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ORIGINAL ARTICLE

MiR-335-5p inhibits cell proliferation, migration and invasion in colorectal cancer through downregulating LDHB

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Summary

Purpose: *Colorectal cancer (CRC) is a common malignancy with high mortality rate worldwide. The advancement of new therapeutic strategies is crucial for the efficient treatment of CRC. Many miRNAs play a central role in the progression of cancer cells. There are still few studies on miR-335-5p and CRC. In this study, the potential of miR-335-5p in CRC development and progression was explored.*

Methods: *The expression level of miR-335-5p in CRC cell lines and tissues was detected by quantitative real-time polymerase chain reaction (qRT-PCR) assay. Cell counting kit-8 (CCK8) assay and colony formation assay were applied for evaluating the ability of cell proliferation. Wound healing assay and Transwell assay were applied for detecting cell migration and invasion ability. Moreover, dual luciferase reporter assay was performed to validate if lactic dehydrogenase B (LDHB) is a downstream target of miR-335-5p. Western blotting was used to estimate the protein expression of LDHB.*

Results: *The expression of miR-335-5p was significantly low in CRC tissues and cells. To investigate the function of miR-335-5p in CRC, two CRC cell lines (HCT-116 and SW620) were selected for further experiments. After transfection with mimics and inhibitors to up-regulate or down-regulate miR-335-5p, it was found that overexpression of miR-335-5p decreased cell proliferation and inhibited migration ability and invasion, while the knockdown of miR-335-5p showed the opposite results. Then, it was validated in dual luciferase reporter assay that LDHB could be a potential directive target gene of miR-335-5p. Moreover, the rescue assay confirmed that miR-335-5p executed its function as a tumor suppressor through targeting LDHB in CRC.*

Conclusions: *The present study demonstrated that miR-335-5p regulates cell proliferation, migration as well as invasion of CRC cells through down-regulating LDHB, and demonstrated that miR-335-5p/LDHB axis may be an underlying therapeutic strategy in CRC.*

Key words: *miR-335-5p, proliferation, migration, invasion, LDHB*

Introduction

Colorectal cancer (CRC) is a digestive system tumor with highest incidence rate worldwide. It is the third major cancer and the second leading cause of cancer–related death [1,2]. About 50% of patients die of distant metastasis. Due to changes in people's lifestyles and eating habits and the increase in other risk factors, the incidence of CRC has been continuously increasing [3,4]. Although great progress has been made in the diagnostic techniques and treatment methods for CRC [5], the postoperative recurrence rate of early-stage patients and the survival after radiotherapy and chemotherapy in advanced stages have not been significantly improved. In recent years, some important tumor-related genes have been discovered and gradually used in tumor gene therapy and targeted drug therapy [6]. The advancement of new therapeutic strategies is crucial for the efficient treatment of CRC.

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Micro ribonucleic acids (miRNAs) as highly-conserved small non-coding RNAs, possess the function of post-transcriptional regulation of genes [7], widely involved in cell proliferation, development, metastasis and treatment [8-11], which has been a hot spot in cancer research currently. A class of miRNA has been closely related to CRC [12,13], and can be used as a predictive target for tumors.

The primary purpose of this study was to investigate the role of miR-335-5p in the development and progression of CRC and its underlying mechanism.

Methods

Tissue specimens

This study was approved by the Ethics Committee of Beijing Chaoyang Hospital and informed consent was obtained from all participants before the study entry. All CRC tissue specimens were stored in liquid nitrogen for later use.

Cell culture

Normal human intestinal epithelial cells (HIECs) were purchased from the American Type Culture Collection (Manassas, VA, USA). The CRC cell lines including HCT116, DLD1, HT29, SW480, SW620 and LS174T were all purchased from Shanghai Model Cell Bank (Shanghai, China). All of the cells were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA).

Cell transfection

Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection according to the guidelines. The miR-335-5p mimics for overexpression, miR-335-5p inhibitor for down-regulation and miRNA negative control (NC) for control were purchased from GenePharma (Shanghai, China). Plasmid was transfected into cell lines in order to overexpress LDHB, while siRNA was used to down-regulate LDHB. The transfection efficiency was evaluated via qRT-PCR.

RNA isolation and qRT-PCR

Total RNA was extracted from cells and tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) and synthesized into cDNA according to the instructions of reverse transcriptase Kit (TaKaRa, Dalian, China). Then, the LDHB mRNA expression level was normalized with glyceraldheyde 3-phosphate dehydrogenase (GAPDH), while U6 was used as control for the miR-335-5p expression level.

Cell counting kit-8 (CCK-8) assay

CCK8 assay was used to examine the cell proliferation ability. After transfection, the cells were inoculated into 96-well plates (4×10^3 cells/well). After culture for 24, 48 and 72 h, CCK8 solution (Yisheng, Shanghai, China) was added to each well for culture for another 2 h at 37°C. The optical density (OD) value (490 nm) was detected using a spectrophotometer (Thermo Scientific, Rockford, IL, USA).

Colony formation assay

Cells were transfected and inoculated into 6-well plates (80 cells/well). After 14 days, colonies were visible, and then the cells were fixed with 1% trichloroacetic acid for 3 h at room temperature, and stained with aniline violet. Finally, the colonies were counted under a light microscope.

Transwell assay

Chambers and Matrigel were purchased from Millipore. The cells were suspended in DMEM with 2% fetal bovine serum (FBS) (about 1×10^5 cells/mL). Then, 200 µL cell suspension was added into the upper chamber with Matrigel, and 600 µL DMEM with 10% FBS was added into 24-well plates, followed by culture for 20 h at 37°C. After that, the chamber was taken out, added with methanol, stained with crystal violet, washed and dried. Images were acquired under light microscope. Chambers were decolorized with 33% acetic acid and the number of penetrating cells was counted based on the OD value (490nm).

Wound healing test

The transfected cells were inoculated on 6-well plates (1×10^5 cells/well) with 5% CO_2 at 37°C. After 24 h, the plate was scratched using a pipette. Then the cells were washed and incubated with fresh serum-free DMEM in the incubator and observed at 0 h, 24 h and 48 h. Images were acquired under an inverted microscope.

Western blot

Protein was abstracted by radioimmunoprecipitation assay (RIPA) buffer and Phenylmethanesulfonyl fluoride (PMSF) (Beyotime, Shanghai, China). Protein lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After that, the membranes were sealed with primary antibodies at 4°C overnight and then incubated with specific secondary antibodies 2 h at room temperature. Antibodies against LDHB, GAPDH and Goat anti-rabbit IgG antibody were purchased from Abcam (Abcam, Cambridge, MA, USA). Protein relative expression level was evaluated by Image J software.

Dual luciferase reporter assay

The wild-type LDHB 3'-UTR sequence or the mutant-type LDHB 3'-UTR sequence was combined to pGL3 promoter vector (Genscript, Nanjing, China). Luciferase activity was determined using the Victor 1420 Multilabel Counter (Wallac, Finland) via Dual-Luciferase Reporter Assay System (Promega, Hercules, CA, USA) according to the manufacturer's instructions.

Statistics

All of the experiments were carried out for at least three times. All the data were expressed as mean ± standard error of mean. Two groups of data were analyzed via independent samples Student's unpaired t-test. P<0.05 suggested statistically significant differences.

Results

MiR-335-5p was lowly expressed in CRC tissues and cell lines

The expression level of miR-335-5p was assessed by qRT-PCR in CRC tissues and cells. As shown in Figure 1A, the expression level of miR-335-5p in tumor tissues was lower than that in adjacent normal tissues. Subsequently, the expression level of miR-335-5p was examined in different types of CRC cell lines including HCT116, DLD1, HT29, SW480, SW620 and LS174T (Figure 1B). The results illustrated that miR-335-5p was down-regulated in all the CRC cells compared with that in HIECs. Two cell lines with the lowest and highest expression of miR-335-5p (HCT116 and SW620) were selected for further studies. Mimics and inhibitor were transfected in the cells to overexpress and down-regulate miR-335-5p, respectively. The transfection efficiency was detected via qRT-PCR (Figure 1C) and showed that the expression of miR-335-5p after transfection of mimics or inhibitor in CRC cells was overexpressed or downregulated, respectively.

MiR-335-5p repressed the proliferation ability of CRC cells

CCK8 assay and colony formation assay were applied to evaluate the influence of miR-335-5p on the proliferation of CRC cells. The results showed that at 48 and 72 h, the proliferation rate in the

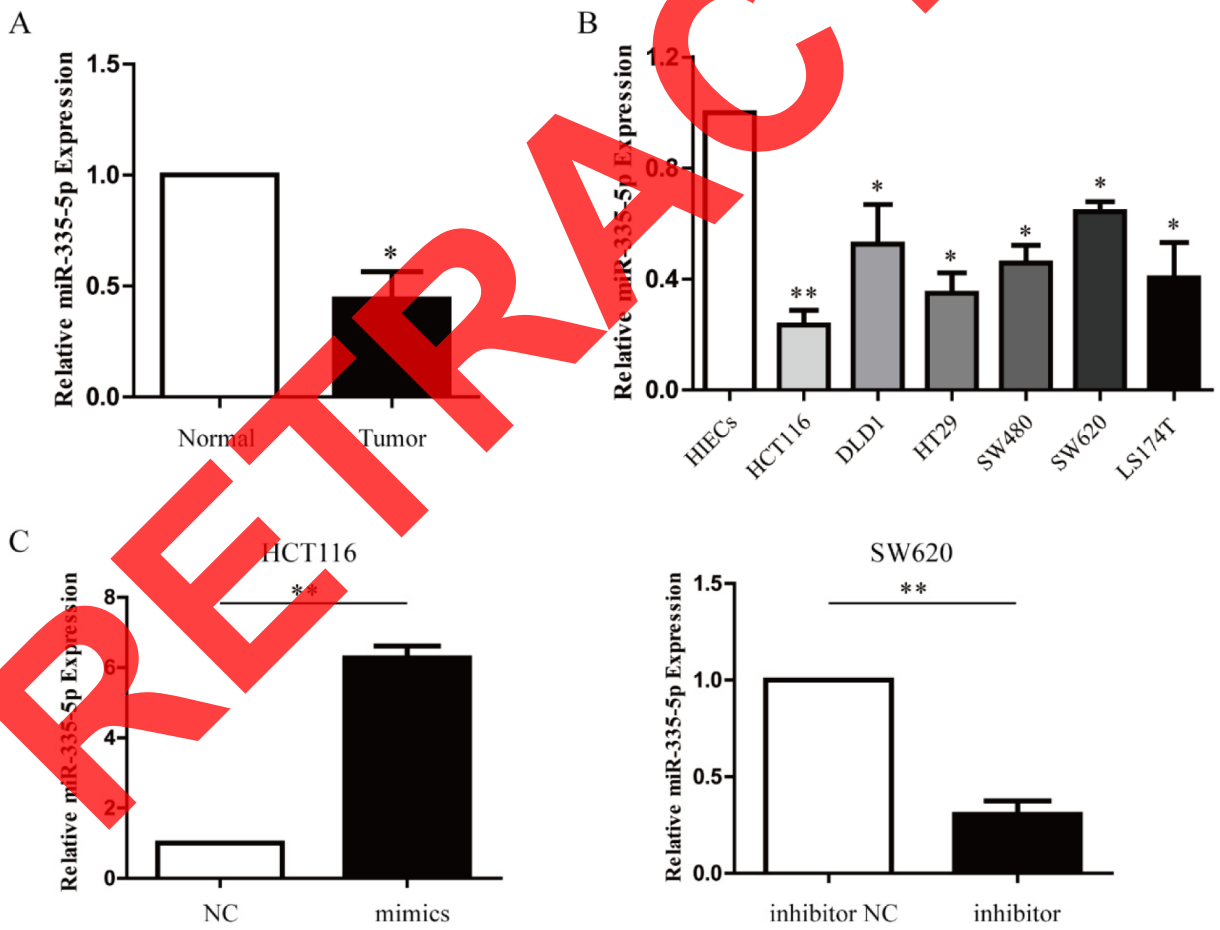

Figure 1. Relative expression of miR-335-5p in CRC and cells transfected. **A:** The relative expression of miR-335-5p is detected in 42 pairs of CRC tissues and adjacent normal tissues. The Figure shows that the expression level of miR-335-5p in tumor tissues was significantly lower than that in adjacent normal tissues. **B:** qRT-PCR is used to verify the expression of miR-335-5p in CRC cell lines. The Figure shows that miR-335-5p was downregulated in all the CRC cells compared with that in HIECs. **C:** Transfection efficiency of mimics and inhibitor was determined by qRT-PCR. The Figure shows that the expression of miR-335-5p after transfection of mimics or inhibitor in CRC cells was overexpressed or downregulated, respectively. *p<0.05, **p<0.01 vs. NC group. The data are expressed as mean ± SEM.

overexpression (mimics) group was obviously lower than that in the control (NC) group, while there was no statistically significant difference at 24 h (Figure 2A). The opposite results were found in down-regulation (inhibitor) group. Similarly, the colony formation rate was lower in the mimics group compared with the NC group (Figure 2B), which was further confirmed by the opposite results in the inhibitor group. The above findings suggest that miR-335-5p inhibits the proliferation of CRC cells.

MiR-335-5p suppressed the migration and invasion of CRC cells

Wound healing assay and Transwell assay were employed to estimate the migration and invasion

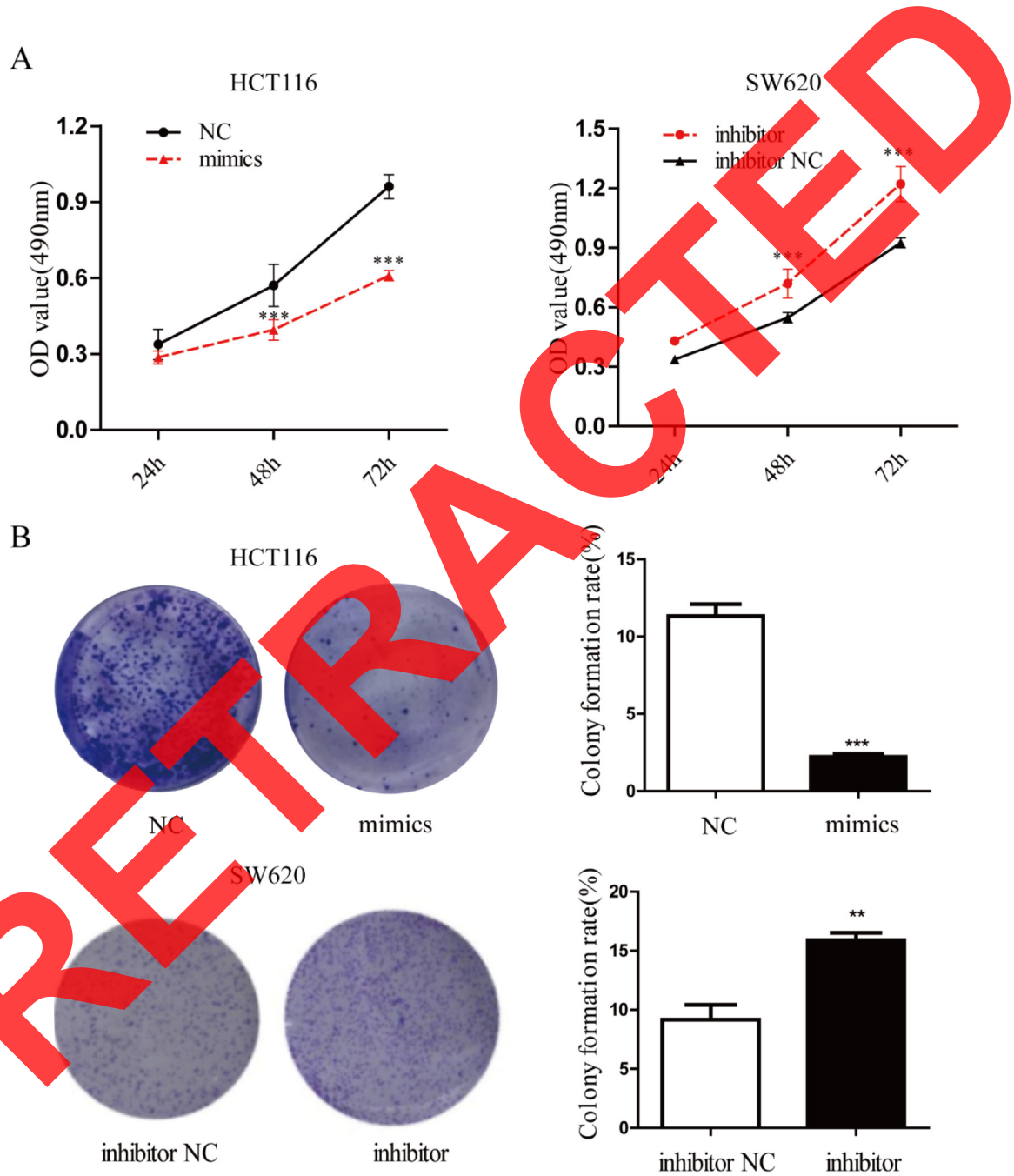

Figure 2. MiR-335-5p suppresses proliferation of CRC cell lines. **A:** CCK8 assay is used to analyze the cell proliferation and higher OD value indicates the higher cell proliferation ability. The Figure shows that at 48 and 72 h, the proliferation rate in the overexpression (mimics) group was obviously lower than that in the control (NC) group, while the opposite results were found in downregulation (inhibitor) group. **B:** Cell proliferation ability was detected. The Figure shows that the colony formation rate was lower in the mimics group compared with the NC group; however, the colony formation rate was higher in the inhibitor group compared with the NC group. **p<0.01, ***p<0.001, compared to control group (NC). The data are expressed as mean ± SEM.

ability of CRC cells. The results of wound healing assay revealed that the migration ability of CRC cells was obviously lower after mimics were transfected compared with the NC group (Figure 3A). The migration ability was improved in the inhibitor group compared with the NC group. Besides, the Transwell assay (Figure 3B) showed that the number of invasive cells in the mimics group was obviously lower than that in the NC group, while it was increased in the inhibitor group compared with the NC group. These results demonstrate that miR-335-5p weakens the migration and invasion of CRC cells.

LDHB was the potential target of miR-335-5p in CRC cells

To identify the target gene of miR-335-5p, several online public available databases were employed in the prediction and LDHB was considered as a potential downstream target of miR-335-5p. In order to further investigate this hypothesis, the dual luciferase reporter assay was designed for validation (Figure 4A). The results revealed that the luciferase activity in the mimics group was noticeably lower than that in the NC group after the co-transfection with LDHB WT-type 3'-UTR, while there was no significant difference after the co-transfection with mutant-type 3'-UTR. Then, western blotting (Figure 4B) and qRT-PCR (Figure 4C) were performed to explore the correlation between LDHB and miR-335-5p. The outcomes demonstrated that when miR-335-5p was up-regulated in the mimics group, the LDHB expression was decreased. On the contrary, the LDHB expression was increased when miR-335-5p was inhibited in the inhibitor group. These results suggest that LDHB was an affirmative target of miR-335-5p.

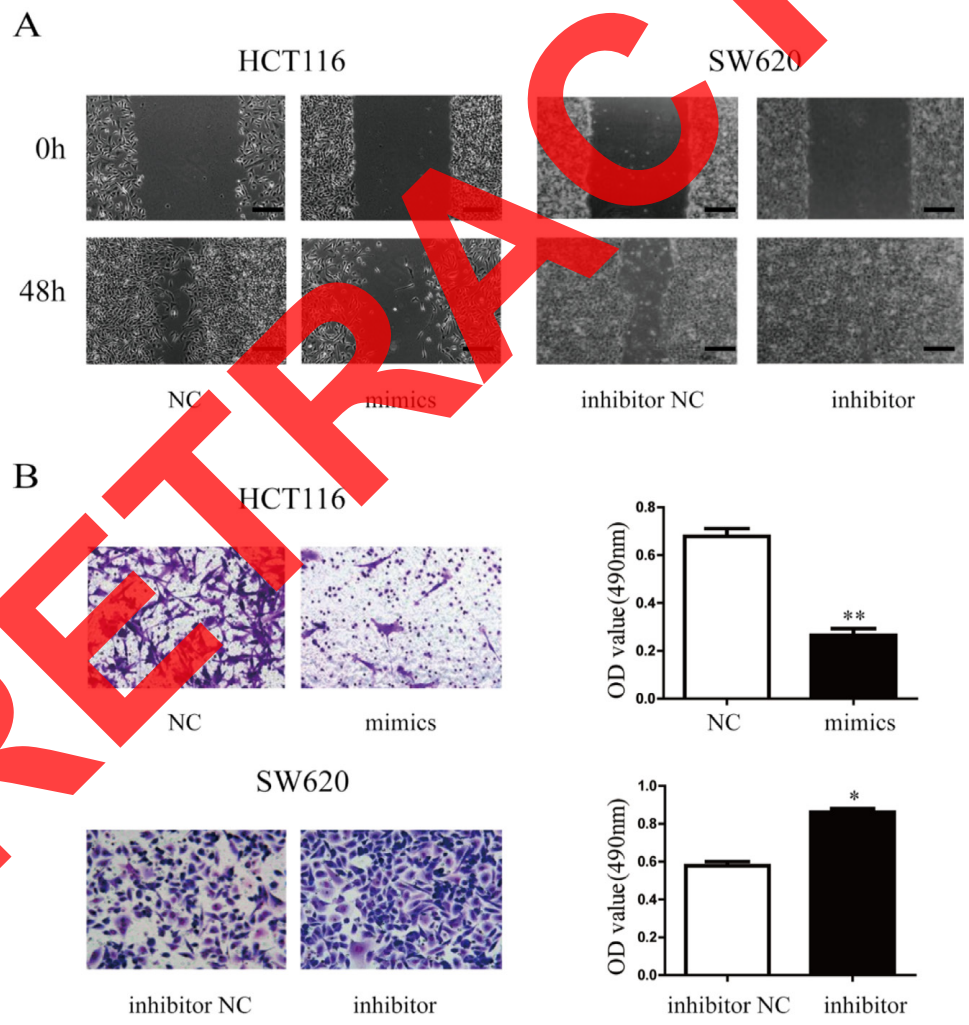

Figure 3. MiR-335-5p inhibits cell migration and invasion in CRC cell lines. **A:** Wound healing assay is performed in transfected cells. The Figure shows that the migration ability of CRC cells was obviously lower after mimics were transfected compared with the NC group. Meanwhile, the migration ability was improved in the inhibitor group compared with the NC group. **B:** Invasive cells were detected by Transwell assay with matrigel. The Figure shows that the number of invasive cells in the mimics group was obviously lower than that in the NC group, while it was increased in the inhibitor group compared with the NC group. *p<0.05, **p<0.01 compared to control group. The data are expressed as mean ± SEM.

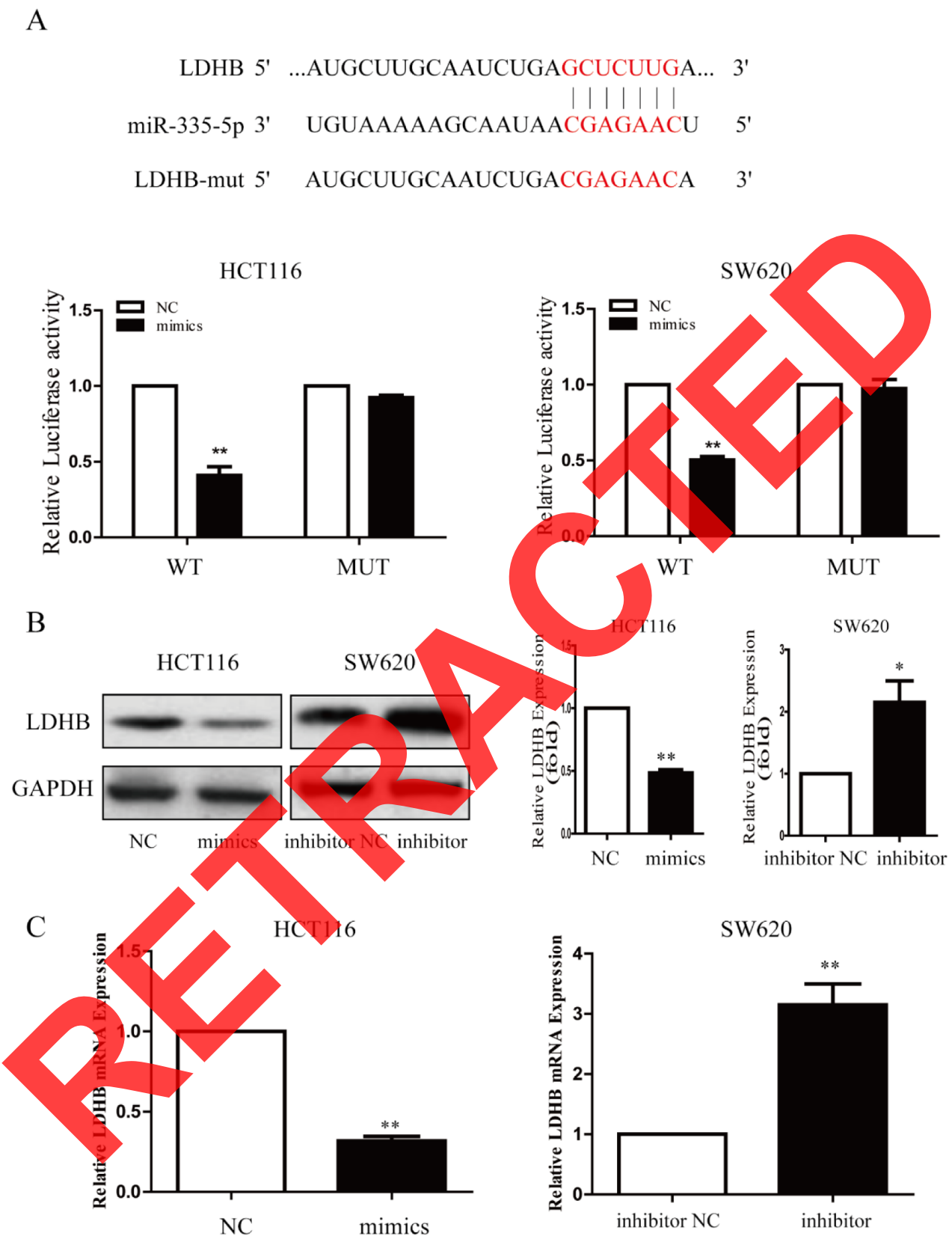

Figure 4. LDHB is proved to be the target gene of miR-335-5p. **A:** Dual luciferase reporter assay is constructed to prove that miR-335-5p directly binds to the 3'-UTR of LDHB. The sequence of wild-type and mutant-type LDHB 3'-UTR is transfected. After the co-transfection with wild-type or mutant-type 3'-UTR reporter plasmids, the relative luciferase activity was detected and recorded by histogram. **B-C:** Western blotting and qRT-PCR were used to test the protein level of LDHB in transfected cell lines. The Figure shows that when miR-335-5p was upregulated in the mimics group, the LDHB expression was decreased. On the contrary, the LDHB expression was increased when miR-335-5p was inhibited in the inhibitor group. *p<0.05, **p<0.01 compared to control group. The data are expressed as mean ± SEM.

MiR-335-5p functioned as a tumor suppressor by regulating LDHB in CRC cells

To further verify the correlation between miR-335-5p and LDHB, the rescue assay was designed. In the mimics and inhibitor groups, the cells were co-transfected with LDHB overexpression plasmid or LDHB siRNA, respectively. CCK8 assay and colony formation assay showed that compared with the NC group (mimics+NC), the proliferation ability was recovered in mimics+LDHB group after transfection with LDHB overexpression plasmid. In contrast, the proliferation ability was suppressed after transfection with LDHB siRNA in the inhibitor+siRNA group (Figure 5A and 5B). Similarly, over-expressing LDHB induced recovery of migration and invasion of CRC cells compared with the mimics+NC group, while the capability was reduced after transfection with LDHB siRNA in inhibitor + siRNA group (Figure 6A and 6B). Taken together, all the findings demonstrated that miR-335-5p regulates cell proliferation, migration and invasion of CRC cells by targeting LDHB.

Discussion

CRC is a quite common tumor with high rates of invasion or metastasis, leading to a poor prognosis [14]. The occurrence of CRC is controlled by a variety of genes and factors, which is a multi-stage complex process. In recent years, some new CRC markers and targets have drawn great attention. With the study on miRNAs, tumor-related miRNAs

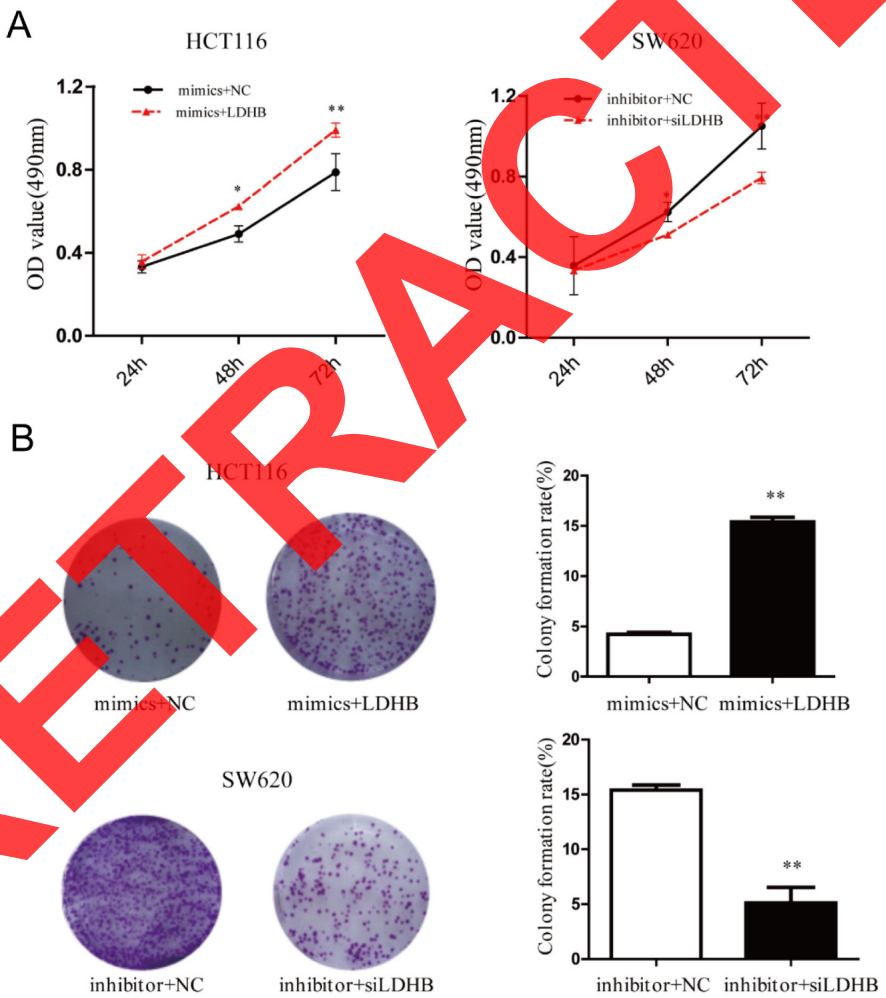

Figure 5. MiR-335-5p functioned as a tumor suppressor by directly targeting LDHB. **A:** CCK-8 assay was performed to elucidate the effect of miR-335-5p on cell proliferation in co-transfected cells. The Figure shows that compared with the NC group (mimics+NC), the viability of CCK-8 was recovered in mimics+LDHB group after transfection with LDHB overexpression plasmid. In contrast, the proliferation ability was suppressed after transfection with LDHB siRNA in the inhibitor+siRNA group. **B:** Colony formation assay was used to examine cell proliferation in co-transfected cells. The Figure shows that compared with the NC group (mimics+NC), the proliferation ability was recovered in mimics+LDHB group after transfection with LDHB overexpression plasmid. In contrast, the proliferation ability was suppressed after transfection with LDHB siRNA in the inhibitor + siRNA group. *p<0.05, **p<0.01, ***p<0.001, compared to control group. The data are expressed as mean ± SEM.

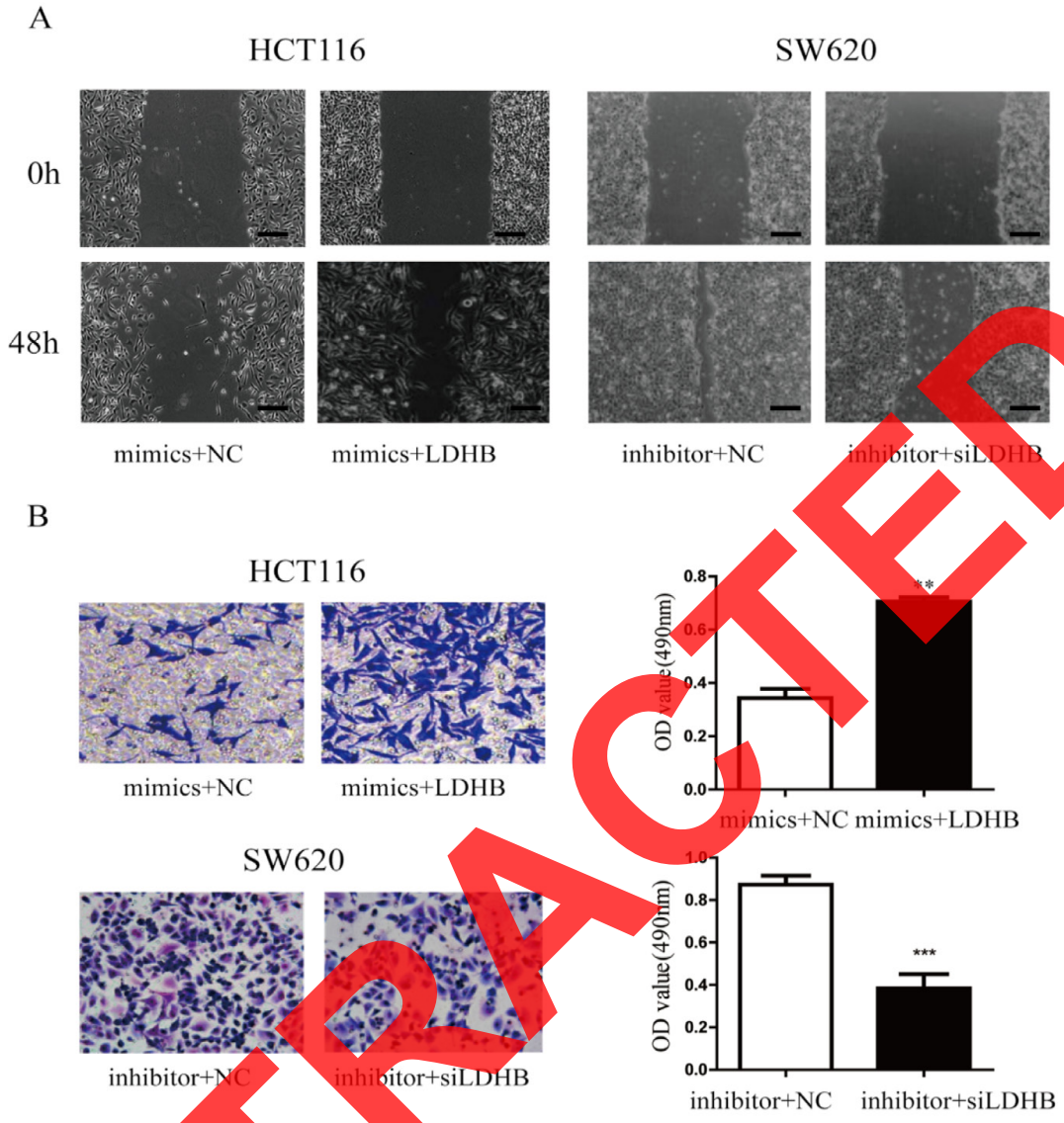

Figure 6. MiR-335-5p suppresses cell migration and invasion by negatively regulating LDHB. **A:** Wound healing assay is employed to examine the migration ability of co-transfected cells. The Figure shows that overexpressing LDHB induced recovery of migration and invasion of CRC cells compared with the mimics+NC group, while the capability was reduced after transfection with LDHB siRNA in the inhibitor + siRNA group. **B:** Invasive cells are detected by Matrigel assay. The Figure also shows that overexpressing LDHB induced recovery of migration and invasion of CRC cells compared with the mimics+NC group, while the capability was reduced after transfection with LDHB siRNA in the inhibitor + siRNA group. **p<0.01, ***p<0.001 compared to control group. The data are expressed as mean ± SEM.

have become a new hotspot [15]. It was reported that the dysregulation of miR-335-5p is inseparably related to the development of many tumors [16]. Luo et al. [17] reported that miR-335-5p can inhibit the metastasis of thyroid cancers and it has similar function in osteosarcoma [18] and gastric cancer [19]. Moreover, it also inhibits cell proliferation and migration of non-small cell lung cancer [20]. Another study has shown that miR-335-5p can strengthen the chemosensitivity of ovarian cancer [21]. However, the potential molecular mechanism of miR-335-5p in CRC remains to be elucitated.

In this study, it was first confirmed that the expression level of miR-335-5p is significantly low in CRC tissues and cells compared with the adjacent normal tissues and HIECs. Subsequently, miR-335-5p was overexpressed in HCT116 and down-regulated in SW620 to inspect the role of miR-335-5p in CRC. After transfection with mimics and inhibitor, CCK8 assay and colony formation assay were performed and it was found that miR-335-5p could suppress cell proliferation. Furthermore, it was also shown that miR-335-5p could reduce the migration and invasion of CRC in wound healing assay and Transwell assay. Next, several online databases were employed to predict the downstream target of miR-335-5p and LDHB was considered as the potential target of miR-335-5p. This conjec-

ture was further proved by dual luciferase reporter assay. Finally, further rescue assay indicated that miR-335-5p functioned as a tumor suppressor in CRC by regulating LDHB.

Conclusions

In conclusion, the results suggest that the expression of miR-335-5p was down-regulated in both CRC tissues and cells. To further find the features of miR-335-5p, its mimics and inhibitor were transfected to overexpress or down-regulate miR-335-5p, respectively, in different cell lines. After transfection, cell functional assays were subsequently performed and it was found that miR-335-5p could reduce the proliferation, migration and invasion of CRC cells. Moreover, the databases and dual luciferase reporter assay revealed that miR-335-5p could target LDHB at 3'-UTR, demonstrating that LDHB can be a potential target of miR-335-5p. Further rescue assay confirmed that miR-335-5p might influence the development and progression of CRC by regulating LDHB. The above findings indicate that miR-335-5p/LDHB axis may be a novel therapeutic strategy in CRC. The molecular mechanism of miR-335-5p in CRC and its significance in clinical diagnosis and treatment remain to be further studied.

Conflict of interests

The authors declare no conflict of interests.

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