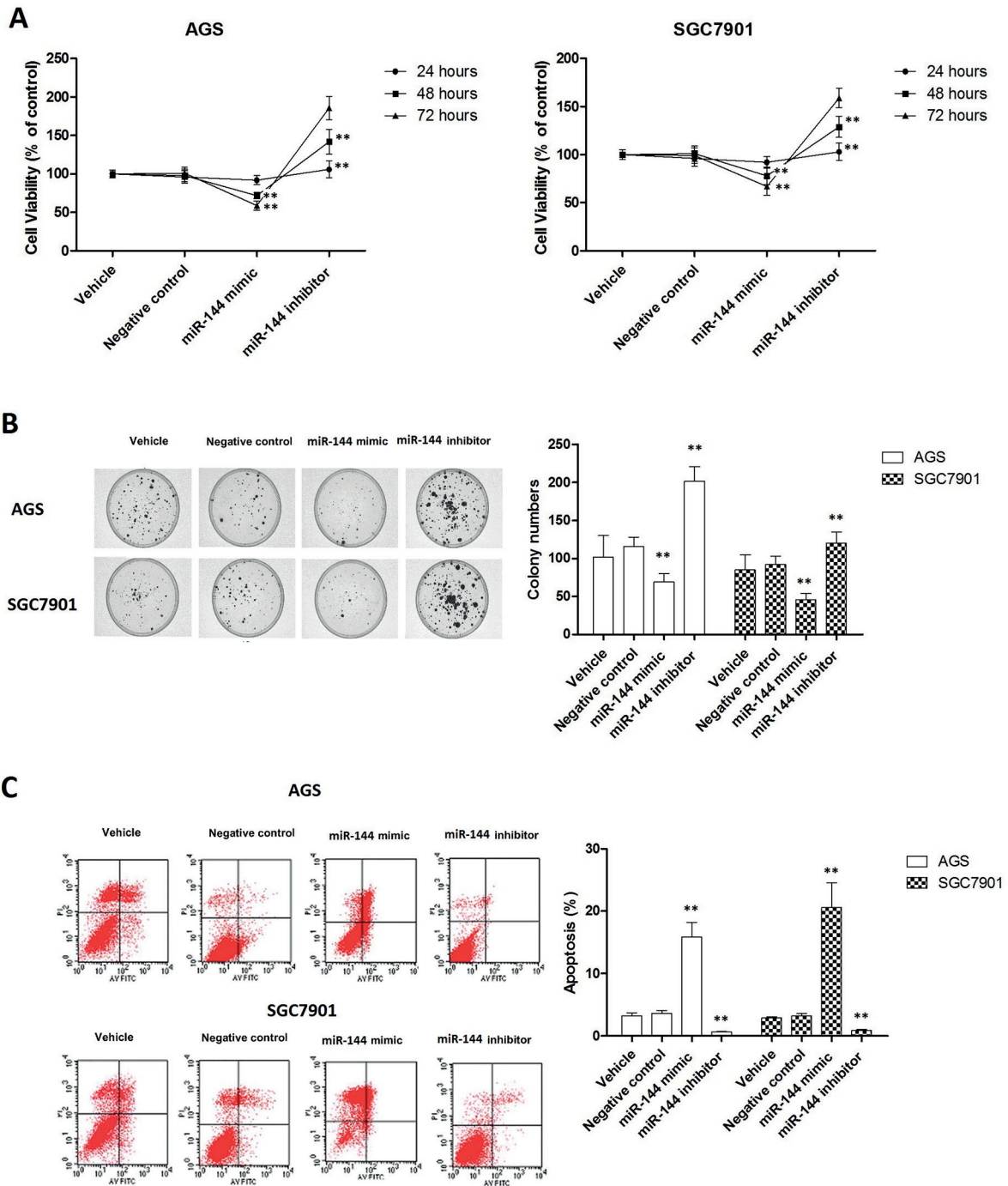
**Results**

**Association Between miR-144 Levels and Clinicopathological Features**

Quantitative RT-PCR was conducted to determine the expression of miR-144 in GC tissue. As shown in Figure 1A, the decreased level of miR-144 was observed in HCC tissue compared to the matched normal tissue ( $p < 0.01$ ), suggesting that miR-144 may act as tumor suppressor in GC. By adopting a cut-off value corresponding to the median miR-144 level, patients were allocated into two groups: 34 patients had low levels of miR-144 and 33 patients had high levels of miR-144. After grouping, the association of miR-144 level with clinicopathological characteristics was analyzed. As presented in Table I, the low miR-144 level was significantly correlated with the lymph node metastasis, the TNM stage (stages III and IV) and the differentiation degree; however, no significant relation was found between miR-144 expression and gender, age, lymphatic invasion and venous invasion.

**Ectopic Overexpression of miR-144 Suppresses cell Growth and Induces Apoptosis**

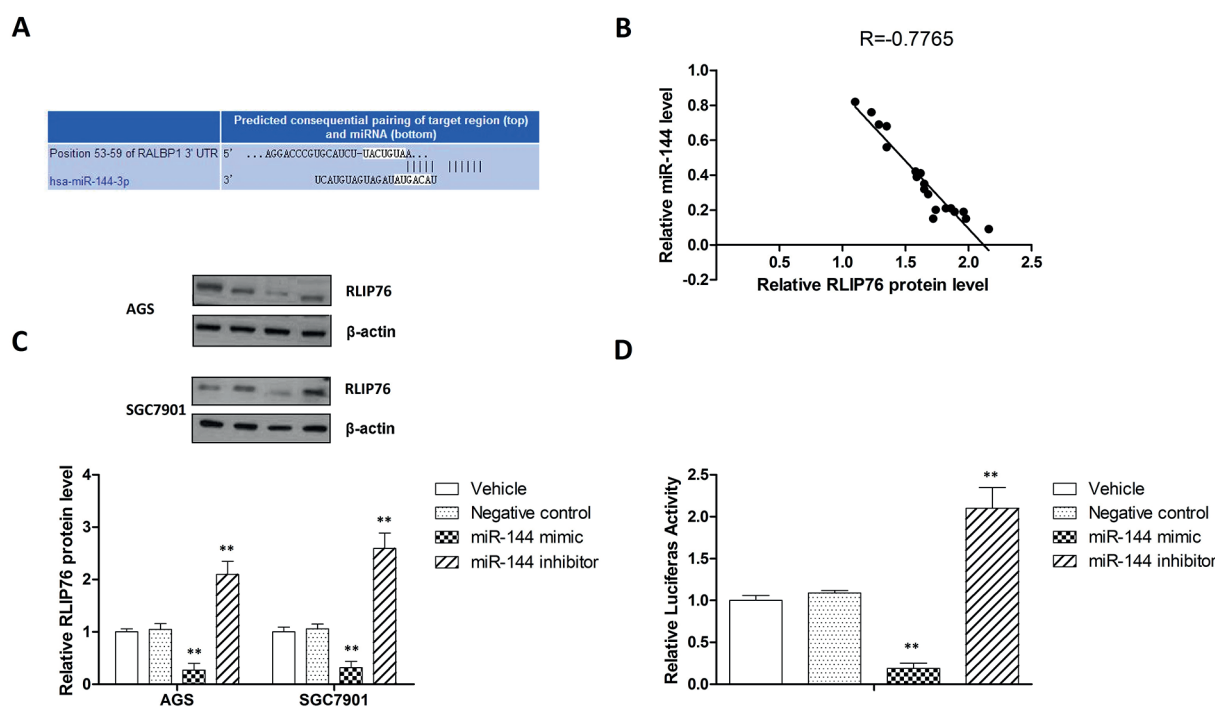
The role of miR-144 in GC was studied in GC cell lines SGC7901 and AGS. MiR-144 mimic and inhibitor as well as negative control (NC) were transfected into GC cells to assess the effect of miR-144 on cell growth and apoptosis. As shown in Figure 1B, miR-144 mimic was able to significantly increase the expression of miR-144 while the miR-144 inhibitor transfection was associated with the markedly decreased level of miR-144.



**Figure 2.** MiR-144 functions as tumor suppressor in GC cell lines. \*\* $p < 0.01$  vs. vehicle.

Results from CCK-8 assay showed that the over-expression of miR-144 significantly inhibited the proliferation of GC cells more than the control cells while the miR-144 knockdown enhanced the proliferation of both tested cell lines (Figure 2A). The anti-proliferative effect of miR-144 was also

confirmed by colony formation assay, as shown in Figure 2B. Next, we examined whether or not the miR-144 was involved in the apoptosis of GC cells. As shown in Figure 2C, the miR-144 significantly increased apoptotic population while the miR-144 inhibitor led to resistance to apoptosis.



**Figure 3.** MiR-144 regulates the expression of RLIP76 by directly targeting the 3'-UTR. \*\* $p < 0.01$  vs. vehicle.

### RLIP76 is a Direct Target of miR-144

It is well established that the miRNA regulates the expression of target genes post-transcriptionally; therefore, two computational algorithms, TargetScan<sup>24</sup> and microRNA.org<sup>25</sup>, were used in combination to study the potential target that mediated the anti-tumor effect of miR-144 in melanoma. Both methods predicted an interaction between miR-144 and the target sites in the RLIP76 3'-UTR, as shown in Figure 3A. Then, the miR-144 expression levels and protein expression of RLIP76 in the tissue of 20 patients randomly chosen from the specimen pool were examined to explore the possible correlation. As shown in Figure 3B, our results established an inverse correlation between the protein levels of RLIP76 and miR-144 in patients' tissue. To further elucidate the regulatory effect of miR-144 on RLIP76, the protein level of RLIP76 was examined in cells transfected with miR-144 mimic or miR-144 inhibitor. As shown in Figure 3C, the transfection with miR-144 mimic led to a significant decrease in protein expression of RLIP76. In contrast, the miR-144 inhibitor correlated with the significant increase of the RLIP76 protein level. Next, the luciferase assay was performed to determine whether miR-144 regulated the expression of RLIP76 by directly targeting the 3'-UTR of RLIP76. As shown in Figure 3D, miR-144

mimic significantly decreased the luciferase reporter activity while miR-144 inhibitor significantly increased the reporter activity, compared with controls, indicating that RLIP76 shall be a direct target of miR-144. Taken together, our findings showed that miR-144 regulated the expression of RLIP76 post-transcriptional through directly targeting the 3'-UTR of the mRNA.

### MiR-144 Exerts Anti-tumor Effect Through Modulating RLIP76

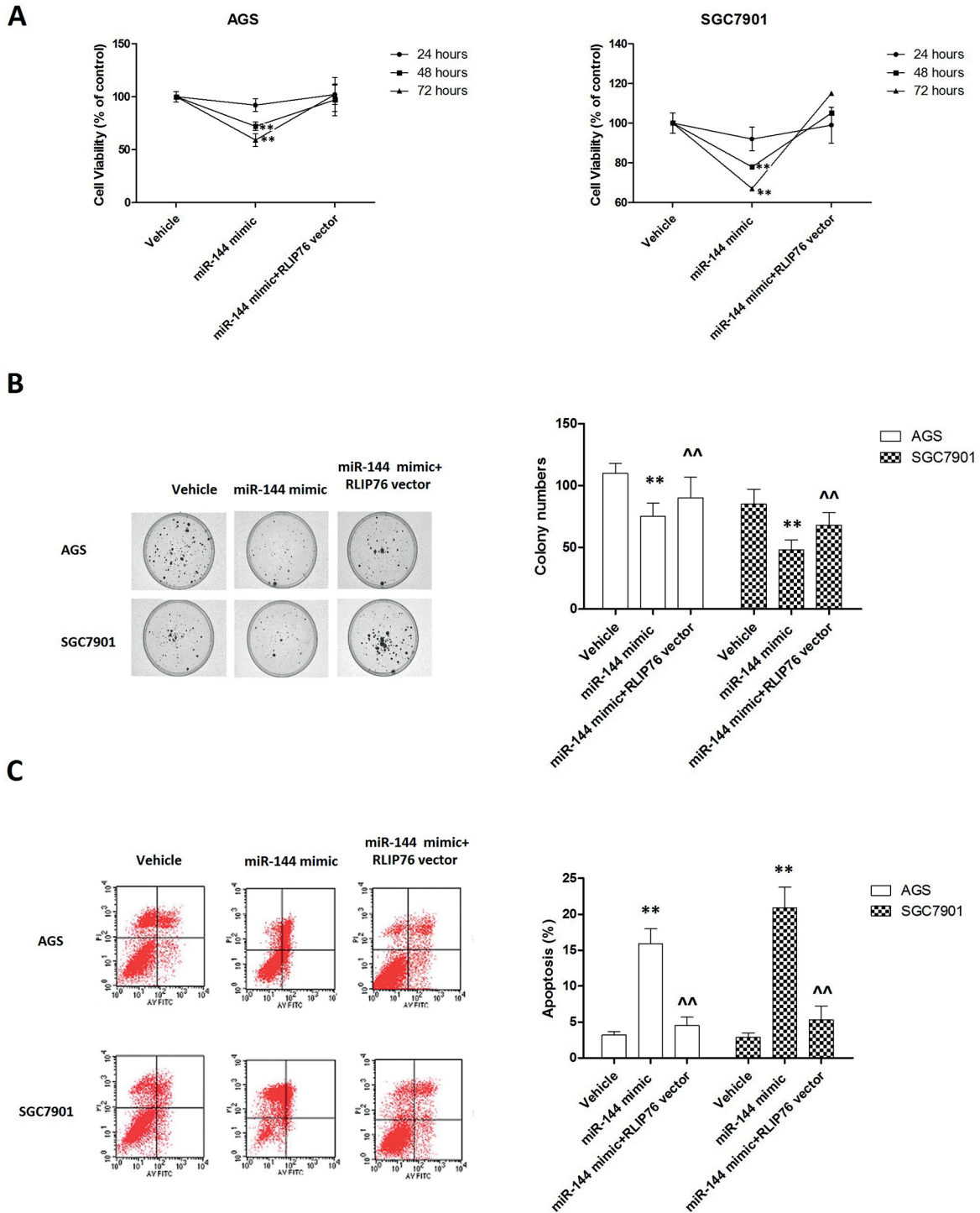
To verify the role of RLIP76 in the anti-tumor effect of miR-144, RLIP76 was overexpressed in both GC cells by using overexpression plasmid. As shown in Figure 4A, the level of RLIP76 protein was markedly increased in cells transfected with overexpressing plasmid. In addition, the ectopic overexpression of RLIP76 significantly attenuated the suppression of proliferation and apoptosis as induced by miR-144 mimic (Figure 4A, 4B and 4C), which provided strong evidence that the anti-tumor effect of miR-144 was mediated by RLIP76.

## Discussion

MicroRNAs (miRNAs), accounting for a family of small non-coding RNAs with a length of

21-25 nucleotides, play an important role in a variety of physiological activities of cells, including proliferation, development, apoptosis and differentiation through post-transcriptionally regula-

ting expression of their target genes<sup>26</sup>. As early as in 2006, miRNAs have been reported to play a role in the development and progression of GC<sup>27</sup>. Then, in the next decade, the correlation between



**Figure 4.** The ectopic overexpression of RLIP76 significantly attenuated the suppression of proliferation and apoptosis induced by miR-144 mimic. \*\* $p < 0.01$  vs. vehicle, ^^ $p < 0.01$  vs. miR-144 mimic.

a number of miRNAs and clinical outcomes has been explored. For instance, miR-340 is reported to promote the tumor growth of GC cells<sup>28</sup>. On the other hand, a number of miRNAs, such as miR-218, have been found to play a role of tumor suppressors in GC<sup>29</sup>. In the current study, we showed that miR-144 was aberrantly down-regulated in GC tissues compared with the corresponding normal control tissues, suggesting the role of miR-144 as tumor suppressor in GC. Moreover, the association between miR-144 levels with clinicopathological features was explored. We found that low expression of miR-144 was significantly associated with the lymph node metastasis, the TNM stage (stages III and IV) and the differentiation degree, indicating the involvement of miR-144 in tumor invasion, which is consistent with previous studies<sup>11</sup>. Our findings here provided experimental evidence that miR-144 functioned as tumor suppressor in GC.

The aberrant down-regulation of MiR-144 has been documented in a variety of human malignancies, including cholangiocarcinoma<sup>30</sup>, colorectal cancer<sup>31</sup>, bladder cancer<sup>32</sup>, and thyroid cancer<sup>33</sup>, and a number of studies have investigated the role of miR-144 in tumor growth and metastasis. Although Liu et al<sup>11</sup> demonstrated that miR-144 might suppress the metastasis of GC by targeting the MET expression; the role of miR-144 in GC has not been fully understood. Therefore, the present work was carried out to further investigate the functional role of miR-144 in GC. The findings of the present study indicated that the reduced expression of miR-144 in cancer cell lines and human tissues is negatively correlated with the progression of GC. Next, we conducted experiments to examine the effect of overexpression of miR-144 in GC cells. We found that miR-144 significantly suppressed the proliferation and induces apoptosis. Moreover, we identified RLIP76 as a novel target of miR-144 in GC cell lines.

RLIP76 has been implicated in a variety of human malignancies<sup>19,21,34</sup>. Notably, a recent study<sup>35</sup> revealed the oncogenic role of RLIP76 in GC. In agreement with these data, our results also showed that inhibition of RLIP76 could suppress proliferation and enhance apoptosis in GC cells, highlighting the potential of RLIP76 as a therapeutic target in GC; however, the understanding of the regulatory mechanism of RLIP76 in cancer cells still remained limited. A histone acetyltransferase and a transcriptional co-activator, P300, was the first one found to be able of regulating RLIP76 expression in the breast cancer

cells<sup>36</sup>. The participation of miR-101 in regulating RLIP76 in prostate cancer cells has been reported<sup>23</sup>, suggesting that miRNAs might act as the upstream signaling regulating the expression of RLIP76. In the present investigation, we showed that miR-144 suppressed cell growth and induced apoptosis in GC cells by modulating RLIP76 and demonstrated that the anti-tumor effect of miR-144 was reversed by RLIP76 overexpressing plasmid. Moreover, RLIP76 has been identified as a novel target of miR-144; miR-144 regulated the expression of RLIP76 at post-transcription level; besides, the cell proliferation and apoptosis, the involvement of RLIP76 in the angiogenesis and chemoresistance of solid tumor has also been evidenced<sup>37-39</sup>. Hence, by targeting RLIP76, miR-144 might also be able to suppress the metastasis and angiogenesis of GC as well as enhance the chemosensitivity.

## Conclusions

We observed that miR-144 is aberrantly down-regulated in GC tissue and miR-144 level was significantly related to the advanced cancer stage. Moreover, our findings indicated that miR-144 functioned as a tumor suppressor gene in GC and identified RLIP76 as a direct target of miR-144. We suggest the potential value of miR-144 and RLIP76 as new therapeutic targets for GC.

## Conflict of interest

The authors declare no conflicts of interest.

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