miRNA-302b Suppresses Human Hepatocellular Carcinoma by Targeting AKT2

Lumin Wang1, Jiayi Yao1, Xiaogang Zhang2, Bo Guo1, Xiaofeng Le6, Mark Rubbery7, Zongfang Li8, Kejun Nan9, Tusheng Song1, and Chen Huang1,5

Abstract

miRNAs (miR) play a critical role in human cancers, including hepatocellular carcinoma. Although miR-302b has been suggested to function as a tumor repressor in other cancers, its role in hepatocellular carcinoma is unknown. This study investigated the expression and functional role of miR-302b in human hepatocellular carcinoma. The expression level of miR-302b is dramatically decreased in clinical hepatocellular carcinoma specimens, as compared with their respective nonneoplastic counterparts, and in hepatocellular carcinoma cell lines. Overexpression of miR-302b suppressed hepatocellular carcinoma cell proliferation and G1–S transition in vitro, whereas inhibition of miR-302b promoted hepatocellular carcinoma cell proliferation and G1–S transition. Using a luciferase reporter assay, AKT2 was determined to be a direct target of miR-302b. Subsequent investigation revealed that miR-302b expression was inversely correlated with AKT2 expression in hepatocellular carcinoma tissue samples. Importantly, silencing AKT2 recapitulated the cellular and molecular effects seen upon miR-302b overexpression, which included inhibiting hepatocellular carcinoma cell proliferation, suppressing G1 regulators (Cyclin A, Cyclin D1, CDK2) and increasing p27Kip1 phosphorylation at Ser10. Restoration of AKT2 counteracted the effects of miR-302b expression. Moreover, miR-302b was able to repress tumor growth of hepatocellular carcinoma cells in vivo.

Implications: Taken together, miR-302b inhibits HCC cell proliferation and growth in vitro and in vivo by targeting AKT2. Mol Cancer Res; 12(2); 190–202. ©2013 AACR.

Introduction

Hepatocellular carcinoma is the sixth most prevalent cancer and the third most frequent cause of cancer mortality worldwide. It is thought to develop in a multistep process requiring the accumulation of several structural and genomic alterations and affecting many different pathways (1). It is well documented that a defect in cell-cycle control is an essential step in the development and progression of human cancer. A number of oncogenes and tumor suppressors involved in cell-cycle regulation are often aberrant in hepatocellular carcinoma, thereby promoting cancer cell proliferation (2).

Cell-cycle–related genes such as cyclin D1, cyclin E, cyclin A, p53, p14, p16, p19, c-Jun, and Pten have been implicated in hepatocarcinogenesis. Among these, AKT2 involvement has been investigated thoroughly in the induction and progression of hepatocellular carcinoma both in cancer cell proliferation and migration (3, 4). AKT2, a key downstream effector of the phosphatidylinositol 3-kinase (PI3K) signaling pathway, modulates the function of numerous substrates related to cell-cycle progression at the G1–S and G2–M transitions, either by direct phosphorylation of the target proteins themselves or, indirectly, by regulating protein expression levels (5). In the G1–S phase, AKT2 controls the transcription and/or stabilization of c-Myc and cyclin D1, whereas it also suppresses the expression of multiple negative cell-cycle regulators such as p21Cip1, p27Kip1, and p15INK4b by phosphorylation of their substrates, respectively (6, 7). In addition, AKT binds to and phosphorylates Mdm2 and MdmX at Ser166, Ser186, and Ser367 to enhance protein stability and facilitate the function of the heterocomplex of Mdm2-MdmX; which is to induce p53 ubiquitination, therefore bypassing the G1 checkpoint (8, 9). In the G2–M phase, AKT2 is necessary for efficient entry into mitosis during unperturbed cell cycles by mechanisms such as phosphorylation of CDK1 and CDK2 and cytoplasmic accumulation of CDC25B (10, 11). Thus, phosphorylation by AKT regulates compartmentalization...
of multiple substrates involved in cell-cycle progression in hepatocellular carcinoma proliferation.

MicroRNAs (miRNA) are a class of non-protein-coding, endogenous, small RNAs that regulate gene expression by translational repression, mRNA cleavage, and decay initiated by miRNA-guided rapid deadenylation (12). More than the past 10 years, miRNAs are particularly important in nearly all cancer development studies in that they can be targets of genomic lesions, controlled by classic tumor signals, and they themselves present as a class of oncogenes or tumor suppressors (13). Importantly, miRNAs have been demonstrated to have essential roles in hepatocellular carcinoma progression by affecting cell proliferation and metastasis by targeting tumor-related gene (14–16). The present studies proved that miR-122, miR-26, and miR-223 suppressed tumor proliferation, whereas miR-130b, miR-221, miR-222 promoted tumor development in hepatocellular carcinoma (17–21). Ever since the adoption of the miRNA array technique, many more precursors and mature miRNAs have been found to be aberrantly regulated in hepatocellular carcinoma progression; among which, the miR-302 cluster has garnered much attention (15). The miR-302 cluster, which consists of miR-302a, -302b, -302b*, -302c, -302c*, -302d, -302e, and -302f, was first found to be functionally correlated with self-renewal and proliferation properties in the stemness maintenance of embryonic stem cells (ESC; refs. 22 and 23). Further studies confirmed that the ESC-specific transcription factors Otx4, Sox2, Nanog, and Rex-1 had binding sites on the miR-302 promoter, thus regulating its expression (24–26). In addition, miR-302-367 has been identified to posttranscriptionally regulate CYCLIND1 and CDK4, therefore affecting cell-cycle progression (25). More recently, miR-302 has been reported to be a new tumor marker used to predict the malignant behavior of different types of tumor. Lin and colleagues demonstrated the tumor suppressive activity of miR-302 in human pluripotent stem cells by both the cyclin E-Cdk2 and cyclin D-Cdk4/6 pathways in the G1-S cell-cycle transition. More importantly, miR-302 was found to target Bmi-1, thus promoting the tumor suppressor functions of p16Ink4a and p14/p19Arf directed against Cdk4/6 (27). Furthermore, tumor-related miRNA studies demonstrated the potential tumor-suppressor role for miR-302b in attenuating cell growth, promoting cell apoptosis, and subsequently interrupted the AKT2-related cell-cycle pathway. Silencing of AKT2 produced similar cellular and molecular effects as miR-302b overexpression, whereas restoration of AKT2 counteracted the effects of miR-302b expression. These findings illustrated the tumor suppressor role of miR-302b in the control of the G1-S cell cycle and therefore suppressed hepatocellular carcinoma progression, which may be a beneficial strategy for future cancer therapy.

**Materials and Methods**

**Cell line and tissue specimens**

Human liver cancer cell line SMMC-7721, Bel-7404, HuH7, and normal liver cell HL-7702 cells were maintained in 1640 medium (1640; PAA Laboratories GmbH) supplemented with 10% FBS (PAA Laboratories GmbH). Normal liver tissues were collected from patients undergoing resection of hepatic hemangiomas. Twenty-seven paired hepatocellular carcinoma and adjacent nontumor liver tissues were collected from patients undergoing resection of hepatocellular carcinoma at the hepatobiliary surgery department of the First Affiliated Hospital (Xi’an Jiaotong University, Xi’an, China). The relevant characteristics of the studied subjects were shown in Table 1. No local or systemic treatment had been conducted before operation. Tissue samples were immediately snap frozen in liquid nitrogen until RNA extraction. Both tumor and nontumor tissues were histologically confirmed. Informed consent was obtained from each patient and was approved by the Institute Research Ethics Committee at Cancer Center (Xi’an Jiaotong University).

**RNA extraction, cDNA synthesis, and quantitative real-time PCR**

Total RNA was extracted from prepared liver samples with TRIzol reagent (Invitrogen) and cDNA was synthesized according to the manufacturer’s protocol (MBI Fermentas). Quantitative real-time PCR (qRT-PCR) was performed using a Maxima SYBR Green qPCR Master Mixes (MBI Fermentas), and PCR-specific amplification was conducted in the IQ5 Optical System real-time PCR machine. The relative expression of genes (miR-302b, U6, Akt2, β-actin) was calculated with the 2−ΔΔCt method (30). The primers used are listed here (qRT-PCR, miR-302b-F 5’-ATCCAGCTCGTCTCGTG-3’, miR-302b-R 5’-TGCTTAAAGTGCTTTCCATGTT-3’; AKT2-F 5’-CTCACACACAGCTCACCGAGACC-3’, AKT2-R 5’-TGGGTCTGTGGAAGGCATACTTCT-3’; U6-F 5’-TGCGGGTGCTGCTCGCTTCG-3’, U6-R 5’-CCAGAGGCTGCTCGTTCCAGG-3’; β-actin-F 5’-CGTTGCATTTAAGGAGAAGCT-3’, β-actin-R 5’-CTAGAAGCATTTGCGGTGG-3’).

**Construction of expression plasmids**

Human miR-302b precursor (pri-miR-302b) was cloned into pSilencerTM4.1-CMV vector (Ambion, Geneworks) according to the manufacturer’s instructions: miR-302b-F 5’-AATTTGCGTCCCTTCAACTTTAACATGG-AAAGTGCTTTCTGTGACTTTAAAGTAAGTGCTTCCATGTT-3’,miR-302b-R 5’-TGCGGGTGCTGCTCGCTTCG-3’, R 5’-AGCTTACTCCTACTAAACATGGAGGACACACTTTAATGG-TCAGAAAGGAGCATTCTCCATGTTAAAGTTGGAAGGGAGCCG-3’. Primers contained 5’ EcoRI and 3’ HindIII restriction sites to facilitate cloning into the vector. We also commercially synthesized a 2’-O-methyl-modified antisense oligonucleotide of miR-302b (ASO-miR-302b) as an inhibitor of miR-302b (BGI), GV141 vector (GeneChem) was
used to construct vector of re-expression AKT2. The cDNA of AKT2 was chemically synthesized and cloned into GV141 vector between the XhoI and KpnI sites.

Analysis of clonogenicity in vitro and tumorigenicity in vivo

For clonogenicity analysis, 24 hours after transfection, HL-7702, SMMC-7721, Huh7, and Bel-7404 cells were resuspended and seeded onto 12-well plates at density of 2,000 cells/well, incubated 2 weeks later, colonies were stained with 0.5% crystal violet.

All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals and were performed according to the institutional ethical guidelines for animal experiment. Viable miR-302b and negative control (NC)–transfected SMMC-7721 viable cells were suspended in 100 μL PBS and then injected subcutaneously into either side of the posterior flank of the same female nude mouse at 4 to 5 weeks of age. Tumor growth was examined every 3 days for 4 weeks. Tumor volume (V) was monitored by measuring the length (L) and width (W) with calipers and calculated with the formula (L × W²) × 0.5.

miRNA and RNA interference

pri-miR-302b, the ASO-miR-302b control vector or oligonucleotides, ASO-NC, siRNAs duplexes targeting human AKT2 were synthesized and purified by BGI. siRNA duplexes with nonspecific sequences were used as siRNA negative control. RNA oligonucleotides were transfected by using Lipofectamine RNAi-MAX (Invitrogen) and medium was replaced 6 hours after transfection. A final concentration of 100 nmol/L miRNA or siRNA was used unless indicated. RNA transfection efficiency is approximately 70% to 80% and the overexpression of miRNA or siRNA persists for at least 48 hours. Lipofectamine 2000 (Invitrogen) was used for transfection of plasmid alone or together with RNA oligonucleotides. All oligonucleotide sequences are listed here (ASO-NC 5′-TGACCTGACTGAACTCTGACTG-3′. ASO-miR-302b 5′-TGATTTTGTACTTCTGGAAACGTGAGCTTCGGAGAA-3′. siRNA-ctrl-F 5′-AAATTCTCCGAACGTGTCACGT-3′. siRNA-ctrl-R 5′-AGGTGACAGTCTGGAGAAATT-3′. siAKT2-F 5′-AAGGATGAAATGCGGTCAACA-3′ siAKT2-R 5′-TGTTGAGCGACCTCATCCT-3′).

MTT assay for cell proliferation

Cells were washed with warm 1640 and MTT (Sigma) working solution was added into wells. Cells were incubated at 37°C for 4 hours, and then solubilized the converted dye with acidic isopropanol (0.04 M HCl in absolute isopropanol). Absorbance of the converted dye was measured at a wavelength of 490 nm with FLUOstar OPTIMA (BMG).

Cell-cycle analysis

Cells were harvested by trypsinization and 1 × 10⁶ cells were used for analysis. The cells were washed in PBS and fixed in ice-cold ethanol overnight at 4°C. The cells were then washed in PBS and incubated in 1 mL staining solution (20 μg/mL propidium iodide and 10 U/mL RNaseA) for 30 minutes at room temperature. The cells were examined by fluorescence-activated cell sorting (FACS) using a flow cytometer (FACSort; Becton), and the cell-cycle populations were determined by ModFit software.

Dual-luciferase reporter gene assay

Luciferase reporter gene assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Cells of 90% confluence were seeded in 96-well plates. For AKT2 3′-untranslated region (UTR) luciferase reporter assay, wild-type or mutant reporter constructs (termed WT or Mut) were cotransfected into SMMC-7721 cells in 96-well plates with 100 nmol/L miR-302b or 100 nmol/L miR-NC and Renilla plasmid by using lipofectamine 2000 (Invitrogen). Renilla gene assays were performed 24 hours posttransfection using the Dual-Luciferase Assay System (Promega). Firefly luciferase activity was normalized for transfection efficiency using the corresponding Renilla luciferase activity. All experiments were performed at least 3 times.

In vivo bioluminescence imaging

At 4 weeks after injection, mice from the miR-302b group (n = 4) and control group (n = 4) were subjected to in vivo bioluminescence imaging (31, 32). Briefly, the animals, anesthetized by isoflurane as described previously (33), were intraperitoneally injected with d-luciferin (Biotium) in a concentration of 150 mg/kg, and 20 minutes later, were subjected to the in vivo bioluminescence imaging using the system of IVIS Spectrum.

Western blot analysis

Cell protein lysates were separated in 10% SDS-PAGE transferred to polyvinylidene difluoride membranes (Roche), then detected with rabbit polyclonal antibody specific for AKT2 (Bioworld Biotechnology) and commercial ECL Kit (Pierce). Protein loading was estimated by using human anti-β-actin monoclonal antibody (Bioworld Biotechnology).

Immunohistochemistry

Immunohistochemistry (IHC) was performed according to the methods described previously (34). The sections were pretreated with microwave, blocked, and incubated using polyclonal rabbit anti-human AKT2 (Bioworld Biotechnology). Staining intensity was assessed by Leica Q550 image analysis system.

Immunofluorescence microscopy

To determine the effect of miR-302b on the protein level of AKT2/Ki-67, we also performed immunofluorescence staining using the AKT2 (Abscam) or Ki-67 antibody (Millipore). After 48 hours, the transfected SMMC-7721, Huh7, and Bel-7404 cell lines were fixed with 4% formaldehyde for 20 minutes, then incubated with 0.5% Triton X-100. Rabbit/mouse anti-AKT2/Ki-67 antibody was used for immunofluorescence staining. After washed 3 times with PBS, the cells were incubated with a goat anti-rabbit/mouse
antibody (Millipore), and measured by immunofluorescence microscopy.

**Statistical analysis**

Data are expressed as the mean ± SEM from at least 3 separate experiments. Unless otherwise noted, the differences between groups were analyzed using Student t test when only 2 groups. All tests performed were two-sided. Differences were considered statistically significant at P < 0.05. All statistical analysis was performed using SPSS13.0 software (SPSS Inc.). The linear correlation coefficient (Pearson r) was calculated to estimate the correlation between miR-302b values and AKT2 levels in the matched hepatocellular carcinoma tumor specimens.

**Results**

miR-302b is frequently reduced in human hepatocellular carcinoma tissue samples and hepatocellular carcinoma cell lines

To explore the role of miR-302b in liver cancer, we analyzed the expression of miR-302b in 27 paired hepatocellular carcinoma and adjacent noncancerous liver tissues by real-time PCR. Compared with their peritumor counterparts, significant downregulation of miR-302b was observed in 74% (20/27) of hepatocellular carcinoma samples (Fig. 1A). Next, we found that miR-302b was downregulated in examined hepatocellular carcinoma cells compared with normal hepatocytes (Fig. 1B). This observation was consistent with the results of the expression of miR-302b in hepatocellular carcinoma tissues at RNA levels. Meanwhile, we examined the correlation of 302b levels with grading and tumor stage and found that the expression of 302b was downregulated in 16 (84%) of poorly differentiated tumor tissues (Supplementary Table S1) or in 11 (55%) of the tumor stage III (Supplementary Table S2), thus indicating that the miR-302b may act as a tumor suppressor in the hepatocellular carcinoma.

miR-302b decreases SMMC-7721/Bel-7404 cells growth, induces G1–S arrest in vitro

miR-302b, a highly conserved sequence across species, is located in intron 8 of the Larp7 gene on chromosome 3, sharing the same seed sequence with miR-302a/c/d (25). To explore the tumor suppressor role of miR-302b in

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**Table 1. Background data among 27 patients with hepatocellular carcinoma**

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; TNM, tumor-node-metastasis staging system.
hepatocellular carcinoma, SMMC-7721/Bel-7404 cells were transfected with miR-302b precursor or its negative controls. The efficiency of transfection was monitored with a GFP-labeled oligo and an average 80% efficiency was observed at a concentration of 100 nmol/L without obvious toxicity (data not shown). qRT-PCR was performed to examine the expression levels of miR-302b after transfection of the miR-302b expression construct, or its negative control (empty vector). As expected, a 30-fold increase in the expression of miR-302b was observed in the SMMC-7721 cells transfected with 100 nmol/L of miR-302b relative to the cells transfected with 100 nmol/L of miR-302b negative control and a 20-fold increase of the miR-302b expression was shown in Bel-7404 cells (Fig. 2A). To study the role of miR-302b in hepatocellular carcinoma cell proliferation, MTT assays were used. Results demonstrated that transient overexpression of miR-302b inhibited the proliferation of SMMC-7721/Bel-7404 at 48 and 72 hours after transfection (Fig. 2B). To further examine the inhibitory role of miR-302b in hepatocellular carcinoma cells, a colony formation assay was used after similar transient transfection. Notably, miR-302b–transfected cells displayed fewer and smaller colonies compared with control NC-transfectants (Fig. 2C). Meanwhile, we confirmed that miR-302b–mediated repression of AKT2 or ki-67 by immunofluorescence staining. The red signal of AKT2 or green signal ki-67 in the miR-302b–transfected SMMC-7721/Bel-7404 cells was visibly low compared with that of the cells infected with miR-ctrl (Fig. 2D), suggesting a growth-inhibitory role of miR-302b. Taken together, MTT, colony formation, and immunofluorescence staining assays demonstrated that miR-302b was able to inhibit the proliferation of hepatocellular carcinoma cells in vitro.

To further investigate the mechanisms by which miR-302b inhibits hepatocellular carcinoma cells proliferation, we adopted a serum starvation–stimulation strategy to clarify whether miR-302b–induced inhibition of cell proliferation resulted from a blocked cell-cycle checkpoint. SMMC-7721/Bel-7404 cells were transfected with a miR-302b expression construct or an empty vector for 24 hours, followed by synchronization with serum deprivation for 24 hours and then stimulation to enter S-phase by serum readdition. Overexpression of miR-302b resulted in a marked accumulation of the G1-population in the human hepatocellular carcinoma cell line SMMC-7721/Bel-7404 (Fig. 2E), suggesting that miR-302b blocked the G1–S transition.

To examine the antiproliferative role of miR-302b in human hepatoma cells, we eliminated endogenous miR-302b in SMMC-7721/Bel-7404 cells by using its inhibitor, ASO-miR-302b. Reduced endogenous expression of miR-302b in the SMMC-7721/Bel-7404 cells by ASO-miR-302b resulted in increased cell viability, colony formation potential, and expression of AKT2 and Ki-67 (Fig. 2F–I). Further investigation of ASO-miR-302b on cell-cycle progression of the SMMC-7721/Bel-7404 cells proved that ASO-transfected cells were more inclined to enter S-phase (Fig. 2J). These results suggested that the baseline expression of miR-302b would initiate its antiproliferation potential at G1–S phase in hepatoma cells, thus preventing further malignancy progression.

**miR-302b inhibits hepatocellular carcinoma cell proliferation via directly targeting AKT2**

To uncover the mechanisms by which miR-302b induced G1–S arrest, we searched for the target genes of miR-302b. Among the predicted target genes of miR-302b in the TargetScan (http://www.targetscan.org/), DIANA (http://diana.cslab.ece.ntua.gr/microT/), and PicTar (http://pictar.mdc-berlin.de/) databases, we found that AKT2, but not AKT1 or AKT3. AKT2 was one of the top candidates and of particular interest because of its essential role in different cancers (5). As shown in Fig. 3A, the region complementary to the miR-302b seed region was found in the 3’-UTR of human AKT2. To validate whether AKT2 was the direct target gene of miR-302b, a Dual-Luciferase Reporter System

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**Figure 1.** Dysregulated miR-302b in hepatocarcinoma tissues and cells. A, qRT-PCR was performed to examine miR-302b expression in 27 paired human hepatocellular carcinoma and adjacent nontumor tissues. B, qRT-PCR was used to analysis of miR-302b expression in normal hepatocytes and hepatocellular carcinoma cells (*, \( P < 0.01; \), \( P < 0.05; \) Student t test).
containing wild-type or mutant 3′-UTR of AKT2 was used. The luciferase assay showed that miR-302b significantly led to the suppression of luciferase activity, indicating that miR-302b directly bound to its predicted binding site on AKT2. In contrast, miR-302b had no effect on the luciferase activity of a mutant 3′-UTR of AKT2 construct (Fig. 3B), indicating that miR-302b specifically binds to the seed sequence at the 3′-UTR of AKT2. To test whether miR-302b expression affected endogenous AKT2 expression, we transfected the miR-302b expression construct, ASO-miR-302b, empty vector, or ASO-NC into SMMC-7721 cells. We observed a decrease of AKT2 at both of the mRNA and protein levels.
after transfection with miR-302b, whereas knockdown of miR-302b enhanced AKT2 protein expression (Fig. 3C). To further investigate the relationship between AKT2 and miR-302b, we also examined the expression of AKT2 and miR-302b in 27 paired samples of hepatocellular carcinoma and their adjacent normal tissues. We found that AKT2 expression was higher in hepatocellular carcinoma tissues compared with their normal counterparts (Fig. 3D). MiR-302b levels were inversely correlated with AKT2 expression (Fig. 3E). The expression of AKT2 was upregulated in 4 pairs of tissues and in hepatocellular carcinoma cell lines compared with their normal counterparts, separately (Fig. 3F). Taken together, the above data suggests that miR-302b is able to directly regulate AKT2 expression in hepatocellular carcinoma cells.

Knockdown of AKT2 produces similar effects to that of miR-302b overexpression in hepatocellular carcinoma cells

Next, we silenced AKT2 expression by RNAi method to test whether AKT2 is involved in the antitumor effects of miR-302b. From mRNA and protein expression levels, AKT2 can be specifically knocked-down by siRNA in SMMC-7721 and Bel-7404 cells at 35% and 42%, separately (Fig. 4A and G). As shown in Fig. 4B–E, silencing of AKT2 resulted in suppressed cell proliferation and induced G1–S arrest, which was in line with the effects of miR-302b overexpression in SMMC-7721 cells. Similar antiproliferation effects by AKT2 silencing was also evident in Bel-7404 cells, which was in line with that of the miR-302b overexpression (Fig. 4G–K). Next, we analyzed protein levels of AKT2...
related G1 regulators after overexpressing miR-302b or silencing AKT2 in SMMC-7721 and Bel-7404 cells by Western blot analysis. As shown in Fig. 4F and L, both miR-302b expression and AKT2 siRNA could reduce the expression of CYCLINA, CYCLIND, and CDK2, which are 3 essential regulators of the G1–S phase transition.
Notably, miR-302b expression and AKT2 knockdown clearly decreased CYCLIND1 phosphorylation at Thr286, which is regulated by the AKT2-glycogen synthase kinase-3β pathway (5). In addition, miR-302b expression and AKT2 knockdown increased the Ser10 phosphorylation of p27Kip1. Moreover, reducing the expression of miR-302b by ASO-miR-302b had adverse results. These results demonstrate that miR-302b and its target gene AKT2 share similar cellular and molecular effects in SMMC-7721 and Bel-7404 cells.

Overexpression of AKT2 eliminated the effects of miR-302b on hepatocellular carcinoma cells

To further demonstrate that the miR-302b exhibited tumor suppressor function through AKT2, we constructed AKT2 overexpression vector, which was cotransfected with miR-ctrl or miR-302b into SMMC-7721 or Bel-7404 cells. Overexpression of AKT2 in SMMC-7721 or Bel-7404 cells rescued AKT2 expression level reduced by miR-302b (Fig. 5A). Moreover, after cotransfected with the miR-302b and AKT2 vector, we found that the overexpression of AKT2 counterbalanced the tumor suppressor effect of miR-302b in hepatocellular carcinoma cells at cell proliferation (Fig. 5B). To investigate the effect of overexpression of AKT2 on cell-cycle progression of the SMMC-7721/Bel-7404 cells, cell-cycle assay was used. From the Fig. 5C, we found that overexpression of AKT2 was in cline to re-enter S-phase in both SMMC-7721 and Bel-7404 cells. Meanwhile, compared with cotransfected with miR-302b and AKT2-ctrl, cotransfected with miR-302b and AKT2 could be able to re-enter S-phase in hepatocellular carcinoma cells also. Furthermore, cell-cycles regulators expression levels were tested by Western blot analysis after cotransfected with miR-ctrl and AKT2 or cotransfected with miR-302b and AKT2 in hepatocellular carcinoma cells. The expression of CYCLINA, CYCLIND1, and CDK2 were upregulated after transfection of AKT2. Meanwhile, compared with cotransfected with miR-302b and AKT2-ctrl, the expression of regulators was unregulated after cotransfected with miR-302b and AKT2 (Fig. 5D). These results further suggest that
miR-302b exhibit tumor suppressor role by direct targeting at AKT2.

**miR-302b inhibits hepatocellular carcinoma tumor growth in vivo**

To further confirm the growth inhibitory function of miR-302b in hepatocellular carcinoma, we tested the effects of miR-302b on tumor growth in an in vivo xenograft model. miR-302b and miR-control–transfected SMMC-7721 cells were injected subcutaneously into either posterior flank of the same nude mice. The mice were followed for observation of xenograft growth for 4 weeks. As shown in Fig. 5A, the tumors from mice that were intratumorally injected with synthetic miR-302b were significantly smaller than those in control mice on day 15 after the first injection. On day 30, the average volume of miR-302b–treated tumors was much smaller than that in control tumors (Fig. 6A and B). The average tumor weights for the control- and the miR-302b groups on day 30 were 0.25 and 0.04 g, respectively (Fig. 6C). Furthermore, the expression levels of miR-302b and AKT2 in the tumor tissues were examined by qRT-PCR and Western blot analyses. Consistent with the in vitro data, the in vivo data showed that the expression of miR-302b was increased and the expression of the AKT2 protein was reduced in miR-302–treated tumors (Fig. 6D). Immunohistochemical analysis also demonstrated decreased proliferation potential and AKT2 levels in the tumor tissue treated with miR-302b (Fig. 6E and F).

These data indicated that miR-302b expression is capable of inhibiting tumor growth and AKT2 expression levels in vivo.

**Discussion**

In the past 10 years, dysregulation of miRNAs has been shown to be a common event that can control cell proliferation in hepatocellular carcinoma development and progression (15, 16, 29). In this study, we have shown that miR-302b was frequently downregulated in both hepatocellular carcinoma tissues and cell lines. Enforced expression of miR-302b inhibited hepatocellular carcinoma cells proliferation and cell viability by blocking the G1–S transition in vitro. Moreover, gain- and loss-of-function of miR-302b and cotransfected with miR-302b and AKT2 studies revealed that miR-302b suppressed cell proliferation by directly targeting AKT2-related cell-cycle signaling. Furthermore, tumor formation assays demonstrated that miR-302b–targeting AKT2 was responsible for suppression of tumor growth in vivo. Therefore, our data provided a more comprehensive understanding of the tumor suppressor role of miR-302b during hepatocellular carcinoma development.

In a previous miRNA microarray study, we found that miR-302b was downregulated in clinical hepatocellular carcinoma samples. After we examined the expression of miR-302b in hepatocellular carcinoma cell lines and tissues in this research, we further confirmed the previous result that miR-302b was frequently suppressed in hepatocellular carcinoma, suggesting that miR-302b might be a novel tumor suppressor.
supressor. Notably, miR-302b is located at chromosome 4q25, a region that is controlled by altered levels of transcriptional factors such as NANOG and OCT3/4, but not DNA copy number alteration. Of particular interest, over-expression of OCT4, a master regulator of stemness and self-renewal in embryonic stem cells, has been reported to be evidence of a higher grade of malignant cancers, including breast cancer, bladder cancer, and hepatocellular carcinoma cancer cells (35–37). Although the role of stemness and reprogramming potential of miR-302b in ES cells has been identified, the underlying mechanism responsible for decreased expression of miR-302b in hepatocellular carcinoma is still unknown. Thus, in this study we have evaluated a potential alteration in the expression of miR-302b, the main regulatory miRNA in hepatocellular carcinoma tumor samples. We found that miR-302b was significantly downregulated in liver cancer cell lines and hepatocellular carcinoma tissues. From gain- and loss-of-function studies, miR-302b in liver cancer cells has been proven to decrease cell proliferation, clonogenicity, and also induced G1–S arrest in vitro. Also, ectopic expression of miR-302b in SMCC-7721 and Bel-7404 cells inhibited proliferation of liver tumors in vivo. Therefore, our data provided a more comprehensive understanding of the tumor suppressor role of miR-302b during hepatocellular carcinoma development.

AKT2 is a homolog of the v-akt oncogene, a protein serine/threonine kinase prosurvival protein, which is member of the AKT family of proteins (AKT1, 2, and 3) that are activated by the PI3K pathway (11). The PI3K pathway is one of the most potent prosurvival pathways in cancer (38). The deregulation of the AKT signaling pathway has been associated with numerous other cancers, including glioblastoma, breast, prostate, lung, and liver cancers (3, 5). Numerous investigators have reported correlations between tumor AKT activity and various clinicopathologic parameters (39). In particular, AKT activation has been shown to correlate with advanced disease and/or poor prognosis in some tumor types (40). In light of its importance in the regulation of cell proliferation, AKT2 is becoming increasingly recognized as an essential target for potential anticancer inhibitors to promote progression through normal, unperturbed cell cycles. This is achieved by acting on diverse downstream factors involved in controlling the G1–S and G2–M transitions (5). Here, we demonstrated that miR-302b suppressed AKT2 expression by binding directly to the 3′-UTR of AKT2, and an inverse correlation was observed between miR-302b and AKT2 expression in hepatocellular carcinoma tissues. We showed that AKT was upregulated in hepatocellular carcinoma tissues and that silencing AKT by RNAi inhibited tumor properties of liver cancer cells, similar to that of miR-302b overexpression (Figs. 1 and 4). Meanwhile, overexpression of AKT2 could eliminate the effect of miR-302b on hepatocellular carcinoma cells (Fig. 5). Therefore, upregulation of AKT2 in hepatocellular carcinoma because of miR-302b deprivation may, at least partially, explain the antitumor effects of miR-302b involved in hepatocellular carcinoma. In a word, the reported targets of miR-302b and our observations indicated that miR-302b might regulate AKT2-related signaling pathways, and loss of miR-302b would lead to the tumor progression in hepatocellular carcinoma. There is an interesting thing, from the results of Figs. 2, 4, and 5, we found that AKT2 might not be the only relevant miR-302b target for the observed phenotype. Subramanyam and colleagues demonstrated that embryonic stem cell-specific cell-cycle-regulating (ESCC) miRNAs could have hundreds of target gene and play the roles from many different functional modules, for instance, cell-cycle regulation, vesicular transition, epigenetic regulation, cell signaling, and epithelial–mesenchymal transition. miR-302b as a number of ESCC miRNAs, it might target and regulate other cell cycles regulators, such as CDKN1A, RBL2, and CDC2L6 in the process of cell proliferation (41).

AKT2 is essential for progression from G0–G1 to S-phase by activating the positive regulator of G1–S transition, including cyclin D1, cyclin D2, cyclin E1, during cell-cycle progression (31). Here, we showed that activated AKT2 phosphorylates various substrates at serine/threonine residues directly or indirectly by regulating the expression of various substrates, including P27kip1, CDK2, CYCLIND1, and CYCLINA. In this research, miR-302b and siRNA targeted at AKT2 could reduce both the expression levels and phosphorylation of CYCLIND1 at threonine 286, suggesting a potent cell-cycle progression role of miR-302b. Moreover, miR-302b and si-AKT2 could not abolish the expression levels of P27, whereas they could increase the phosphorylation of P27 at Ser10 (Fig. 4F and L). CDK2, an S-phase cyclin-dependent kinase, is the target for AKT phosphorylation of P27 at Ser10 (Fig. 4F and L). CDK2, an S-phase cyclin-dependent kinase, is the target for AKT2 during the cell-cycle progression. In previous research, CDK2 was reported to be phosphorylated directly by AKT2 at threonine 39 residues both in vitro and in vivo (11). Here, reduced expression levels of CDK2 were detected after miR-302b and si-AKT2, suggesting an indirect effect induced by the elimination of AKT2. Our findings suggest that miR-302 may function as a cell-cycle suppressor by targeting AKT2 in hepatocellular carcinoma.

The above results suggested that miR-302b could act as a potent therapeutic intervention of hepatocellular carcinoma. Further animal studies indicated that miR-302b could suppress the growth of hepatocellular carcinoma xenografts in nude mice and decrease the expression of AKT2 in treated tumors (Fig. 6). The in vivo studies support our observations that miR-302b targets AKT2 and suppresses liver cancer cell growth in vitro. Further systemic administration of miR-302b delivered by cholesterol conjugated 2′-O-methyl-modified oligonucleotides, lentivirus vector (42), AAV vectors (43), or locked nucleic acid-modified oligonucleotides (44) could be performed for further determination of the anti-cancer potential of hepatocellular carcinoma.

In summary, we investigated the roles of miR-302b and its targeted gene, AKT2, in their control of the cell cycle and their potential implication in pathologic processes. Our findings suggest that miR-302b may be a novel tumor suppressor that blocks the growth of hepatocellular carcinoma cells through targeting AKT2-related cell-cycle progression signaling pathways. Our findings highlight the functional association of miR-302b and their host genes, provide
new insight into the regulatory network of the cell cycle, and open the possibility for future therapeutic interventions.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: L. Wang, J. Yao, X. Le, K. Nan, T. Song, C. Huang
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