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Liver X Receptor Agonism Sensitizes a Subset of Hepatocellular Carcinoma to Sorafenib by Dual-Inhibiting MET and EGFR

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Abstract

Sorafenib is the first approved systemic therapy for advanced hepatocellular carcinoma (HCC) and is the firstline choice in clinic. Sustained activation of receptor tyrosine kinases (RTKs) is associated with low efficacy of sorafenib in HCC. Activation of liver X receptor (LXR) has been reported to inhibit some RTKs. In this study, we found that the LXR agonist enhanced the antitumor activity of sorafenib in a subset of HCC cells with high LXR/α gene expression ratio. Mechanically, the activation of LXR suppressed sorafenib dependent recruitment of MET and epidermal growth factor receptor (EGFR) in lipid rafts through cholesterol efflux. Our findings imply that LXR agonist can serve as a potential sensitizer to enhance the antitumor effect of sorafenib.

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Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancerrelated death worldwide [1]. Sorafenib, a tyrosine kinase inhibitor (TKI), is the first approved systemic therapy for advanced HCC and is the firstline choice in clinic. However, only a small part of HCC patients are sensitive to sorafenib [2,3]. Combination of sorafenib with other drugs or compounds maybe a way to enhance the sensitivity of sorafenib. Recent studies have shown that aberrant activations of several receptor tyrosine kinases (RTKs) and their downstream pathways are strongly correlated with the disrupted efficacy of sorafenib [4–7]. Among these kinases, MET and epidermal growth factor receptor (EGFR) are presumed to be the most promising targets, as strategies combining MET or EGFR inhibitors with sorafenib have shown benefits in preclinical models [8–10]. However, compensatory activation of untargeted kinases and unpredictable crosstalk between them have limited further progression of combination strategy [11]. All these observations highlight the necessity of elucidating the mechanism behind overactivation of RTKs and seeking solutions that can block multiple RTKs.

Liver X receptor (LXR) is a member of the nuclear receptor (NR) superfamily of liganddependent transcription factors, which has a key function in regulating cholesterol homeostasis [12]. Recently, accumulating evidences have demonstrated that LXR is involved in a variety of malignancies and is considered highly druggable therapeutic targets [13–17]. Agonists of LXR have shown broadspectrum antitumor effects in various cancers by inhibiting RTKs, such as EGFR and vascular endothelial growth factor receptor 2 (VEGFR2) [18,19]. However, the effect of LXR activation on other RTKs like MET and the mechanism by which LXR inhibits these kinases remain unknown. RTKs and other growth factors depend on complete and stable cytomembrane to promote growth. Since LXRs can regulate membrane composition and function by modulating cholesterol and other lipid metabolism, we suggest that LXRs can inhibit multiple RTKs and the inhibition is related to cholesterol metabolism [19–21]. More recently, increasing malignancies depend heavily on cellular cholesterol to support their growth and metastasis, and LXR agonists have shown remarkable anticancer effects in these tumors by reducing cellular cholesterol [22,23]. But whether cholesterol metabolism plays a central role in the antitumor effects of LXR agonists requires further investigations. And the effect of LXRmediated inhibition of RTKs on sorafenib's efficacy remains to be elucidated.

In this study, we determine the effects of the combination of an LXR agonist, T0901317, and sorafenib on the growth of a subset of HCC cells and their xenografts, and further reveal the underlying mechanism.

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Materials and Methods

Reagents Sorafenib (multikinase inhibitor), T0901317 (LXR panagonist), GW3965 (LXR panagonist), PF04217903 (ATPcompetitive Met inhibitor), Gefitinib (EGFRtyrosine kinase inhibitor), MK2206 (Akt1/2/3 inhibitor), SCH772984 (ERK1/2 inhibitor), and SB202190 (p38 MAPK Inhibitor) were purchased from Selleck Chemicals (Houston, TX, US). Antibodies against LXRα and LXR were purchased from Abcam cooperation (Cambridge, UK). All other antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Puromycin and TRIzol reagent were purchased from Thermo Fisher Scientific (Grand Island, NY, USA). Cell Counting Kit8 (CCK8) was purchased from Dojindo Laboratories (Mashikimachi, kamimashiki gun Kumamoto, JAPAN). PI/RNase Staining Buffer and FITC Annexin V Apoptosis Detection Kit were purchased from BD Biosciences (San Diego, CA, USA). Cholesterol Assay Kit was purchased from Invitrogen (San Diego, CA, USA). BCA Protein Assay Reagent was purchased from Beyotime Biotechnology (Shanghai, China).

Cell Culture

Human HCC cell lines MHCC97H, HCCLM3 were obtained from Liver Cancer Institute, Fudan University, Shanghai, China. Other HCC cell lines Hep3B and HepG2 were purchased from Cell Resources Center, Chinese Academy of Sciences, Shanghai, China. All cell lines were cultured in highglucose DMEM supplemented with 10% FBS in an atmosphere of 5% CO_2 at 37 C.

Clinical Specimens

HCC tumor and nontumor specimens were collected during surgical resection. This study contains 36 HCC patients. None of the patients received any preoperative cancer treatment. Use of HCC specimens was approved by the Ethics Committee of Fudan University.

Growth Assay

Cell proliferation was counted with a CCK8 assay following the manufacturer's instructions. Briefly, 5 10^3 cells were seeded in triplicate in 96well plates and incubated in the presence of T0901317, GW3965, or T0901317 with sorafenib at indicated concentrations for 72 h. Then viable cells were counted by detecting the absorbance at OD 450 nm.

Flow Cytometry Assay

For cell cycle analysis, after treatment with DMSO, T0901317 (1 M), sorafenib (3 M) or combination therapy for 72 h, MHCC97H cells were harvested and fixed and stained with PI/RNase Staining Buffer for 15 min at room temperature before analysis. For apoptosis analysis, cells were harvested and resuspended in 1 Binding Buffer, and stained with PI and FITC Annexin V at room temperature for 15 min before analysis. Data were acquired using a BD FACSCalibur and analyzed by CellQuest software.

Cholesterol Assay

Cholesterol was extracted using chloroform/methanol (2:1) and quantified by Cholesterol Assay Kit following the manufacturer's instructions. After homogenization and spinning, the organic phase was dried and suspended in a reaction mix containing HRP, cholesterol oxidase, and cholesterol esterase. Absorbance at 590 nm was finally measured in a microplate reader.

Lipid Rafts Extraction

Membrane lipid rafts were isolated by ultracentrifugation on sucrose gradients. In general, after treatment with DMSO, T0901317 (1 M), sorafenib (3 M) or combination therapy for 72 h, MHCC97H cells were harvested and resuspended in 0.5 M Na_2CO_3. A discontinuous sucrose gradient of 40%, 35%, 22%, and 5% sucrose were formed. Tubes were subjected to ultracentrifugation at 200000 g for 20 h in Beckman Coulter Optima LE80 K swinging rotor SW 40Ti (Beckman) at 4 C. 24 fractions were collected from the top of the gradient. Samples were then subjected to western blot analysis.

Immunohistochemistry

Immunohistochemistry staining of paraffin sections was performed using a twostep protocol with Novolink Polymer Detection System (NovoLink. TM. Detection System, Leica, UK) and the GTVision II Detection Kit (Gene Tech, Shanghai, China). After antigen retrieval, the slices were incubated with the primary antibody overnight at 4 C, followed by incubation with the secondary antibody at 37 C for 30 min. The sections were stained with DAB and counterstained with hematoxylin (Dako, Glostrup, Denmark), dehydrated in ethanol, mounted in dimethyl benzene, and placed under a coverslip.

Western Blot

Cells were harvested in 1x sample buffer, and then boiled for 15 min. The protein concentration was quantified using BCA protein assay reagent. All proteins were separated on 8–12% SDSPAGE. GAPDH or actin was used as control.

Quantitative Real-Time PCR

Total RNA was isolated using the TRIzol Reagent following the manufacturer's protocol. Reverse transcription was performed with PrimeScript RT Master Mix (Takara Bio). Realtime PCR was performed on a 7900HT Fast RealTime PCR System (Applied Biosystems) using the PrimeSTAR HS DNA Polymerase (Takara Bio). The primers used for amplification of human genes were shown in supplementary materials (Table S1).

Lentiviral Transduction

MHCC97H cells were seeded at a density of 5 10^5 cells/well in 6well plates and incubated until they reached 50% confluence. Then cells were infected with lentiviral media containing shRNA for LXRα (shLXRα), LXR (shLXR) and an empty vector as a negative control (shCTRL). Lentiviral selection was performed by culturing the cells in the presence of 3 g/ml of puromycin for 1 week. The shRNA sequences were shown in supplementary materials (Table S2).

Animal Experiments

Male, 4weekold BALBc nu/nu mice were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Science. All mice were bred in laminar flow cabinets under specific pathogenfree conditions. The experimental protocol was approved by the Shanghai Medical Experimental Animal Care Committee. MHCC97H cells (1 10 [7]) were subcutaneously inoculated into the right flanks of the nude mice. When palpable tumors were formed, mice were randomly assigned to 4 groups (n = 6 for each group), which received a daily oral dose of PBS (control group), 30 mg/kg sorafenib, 10 mg/kg T0901317, or combination ther-

apy for 3 weeks, and tumor samples were then extracted for further analysis. T0901317 was dissolved in Cremophor EL, and sorafenib was in Cremophor EL/ethanol/water (12.5:12.5:75) mixture.

Data Analysis

Data are presented as the mean SEM of three experiments or are representative of experiments repeated at least three times. The data were analyzed using student's T test or Mann–Whitney's test with significance determined as $P < .05$.

Results

LXR Agonist Enhances the Anti-Tumor Effect of Sorafenib in a Subset of HCC Cells

LXR is a key regulator of cholesterol homeostasis and has broad antitumor effects *via* inhibition of multiple RTKs [12,24,25]. We first examined the effect of an LXR agonist, T0901317, on proliferation of four human HCC cells, MHCC97H, HCCLM3, Hep3B, and HepG2, and found that T0901317 treatment had no significant effect on cell proliferation of these cells (Fig. 1*A*). We also used another LXR agonist, GW3965, and got similar results (Fig. 1*B*), therefore, T0901317 was used in the following experiments. To determine the effect of T0901317 on the sensitivity of HCC cells to sorafenib, we treated four HCC cells above with combination of sorafenib and T0901317, and found that T0901317 treatment enhanced the suppression effect of sorafenib on the growth of MHCC97H and HCCLM3 cells, but not in Hep3B and HepG2 cells (Fig. 1*C*). Similar discrepancies in the response to combination therapy were also observed between MHCC97L and Huh7 cells (Fig. S1, *A* and *B*). To further confirm the enhancement of LXR agonist on sorafenib's efficacy, we combined GW3965 with sorafenib in MHCC97H cells, and found similar results (Fig. S1*C*). Moreover, sorafenib also improved the antiproliferation effect of T0901317 in MHCC97H cells (Fig. S1*D*). Combination index (CI) was used to evaluate the effect of combination therapy [26]. CI = 1 indicates additive effect, CI < 1 represents synergism, and CI > 1 means antagonism. To further confirm the above findings, we calculated CI of each cell and found synergism in MHCC97H and HCCLM3 cells (Table 1). These results indicate that T0901317 enhances the antitumor effect of sorafenib in a subset of HCC cells.

High LXR-/α Expression Ratio is Responsible for LXR Agonist-Mediated Enhancement of Anti-Tumor Activity of Sorafenib

To identify the LXR isoform that is responsible for the synergistic effect of LXR agonist and sorafenib, we first examined the expression levels of LXRα and LXR in four HCC cells. High LXR/α gene expression ratio was detected in MHCC97H and HCCLM3 cells (Fig. 2*A*). We then conducted growth assays in MHCC97HshLXRα and MHCC97HshLXR cells. Efficiency of knockdown was confirmed by western blot (Fig. 2*B*). Agonism of LXR was proved by detecting mRNA level of cytochrome

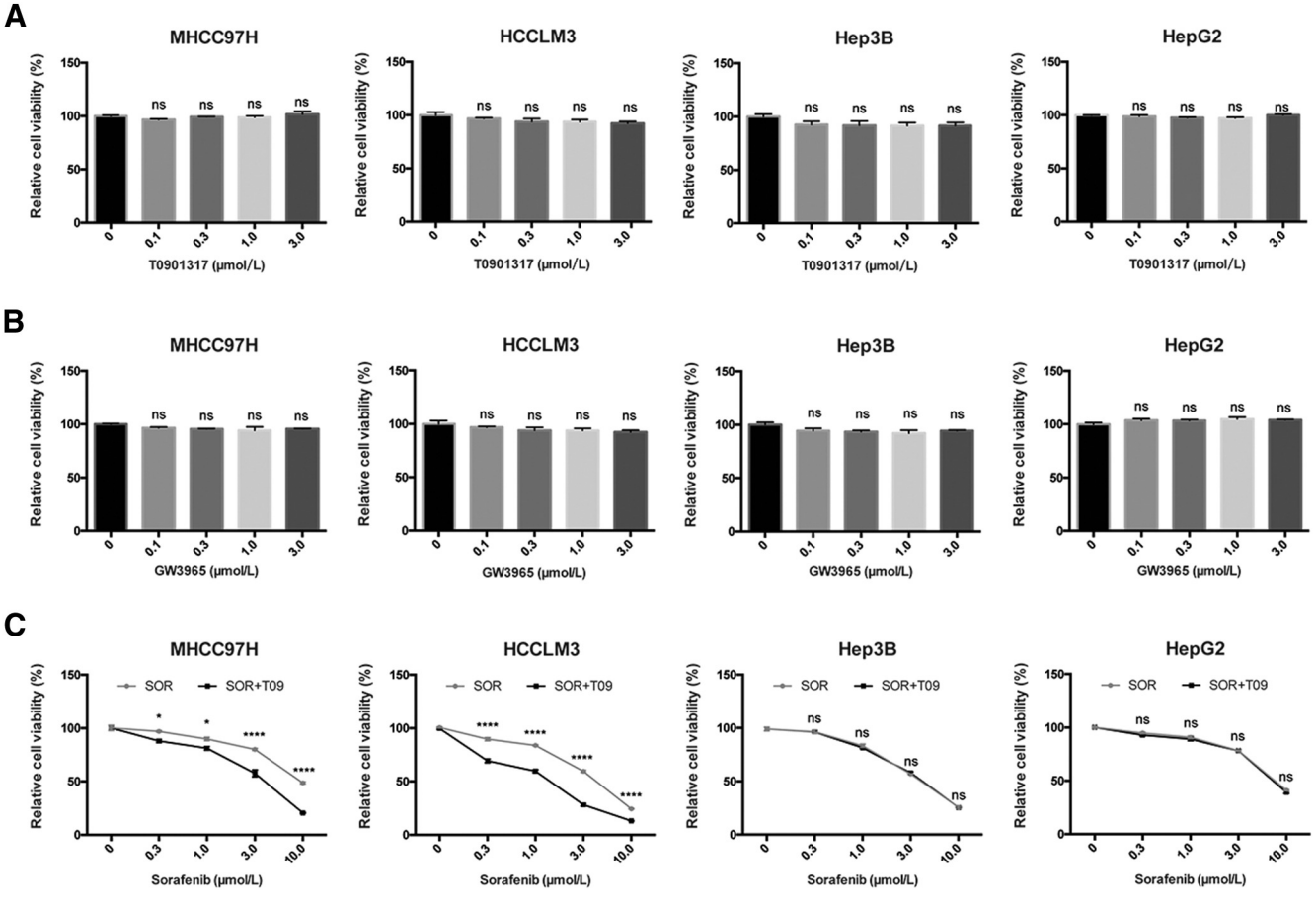

Fig. 1. Anti-proliferative effect of LXR agonists with or without sorafenib in human HCC cells. (A) Effects of T0901317 (T09) on *in vitro* cytotoxicity in MHCC97H, HCCLM3, Hep3B, and HepG2 cells. Cells were incubated in T09 at indicated concentrations for 72 h, then relative viabilities were determined by CCK8 assays. (B) Cells were incubated in GW3965 (GW) at indicated concentrations for 72 h before CCK8 assays. (C) Cells were incubated in sorafenib (SOR) with/without T09 (1 M) for 72 h before detection. N 3, $^{*}P < .05$, $^{****}P < .0001$.

P450 family 7 subfamily A member 1 (CYP7A1), a canonical target gene of LXR (Fig. S2) [27]. The results showed that knockdown of LXR rather than LXRα significantly diminished the synergistic effect (Fig. 2, *C–E*), indicating that high LXR/α expression ratio is responsible for T0901317mediated enhancement of antitumor activity of sorafenib. To further confirm the role of high LXR/α expression ratio in the synergism, we increased the ratio by overexpressing LXR in Hep3B cells (Fig. 2*F*). Synergistic effects were observed in Hep3BLXR cells, which verified the necessity of high LXR/α expression ratio in the synergism (Fig. 2, *G* and *H*). Moreover, a significant correlation between high LXR expression and high tumor grades or poor survival of HCC patient could be observed by using TCGA database (Fig. S3) [28]. Together, these results indicate that high LXR/α expression ratio is responsible for LXR agonistmediated enhancement of antitumor activity of sorafenib.

LXR Agonist Enhances the Anti-Tumor Effect of Sorafenib by the Dual Blockade of MET and EGFR

Previous studies have showed that LXR agonists suppressed tumor growth mainly by inducing proapoptotic effect or cell cycle arrest [29]. However, we did not observe such effects of LXR agonist on apoptosis or cell cycle arrest in our experiment (Fig. S4). Overactivation of RTKs is thought to be involved in impaired efficacy of sorafenib in HCC [30,31]. We found that the expression of MET and EGFR as well as the IC_{50} value of sorafenib were much higher in MHCC97H and HCCLM3 cells than that in Hep3B and HepG2 cells (Fig. S5). LXR activation suppressed both MET and EGFR and their phosphorylation, and therefore inhibited the downstream pathways alone or with sorafenib in MHCC97H cells (Fig. 3, *A–C*). To confirm the role of MET and EGFR pathways in regulating the efficacy of sorafenib, we then examined the effect of inhibitors of MET and EGFR pathways combined with sorafenib on cell growth in MHCC97H cells. The results showed that inhibition of MET, EGFR, Akt and ERK enhanced the antiproliferation effect of sorafenib (Fig. 3, *D–H*). Moreover we found that inhibition of both RTKs and their phosphorylated forms was diminished in MHCC97HshLXR cells comparing with MHCC97HshCTRL or MHCC97HshLXRα cells (Fig. 3*I*). These results demonstrate that LXR agonist enhances the antitumor effect of sorafenib by dual blockade of MET and EGFR.

LXR agonist suppresses recruitment of MET and EGFR in lipid rafts by promoting cholesterol efflux.

Given that LXR is a core regulator of cholesterol homeostasis, we next investigated the role of cholesterol metabolism in LXRmediated RTK sup-

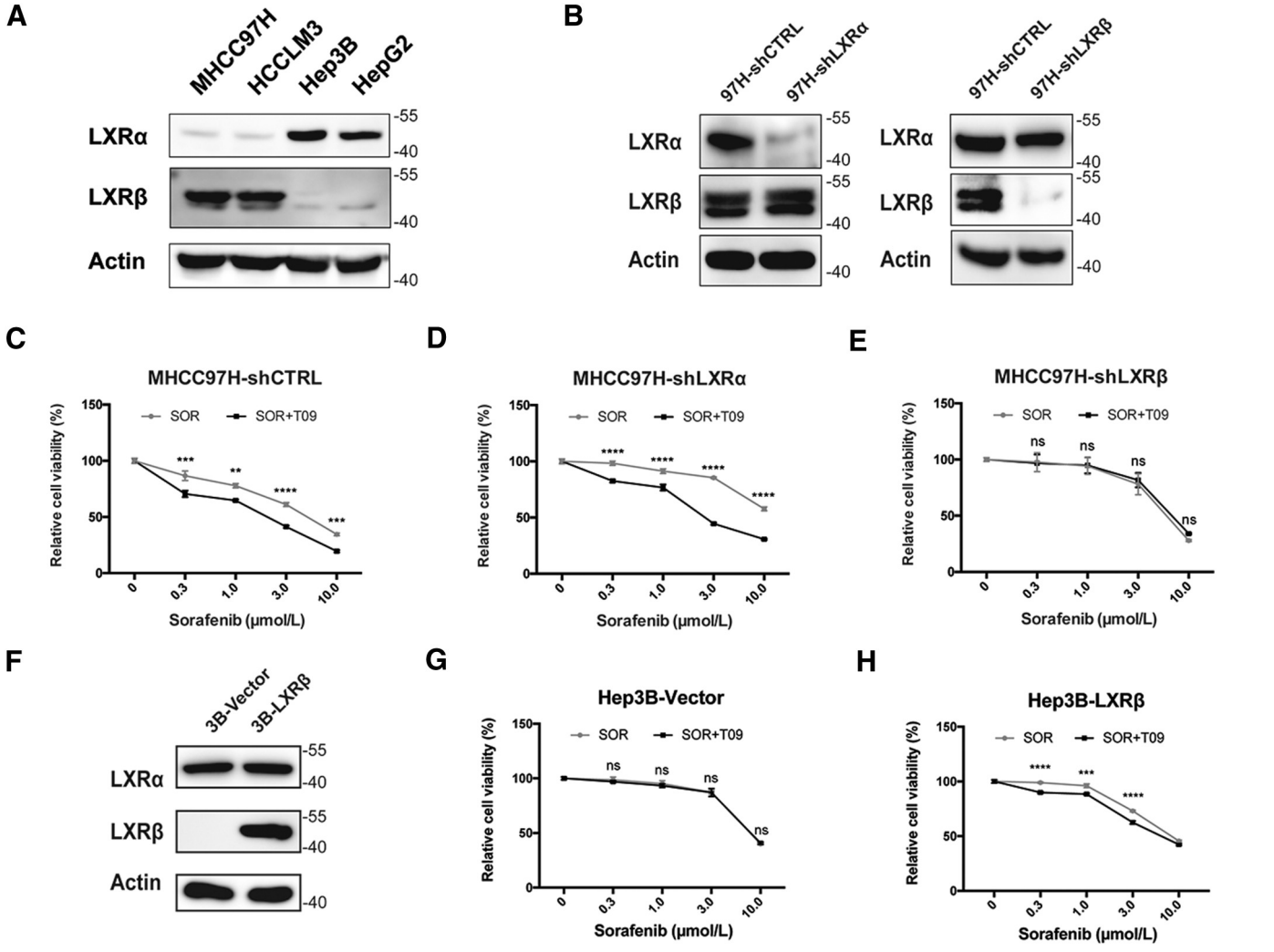

Fig. 2. LXR rather than LXRα exerts the synergistic effect. (A) Expression of LXRs in HCC cell lines detected by western blot analysis. (B) Verification of knockdown efficiency of LXRs in MHCC97H cells by western blot. (C-E) Effects of SOR with or without T09 (1 M) on cell viabilities in MHCC97H-shCTRL (C), MHCC97H-shLXRα (D), and MHCC97H-shLXR cells (E). (F) Verification of LXR overexpression in Hep3B cells by western blot. (G-H) Effects of SOR with or without T09 (1 M) on cell viabilities in Hep3B-Vector (G), and Hep3B-LXR cells (H). N 3, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

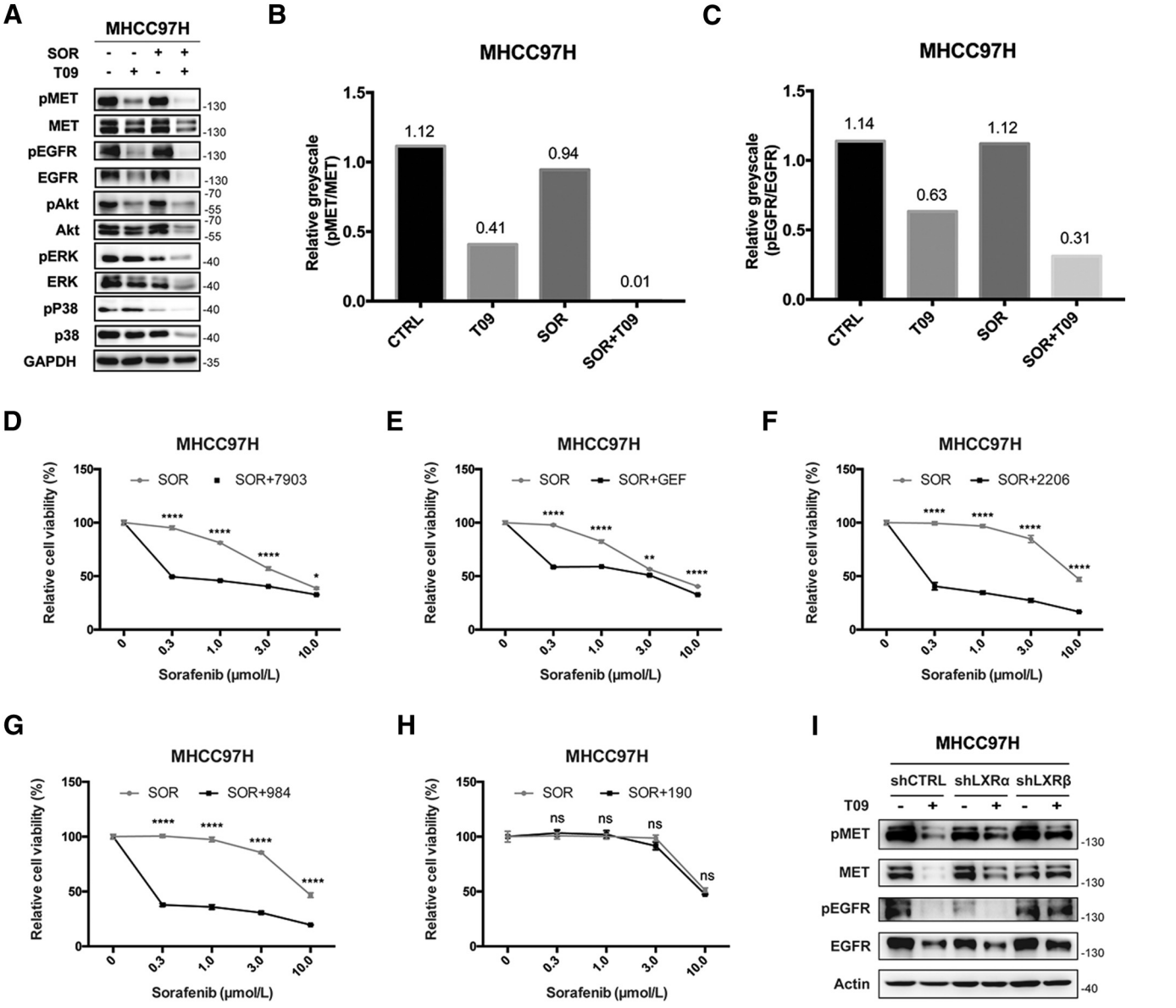

Fig. 3. T0901317 dual-inhibits MET and EGFR. (A) Effects of T09 with or without SOR on MET/EGFR pathways in MHCC97H cells. Cells were incubated in SOR (3 M) with/without T09 (1 M) for 72 h before western blot. (B-C) Quantification of phospho/non-phospho- MET (B) and EGFR (C) in (A). (D-H) Effects of inhibitors targeting MET (D), EGFR (E), Akt (F), ERK (G), and P38 (H) combined with SOR on proliferation of MHCC97H cells. Cells were incubated in SOR with/without PF-04217903 (7903, 10 nM), Gefitinib (GEF, 10 M), MK-2206 (2206, 3 M), SCH772984 (984, 3 M), or SB202190 (190, 3 M) for 72 h before CCK8 assays. (I) Effects of T09 on MET, pMET, EGFR, and pEGFR in MHCC97H-shCTRL, MHCC97H-shLXRα, and MHCC97H-shLXR cells. N 3, $^{*}P < .05$, $^{**}P < .01$, $^{****}P < .0001$.

pression in HCC. We first measured free and total cholesterol levels of MHCC97H cells in each group, and found that T0901317 alone or in combination with sorafenib reduced both free and total cholesterol levels (Fig. 4, *A* and *B*). Lipid rafts are reported to play an important role in the regulation of membrane receptors and their downstream pathways [19,20]. RTKs in lipid rafts are functional forms that promote tumor progression. So, we determined protein levels of MET and EGFR in lipid rafts and found that both RTKs were upregulated after sorafenib treatment and were reinhibited by T0901317 (Fig. 4*C*). Greyscale analysis of MET and EGFR in Fig. 4*C* confirmed the phenomenon (Fig. 4, *D* and *E*). We also examined the expression of ATP binding cassette subfamily A member 1 (ABCA1), a canonical LXR target gene regulating cholesterol efflux [25], and found that ABCA1 gene expression was upregulated by treatment of T0901317 (Fig. 4, *F* and *G*), confirming the activation of LXR. Moreover, we found that cholesterol could rescue reduced cell viability in combination group by restoring MET and EGFR expression (Fig. 4, *H* and *I*). Further analysis using paired tumor and nontumor tis-

Table 1. Combination index (CI) of sorafenib and T0901317 in HCC cell lines

Cell lines	Drug doses (SOR:T09)	CI
MHCC97H	3:1	0.37025
HCCLM3	3:1	0.40034
Hep3B	3:1	1.01512
HepG2	3:1	1.44615

Cells were incubated in sorafenib with T0901317 for 72 h. Cell viability was investigated by CCK8 assay. The CI values of four cells analyzed by CompuSyn software are shown.

sues from 36 patients showed that the cholesterol efflux gene ABCA1 significantly decreased in HCC (Fig. S6*A*), which was consistent with our previous study [32]. Moreover, downregulation of ABCA1 in HCC patients was also observed by using TCGA data (Fig. S6*B*) [28]. These findings demonstrate that LXR agonist suppresses the recruitment of MET and EGFR in lipid rafts by sorafenib *via* promoting cholesterol efflux.

LXR Agonist Enhances the Anti-Tumor Activity of Sorafenib in Xenograft Model

To determine the efficacy of the combination of sorafenib and T0901317 on HCC tumor growth *in vivo*, we established a MHCC97H xenograft model. The combination of T0901317 treatment significantly enhanced the antitumor effects of sorafenib treatment alone (Fig. 5, *A*–*C*). Also, no obvious body weight loss was observed in mice (Fig. 5*D*). These results were coordinated with our previous findings in cell experiments. Since we had uncovered potential mechanisms underline the synergism between sorafenib and T0901317, we then sought to validate dualinhibition of MET and EGFR in mouse model. Given that phosphorylated MET and EGFR were main functional forms which promoted cancer progression, we stained pMET and pEGFR by immunohistochemistry (IHC) in tumor tissues of each group. Data showed that T0901317 alone or with sorafenib significantly decreased both pMET and pEGFR, which was consistent with *in vitro* results (Fig. 5*E*). Further quantification of IHC scores of each group also supported these findings (Fig. 5, *F* and *G*). Besides, upregulation of both pMET and pEGFR were observed in sorafenib group, which indicated compensatory activation. As a multikinase inhibitor, sorafenib was reported to activate various untargeted oncogenic pathways while inhibiting multiple RTKs [33]. And these aberrant activated molecules would provide new targets for combination therapy while counteracting part of sorafenib's antitumor effects. Here in our study, compensatorily activated MET and EGFR were reinhibited by T0901317 in xenograft model. Collectively, our data support the notion that LXR activation enhances the antitumor effects of sorafenib in a subset of HCCs.

Discussion

In the present study, we revealed that reduction of cellular cholesterol *via* LXR activation improved sorafenib's efficacy in a subset of HCC cells with a high LXR/α gene expression ratio. As is reported elsewhere, the two isoforms of LXR have distinct expression patterns and different functions. LXR is ubiquitously expressed at a moderate level in most physiological

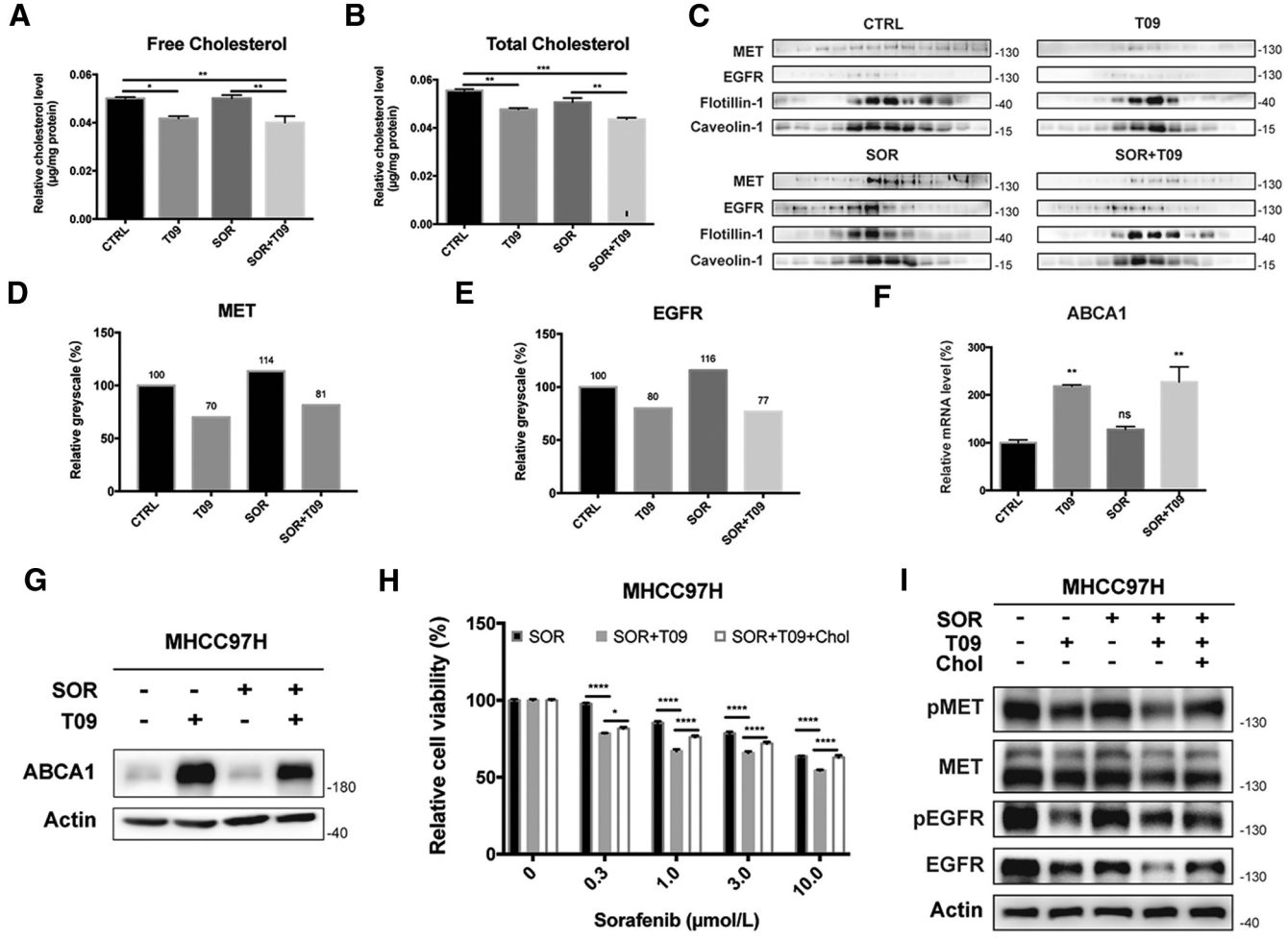

Fig. 4. T0901317 promotes cholesterol efflux by up-regulating ABCA1. (A-B) Quantification of free (A) and total (B) cytoplasmic cholesterol in MHCC97H cells after combination therapy. (C) Western blot analysis of MET and EGFR in lipid rafts in MHCC97H cells incubated in SOR with/without T09. (D-E) Quantification of MET (D) and EGFR (E) in lipid rafts in MHCC97H cells in (C). Expression level in CTRL group was standardized as 100%. (F-G) Quantitative real-time PCR (F) or western blot (G) of ABCA1 expression in MHCC97H cells after combination therapy. (H) Relative cell viabilities of MHCC97H cells treated by SOR, SOR with T09, and combination therapy with Cholesterol (Chol). (H) Effects of T09, SOR, and Chol on MET/EGFR pathways in MHCC97H cells. N 3, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

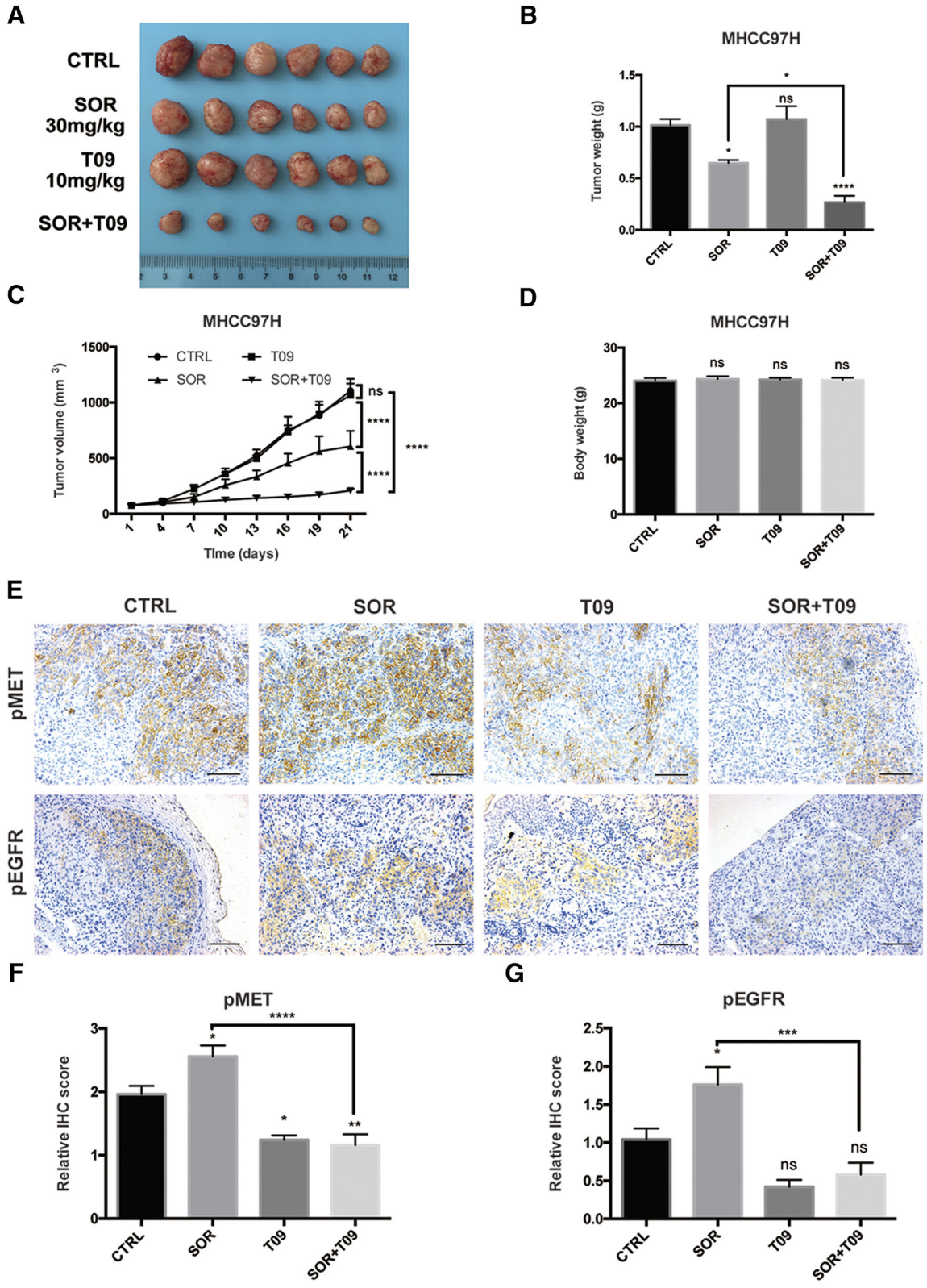

Fig. 5. T0901317 improves the efficacy of sorafenib *in vivo*. (A-B) Tumor volume (A) and tumor weight (B) in MHCC97H xenograft mouse model at day 21 (n = 6). (C) Tumor growth curves in mouse model after SOR and/or T09 treatment. (D) Body weight of the implantation mice at day 21. (E) Representative images of immunohistochemistry (IHC) staining for pMET and pEGFR from MHCC97H xenografts (200 magnification; scale bars, 100 m). (F-G) Quantitative analysis of IHC scores of pMET (F) and pEGFR (G) in each group in (E). N 3, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

systems, whereas LXRα expression is mostly restricted to metabolically active tissues like liver [25]. However, high LXR expression was found in HCC and correlated with high tumor grades and poor survival (Fig. S3) [28]. Such shift from LXRα to LXR may provide new targets for HCC therapy. Besides, despite the highly conserved domains and shared function in cholesterol homeostasis, LXR exerts many more noncanonical effects than LXRα. Recent studies have demonstrated that LXR could interact directly with ABCA1 to modulate cholesterol efflux [34]. These findings hint that high LXR/α gene expression ratio may favor cholesterol efflux under LXR agonism. Other studies have found that activation of LXR increased triglyceride levels mainly through LXRα, while activating of LXR would not increase triglyceride production in the liver [35,36]. Given that increased liver and circulating triglyceride levels are the main side effects precluding further development of LXR agonists, selective activation of LXR is of great importance. GW3965 is a panagonist of LXR with a higher EC50 for LXR [37]. However, no significant differences between GW3965 and T0901317 were observed in their antiproliferation effects in our set (Fig. S1*C*), which was consistent with other studies. One possible reason is that the working concentration of GW3965 is much higher than its EC50 value at both isoforms of LXR. Although we have drawn the conclusion that agonism of LXR can enhance sorafenib's efficacy in high LXR/α expression ratio population, applying LXR specific agonist will further strengthen it.

As is shown above, LXR expression was found to be upregulated in HCC tumor tissues and correlated with high tumor grades and poor survival in TCGA samples (Fig. S3) [28]. Thus, we applied IHC analysis in clinical samples of 10 HCC patients to determine the correlation between LXR and MET or EGFR. However, no significant correlations were observed (data not shown). There are two possible reasons for this phenomenon, one is that LXRs can either be activated or inhibited by different ligands. Therefore, the expression levels of LXRs cannot fully reflect their roles in cancer progression. Another explanation is the heterogeneity of HCC. Due to the low positive incidence of LXR, MET and EGFR, it is reasonable to enlarge cases to determine the correlations.

We have observed that T0901317 with/without sorafenib dual inhibited MET and EGFR in whole cell lysates and lipid rafts (Fig. 3, *A–C* and Fig. 4, *C–E*), which is coordinated with our main conclusion, that is, T0901317 sensitizes HCC cells to sorafenib by dualblockade of MET and EGFR. Another interesting phenomenon was that MET and EGFR in lipid rafts was upregulated after sorafenib treatment (Fig. 4, *C–E*). Combining upregulation of pMET and pEGFR in sorafenib group in mouse model (Fig. 5, *E–G*), we assume that exposure to sorafenib will recruit MET and EGFR in lipid rafts. Since lipid rafts play an important role in signal transduction of membrane receptors including RTKs [38–40], such recruitment by sorafenib is of great significance and requires further investigations.

In conclusion, our results provide new insights suggesting that cholesterol metabolism affects multiple RTKs and their downstream pathways. And targeting cholesterol metabolism improves sorafenib's efficacy in HCC. In addition, this study provides a mechanismbased rationale for the action of targeting cholesterol metabolism in cancer treatment. However, there are still a number of fundamental questions that remain to be answered. Are there other RTKs that response to LXRcholesterol axis, and do they correlate with sorafenib's efficacy? Whether statins or other interventions targeting cholesterol metabolism exhibit similar effects? Does cholesterol metabolism play a central role in regulating the efficacy of other TKIs? All the above questions require further investigations.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2019.08.002.

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