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Metformin inhibits the prometastatic effect of sorafenib in hepatocellular carcinoma by upregulating the expression of TIP30

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Key words

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We previously found that a low dose of sorafenib had a prometastatic effect on hepatocellular carcinoma (HCC), which was caused by downregulation of TIP30 expression. More recently, metformin has been shown to have potential as a preventive and therapeutic agent for different cancers, including HCC. This study evaluated whether the combination of sorafenib and metformin is sufficient to revert the expression of TIP30, thereby simultaneously reducing lung metastasis and improving survival. Our data show that the combination of sorafenib and metformin inhibits proliferation and invasion *in vitro*, prolongs median survival, and reduces lung metastasis of HCC *in vivo*. This effect is closely associated with the upregulation of TIP30, partly through activating AMP-activated protein kinase. Thioredoxin, a prometastasis factor, is negatively regulated by TIP30 and plays an essential role during the process of HCC metastasis. Overall, our results suggest that metformin might be a potent enhancer for the treatment of HCC by using sorafenib.

Hepatocellular carcinoma (HCC), one of the most common malignant tumors, accounts for 80–90% of primary liver cancers.[1] Liver resection is the treatment option for only 10–30% of diagnosed patients, and the 5-year recurrence and metastasis rates are as high as 50–80%. More than 70% of HCC patients have lost the opportunity to receive surgical treatment at the time of diagnosis[2] and can only receive palliative treatment.

Sorafenib, a Raf kinase and receptor tyrosine kinase inhibitor, is a standard first-line therapeutic agent for advanced HCC and functions by inhibiting tumor cell proliferation and angiogenesis.[3] However, studies have shown that sorafenib only increases the median survival of patients with advanced HCC by less than 3 months, indicating that the effect of sorafenib treatment needs to be improved.[4,5] More alarmingly, experimental studies have reported that, although sorafenib (30 mg/kg/day) can prolong the survival of nude mice, the metastatic ability increased significantly.[6,7]

Metformin is a widely recommended oral drug for type 2 diabetes and its antitumor effects have recently attracted attention. Metformin has been shown to exert anticancer activities in several cancers, such as breast cancer,[8] colorectal cancer, pancreatic cancer,[9] lung cancer,[10] and esophageal cancer.[11] Previous studies have shown that the antitumor effect of metformin might be mainly achieved through the activation of AMP-activated protein kinase (AMPK), leading to inhibition of tumor cell proliferation.[12] Recent studies suggest that metformin treatment can reduce the risk of HCC in patients with type 2 diabetes[13] and inhibit HCC invasion and increase drug sensitivity to sorafenib;[14] however, the underlying mechanism remains unclear.

TIP30 is a tumor suppressor that plays an important role in inhibiting HCC invasion and metastasis.[15] We previously showed that downregulation of TIP30 by sorafenib could induce the epithelial–mesenchymal transition (EMT) phenotype of tumor cells, which could promote tumor invasion and metastasis.[6] In the present study, we found that combined treatment with sorafenib and metformin could minimize the prometastatic effects of sorafenib by upregulating the expression of TIP30, which is mediated in part by the activation of AMPK.

Materials and Methods

Cell culture and transfection. The MHCC97H cell line was obtained from the Liver Cancer Institute. MHCC97H-shTXN and MHCC97H-shCON cells were obtained by infecting MHCC97H-wt cells with lentiviral vectors encoding shRNA for thioredoxin-1(TXN) and shCON as a control. The shRNA construct against TXN (Cat. No. HSH018335-4-LVRH1GP), sh-control (Cat. No. CSHCTR001-LVRH1GP), and Lenti-PacHIV Expression Packaging Kit (Cat. No. HPK-LvTR-20) were all obtained from the GeneCopoeia (Rockville, MD, USA). *TIP30* gene expression was silenced with TIP30 siRNA, which was obtained from Suzhou GenePharma China (Suzhou, China), and synthesized as follows: TIP30-219 siRNA, 5′-GC AGAAUAAAUCCGUCUUUTT-3′(sense) and 5′-AAAGACG GAUUUAUUCUGCTT-3′(antisense); TIP30-474 siRNA, 5′-GGAGGGAUUUGUUCGUGUUTT-3′(sense) and 5′-AACAC GAACAAAUCCCUCCTT-3′(antisense); TIP30-706 siRNA, 5′-CCAGGUGAAUGGCUGGUUATT-3′(sense) and 5′-UAAC CAGCCAUUCACCUGGTT-3′(antisense); and si-control, 5′-U UCUCCGAACGUGUCACGUTT-3′(sense) and 5′-ACGUGA CACGUUCGGAGAATT-3′(antisense). The siRNAs (50 pmol) were incorporated into MHCC97H cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfected cells were cultured to further experiments at 48 h and 72 h after siRNA treatment. MHCC97H-siTIP30 and MHCC97H-siCON cells were obtained by transfecting MHCC97H-wt cells with siRNA. All cells were maintained in DMEM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS (Gibco BRL).

Mice and reagents. The animals were 5-week-old BALB/c male nude mice obtained from the Beijing Vital River Company (Beijing, China). Sorafenib (Bayer Healthcare, Leverkusen, Germany) and metformin (1,1-dimethylbiguanide hydrochloride, Sino-American Shanghai Squibb, Shanghai, China) were dissolved in normal saline and were given to mice daily orally. For *in vitro* experiments, sorafenib was resuspended in DMSO and used at a 5-μmol/L concentration; metformin was resuspended in PBS and used at 5 mmol/L or 10 mmol/L concentrations.

Animal models and treatment. All surgical procedures and care given to the animals were in accordance with institutional ethics guidelines. MHCC97H cells were cultured in DMEM containing 10% FBS. After harvesting, 1×10^7 cells were injected s.c. into the posterior flank of nude mice. Four weeks later, the tumors were removed aseptically, rinsed with normal saline, and dissected into 1-mm^3 blocks. After anesthetizing the new recipient mice, we disinfected the skin and cut an incision about 1.5 cm to expose the liver, then implanted one piece of tumor tissue into the liver orthotopically. After surgery, the mice were randomly divided into eight groups ($n = 6$, each group): the control group (normal saline); the metformin group (200 mg/kg); the sorafenib group (30 mg/kg); and the combination group (30 mg/kg sorafenib and 200 mg/kg metformin). Treatment was started 1 week after the surgery and lasted for 4 weeks, after which tumor samples and lung tissues were extracted for further examination. All the drugs were given daily orally. The other four groups ($n = 6$ for each group, used as the survival observation group) received the same treatment, and were used to assess survival. Ethical approval was provided by the Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) research ethics committee.

Examination of lung metastases by H&E staining. Orthotopic tumors were extracted and measured to obtain tumor volumes (V) according to the formula $V = ab^2/2$, where a and b are the largest and smallest diameters, respectively. The lungs were also extracted and fixed with 4% formaldehyde. Serial sections were cut for histologic study at 5 μm. Intermittently, sections were selected and examined for lung metastasis. The number of lung metastases was directly evaluated.

Western blot analyses. Tumor and cell proteins were extracted and the concentrations were measured using a BCA protein assay (Thermo, Fisher Scientific, Massachusetts, USA). Proteins were subjected to Western blot analysis using anti-phospho-AMPK (Cell Signaling Technology, Massachusetts, USA), anti-AMPK (Cell Signaling Technology), anti-TIP30 (Abcam, Cambridge, UK), anti-TXN (Abcam), and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell proliferation and transwell invasion assays. Cell proliferation was examined using a CCK-8 assay (Dojindo, Tokyo, Japan). MHCC97H cells were cultured in 96-well plates (2000 cells/well) in sorafenib (5 μM), metformin (10 mM), and both in combination for 96 h. The procedure for calculating cell proliferation has been described previously.[16] We used transwell chamber inserts (Corning, Corning, NY, USA) to assess cell invasion. Chambers were first incubated with 50 μL diluted Matrigel for 1 h in an incubator. Next, MHCC97H cells were prepared at a concentration of 5×10^6/mL in DMEM only and incubated with sorafenib (5 μmol/L), metformin (10 mmol/L), or both in combination, and were subsequently added in a 200-μL volume to the upper chamber. Then DMEM that contained 10% FBS was added to the lower chamber. After 48 h, cells that had migrated to the other side of the membrane were stained with Giemsa, imaged at ×20 magnification using a light microscope, and counted.

Statistical analysis. Continuous data were expressed as means ± SD. Significant differences between two groups were analyzed by ANOVA. The data were considered statistically significant when P-values were less than 0.05. Statistical tests were carried out with SPSS 17.0 (SPSS, Chicago, IL, USA).

Results

Sorafenib and metformin in combination prolong median survival and reduce tumor volume and lung metastasis in orthotopic MHCC97H models. In the orthotopic MHCC97H models, sorafenib treatment (30 mg/kg/day) reduced tumor volume and prolonged median survival (Fig. 1a,b), but increased the number of lung metastases (Fig. 1c), indicating that the therapeutic effects of sorafenib also result in a concomitant enhancement of invasion and metastasis. When mice were treated with the combination of sorafenib (30 mg/kg/day) and metformin (200 mg/kg/day), median survival, tumor volume, and the number of lung metastases (Fig. 1) were all reduced compared with sorafenib treatment alone. These data suggest a potential synergistic effect of sorafenib and metformin, as the metformin treatment group had no statistically significant differences compared with the control group (Fig. 1). Overall, our data indicate that the combination of sorafenib with metformin can prolong median survival and reduce tumor volume and lung metastasis in orthotopic MHCC97H models.

Metformin increases expression of TIP30 by activating AMPK. We previously showed that TIP30 is a crucial protein down-regulated by sorafenib treatment, which leads to an enhanced metastatic and invasive potential in HCC. Recent work has also pointed to the activation of AMPK as an avenue to inhibit HCC invasion and to increase the drug sensitivity of sorafe-

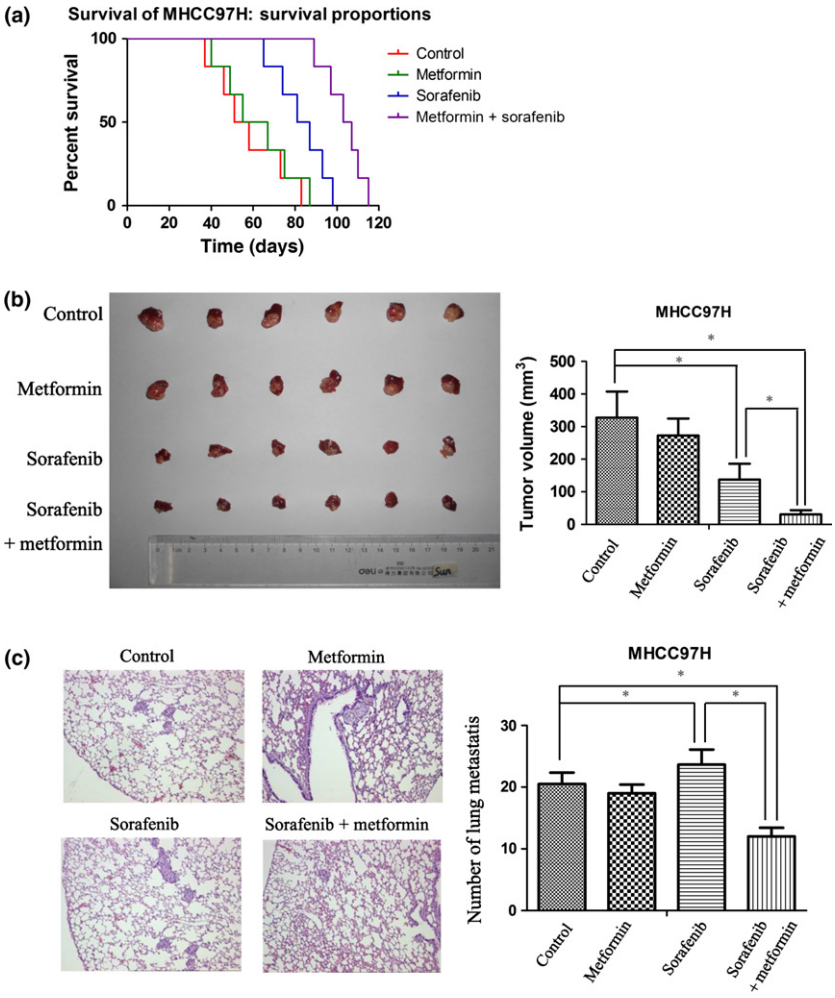

Fig. 1. Combination treatment with sorafenib and metformin prolonged median survival and reduced tumor volume and lung metastasis in orthotopic MHCC97H models. (a) Sorafenib treatment with or without metformin improved median survival in orthotopic MHCC97H models significantly. The median survival was 58.0 ± 17.23 days in the control group, 83.0 ± 12.25 days in the sorafenib group, and 103.5 ± 9.38 days in the sorafenib plus metformin group ($P = 0.007$, $P < 0.001$ versus control group, respectively). Compared with the sorafenib alone, the combination of sorafenib with metforminalso prolonged the median survival significantly ($P = 0.024$). (b) Tumor sizes were 327.52 ± 80.28, 137.50 ± 48.75, and 30.82 ± 12.59 mm^3 in control, sorafenib, and sorafenib plus metformin groups, respectively ($P < 0.001$, $P < 0.001$ versus control group, respectively). Compared to sorafenib treatment alone, the combined treatment group reduced tumor sizes significantly ($P = 0.003$). Sorafenib treatment significantly inhibited the tumor growth, and metformin could further enhance this effect. (c) Number of lung metastases was significantly reduced by the combination treatment, whereassorafenib treatment alone increased the number of lung metastases. The number of lung metastases was 20.5 ± 1.87, 23.67 ± 2.42, and 12.0 ± 1.41 in control, sorafenib, and combined treatment groups, respectively ($P = 0.007$, $P < 0.001$ versus control group, respectively). Data are presented as mean ± SD.

nib.[14,17] Therefore, we assessed whether the increased therapeutic effects we observed with a combination of sorafenib and metformin were mediated by increases in TIP30 expression as well as AMPK activation. We found that metformin markedly upregulated the expression of TIP30 in tumor tissues. Moreover, while sorafenib treatment alone downregulated the expression of TIP30 when compared to the control group in tumor tissues, the combination of sorafenib with metformin increased AMPK activity and upregulated TIP30 markedly, as assayed by Western blotting (Fig. 2a). The expression level of these proteins in the other three groups of orthotopic models are shown in Figure S1. Consistent with the *in vivo* results, sorafenib treatment alone (5 μM) downregulated the expression of TIP30, whereas combined treatment with metformin (5 mM or 10 mM) increased the activity of AMPK and expression of TIP30 in MHCC97H cells (Fig. 2b). It is worth noting that metformin treatment alone upregulated the expression of TIP30 in a dose-dependent manner (Fig. 2b). In sum, our data suggests that metformin upregulates the expression of TIP30 in MHCC97H cells, may be in part by activating AMPK.

Sorafenib combined with metformin inhibits proliferation and invasiveness in MHCC97H cells by upregulating expression of TIP30. The effects of sorafenib and metformin on the viability of MHCC97H cells were studied by CCK-8 assay. As shown in Figure 3(a), the combination of sorafenib (5 μM) with metformin (10 mM) strongly inhibited the proliferation of MHCC97H cells on day 5 of treatment compared with the control group and the sorafenib or metformin treatment alone groups.

In addition, the Matrigel invasion assay was used to detect the ability of invasiveness. As shown in Figure 3(b), sorafenib (5 μM) treatment lowered TIP30 expression and promoted invasion of MHCC97H cells, whereas the combination of sorafenib (5 μM) with metformin (10 mM) increased the expression of TIP30 and repressed invasiveness compared to the control group and the metformin treatment group (Fig. 3b). Furthermore, knockdown of TIP30 by specific siRNAs significantly increased the invasiveness of MHCC97H cells compared with the MHCC97H-siCON group. However, when the cells were treated with sorafenib (5 μM), the invasiveness of MHCC97H-siTIP30 was not markedly upregulated (Fig. 3c), which was consistent with our previous study.[6] Taken together, the combination of sorafenib with metformin inhibits the proliferation and invasiveness of MHCC97H cells, partly by upregulating the expression of TIP30.

Thioredoxin plays an important role in tumor metastasis and is negatively regulated by TIP30. A previous study found that tumor metastasis is associated with the overexpression of TXN *in situ* in the microenvironment of carcinoma and the overexpression of TXN enhances the metastatic potential of tumors.[18] Our data showed that sorafenib treatment alone enhanced pulmonary metastasis, whereas the combination treatment caused a reduction both in inoculated tumor and lung metastasis (Fig. 1). Therefore, we examined the correlation of

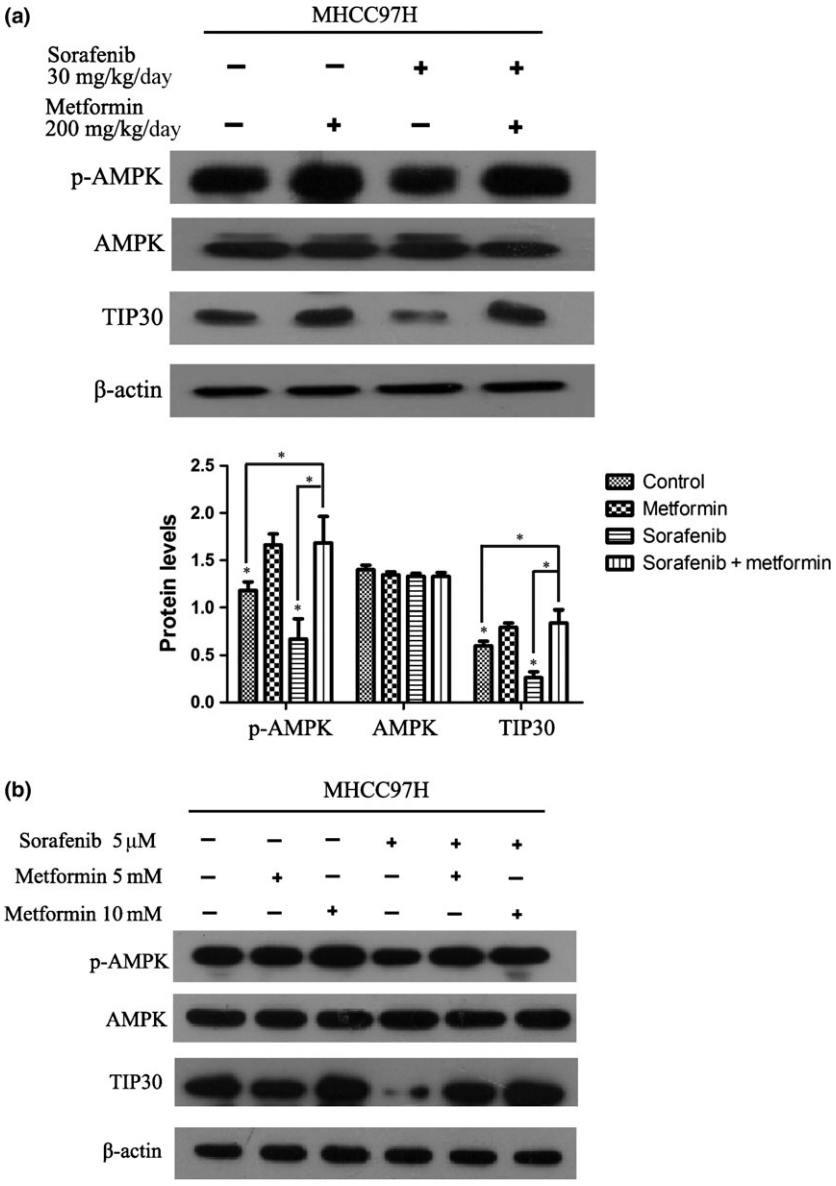

Fig. 2. Metformin upregulated the expression of TIP30 in part by activating AMP-activated protein kinase (AMPK). (a) Expression of p-AMPK and TIP30 were confirmed by Western blotting in orthotopic modelsand normalized with β-actin. The relative levels of protein were compared. Significant differences were analyzed by ANOVA. Data are shown as mean ± SD of three independent experiments, $*P < 0.05$. (b) Expression of p-AMPK, AMPK, and TIP30 was confirmed by Western blotting in MHCC97H cellstreated with sorafenib (5 μmol/L) and metformin (5 mmol/L or 10 mmol/L) for 48 h.

metastatic ability with the expression of TXN in the four treatment groups and found that TXN was upregulated in the sorafenib group and significantly downregulated in the combination treatment group (Fig. 4a). Our *in vitro* experiments also showed a similar result, with sorafenib treatment alone upregulating the expression of TXN and the combination treatment downregulating the expression significantly compared to the control group (Fig. 4b).

Recent work indicated that metformin can increase the expression of TXN through the AMPK pathway.[19] Our data showed that a combination of sorafenib with metformin reduced the expression of TXN, where as sorafenib alone increased the expression of TXN (Fig. 4a). The expression level of TXN in the other three groups of orthotopic models are shown in Figure S1. Thus, we asked whether TXN is regulated by TIP30. Knockdown of TIP30 by specific siRNAs increased the expression of TXN without affecting AMPK activity (Fig. 4c), while shRNA-mediated knockdown of TXN did not affect the expression of TIP30 or AMPK activity (Fig. 4d), suggesting that TXN is downstream of TIP30. Furthermore, when MHCC97H-shTXN cells were treated with sorafenib, the invasiveness was blunted compared to wild-type MHCC97H cells (Fig. 4e). Thus, we speculate that TXN plays an important role in tumor invasiveness and cell motility and is negatively regulated by TIP30.

Discussion

Sorafenib has been proven to be a standard first-line therapeutic agent for advanced HCC. However, the survival benefits in patients are still not satisfactory. Our previous study has shown that low-dose sorafenib treatment, which is quite common in patients who cannot tolerate its severe side-effects, significantly increases the number of lung metastases in mice due to downregulation of TIP30 expression.[6,7]

TIP30 was first identified as a tumor suppressor in small-cell lung cancer,[20] owing to its kinase activity[21] and ability to inhibit tumor cell proliferation and metastasis. Interestingly, lower expression of TIP30 protein was found in 33% of HCC cases.[22] Zhang *et al.*[23] identified that the expression of TIP30 protein in highly differentiated liver cancer tissues was higher than that in poorly differentiated cancerous tissues. Our

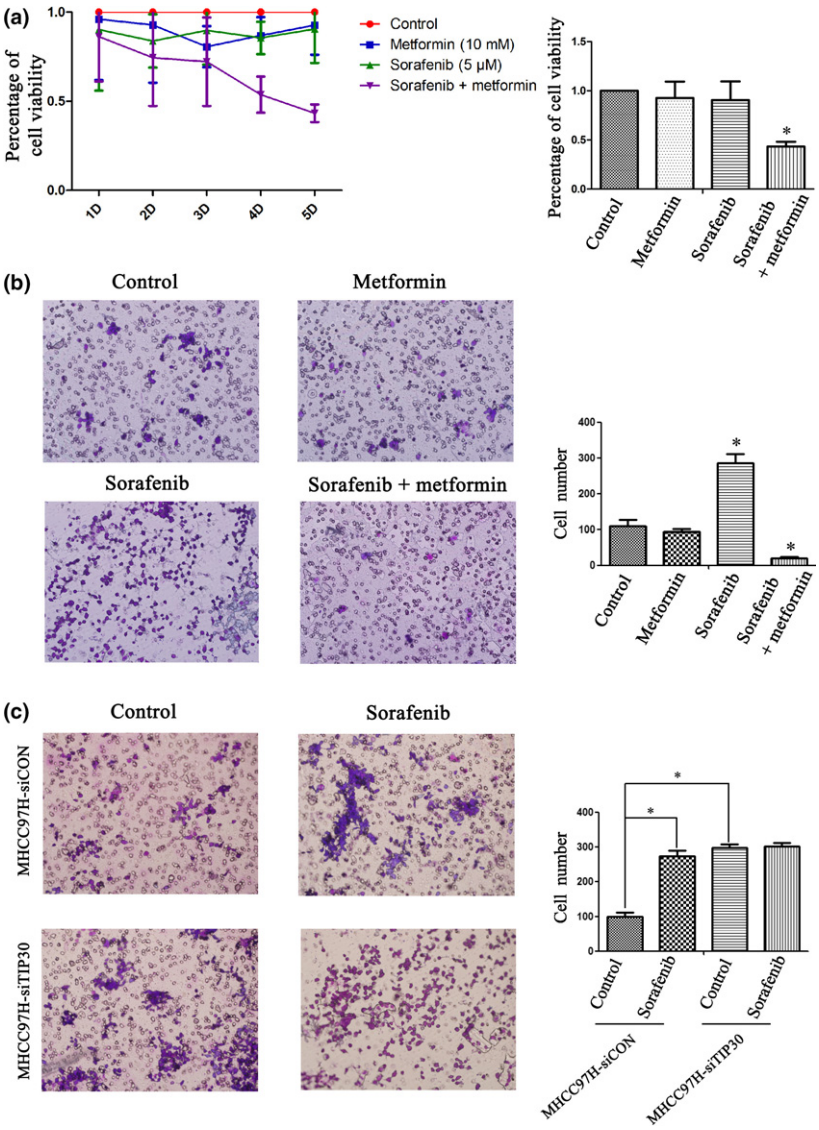

Fig. 3. Combination of sorafenib with metformin inhibited the proliferation and invasiveness of MHCC97H cells, partly by upregulating the expression of TIP30. (a) CCK-8 assay showed that the proliferation was significantly inhibited by combined treatment. (b) Sorafenib alone enhanced the invasiveness of hepatocellular carcinoma cells. The combination of sorafenib and metformin significantly alleviated invasion. (c) Knockdown of TIP30 led to a remarkable increase of invasiveness, but neutralized the effect of sorafenib. The invasion of MHCC97H cells was determined using a Matrigelinvasion assay, photographed at ×20 magnification using a light microscope. Data were represented as mean ± SD of three independent experiments. *$P < 0.05$ versus control.

previous work showed that a low dose of sorafenib had a pro-metastatic effect on HCC, which was caused by downregulation of TIP30 expression.[6] Therefore, TIP30 expression levels are significantly associated with invasion and metastasis of liver cancer. Recent studies have found that TIP30 could not only regulate cell proliferation and apoptotic genes,[15] but was also involved in cell glucose tolerance.[24] In the present study, we used the combination of sorafenib and metformin to treat HCC in a nude mouse model. Surprisingly, our results show that the combined treatment not only prolongs median survival but also reduces tumor volume and lung metastasis by upregulating the expression of TIP30. Although the evaluation method of lung metastasis is different to our previous study,[6] the significant difference does exist. *In vitro* results confirm that the combination therapy inhibits the proliferation and invasion of HCC cells.

The role of metformin in cancer therapy is increasingly prominent. Donadon *et al.*[13] investigated the incidence of HCC in 2924 patients with type 2 diabetes and discovered that metformin significantly reduced the risk of primary hepatic carcinoma compared with insulin. Similarly, Chen *et al.*[25] tracked patients with type 2 diabetes and primary hepatic carcinoma to observe the relationship between diabetes treatment and liver cancer prognosis. However, metformin's specific anticancer mechanism has not been clarified. Some studies suggest that metformin can activate the tumor suppressors in the AMPK metabolic pathway and prompt downstream factors such as mTOR, p53, and FOXO3 to adjust metabolism, growth rate, and the cell cycle.[26–29] Our results confirmed that a combination of sorafenib and metformin upregulated the activity of AMPK and expression of TIP30. Noticeably, downregulation of TIP30 significantly enhanced the expression of TXN, but had no effect on the expression of p-AMPK.

Thioredoxin-1 (also known as thioredoxin or TXN), an important part of the thioredoxin system, is mostly upregulated in cancerous tissue, which may contribute to tumor cells' ability to adjust to the microenvironment and maintain their malignant potential.[30] A report has shown that TXN can scavenge reactive oxygen species and regulate cellular proliferation and apoptosis, and cells expressing a high level of TXN will have the higher antioxidant phenotype correlating with a higher level of aggressiveness.[31] Our previous study has shown that overexpression of TXN could protect HCC cells from DNA

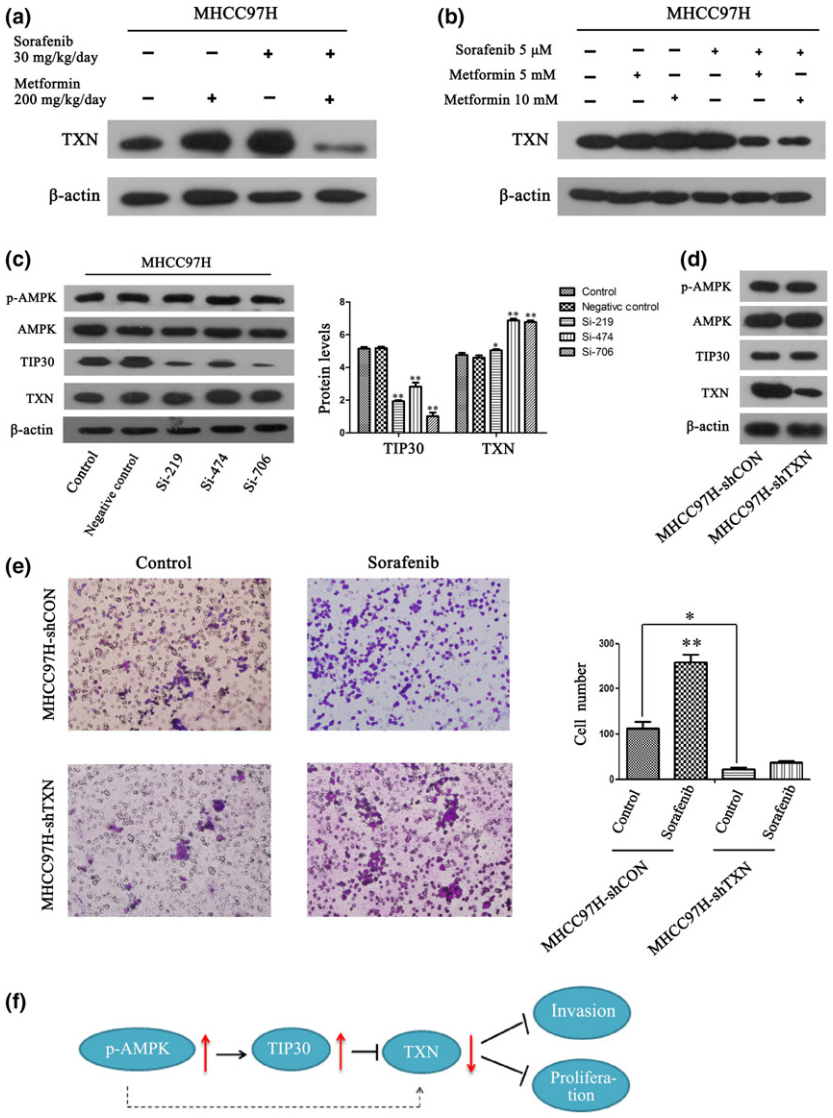

Fig. 4. Thioredoxin-1 (TXN) plays an important role in hepatocellular tumor metastasis, which is negatively regulated by TIP30. (a) Expression of TXN was confirmed at the protein level in orthotopic MHCC97H models. (b) Expression of TXN was evaluated at the protein level in the MHCC97H cell line. (c) MHCC97H cells were transfected with TIP30 siRNA. After 72 h, the expression of AMP-activated protein kinase (AMPK), p-AMPK, TIP30, and TXN were measured by Western blot analysis. The expression of TIP30 and TXN were normalized with β-actin. The relative levels of protein were compared. Significant differences between two groups were analyzed by ANOVA. Data are shown as mean ± SD of three independent experiments. $*P < 0.05$; $**P < 0.01$. (d) Expression of TIP30, p-AMPK, and TXN was confirmed at the protein level in MHCC97H-shCON and MHCC97H-shTXN cell lines. (e) Invasion of the MHCC97H-shTXN cell line after treatment with sorafenib (5 μM) was determined using the Matrigelinvasion assay. Left panel, migrated MHCC97H and MHCC97H-shTXN cells, photographed at ×20 magnification using a light microscope. Right panel, quantification of invasion assay. Data are shown as mean ± SD of three independent experiments. $*P < 0.05$; $**P < 0.01$. (f) Depiction of the interaction between p-AMPK, TIP30, and TXN. Activation of p-AMPK upregulates the expression of TIP30, which reduces the invasion of MHCC97H cells. TXN, positively regulated by AMPK and negatively regulated by TIP30, plays an essential role during the process of metastasis.

damage, and knockdown of TXN inhibits cell proliferation.[32] Kakolyris *et al.*[33] found that excessive expression of TXN correlated with an aggressive phenotype and poor prognosis in non-small-cell lung cancer. We detected the expression of TXN in our four treatment groups and found that TXN was upregulated in the sorafenib group and downregulated in the combination treatment group. *In vitro* experiments showed similar results. Knockdown of TXN led to impaired proliferation and invasion, but had no impact on the expression of TIP30. Therefore, we propose that TXN may act as a downstream factor of TIP30 and execute an important role in the metastasis of HCC (Fig. 4f).

This study shows that metformin can improve the efficacy of sorafenib by reversing the downregulated expression of TIP30. Activation of the AMPK pathway and negative regulation of TXN are mainly responsible for such a modulation. Therefore, metformin might be a potential complement for the treatment of HCC with sorafenib.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Expression of phosphorylated AMP-activated protein kinase (p-AMPK), AMPK, TIP30, and thioredoxin-1 (TXN) in three other groups of orthotopic models.