miR-224 Regulates the Aggressiveness of Hepatoma Cells Through the IL-6/STAT3/SMAD4 Pathway

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ABSTRACT

Background: *Previous studies have shown that miR-224 regulates the progression of liver cancer. The aim of this study was to investigate the underlying mechanisms.*

Methods: *The miR-224, p-STAT3 and SMAD4 expression levels were checked with tissue or/and serum samples of HCC patients by qRT-PCR or IHC methods. The regulatory role of IL-6 in p-STAT3 and SMAD4 was investigated by Western-blot. The targeted gene of miR-224 was verified by both Western-blot and luciferase reporter assay. Furthermore, the carcinogenesis of miR-224 in HCC was investigated by cell experiments in vitro and mouse xenograft model and in vivo imaging in vivo.*

Results: *It was found miR-224 was elevated in both tissue and serum of HCC patients. The p-STAT3 expression was higher but the SMAD4 was lower in the HCC tumor tissues. Moreover, IL-6 can induce the p-STAT3/STAT3 and miR-224 expression in HCC cells and STAT3 played the bridge role between IL-6 and miR-224. Target gene studies found miR-224 targeted the 3'UTR of SMAD4. Finally, the promoting roles of miR-224in the growth, proliferation, invasion and migration of HCC were discovered by in vitro and in vivo studies.*

Conclusion: *It implies that miR-224 may potentially represent a new target for developing novel anti-HCC therapeutics.*

Keywords: *Hepatocellular carcinoma, miR-224, STAT3, SMAD4, invasion and metastasis*

INTRODUCTION

Hepatocellular carcinoma (HCC) constitutes the majority of primary liver cancer cases, ranking as the second leading cause of cancer-related deaths worldwide.[1] Chronic hepatitis B virus and hepatitis C virus infection as well as nonalcoholic fatty liver disease are the main causes of HCC. However, the pathogenesis of HCC may involve common signaling pathways independent of HCC etiological agents.

The signal transducer and activator of transcription 3 (STAT3) protein, a member of the STAT3 transcription factor family, is implicated in the development of HCC.[2] Activated STAT3 has been shown to upregulate the expression of other genes involved in anti-apoptosis, proliferation, invasion, migration, and angiogenesis during tumorigenesis. Therefore, inhibition of STAT3 activation may potentially be used to prevent and treat HCC.[3-6]

Interleukin-6 (IL-6) is a known proinflammatory cytokine that facilitates hepatic tumorigenesis.[7] IL-6 functions primarily through activating STAT3.[8] Meanwhile, miR-224 is mainly expressed by liver tissue and is considered oncogenic as it promotes HCC progression. In addition, miR-224 may potentially serve as a biomarker for HCC diagnosis and as a therapeutic target.[9] Moreover, it has been demonstrated that STAT3 can be regulated by miRNAs in HCC.[10] Our previous study found that IL-6 induces miR-224 expression as well as promotes the invasion and metastasis of cholangiocarcinoma.[11] Importantly, miR-224 is upregulated in the tumor tissue of liver cancer.[12]

Studies have revealed that miR-224 expression in tumor tissue, as well as miR-224 released in the serum, can be detected in HCC patients,[13-15] however, the regulatory functions of miR-224 in HCC have not been investigated using both in vitro and in vivo systems very well. STAT3 is recruited in the signal transduction cascade initiated by different inflammatory cytokines. For instance, STAT3 phosphorylation has been shown to be activated by IL-6,[16] and an elevated miR-224 expression level has

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been detected in HCC cells with IL-6 induction.[17] In these studies, it has been suggested that STAT3 mediates the transcriptional regulation of miR-224 expression. Finally, SMAD4 is a downstream protein in the IL-6/STAT3 signaling pathway that may function as a tumor suppressor gene in HCC tumorigenesis.[18] SMAD4 is predicted to be a target gene of miR-224.

In this study, we investigated the impact of an elevated miR-224 expression level on HCC growth and metastasis using both in vitro and in vivo systems. We also explored the signal transduction pathways mediating the tumor growth activity of the expressed miR-224.

MATERIALS AND METHODS

Ethics Statement

The human study protocol was approved by the Institutional Review Board of Nanjing Medical University. All patients provided written informed consent prior to their enrolment.

Collection of Human Serum and Tissue Samples

Blood samples as well as tumor and matched adjacent nontumorous tissues were collected from 21 HCC patients (the clinic data of HCC patients was shown in Table 1), 20 healthy subjects. Both the HCC patients and healthy subjects were admitted to or recruited by Wuxi People's Hospital. All liver specimens, once obtained, were immediately stored at −80°C until analysis.

Table 1. Clinic Data of HCC Patients

	Mean ± SD
Gender	Male/female (16/5)
Age (years)	60.95 ± 13.09
HBV positive	10
HCV positive	2
Liver cirrhosis	15
AFP (μg/L)	193.15 ± 440.96
TBil (μmol/L)	16.57 ± 7.8
Alb (g/L)	37.77 ± 3.24
ALT (U/L)	28.23 ± 11.78
AST (U/L)	27.38 ± 12.32
GGT (U/L)	51.24 ± 51.85
Pathological grading	
High (I)	5
Moderate (II)	10
Low (III)	6

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Detection of miRNAs in Serum and Tissue

Total RNA was isolated from the blood and tissue samples using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A bulge-loop™ miRNA qRT-PCR primer set (1 RT primer and 2 PCR primers for miR-224) was designed and synthesized by RiboBio (Guangzhou, China). 5S Ribosomal RNA was used as an internal control for the serum miR-224 level, and U6 RNA was used as an internal control for the hepatic miR-224 level. The iQTM SYBR® Green Supermix (Bio-Rad) was used to prepare the PCR mixture, which contained specific primers for each of the target genes, and qRT-PCR was performed using an ABI 7500 qPCR system (Applied Biosystems, Foster City, CA, USA). The relative expression level of each gene was calculated using the $2^{-\Delta\Delta CT}$ method. Each PCR sample was run in triplicate, and all qRT-PCR assays were repeated 3 times.

Tissue Chip and Immunohistochemical Staining

The tumor and adjacent tissues chip from HCC patients were supplied by Zuocheng biotechnology (Zuocheng Biotechnology Co., Ltd, Shanghai). The expression of phospho-STAT3 (p-STAT3) and SMAD4 in sections of HCC tumors, adjacent nontumorous tissues, and mouse subcutaneous tumor tissues were detected by immunohistochemical staining, which employed rabbit anti-human p-STAT3 and SMAD4 antibodies (Sigma, USA). In brief, the retrieval of antigen was first performed in the sections using Tris-EDTA Buffer (pH 9.0, Dako, Australia) at 100°C for 10 min. The sections were first probed with the corresponding specific antibody, followed by Envision-labeled polymer-horseradish peroxidase rabbit antibody (Dako). The expressed proteins were stained with diaminobenzidine. The expression signal was quantified by HlivH150CS04 software. The positive percentage = positive cells number/total cells number%, the positive percentage score (A) was 0, 1, 2, 3, or 4, $A < 5\%$ as 0, $5\% \leq A < 25\%$ as 1, $25\% \leq A < 50\%$ as 2, $50\% \leq A < 75\%$ as 3, and $A \geq 75\%$ as 4, the positive staining intensity score (B) was 0, 1, 2, and 3, 0 = negative, 1 = mild positive, 2 = moderately positive, 3 = strong positive, and the staining score (C) = A × B. The results were judged by 2 pathologists at the same times.

Cell Culture and Transfection

Human hepatoma cell lines (SK-Hep-1 and Huh7) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS),

100 g/mL streptomycin, and 100 U/mL penicillin. The miR-224 mimic (224M) and nonspecific mimic (NSM) were synthesized by RiboBio (Guangzhou, China). Cells grown to approximately 70% confluency were transfected using 20 nM 224M or NSM mixed with Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA) separately. The cells were cultured after transfection for the following studies.

IL-6 Treatment

Approximately 5×10^4 cells per well were plated in 6-well plates in growth medium containing 10 ng/mL recombinant human IL-6 (Cat# PHC0064; Invitrogen, Carlsbad, CA, USA). The IL-6-containing medium was changed daily. Seventy-two hours after transfection the cells were harvested for protein or total RNA extraction.

Huh7 cells were transfected with the STAT3 siRNA (Shanghai Genechem Co., Ltd), 72 h after transfection, the cells were then induced by IL-6 (10 ng/mL), 72 h after induction, the cells were harvested and the total RNA was extracted to check the miR-224 expression.

Assays for Cell Growth, Proliferation, Invasion, and Wound Healing

A total of 100 000 transfected SK-Hep-1 and Huh7 cells got above were seeded in 24-well plates separately (Corning, USA). The numbers of cells grown over the observation period were estimated every other day after trypsin treatment to determine the cell growth kinetics.

For the cell invasion assay, 2.5×10^4 transfected SK-Hep-1 and Huh7 cells got above were seeded in 24-well plates with inserts. This 2-chamber system was equipped with a cell-permeable membrane coated with Matrigel (R&D Systems, USA). After culturing for 24 h, the cells were fixed and stained with crystal violet. The cells in the bottom chamber were considered invaded and counted.

A wound healing assay was conducted to determine the cell migration ability. Briefly, the cell monolayer was scratched after 2.5×10^4 transfected cells/well reached 90% confluency in 24-well plates. Images of the same 10 fields under a microscope were photographed at 0, 24, and 48 h post-scratching.

The cell proliferation capacity was assayed through the incorporation of Cell-Light™ Edu Apollo 567, SK-Hep-1, and Huh7 cells at a density of 3×10^3/well in 96-well plates were incubated for 24 h and transfected with 20 nM NSM or 224M for 6 h. EdU and 4′,6-diamidino-2-phenylindole (DAPI) staining was conducted in all wells, respectively (Staining kit, RiboBio). The proliferative cells marked by EdU incorporation were counted under a fluorescence microscope, and the percentage of EdU-positive cells was calculated using the following formula: (number of proliferative cells/total cells) × 100%.

Protein Extraction and Western Blot

Total cellular protein was extracted by adding lysis buffer and quantified using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Protein samples were then separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were first blocked in 5% dry milk for 1 h in Tris-buffered saline containing 0.1% Tween-20 and then incubated with rabbit anti-human phospho-STAT3 (p-STAT3), STAT3, or SMAD4 antibody at 1:2000 (Sigma, USA), or a rabbit anti-human glyceraldehyde 3-phosphate dehydrogenase antibody at 1:5000 dilution (Sigma, USA) at 4°C overnight. The membranes were then treated with horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:2000 (Sigma Aldrich, St. Louis, MO, USA). The protein bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) and quantified using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

Plasmid Construction for the Luciferase Reporter Assay

Wild-type and mutated 3′-untranslated region (3′UTR) of SMAD4 cDNA were cloned into the pmiR-RB-REPORT vector (RioBio). Briefly, a 780-bp fragment of the 3′UTR of human SMAD4 cDNA containing the putative target site of miR-224 was amplified by PCR using the genomic DNA isolated from HEK293T cells and cloned to the upstream location of the *Renilla* luciferase reporter gene in the pmiR-RB-REPORT vector. The primer sequences for wild-type SMAD4 were 5′-GGCGGCTCGAGTTTTAGGGTGGTTAGGACA-3′ (sense) and 5′-AATGCGGCCGCAGACAGGACAGGCT TTATG-3′ (anti-sense). The 2 primer sequences contained *Xho*I and *Not*I sites, respectively. The mutants were generated by replacing 7 nucleotides of the miR-224-binding site using primers containing 7 mutated bases at the 1047-1053 bp position (5′-TTAATGGGAAC CCTCTCTTGGCCAAGGAAGAAT-3′ and 5′-GGCCAAGA GAGGGTTCCCATTAAATGGTACAAA-3′) and 7 mutated bases at the 1194-1200 bp position (5′-TTAATGGGA

ACCCTCTCTTGGCCAAGGAAGAAT-3′ and 5′-AATGC GGCCGCAGACAGGACAGGCTTTATGTCCCCCATTC CTCC GAGGGTTCTCCAGGGACATTGAAAG-3′). The QuikChange XL Site-Directed Mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). The mutated DNA sequences were confirmed by sequencing after the completion of mutagenesis.

For the luciferase reporter assay, 1.5×10^4 HEK293T cells/well were grown in a 96-well plate for 24 h and then transfected with Lipofectamine 2000 (Invitrogen) mixed with pmiR-RBREPORT luciferase reporter plasmids of either the wild-type or the mutated SMAD4 3′UTR and 224M or NSM for 48 h. The Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA), which utilizes Firefly luciferase activity to normalize the *Renilla* luciferase activity, was used to measure the luciferase activity in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Each experiment was conducted in triplicate and repeated 3 times.

The In Vivo Experiment

Establishment of a Cell Line With Stable miR-224 Expression

Lentiviral vectors hU6-MCS-Ubiquitin-firefly-Luciferase-IRES-purinomycin-miR-224 and hU6-MCS-Ubiquitin-firefly-Luciferase-IRES-purinomycin-anti-miR-224 were purchased from GENECHEM (Shanghai, China). The SK-Hep-1 cell line was transduced by the lentiviruses for 72 h, and the transduction efficiency was determined by fluorescence microscopy. Puromycin was then added to the cells (2 μg/mL) every 3 days until all the cells (cells without lentivirus) in the control wells died.

Establishment of the Mouse Xenograft Tumor Model

Female nude mice (6–8 weeks old) were obtained from Changzhou Kaiwen Laboratory Animal Co., Ltd. (Jiangsu, China) and housed in isolated vented cages under specific pathogen-free conditions in the Animal Facility of Wuxi Hospital. The mice were acclimated for 1 week with a standard chow diet. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

The SK-Hep-1 cell lines, as well as cells stably expressing miR-224, were grown to 4×10^7 cells in DMEM containing 10% FBS, harvested separately, resuspended in 100 μL of saline, and implanted subcutaneously into the mice. The control cells were injected into the left lateral torso of the mice, and the miR-224-overexpressing cells were injected into the right lateral torso of the mice ($n = 3$). When the tumors reached about 200 mm^3 in size, the mice were sacrificed and the histopathological evaluation and immunohistochemical staining were performed.

Noninvasive Detection of Luciferase Activity in Mice

The SK-Hep-1 cell line overexpressing mR-224 was injected into mice through the caudal vein, the nude mice were weakly anesthetized with isoflurane, and then the mice were visualized with an In Vivo Imaging System (IVIS) (PerkinElmer). Before imaging, the mice were intraperitoneally injected with 150 mg/kg D-luciferin (Promega). After 5 min, the mice were moved into the IVIS chamber, and data were collected and analyzed using Living Image software (Xenogen). Luminescence signals were determined using the region of interest (ROI) function of the Living Image software. Total flux within a ROI was defined as the signal intensity. For ex vivo imaging, the excised tissues were submerged in 300 μg/mL D-luciferin and analyzed using the IVIS platform. The luminescence signal in ex vivo samples was quantified using an area drawn around the dish containing the specimen.

Statistical Analysis

Statistical analysis was performed with a 1-way or 2-way analysis of variance (GraphPad Prism 5). All data are presented as the mean ± standard error of the mean. Differences with $P < .05$ between groups were considered statistically significant. The correlations were assessed by Pearson's correlation coefficients.

RESULTS

The miR-224 Level was Elevated in HCC Serum and Tumor Tissue, Accompanied by Activated STAT3, and Reduced SMAD4 Levels in HCC Tumor Tissues

As shown by qRT-PCR, the miR-224 levels in both the serum and tumor samples were elevated in the HCC patients (Figure 1A and B). According to the immunohistochemistry results, the percentage of cells with p-STAT3 expression was higher in the tumor tissues (Figure 1C–E) and the percentage of cells with SMAD4 expression was lower in the tumor tissues, compared to the adjacent nontumorous tissues.

MiR-224 Promoted the Ability of HCC Cells to Grow and Proliferate In Vitro

SK-Hep-1 and Huh7 cells were transfected with 224M or NSM, after transfection. Cell counting assay showed

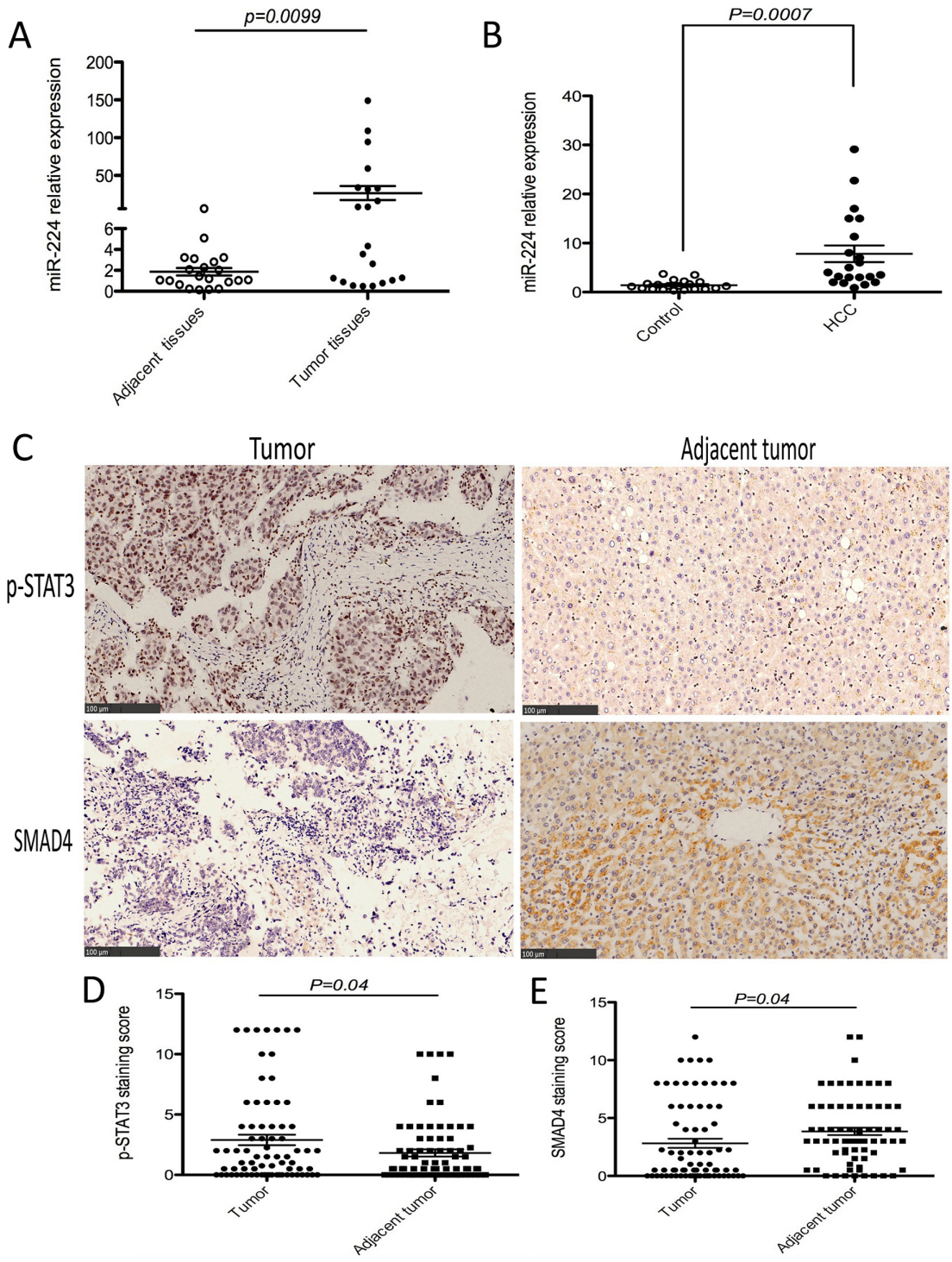

Figure 1. The expression of miR-224, p-STAT3, and SMAD4 in HCC patients. (A) The expression of miR-224 in tumor tissues and (B) serum samples from 21 HCC patients as detected by qRT-PCR. The horizontal bars represent the mean ± SEM of the miR-224 level. Each sample was amplified in triplicate and retested 3 times. (C) The expression of p-STAT3 (upper panel) and SMAD4 (down panel) were detected by immunohistochemistry with 75 tumor or adjacent tumor tissues from tissue chip. Positive expression appears as a brown color. (D) and (E) The quantified staining score of the 2 markers above. SEM, standard error of the mean; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

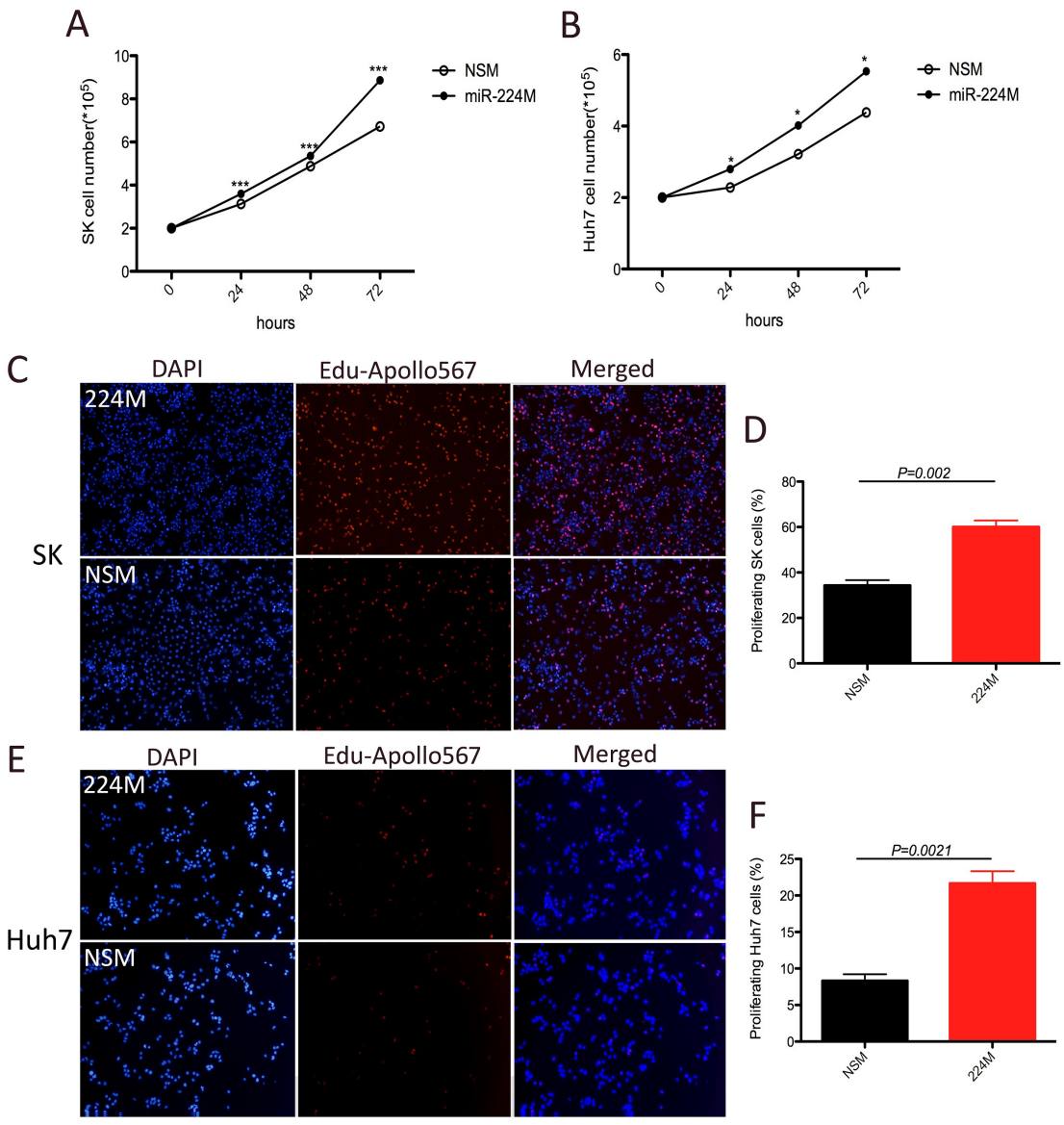

Figure 2. The proliferation promotion of miR-224 expression in hepatoma cell. (A) and (B) The cell growth study. SK-Hep-1 (left panel) and Huh7 (right panel) cells (100 000) transfected with miR-224 mimic (miR-224M) or NSM were counted at 0, 24, 48, and 72 h. The mean (±SEM) values of 3 independent experiments are plotted; *P < .05, ***P < .001. (C and E) Proliferation study with EdU incorporation. A total of 3 × 10^3 SK-Hep-1 (upper panel) or Huh7 (down panel) cells per well in 96-well plates were cultured for 24 h and then transfected with miR-224M or NSM for 48 h. EdU-positive cells were stained red, and nuclei were stained with DAPI (blue). (D and F) The percentage of EdU-positive cells in 10 randomly selected microscopic fields was manually counted. Mean (±SEM) values of 3 independent experiments are presented. SEM, standard error of the mean.

over expression of miR-224 can accelerate the growth rate of SK-Hep-1 and Huh7 cells compared to the NSM-transfected cells (P < .05; Figure 2A and B). The EdU incorporation assays showed that 224M transfection increased the proliferation rate of SK-Hep-1 and Huh7 cells, compared to the NSM-transfected cells (P < .05; Figure 2C–F).

MiR-224 Promoted the Invade and Migrate of HCC Cells In Vitro

In addition, wound healing and transwell assays were used to analyze the effect of miR-224 on the in vitro invasion and metastasis of SK-Hep-1 and Huh7 cells, respectively. The wound healing scratch test revealed that the transfection of miR-224 also accelerated

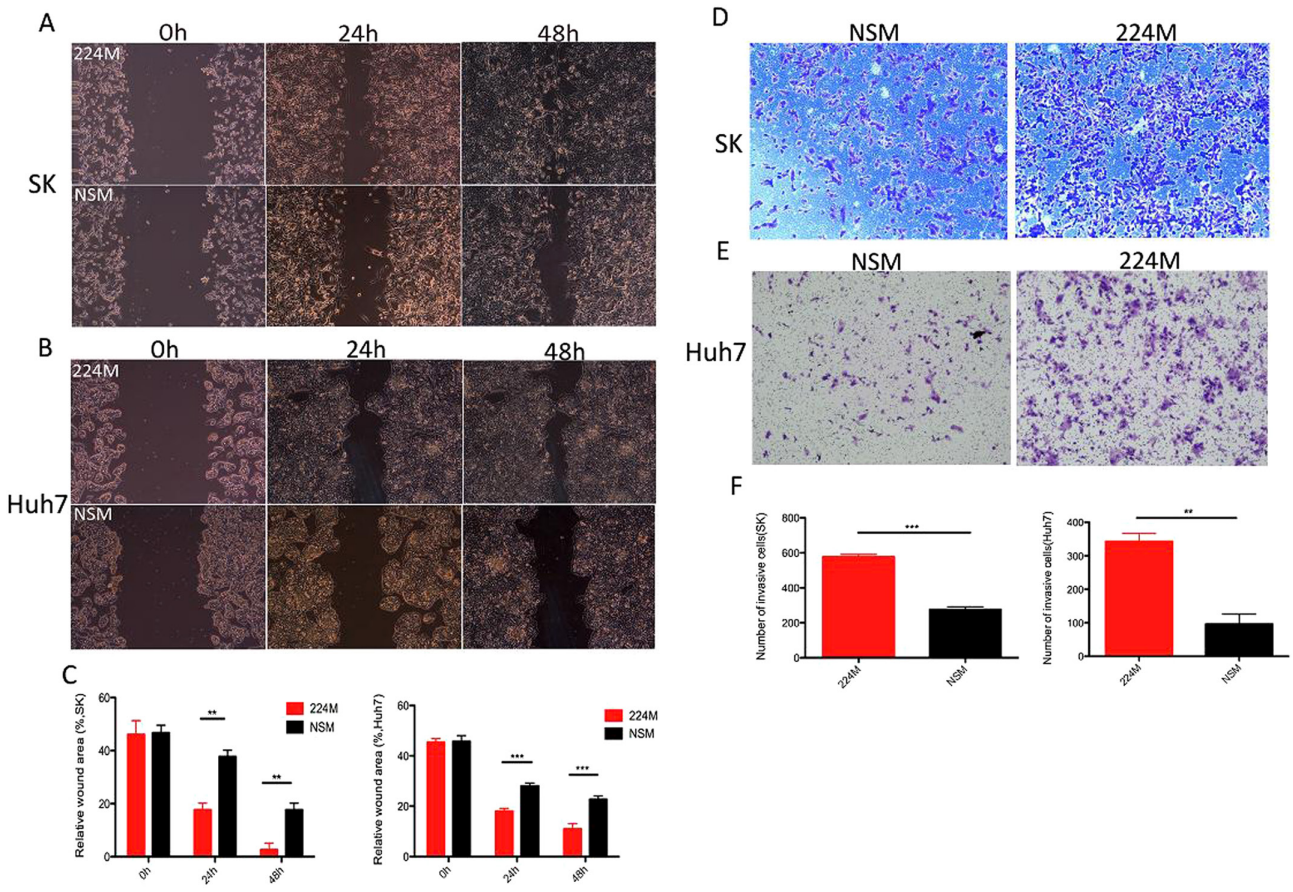

Figure 3. The role of miR-224 expression in hepatoma cell invasion and migration. (A and B) Wound healing assay to detect cell migration. A total of 2.5 × 10^4 transfected SK-Hep-1 (upper panel) or Huh7 (down panel) cells were seeded in 24-well plates. The cell layer of approximately 90% confluence was scratched using a sterile 50-μL pipette tip. The healing/filling of the scratched space was monitored under an inverted microscope, and images were captured at 0, 24, and 48 h after scratching. (C) Data are shown as the mean value of 3 independent experiments of A and B, bars represent the mean ± SEM (n=3) of the area % of the migrated cells per field; $^{**}P < .01$, $^{***}P < .001$. (D and E) Transwell assay showing cell invasion. After transfection for 48 h, 2.5 × 10^4 transfected SK-Hep-1 (upper panel) or Huh7 cells (down panel) per well in 24-well plates were monitored for migration through Matrigel-coated membranes. Cells in the bottom chamber were stained with crystal violet after being fixed with formalin. (F) The number of cells in 10 randomly selected fields was counted. Data are shown as the mean value of 3 independent experiments. Bars represent the mean (±SEM) number of invaded cells per field; $^{**}P < .01$, $^{***}P < .001$. SEM, standard error of the mean.

the mobility of SK-Hep-1 and Huh7 cells at 48 h after scratching or in the transwell chamber, compared with the NSM-transfected cells ($P < .01$) (Figure 3A–C). Meanwhile, a transwell study showed overexpression of miR-224 in SK-Hep-1 and Huh7 cells were associated with a significantly increased cell invasion ability ($P < .01$) (Figure 3D–F).

IL-6 Induced p-STAT3 Expression, the Elevated miR-224 Expression Inhibited SMAD4 Expression

IL-6 and STAT3 are believed to be the key signaling pathway components involved in the development of HCC. To explore whether the IL-6/STAT3 pathway induces miR-224 expression, IL-6 was added to SK-Hep-1 and Huh7 cells, and the STAT3 and p-STAT3 expression level were assayed by western blot. As shown in Figure 4A, the addition of IL-6 activated STAT3 in both cell lines. Our previous study showed that IL-6 upregulates miR-224 expression in cholangiocarcinoma. In this study, it was found IL-6 can also induce the miR-224 expression in HCC cell line Huh7 cells, but this induction effect can be inhibited by STAT3 siRNA ($P < .001$) (Figure 4B).

Bioinformatics analysis suggested that 7 bases in the seed region of miR-224 are complementary to

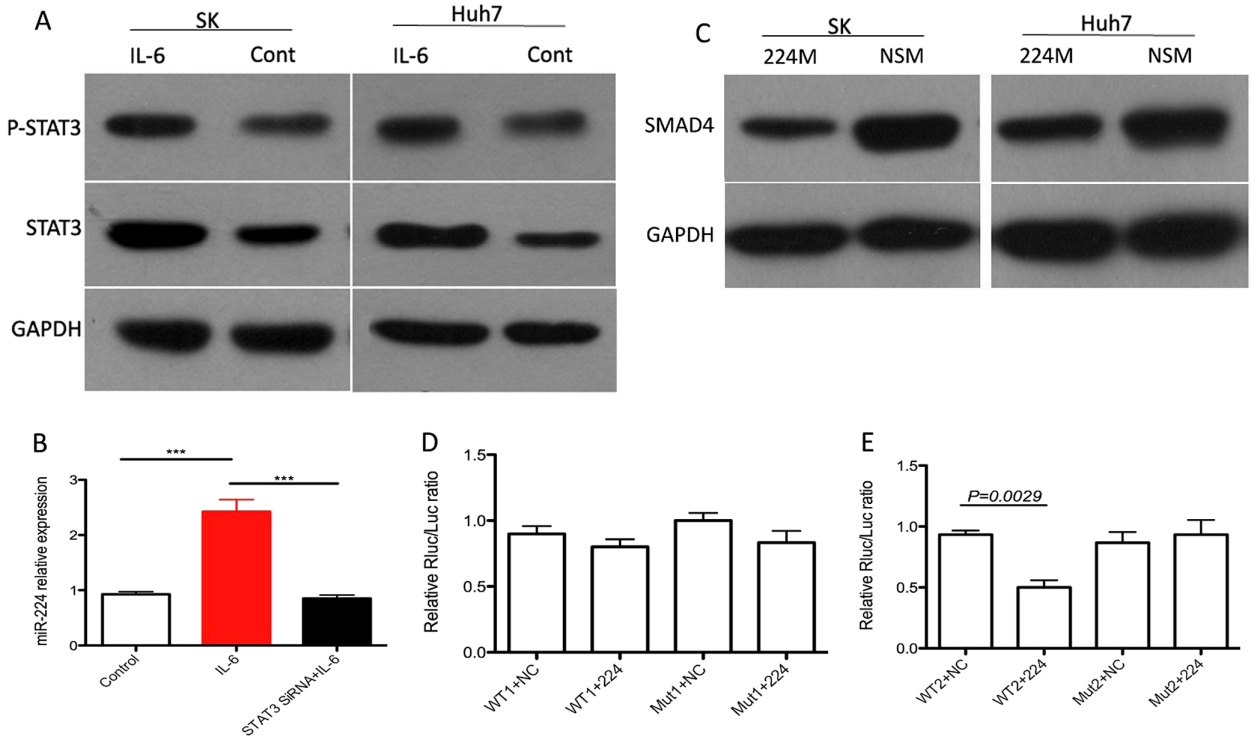

Figure 4. IL-6/STAT3/SMAD4 and miR-224 interaction. IL-6 at a final concentration of 10 ng/mL was added to the cell culture, (A) IL-6 induced the STAT3 and Phosphorated STAT3 expression in both SK-Hep-1 and Huh7 cell lines. Equal amounts of protein were loaded using GAPDH as the reference. (B) IL-6 was applied to induce the Huh7 cells or the cells transfected with STAT3 small interfere RNA (STAT3 SIRNA) before, DEPC water was used as the control, the miR-224 expression was detected after induction. Bars represent the mean ± SEM of 3 independent experiments; ***$P < .001$. (C) An elevated miR-224 expression level inhibited SMAD4 expression. SK-Hep-1 and Huh7 cell lines were transfected with miR-224M or NSM for 72 h and then subjected to western blot analysis of SMAD4 expression. (D and E) Confirmation of the target gene predicted by the Luciferase reporter assay. HEK293T cells were cotransfected with miR-224M or NSM plus wild-type (WT) of the 1047–1053-bp region of the SMAD4 3′UTR (SMAD4-WT1), mutant 3′UTR (SMAD4-Mut1), wild-type (WT) of the 1194-1200 bp region of the SMAD4 3′UTR (SMAD4-WT2) or mutant 3′UTR (SMAD4-Mut2), and then *Renilla* luciferase activity was assayed. Data represent the mean value of 3 independent experiments ± SEM. *$P < .05$, **$P < .01$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.

nucleotides at 1047-1053 bp and 1194-1200 bp of the SMAD4 mRNA 3′UTR, respectively, implying that miR-224 may regulate the SMAD4 protein level; therefore, western blot assays were performed. As shown in Figure 4C, miR-224 overexpression significantly inhibited the expression of SMAD4 protein in the HCC cell lines SK-Hep-1 and Huh7. Furthermore, luciferase reporter assays suggested that miR-224 directly binds to the 2 identified sites at the 3′UTR of SMAD4 as luciferase activity was decreased in the cells cotransfected with the 224M and the wild-type of the 1194-1200 bp (site2) section of the SMAD4 3′UTR, compared to the control cells. In contrast, luciferase activity was higher in the cells cotransfected with 224M and the mutant 3′UTR of SMAD4 (Figure 4D–E).

miR-224 Promoted Subcutaneous Tumor Growth and Metastasis in Nude Mice

The hepatoma cell line SK-Hep-1 with stable expression of miR-224 was subcutaneously injected into the right lateral torso of nude mice, and the cells transfected with control lentivirus was injected into the left lateral torso. The implanted cells formed masses that grew as big as 2 cm^3 in 25 days. However, the tumor sizes on the right side were larger than those on the left side, indicating that miR-224 may have accelerated the tumor growth (Figure 5A). The subcutaneous tumor tissues were stained for p-STAT3 and SMAD4 expression, which were higher or lower in the tumors expressing miR-224 compared with the control tumors, respectively (Figure 5B).

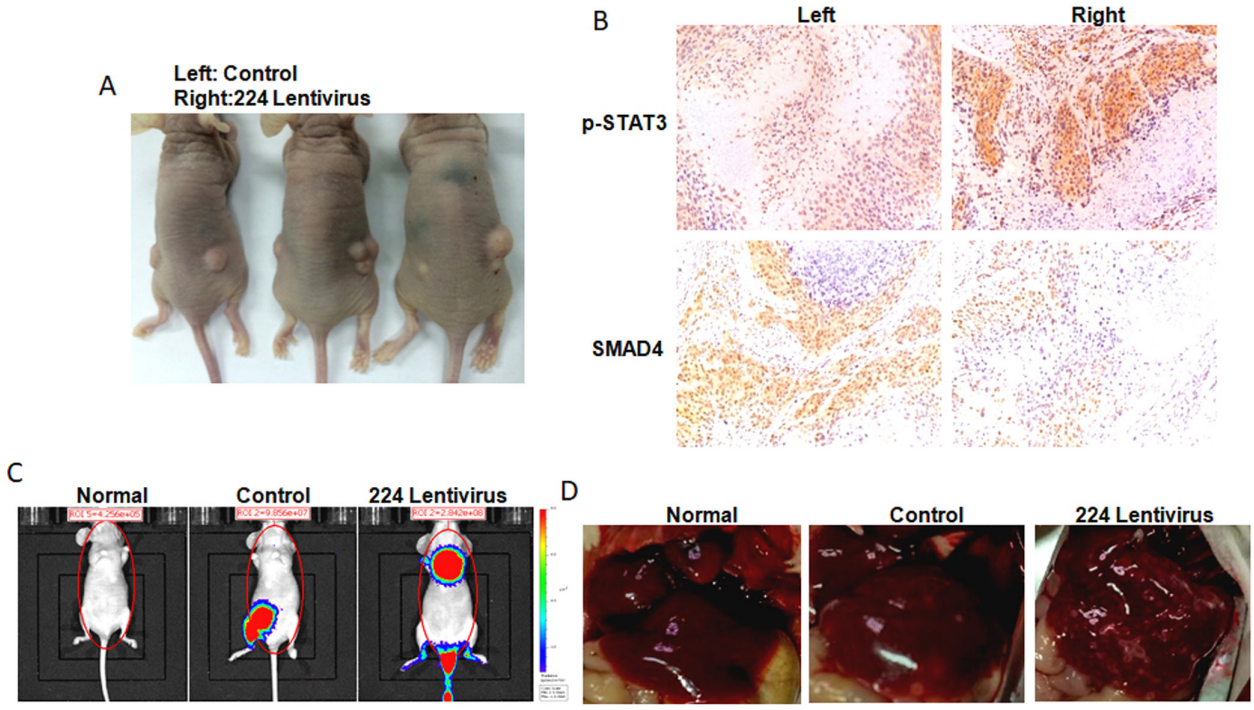

Figure 5. The in vivo functions of miR-224. (A) Nude mice were subcutaneously injected into the right lateral torso with SK-Hep-1 cells that stably express miR-224, and the empty vector-transfected cells were injected into the left lateral torso as the control. (B) Immunohistochemical staining for p-STAT3 and SMAD4 expression in subcutaneous tumor tissues (200× magnification); positive protein expression is indicated by the brown color. (C) The nude mice that survived caudal vein injection with *miR-224-* or empty vehicle-infected SK-Hep-1 cells. The ventral images shown (taken 31 days after injection) are representative of 2 experiments ($n=3$). Image intensity is represented by a color scale ranging from blue (just above the background noise; set to 6000 photons/s/cm^2/sr) to red (at least 1 × 10^5 photons/s/cm^2/sr). (D) The histopathological changes showed the normal liver looked smooth, and the color of the surface became deepened and spotty (controls). Significant nodular lesions appeared on the surface of the liver in the mice in the miR-224 group.

On day 31 after treatment, the live imaging study detected the expressed luciferase protein in the treated mice. The total ROI was higher in the mice overexpressing miR-224 compared with controls (Figure 5C). Histopathological analysis showed infiltration with massive heterogeneous cells in the liver in the mice with miR-224 overexpression (Figure 5D).

DISCUSSION

MiR-224 is considered an oncogenic miRNA and implicated in HCC carcinogenesis; it is possibly mediated by the tumor suppressor gene.[19] The causal relationship between the miR-224 expression level and HCC growth also has been demonstrated by the findings that autophagy-mediated miR-224 degradation suppresses HCC.[20] In addition, it has been shown that the plasma miR-224 level can be used as a noninvasive biomarker for the early development of HCC.[15,21-23] Moreover, miR-224 has been suggested as a novel therapeutic target for HCC treatment.[24] All of the studies above showed miR-224 plays the key roles in HCC progression, but the further mechanism still kept unclear at present, indeed, miR-224 warrants further detailed investigations.

Our previous study demonstrated that miR-224 expression was upregulated in a cohort of cholangiocarcinoma patients and that miR-224 expression promoted the growth of both cholangiocarcinoma and HCC cells.[12] This study found the following: (1) the miR-224 level in both tumor tissue and serum was elevated in HCC patients, (2) miR-224 overexpression increased the ability of hepatoma cell lines to grow, proliferate, invade, and migrate in in vitro experiments, and (3) miR-224 expression promoted the growth of implanted hepatoma cells in an in vivo nude mouse model. Taken together, our findings provide new insights into the biological functions of miR-224 in HCC carcinogenesis.

Figure 6. The signaling pathway diagram regulated by miR-224 in HCC tumor migration and invasion was shown, the activation role was indicated by the red arrow and the inhibition role was indicated by the red suppress symbol.

Previous studies have noted that several transcription factors regulate miR-224 expression. For instance, p53 and NF-κB p65 regulate miRNA-224 transcription in mouse ovarian granulosa cells.[25] In addition, p65/NFκB may function as a direct transcriptional regulator of miR-224 expression, and the elevated miR-224 expression level was linked with cell migration/invasion in HCC.[26] Moreover, the combination of an elevated miR-224 level with protein kinase B (AKT) expression could effectively predict a poor prognosis for HCC patients.[27] Furthermore, STAT3 is a key transcription factor involved in HCC carcinogenesis and can be activated by inflammatory cytokines. The IL-6/STAT3 pathway also is emerging as a target for the treatment of HCC.[28] This study showed that IL-6 induced both STAT3 and miR-224 expression in hepatoma cell lines and STAT3 played the bridge role between IL-6 and miR-224, our findings suggested that IL-6 induces the hepatic miR-224 expression through the STAT3 pathway.

SMAD4 is the downstream gene of STAT3, it has been suggested SMAD4 is an important HCC suppressor protein.[29] Several miRNAs may potentially target SMAD4 and regulate tumorigeneses, for instance, miR-130a-3p promotes cell migration and invasion through inhibiting SMAD4 in gemcitabine-resistant hepatoma cells.[30] MiR-224 is predicted to bind to 2 sites in the 3′UTR of SMAD4. The results from this study showed that miR-224 directly binds to the SMAD4 3′UTR, it was reported previously miR-224 can target to SMAD4 protein and then promote the tumor progression in HCC patients,[31] in this study, we got the same results by luciferase reporter assay, then we used the different HCC cell lines SK-Hep-1 and Huh7 cells to verified the results.

Taken together, it was showed that IL-6 activates STAT3 phosphorylation, then induces miR-224 expression, furthermore, the elevated miR-224 expression facilitates HCC carcinogenesis by targeting to the tumor suppressor gene SMAD4 (Figure 6). It was speculated that IL-6/STAT3/miR-224/SMAD4 is the new signaling pathway in HCC progression.

Ethics Committee Approval: This study was approved by the Nanjing Medical University Clinic Institutional Review Board (IRB) protocols.

Informed Consent: All patients provided written informed consent prior to their enrolment.

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