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ORIGINAL RESEARCH

MiRNA-124 inhibits the proliferation, migration and invasion of cancer cell in hepatocellular carcinoma by downregulating lncRNA-UCA1

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Purpose: It has been reported that miRNA-124 inhibits hepatocellular carcinoma (HCC) progression, while lncRNA-UCA1 promotes HCC. The aim of this study is to find whether miRNA-124, as a tumor suppressor in HCC can inhibit lncRNA-UCA1 in HCC cell.

Methods: Tumor tissues and adjacent healthy tissues were obtained from 66 patients diagnosed with HCC in Binzhou Medical University Hospital from January 2011 to January 2013. Cell proliferation assay, in vitro cell migration and invasion assay were applied for the research.

Results: In the present study we found that miRNA-124 was downregulated, while lncRNA-UCA1 was upregulated in tumor tissues comparing to adjacent healthy tissues of HCC patients. Expression of miRNA-124 and lncRNA-UCA1 in tumor tissues were not affected by HBV or HCV infection. Analysis of followed-up data revealed that low miRNA-124 level and high lncRNA-UCA1 level were closely correlated with poor survival. Overexpression of miRNA-124 led to inhibited lncRNA-UCA1 expression in cells of HCC cell lines, while overexpression of lncRNA-UCA1 failed to significantly affect miRNA-124 expression. Expression levels of miRNA-124 and lncRNA-UCA1 were inversely and significantly correlated in tumor tissues but not in adjacent healthy tissues. Overexpression of miRNA-124 led to inhibited, while overexpression of lncRNA-UCA1 led to increased proliferation, migration and invasion rates of HCC cell lines. In addition, lncRNA-UCA1 overexpression attenuated the inhibitory effects of miRNA-124 overexpression on cancer cell proliferation, migration and invasion.

Conclusion: Therefore, miRNA-124 may inhibit the proliferation, migration and invasion of cancer cell in hepatocellular carcinoma by downregulating lncRNA-UCA1.

Keywords: hepatocellular carcinoma, lncRNA-UCA1, miRNA-124, survival

Introduction

Liver cancer is a deadly and highly prevalent malignancy in the world.[1] Liver cancer affects about 800,000 new cases every year and the incidence is increasing trend.[2] As the most common type of liver cancer, hepatocellular carcinoma accounts for about 80% cases of liver cancer.[3] In spite of the efforts made on the treatment of HCC, recurrence rate is still high,[4] leading to poor survival. At present, the overall 5-year survival rate of HCC is still below 20%.[5]

Non-coding RNAs (ncRNAs) are non-protein coding RNA transcripts with critical functions in both physiological and pathological processes.[6,7] ncRNAs are divided into different subgroups according to their sizes and functions.[6,7] Long

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non-coding RNAs, or lncRNAs, are non-coding RNA transcripts longer than 200 nucleotides.[8] LncRNAs are key players in human cancers including HCC.[9] It is known that lncRNAs may interact with microRNAs (miRNAs), another subgroup of ncRNAs, to participate in cancer biology.[10] It is known that miRNA-124 inhibits hepatocellular carcinoma (HCC) progression,[11,12] while lncRNA-UCA1 promotes HCC.[13] Our data showed that miRNA-124 inhibited the proliferation, migration and invasion of cancer cell in hepatocellular carcinoma possibly by down-regulating lncRNA-UCA1.

Materials and methods

Human specimens

Tumor tissues and adjacent healthy tissues were obtained from 66 patients diagnosed with HCC in Binzhou Medical University Hospital from January 2011 to January 2013. Inclusion criteria: 1) newly diagnosed HCC cases through pathological biopsies; 2) willing to and completed a 5-year follow-up after admission; 3) patients provided informed consent. Exclusion criteria: 1) patients who were not willing to receive liver biopsy; 2) patients who were diagnosed with multiple diseases; 3) death occurred by not by HCC. These patients included 36 males and 30 females (33–72 years, 49.4±4.8 years). HBV or HCV infections were detected by sensitive PCR. There were 30 cases of HBV-positive, 19 cases of HCV-positive and 17 cases of negative for both HBV and HCV. Ethics Committee of Binzhou Medical University Hospital approved this study.

Follow-up

A 5-year follow-up was performed after admission. Overall survival of patients was recorded to be used to plot survival curves.

Real-time quantitative PCR (RT-qPCR)

To detect miRNA-124, mirVana miRNA isolation Kit (Thermo Fisher Scientific) was used to extract miRNAs, and reverse transcriptions were performed using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). PCR mixtures were made using Agilent miRNA QRT-PCR Detection Kit (Agilent). To detect the expression of lncRNA-UCA1, MPure™ Total RNA Extraction Kit (117022160, MP Biomedicals) was used to extract total RNA and MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Lucigen) was used to perform reverse transcription. PCR mixtures were made using SYBR® Green Quantitative RT-qPCR Kit (Sigma-Aldrich). Primers of miRNA-124, lncRNA-UCA1 as well as endogenous control β-actin and U6 were designed and synthesized by GenePharma (Shanghai, China). Sequences of primers were: 5′-TCGGGTAACTCTTACGGT-3′ (forward) and 5′-GGTCCATTGAGGCTGTAG-3′ (reverse) for UCA1; 5′-GACCTCTATGCCAACACAGT-3′ (forward) and 5′AGTACTTGCGCTCAGGAGGA-3′ (reverse) for β-actin; 5′-CTAGCCTGCAGGCGTGCTG-3′ (forward) for miRNA-124. miRNA-124 reverse primer and U6 primers were included in the qPCR kit. Using $2^{-\Delta\Delta CT}$ method, lncRNA-UCA1 was normalized to endogenous control β-actin and miRNA-124 expression was normalized to endogenous control U6.

HCC cell lines, vectors and cell transfection

Cells of SNU-398 and SNU-449 HCC cell lines were from ATCC (USA). RPMI-1640 Medium (Catalog No. 30-2001) containing 10% heat-inactivated fetal bovine serum (FBS) was used as cell culture medium. Vectors expressing lncRNA-UCA1 and ROCK1 were purchased from Sangon (Shanghai, China). miR-124 mimic and Scrambled negative control miRNA were bought for Sigma-Aldrich. Lipofectamine 3000 reagent (Thermo Fisher Scientific) was used to achieve transient transfections. All operations were performed according to the manufacturer's instructions. Doses of miRNA and vectors were 40 and 10 nM, respectively. Cells with no transfections were control cells (control, C). Empty vector- or Scrambled negative control miRNA-transfected cells were negative control cells (negative control, NC).

Cell proliferation assay

Expression of miRNA-124 and lncRNA-UCA1 was detected by RT-qPCR. Cell proliferation was detected only in cases of overexpression rates of miRNA-124 and lncRNA-UCA1 were higher than 200%. Single cell suspensions (3×10^4 cells/mL) were prepared using RPMI-1640 Medium (10% FBS). Cell suspensions were transferred to each well of a 96-well plate with 0.1 mL for each well and cells were cultivated under normal conditions, followed by addition of CCK-8 solution (10 μL) every 24 hrs for 4 times. After that, cells were cultivated for further 4 hrs and cell proliferation was represented by OD values at 450 nm.

In vitro cell migration and invasion assay

Expression of miRNA-124 and lncRNA-UCA1 was detected by RT-qPCR. Cell migration and invasion were detected only in cases of overexpression rates of miRNA-124 and lncRNA-UCA1 were higher than 200%. Single cell suspensions (3×10^4 cells/mL) were prepared using RPMI-1640 Medium (1% FBS). Cell suspensions were transferred to the upper chamber with 0.1 mL for each well, and RPMI-1640 Medium containing 20% FBS was used to fill the lower chamber. Cell culture was performed under normal conditions for 24 hrs and 0.5% crystal violet (Sigma-Aldrich, USA) was performed at room temperature for 10 mins. The same protocol was used for both migration and invasion assay, but the upper chamber was coated with Matrigel (356,234, Millipore, USA) prior to invaison assay. Cell migration and invasion were normalized to cell proliferation. Control group was set to 100%, and all other groups were normalized to this group.

Statistical analysis

All statistical analyses were performed by GraphPad Prism 6 software. Correlations were analyzed by Pearson's correlation coefficient. Based on follow-up data, Kaplan–Meier method and log-rank test were used to plot and compare survival curves. Comparisons of expression levels of miRNA-124 and lncRNA-UCA1 were compared with paired *t*-test. Comparisons between 2 groups were compared by unpaired *t*-test. One-way ANOVA and Tukey test was used for comparisons among 3 groups. $p<0.05$ was statistically significant.

Results

miRNA-124 was downregulated and lncRNA-UCA1 was upregulated in HCC

miRNA-124 and lncRNA-UCA1 in 66 patients with HCC were detected by RT-qPCR. It was observed that miRNA-124 was significantly downregulated (Figure 1A) and lncRNA-UCA1 was significantly upregulated (Figure 1B) in tumor tissues of HCC patients compared to healthy tissues ($p<0.05$).

Expression of miRNA-124 and lncRNA-UCA1 in tumor tissues was not affected by HBV or HCV infection

Among the 66 patients with HCC, there were 30 cases of HBV-positive, 19 cases of HCV-positive and 17 cases of negative for both HBV and HCV. As shown in Figure 2, no significant differences in expression levels of miRNA-124 (Figure 2A) and lncRNA-UCA1 (Figure 2B) were found among HBV-positive, HCV-negative and non-infection groups.

Low miRNA-124 level and high lncRNA-UCA1 level were closely correlated with poor survival of HCC patients

Patients were divided into low (n=35) and high (n=31) lncRNA-UCA1 (cutoff value =4.12) as well as low (n=32) and high (n=34) miR-124 (2.04) groups. Survival curve analyses were performed. As shown in Figure 3, overall survival rate was significantly lower in low miRNA-124 level (Figure 3A) and high lncRNA-UCA1 level group (Figure 3B).

Expression levels of miRNA-124 and lncRNA-UCA1 were inversely and significantly correlated in HCC

Correlations between miRNA-124 and lncRNA-UCA1 were analyzed by Pearson's correlation coefficient. As

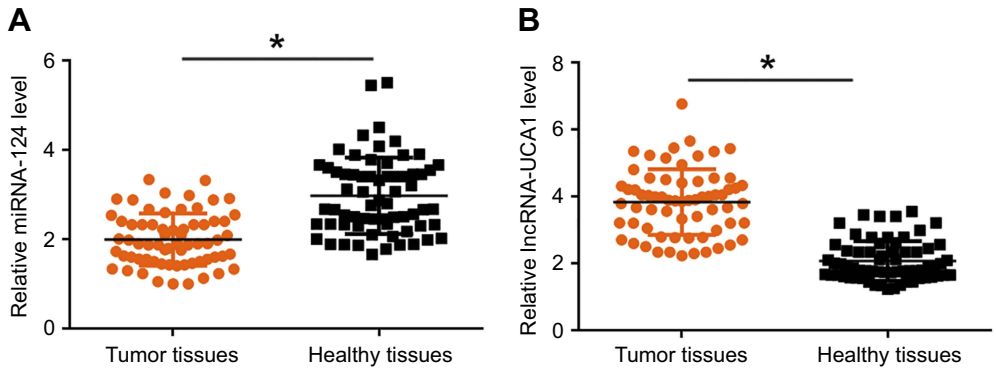

Figure 1 MiRNA-124 was downregulated and lncRNA-UCA1 was upregulated in tumor tissues of HCC patients. RT-qPCR results showed that miRNA-124 was significantly downregulated (**A**), and lncRNA-UCA1 was significantly upregulated (**B**) in tumor tissues than in adjacent healthy tissues of HCC patients (*$p<0.05$).

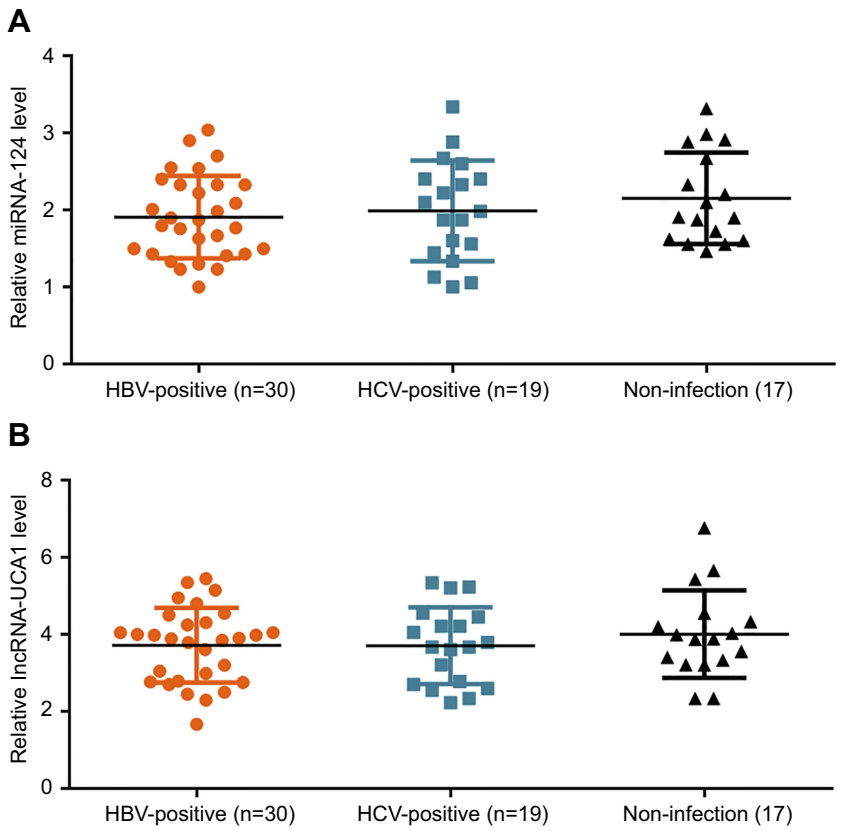

Figure 2 Expression of miRNA-124 and lncRNA-UCA1 in tumor tissues were not affected by HBV or HCV infection. No significant differences in expression levels of miRNA-124 (**A**) and lncRNA-UCA1 (**B**) were found among HBV-postive, HCV-negative and non-infection groups.

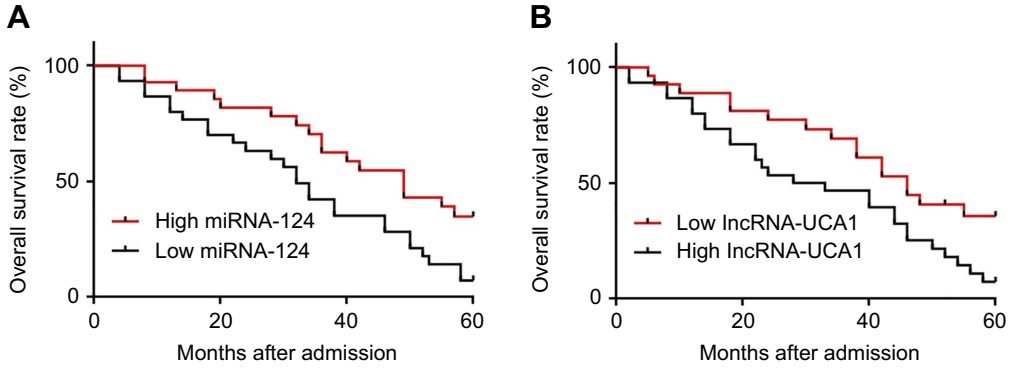

Figure 3 Low miRNA-124 level and high lncRNA-UCA1 level were closely correlated with poor survival of HCC patients. ROC curve analysis showed that low miRNA-124 level (**A**) and high lncRNA-UCA1 level (**B**) were closely correlated with poor survival of HCC patients.

shown in Figure 4, expression levels of miRNA-124 and lncRNA-UCA1 were inversely and significantly correlated in tumor tissues (Figure 4A) but not in adjacent healthy tissues (Figure 4B).

Overexpression of miRNA-124 led to inhibited lncRNA-UCA1 expression

To further confirm the correlation between miRNA-124 and lncRNA-UCA1, miRNA-124 mimic and lncRNA-UCA1 expression vectors were transfected into cells of both SNU-398 and SNU-449 HCC cell lines. Overexpression rates of miRNA-124 (255–375%) and lncRNA-UCA1 (230–340%) were higher than 200% (data not shown). Comparing to C and NC groups, overexpression of miRNA-124 led to inhibited lncRNA-UCA1 expression in cells of both HCC cell lines (Figure 5A, $p<0.05$), while overexpression of lncRNA-UCA1 failed to significantly affect miRNA-124 expression (Figure 5B).

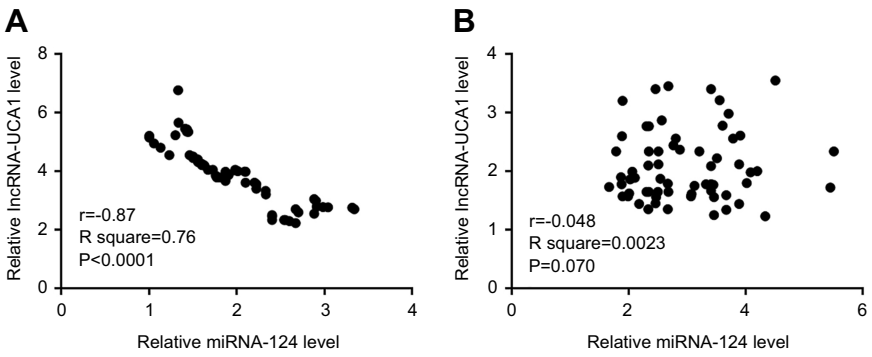

Figure 4 Expression levels of miRNA-124 and lncRNA-UCA1 were inversely and significantly correlated in tumor tissues. Pearson's correlation coefficient analysis showed that expression levels of miRNA-124 and lncRNA-UCA1 were inversely and significantly correlated in tumor tissues (Figure 4A) but not in adjacent healthy tissues (Figure 4B).

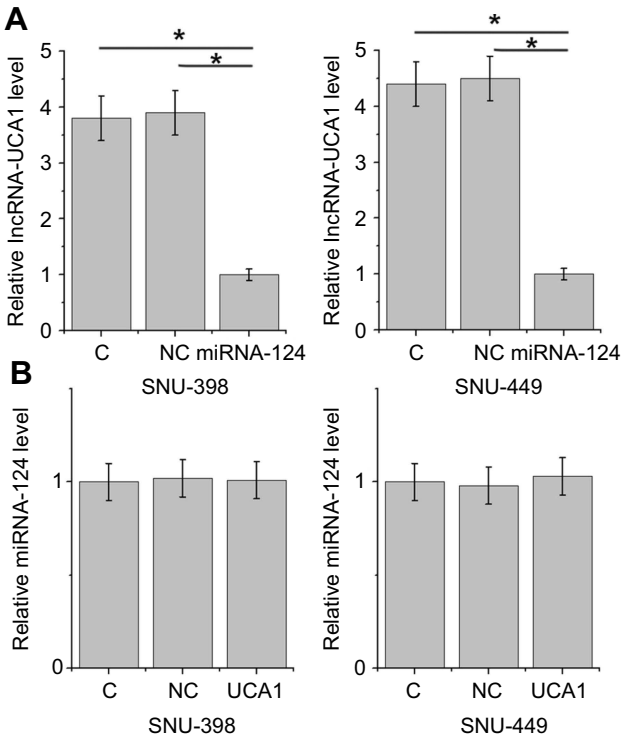

Figure 5 Overexpression of miRNA-124 led to inhibited lncRNA-UCA1 expression. Overexpression of miRNA-124 led to inhibited lncRNA-UCA1 expression in cells of both HCC cell lines (**A**) (*p<0.05), while overexpression of lncRNA-UCA1 failed to significantly affect miRNA-124 expression (**B**).

miRNA-124 led to inhibited proliferation, migration and invasion of HCC cells by inhibiting lncRNA-UCA1

Comparing to C and NC groups, overexpression of miRNA-124 led to inhibited, while overexpression of lncRNA-UCA 1 led to increased proliferation (Figure 6A), migration (Figure 6B) and invasion (Figure 6C) rates of cells of HCC cell lines SNU-398 and SNU-449 (p<0.05). In addition, lncRNA-UCA1 overexpression attenuated the inhibitory effects of miRNA-124 overexpression (p<0.05).

miRNA-124 downregulated ROCK1, which upregulated UCA1

Comparing to two controls, overexpression of ROCK1 resulted in the downregulation of ROCK1 mRNA in cells of HCC cell lines SNU-398 and SNU-449 (Figure 7A, p<0.05). In contrast, ROCK1 overexpression mediated the upregulation of UCA1 in cells of both cell lines (Figure 7B, p<0.05).

Discussion

It is well known that miRNAs and lncRNAs are essential players in cancer biology. The involvement of miRNA–lncRNA interactions in cancer has also been reported. We first showed that miRNA-124, as a tumor suppressor in HCC, may inhibit lncRNA-UCA1, an oncogene in HCC, to through ROCK1 to inhibit HCC.

MiRNA-124 is a well-characterized tumor suppression miRNA in many different types of human cancers, including HCC.[11,12,14] Our study further confirmed that miRNA-124 was downregulated in HCC tissues than in adjacent tissues, and the low expression level of miRNA-124 is closely correlated with poor overall survival of HCC patients. In addition, overexpression of miRNA-124 led to inhibited behaviors of HCC cells. Our study further confirmed the role of miRNA-124 in HCC and suggested that miRNA may serve as a prognostic marker for HCC.

MiRNA-124 participates in cancer biology through the interactions with multiple signaling molecules.[11,12,14] A recent study reported that miRNA-124 interacted with lncRNA MALAT1 to regulate the growth of tongue cancer.[15] LncRNA-UCA1 exerts oncogenic effects on different types of cancers, and it has been reported that lncRNA-UCA1 may inhibit miR-216b,[13] miR-193a-3p[16] and miR-204-5p[17] to promote cancer development.

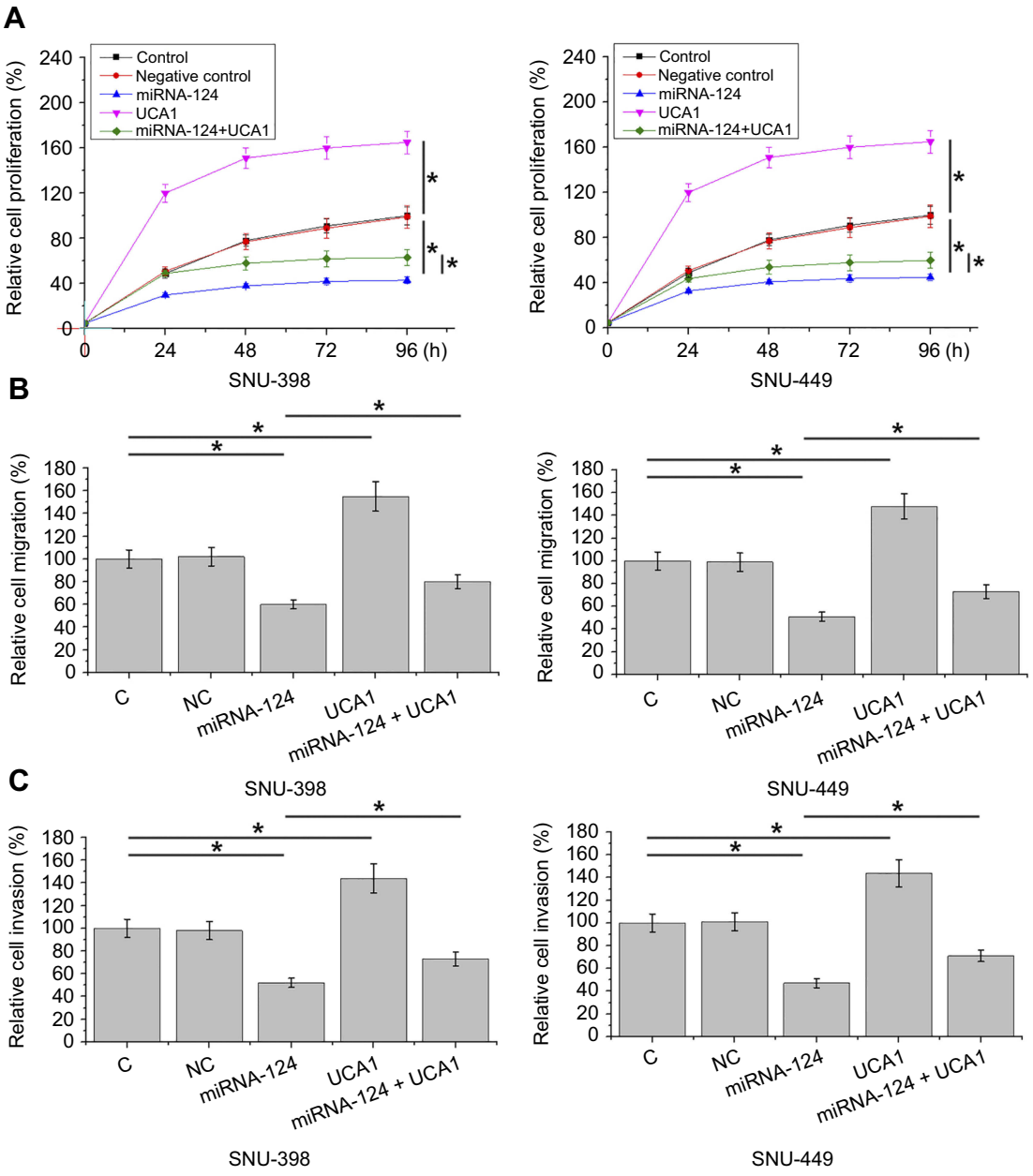

Figure 6 MiRNA-124 led to inhibited proliferation, migration and invasion of HCC cells by inhibiting lncRNA-UCA1. Overexpression of miRNA-124 led to inhibited, while overexpression of lncRNA-UCA1 led to accelerated proliferation (**A**), migration (**B**) and invasion (**C**) of cells of HCC cell lines SNU-398 and SNU-449. In addition, lncRNA-UCA1 overexpression attenuated the inhibitory effects of miRNA-124 overexpression on cancer cell proliferation, migration and invasion (*p<0.05).

However, the inhibition of lncRNA-UCA1 by miRNAs has not been reported. In this study, we first reported that miRNA-124 may serve as an upstream inhibitor of lncRNA-UCA1, and the inhibition of lncRNA-UCA1 by miRNA-124 is involved in the regulation of behaviors of HCC cells. However, significantly inverse correlation between lncRNA-UCA1 and miRNA-124 was only observed in tumor tissues but not in adjacent cancer tissues. Therefore, the interaction between lncRNA-UCA 1 and miRNA-124 may be disease-specific. In addition, our study also observed that expression of lncRNA-UCA1 and miRNA-124 in tumor tissues was not affected by HBV or HCV infections, which are common causes of HCC.[18] Therefore, lncRNA-UCA1 and miRNA-124 may interact to participate in HCC through HBV- and HCV-independent pathways. It has been reported that TGF-β

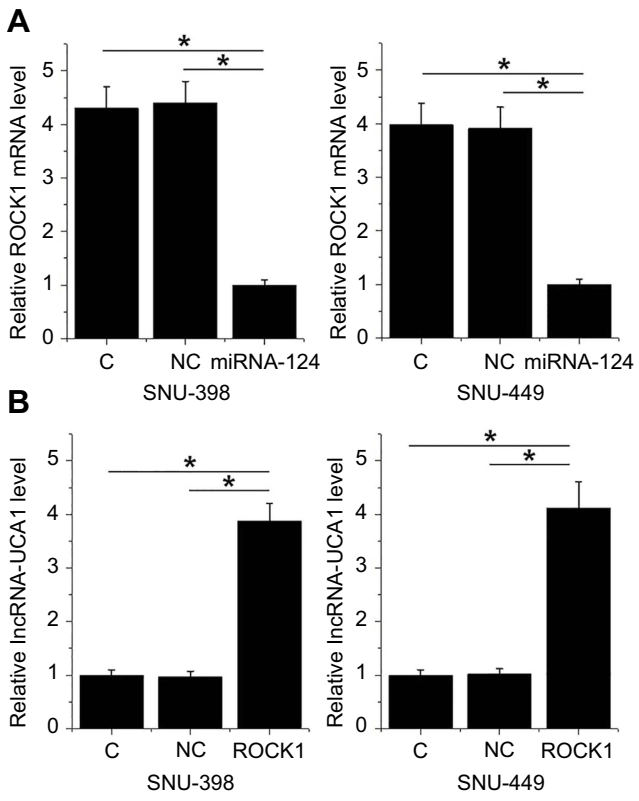

Figure 7 MiRNA-124 downregulated ROCK1, which upregulated UCA1. Comparing to two controls, overexpression of ROCK1 resulted in the downregulation of ROCK1 mRNA in cells of HCC cell lines SNU-398 and SNU-449 (**A**). In contrast, ROCK1 overexpression mediated the upregulation of UCA1 in cells of both cell lines (**B**) (*p<0.05).

pathway can induce the expression of lncRNA-UCA1,[19] while which TGF-β pathway branch mediates this action is unknown. ROCK1, a branch of TGF-β pathway, is a direct target of miR-124.[20] In the present study, we showed that ROCK1 overexpression resulted in upregulated lncRNA-UCA1, while miR-124 overexpression caused downregulated ROCK1 in HCC cells. Therefore, we characterized a novel miR-124/ROCK1/lncRNA-UCA1 pathway in HCC.

Conclusion

In conclusion, our study confirmed the roles of lncRNA-UCA 1 and miRNA-124 in HCC and revealed that miRNA-124 may serve as an upstream inhibitor of lncRNA-UCA1 to inhibit the proliferation, migration and invasion of HCC cells.

Ethical approval and informed consent

We followed ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Acknowledgments

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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