Am J Transl Res 2021;13(12):14074-14080
www.ajtr.org /ISSN:1943-8141/AJTR0128676

Original Article

miR-125a-5p-targeted regulation of TRA2β expression inhibits proliferation and metastasis of hepatocellular carcinoma cells

Junde Zhou[1*], Linna Wang[2*], Yunfu Cui[3], Lanhua Tang[4]

[1]Ward 3 of General Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang, China; [2]The Sixth Department of Oncology, General Hospital of Heilongjiang General Bureau of Land Reclamation, Harbin 150088, Heilongjiang, China; [3]Ward 1 of General Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang, China; [4]Department of Oncology, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China. [*]Equal contributors and co-first authors.

Received December 21, 2020; Accepted August 3, 2021; Epub December 15, 2021; Published December 30, 2021

Abstract: Objective: To explore the regulation of miR-125a-5p in hepatocellular carcinoma (HCC) and its mechanisms. Methods: By transfecting a miR-125a-5p sequence and an interfering sequence of miR-125a-5p-s into human HCC cell lines HCC-LM3 and HepG2, miR-125a-5p-related levels were assesed by Western blot. The abilities of cell proliferation and migration were assessed by cell culture and Transwell assay, respectively. Results: HepG2 cells showed increased miR-125a-5p levels compared with HCC-LM3 cells ($P < 0.01$). However, compared with QZG cells, the level of miR-125a-5p in HepG2 and HCC-LM3 cells was down-regulated. Compared with miR-125a-5p groups, miR-125a-5p-s groups showed increased colony formation rate and mobility ($P < 0.01$). After being transfected with miR-125a-5p, the transformation factor 2β (TRA2β) and mRNA levels were decreased, whereas 5p-s expression was increased ($P < 0.01$). Inhibition of TRA2β by small interfering RNA (siRNA) diminished the ability of cells. Conclusion: miR-125a-5p inhibits the invasive capacity of HCC cells through targeting the TRA2β pathway.

Keywords: microRNA-125a, hepatocellular carcinoma, TRA2β, invasion

Introduction

Hepatocellular carcinoma (HCC) poses a serious threat to public health [1]. At present, the diagnosis and treatment of liver cancer is still a big problem [2]. The origin and development of HCC and the search for new therapeutic targets have become the focus of HCC research in recent years [3].

miRNAs play a critical role in the regulation of physiological processes such as development, differentiation, apoptosis, proliferation, and metabolism. Abnormal expression of miRNAs is involved in various human diseases [4]. miRNAs are located in vulnerable, abnormal genomic regions and have oncogenic or inhibitory effects in cancer [5]. It is noteworthy that different expression levels of the same miRNA may be expressed differently in multiple conditions. For example, miR-125a-5p may be expressed at different levels in hepatic fibrosis and HCC [6, 7]. miR-125a is located on chromosome 19q13 and is often absent in human cancers. Studies have shown that miR-125a-5p expression is decreased in HCC cells and it is associated with aggressive forms of cancer. It is clear from these studies that miR-125a-5p is a valuable biomarker for HCC.

The correlation between miR-125a-5p and HCC correlation needs further elucidation. In order to clarify the character of miR-125a-5p in regulating HCC cell invasion and elucidate its underlying mechanisms, this study investigated the connection between miR-125a-5p expression and the migration of human HCC HepG2 and HCC-LM3, as well as its effects on proliferation and metastasis.

Materials and methods

Main reagents and materials

Human HCC cell lines (HepG2 and HCC-LM3) and the non-malignant human epithelioid he-

patic cell line QZG were purchased from the Cell Bank of the Chinese Academy of Sciences (Beijing), and the use of these cell lines was in accordance with the ethical requirements. Transforming factor 2β (TRA2β) polyclonal antibody was bought from Abcam (Cambridge, UK), while fluorescence detection kit, RPMI-1640, Opti-MEM medium were bought from Hyclone (Logan, Utah, USA), and fluorescent dye FAM was bought from Shanghai Chutai Biotechnology Co. Wright-Giemsa stain was bought from Shanghai Yuanmu Biotech (Shanghai, China), and TRIzovl reagent and Lipofectamine 2000 transfection reagent were bought from Invitrogen (Grand Island, NY, USA). SDS-PAGE was bought from Sigma-Aldrich (St. Louis, Missouri, USA). THPCR kit was bought from Toyobo Scientific Research (Tokyo, Japan). PBS buffer was bought from Beyotime Institute of Biotechnology (Jiangsu, China). TRA2β small interfering RNA (siRNA) was bought from Santa Cruz Biotechnology (Shanghai, China).

Cell culture and proliferation

The frozen cells were thawed in a 37°C water bath, and centrifuged at 4°C at 300 r/min. Cells were resuspended in modified RPMI-1640 medium containing 10% FBS. Cells in the logarithmic growth period were diluted into 1×10^3 cells/mL. The agar was mixed thoroughly with the medium (1:9), added to the plate, placed at 25°C, and then inoculated with cell suspension mixture and 15 mL of 0.5% soft agar. The plates were placed in a 37°C, 5% CO_2 incubator for 2 weeks to obtain the appropriate cell density. The colony formation rate (%) = (number of colonies)/number of cells incubated) × 100%. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University.

miR-125a-5p gene transfection

The 2'-O-methyl oligonucleotide of miR-125a-5p was synthesized by Biotech (Shanghai, China): the forward sequence was 5'-UCCCUGAGACCC UUUAACCUGUGA-3' and the reverse sequence was 5'-UCA CAGGUUAAAGGGUCUCAGGGA-3'; the 2'-O-Me-scramble-5p sequence was 5'-GGACGGCGAUCAGAUAAGAGUUUCU-3'. The fluorescent dye FAM was the fluorescent tracer for oligonucleotides. The transfection procedure was performed according to Lipofectamine 2000 instructions. A cell volume of 3×10^4 including modified RPMI-1640 medium and 500 μL of 10% FBS was inoculated in 24-well plates and cultured to 70%-90%. A total of 100 pmol of oligonucleotides was added to 50 μL Opti-MEM serum-free medium and shaken to mix. Then, Opti-MEM serum-free medium was used to dilute the Lipofectamine 2000 to 1:50. After shaking, it was let stand for 5 min at 25°C. The above two dilutions were mixed and the mixture was added to each well. After incubation at 37°C for 5 h, 500 μL of fresh modified RPMI-1640 medium replaced the original medium. After incubating in the incubator for 24 h, cells were observed by the fluorescence bio-microscope (CX41-32RFL, Olympus Corporation, Tokyo, Japan). Lipofectamine 2000 and PBS were added to create a blank control group using the same protocol. To interfere with TRA2β protein expression, TRA2β siRNA (50 pmol) was added to the medium, and the control group was treated with interference fragment siNC.

Reverse transcription and qPCR analysis

TRIzovl reagent was added and the cultured cells were harvested for extraction. The reverse transcription kit was operated referring to the ABI (Abilene, Texas, USA) reverse transcription kit instructions. Using cDNA as the template, the reaction system and conditions were: 10 μL deionized water, 1 μL, oligo (dT), 2 μL RNA incubated at 16°C for 30 min, 2 μL deoxyribonucleotide triphosphate, 1 μL RNA inhibitor, 4 μL loading buffer, incubated at 42°C for 30 min, heated at 85°C for 5 min, rapidly cooled and stored at 4°C. The fluorescence quantification PCR was performed using TaqMan Universal PCR Master Mix 20 μL reaction system, operated on a Roche (Switzerland) LightCycler480 instrument, with β-action as the internal reference. Pre-denaturation, 10 min; denaturation 95°C for 15 s; annealing 60°C for 30 s, and 70°C for 30 s over 40 cycles of amplification (**Table 1**).

Western blot analysis

A volume of 50 μg of cellular protein was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane, which was incubated overnight at 4°C after TRA2β diluted 1:3000 with rat anti-human monoclonal antibody. After washing three times with PBS, the membrane was treated with horseradish perox-

Table 1. Quantitative polymerase chain reaction primers and miRNA sequences

Gene	Primer, 5'→3'
miR-125a	Sense: CTATGTTTGAATGAGGCTTCAG Antisense: CGCGTCGCCGCGTGTTTAAACG
TRA2β	Sense: TCTACTCGCAGGCGTCATGTTG Antisense: AGCTCAGCCCAAATACTCCAAGAC
β-action	Sense: ATCATGTTTGAGACCTTCAACA Antisense: CATCTCTTGGTCGAAGTCCA
miRNA-125a 5p	ccctgagacccttaacctgtga
miRNA-125a 5p-s	ucccugagacccuuuaaccuguga

idase-conjugated goat anti-rat secondary antibody at a dilution of 1:3000 at 25°C for 30 min, and then washed for removal of all uncoupled Abs. Western blotting detection reagents were used for staining and image optical density was observed.

Transwell invasion assay in vitro

Cell invasion was measured using the Transwell migration assay *in vitro*. The cells at a concentration of 1 × 10^5 cells/mL were suspended and 50 μL of it was taken into the upper chamber. A suspension of 50 μL was inoculated into the upper chamber with 1 × 10^5 cells/mL. RPMI-1640 medium containing 10% FBS was placed in the lower chamber. After 24 h of incubation, cells attached to the lower chamber were fixed with 10% formalin and cell migration was quantified by Giemsa staining.

Statistical analysis

SPSS 21.2 software was used and the data were expressed as mean ± standard deviation and analyzed by data Student *t*-test. $P < 0.05$ indicated a significant difference. Graphpad Prism 8 was used to illustrate the statistical figures.

Results

The expression of Mir-125A-5p in HCC cells was decreased

The mRNA expression was detected by qPCR. Compared with the QZG cell lines, miR-125a-5p expression was down-regulated in HepG2 and HCC-LM3 cells ($P < 0.01$). Moreover, HCC-LM3 cells had the lowest expression, which was related to the high metastasis ability of cell lines. Compared with negative controls, miR-125a mRNA expression was increased ($P < 0.01$). miR-125a-5p expression was the highest in non-malignant QZG cells, while HCC-LM3 expression was the lowest in malignant cell lines, indicating that miR-125a-5p could be related to HCC and miR-125a-5p was involved in regulation in HCC cells by transfection (**Figure 1**).

miR-125a-5p restrained the proliferation of HCC cells

HepG2, HCC-LM3 and QZG cell lines were transfected with miR-125a-5p. After resuspending growth in modified RPMI-1640 medium containing 10% FBS and culture in an incubator at 37°C and 5% CO_2, the cells with exponential were collected for colony formation assay to evaluate the proliferation ability of each cell line. The results showed that the cell proliferation rates of HepG2, HCC-LM3 and QZG cell lines transfected with miR-125a-5p were significantly reduced, and the colony formation assay showed that cell density was significantly reduced compared with the negative control group ($P < 0.01$, **Figure 2**).

miR-125a-5p restrained the migration of HCC cells

Different miR-125a-5p and -5ps sequences were transfected into cells, and Transwell analysis was used 48 h after transfection to explore cell migration ability. The results showed that the migration rate of HepG2 and HCC-LM3 cells was significantly higher than that of QZG cells ($P < 0.05$). miR-125a-5p transfection could significantly inhibit cell migration compared to negative controls, while miR-125a-5p-s exhibited inverse results ($P < 0.01$; **Figure 3**).

miR-125a-5p regulated TRA2β

Western-blot results showed that TRA2β in HCC-LM3 cells after miR-125a-5p transfection were 0.13 ± 0.24, which was lower than 0.44 ± 8.78 in the negative control group ($P < 0.01$). However, the TRA2β level increased significantly by 0.68 ± 0.13 in miR-125a-5p-s groups, which was significantly higher than that of the negative control group ($P < 0.01$). The miR-125a-5p transfection down-regulated the TRA-2β mRNA expression in HCC-LM3 cells, whereas the opposite results were observed after miR-125a-5p-s transfection ($P < 0.01$; **Figure 4**).

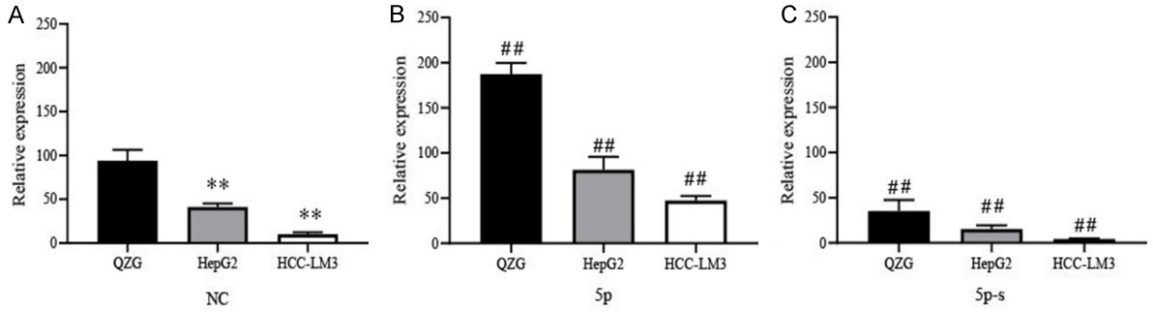

Figure 1. RNA expression after transfection. Note: $^{**}P < 0.01$ for compared to NC; $^{\#\#}P < 0.01$ compared to QZG NC.

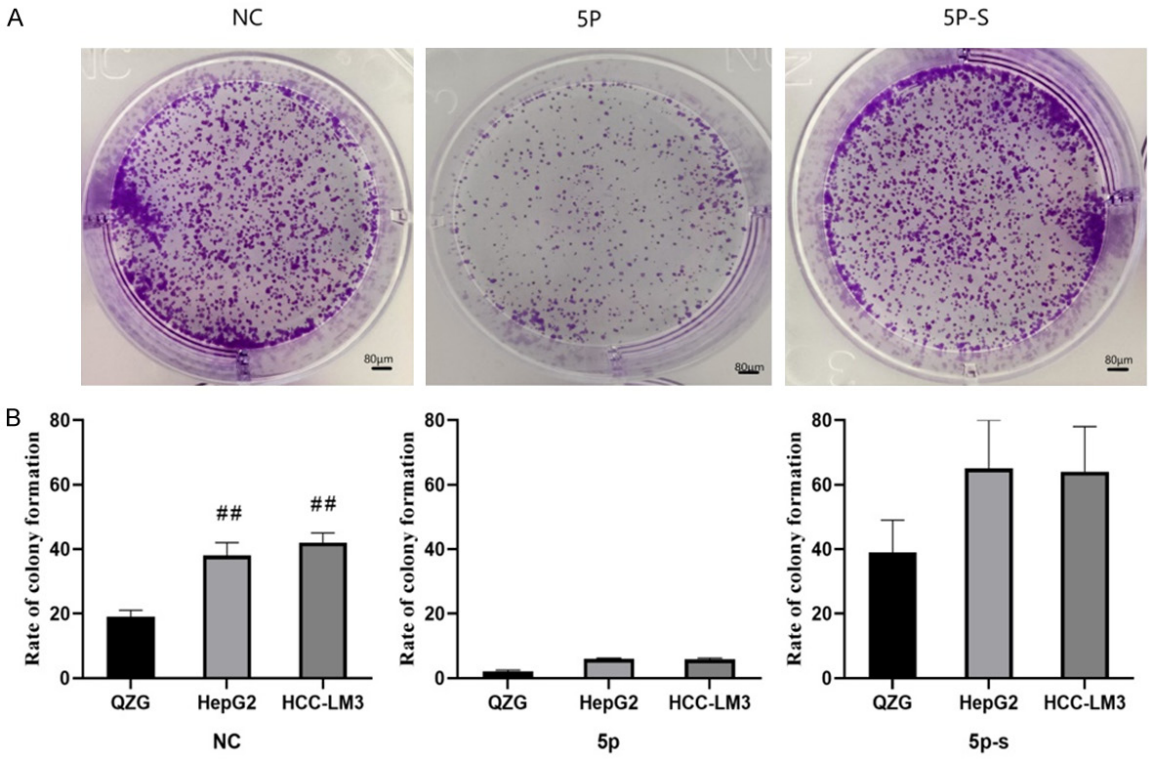

Figure 2. HCC-LM3 cell proliferation. Note: (A) Proliferation assay showed that transfection of miR-125a-5p inhibited the proliferation rate of HCC-LM3 and HepG2 cells. The opposite results were observed after transfection of miR-125a-5p-s ($P < 0.01$). (B) $^{\#\#}P < 0.01$, compared with NC.

TRA2β inhibited the proliferation and migration of HCC

In order to elucidate and analyze the correlation between TRA2β and HCC cell proliferation and migration, TRA2β-specific siRNA was used in HCC-LM3 cell lines. The results showed that the addition of siRNA inhibitors significantly reduced the colony formation and cell density of HCC-LM3 cell lines (**Figure 5A**). Meanwhile, Transwell analysis showed that siRNA inhibitors also significantly reduced the migration ability of cells (**Figure 5B**).

Discussion

Aberrant miRNA expression was found to be involved in the development and metastasis of several cancers [8-12]. miR-125a has been shown to inhibit the progression of breast cancer cells, and has also been reported to predict the onset of ovarian cancer [13, 14]. Also, miR-125a is associated with the replication and development of hepatitis B virus [15, 16]. It has been found that miR-125a inhibits the proliferation and migration of HCC cells by vascular endothelial growth factor and regula-

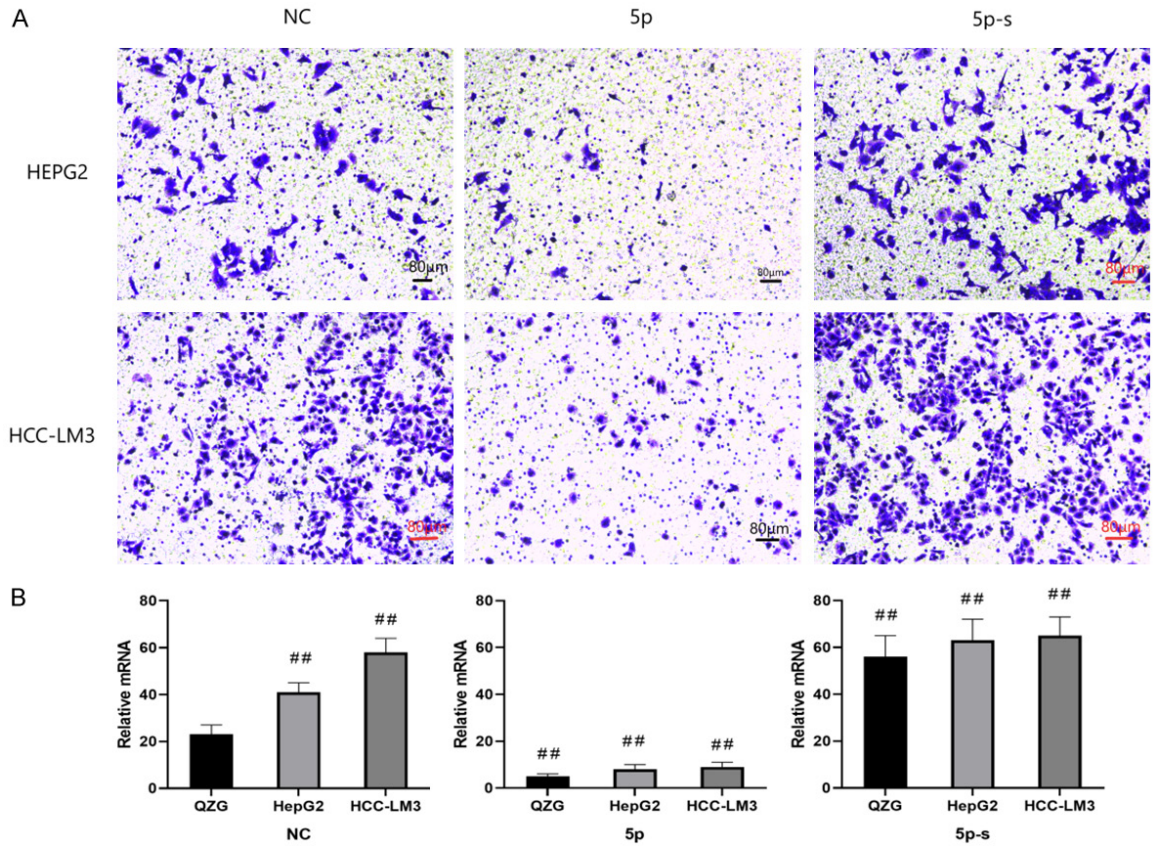

Figure 3. miR-125a-5p inhibited the migration of hepatocytes. Note: (A) miR-125a-5p transfection inhibited cell migration, whereas the opposite results were detected upon transfection with miR-125a-5p-s. (B) $^{\#\#}P < 0.01$ compared with NC.

tion of the mitochondrial membrane potential. The phosphatidylinositol 3-kinase (PI3K)/AKT pathway accelerates cell proliferation, invasion, and migration [17]. Moreover, miR-21 has been shown to inhibit HCC cells by modulating the PI3K/AKT pathway [18].

HepG2 and HCC-LM3 cells have different metastatic potentials in HCC, and HCC-LM3 cells have greater migration and invasion ability than HepG2 cells. This study showed that miR-125a-5p was greatly decreased in HCC-LM3 cells compared with HepG2 cells. QZG has hepatocellular specificity. The levels of miR-125a-5p in the cells were changed by miR-125a-5p and miR-125a-5p-s transfection. Transfection with miR-125a-5p increased its level in HCC cells, as miR-125a-5p-s decreased its level in hepatoma cells. These results suggested that miR-125a-5p was correlated with the invasion and migration ability of HCC cells. The expression of miR-125a-5p in HCC cell lines with varying invasive abilities was detected by immunohistochemistry, WB and qPCR. It has been demonstrated that HCC tissues show down-regulated miR-125a-5p with high invasive capacity, suggesting that miR-125a-5p expression was related to invasion and migration of HCC and could be used as a prognostic marker for patients with HCC. The part of miR-125a-5p in HCC progression was further demonstrated.

In this study, Transwell assay was conducted to verify the role of miR-125a-5p in the regulation of proliferation and migration of HCC. Cell proliferation assays can be performed to monitor tumor growth after fixation and determine the degree of malignancy, and in addition, there is a correlation between the strength of invasive capacity of tumor cells and the number of cell colonies [19-21]. Tumor ability depends on the ECM in which it grows; therefore, the Transwell compartments that mimic the ECM are a reliable method to determine cell invasion capacity. In the present study, a significant decrease in colony formation of hepatoma cells was detected after miR-125a-5p overexpression, while it was increased after inhibiting miR-

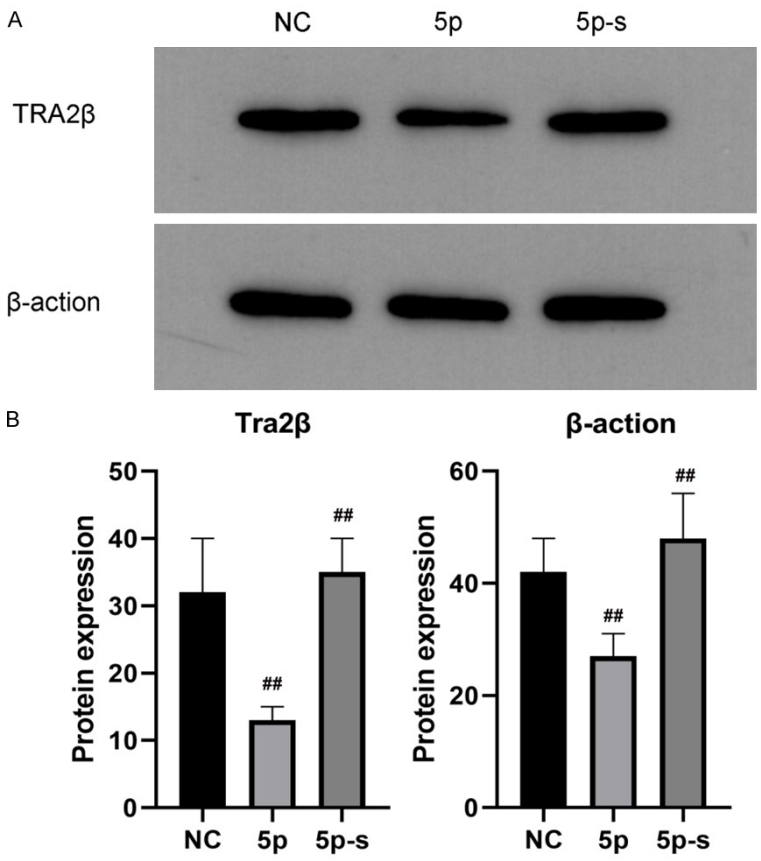

Figure 4. Western Blot results. Note: (A) Western Blot images. (B) The expression of TRA2β protein decreased in HCC-LM3 cells after miR-125a-5p transfection, but increased after transfection with miR-125a-5p-s. $^{##}P < 0.01$ compared with NC.

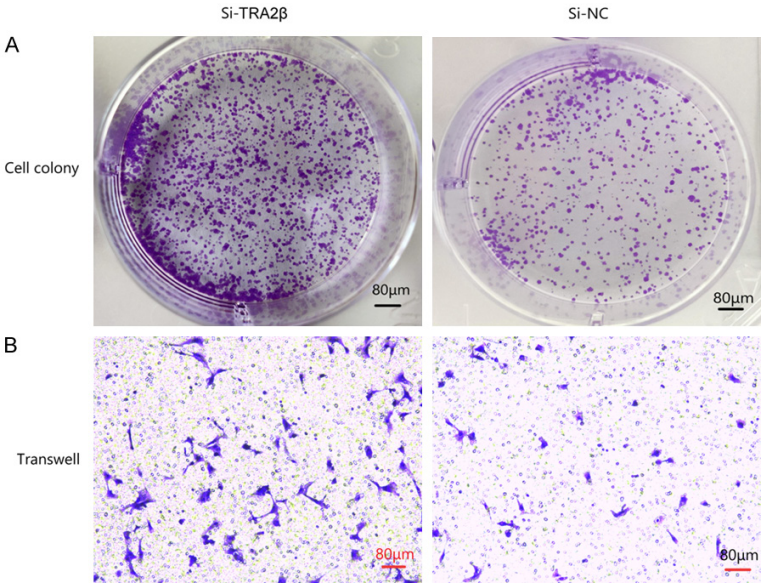

Figure 5. Proliferation and migration after si-RNA inhibition. A. Inhibition of cell proliferation after siRNA transfection. B. siRNA transfection inhibits cell migration.

125a-5p. Meanwhile, the overexpression of miR-125a-5p decreased and the migration ability of HCC cells, whereas after the inhibition of miR-125a-5p, the results were inversed. The above data suggest that the overexpression of miR-125a-5p may inhibit the progress of HCC.

It is shown that miR-125a-5p regulates the invasion and migration of HCC, but the underlying mechanism remains to be elucidated. This study showed that TRA2β mRNA and protein levels decreased after miR-125a-5p overexpression, but levels were upregulated after miR-125a-5p-stransfection. Therefore, miR-125a-5p may inhibit HCC progression by inhibiting the TRA2 pathway. In order to confirm this idea, the cells were transfected with siRNA. This showed that the proliferation and migration of HCC decreased after inhibition of TRA2β expression by si-TRA2β transfection.

In conclusion, miR-125a-5p expression can inhibit the proliferation and migration of HCC, which is related to its inhibition of TRA2β protein expression. Therefore, miR-125a-5p may be a new therapeutic target for HCC.

Acknowledgements

This work was supported by the clinical safety and prognosis of laparoscopic combined with open hepatectomy for primary hepatocellular carcinoma (Grant Number 2019XJ92).

Disclosure of conflict of interest

None.

Address correspondence to: Lanhua Tang, Department of Oncology, Xiangya Hospital, Central South University, No. 87, Xiangya Road, Changsha 410008, Hunan, China. Tel: +86-15273138751; E-mail: caojunronga@163.com; Yunfu Cui, Ward 1 of General Surgery, The Second Affiliated Hospital of Harbin Medical University, No. 246, Xuefu Road, Nangang District, Harbin 150086, Heilongjiang, China. E-mail: yfcui@hotmail.com

References

[1] Liu Z, Sun J, Wang X and Cao Z. MicroRNA-129-5p promotes proliferation and metastasis of hepatocellular carcinoma by regulating the BMP2 gene. Exp Ther Med 2021; 21: 257.

[2] Giannini EG, Farinati F, Ciccarese F, Pecorelli A, Rapaccini GL, Di Marco M, Benvegnù L, Caturelli E, Zoli M, Borzio F, Chiaramonte M and Trevisani F. Prognosis of untreated hepatocellular carcinoma. Hepatology 2015; 61: 184-190.

[3] Deng GL, Zeng S and Shen H. Chemotherapy and target therapy for hepatocellular carcinoma: new advances and challenges. World J Hepatol 2015; 7: 787-798.

[4] Li L, Guo Z, Wang J, Mao Y and Gao Q. Serum miR-18a: a potential marker for hepatitis B virus-related hepatocellular carcinoma screening. Dig Dis Sci 2012; 57: 2910-2916.

[5] Ding J, Huang S, Wu S, Zhao Y, Liang L, Yan M, Ge C, Yao J, Chen T, Wan D, Wang H, Gu J, Yao M, Li J, Tu H and He X. Gain of miR-151 on chromosome 8q24.3 facilitates tumour cell migration and spreading through downregulating RhoGDIA. Nat Cell Biol 2010; 12: 390-399.

[6] Bi Q, Tang S, Xia L, Du R, Fan R, Gao L, Jin J, Liang S, Chen Z, Xu G, Nie Y, Wu K, Liu J, Shi Y, Ding J and Fan D. Ectopic expression of MiR-125a inhibits the proliferation and metastasis of hepatocellular carcinoma by targeting MMP-11 and VEGF. PLoS One 2012; 7: e40169.

[7] Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, Bentwich Z, Hod M, Goren Y and Chajut A. Serum microRNAs are promising novel biomarkers. PLoS One 2008; 3: e3148.

[8] Gao G, Gay HA, Chernock RD, Zhang TR, Luo J, Thorstad WL, Lewis JS Jr and Wang X. A microRNA expression signature for the prognosis of oropharyngeal squamous cell carcinoma. Cancer 2013; 119: 72-80.

[9] Chen S, Xue Y, Wu X, Le C, Bhutkar A, Bell EL, Zhang F, Langer R and Sharp PA. Global microRNA depletion suppresses tumor angiogenesis. Genes Dev 2014; 28: 1054-1067.

[10] Vriens MR, Weng J, Suh I, Huynh N, Guerrero MA, Shen WT, Duh QY, Clark OH and Kebebew E. MicroRNA expression profiling is a potential diagnostic tool for thyroid cancer. Cancer 2012; 118: 3426-3432.

[11] van Kouwenhove M, Kedde M and Agami R. MicroRNA regulation by RNA-binding proteins and its implications for cancer. Nat Rev Cancer 2011; 11: 644-656.

[12] Calin GA and Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-866.

[13] Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS and Benz CC. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. J Biol Chem 2007; 282: 1479-1486.

[14] Cowden Dahl KD, Dahl R, Kruichak JN and Hudson LG. The epidermal growth factor receptor responsive miR-125a represses mesenchymal morphology in ovarian cancer cells. Neoplasia 2009; 11: 1208-1215.

[15] Coppola N, Potenza N, Pisaturo M, Mosca N, Tonziello G, Signoriello G, Messina V, Sagnelli C, Russo A and Sagnelli E. Liver microRNA hsa-miR-125a-5p in HBV chronic infection: correlation with HBV replication and disease progression. PLoS One 2013; 8: e65336.

[16] Potenza N, Papa U, Mosca N, Zerbini F, Nobile V and Russo A. Human microRNA hsa-miR-125a-5p interferes with expression of hepatitis B virus surface antigen. Nucleic Acids Res 2011; 39: 5157-5163.

[17] Martini M, De Santis M, Braccini L, Gulluni F and Hirsch E. PI3K/AKT signaling pathway and cancer: an updated review. Ann Med 2014; 46: 1-12.

[18] Bai YN, Zhao-Yan Y, Luo LX, Jiang Y, Qing-Jie X and Yong Z. MicroRNA-21 accelerates hepatocyte proliferation in vitro via PI3K/Akt signaling by targeting PTEN. Biochem Biophys Res Commun 2013; 443: 802-807.

[19] Wang RY, Chen L, Chen HY, Hu L, Li L, Sun HY, Jiang F, Zhao J, Liu GM, Tang J, Chen CY, Yang YC, Chang YX, Liu H, Zhang J, Yang Y, Huang G, Shen F, Wu MC, Zhou WP and Wang HY. MUC15 inhibits dimerization of EGFR and PI3K-AKT signaling and is associated with aggressive hepatocellular carcinomas in patients. Gastroenterology 2013; 145: 1436-1448, e1412.

[20] Abbud-Antaki RA, Marhefka JN, DeLuca AL and Zuromskis MP. The Cancer BioChip System: a functional genomic assay for anchorage-independent three-dimensional breast cancer cell growth. Horm Cancer 2012; 3: 261-270.

[21] Guadamillas MC, Cerezo A and Del Pozo MA. Overcoming anoikis--pathways to anchorage-independent growth in cancer. J Cell Sci 2011; 124: 3189-3197.