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miR-340 suppresses tumor growth and enhances chemosensitivity of colorectal cancer by targeting RLIP76

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Abstract. – OBJECTIVE: Colorectal cancer (CRC) is a common human malignancy and is the second leading cause of cancer deaths worldwide with a dismal prognosis. Previous investigations have shown that miR-340 can modulate the metabolism of CRC cells. The aim of this report is to study the role of miR-340 in the development and progression of CRC.

PATIENTS AND METHODS: The level of miR-340 in CRC cells was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting. CRC cell lines were used as model cell lines and the anti-tumor effect of miR-340 in vitro was examined. The luciferase reporter assay was performed. The level of miR-340 was restored in CRC cells by the usage of the miR-340 mimic. Re-expression of RLIP76 in CRC cells was then constructed. Moreover, the target gene of miR-340 was identified through the experiment of *in vivo* xenograft model.

RESULTS: The aberrant downregulation of miR-340 is correlated with advanced stage of CRC. Furthermore, the ectopic overexpression of miR-340 in CRC cell lines resulted in growth inhibition, apoptosis and enhanced chemosensitivity *in vitro* and *in vivo*, which was mediated by directly targeting RLIP76.

CONCLUSIONS: miR-340 acts as a tumor suppressor in CRC and is involved in the chemoresistance of CRC.

Key Words:
Colorectal cancer, Chemosensitivity, miR-340, RLIP76.

Introduction

Based on the statistical data of World Health Organization (WHO), the colorectal cancer (CRC) is the 2nd most common malignancy in female population and the 3rd most common cancer in male population[1]. CRC is also the second leading cause of cancer related deaths worldwide, and accounts for 1 million new diagnoses along with over 600,000 death cases in each year[2]. Patients who are diagnosed early with localized disease can be treated and possibly cured with surgery; approximately 20-25% of patients who are diagnosed with metastatic disease, are commonly treated with chemotherapeutics. Despite the advances in therapeutic strategies, the prognoses of patients with metastatic CRC remain dismal due to resistance to chemotherapeutics[3]. Therefore, the identification of the factors which are involved in the CRC progression and chemoresistance is imperative to determine the potential novel targets for improving the clinical outcome of the metastatic CRC patients. RLIP76 is a multi-functional protein, which is involved in the ATP-dependent transport of glutathione conjugates and chemotherapy drugs[4-6]. The majority of the previous studies focused on the study of the transporter activity of RLIP76. Accumulating evidence suggested that RLIP76 is involved in cell proliferation, metastasis and ligand-dependent receptor endocytosis[7-10]. Furthermore, RLIP76 is a stress-inducible non-ABC (ATP-binding cassette) transporter, which overexpressed in the most cancer cell lines of many human cancers[11]. *In vitro* studies[12] showed that the RLIP76 blocked by siRNA inhibited the proliferation, enhanced the apoptosis, and suppressed the invasion of colon cancer cells. RLIP76-targeted therapies which using antibody, short hairpin RNA (shRNA) or antisense always lead to durable and complete remission in xenografts of colon cancer[13]. Moreover, Wang et al[14] revealed that the RLIP76 was associated with drug-resistance of colorectal cancer cells. Collectively, these findings highlighted the potential of RLIP76, which is an important target to improve drug resistance and tumor treatment of CRC. microRNAs (miRNAs) is a family of endogenous non-coding mRNA molecules with

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length of 18-25 nucleotides which has attracted a lot of attention due to the roles in tumorigenicity and tumor progression[15]. miRNAs are functioning either as oncogenes or as tumor suppressors by regulating the expression of target proteins at the post-transcriptional level by binding the 3′-UTR portion of mRNAs through translational repression or degradation[16]. By modulating different target genes, miRNAs were found as an important role in a variety of cellular activities such as cell apoptosis, cell proliferation, invasion, migration and stem cell differentiation[17-19]. Regarding miR-340, a previous report[20] has shown that miR-340 suppressed the growth of CRC cells by modulating metabolism. The correlation of decreased miR-340 in bone marrow with liver metastasis of CRC was established[21]. In this work, we found that an aberrant down-regulation of miR-340 is correlated with the advanced stage of CRC. Moreover, the results showed that ectopic overexpression of miR-340 in CRC cell lines resulted in growth inhibition, apoptosis and enhanced chemosensitivity *in vitro* and *in vivo*, which was mediated by directly targeting RLIP76.

Patients and Methods

Clinical Tissue Samples

The clinical study was reviewed and approved by Medical Ethics Committee of Peking University International Hospital (Beijing, China) and consent forms were signed by all patients or guardians. From December 2014 to December 2016, 91 samples along with matched normal tissues from CRC patients were collected at Department of Medical Oncology, Peking University International Hospital (Beijing, China). Patients who undergone chemotherapy or radiotherapy before the surgery or with familial adenomatous polyposis CRC were excluded from the study. The collected tissue was snapped frozen and kept in liquid (-70°C) until the analysis. The expression level of miR-340 in tissue was determined by quantitative reverse transcription Polymerase Chain Reaction (qRT-PCR). The expression of RLIP76 was analyzed by automated capillary Western blot (WES)[22].

Cell Lines and Cultures

Human CRC cells HT-29 and HCT116 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Normal human colon cell NCM460 cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) Invitrogen (Carlsbad, CA, USA) which containing 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and 10% fetal bovine serum (FBS) Invitrogen (Carlsbad, CA, USA). Cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Quantitative Real-time PCR (qRT-PCR)

TRIzol Reagent (Life Technology, Carlsbad, CA, USA) was used to isolate the total RNA from cultured CRC cells. The PrimeScript™ RT Master Mix (TaKaRa, Dalian, China) was used to synthesize first strand cDNA. Then the expression level of RLIP 76 mRNA was determined by RT-PCR using Power SYBR Green PCR Master Mix (Carlsbad, CA, USA) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control. The forward and reversed primer sequences were synthesized by Sangon (Shanghai, China) based on published sequence[23]. To assess the miRNA-340 in cells and tissues, mirVana Kit (Ambion, Carlsbad, MA, USA) was utilized to isolate miRNA. Then miR-340 level was measured by RT-PCR using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) with U6 as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression of target genes[24].

Western Blotting Analysis

The tissues from the involved patients were isolated and cultured by lysis buffer (Beyotime, Shanghai, China). Extracted proteins were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for the separation before transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) for protein probing. Specific primary antibodies against RLIP76 and β-actin were purchased from Abcam (Cambridge, MA, USA). Goat anti-rabbit IgG-HRP used as the second antibodies were purchased from Beyotime (Shanghai, China). Protein blots were visualized with enhanced chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by BandScan software (Glyko, Novato, CA, USA).

Luciferase Reporter Assay

The luciferase reporter assay was performed as mentioned by a previous study[25]. Briefly, the RLIP76 3'UTR region including miR-340 bind-

ing sites was amplified by PCR from HCT116 cells and then inserted into a pMIR-REPORT vector (Applied Biosystems, Carlsbad, CA, USA). HCT116 cells were co-transfected with luciferase reporter vector and miR-340 mimic (or negative control) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 hours post transfection, the luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI, USA).

Transfection of miR-340 Mimic

The lentiviral constructed of miR-340 mimics, anti-miR-340 (miR-340 inhibitor) and NC were synthesized by Genepharma (Shanghai, China). HCT116 and HT-29 cells were infected with the constructed lentiviral to induce ectopic expression of miR-340 or suppress the constitutive expression of miR-340.

Re-Expression of RLIP76 in CRC Cells

The expression of RLIP76 was restored with a vector constructed with an empty vector as control as described by a previous study (10). HCT116 and HT-29 cells were transfected with vectors using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Cell Proliferation Assay

Cell Counting Kit-8 (Beyotime, Shanghai, China) was used to assess the cell proliferation. Briefly, a total of 1×10^5 cells were plated in culture plates. Then, the cells were put in incubation for 48 hours. The viable cells were examined by measuring absorbance at 450 nm (Tecan Group Ltd, Männedorf, Switzerland).

Flow Cytometry

HCT116 and HT-29 cells were stained with Annexin V-PE and propidium iodide using an Apoptosis kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Then the apoptotic percentage of treated cells was determined by a flow cytometer (Beckman Coulter Inc., Miami, FL, USA).

In vivo Xenograft Model

The protocol of animal experiments was reviewed and approved by Medical Ethics Committee of Peking University International Hospital (Beijing, China). BALB/c nude mice (4 weeks) were used for the CRC xenograft models. A suspension of 1×10^7 HCT116 cells was injected subcutaneously into the left posterior flank region of each mouse. Two weeks after transplantation, the established tumors were treated, miR-370 mimic suspended in atelocollagen (Koken, Tokyo, Japan), alone or combined were injected i.p. (intraperitoneally) into mice daily for 18 days. Then, the mice were sacrificed after 28 days. The volumes and weights of xenograft tumors were measured. Immunohistochemistry analysis was performed to determine the expression of RLIP76 in tumor tissues following standard protocol.

Statistical Analysis

Values were presented as the mean ± SD. The comparison of miR-340 levels in tumors and normal tissues were performed by student's *t*-test. Statistical comparisons between cell lines were performed by one-way ANOVA followed by Dunnett's *t*-test. GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) was used to analyze experimental data and a *p*-value less than 0.05 was considered to be statistically significant.

Results

miR-340 is Downregulated in CRC Tissues and Cell Lines

The levels of miR-340 in collected sample tissues and the matched non-neoplastic tissues were measured by qRT-PCR. As shown in Figure 1A, the expression of miR-340 was significantly lower in the CRC tissues compared with matched non-neoplastic tissues. Moreover, the correlation between the tumor stages and miR-340 expression was explored. As shown in Figure 1B, it showed that miR-340 was decreased with the progression of CRC. The levels of miR-340 in CRC cells lines were also compared with normal colon cell NCM460, which showed that the miR-340 was expressed at a significantly lower level than normal colon cells (Figure 1C). Collectively, these results suggested that miR-340 was downregulated in CRC and may function as a tumor suppressor.

Downregulation of miR-340 Correlates with Poor Clinicopathological Characteristics

The correlation between levels of miR-340 and clinicopathological parameters was analyzed. The expression levels of miR-340 of CRC tissues were less than the expression levels in

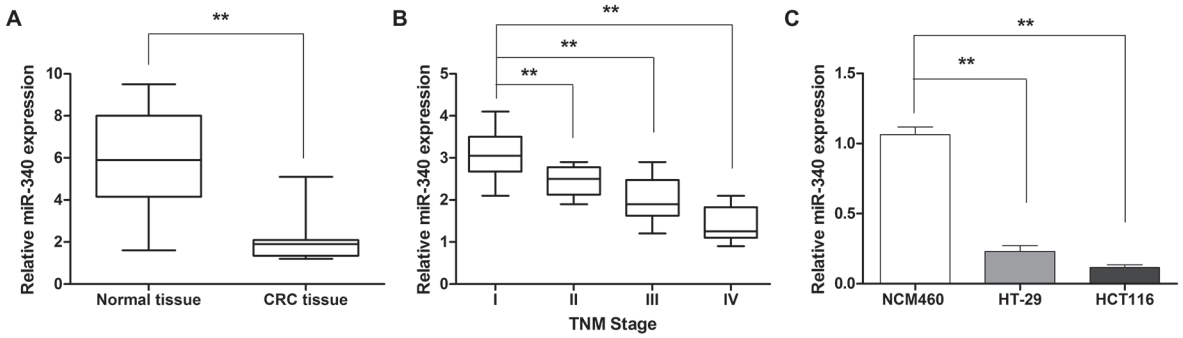

Figure 1. miR-340 is aberrantly downregulated in CRC tissue and cell lines. ***A,*** miR-340 is abnormally downregulated in CRC tissue compared with normal tissue. ***B,*** miR-340 expression correlates with the TNM stage. ***C,*** CRC cell lines present significantly lower level of miR-340. $^{**}p<0.01$.

median. As listed in Table I, the statistic analysis of 91 CRC cases showed that low levels of miR-340 were significantly correlated with advanced tumor stage and lymph node metastasis. In contrast, no significant correlation was found between miR-340 levels with other clinicopathological features.

miR-340 Suppresses CRC Cell Growth in vitro and Sensitizes CRC Cells to Oxaliplatin

To determine the affection of miR-340 on the proliferation of CRC cells, miR-340 mimic and inhibitor were used to establish cell lines with ectopic overexpression of miR-340 or miR-340 knockdown. As shown in Figure 2A, the levels of miR-340 in both cells were significantly elevated by miR-340 mimic while repressed by miR-340 inhibitor. The effect of miR-340 on cell growth was examined by CCK-8 (Cell Counting Kit) assay in Figure 2B, which showed that miR-340 significantly suppressed the cell growth whereas miR-340 knockdown promoted the proliferation of CRC cells. Then the effect of miR-340 on cell apoptosis was also assessed. As shown in Figure 2C, up-regulation of miR-340 resulted in a significant increase in apoptotic cell population while the repressing miR-340 led to less apoptotic cells. Then the effect of miR-340 on the sensitivity of CRC cell to oxaliplatin was examined. As shown in Figure 2D and 2E, miR-340 rendered CRC cells were significantly more sensitive to oxaliplatin, while CRC cells with miR-340 knockdown were correlated with the resistance to oxaliplatin. Therefore, the results showed that the miR-340 correlated with the apoptotic potential of CRC cells.

RLIP76 is a Direct Target of miR-340 in CRC Cells

Combined the searching results in bioinformatics database (Microcosm and Target scan), we postulated that the miR-340 may exert an anti-tumor effect by targeting RLIP76. The putative binding sites for miR-340 in the 3'-UTR of RLIP76 were shown in Figure 3A. To further investigate the correlation between the expression levels of miR-340 and RLIP76, 20 tissue specimens were randomly chosen for RT-PCR and simple Western blot analysis. As shown in

Table I. The statistic analysis of 91 CRC cases.

	miR-340 expression		
Variables	**High (47)**	**Low (44)**	***p*-value**
Age			0.8212
≤ 50	27	18	
> 50	20	16	
Sex			0.3516
Male	32	19	
Female	15	15	
Tumor size			0.5304
≤ 5cm	24	19	
> 5cm	23	25	
Histological grade			0.5016
Well, moderate	22	19	
Poor	25	15	
TNM stage			0.0063**
I and II	30	15	
III and IV	17	29	
Site			0.6532
Colon	23	19	
Rectum	24	15	
Lymph node metastasis			0.0340*
Absence	30	18	
Presence	16	26	

$^{\#}p<0.05$, $^{**}p<0.01$.

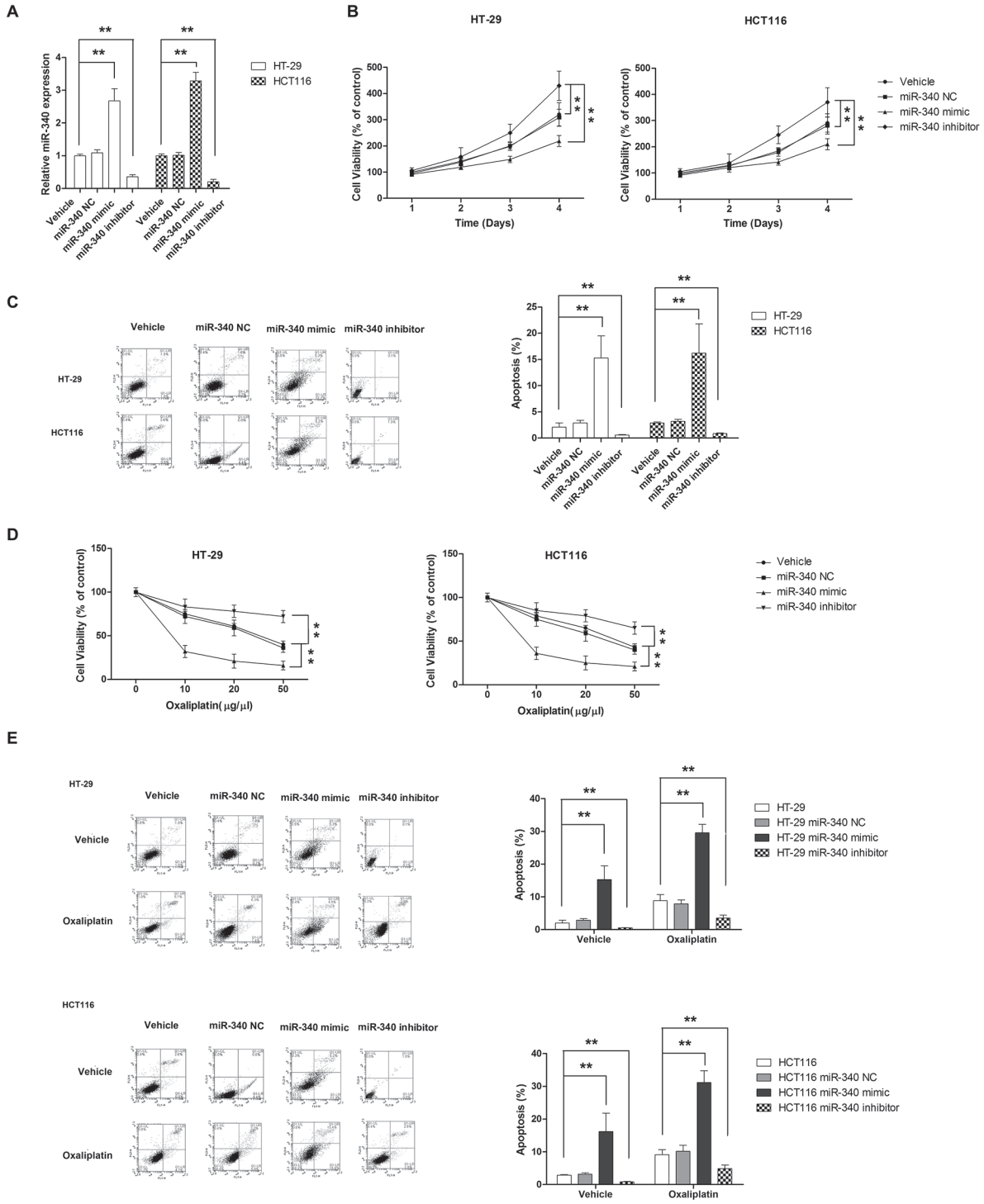

Figure 2. miR-340 acts as tumor suppressor and chemosensitizer. ***A***, The miR-340 level is significantly elevated by miR-340 mimic and suppressed by the miR-340 inhibitor. ***B,*** miR-340 mimic suppresses cell proliferation. ***C,*** miR-340 induces apoptosis in CRC cells lines. ***D,*** miR-340 mimic increases the chemosensitivity of CRC cells to oxaliplatin. ***E,*** miR-340 mimic enhances oxaliplatin-induced apoptosis. $^{**}p<0.01$.

Figure 3B and 3C, both the protein and mRNA levels of RLIP76 were significantly and inversely correlated with miR-340 levels. Moreover, the mRNA and protein levels of RLIP76 in CRC cells transfected with miR-340 mimic and miR-124 mimic were also examined to further

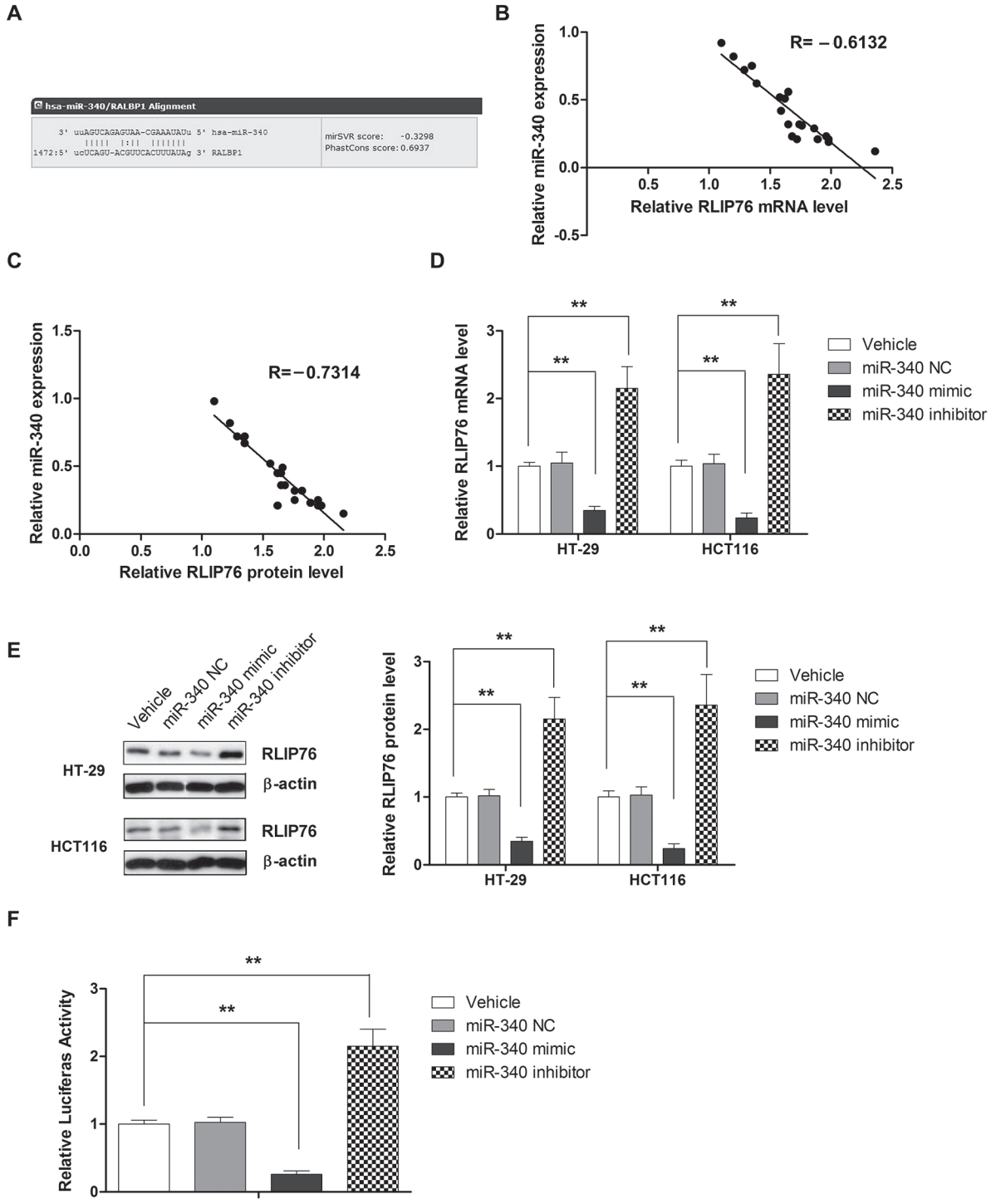

Figure 3. miR-340 directly targets the RLIP76 gene. ***A,*** Schematic illustration of the hypothesized duplexes formed from interactions between the RLIP76 3'-UTR binding sites and miR-340. ***B,*** Inverse correlation between miR-340 and RLIP76 mRNA level in CRC tissues. ***C,*** Inverse correlation between miR-340 and RLIP76 protein level in CRC tissues. ***D,*** miR-340 mimic significantly elevates the expression of RLIP76 mRNA in CRC cells. ***E,*** miR-340 mimic significantly elevates the expression of the RLIP76 protein in CRC cells. ***F,*** Luciferase activity is increased by miR-340 inhibitor and suppressed by miR-340 mimic. $^{**}p<0.01$.

demonstration of the regulatory role of miR-340 on RLIP76. As expected, the miR-340 overexpression led to a significant decrease in RLIP76 mRNA and protein expression compared with parental cell lines. On the other hand, downregulation of miR-340 was correlated with significantly higher expression of both RLIP76 mRNA and proteins (Figure 3D and 3E). Therefore, these data suggested that miR-340 may be involved in the degradation of mRNA of RLIP76 other than post-transcriptional regulation of protein expression of RLIP76. Then, luciferase assay was performed to determine whether RLIP76 was a direct target of miR-340. As shown in Figure 3F, luciferase reporter activity was significantly increased in cells transfected with miR-340 mimic while decreased in cells transfected with miR-340 inhibitor, providing direct evidence that RLIP76 was directly targeted by miR-340.

miR-340 Suppresses cell Growth, Induces Apoptosis and Enhances Chemosensitivity by Repressing RLIP76

The role of RLIP76 in the anti-tumor effect of miR-340 was explored. Both HT-29 and HCT116 cells were transfected with a vector to introduce

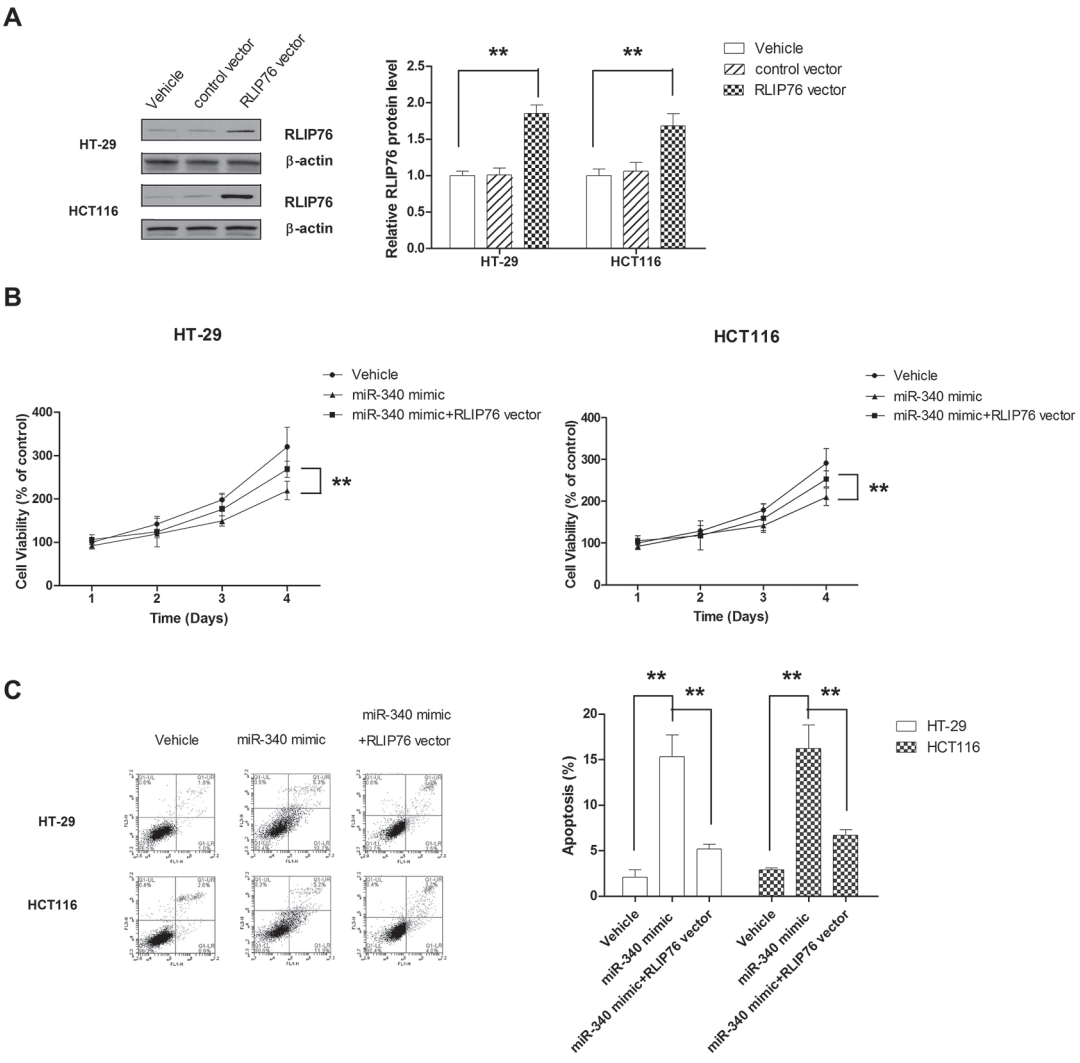

Figure 4. RLIP76 vector attenuates the anti-tumor effect of miR-340 mimic in CRC cells. ***A,*** RLIP76 vector successfully introduces ectopic overexpression of RLIP76. ***B,*** Overexpression of RLIP76 in cells transfected with miR-340 mimic leads to impaired anti-proliferative effect. ***C,*** Overexpression of RLIP76 in cells transfected with miR-340 mimic leads to less apoptosis in CRC cells.

(Figure Continued).

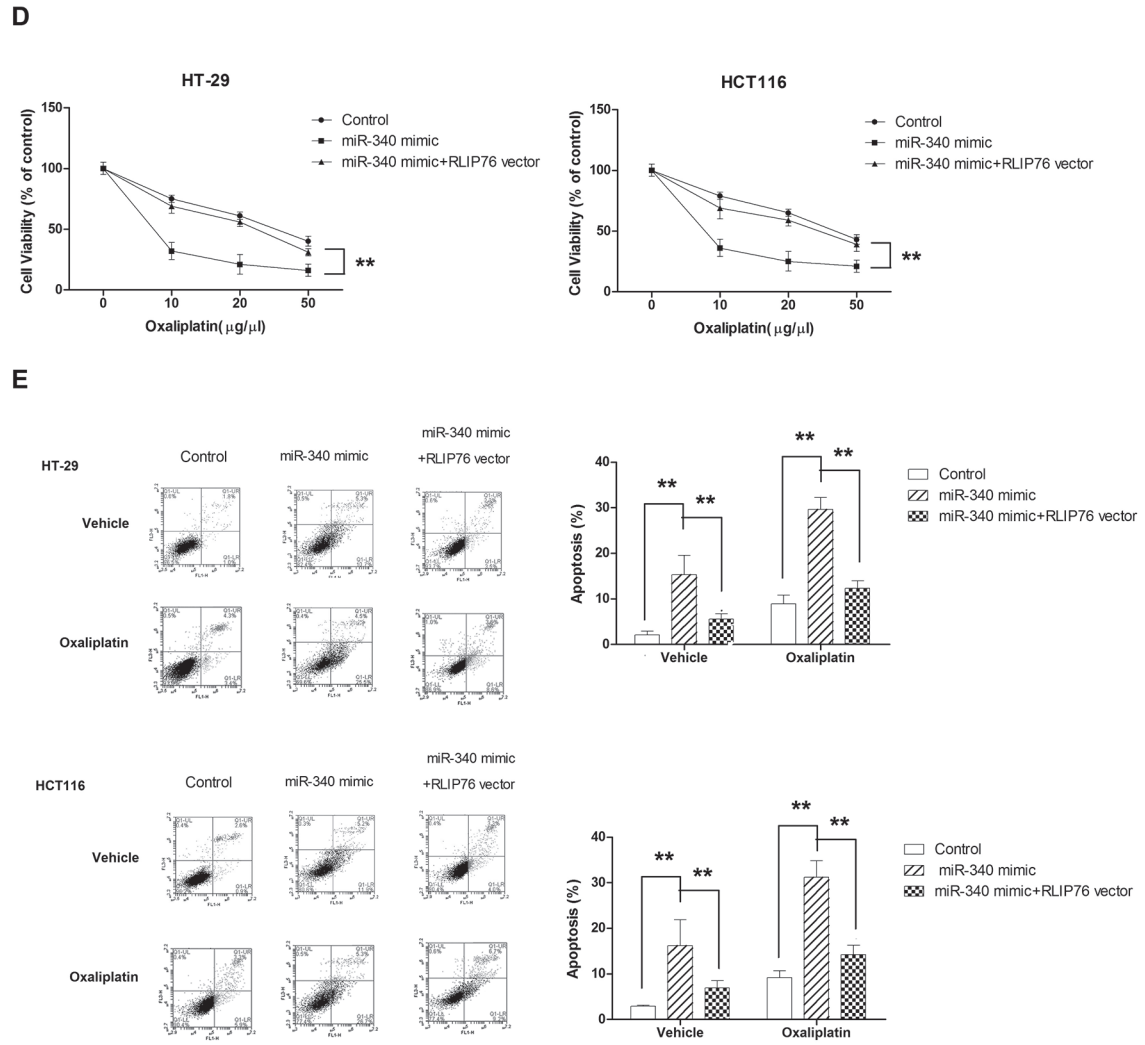

Figure 4. (*Continued).* ***D,*** Overexpression of RLIP76 in cells transfected with miR-340 mimic correlates with lower sensitivity to oxaliplatin. ***E,*** Overexpression of RLIP76 in cells transfected with miR-340 mimic reverses the enhancing effect of miR-340 on oxaliplatin-induced apoptosis. $^{**}p<0.01$.

ectopic overexpression of RLIP76 (Figure 4A). As shown in Figure 4B and 4C, the overexpression of RLIP76 significantly attenuated the inhibitory effect of miR-340 mimic on cell growth in both HT-29 and HCT116 cells. Meanwhile, the apoptosis-inducing effect of miR-340 in both cell lines was also reversed by the RLIP76 vector (Figure 4C). Then CCK-8 assay and flow cytometry assay were conducted to investigate whether RLIP76 also mediated the sensitizing effect of miR-340 to chemotherapeutic. As shown in Figure 4D and 4E, overexpression RLIP76 significantly decreased the chemosensitivity of CRC cells transfected with miR-340 mimic, which confirmed that miR-340 enhanced the sensitivity of CRC cells to oxaliplatin through targeting RLIP76.

miR-340 Inhibits Tumor Growth and Potentiates the Effectiveness of Oxaliplatin in vivo

To validate the regulatory role of miR-340 in tumor growth and chemoresistance *in vivo*, we examined the effect of miR-340 mimic on tumor growth and chemoresistance of HCT116 cells by using xenograft mouse model. As shown in Figure 5A, the treatment with oxaliplatin alone did not suppress the tumor growth in mice. However,

when combined with miR-340 mimic, oxaliplatin treatment significantly inhibited the increase in tumor volume and tumor weight (Figure 5A and 5B). Immunohistochemistry (IHC) analysis showed that the xenografts derived from mice administered with miR-340 mimic had repressed expression of RLIP76 compared to the control xenografts (Figure 5C). Therefore, the results suggested that miR-340 can function as a tumor suppressor and regulate chemoresistance by modulating its downstream target gene RLIP76 *in vivo*.

Discussion

In the past two decades, more and more emerging studies have demonstrated that miRNAs were involved in the development of human malignancies[26]. Regarding the CRC, miRNAs are considered to be aberrantly expressed in tumor tissues and implicated in tumor initiation, progression, and metastasis[27]. miRNAs can regulate multiple signaling pathways that contribute to CRC, including inflammation, cell survival, cell death, invasion, and metastasis, which render miRNAs promising biomarkers for predicting prognosis. In fact, some miRNAs are found to correlate with advanced disease stages of CRC with poor clinical outcomes. For instance, Zhang et al[28] have reported that miR-378 was significantly downregulated in CRC tissues and can be taken as an independent prognostic factor in CRC. Another group reported that an elevated level of miR-183 was closely related to advanced clinical stage, lymph node and distant metastases, and poor prognosis of CRC[29]. In 2012, Sun et al[20] found that miR-340 can function as a tumor suppressor and exerted an anti-proliferative effect on CRC cells by modulating metabolism. The correlation of decreased miR-340 in bone marrow with liver metastasis of CRC has also been established[21]. However, the correlation between clinical features and miR-340 has never been investigated. In this study, we found that the aberrant low expression of miR-340 was correlated with advanced TNM (Malignant Tumors) stages and lymph node metastasis. Meanwhile, in consistent with the previous findings, our results confirmed the role of miR-340 as a tumor suppressor in CRC by showing that ectopic overexpression of miR-340 suppressed the cell growth and induced apoptosis in CRC cell lines. Moreover, our results also provided experimental evidence that miR-340 conferred to the chemoresistance of CRC. Therefore, our data highlighted the potential of miR-340 as a prognostic biomarker and therapeutic target. In 2009, Baffa et al[30] pointed out that miR-340 played a role in human cancers by analyzing 43 paired primary and lymph node-metastatic tumors by using miRNA microarray. Since then, aberrant expression of miR-340 has been detected in a

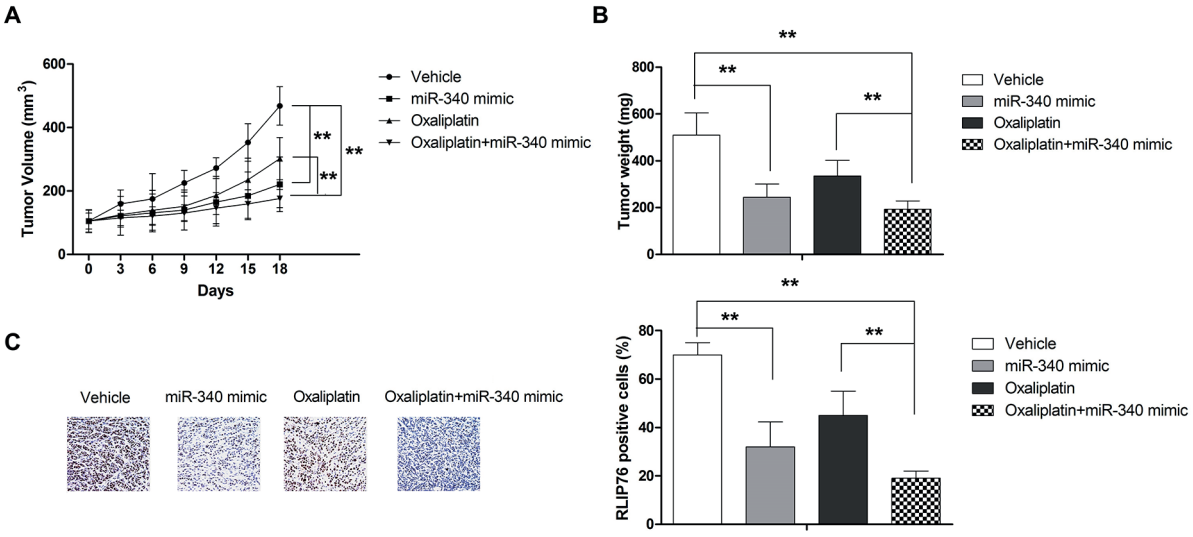

Figure 5. miR-340 exerts an anti-tumor effect *in vivo*. ***A,*** miR-340 treatment significantly inhibits tumor growth and enhances the effect of oxaliplatin. ***B,*** Tumor weight is decreased corresponding to tumor volume. ***C,*** The anti-tumor effect of miR-340 is associated with low expression of RLIP76. (N=8 per group). $^{**}p<0.01$.

variety of human malignancies, such as hepatocellular carcinoma[31], melanoma[32], and glioblastoma multiforme[33]. miR-340 is found to play a regulatory role in various physiological processes of tumor cells including proliferation, apoptosis, metastatic behavior, and metabolism, and a number of molecules have been identified as the direct target gene of miR-340. For instance, phosphoserine aminotransferase 1 was found to be responsible for the anti-tumor effect of miR-340 on esophageal cancer cell[34]. In osteosarcoma, miR-340 has been found to suppress the tumor growth and metastasis by directly targeting rock1[35]. The role of miR-340 in the metabolism of cancer cells has also been found in oral squamous cell carcinoma[36]. In this present study, RLIP76 was identified as a novel target of miR-340 and mediates the anti-proliferative and apoptosis-inducing effect of miR-340 in CRC cells, further supporting the concept that miRNAs could regulate the cellular activities of cancer cells by targeting different target genes. Other than tumor growth and metastasis, the involvement of miR-340 in the chemoresistance of cancer has also been confirmed in hepatocellular carcinoma, by showing that upregulation of miR-340 reverses cisplatin resistance of hepatocellular carcinoma cell lines by targeting Nrf2-dependent antioxidant pathway[31]. In consistent with their work, our findings also showed that miR-340 confers to the chemoresistance in CRC, highlighting the possible clinical benefits from combining miR-340 target gene therapy with conventional chemotherapy. Aberrant upregulation of RLIP76 was detected in both cancer tissues and malignant cancer cells, which suggested the role of RLIP76 as an oncogene[11,13,37,38]. Preclinical researches with xenograft mice models also showed that the RLIP76-targeted therapy was effective in treating CRC[39]. Moreover, some studies have also found that RLIP76 was a crucial mediator in the metastasis and angiogenesis process of CRC[12]. In breast cancer cell, the promoter activity and expression of RLIP76 were regulated by p300, suggesting a transcriptional regulatory mechanism of RLIP76[40]. A very recent study by Yang et al[25] reported that RLIP76 was regulated by miR-101 at post-transcriptional level in prostate cancer cells. In this paper, it also showed that RLIP76 was the key factor mediating the anti-tumor effect of miR-340 in CRC, further supporting the important role of post-transcriptional regulation in RLIP76 expression in cancer cells.

Conclusions

We identified an important tumor-suppressive miRNA, miR-340, that is correlated with clinical features of CRC patients. miR-340 plays a key role in the tumor growth by repressing colorectal cancer cell growth as well as by sensitizing CRC cells to a chemotherapeutic drug through directly targeting RLIP76. Hence, we demonstrated the importance of miR-340/RLIP76 signaling in CRC development and suggests that targeting this signaling may provide a new therapeutic approach for the treatment of CRC.

Conflict of interest

All the authors contributed to the work have read and approved the final manuscript for submission, and no conflicts declared.

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