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ORIGINAL ARTICLE

MiR-378a-3p represses malignant behaviors in liver hepatocellular carcinoma via targeting SIRT6

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Summary

Purpose: *Liver hepatocellular carcinoma (LIHC) is characterized with high recurrence and poor prognosis. This study aimed to explore how miR-378a-3p affects the progression of LIHC.*

Methods: *The expression of miR-378a-3p in LIHC and normal tissues, as well as in LIHC cell lines HepG2, 97H, SNU423, SNU449 and SMMC-7721 were detected. Then, miR-378a-3p was overexpressed to evaluate its function on LIHC cells proliferation, migration and invasion. Bioinformatics analysis was used to predict the target of miR-378a-3p. Subsequently, the effects of SIRT6 on LIHC cells proliferation, migration and invasion, and the interaction between miR-378a-3p and SIRT6 were investigated. Xenograft models were established to conduct the functional experiment in vivo.*

Results: *miR-378a-3p was lowly expressed in LIHC. Moreover, miR-378a-3p overexpression could repress LIHC cells proliferation, migration, invasion and epithelial-mesenchymal transition (EMT), and meanwhile facilitates its apoptosis. Mechanistically, we determined that miR-378a-3p could directly bind to the 3'-UTR of Human sirtuin 6 (SIRT6). Based on rescue experiment, we found that miR-378a-3p overexpression restrained LIHC cells malignant behaviors, including proliferation, invasion and EMT process, while which could be remedied by SIRT6 overexpression. More importantly, through injecting miR-378a-3p-agomir into nude mice, we noticed that miR-378a-3p overexpression efficiently suppressed the weight and volume of xenograft tumor, and meanwhile prevented the Ki67 expression and EMT process in tumor formed by miR-378a-3p-overexpressed 97H cells.*

Conclusions: *miR-378a-3p/SIRT6 axis could serve as a potential candidate for the treatment of LIHC.*

Key words: *liver hepatocellular carcinoma, miR-378a-3p, SIRT6, tumor, apoptosis, migration, invasion, EMT*

Introduction

Liver hepatocellular carcinoma (LIHC) is identified as the most prevalent cancer type, almost accounting for 80-90% of all cases of primary liver cancers [1,2]. It has been reported that the triggers causing LIHC mainly including genetic factors, aflatoxin, hepatitis B or C virus (HBV or HCV) and blue-green algae [3,4]. Recently, liver transplantation has been confirmed as the most efficient method for the treatment of LIHC [5,6], nevertheless, the high metastasis and recurrence rate significantly contributed to its poor prognosis [7]. Therefore, uncovering the potential biomarkers predicting LIHC recurrence and investigating the molecular mechanism underlying tumorigenesis are urgently required for LIHC.

As the small noncoding single-stranded RNA molecules [8], microRNAs (miRNAs/miRs) have been proved to play functional roles through

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binding to the 3'-UTR of messenger RNA [9]. Consistently, microRNA-378a-3p (miR-378a-3p) significantly contributed to the pathogenesis and progression of multiple cancers via mediating its target gene based on extensive researches. For instance, Kong et al [10] clarified that lncRNA-ZXF1-sponged miR-378a-3p could facilitate the tumorigenesis in endometrioid endometrial cancer (EEC) through suppressing PCDHA3, and Chanjiao et al [11] suggested that miRNA-378a-3p induced the ovarian cancer cells growth via inhibiting PDIA4. In addition, Qian F et al identified miR-378a-3p could act as an indicator for the diagnosis and overall survival of LIHC based on computational screen [12]. However, the fundamental mechanism through which miR-378a-3p affects the progression of LIHC remains insufficient.

The silent information regulator protein (SIRT) family is composed of 7 members ranging from SIRT1 to SIRT7 [13,14]. As a member of sirtuin family,silent information regulator protein 6 (SIRT6) is characterized with NAD^+-dependent histone deacetylase and single ADP ribosyltransferase [15,16], and through these two functions SIRT6 has been proved to participate in the DNA repair [17] and telomere maintenance [18], and played a critical role in the metabolism of glucose and lipids [19], inflammation [20], and the occurrence and progression in cancers [21], like ccRCC [22], colorectal cancer [23] and lung cancer [24].Interestingly, SIRT6 has been proved to be involved in the progression of LIHC [25]. However, whether and how miR-378a-3p mediates SIRT6 to participate in the progression of LIHC has not been clarified.

Table 1. Sequences of siRNA against specific targets

si-SIRT6-1	5'-3'	CACTTTGTTACTTGTTTCTGTCC
si-SIRT6-2	5'-3'	GTCTCACTTTGTTACTTGTTTCT

Thus, we examined the expression levels of miR-378a-3p and SIRT6 in LIHC, and revealed the correlation between them. Furthermore, we explored the functional role of miR-378a-3p/SIRT6 axis in LIHC cells progression, which may be considered as a novel mechanism explaining the progression of LIHC.

Methods

Tissue collection

LIHC tissues and corresponding normal tissues were provided by the Affiliated Hospital of Shaoxing University, and this research plan was performed based on the World Medical Association Declaration of Helsinki and approved by the Affiliated Hospital of Shaoxing University ethics committee. All patients signed the informed consent form.

Cell culture and treatment

Human liver cancer cell lines, HepG2, 97H, SNU423, SNU449 and SMMC-7721 these as well as human normal liver cell LO2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and incubated in the medium containing 10% FBS formulated by ATCC. The miR-378a-3p-mimics, si-SIRT6-1, si-SIRT6-2, pcDNA-SIRT6 were obtained and designed by Gene Chem Corporation (Shanghai, China) (Table 1).

Cell counting kit-8 (CCK-8)

97H and HepG2 cells were respectively planted into 96-well plates containing 10% CCK-8 solution (cell counting-kit) (Sigma, St. Louis, MO, USA). The absorbance at 450 nm was examined using cell counting kit reagent (Beyotime Biotechnology, Shanghai, China).

Colony formation assay

For the analysis of cloned cells, cells were incubated into 6-well plates for 2 weeks at 37°C, then 4% paraformaldehyde and 1% crystal violet were adopted for the fixation and stain. A microscope was utilized for the visualization and measurement of colonies.

Table 2. Sequences of PCR primers used in this study

Gene		*Primer sequences*
miR-378a-3p	Forward(5'-3')	GACTGGACTTGGAGTCAGA
	Reverse(5'-3')	CCAGTTTTTTTTTTTTTTTGCCTTCT
SIRT6	Forward(5'-3')	CCCACGGAGTCTGGACCAT
	Reverse(5'-3')	CTCTGCCAGTTTGTCCCTG
GAPDH	Forward(5'-3')	AATGGACAACTGGTCGTGGAC
	Reverse(5'-3')	CCCTCCAGGGGATCTGTTTG
U6	Forward(5'-3')	CGCTTCACGAATTTGCGTGTCAT
	Reverse(5'-3')	GCTTCGGCAGCACATATACTAAAAT

TUNEL

In-Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) was employed for the assessment of cells apoptosis. In consistence with the protocols, 97H and HepG2 cells were respectively mixed with terminal deoxynucleotidyl transferase (TdT), followed by staining by fluorescein. The nuclei of apoptotic cells could stained into green by TUNEL assay, while DAPI all nuclei of 97H and HepG2 cells could be stained into blue by 4',6-diamidino-2-phenylindole (DAPI).

Transwell assay

To estimate the cells'invasive ability, 97H and HepG2 cells were cultured in the upper chambers loaded with Matrigel (BD, Franklin Lakes, NJ, USA); meanwhile, Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) was added into the lower chambers. Then, 97H and HepG2 cells were removed, and dyed with 0.1% crystal violet after fixing by 4% paraformaldehyde. A microscope (Zeiss, Oberkochen, Germany) was employed for the visualization and calculation of migratory and invasive cells.

Wound healing assay

For the wound scratch evaluation, 97H and HepG2 cells were scratched by a sterile pipette tip after incubating for 24 h in 12-well plates. A microscope was utilized to observe and detect the migratory cells.

Luciferase reporter assay

The binding site of miR-378a-3p and SIRT6 was predicted using TargetScan database. To examine the luciferase activity, miR-378a-3p mimics or NC mimics were co-transfected with SIRT6-MUT or SIRT6-WT into 97H and HepG2 cells. Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was conducted to estimate the luciferase activity of SIRT6.

RNA immunoprecipitation (RIP)

To uncover the correlation between miR-378a-3p and SIRT6, Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) was employed and Anti-AGO2 (ab186733, 1:50, Abcam, Cambridge, MA, USA) was used. Anti-IgG was determined as isotype control, and the bound complexes of RNA were detected using real time PCR (RT-PCR).

qRT-PCR

mirVana™PARIS™Kit (Life Technologies, Gaithersburg, MD, USA) was adopted to collect the RNA. The relative expression was detected using in consistence with manufacturer's protocols. GAPDH and U6 were adopted as the internal reference of relative gene expression. The detailed primer sequences are listed in Table 2.

Western blotting (WB)

Total proteins were obtained by radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). After isolating the extracted samples using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein samples were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and covered by phosphate buffered saline (PBS) containing 5% skimmed milk for 60 min, and then cultured with primary antibodies at 4°C overnight, followed by incubating with the anti-rabbit secondary antibodies conjugated with horseradish peroxidase. Electrochemiluminescence (ECL) reagent (Millipore, Billerica, MA, USA) was employed for the visualization of blot bands and the measurement of protein expression. GAPDH (ab8245, 1:1000, abcam, Cambridge, MA, USA) was determined for the normalization. The primary antibodies bax (ab32503, 1:2000), caspase-3 (ab32351, 1:5000), cleaved caspase-3 (ab32042, 1:500),caspase-9 (ab32539, 1:1000), cleaved caspase-9 (ab2324, 1 μg/ml), bcl-2 (ab32124, 1:1000), and SIRT6 (ab191385, 1:2000), E-cadherin (ab76055, 1:200), N-cadherin (ab76011, 1:5000), Vimentin (ab92547, 1:2000) and MMP-9 (ab76003, 1:5000) were obtained from ABCAM (Cambridge, MA, USA).

Xenograft model

To conduct the functional experiment *in vivo*, BALB/c nude mice (5 weeks old, 20 g) were subcutaneously in-

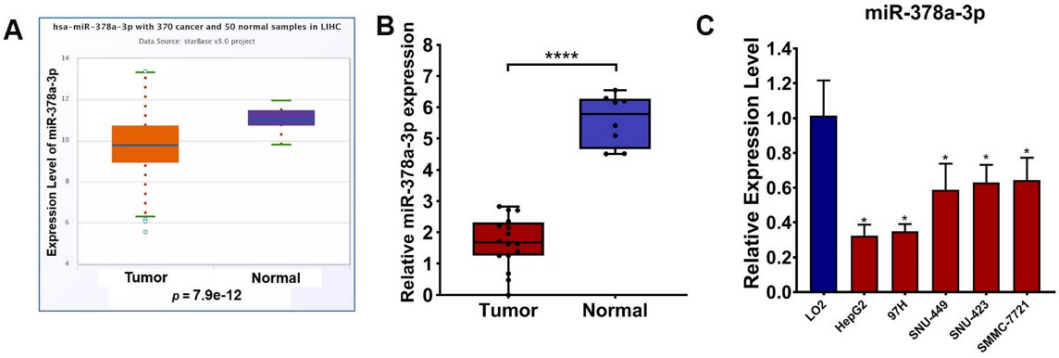

Figure 1. miR-378a-3p is lowly expressed in LIHC. **A:** TCGA database was utilized to analyze the different expression of miR-378a-3p in LIHC. **B**: q-RT-PCR was adopted to measure miR-378a-3p expression in LIHC tissues. **C:** miR-378a-3p expression was measured using qRT-PCR in LIHC cell lines HepG2, 97H, SNU423, SNU449 and SMMC-7721, and human normal liver cells LO2. *p<0.05 and ****p<0.001.

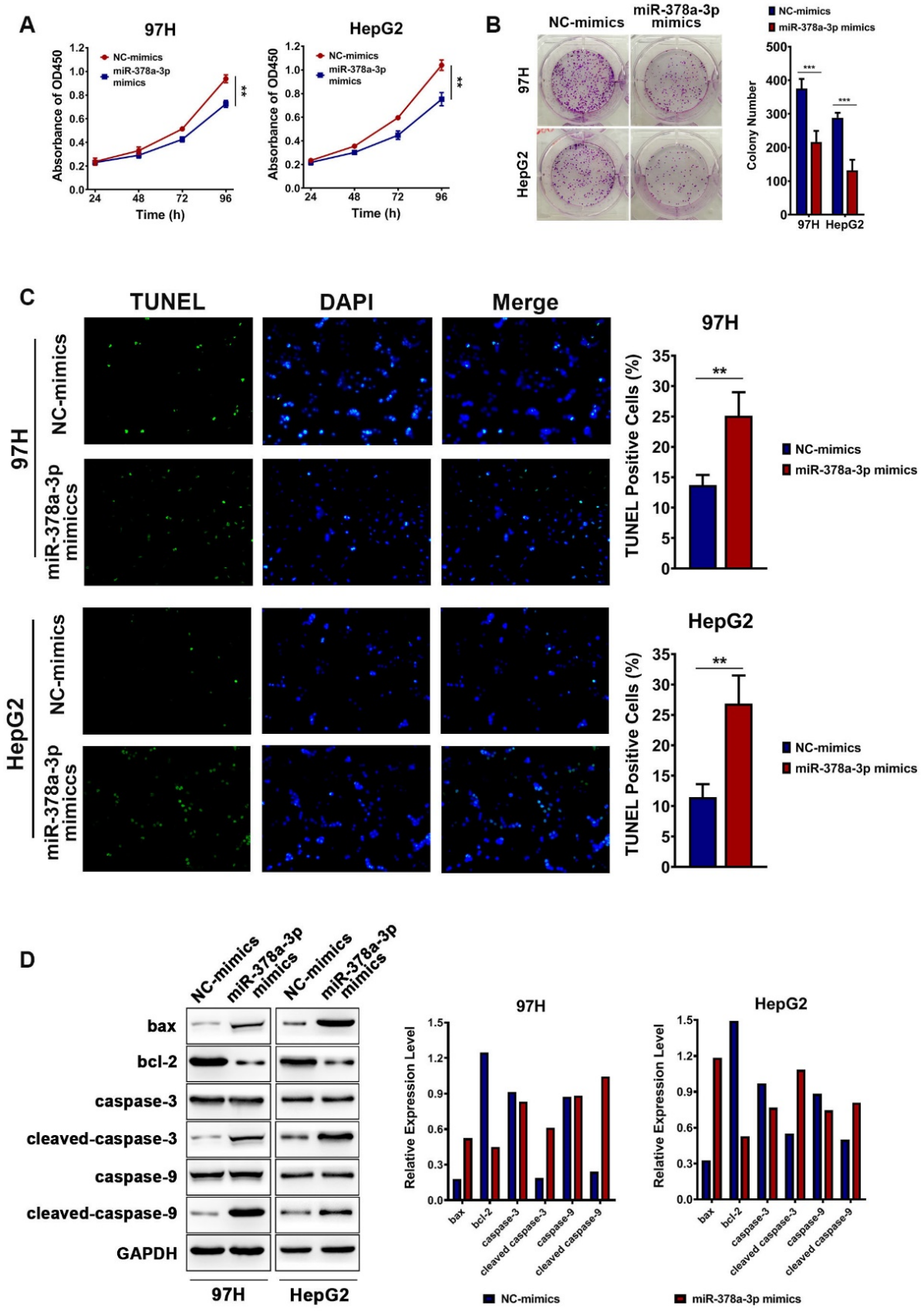

Figure 2. Upregulated miR-378a-3p suppresses LIHC cells proliferation and facilitates its apoptosis. For functional exploration, 97H and HepG2 cells were transiently infected withmiR-378a-3p mimics. CCK-8 **(A)** and colony formation **(B)** assays were employed to examine 97H and HepG2 cells proliferation rate. **(C)** TUNEL was utilized to detect the apoptosis rate. **(D)** Western blotting and qRT-PCR were performed for the detection of apoptosis-related proteins expression. **p<0.01, ***p<0.001.

jected with 97H cells into the flanks, followed by the intratumor injection with miR-378a-3p-agomir or NC-agomir. The tumor volume was monitored. Following the 28 day injection, the xenografts were weighted and the tumor tissues were collected for further examination. The $(L\times W^2)/2$ formula was employed for the volume calculation. In this strategy, all operations were finished based on the approval of Affiliated Hospital of Shaoxing University Committee.

Immunohistochemistry (IHC)

IHC was introduced into the detection of Ki67 in tumor. In brief, the collected xenograft antigen retrieval was performed through boiling in citrate buffer. 0.2% Triton-X was implemented to permeabilize it. Ki67 (ab92742, 1:500, Abcam, Cambridge, MA, USA) was then maintained overnight at 4°C with the treated tumor tissues, followed by incubating with secondary antibody. The stain was finished by 3,3'-diaminobenzidine substrate (DAB). A light microscope was applied to observe the Ki67-positive cells.

Statistics

The data were presented as the mean±standard deviation (SD). All data-related calculations were conducted using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and

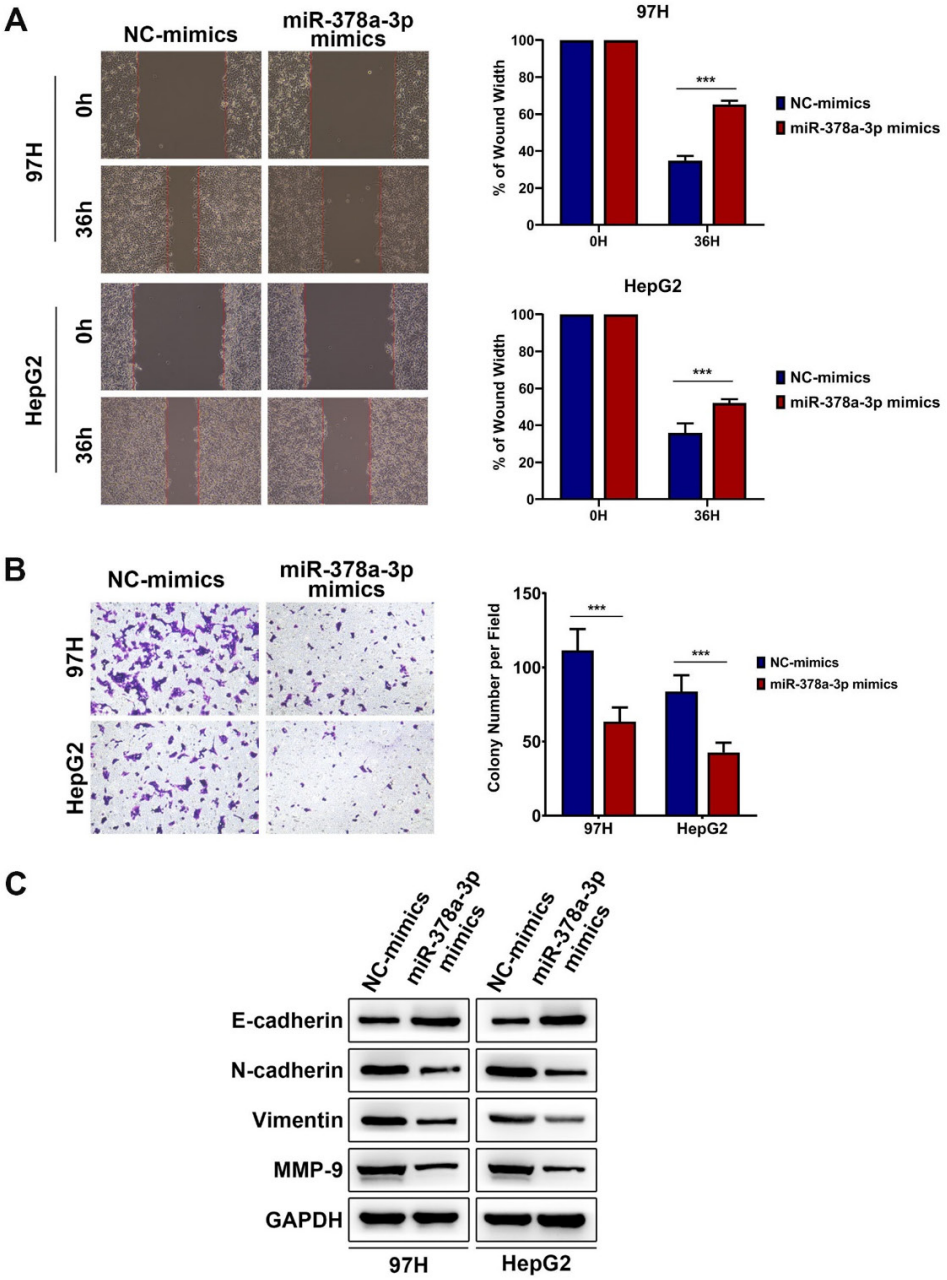

Figure 3. miR-378a-3p overexpression represses LIHC cells migration, invasion and EMT. Wound healing **(A)** and transwell **(B)** were utilized to measure cells migration and invasion. **(C)** Western blotting was implemented to examine EMT-related indicators. ***p<0.001.

GraphPad Prism (Version X; La Jolla, CA, USA). Student's t-test and one-way analysis of variance (ANOVA) were employed for the analysis of differences internal groups. Kaplan-Meier method was adopted to estimate the overall survival. P<0.05 showed statistical significance.

Results

miR-378a-3p is lowly expressed in LIHC

To uncover the association between miR-378a-3p and LIHC, we first assessed the expression levels of miR-378a-3p based on TCGA database, and observed miR-378a-3p was downregulated in LIHC tissues than in normal tissues (Figure 1A). In addition, q-RT-PCR further confirmed that miR-378a-3p was lowly expressed in frozen LIHC tissues (Figure 1B), as well as LIHC in cell lines HepG2, 97H, SNU423, SNU449 and SMMC-7721 (Figure 1C). These findings suggested that miR-378a-3p was lowly expressed in LIHC.

miR-378a-3p overexpression represses LIHC cells proliferation and drives its apoptosis

To evaluate the functional role of miR-378a-3p in LIHC cells, we generated miR-378a-3p-upregulated 97H and HepG2 cells. Based on proliferation analysis-relative experiments, we observed overexpressing miR-378a-3p significantly repressed the proliferation rate of 97H and HepG2 cells (Figure 2A and 2B). Reversely, TUNEL assay exhibited that miR-378a-3p overexpression caused an obvious rise in the percentage of TUNEL-positive apoptotic cells (Figure 2C), which could be specifically embodied in the upregulation of pro-apoptosis pro-

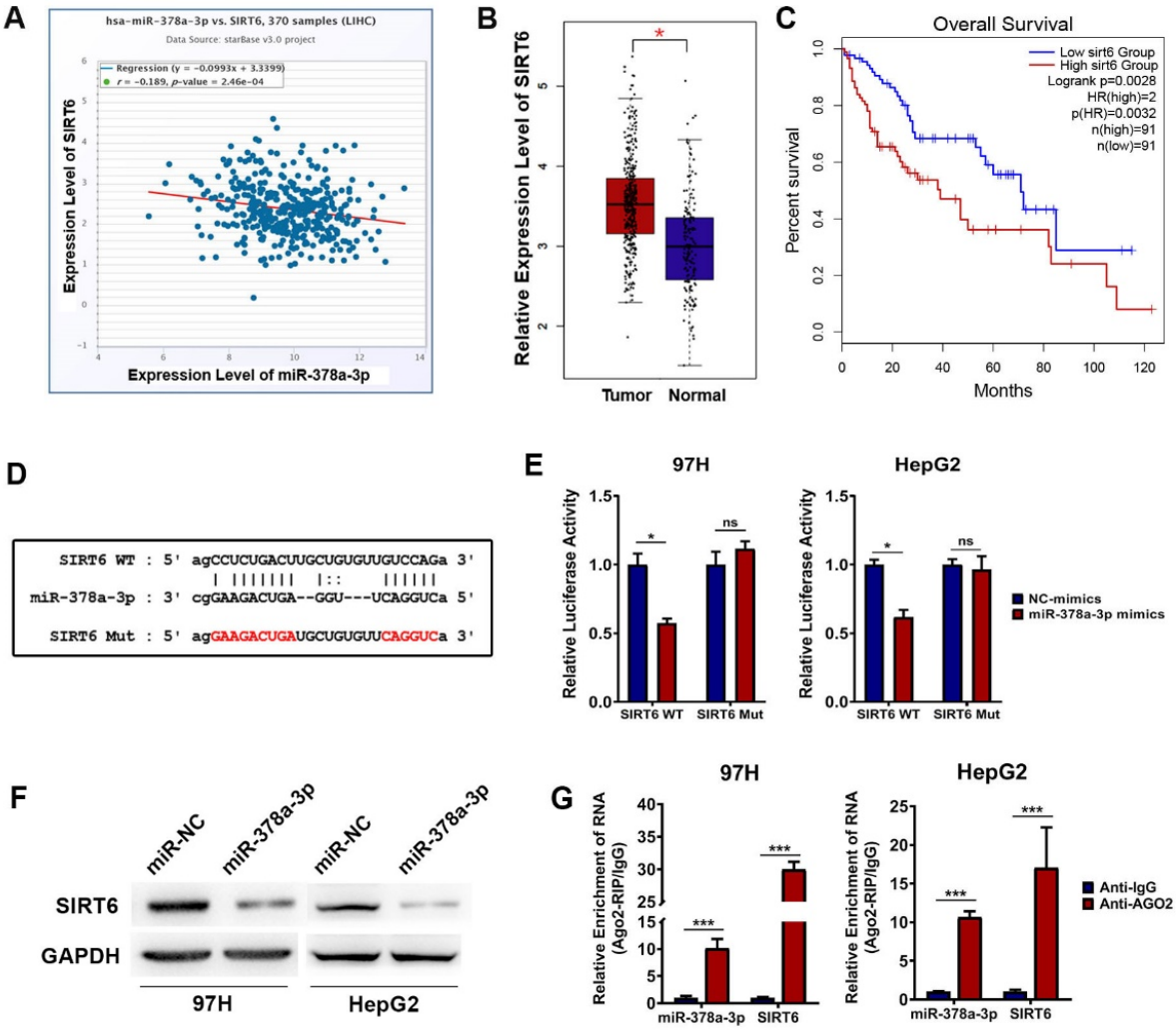

Figure 4. miR-378a-3p targets SIRT6 in LIHC. StarBase **(A)** and TCGA database **(B)** were respectively adopted analyzed the relationship of miR-378a-3p and SIRT6 and the different expression of SIRT6 in LIHC. **(C)** The overall survival analysis in the LIHC patients based on SIRT6 expression. **(D)** StarBase exhibited a binding site. Dual luciferase reporter **(E)**, Western blotting **(F)** and RIP **(G)** assays were utilized to validate the binding between miR-378a-3p and SIRT6. *p<0.05, ***p<0.001.

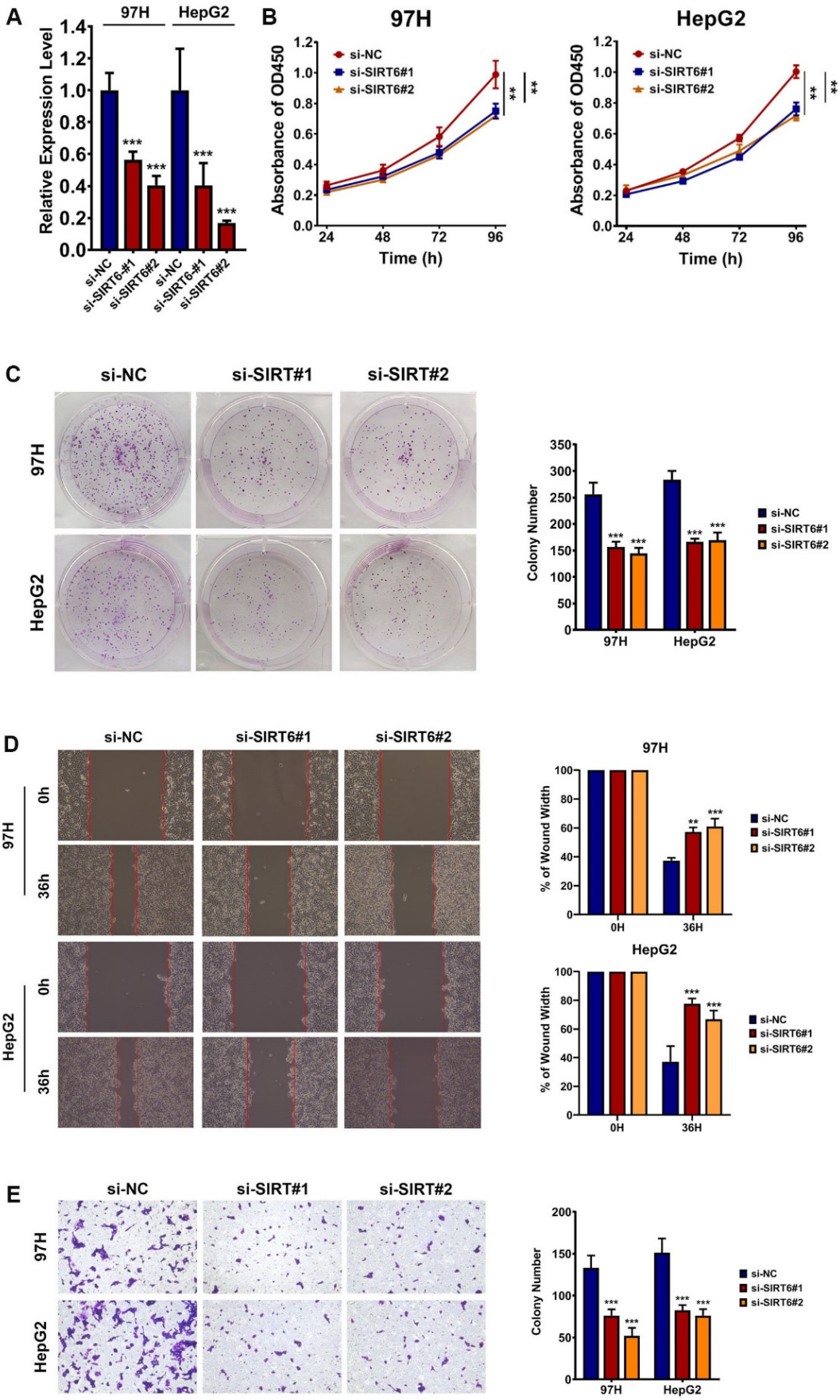

Figure 5. Depleted SIRT6 suppresses LIHC cells proliferation and invasion. 97H and HepG2 cells were transiently infected with SIRT6 siRNA, and qRT-PCR was utilized to examine the transfection efficacy **(A)**. Cells proliferation, migration and invasion were analyzed using CCK-8 **(B)**, colony formation **(C)** Wound healing **(D)** and transwell **(E)**. **p<0.01, ***p<0.001.

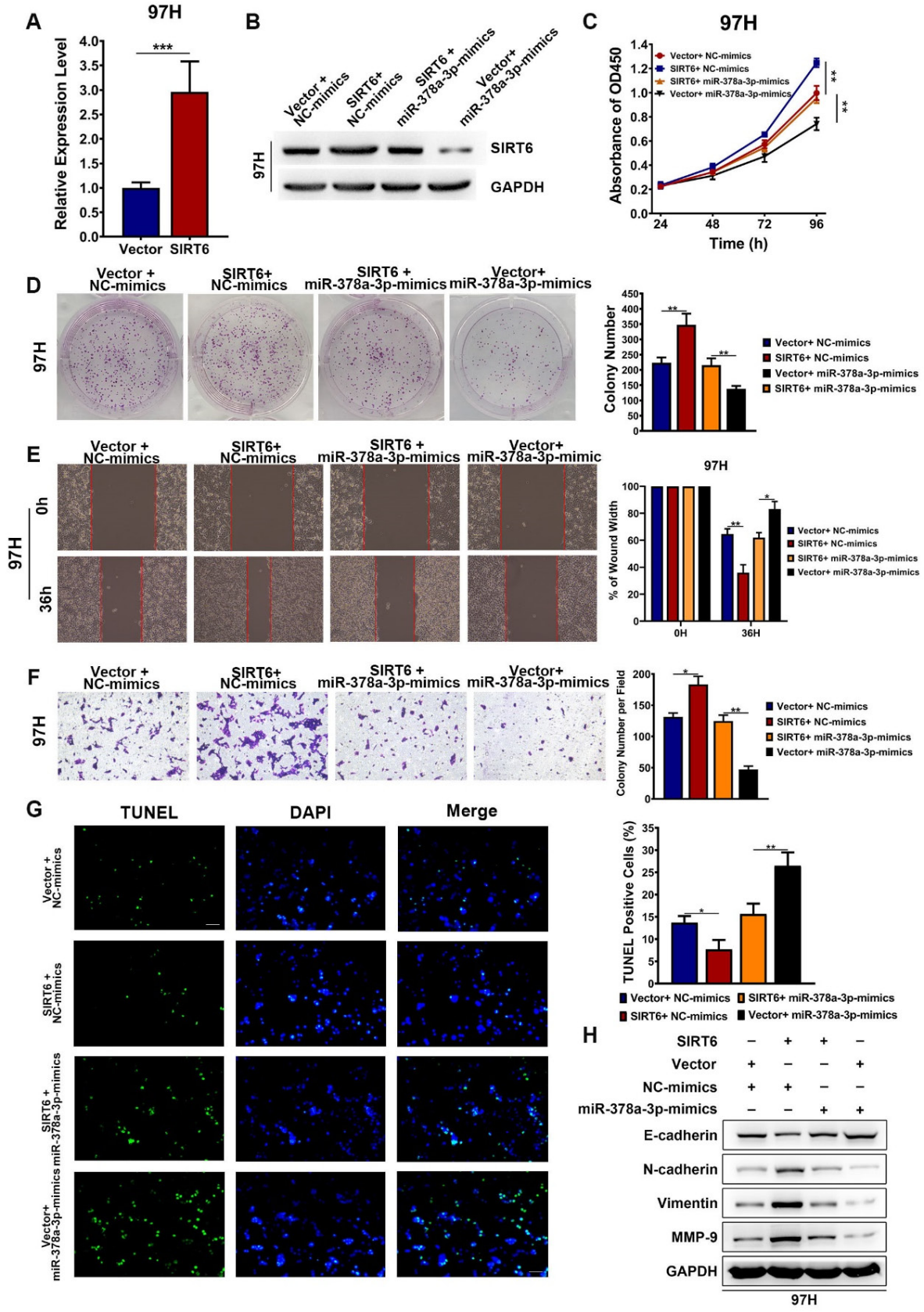

Figure 6. miR-378a-3p represses LIHC cells progress via inhibiting SIRT6. To validate whether miR-378a-3p regulates LIHC progression in a SIRT6-dependent manner, 97H cells were performed SIRT6 overexpression and were cotransfected with miR-378a-3p and SIRT6 overexpression plasmids, q-PCR **(A)** and WB **(B)** were adopted to detect the corresponding transfection efficacy. Cells proliferation and metastasis were measured by CCK-8 **(C)**, colony formation **(D)** Wound healing **(E)** and transwell **(F)**. **(G)** The cells apoptosis was evaluated using TUNEL assay. **(H)** Western blotting was implemented to examine EMT-related indicators. *p < 0.05, **p<0.01, ***p<0.001.

teins bax, cleaved caspase-3, and cleaved caspase-9, and the downregulation of anti-apoptosis proteins bcl-2 (Figure 2D).

miR-378a-3p prevents LIHC cells migaration, invasion and EMT

Subsequently, we performed wound healing (Figure 3A) and transwell (Figure 3B) assays to investigate the functional role of miR-378a-3p in LIHC cells metastasis and we revealed that upregulated miR-378a-3p could remarkably restrain the migratory and invasive capacities of 97H and HepG2 cells. In addition, overexpressed miR-378a-3p efficiently blocked the EMT process in LIHC cells, which could be specifically reflected in the rise of E-cadherin and the reduction of N-cadherin, Vimentin and MMP-9. Overall, these findings clarified that upregulated miR-378a-3p could remarkably suppress LIHC cells malignant behaviors.

miR-378a-3p inhibits SIRT6 in LIHC

Next, we tried to reveal the fundamental mechanism underlying the inhibited role of miR-378a-3p in LIHC cells progression. SIRT6 has been proved to contribute to the development of LIHC [26], and, interestingly, in our study, we found miR-378a-3p expression was negatively associated with SIRT6 (Figure 4A), and SIRT6 was upregulated in LIHC tissues based on the TCGA database (Figure 4B). Moreover, we observed high expression of SIRT6 indicating poor overall survival (Figure 4C).

Subsequently, to reveal the regulatory mechanism between miR-378a-3p and SIRT6, we predicted the binding site between them using a bioinformatics StarBase databases (Figure 4D). Additionally, it was observed that miR-378a-3p mimics efficiently repressed the luciferase activity in SIRT6-WT plasmid transfected 97H and HepG2cells, while there was no change in cells transfected with SIRT6-MUT plasmid (Figure 4E). Furthermore, we observed an obvious downregulation of SIRT6 in miR-378a-3p-upregulated 97H and HepG2 cells (Figure 4F). RIP assay further validated that miR-378a-3p and SIRT6 could be considerably enriched in anti-AGO2 complexes in 97H and HepG2 cells, in comparison with the anti-IgG group (Figure 4G). Overall, these data indicated that miR-378a-3p negatively regulated the expression of SIRT6 in LIHC.

SIRT6 depletion represses LIHC cells proliferation, migration and invasion

To investigate the role of SIRT6 in the development of LIHC, we performed stable transfec-

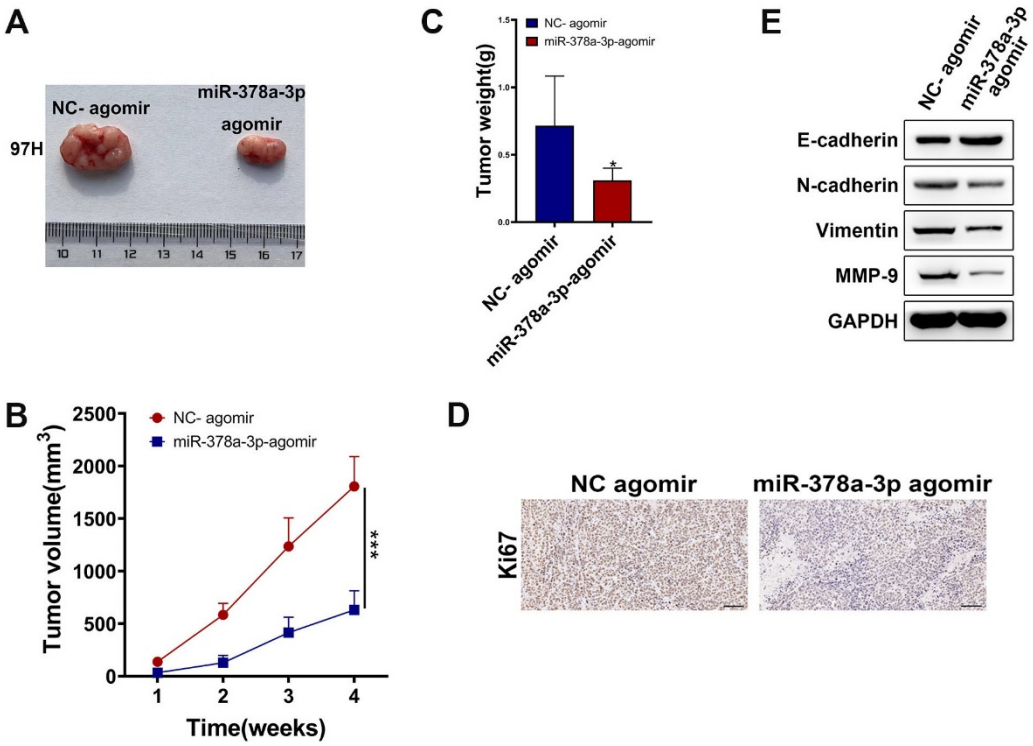

Figure 7. miR-378a-3p prevents tumor growth and EMT in vivo. The flanks of nude mice was subcutaneously injected with 97H cells and followed by the intratumor injection of miR-378a-3p-agomir or NC-agomir. The tumor volume **(A and B)** and weight were monitored **(C)**. **(D)** IHC was applied to detect the Ki67 expression in tumor. **(E)** Western blotting was implemented to examine EMT-related indicators. *p<0.05, ***p<0.01.

tion in 97H and HepG2 cells with SIRT6 siRNA (Figure 5A), and observed SIRT6 depletion could efficiently repress the proliferation rate of 97H and HepG2 cells (Figure 5B and 5C). Similarly, wound scratch and transwell experiments also exhibited the suppressive effect on cells migration and invasion mediated by silencing SIRT6 (Figure 5C and 5D). Collectively, these findings demonstrated that siRNA-mediated SIRT6 depletion could repress the growth and metastasis of 97H and HepG2 cells.

miR-378a-3p represses LIHC cells malignant progress via inhibiting SIRT6

To validate whether miR-378a-3p regulates LIHC progression in a SIRT6-dependent manner, we (Figure 6A) cotransfected miR-378a-3p mimics and SIRT6 overexpression plasmids ((Figure 6A and 6B) into LIHC cells. It was shown that miR-378a-3p overexpression suppressed the 97H cells proliferation rate, which could be rescued by SIRT6 overexpression (Figure 6C and 6D). Consistently, miR-378a-3p overexpression-mediated repressive effect on 97H cells migration and invasion could be efficiently rescued by overexpressing SIRT6 (Figure 6E and 6F), while SIRT6 overexpression obviously blocked the 97H cells apoptosis induced by overexpressing miR-378a-3p (Figure 6G). Additionally, upregulated SIRT6 also played as a crucial role in recovering miR-378a-3p overexpression-blocked EMT process (Figure 6H). Our data implied that the miR-378a-3p could suppress the LIHC cells proliferation, migration, invasion and EMT, and facilitate their apoptosis via targeting SIRT6.

miR-378a-3p prevents tumor growth and EMT process in vivo

Through subcutaneously injecting 97H cells into the flanks of nude mice, followed by the intratumor injection of miR-378a-3p-agomir or NC-agomir, we noticed that miR-378a-3p overexpression efficiently prevented the growth of tumor (Figure 7A), which could be observed in the reduction of tumor volume (Figure 7B) and weight (Figure 7C). Moreover, the Ki67 expression (Figure 7D) and EMT process (Figure 7E) in tumor were inhibited by miR-378a-3p overexpression, which further confirmed that miR-378a-3p could attenuated the malignant behaviors of LIHC *in vivo*.

Discussion

In this study, our findings demonstrated miR-378a-3p as a suppressor in LIHC cells proliferation, migration and invasion. Moreover, we identified SIRT6 as downstream targeting protein of miR-378a-3p, and found that SIRT6 depletion exerted repressive effect on LIHC cell. Thus, we revealed the miR-378a-3p/SIRT6 axis participated in the progression of LIHC.

Accumulating researches have proved that miR-378a-3p functionally acted as an inhibitor in the progression of diverse cancers, like papillary thyroid cancer (PTC) [27], breast cancer [28] and colorectal cancer [29]. In addition, miR-378a-3p has been demonstrated to regulate cells proliferation, migration and apoptosis. For example, Tan et al revealed that miR-378a-3p could restrain H9C2 cells apoptosis induced by ischemia/reperfusion [30], and Zhang et al [31] suggested that micRNA-378a-3p could generate repressive effect on retinoblastoma cells proliferation, while facilitating cells apoptosis through inhibiting FOXG1. Consistent with previous studies, our results showed that miR-378a-3p was downregulated in LIHC. Furthermore, we validated miR-378a-3p overexpression could suppress the LIHC cells proliferation, metastasis and EMT, while inducing their apoptosis.

As referred above, SIRT6 was classified to the SIRT family, and accumulating studies have suggested that SIRT6 contributed to the occurrence and development of various cancers, mainly owning to the NAD^+-dependent histone deacetylase and single ADP ribosyltransferase. Specifically, Fu et al [32] suggested that SIRT3 and SIRT6 could facilitate the progression of prostate cancer via repressing the innate immune response mediated by necroptosis, and Becherini et al [33] revealed that SIRT6 could significantly facilitate the oxidative phosphorylation and the tumor progression in breast cancer. In addition, Yang et al reported Sirt6 could play a promoting role in the drug resistance and the growth and metastasis of tumor in diffuse large B-cell lymphoma via PI3K/Akt pathway [34]. Herein, we confirmed that miR-378a-3p could inhibit SIRT6 expression, and found that SIRT6 was highly expressed in LIHC and the high expression of SIRT6 indicated poor LIHC prognosis. Moreover, siRNA-mediated depletion of SIRT6 remarkably restrained the growth, metastasis and EMT of LIHC cells, and miR-378a-3p inhibited LIHC cells progression via inhibiting SIRT6. *In vivo*, miR-378a-3p overexpression significantly repressed the growth and EMT process of tumor, which further confirmed the inhibitory role of miR-378a-3p in the malignant behaviors of LIHC.

Conclusions

To summarize, we observed miR-378a-3p was lowly expressed in LIHC and upregulated miR-378a-3p restrained LIHC cells growth, metastasis

and EMT process, while accelerated their apoptosis. Moreover, miR-125b-5p negatively regulated the expression of SIRT6 in LIHC, and exerted repressive effect on LIHC progression via inhibiting SIRT6. More importantly, miR-125b-5p also prevented the growth and EMT process of tumor *in vivo*. These findings provided the rationale for considering miR-378a-3p/SIRT6 axis as a novel therapeutic strategy against progression of LIHC.

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Conflict of interests

The authors declare no conflict of interests.

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