Original Article

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FOXA2 plays a critical role in hepatocellular carcinoma progression and lenvatinib-associated drug resistance

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SUMMARY Hepatic forkhead box protein A2 (FOXA2) is a crucial transcription factor for liver development and metabolic homeostasis. However, its role in hepatocellular carcinoma (HCC) progression and lenvatinib-related drug resistance remains unknown. In this study, the level of FOXA2 expression was found to be lower in HCC tissues than in paired adjacent tumor tissues. A low level of FOXA2 expression was associated with aggressive tumor characteristics (vascular invasion and poor differentiation). A low level of FOXA2 expression was found to be an independent risk factor for tumor recurrence (hazard ratio (HR): 1.899, $P < 0.001$) and long-term survival (HR: 2.011, $P = 0.003$) in HCC patients after hepatectomy. In xenograft animal models, FOXA2 overexpression significantly inhibited tumor growth. Moreover, FOXA2 overexpression was found to enhance the inhibitory effect of lenvatinib on HCC cells by upregulating the adenosine monophosphate-activated protein kinase-mechanistic target of rapamycin (AMPK-mTOR) pathway. Conversely, inhibition of adenosine monophosphate-activated protein kinase (AMPK) or stimulation of mechanistic target of rapamycin (mTOR) attenuated the sensitization of cells overexpressing FOXA2 to lenvatinib. Similarly, FOXA2 overexpression augmented the antitumor effect of lenvatinib in animal models with xenograft tumors. FOXA2 overexpression increased autophagy in HCC cells treated with lenvatinib. Lenvatinib treatment activated the platelet-derived growth factor receptor-extracellular regulated protein kinase (PDGFR-ERK) pathway in HCC. FOXA2 overexpression further downregulated the PDGFR-ERK pathway through the activation of the AMPK-mTOR axis. In conclusion, FOXA2 was identified as an independent risk factor for HCC after hepatectomy. FOXA2 was found to be closely associated with the biological progression of HCC. By modulating the AMPK-mTOR-autophagy signaling pathway, FOX2 significantly augmented antitumor effect of lenvatinib in HCC.

Keywords hepatocellular carcinoma, FOXA2, lenvatinib, AMPK-mTOR pathway, drug resistance

1. Introduction

Hepatocellular carcinoma (HCC) is the most common liver malignancy, with an incidence of 42.5% in China (*1*). Overall, the 5-year survival rate of HCC patients in China is only 11.7-14.1% (*2*). Therefore, elucidating the mechanisms underlying HCC development is important. The hepatocyte nuclear factor 3 (HNF3) family, which includes the transcription factors hepatic forkhead box protein A1 (FOXA1), hepatic forkhead box protein A2 (FOXA2), and hepatic forkhead box protein A3 (FOXA3), was first identified in the liver (*3*). The HNF3 family regulates the expression of over 50% of functional genes in the liver, including those involved in liver development, glucose and lipid metabolism, and bile metabolism (*4-6*). Unlike FOXA1 and FOXA3, FOXA2 knockout in the endoderm resulted in immediate postnatal death in mice. Approximately 43.5% of genes are reported to interact with FOXA2. Its knockdown can affect the transcription of the bile acid transporter gene, leading to intrahepatic cholestasis (*7*). FOXA2 has also been found to be downregulated in many solid tumors, such as pancreatic cancer, prostate cancer, breast cancer, and colon cancer. The expression of FOXA2 has been linked to tumor invasion and metastasis, as well as a poor prognosis. For instance, Smith *et al*. (*8*) found that FOXA2 had a high number of mutations in endometrial cancer tissues and was closely related

to tumor progression. Vorvis *et al*. (*9*) reported that FOXA2 knockdown in prostate cancer cells significantly inhibited cell growth. McDaniel *et al.* (*10*) found that FOXA2 regulated the development and differentiation of biliary progenitor cells. Similarly, a previous study by the current authors indicated that downregulation of FOXA2 promoted intrahepatic cholangiocarcinoma development (*11*). A number of studies have investigated the role of FOXA2 in HCC. The inactivation of FOXA2 has been found to regulate the Notch pathway, thereby promoting the progression of HCC (*12*). FOXA2 also regulated the transcription of p53 and p21, thereby affecting the proliferation of HCC cells (*13,14*). FOXA2 was found to be associated with the epithelial-mesenchymal transition (EMT) by downregulating the expression of E-cadherin (*15*). All of the aforementioned findings indicate that FOXA2 plays a critical role in both liver development and liver tumors.

Lenvatinib, a small-molecule inhibitor of multiple receptor tyrosine kinases, was approved for the first-line treatment of patients with unresectable HCC (*16*). Despite the high objective response rate in the treatment of HCC, drug resistance was inevitable. Because lenvatinib's potent antiangiogenic ability depended on various tyrosine kinase inhibitors, the mechanisms of lenvatinib resistance were complex. Previous studies have indicated that neurofibromin 1 (NF1), dual-specificity phosphatase 9 (DUSP9), and dual-specificity phosphatase 4 (DUSP4) were critical drivers of lenvatinib resistance in HCC (*17,18*). Inhibition of epidermal growth factor receptor (EGFR) might enhance the sensitivity of HCC to lenvatinib (*19*). The activation of signal transducers and activators of transcription 3 (STAT3) / ATP binding cassette subfamily B member 1 (ABCB1) signaling contributed to lenvatinib resistance, whereas the inhibition of EGFR reversed this process (*20*). Targeting pathways, such as the Integrin beta 8 (ITGB8)/ heat shock protein 90 (HSP90) / protein kinase B (AKT) axis or the AKT/mechanistic target of rapamycin (mTOR) and extracellular regulated protein kinase (ERK) signaling pathways, might enhance the sensitivity of lenvatinib in HCC patients (*21,22*). Further understanding the mechanism of lenvatinib resistance to HCC could provide a potential treatment strategy in the event of unsatisfactory clinical benefits from lenvatinib. Since FOXA2 interacts with numerous genes in HCC, the effect of FOXA2 on lenvatinib resistance in HCC required investigation.

2. Materials and Methods

2.1. Human tumor tissues and follow-up

In total, 290 HCC patients with complete clinical data who underwent surgical resection at the West China Hospital of Sichuan University between January 2010 and December 2016 were included in this study. Overall survival (OS) was defined as the duration between the initial surgery and the patient's death. Recurrence-free survival (RFS) was defined as the duration between the date of curative surgery and the date of relapse. Tissue microarray (TMA) cores (1.5 mm in diameter) were derived from formalin-fixed, paraffin-embedded samples. Immunohistochemical (IHC) staining was performed on the TMA slides, and the results were interpreted by three pathologists using a blinded method. The level of FOXA2 expression was scored based on the distribution and intensity of the signal. At least five areas were examined under 400x magnification and scored based on their signal distribution as follows: 1, ≤ 25% stained; 2, 26-50% stained; 3, 51-75% stained; and 4, > 75% stained. The staining intensity was scored as follows: 0, negative; 1, weakly positive (light yellow); 2, positive (brownish yellow); and 3, intensely positive (brown). Tissues with an IHC score of less than or equal to 7 were designated as having a low level of expression, while those with a score of more than seven were designated as having a high level of expression. To investigate the relationship between FOXA2 expression and HCC, classic clinical characteristics, such as age, alpha fetoprotein (AFP) level, hepatitis B virus (HBV) infection status, albumin, total bilirubin, degree of differentiation, tumor size, satellite lesion, vascular invasion, and cirrhosis of the liver, were included. This study was approved by the Ethics Committee of West China Hospital, Sichuan University, and written informed consent was obtained from each participant.

2.2. Animals and treatments

Subcutaneously, 5×10^6 cells infected with Lenti-FOXA2 or Lenti-GFP were inoculated into the right forelimb of nude mice (*n* = 15). HCC tumors were successfully grafted to the majority of mice after three weeks. Lenvatinib (Selleck Chemicals, USA, cat# S1164) was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) according to the manufacturer's instructions to produce a 20-mM stock solution. The 20-mM stock solution was diluted to 1 mM in a 0.3% sodium carboxymethyl cellulose solution. Mice were administered either DMSO or lenvatinib (200 μL). DMSO (diluted in 0.3% sodium carboxymethyl cellulose solution) was used as an untreated control. Mice were randomly assigned to one of four groups: green fluorescent protein (GFP)-vehicle (*n* = 5), a GFP-lenvatinib (*n* = 5), a FOXA2-vehicle (*n* = 5), and FOXA2-lenvatinib (*n* = 5). The tumor size was recorded every 5 days. The tumor weight was recoded at sacrifice. Animal experiments were conducted in accordance with national and international laws and policies, and they were approved by the Department of Animal Care and Use Committee of Sichuan University.

2.3. Western blotting and immunohistochemistry

Western blotting was performed in accordance with the standard protocols. Proteins were extracted from cells or tissues using RIPA buffer containing a protease inhibitor cocktail, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Millipore). The antibodies used in this study were as follows: FOXA2 (1:1000; Abcam, UK, cat# EPR22919-71), FLAG-tag (1:1500; Proteintech, USA, cat# 80010-1-RR), Actin (1:1500; Proteintech, USA, cat# 66009-1-Ig), PDGFR: platelet-derived growth factor receptor (1:1000; Abcam, UK, cat# ab215978), p-PDGFR (1:1000; Abcam, UK, cat# ab134048), p-ERK1/2 (1:500; Abcam, UK, cat# ab76299), ERK1/2: extracellular regulated protein kinases 1/2 (1:1000; Abcam, UK, cat# ab184699), p-AMPK (T172) (1:500; Abcam, UK, cat# ab133448), adenosine monophosphate-activated protein kinase (AMPK) (1:500; Abcam, UK, cat# ab32047), p-mTOR (S2448) (1:500; Abcam, UK, cat# ab109268), ribosomal protein S6 kinase (S6K) (1:500; Abcam, UK, cat# ab186753), p-S6K (1:500; Abcam, UK, cat# ab60948), cleaved-caspase 3 (1:500; Abcam, UK, cat# ab32042), cleaved-PARP1 (1:500; Abcam, UK, cat# ab32561), microtubule-associated protein light chain 3II (LC3II) (1:500; Abcam, UK, cat# ab192890), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1500; Proteintech, USA, cat# 60004-1-Ig). Immunohistochemistry (IHC) was performed as previously described (*11*).

2.4. Cell culture, transfection, cell proliferation, and invasion

The HCC cell lines Hep3B and Huh7 were obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal calf serum. The small interfering RNA (siRNA) against FoxA2 (target sequence: CCATGAACATGTCGTCGTA) was synthesized by RiboBio Co., Ltd. (Shanghai, China). A FOXA2 overexpression plasmid (pcDNA3.1-FLAG-FOXA2) and theplvx-FOXA2 lentivirus were obtained from Tsingke Biotechnology Co., Ltd. (Beijing, China). Tumor cells (3×10^3 cells/well) were seeded in 96-well plates and grown for 24 h. Hep3B and Huh7 cells were transfected with the siRNA or FOXA2 overexpression plasmid and incubated for 72 h. A cell proliferation assay (CCK-8 kit, BD Biosciences, USA) was performed to assess Hep3B and Huh7 cell proliferation. A wound healing assay was performed to evaluate tumor cell invasion. The scratch wound healing assay and the transwell migration assay (BD Bioscience) were used to assess the migration of tumor cells. The results were analyzed using the mean number of cells in three fields for each sample.

2.5. Statistical analysis

The software R (3.6.1 Windows version) was used for statistical analyses. The continuous variables were expressed as the median ± interquartile range (IQR), and the difference between groups was tested using the Mann–Whitney *U* test. Categorical variables were expressed as percentages, and statistical analyses were performed using the χ^2 test or Fisher's exact test. The Kaplan-Meier test was used for survival analysis and the log-rank test was used for difference analysis. The risk factors were analyzed using a stepwise Cox regression model. The potential risk factors ($P < 0.1$) were selected in univariate analysis and then included in multivariate analysis. The gray values of protein bands in Western blots were measured and analyzed using the software ImageJ (LOCI, University of Wisconsin, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. FOXA2-associated tumor features and the prognosis for HCC patients

Western blotting (WB) revealed that FOXA2 was significantly downregulated in tumor tissue compared to adjacent tumor tissue (Figure 1A). Based on the IHC score (Figure 1B), 150 patients had a high level of FOXA2 expression and 140 had a low level of FOXA2 expression. The study included 290 patients with HCC after hepatectomy, as shown in Supplementary Table S1 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=137*). There were significant differences in portal vein tumor thrombus (PVTT), poorly differentiated tumors, and serum alpha-fetoprotein (AFP) levels ($P < 0.05$). In comparison to the group with a high level of FOXA2 expression, the group with a low level of FOXA2 expression had a higher incidence of PVTT (12.9% *vs*. 5.3%), a higher proportion of poorly differentiated tumors (8.6% *vs*. 34.0%), and higher AFP levels (> 400 ng/mL). Based on survival analysis, the 1-year, 3-year, and 5-year recurrence-free survival (RFS) in the group with a low level of FOXA2 expression was 48.6%, 28.7%, and 17.0%, respectively, whereas the 1-year, 3-year, and 5-year RFS in the group with a high level of FOXA2 expression was 70.6%, 45.8%, and 34.6%, respectively ($P < 0.001$). The 1-year, 3-year, and 5-year OS in the group with a low level of FOXA2 expression was 92.7%, 76.7%, and 49.1%, respectively, whereas the 1-year, 3-year, and 5-year OS in the group with a high level of FOXA2 expression was 72.9%, 47.7%, and 25.9%, respectively ($P < 0.001$) (Figure 1C). A low level of FOXA2 expression was an independent risk factor for tumor recurrence (hazard ratio (HR): 1.899, $P < 0.001$) and long-term survival (HR: 2.011, $P = 0.003$) in HCC patients after hepatectomy (Figure 1D). Therefore, a low level of FOXA2 expression in HCC indicated aggressive tumors.

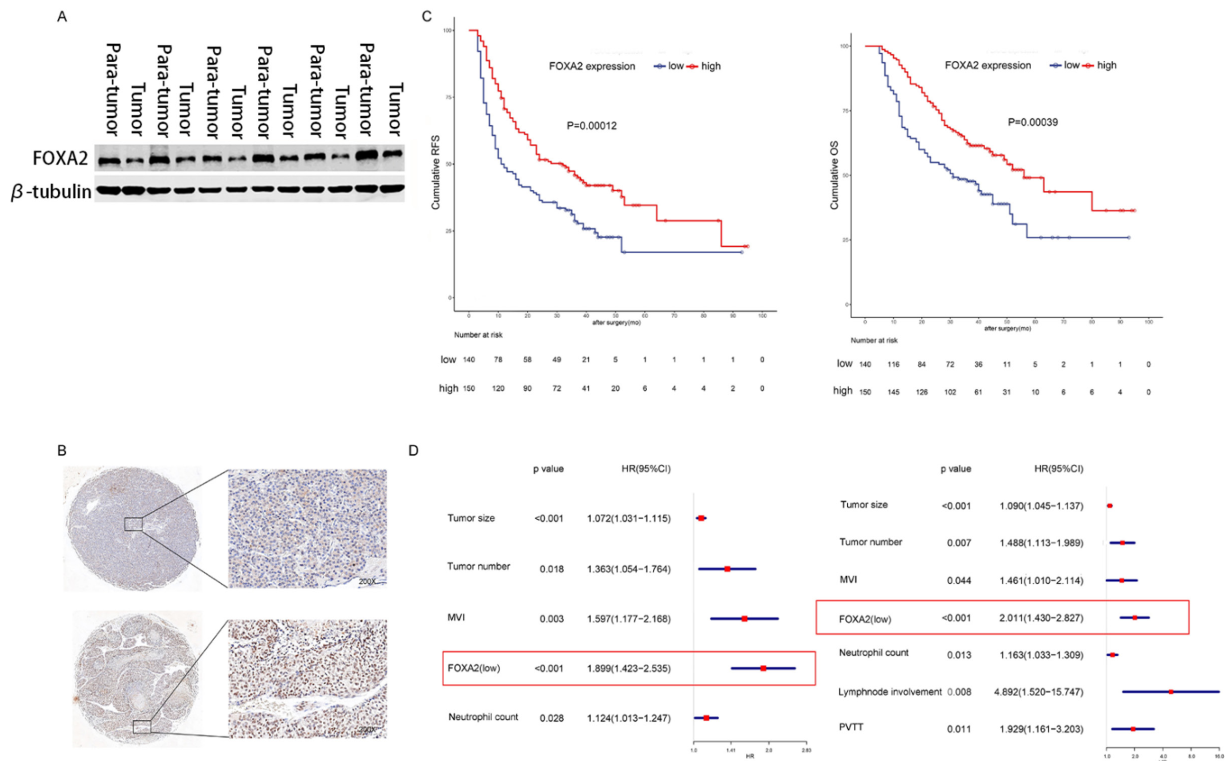

Figure 1. Clinicopathological features correlated with the expression of FOXA2. A: Western blots indicating FOXA2 expression in paired tumor and para-tumor tissue (*n* = 6). B: Representative images of low and high levels of FOXA2 expression in HCCs. C: survival analysis of HCC patients with a high or low level of FOXA2 expression; D: Multivariate analysis of recurrence-free survival (left) and overall survival (right) after HCC resection.

3.2. FOXA2 expression influenced HCC cell proliferation and invasion

FOXA2 was overexpressed and knocked down in Hep3B by plasmid transfection and in Huh7 cells by siRNA transfection (Supplementary Figure S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=137*). The cell cloning assay revealed that the proliferation of Hep3B and Huh7 cells was significantly enhanced after FOXA2 knockdown but inhibited when FOXA2 was overexpressed (Figure 2G and 2H). The scratch wound healing and transwell migration assays revealed that the knockdown of FOXA2 increased the invasion of Hep3B and Huh7 cells while the overexpression of FOXA2 inhibited it (Figure A-F). These findings suggest that a low level of FOXA2 expression might promote HCC progression.

3.3. Effects of FOXA2 expression on the cytotoxicity of lenvatinib in HCC cells

Hep3B and Huh7 cells were divided into two groups, with one group transfected with an empty plasmid and the other group transfected with a FLAG-FOXA2 plasmid. Both groups received varying concentrations of lenvatinib (0 μM, 1 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM). Cell death occurred at 5 μM, 10 μM, and 20 μM and the number of dead cells increased with an increase in the lenvatinib concentration (Figure 3A and 3B). FOXA2 overexpression was found to increase the sensitivity of HCC cells to lenvatinib. Hep3B and Huh7 cells were transfected with FLAG-FOXA2 and treated with lenvatinib (200 nM); clone proliferation decreased significantly compared to that in the FOXA2 overexpression + lenvatinib group (Figure 3C and 3D). These findings suggest that FOXA2 overexpression augmented the ability of lenvatinib to promote cell death.

3.4. FOXA2 increased the antitumor effect of lenvatinib *via* the AMPK-mTOR pathway

Stemness and drug resistance were found to be closely related to the AMPK-mediated pathway (*23,24*). WB revealed that FOXA2 overexpression and lenvatinib treatment upregulated p-APMK expression in a synergistic manner (Figure 4A and 4B). Lenvatinib has been found to effectively inhibit HCC cell invasion. Following lenvatinib treatment, Hep3B and Huh7 cells overexpressing or not overexpressing FOXA2 were treated with AMPK inhibitor compound C (2 μM). Their ability to invade cells was significantly restored, with no significant differences between the groups (Figure 4C). When the mTOR activator MHY1485 (5 μm) was added to Hep3B/Huh7 cells overexpressing FOXA2 after treatment with lenvatinib (10 μm), the antitumor effect of lenvatinib that was enhanced by FOXA2 was attenuated

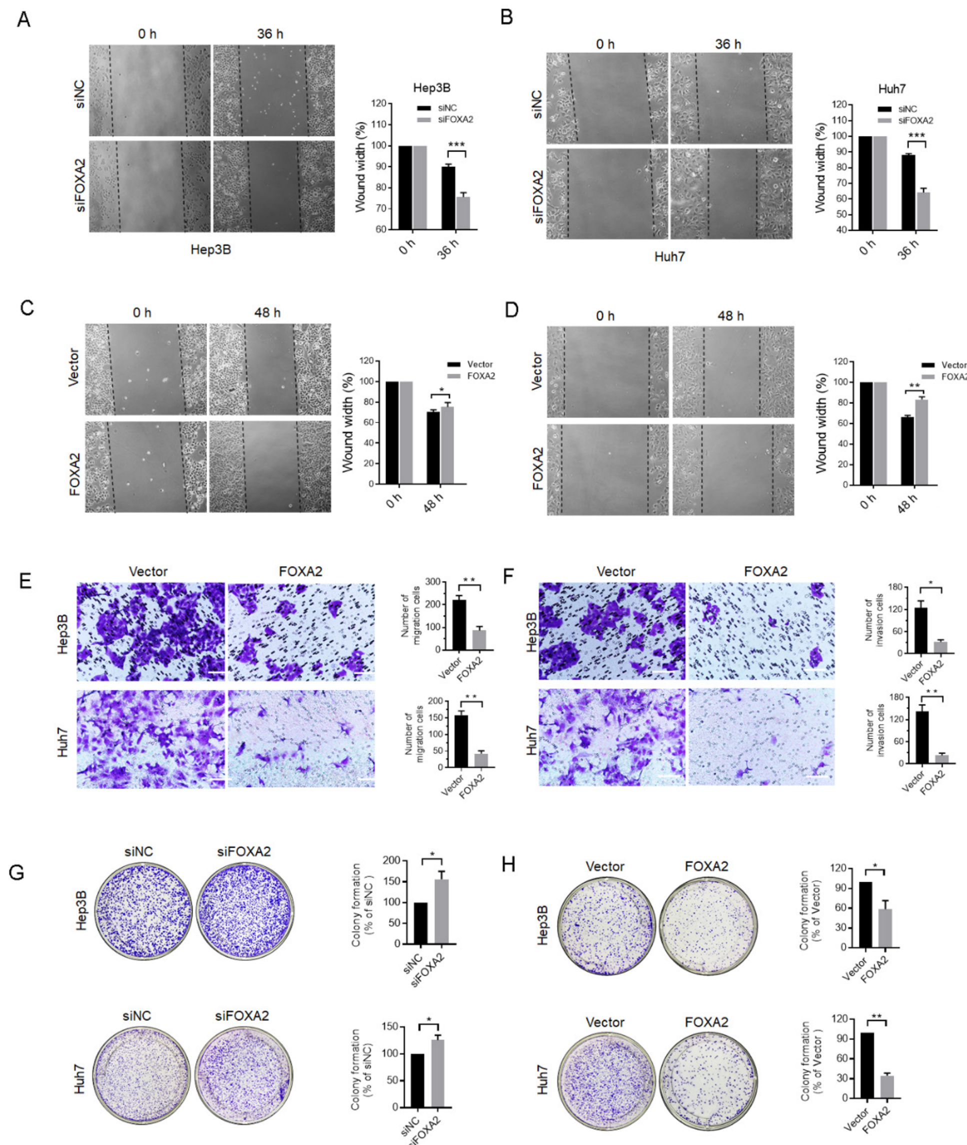

Figure 2. FOXA2 inhibited HCC cell (Huh7/Hep3B) invasion. Scratch wound and transwell assay suggested that FOXA2 inhibited or promoted HCC cell migration after FOXA2 overexpression or knockdown **(A-F)**; Clonal formation assays indicated that cell proliferation decreased after FOXA2 overexpression and increased after FOXA2 knockdown **(G** and **H)**.

(Figure 4G). Similar results have been observed in cloning assay (Figure 4 E-F). This suggested that the inhibitory effect of FOXA2 on HCC was associated with AMPK activation and mTOR inhibition. The levels of p-mTOR and p-S6K expression decreased, while the level of p-AMPK expression increased. Mammalian target of rapamycin C1 (mTORC1) is an important AMPK substrate. FOXA2 overexpression combined with lenvatinib inhibited mTORC1 activation, whereas AMPK inhibitor compound C (2 uM) significantly restored p-mTOR expression (Figure 4H). These findings indicated that FOXA2 overexpression inhibited the mTOR pathway by activating AMPK (Figure 9).

3.5. Lenvatinib combined with FOXA2 overexpression upregulated the STRADα- LKB1 axis

Activation of AMPK or inhibition of mTOR in HCC may enhance the antitumor effect of lenvatinib (*25,26*). Lenvatinib combined with FOXA2 overexpression was found to activate AMPK. Moreover, metformin activated AMPK in HCC cell lines and indeed enhanced the antitumor effect of lenvatinib in HCC cell lines. LKB1 is an important upstream regulator of AMPK (Supplementary Figure S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=137*). A previous study reported that the nuclear residence of LKB1 in HCC cells attenuated activation of AMPK (*27*). Ste20-related adaptor alpha (STRADα) may promote the nuclear - cytoplasmic translocation of liver kinase B1(LKB1) (*28*). Compared to lenvatinib treatment alone, lenvatinib combined with FOXA2 overexpression lead to significantly increased LKB1 phosphorylation. The expression of the LKB1 regulator STRADα was up-regulated (Figure 5A). After FOXA2 overexpression, the levels of LKB1 and STRADα protein in the

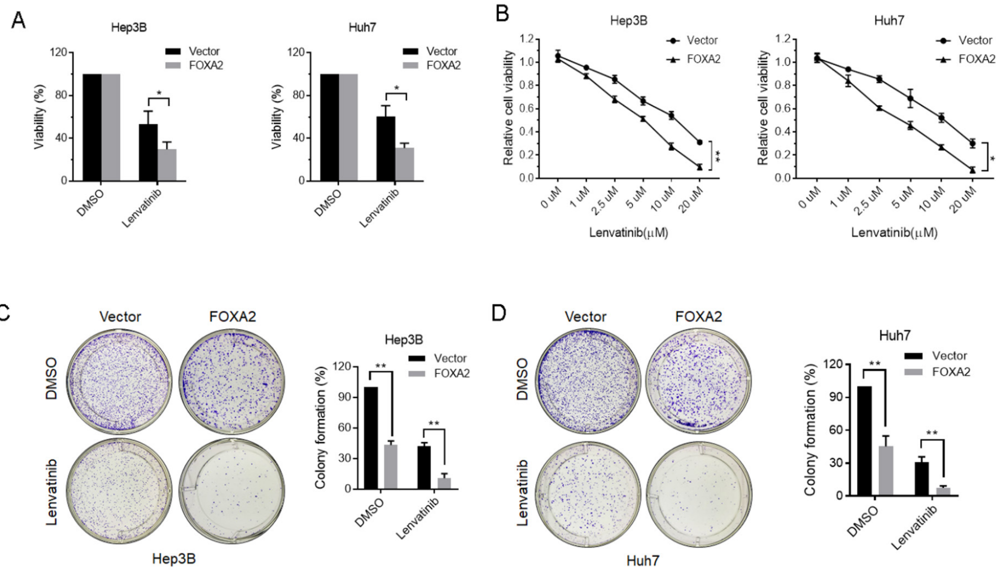

Figure 3. FOXA2 increased HCC cell (Hep3B/Huh7) sensitivity to lenvatinib. The viability of HCC cell lines was significantly reduced by lenvatinib and further inhibited after FOXA2 overexpression **(A** and **B)**. Clonal formation assays suggested that FOXA2 overexpression further inhibited HCC cell proliferation **(C** and **D)**.

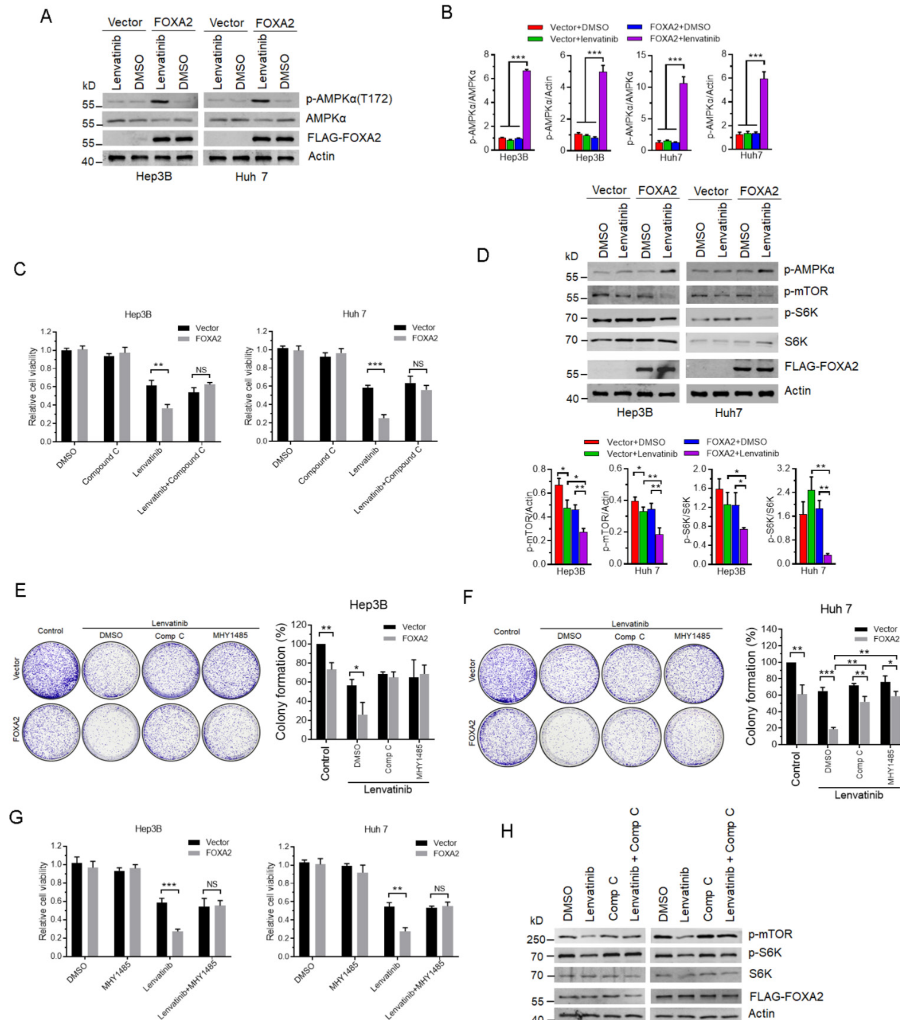

Figure 4. FOXA2 increased HCC cell (Hep3B/Huh7) sensitivity to lenvatinib via the AMPK signaling pathway. p-AMPK significantly was up-regulated in HCC cell lines overexpressing FOXA2 after treatment with lenvatinib **(A** and **B)**. After adding AMPK inhibitor Compound C (2 uM) for 48 h, a CCK-8 assay indicated that the cell viability was restored by treatment with lenvatinib **(C)**. The levels of p-mTOR and p-S6K protein were significantly down-regulated in HCC cell lines overexpressing FOXA2 after treatment with lenvatinib **(D)**. Colony assay and CCK-8 indicated that inhibition of AMPK or activation of mTOR impaired HCC cell line sensitivity to lenvatinib by **FOXA2 (E-G)**. Inhibition of AMPK upregulated the levels of p-mTOR or p-S6K protein in HCC cell lines treating with lenvatinib and FOXA2 overexpression **(H)**.

cytoplasm were up-regulated, while the levels of LKB1 and STRADα protein in the nucleus were down-regulated (Figure 5B). This suggested that lenvatinib combined with FOXA2 overexpression further promoted LKB1 cytoplasmic residence and activated AMPK by upregulating the level of STRADα expression (Figure 9).

3.6. Autophagy increased with a high level of FOXA2 expression and lenvatinib treatment

The AMPK-mTOR pathway plays an important role in autophagy. To determine whether FOXA2-induced lenvatinib sensitization occurs *via* AMPK-mediated autophagy, the level of expression of LC3-II, a key molecule of autophagy, was measured. When cells overexpressing FOXA2 were treated with lenvatinib, the level of LC3-II expression increased. When the cells overexpressing FOXA2 were treated with an autophagy inhibitor (bafilomycin A1) after lenvatinib treatment, the level of LC3-II expression continued to increase (Figure 6A). These results revealed that LC3-II accumulation was caused by an increase in autophagy rather than inhibition of autophagy clearance. Hep3B cells were co-transfected with GFP-LC3 and FLAG-FOXA2

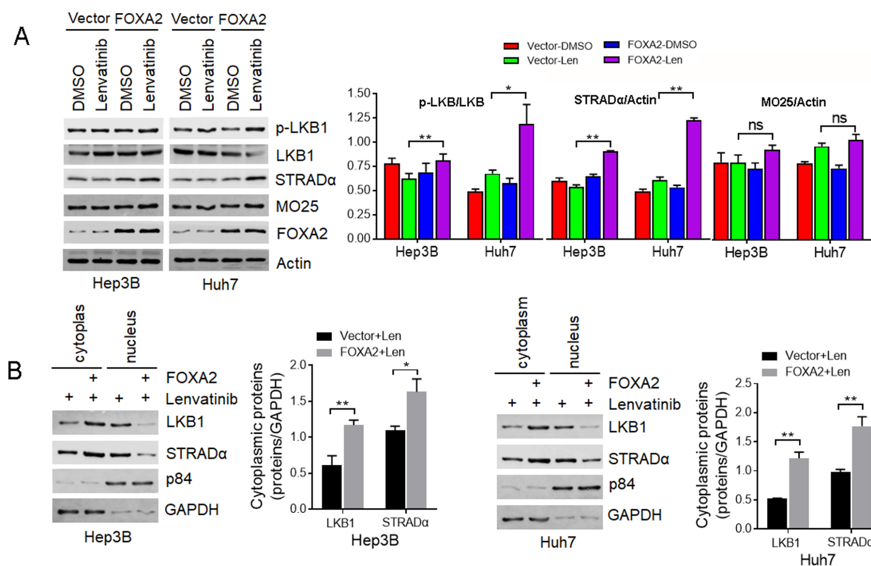

Figure 5. Lenvatinib combined with FOXA2 overexpression upregulated p-LKB1 and the STRADα-LKB1 axis. The level of LKB1 phosphorylation increased and the level of expression of the LKB1 regulator STRADα was up-regulated **(A)**. After FOXA2 overexpression, the levels of LKB1 and STRADα protein in the cytoplasm were up-regulated, and the levels of LKB1 and STRADα protein in the nucleus were down-regulated **(B)**. MO25: Liver kinase B1 (LKB1, also known as STK11), MO25, and STRAD. p84: Nuclear matrix protein p84.

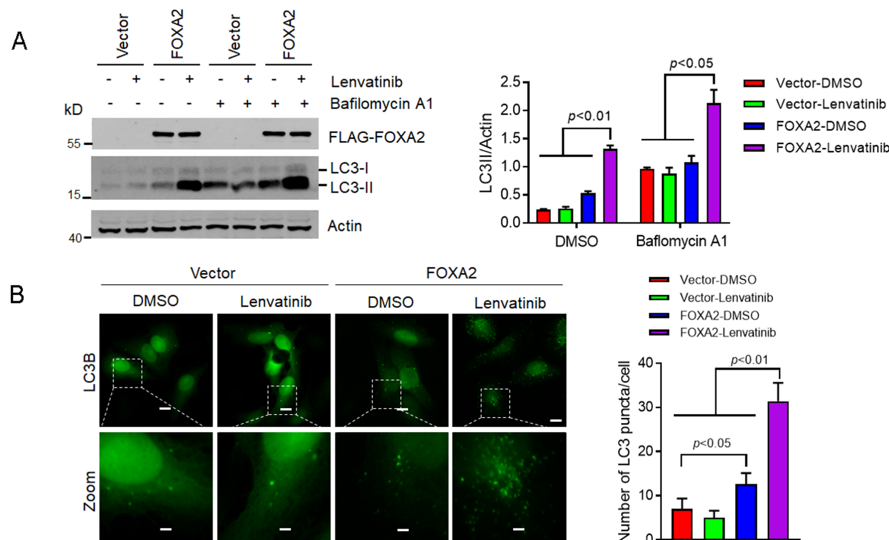

Figure 6. Lenvatinib combined with FOXA2 overexpression promoted autophagy. Microtubule-associated protein light chain 3II (LC3II) was significantly up-regulated in HCC cells overexpressing FOXA2 after lenvatinib treatment. After adding bafilomycin A1, LC3II was further up-regulated **(A)**. The number of LC3 puncta/cell was highest in the FOXA2+lenvatinib group. This suggested that autophagy was enhanced **(B)**. LC3-I: microtubule-associated protein light chain 3 I.

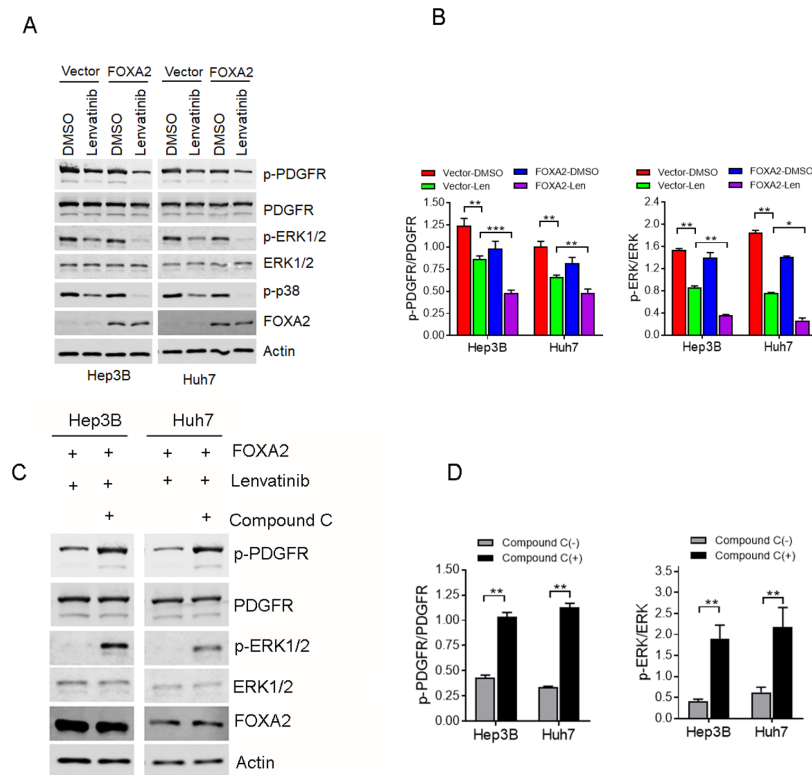

Figure 7. Lenvatinib combined with FOXA2 overexpression inhibits the PDGFR-ERK pathway via the AMPK signaling pathway. After HCC cell lines were transfected with FOXA2 for 24 hours, they were treated with lenvatinib (10 uM) and AMPK inhibitor Compound C (2 uM) for 48 hours. Western blotting indicated that the levels of p-PDGFR and p-ERK1/2 protein were significantly down-regulated compared to lenvatinib or FOXA2 overexpression alone **(A** and **B)**. After adding AMPK inhibitor Compound C, the levels of p-PDGFR and p-ERK1/2 protein were up-regulated **(C** and **D)**.

vectors and incubated for 24 h, followed by treatment with lenvatinib (10 μM) for 24 h. The results suggested that FOXA2 overexpression combined with lenvatinib treatment caused a significant increase in autophagy (Figure 6B).

3.7. Lenvatinib treatment combined with FOXA2 overexpression inhibited the PDGFR-ERK pathway

PDGFR plays a significant role in anti-tumor drug resistance. Lenvatinib inhibits tumor progression by targeting PDGFR. Results indicated that FOXA2 overexpression significantly inhibited the expression of p-PDGFR, implying that the enhanced antitumor effect of lenvatinib by forced FOXA2 expression may depend on modification of PDGFR (Figure 7A-B). When the AMPK pathway was inhibited, the effect of forced FOXA2 expression on the sensitivity of HCC cells to lenvatinib was diminished. Interestingly, expression of p-PDGFR and p-ERK1/2 was reversed (Figure 7C-D). These results suggested that FOXA2 may regulate the PDGFR-ERK pathway *via* the AMPK signaling axis and contribute to the antitumor effect of lenvatinib (Figure 9).

3.8. FOXA2 further inhibited the growth of HCC treated with lenvatinib *via* the AMPK signaling pathway

Hep3B cells were divided into two groups; one group was transfected with lentivirus-green fluorescent protein (Lenti-GFP), and the other group was transfected with lentivirus-FOXA2 (Lenti-FOXA2). Two group cells were seeded in nude mice and observed for 4 weeks. The results indicated that the tumorigenic ability diminished significantly in the group overexpressing FOXA2. Then, two groups were treated with lenvatinib. The weakest tumorigenicity and very slow tumor growth were observed in the group overexpressing FOXA2 (Figure 8A-C). Protein detection in tumor tissues indicated that the level of p-AMPK expression increased significantly in the group overexpressing FOXA2 (Figure 8D-E). This confirmed that FOXA2 enhanced the antitumor effect of lenvatinib *via* the AMPK-mTOR pathway.

4. Discussion

Results indicated that the expression of FOXA2 in tumor tissue was downregulated compared to that in adjacent tumor tissue. Consistent with the current findings, data from high-throughput sequencing expression profiling suggested that the content of FOXA2 mRNA in tumor samples was significantly lower than that in paired non-cancer tissues (GSE119336, by Cao Peng and Li Yong). The level of FOXA2 expression in a variety of human

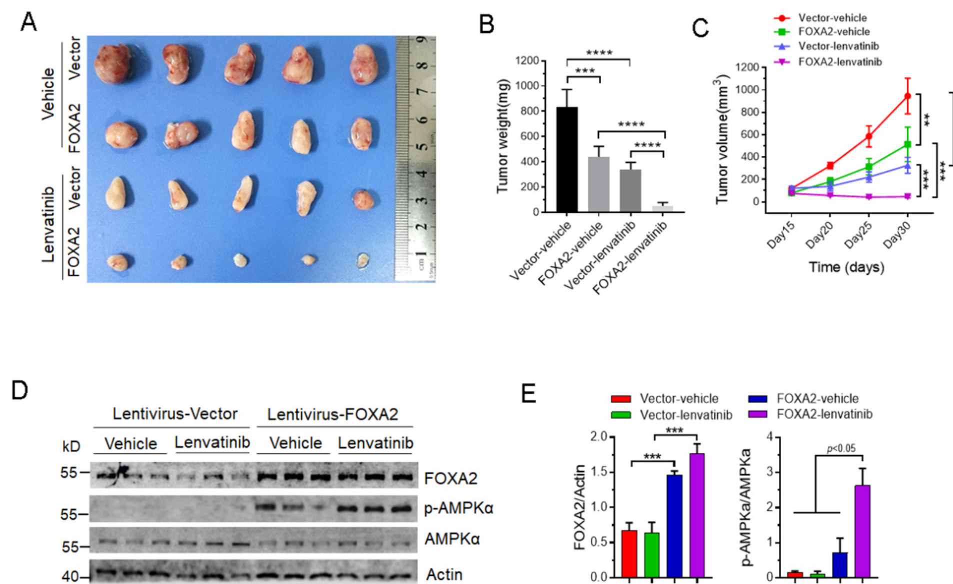

Figure 8. FOXA2 further inhibited the growth of HCC treated with lenvatinib via the AMPK signaling pathway. FOXA2 overexpression enhanced the antitumor effect of lenvatinib **(A)**. Tumor weight **(B)** and volume **(C)** of subcutaneous implant models in nude mice produced by injecting HCC cells stably expressing FOXA2 (n = 10, lenvatinib: n = 5; control: n = 5) and an empty vector (n = 10, lenvatinib: n = 5; control: n = 5); FOXA2 overexpression combined with lenvatinib up-regulated p-AMPK **(D** and **E)**.

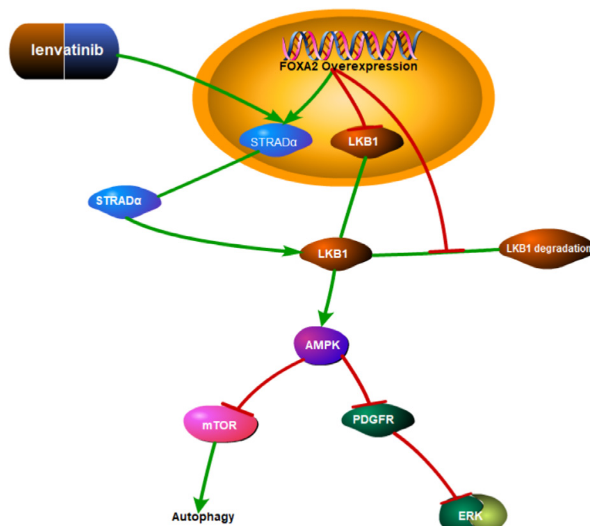

Figure 9. FOXA2 overexpression increased the antitumor effect of lenvatinib via the AMPK-mTOR-Autophagy pathway. Lenvatinib treatment combined with FOXA2 overexpression inhibited the PDGFR-ERK pathway via activation of AMPK. The levels of LKB1 and STRADα protein in the nucleus were down-regulated. FOXA2: forkhead box protein A2; STRADα: ste20-related adaptor alpha; LKB1: liver kinase B1; AMPK: adenosine monophosphate-activated protein kinase; mTOR: mechanistic target of rapamycin; PDGFR: platelet-derived growth factor receptor; ERK: extracellular regulated protein kinases.

HCC cell lines has been reported to be significantly lower than that in normal human fetal hepatocytes (LO-2) (*29*). According to IHC, 48.3% of patients had a low level of FOXA2 expression. Patients with a low level of FOXA2 expression had a higher incidence of portal vein invasion and poorly differentiated tumors. This suggested that the expression of FOXA2 was associated with tumor aggressiveness. In terms of prognosis, patients with a high level of FOXA2 expression had a significantly higher 5-year RFS and OS than patients with a low level of FOXA2 expression. A low level of FOXA2 expression was identified as an independent risk factor for tumor recurrence. In addition, tumor size, the number of tumors, MVI, PVTT, and lymph node status have been found to be independent risk factors for patients with HCC (*30*). This indicates that a low level of FOXA2 expression was associated with a highly invasive tumor.

The proliferation and invasion capacity of HCC cells were found to be significantly enhanced by a decrease in FOXA2 expression but increased after forced FOXA2 expression. Previous studies have found that patients with higher levels of FOXA2 expression have a better prognosis for breast carcinoma and gastric cancer (*31,32*). A study has also found that FOXA2 acted as a tumor suppressor and inhibited the invasion of various tumors (*13*). FOXA2 regulated tumor behavior by interacting with various genes, including the cadherin 1(CDH1) promoter, forkhead box protein P2 (FOXP2), and staphylococcal nuclease domain containing 1 (SND1) (*29,33,34*). The current authors previously reported that FOXA2 promotes intrahepatic cholesteatoma by activating the MAPK signaling pathway (*11*). According to preliminary findings and previous research (*11*), FOXA2 contributed to tumor progression in HCC cells. Lenvatinib is a small-molecule inhibitor of multiple receptor tyrosine kinases that is widely used to treat patients with unresectable HCC. However, this drug has only a limited clinical benefit. Numerous studies have been conducted to investigate potential targets to

improve prognosis (*17-19,21*). The current study found that overexpression of FOXA2 enhanced the ability of lenvatinib to promote cell death in lenvatinib-sensitive HCC cells.

FOXA2 mutations have been reported to be associated with the AMPK signaling pathway (*35*). The AMPK pathway is closely involved in cancer drug resistance *via* several mechanisms of antitumor drug resistance (*24,36*). An AMPK activator enhanced the antitumor effect of cisplatin in meningiomas *via* the AMPK/mTOR signaling pathway (*37*). AMPK/mTOR-mediated autophagy was found to contribute to docetaxel resistance in castration-resistant prostate cancer (*38*). These findings suggest that the contribution of FOXA2 to lenvatinib sensitivity in HCC might be dependent on the AMPK signaling pathway. The enhanced antitumor effect of lenvatinib by forced overexpression of FOXA2 was found to be attenuated when AMPK was inhibited. AMPK phosphorylation increased significantly in HCC cells following lenvatinib treatment and overexpression of FOXA2; however, the levels of p-mTOR and p-S6K expression decreased. These findings indicate that the AMPK/mTOR signaling pathway played a critical role in increasing lenvatinib sensitization by FOXA2 in HCC cells. The AMPK enzyme is a critical energy and nutrient sensor in cells. AMPK activation might regulate the metabolic reprogramming and self-regeneration of cancer stem cells. Therefore, targeting the AMPK signaling pathway is a potential strategy for overcoming cancer drug resistance (*24,39*). The current study found that FOXA2 might be a key target for overcoming lenvatinib resistance.

Autophagy is a physiological cell survival mechanism utilized by tumor cells to prevent cell death and induce drug resistance. FOXA2 has been found to be closely related to autophagy (*40*). mTOR-mediated cancer drug resistance inhibits autophagy and generates a druggable metabolic vulnerability (*41*). AMPK phosphorylation has a critical role in mediating autophagy (*39*). Chloroquine is an autophagy inducer that increases microtubule-associated protein light chain 3B-II protein expression. Cisplatin induced autophagy and contributed to drug resistance by activating the AMPK/mTOR signaling pathway. Cisplatin in combination with chloroquine decreased the level of p-AMPK expression and increased the level of p-mTOR expression, which inhibited the activation of autophagy (*42*). Cisplatin in combination with chloroquine was also found to increase the cisplatin-induced apoptosis and growth of lung adenocarcinoma cells. This indicates that the AMPK-mTOR pathway plays an important role in autophagy. The current study indicated that FOXA2 in combination with lenvatinib activated the AMPK signaling pathway and enhanced autophagy. PDGFR is one of the key targets of lenvatinib (*43,44*). In the current study, lenvatinib and FOXA2 overexpression significantly reduced the expression of p-PDGFR compared to lenvatinib alone or overexpressed FOXA2 alone. However, this phenomenon was reversed by an AMPK inhibitor. This may be why FOXA2 overexpression promotes lenvatinib sensitivity in HCC. However, the current study had several limitations. First, lenvatinib inhibits HCC *via* many targets. This study focused on the PDGFR-ERK pathway in drug resistance. Second, the relationship between the AMPK signaling pathway and autophagy has been well-established, but autophagy in lenvatinib-related drug resistance was not fully investigated. Therefore, the hope is that additional quality research will delve into this issue.

In conclusion, results indicated that a low level of FOXA2 expression is significantly associated with poor tumor biology. Results also revealed that overexpressed FOXA2 enhanced HCC susceptibility to lenvatinib, which was mediated by the AMPK/PDGFR signaling pathway. These findings indicate that FOXA2 may be a promising target to treat lenvatinib-associated drug resistance.

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Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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