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MiR-144-3p regulates invasion and proliferation activity in hepatocellular carcinoma by targeting SLITRK4

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► ***Original article***

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ABSTRACT

Background: miR-144-3p exerts inhibitory roles in hepatocellular carcinoma (HCC). This paper aims to explore that miR-144-3p and its predictive target gene, SLIT- and NTRK-like family member 4 (SLITRK4), in HCC progression. ***Materials and Methods:*** The levels of SLITRK4 and miR-144-3p were determined by western blot and qPCR. A loss- and gain-of-function test was used to test whether the relationship between miR-144-3p and SlitrK4 affected the growth of HCC cells. StarBase examined the miR-144-3p target prediction using SLITRK4. Through the use of a luciferase reporter experiment, miR-144-3p's targeting SLITRK4 was identified. The salvage experiment confirmed that miR-144-3p interacts SLITRK4 to regulate the progression of HCC. ***Results:*** Findings showned that the expression of miR-144-3p decreased in HCC tissue and cells. MiR-144-3p inhibits deterioration progression of HCC cell progression. MiR-144-3p targeted SLITRK4 and negatively modulated SLITRK4 levels. Besides, miR-144-3p upregulation decreased HCC migration and invasion activity, while SLITRK4 overexpression reduced this inhibition effects. ***Conclusions:*** MiR-144-3p regulates invasion and proliferation activity in HCC by targeting SLITRK4. MiR-144-3P/ SLITRK4 axis might provide a potential anti-cancer therapy pathway for clinical diagnosis, treatment and prognosis in hepatoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common cancer diagnosed and regarded to be related to cancer mortality all around the world. It is estimated that more than 1 million HCC patients are newly diagnosed annually and more than 700,000 cases die from HCC [1, 2]. In the past 20 years, several different therapies, including tumor section, microwave ablation and chemotherapy, have been developed to treat HCC. Nonetheless, the five-year survival rate in patients with HCC is still unpleasing [3, 4]. Thus, looking for new HCC treatment is the key to combat the situation of relapse and high frequency of metastasis [5].

MicroRNA (miRNA) is a small and non-coding RNA that could bind and target mRNA molecules at complementary sites by complementary base pairing so as to promote target degradation or translation inhibition [6, 7]. Multiple studies indicate that miRNAs play essential roles in cellular immunity, proliferation, invasion, cellular death and many other biological processes [8, 9]. Data demonstrated that miR-144-3p impacts on suppressing breast cancer cellular apoptosis through targeting Karyopherin subunit alpha 2 (KPNA2) [10] and on rectal cancers through mediating BCL6 [11]. Besides, the level of it was found to decrease in GC, which may enhance the sensitivity to radiation of those cells [12]. MiRNA-144-3p guards against the stimulation of ovarian granulosa death of cells brought on by chemotherapy [13]. Nevertheless, detailed biological functions of miR-144-3p in HCC deserve deep research.

SLIT and NTRK-like family number 4 (SLITRK4) is located on Xq27.3, belonging to one six-member synapse tissue family. This family controls the formation of excitatory and inhibitory synapses through trans-synaptic adhesion with LAR receptor protein tyrosine phosphatases (PTPs) [14, 15]. High SLITRK4 expresses in adrenal glands, brain and other tissues. Studies have suggested that SLITRK4 is related to uterine leiomyosarcoma, brain cancers and neuropsychological diseases [16-18]. Previous studies have verified that SLITRK4 exerts tumor promotion effects in HCC. However, mechanism of SLITRK4 on HCC in details still needs to be studied.

This study evaluated the miR-144-3p profile expression and studied miR-144-3p/SLITRK4 interaction in HCC proliferation and invasion. We explored the underlying molecular mechanisms of

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hepatocellular carcinoma and provided new predictive targets for hepatocellular carcinoma.

MATERIALS AND METHODS

Human HCC tissues

We resected 64 specimens of HCC tumors from patients in the The First Affiliated Hospital of University of South China. Patients provided informed consent. The table 1 showed Clinicopathological features from HCC patients. The ethical committee of the Hospital approved this study (2020-LY-k087).

Table 1. Clinicopathological features from HCC patients.

Variables	HCC patients (n=64)
Age	60.93 (35-65)
Sex	
Male	40
Female	24
Pathological grade	
I–II	31
III–IV	33
Lymph node metastasis	
Yes	41
No	23

Cell culture

Two strains of HCC cells, Huh7 and MHCC-LM3, were provided by Chinese Academy of Sciences (Shanghai, China). We used RPMI-1640 with 10% FBS and 1% streptomycin and penicillin to culture cells (GE Healthcare Life Sciences, USA). Briefly, cells were cultured with following conditions: 5%CO_2, 37℃.

Cell transfection

Transfections with inhibitor-miR-144-3p or NC and mimic-miR-144-3p or NC were performed on Huh7 and MHCC-LM3 cells. There were effects on SLITRK4 expression. Next, we co-transfected LV-miR-144-3p and LV-SLITRK4 or LV-NC lentiviruses. All the oligonucleotides used in our study were offered by GenePharma (Shanghai, China). Transfection reagent Lipofectamine 2000 (Invitrogen, USA) was utilized. Briefly, Lipofectamine was mixed with oligonucleotides for 20 minutes in the presence of Opti-MEM media (Invitrogen, USA). After 20 minutes, the solution was transferred to the cells. Cells were collected after 48 hours for the succeeding experiments.

RT-qPCR

Firstly, Trizol (Sigma, USA) was used to extract total RNA in Huh7 as well as MHCC-LM3 cells. Total RNA was quantified using the Nanodrop 2000 (ThermoFisher Scientific, USA). Later, miScript II RT Kit (QIAGEN, USA) was adopted to synthesize cDNA. For U6 (5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGG-3') and miR-144-3p (5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAGTACA-3') reverse transcription. RT-qPCR primers (IDT company) used in the study are: MiR-144-3p Forward primer: 5'-TGCGGTACAGTATAGATG AT-3'; MiR-144-3p Reverse primer: 5'-CCAGTGCAG GGTCCGAGGT-3'; U6-F: 5'-TGCGGGTGCTCGCTTCGG CAGC-3'; U6-R: 5'-CCAGTGCAGGGTCCGAGGT-3; SLITRK4 Forward primer : 5'- GGAAATCTCAGCAGG CACCTTTG-3'; SLITRK4 Reverse primer : 5'- CCAC-TGACAGGCAGGTACATGA-3'; GAPDH Forward primer : 5'- GTCTCCTCTGACTTCAACAGCG-3'; GAPDH Reverse primer : 5'- ACCACCCTGTTGCTGTAGCCAA-3'. Applied bio-system7500 real-time PCR (Applied Biosystems, USA) was utilized in RT-qPCR process. The 2−ΔΔCt technique was utilized to figure out the relative expression.

Western blot

RIPA was added to the cells (Beyotime, Jiangsu, China) in combination with the protease inhibitor (1:1000) to extract protein. Protein was measured using BCA assay (ThermoFisher Scientific, USA), separated by 10% SDS-PAGE, loaded onto PVDF membrane, and blocked in 5% skim milk for 2 hours. Later, specific SLITRK4 or GADPH primary antibody (Abcam, USA) was added at 4℃ overnight, and respective secondary antibodies were reacted for 2 hours. Protein extracts were exposed to ECL Western Blotting Substrate and protein expression was quantified by ImageJ.

CCK-8 experiment

Briefly, 3 × 10^3 cells were cultured in 96-well plate and treated with oligonucleotides as mentioned previously. Later, The CCK-8 reagent (Beyotime, China) was used in 10 μL. The absorbance at 450 nm wavelength was measured using a Thermo Scientific Fluoroskan Ascent.

Colony formation assay

HCC cells, cultured in 96 well plate, were transfected as mentioned previously. Colonies were preserved with alcohol for 15 minutes after 48 hours of transfection. They were then stained for twenty minutes. With crystal violet (Sigma, Germany) before being counted. Leica CTR MIC microscope was employed to count HCC cell colony images in each well. Image J Version 1.49 software was employed for quantitative analysis on colony numbers.

Transwell assay

MiR-144-3p and SLITRK4's effects on HCC migration and invasion were detected through Transwell assay by using the Transwell tiny chamber (24-well plate, diameter 8-μm). 100μL cells (1 × 10^5/mL) were inoculated in the upper chamber, and Matrigel (BD Biosciences, San Jose, California, USA) was laid on tiny upper room for invasion experiment. 600μL RPMI1640 culture medium that contained 20% fetal bovine serum was added to 24-well plates

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in the lower chamber, in which cells were incubated for one day. Cotton swabs were used to gently wipe cells that did not migrate or invade in the upper chamber and 70% formaldehyde was used for half an hour to fix migrated cells and invaded cells. 0.1% crystal violet was utilized to dye cells for twenty minutes. Finally, the microscope was used to capture images of invasion or migration cells in each hole.

Luciferase reporter experiment

StarBase predicted potential miR-136-5p binding sites in SLITRK4 3 'UTR. Synthesized sequences comprising either the mutant (MUT-SLITRK4) or wild -type (WT-SLITRK4) seed sections of SLITRK4 have been inserted into luciferase reporter plasmids and infected with mimic-NC or mimic-miR-144-3p into Huh7 or MHCC-LM3 cells. The level of luciferase activity was measured 24 hours later using a luciferase assay kit (E1500, Promega).

Statistical analysis

The average of three dependent experiments results was calculated, and expressed as mean ± standard deviation (X±SD). The quantity variable was compared using the t-test. R STUDIO (version 3.6.1) was the software used for the statistical studies. If a P-value was less than 0.05, it was deemed statistically significant.

RESULTS

MiR-144-3p levels decline in HCC

We firstly measured the level of miR-144-3p profile in HCC cells and human tissues. RT-qPCR displayed that miR-144-3p decreased in HCC tissues (figure 1A) as well as HCC cell lines (figure 1B), compared with adjacent tissues and normal human hepatocytes QSG-7701.

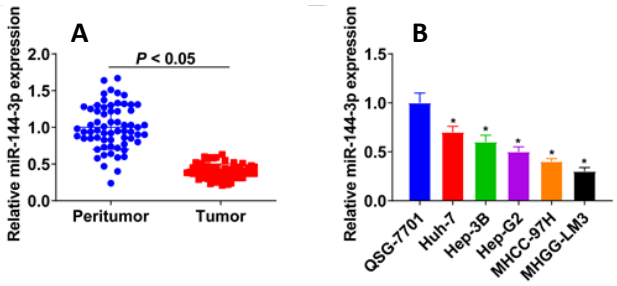

Figure 1. MiR-144-3p levels decrease in HCC. **A)** RT-qPCR determined miR-144-3p in HCC tumor tissues and peritumor tissue isolated from HCC humans (n=64). **B)** RT-qPCR measured miR-144-3p among HCC cell lines and normal cell (N=3). * vs. QSG-7701, P<0.05

SLITRK4 is miR-144-3p's downstream gene and is modulated negatively through miR-144-3p

Previously, several studies have shown that SLITRK4 mRNA is targeted by miR-144-3p [19, 20]. StarBase found the existence of targeted target sites between miR-144-3p and SLITRK4, and luciferase reporter experiment verified this targeting relationship (figure 2A). We used Western blot and RT-qPCR to validate miR-144-3p's effects on SLITRK4 expression in HCC cell lines. Mimic-miR-144-3p decreased SLITRK4 levels in Huh7 and MHCC-LM3 cells (Figure 2B-D). Meanwhile, inhibitor-miR-144-3p elevated SLITRK4 expression (figure 2E-2G). Overall, SLITRK4 is miR-144-3p's downstream gene and is modulated negatively through miR-144-3p.

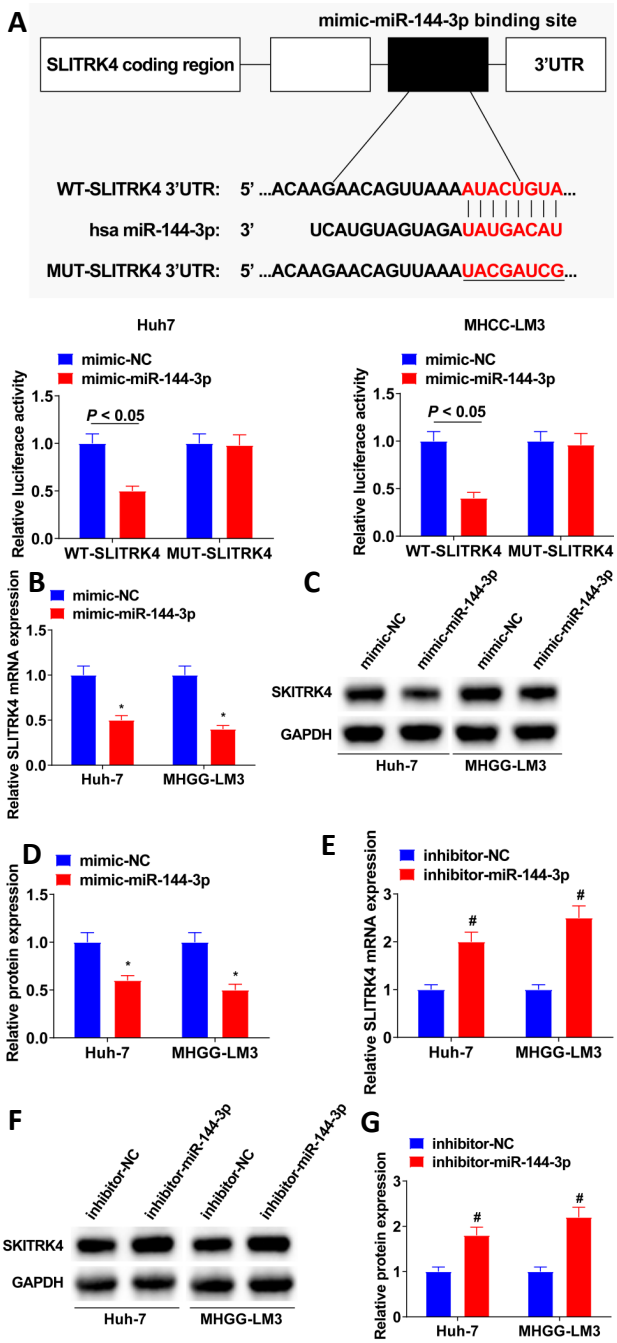

Figure 2. MiR-144-3p negatively targets SLITRK4. **A)** Bio-information websites displayed that miR-144-3p had combination sites with SLITRK4, luciferase reporter gene assay verified this targeting relationship. **B** RT-qPCR determined SLITRK4 levels in Huh7 and MHCC-LM3 cells after overexpression miR-144-3p. **C-D)** Western blot determined SLITRK4 levels in Huh7 and MHCC-LM3 cells after overexpression miR-144-3p. **E** RT-qPCR determined SLITRK4 levels in Huh7 and MHCC-LM3 cells after knockdown miR-144-3p. **F-G** Western blot tested SLITRK4 levels in Huh7 and MHCC -LM3 cells after knockdown miR-144-3p. N=3. * vs. mimic-NC; P<0.05; # vs. inhibitor-NC, P<0.05.

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SLITRK4 suppresses anti-proliferative influences of miR-144-3p on HCC

Findings implied that SLITRK4 levels in cells co-transfected with LV-miR-144-3p and LV-SLITRK4 were higher than those transfected with LV-miR-144-3p only by western Blot and RT-qPCR (figure 3A-3B). We further performed colony formation assay and CCK-8 assay to observe miR-144-3p and SLITRK4 modulation effects on HCC proliferation. Co-transfection of cells with LV-miR-144-3p and LV-SLITRK4 greatly increased cell proliferation activity in HCC cells as compared to LV-miR-144-3p (figure 4A-D). SLITRK4 suppresses anti-proliferative influences of miR-144-3p on HCC.

MiR-144-3p hampers HCC migration and invasion, while SLITRK4 suppresses this inhibition effects

Transwell assay was employed to study miR-144-3p and SLITRK4 modulation effects on HCC migration and invasion. In Huh7 cells transfected with LV-miR-144-3p and LV-SLITRK4 together, we have seen that the number of cell migration and invasions is higher than in LV-miR-144-3p (figure 5A-5B). Similarly, the co-transfection in MHCC-LM3 showed similar results (figure 5C-5D). MiR-144-3p was capable of inhibiting HCC migration and invasion, while SLITRK4 reversed this inhibition effect.

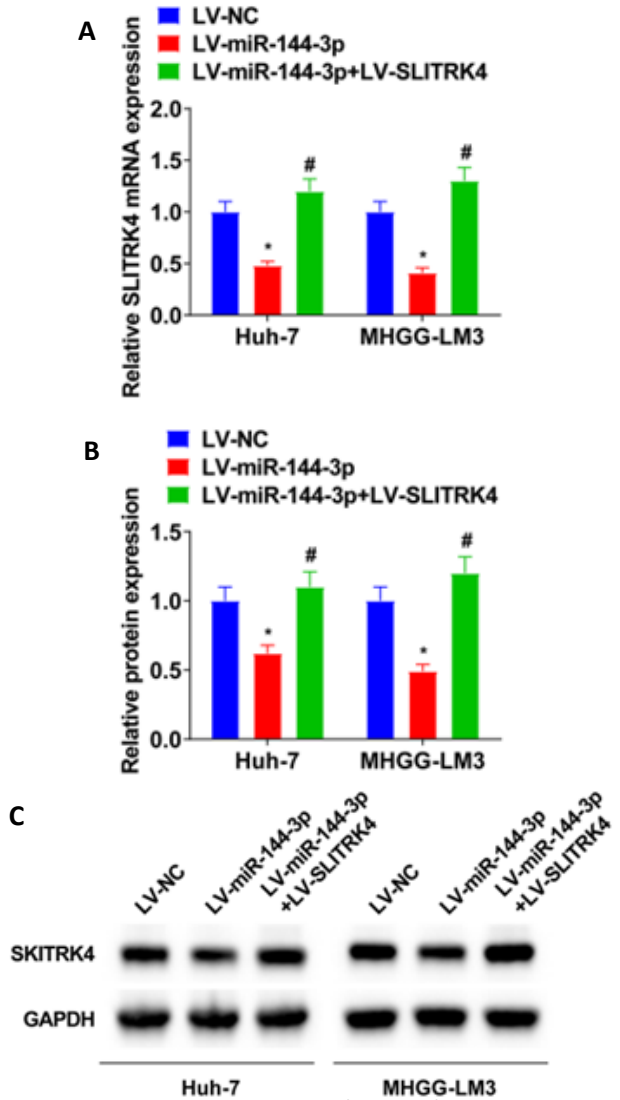

Figure 3. MiR-144-3p up-regulation reduces SLITRK4 expression. **A)** RT-qPCR detected SLITRK4 in Huh7 and MHCC-LM3 cells in rescue co-transfection. **B-C)** Western Blot detected SLITRK4 in Huh7 and MHCC-LM3 cells in rescue co-transfection. N=3. * vs. LV-NC, P<0.05; # vs. LV-miR-144-3p, P<0.05.

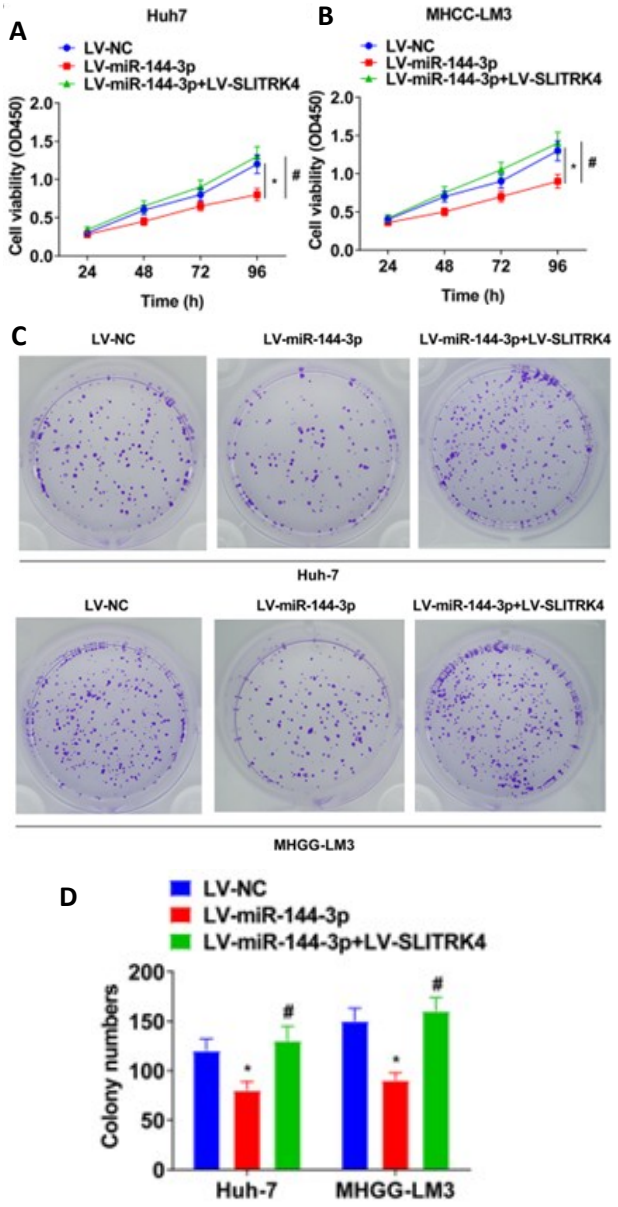

Figure 4. MiR-144-3p inhibits HCC proliferation while SLITRK4 reverses this inhibition effect. **A-B)** CCK-8 method detected Huh7 and MHCC-LM3 cell activity in cells after rescue co-transfection. **C-D)** Colony formation experiment detected Huh7 and MHCC-LM3 colony numbers in cells after rescue co-transfection. N=3. * vs. LV-NC, P<0.05; # vs. LV-miR-144-3p, P<0.05.

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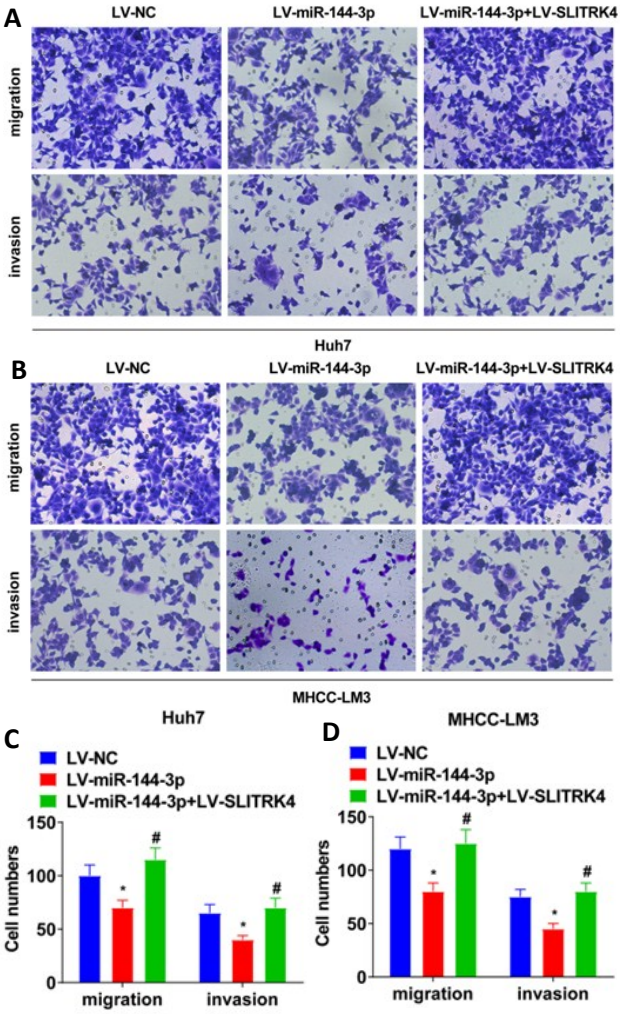

Figure 5. MiR-144-3p inhibits HCC invasion as well as migration while SLITRK4 abolishes this inhibition effect. **A)** Transwell method detected Huh7 cellular invasion images and migration images as well as cell numbers in cells after rescue co-transfection. **B)** Transwell method detected MHCC-LM3 cellular invasion images and migration images as well as numbers of cells in cells after rescue co-transfection. **C)** The number of migratory and invasive Huh7 cells after rescue co-transfection. **D)** The number of migratory and invasive MHCC-LM3 cells after rescue co-transfection. N=3. * vs. LV-NC, P<0.05; # vs. LV-miR-144-3p, P<0.05.

DISCUSSION

Worldwide, hepatocellular carcinoma (HCC) is a frequently diagnosed malignancy that is thought to be associated with cancer mortality [21]. Prior research has unequivocally demonstrated that miRNAs are frequently erroneously produced and regulated in cancers, indicating that miRNAs may now be used as a target for co-diagnosis and therapeutic medication development in the future [22]. It has been suggested that microRNAs (miRNAs) play roles in HCC pathogenesis based on previous studies [23-25].

MiR-144-3p transcripted from the chromosome 1p36 was verified to be positively modulated by the well-known anti-tumor gene p53 [26]. Several studies have regarded miR-144-3p as one gene which could inhibit tumors and exist among different tumors including multiple myeloma [27], glioma [28], oral squamous cell carcinoma (SCC) [29], esophageal SCC [30], endometrial cancer [31]. Especially some researches indicated that miR-144-3p inhibited tumors using methods of modulating downstream genes like MAPK6 [19], PTEN [32], HOXA7 [33], ERO1L [29], ATF2 [34] and PAX8 [35] expression. According to our study, initially, our work shows that we first looked at the expression of miR-18a-5p in HCC to determine its role in promoting cancer cell genesis. The expression of miR-18a-5p declined in HCC, and its overexpression significantly slowed down cell proliferation. The aforementioned findings support earlier research and demonstrate that miR-18a-5p is an oncogene for HCC. They propose that patients with HCC can benefit from immunotherapy targeting miR-144-3p. The SLITRK4 gene codes trans-membrane proteins, belonging to SLITRK family. It has reported that SLITRK family members possess two repeated domains containing abundant leucine in N terminal, which are similar to Sit, one protein controlling axon growth and possessing C-terminal region similar to neurotrophin receptors. SLITRK4 expresses in many different tissues. The highest expression was located in the adrenal gland and brain tissues. So far, there are few reports about SLITRK4 function. Many of them are related to diseases about the nervous system [18, 36, 37]. Previous studies also verified that SLITRK4 played the role of cancer promotion in HCC [38]. This study explored the relationship between SLITRK4 and miR-144-3p as well as their relationship with HCC pathogenesis and progression. Our research investigated that SLITRK4 was a downstream gene for miR-144-3p related to HCC pathogenesis. In contrast, overexpression of SLITRK4 reversed these inhibition effects, indicating that miR-144-3p plays tumor-killing impacts, and SLITRK4 exerts cancer promotion. These results suggest that HCC migration as well as proliferation were inhibited through down-regulating SLITRK4. The study on SLITRK4 and miR-144-3p interaction provided a new viewpoint for researchers to understand HCC pathogenesis

CONCLUSION

MiR-144-3p was capable of inhibiting HCC invasion, migration, and proliferation through modulating SLITRK4. Although there have been some detailed HCC studies on solely miR-144-3p or SLITRK4, and miR-144-3p/SLITRK4 axis could become one potential target spot for clinical diagnosis, treatment as well as prognosis in HCC.

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Not applicable.

Data availability: The figures and tables used to

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support the findings of this study are included in the article.

***Conflicts of interest*:** The authors declare that they have no conflicts of interest.

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***Ethical approval*:** The present study adhered to ethical norms set forth by the institutional and/or national research committee, the 1964 Helsinki Declaration and its subsequent revisions, or equivalent standards of care in all procedures involving human subjects. The First Affiliated Hospital of the University of South China granted approval for all subjects (2020-LY-k087).

***Author contribution*:** FengFeng Zhu conceived and designed the experiments. DianBing Xiao and Hao Xu contributed significantly to the experiments and arranging data. JianCheng Li, KangKang Peng, XinLiang Jiang and DianXiu Wang performed data analyses. DianBing Xiao and Hao Xu wrote the draft manuscript. FengFeng Zhu revised the manuscript. All authors read and approved the final manuscript.

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