MicroRNA-92a contributes to tumor growth of human hepatocellular carcinoma by targeting FBXW7

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Abstract. Deregulation of microRNA-92a (miR-92a) has been reported in several human cancers and is associated with prognosis of patients. However, the clinical significance of miR-92a and the underlying mechanisms involved in hepatocarcinogenesis remain to be determined. The aim of the present study was to determine the role of miR-92a in hepatocellular carcinoma (HCC). The results showed that the expression of miR-92a was upregulated in HCC tissues as compared with matched tumor-adjacent tissues. A high expression of miR-92a was observed in HCC cell lines as compared with a non-transformed hepatic cell line. The gain- and loss-of-function studies revealed that miR-92a significantly promoted proliferation and cell cycle transition from G1 to S phase, and inhibited apoptosis of HCC cell *in vitro*. In tumor-bearing nude mice, the down-regulation of miR-92a suppressed tumor growth of HCC *in vivo*. miR-92a was inversely correlated with F-box and WD repeat domain-containing 7 (FBXW7) expression in HCC tissues. Furthermore, miR-92a negatively regulated FBXW7 abundance in HCC cells. In the present study, FBXW7 was identified as a direct target of miR-92a. Notably, alterations of FBXW7 expression abrogated the effects of miR-92a on HCC cell proliferation, cell cycle and apoptosis. Clinical association analysis revealed that a high expression of miR-92a was correlated with poor prognostic characteristics of HCC. Notably, the high expression of miR-92a conferred a reduced 5-year overall survival (OS) and recurrence-free survival (RFS) of HCC patients. The multivariate Cox regression analysis demonstrated that miR-92a expression was an independent prognostic marker for predicting survival of HCC patients. In conclusion, the results of the present study suggested that miR-92a promotes the tumor growth of HCC by targeting FBXW7 and may serve as a novel prognostic biomarker and therapeutic target for HCC.

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Introduction

MicroRNAs (miRNAs) are a group of endogenous small non-coding RNA molecules, which regulate protein-coding gene expression by interacting with complementary sites within the 3'-untranslated region (UTR) of target mRNAs and targeting mRNAs for cleavage or translational repression (1). Mounting evidence has indicated that miRNAs are involved in tissue morphogenesis, cell processes such as proliferation, cell cycle and apoptosis and major signaling pathways (2-4). Previous studies have identified the critical role of miRNAs in human cancers and suggest that deregulation of miRNAs is involved in tumor development and progression by modulating the expression of oncogenes or tumor suppressors (5).

miR-92a, which belongs to the miR-17-92 cluster, plays a critical role in the progression of lung cancer (6), esophageal squamous cell carcinoma (7), colorectal (8), breast (9), ovarian (10) and cervical cancer (11). Previous findings show that miR-92a functions as a regulator of cell proliferation, apoptosis and invasion in human cancers (11-13). miR-92a promotes lung cancer cell invasion by targeting reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) and a high expression of miR-92a is associated with the poor survival rate of lung cancer patients (6,13). Plasma miR-92a is a promising novel biomarker for the early detection of colorectal cancer and its overexpression is also a prognostic marker for predicting the poor overall and disease-free survival rate of patients (8,14). However, the expression of miR-92a is downregulated in ovarian and breast cancer (9,10). Upregulation of miR-92a inhibits ovarian cancer cell adhesion, invasion and proliferation by suppressing integrin α5 expression (10). Downregulation of miR-92a is associated with aggressive breast cancer features and increased tumor macrophage infiltration (9). Thus, the functional significance of miR-92a in cancer initiation and development seems to be cancer-type specific. Previously, miR-92a was found to be significantly upregulated in hepatocellular carcinoma (HCC) samples and was also identified as hepatitis B virus (HBV)-specific (15,16). The proliferation of HCC-derived cell lines was enhanced by the upregulation of miR-92a and inhibited by the downregulation of miR-92a (16). However,

the clinical significance of miR-92a and the underlying mechanisms involved in the development of HCC remain to be investigated.

The present study aim was to determine the role of miR-92a in HCC. The results showed that the expression of miR-92a was upregulated in HCC tissues. miR-92a promoted proliferation, cell cycle and apoptosis resistance *in vitro*. Moreover, the downregulation of miR-92a inhibited the tumor growth of HCC *in vivo*. Notably, F-box and WD repeat domain-containing 7 (FBXW7) was identified as a direct target of miR-92a. A high expression of miR-92a was associated with poor clinicopathological characteristics and the reduced survival of HCC patients. The results showed a new role for miR-92a in prediction of prognosis and promoting tumor growth of human HCC.

Materials and methods

Clinical samples. HCC and matched normal tumor-adjacent tissues were obtained from 106 patients including 94 males and 12 females, who underwent resection of their primary HCC in the Department of Hepatobiliary Surgery at the First Affiliated Hospital of Xi'an Jiaotong University (Shaanxi, China) from January, 2006 to December, 2009, with a median follow-up period of 38,7 months. None of the patients received preoperative chemo- or radiotherapy. The stage of cancer was determined according to the cancer staging system published in 2010 by the Union for International Cancer Control (UICC). The demographic features and clinicopathological data are shown in Table I. Samples were used after informed consent was obtained. The Xi'an Jiaotong University Ethics Committee approved all the protocols according to the Declaration of Helsinki (as revised in Tokyo 2004).

Reverse transcription-quantitative PCR (RT-qPCR). qPCR primer against mature miRNA hsa-miR-92a-3p (HmiRQP0832) and Homo sapiens snRNA U6 qPCR Primer (HmiRQP9001) were purchased from Genecopoeia (Guangzhou, China). The PCR amplification for the quantification of the miR-92a and U6 was performed using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and TaqMan Human miRNA Assay kit (Applied Biosystems). The relative expression of miR-92a was shown as the fold difference relative to U6.

The PCR amplification for the quantification of the FBXW7 and GAPDH mRNAs was performed using an ABI PRISM 7300 Sequence Detection system (Applied Biosystems) and a SYBR® Premix Ex Taq™ II (Perfect Real Time) kit (Takara Bio, Shiga, Japan), as previously reported (17).

Cell lines and transfection. The human immortalized normal hepatic cell line LO2 and five HCC cell lines (HepG2, Hep3B, Huh7, SMMC-7721 and Bel-7402) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) at 37˚C with 5% CO_2.

miRNA vectors, including miR-92a expression vector (HmiR0204-MR04), the control vector for miR-92a (CmiR0001-MR04 and miR-control), miR-92a inhibitor (HmiR-AN0832-AM04 and anti-miR-92a) and the negative control for the miR-92a inhibitor (CmiR-AN0001-AM04 and anti-miR-NC), were purchased from Genecopoeia. Retroviral vectors pMMP-FBXW7 were generated by inserting the FBXW7 cDNA into pMMP. Retrovirus packaging and transduction were performed as previously described (18). The targeted sequences for FBXW7 siRNA sense, 5'-GGA GUA UGG UCA UCA CAA Att-3' and antisense, 5'-UUU

Table I. Clinicopathological correlation of miR-92a expression in HCC.

Clinicopathological characteristics	miR-92a High expression (n=53)	miR-92a Low expression (n=53)	P-value
Age (years)			
≤50	20	22	0.691
>50	33	31	
Gender			
Male	46	48	0.540
Female	7	5	
HBsAg			
No	3	11	0.022[a]
Yes	50	42	
Serum AFP level (ng/ml)			
≤20	14	18	0.397
>20	39	35	
Tumor size (cm)			
≤5	18	30	0.019[a]
>5	35	23	
No. of tumor nodules			
1	43	45	0.605
≥2	10	8	
Cirrhosis			
Absent	7	17	0.020[a]
Present	46	36	
Venous infiltration			
Absent	24	27	0.560
Present	29	26	
Edmondson-Steiner grading			
I+II	33	47	0.002[a]
III+IV	20	6	
TNM tumor stage			
I+II	34	45	0.014[a]
III+IV	19	8	

[a]Statistically significant. HCC, hepatocellular carcinoma; HBV, hepatitis B virus; AFP, α-fetoprotein; TNM, tumor-node-metastasis.

GUG AUG ACC AUA CUC Cac-3' or a non-specific duplex oligonucleotide as a negative control were produced by Sangon Biotech Co., Ltd. (Shanghai, China). The cells were transfected with the vectors mentioned above using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Cell cycle, proliferation and detection of apoptosis. Flow cytometry was performed using the fluorescence-activated cell sorting (FACS) Calibur and Cell Quest software (both from Becton-Dickinson, San Jose, CA, USA). For the proliferation assay, HCC cells transfected with different vectors were seeded in 96-well plates at a density of $5x10^3$ cells/well for 24 h and assessed using Cell Proliferation ELISA, BrdU kit (5-bromodeoxyuridine) (chemiluminescent) (Roche, Indianapolis, IN, USA). For cell cycle analysis, the cells were seeded in 6-well plates at $2x10^5$/well. Forty-eight hours after transfection, the cells were fixed in 70% ethanol at 4˚C for 24 h and stained with 50 μg/ml propidium iodide (Keygen, Nanjing, China). An Annexin-V-Fluos Staining kit (Roche) was used to analyze apoptosis levels, as previously described (17).

Western blot analysis. The primary antibodies used in the immunoblotting assays were: FBXW7 (WH0055294M2; Sigma) and GAPDH (G8140; US Biological, Swampscott, MA, USA). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibodies (Bio-Rad, Hercules, CA, USA) were used at a 1:1,000-1:5,000 dilution and detected using a Western Blotting Luminol Reagent (sc-2048; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), as described in a previous study (18).

Immunohistochemical staining. Immunohistochemistry was performed on paraformaldehyde-fixed paraffin sections. FBXW7 (WH0055294M2; Sigma) antibody was used in immunohistochemistry using a streptavidin peroxidase-conjugated (SP-IHC) method. Immunohistochemistry was performed as previously reported (19). The percentage of positive tumor cells was graded as: 0, <10%; 1, 10-30%; 2, 31-50%; 3, >50%.

Luciferase reporter assay. The predicted 3'-UTR sequence of FBXW7 that interacted with miR-92a, together with a corresponding mutated sequence within the predicted target sites, were created and inserted into the pRL-TK control vector (Promega, Madison, WI, USA). SMMC-7721 cells that were seeded in a 96-well plate were transfected with 120 ng miR-92a expression vector, miR-92a inhibitor, control vector or negative control. Cells were co-transfected with 30 ng of the wild-type or mutant 3'-UTR of FBXW7 mRNA. Transfections were performed using 0.45 μl of Fugene (Promega). Fourty eight hours after transfection, the cells were collected and measured according to the manufacturer's instructions (Dual-Luciferase Assay System; Promega). The pRL-TK expressing *Renilla* luciferase was cotransfected as an internal control to correct the differences in transfection and harvest efficiencies (20).

In vivo experiments. Four-to-six week-old female BALB/c nude mice (Centre of Laboratory Animals, The Medical College of Xi'an Jiaotong University, Xi'an, China) were used to establish the nude mouse xenograft model. SMMC-7721 ($5x10^6$) cells that were transfected with anti-miR-92a or -miR-NC vectors were mixed in 150 μl of Matrigel and were inoculated subcutaneously into the flank of nude mice. Tumor growth curves were generated as previously described (18). Animal protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

Statistical analysis. Data are presented as the mean ± SEM from at least three independent replicates. Where appropriate, a Pearson's Chi-squared test, a Kaplan-Meier plot, a log-rank test, the multi-variant Cox regression analysis, a Spearman's rank correlation coefficient and a two-tailed Student's t-test were used with the SPSS statistical package for Windows Version 13 (SPSS, Chicago, IL, USA) or GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, CA, USA). $P \leq 0.05$ was considered significant.

Results

Elevated expression of miR-92a is observed in HCC tissues and cells. One hundred and six pairs of HCC tissues and matched tumor-adjacent tissues were tested for miR-92a using RT-qPCR. The results showed that the mean level of miR-92a expression in HCC tissues was significantly higher than that in the non-tumor tissues ($P<0.001$, Fig. 1A). We then analyzed miR-92a expression in a non-transformed hepatic cell line (LO2) and a panel of HCC cell lines (HepG2, Huh7, Hep3B, SMCC-7721 and Bel-7402). As expected, the relative expression of miR-92a was obviously upregulated in the HCC cell lines as compared with that in the non-transformed LO2 hepatic cell line ($P<0.05$, Fig. 1B). The results suggested that an elevated expression of miR-92a may facilitate hepatocarcinogenesis.

Promotive effects of miR-92a on proliferation, cell cycle and apoptosis of HCC cells. To identify the biological function of miR-92a in HCC, we transduced an miR-92a expression plasmid or anti-miR-92a vector into human HCC cell lines with different endogenous expression levels of miR-92a. As measured by RT-qPCR, the expression of miR-92a was significantly altered by the corresponding vector in HCC cells ($P<0.05$, respectively, Fig. 2A). BrdU incorporation assays were performed to determine the effect of altering miR-92a levels on HCC cell proliferation. We found that the downregulation of miR-92a led to a significant reduction of cell proliferation in SMMC-7721 cells ($P<0.05$, Fig. 2B). Furthermore, as determined by flow cytometric analysis, the downregulation of miR-92a resulted in G1 phase arrest in SMMC-7721 cells ($P<0.05$, Fig. 2C). Otherwise, the percentage of apoptotic SMMC-7721 cells was significantly increased following the reduction of miR-92a ($P<0.001$, Fig. 2D). By contrast, the upregulation of miR-92a promoted the proliferation, cell cycle transition from G1 to S phase and apoptosis resistance in Hep3B cells ($P<0.05$, respectively, Fig. 2B-D). Notably, the tumor growth curve revealed that knockdown of miR-92a significantly retarded the tumor growth of human HCC in subcutaneous nude mouse models ($P<0.001$, Fig. 3). These results demonstrated that miR-92a regulates the proliferation, cell cycle and apoptosis of HCC cells.

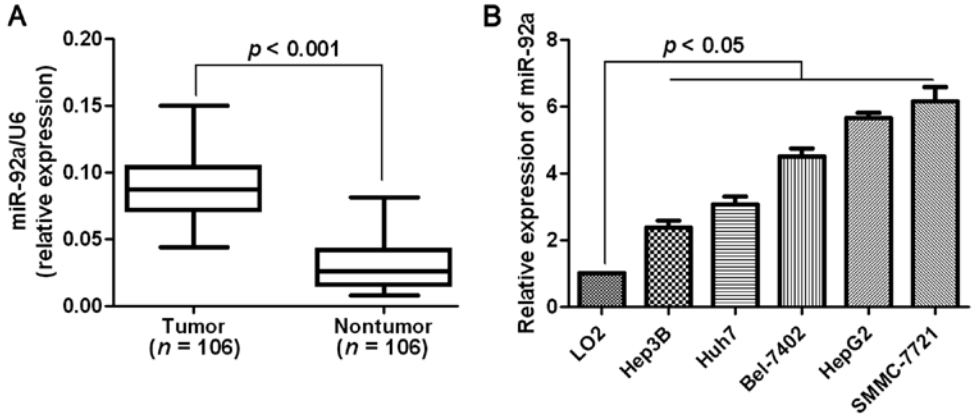

Figure 1. Expression of miR-92a in HCC specimens and cell lines. (A) Quantification of the data revealed that the mean level of miR-92a expression in HCC tissues was significantly higher than that in matched adjacent nontumor tissues. (B) Comparing differences in the expression levels of miR-92a between HCC cell lines with different proliferative potentials and the immortalized hepatic cell line LO2. n = three repeats with similar results.

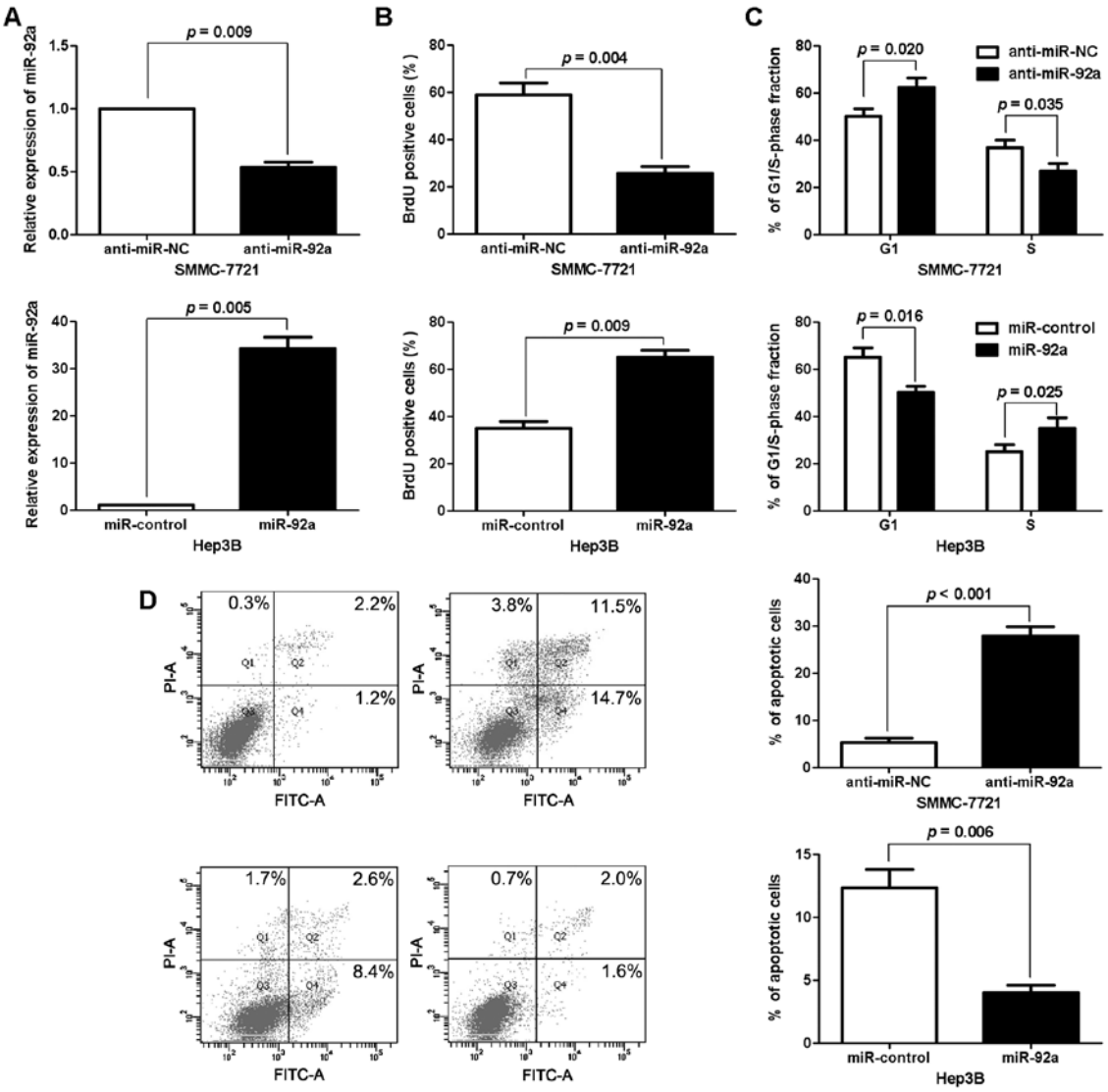

Figure 2. miR-92a promotes HCC cell proliferation, cell cycle transition and apoptosis resistance. (A) SMMC-7721 and Hep3B cells that were transfected with corresponding miRNA vectors were subjected to RT-qPCR for miR-92a. n = three independent experiments. (B) Cell proliferation as measured by BrdU incorporation assays was inhibited by knockdown of miR-92a in SMMC-7721 cells and increased by overexpression of miR-92a in Hep3B cells. n = three independent experiments. (C) As assessed by flow cytometry, knockdown of miR-92a induced G1 phase arrest in SMMC-7721 cells and the overexpression of miR-92a promoted cell cycle transition from G1 to S-phase in Hep3B cells. n = three independent experiments. (D) Quantification of the apoptotic cell population by flow cytometry. miR-92a knockdown increased the percentage of apoptotic SMMC-7721 cells and miR-92a-overexpressing Hep3B cells were composed of a smaller subset of apoptotic cells compared with the control cells. n = three independent experiments.

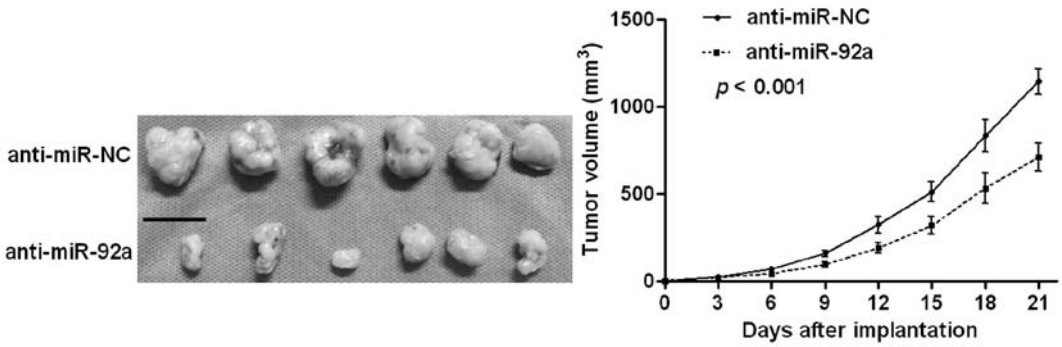

Figure 3. miR-92a suppresses tumor growth *in vivo*. SMMC-7721 cells that were transfected with miR-92a inhibitor (anti-miR-92a, n=six) and negative control (anti-miR-NC, n=six) were implanted into nude mice via subcutaneous injection. Tumor growth curve indicated that the anti-miR-92a-treated group showed a significantly higher tumor-suppressive effect as compared with the control group.

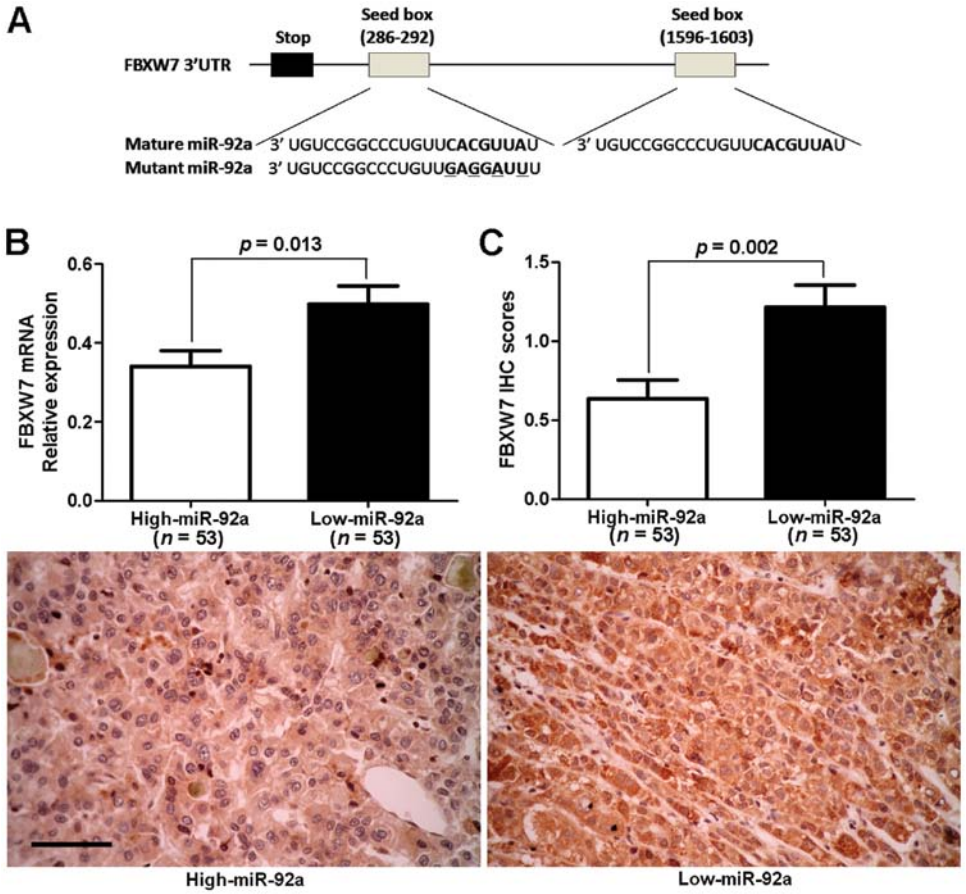

Figure 4. An inverse correlation between miR-92a and FBXW7 expression is observed in HCC. (A) miR-92a and its putative binding sequence in the 3'-UTR of FBXW7. The mutant miR-92a binding site was generated in the complementary site for the seed region of miR-92a. (B) The expression of FBXW7 mRNA in miR-92a high-expressing tumors was significantly lower than that in miR-92a low-expressing tumors. (C) A significant inverse correlation between miR-92a and FBXW7 protein expression was observed in HCC tissues. Representative immunohistochemical staining showed a weak staining of FBXW7 in miR-92a high-expressing HCC tissue and strong staining of FBXW7 in the miR-92a low-expressing tumor. Scale bar: 100 μm.

FBXW7 is a direct downstream target of miR-92a. A search for the candidate target genes of miR-92a was conducted using publically available databases TargetScan 6.2 (http://www.targetscan.org/) and miRanda (microrna.org and miRbase). Of note, the complementary sequence of miR-92a was identified in the 3'-UTR of FBXW7 mRNA, which was selected for subsequent studies (Fig. 4A). One hundred and six samples of HCC tissues were subjected to RT-qPCR and immunostaining for FBXW7 expression. The expression levels of FBXW7 mRNA and protein in the miR-92a high-expressing tumors were significantly lower than those in the miR-92a low-expressing tumors (P<0.05, respectively, Fig. 4B and C). Notably, an obvious inverse correlation between mRNA levels of miR-92a and FBXW7 (r= 0.745, P<0.001) and between miR-92a mRNA and FBXW7 protein (r=-0.632, P<0.001) was revealed by Spearman's correlation analysis in HCC tissues. Furthermore, the expression levels of FBXW7 mRNA and protein were significantly increased by the

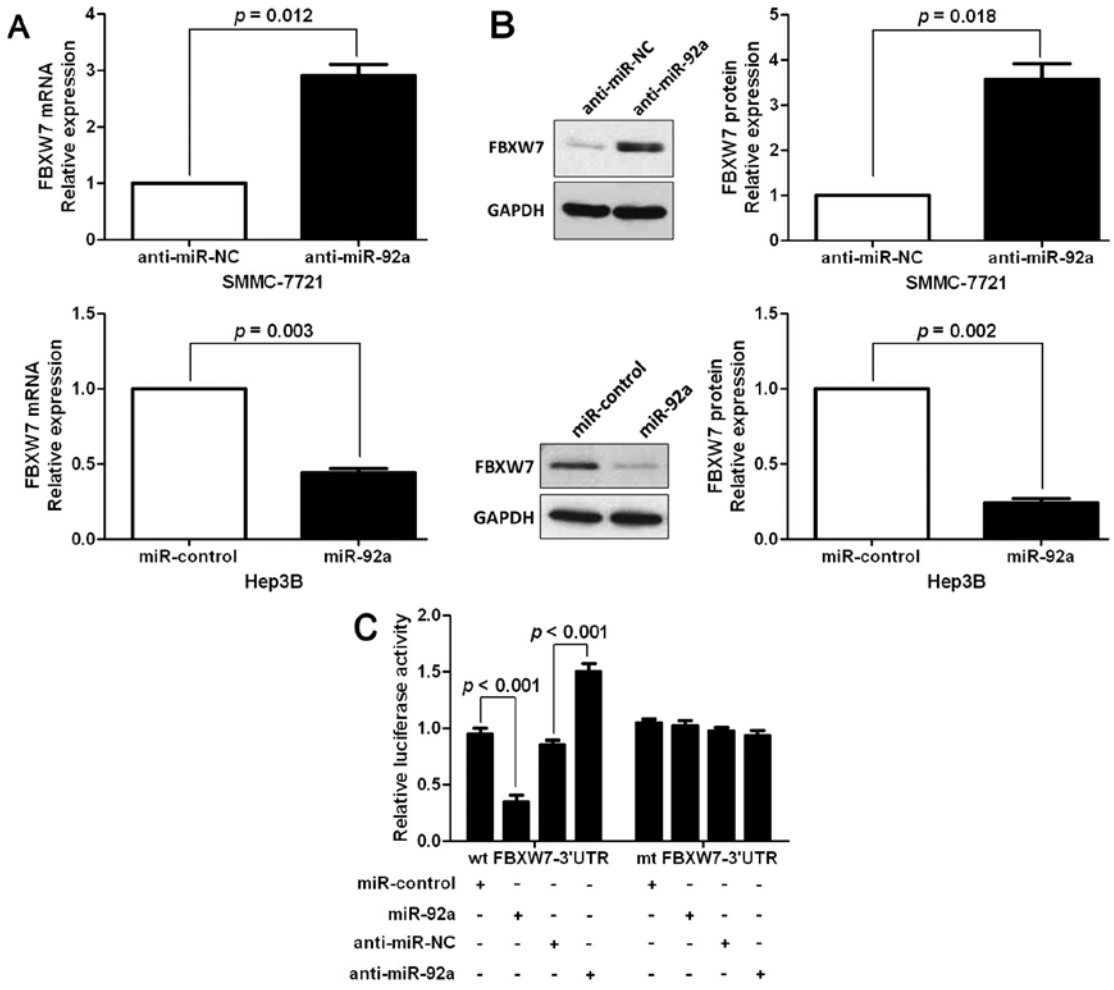

Figure 5. FBXW7 is identified as a direct target of miR-92a in HCC. (A) RT-qPCR analysis of FBXW7 mRNA expression in SMMC-7721 cells with anti-miR-92a or anti-miR-NC vector transfection and Hep3B cells with miR-92a or miR-control vector transfection. n = three repeats with similar results. (B) Knockdown of miR-92a increases the level of FBXW7 protein in SMMC-7721 cells and overexpression of miR-92a reduced the expression of FBXW7 protein in Hep3B cells. n = three repeats with similar results. (C) miR-92a significantly suppresses the luciferase activity that carried wild-type (wt) but not mutant (mt) 3'-UTR of FBXW7. Anti-miR-92a led to a notable increase in the luciferase activity of wt 3'-UTR of FBXW7. n = three repeats with similar results.

downregulation of miR-92a in SMMC-7721 cells (P<0.05, respectively, Fig. 5A and B). By contrast, the overexpression of miR-92a markedly reduced the mRNA and protein levels of FBXW7 in Hep3B cells (P<0.05, respectively, Fig. 5A and B). In addition, the upregulation of miR-92a prominently inhibited the luciferase activity of FBXW7 containing a wild-type (wt) 3'-UTR but did not suppress the activity of FBXW7 with a mutant (mt) 3'-UTR (P<0.001, Fig. 5C). Suppression of miR-92a by anti-miR-92a increased the luciferase activity of wt FBXW7 3'-UTR (P<0.001, Fig. 5C). However, with the mt FBXW7 3'-UTR constructs, there was no relative increase in activity. Collectively, these results strongly suggested that FBXW7 is a downstream target of miR-92a in HCC.

Alterations of FBXW7 levels influence the effects of miR-92a on HCC cells. To confirm that FBXW7 is a functional target of miR-92a, FBXW7 was knocked down by a specific siRNA in miR-92a-suppressive SMMC-7721 cells (P<0.05, Fig. 6A). We found that cell proliferation was significantly increased by FBXW7 knockdown (P<0.05, Fig. 6B). Furthermore, FBXW7 knockdown markedly rescued the downregulation of miR-92a-induced cell cycle arrest and apoptosis (P<0.05, respectively, Fig. 6C and D). Similarly, FBXW7 overexpression inhibited cell proliferation and promoted G1 phase arrest and apoptosis in miR-92a-overexpressing Hep3B cells (P<0.05, respectively, Fig. 6A-D). The results showed that FBXW7 is a downstream mediator of miR-92a in HCC.

Clinical significance of miR-92a expression in HCC cases. The expression of miR-92a was considered as low (n=53) or high (n=53) in accordance with the cut-off value, which was defined as the median of the cohort. The association between clinicopathological characteristics and miR-92a expression was subsequently analyzed. As shown in Table I, a high expression of miR-92a was evidently associated with HBV infection (P=0.022), large tumor size (P=0.019), cirrhosis (P=0.020), high Edmondson-Steiner grading (P=0.002) and advanced tumor-node-metastasis (TNM) tumor stage (P=0.014). Furthermore, the prognostic value of miR-92a was analyzed by Kaplan-Meier estimation. A high expression of miR-92a conferred a poor overall survival (OS) and recurrence-free survival (RFS) of HCC patients (P=0.001, respectively, Fig. 7). The multi-variant Cox regression analysis revealed that miR-92a expression was an independent

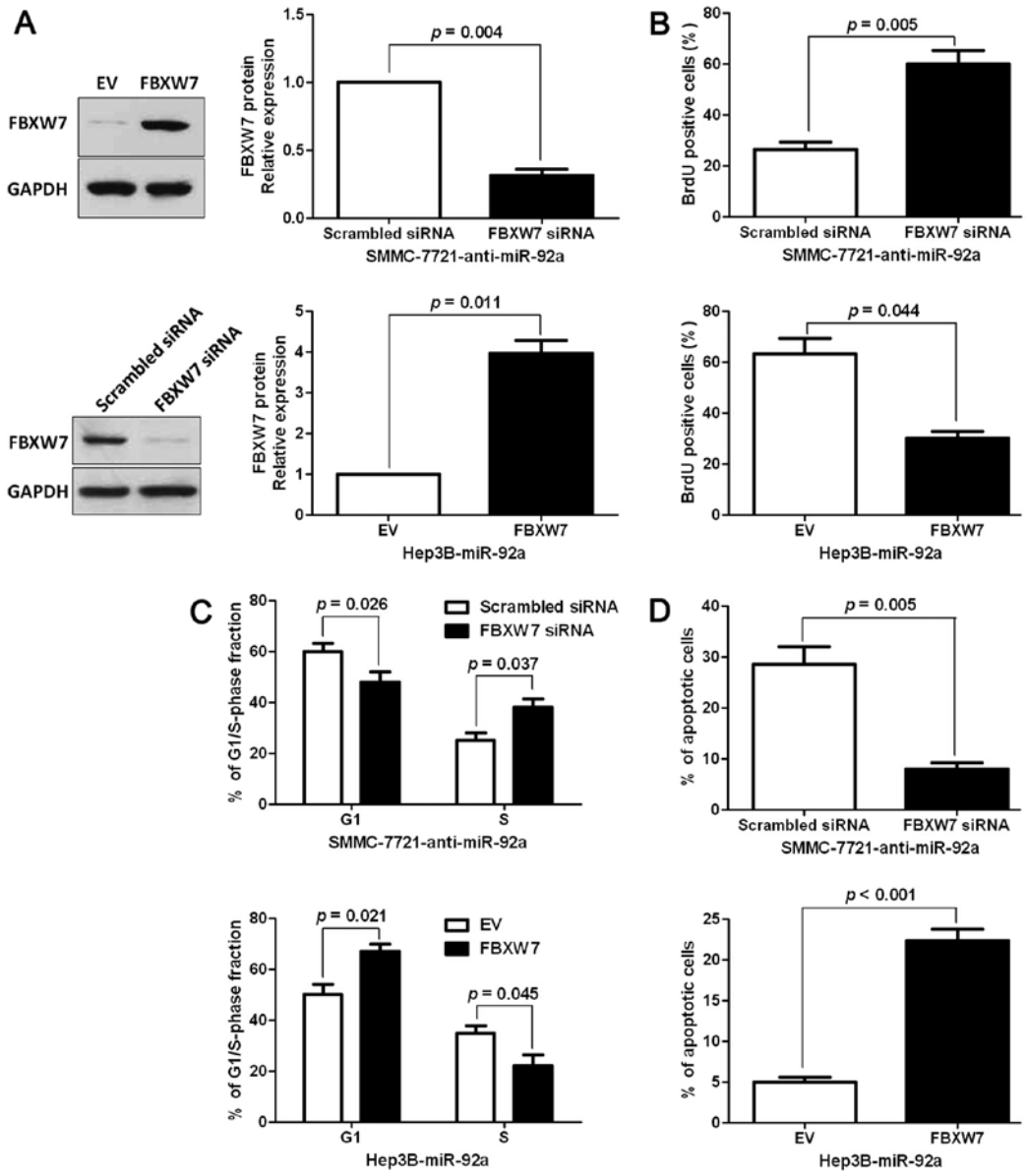

Figure 6. Alterations of FBXW7 partially abolish miR-92a-mediated HCC cell proliferation, cell cycle and apoptosis. (A) miR-92a-suppressive SMMC-7721 cells that were transfected with EV or FBXW7 expression plasmid and miR-92a-overexpressing Hep3B that were transfected with scrambled siRNA or FBXW7 siRNA were subjected to western blot analysis for FBXW7. n = three repeats with similar results. (B) Cell proliferation of the miR-92a-suppressive SMMC-7721 cells was increased by FBXW7 knockdown. Proliferation was significantly decreased after FBXW7 overexpression in miR-92a-overexpressing Hep3B cells. n = three independent experiments. The correlations between miR-92a effects and FBXW7 knockdown or overexpression are shown in the (C) cell cycle distribution and (D) apoptosis. FBXW7 knockdown abrogated the effects of miR-92a knockdown on HCC cells. FBXW7 overexpression induced effects that were opposite to those stimulated by miR-92a. n = three independent experiments. EV, empty vector.

prognostic marker for predicting 5-year OS and RFS in HCC patients (P=0.026 and P=0.042, respectively, Table II). These results showed that miR-92a is a potent biomarker for predicting prognosis of HCC patients.

Discussion

In previous studies, the deregulation of miR-92a has been studied in various human types of cancer and it is a prognostic marker for predicting survival of patients (6,7,9,11,14,16). miR-92a has been found to regulate HCC cell proliferation (16), facilitate G1/S transition (11,21), mediate apoptosis (21) and promote *in vivo* tumor growth (22). The clinical significance of miR-92a and the molecular mechanisms by which miR-92a promotes HCC development remain unclear. In the present study, we identified that the mean level of miR-92a expression was obviously increased in HCC tissues vs. matched tumor-adjacent tissues. Elevated expression of miR-92a was observed in the HCC cell lines as compared with the normal hepatic cell line. The role of miR-92a deregulation in tumor growth was examined via *in vitro* and *in vivo* experiments. We found that the downregulation of miR-92a inhibited HCC cell proliferation and induced G1 phase arrest and apoptosis *in vitro* and suppressed *in vivo* tumor growth. By contrast, the overexpression of miR-92a promoted proliferation and cell cycle transition to S phase and inhibited apoptosis *in vitro*. The

Table II. Multivariate Cox regression analysis of 5-year OS and RFS of 106 HCC patients.

	OS			RFS		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Age	0.853	0.512-1.44	0.523	0.869	0.558-1.362	0.552
Gender	1.373	0.587-3.192	0.454	0.781	0.424-1.479	0.455
HBV	0.934	0.396-2.213	0.869	0.978	0.463-2.156	0.996
No. of tumor nodules	1.761	0.966-3.192	0.063	1.684	1.002-2.849	0.051
Tumor size	3.598	2.041-6.352	<0.001[a]	2.571	1.613-4.076	<0.001[a]
Venous infiltration	4.042	2.362-6.931	<0.001[a]	3.531	2.159-5.782	<0.001[a]
Serum AFP level	1.554	0.872-2.755	0.137	1.463	0.883-2.420	0.140
Cirrhosis	1.008	0.561-1.798	0.964	1.053	0.631-1.753	0.851
Edmondson-Steiner grading	1.241	0.702-2.186	0.450	1.132	0.678-1.888	0.639
TNM tumor stage	2.801	1.348-5.814	0.006[a]	2.198	1.030-4.673	0.032[a]
miR-92a expression	2.283	1.104-4.717	0.026[a]	3.706	1.079-5.155	0.042[a]

[a]Statistically significant. OS, overall survival; RFS, recurrence-free survival; HBV, hepatitis B virus; AFP, α-fetoprotein; TNM, tumor-node-metastasis; HR, hazard ratio; CI, confidence interval.

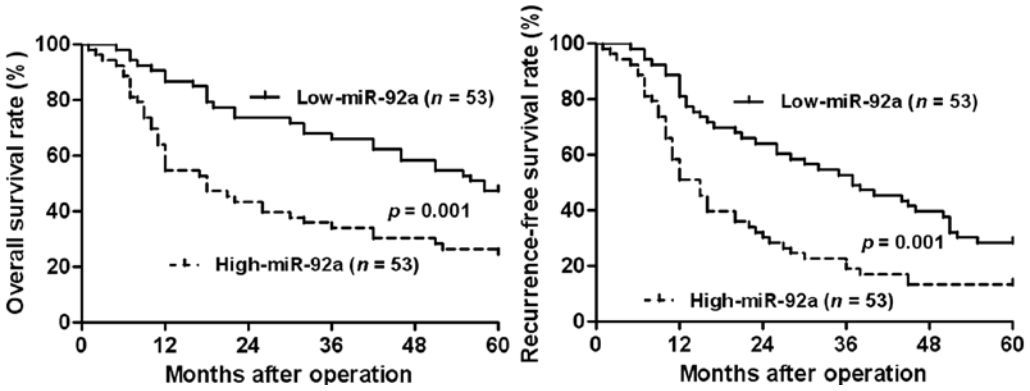

Figure 7. Prognostic value of miR-92a in HCC cases. According to the level of miR-92a expression, Kaplan-Meier 5-year overall and RFS curves of HCC patients showed that high expression of miR-92a was correlated with poor prognosis. The median expression value obtained for miR-92a of the 106 HCC samples detected by qRT-PCR was chosen as the cutoff value. RFS, recurrence-free survival.

results suggested that miR-92a is a novel tumor-suppressive miRNA that plays a critical role in the regulation of tumor growth in HCC.

Results of the present study suggest that miR-92a promoted tumor growth, at least in part, by targeting FBXW7. Firstly, miR-92a was inversely correlated with the levels of both FBXW7 mRNA and protein in HCC tissues. Secondly, miR-92a negatively regulated FBXW7 abundance in HCC cells. Thirdly, the complementary sequence of miR-92a was identified in the 3'-UTR of FBXW7 mRNA. Knockdown of miR-92a increased the luciferase reporter activity of wt 3'-UTR but not mt 3'-UTR of FBXW7. Conversely, the overexpression of miR-92a decreased the luciferase activity of wt 3'-UTR but not mt 3'-UTR of FBXW7. The effects of miR-92a alteration on proliferation, cell cycle and apoptosis of HCC cells were also abolished by FBXW7 modulation. In addition, FBXW7 has been reported to be a direct target of miR-92a in cervical cancer (11). Collectively, our results support FBXW7 as a downstream mediator of miR-92a function in HCC.

Our previous findings have shown that FBXW7 is a potent tumor suppressor and inhibits tumor growth of HCC by inducing growth arrest and apoptosis *in vitro* and *in vivo* (17-19). FBXW7 is a well-known E3 ligase and recognizes target proteins for ubiquitination and degradation. Several oncoproteins have been identified as substrates of FBXW7 including c-Myc (23) and Cyclin E (24). Impaired expression of FBXW7 leads to the accumulation of these oncoproteins, which promote cancer cell proliferation, cell cycle progression and apoptosis resistance. c-Myc protein plays crucial roles in mitogenic signaling and cell growth responses (25) and Cyclin E is a key component of the cell cycle machinery (26). Active Cyclin E-Cdk2 complex is required for G1 and S-phase transition, modulating pRb and thereby activating E2F transcription factors that enable DNA replication (27). It has been reported that c-Myc can induce apoptosis by Caspase-3-dependent and caspase-independent signaling (28). Upregulation of c-Myc and Cyclin E have been reported in HCC and lead to rapid tumor growth *in vitro* and *in vivo* (29,30). Thus, upregulation of c-Myc and Cyclin E may be a mechanism by which miR-92a promotes the proliferation

and cell cycle transition from G1 to S phase and inhibits apoptosis of HCC. Alternatively, our previous results showed that Yes-associated protein (YAP) is a potential target of FBXW7 and is involved in FBXW7-induced growth arrest and apoptosis of HCC (18). It has been shown that YAP plays critical role in hepatocarcinogenesis (31). This mechanism may account for the effects of miR-92a in HCC.

The role of miR-92a in cancer cell proliferation, cell cycle and apoptosis suggests its potential application as a prognostic biomarker of HCC patients. Furthermore, our previous findings showed that a low expression of FBXW7 was an independent prognostic marker for predicting the survival of HCC patients (18). Thus, we demonstrated, for the first time, that high expression of miR-92a was associated with poor prognostic characteristics including HBV infection, large tumor size, cirrhosis, high Edmondson-Steiner grading and advanced TNM tumor stage. We also confirmed that a high expression of miR-92a was an independent prognostic marker for predicting 5-year OS and RFS of HCC patients.

In conclusion, the results show that the expression of miR-92a is reduced in HCC tissues and cell lines. We confirm that miR-92a is an independent prognostic marker for HCC. *In vitro* and *in vivo* studies revealed that miR-92a functions as a novel oncomiRNA by promoting proliferation, cell cycle transition and apoptosis resistance of HCC. Its multiple tumor-promotive effects are mediated by FBXW7. Collectively, the deregulation of miR-92a may play an important role in tumor growth and may be a novel prognostic factor and potential therapeutic target for HCC.

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