Sorafenib Enriches Epithelial Cell Adhesion Molecule–Positive Tumor Initiating Cells and Exacerbates a Subtype of Hepatocellular Carcinoma Through TSC2-AKT Cascade

Dong-Xian Guan, Jie Shi, Yang Zhang, Jiang-Sha Zhao, Ling-Yun Long, Tian-Wei Chen, Er-Bin Zhang, Yuan-Yuan Feng, Wen-Dai Bao, Yue-Zhen Deng, Lin Qiu, Xue-Li Zhang, Yuan-Yuan Feng, Wen-Dai Bao, Yue-Zhen Deng, Lin Qiu, Xue-Li Zhang, H. Phillip Koeffler, Shu-qun Cheng, Jing-Jing Li, and Dong Xie

Sorafenib is a specific adenosine triphosphate–competitive RAF inhibitor used as a first-line treatment of advanced hepatocellular carcinoma (HCC). However, the responses are variable, reflecting heterogeneity of the disease, while the resistance mechanism remains poorly understood. Here, we report that sorafenib treatment can exacerbate disease progression in both patient-derived xenografts and cell line–derived xenografts and that the therapeutic effect of the drug inversely covaries to the ratio of epithelial cell adhesion molecule–positive cells, which may be tumor initiating cells in HCC. The TSC2-AKT cascade mediates this sorafenib resistance. In response to sorafenib treatment, formation of the TSC1/2 complex is enhanced, causing increased phosphorylation of AKT, which contributes to up-regulation of “stemness”–related genes in epithelial cell adhesion molecule–positive cells and enhancement of tumorigenicity. The expression of TSC2 negatively correlated with prognosis in clinical sorafenib therapy. Furthermore, all-trans retinoic acid decreased AKT activity, reduced the epithelial cell adhesion molecule–positive cell population enriched by sorafenib, and potentiated the therapeutic effect of sorafenib in the patient-derived xenograft model.

Conclusion: Our findings suggest that a subtype of HCC is not suitable for sorafenib therapy; this resistance to sorafenib can be predicted by the status of TSC2, and agents inducing differentiation of tumor initiating cells (e.g., all-trans retinoic acid) should improve the prognosis of this subtype of HCC.

Hepatocellular carcinoma (HCC) is currently the fifth most common cancer worldwide and the second leading cause of cancer-related deaths in China. A pathogenic mechanism associated with hepatocarcinogenesis includes cirrhosis related with hepatic regeneration after tissue damage caused by viral infection, toxins, or metabolic dysfunction.2 Activated RAS/RAF/extracellular signal–regulated kinase (ERK) signaling is found in almost all in vivo HCC models and human HCC tissue samples,3-7 promoting the cell cycle, cell survival, resistance to apoptosis, and metastasis.8 This can co-occur with dysregulation of growth factors and

Abbreviations: ATRA, all-trans retinoic acid; CDX, cell line–derived xenograft; EpCAM, epithelial cell adhesion molecule; ERK, extracellular signal–regulated kinase; FACS, fluorescence-activated cell sorting; HCC, hepatocellular carcinoma; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; OS, overall survival; PDX, patient-derived xenograft; PI3K, phosphoinositide 3-kinase; PTEN, phosphate and tensin homolog; TIC, tumor initiating cell.

From the 1Laboratory of Molecular Oncology, Institute for Nutritional Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China; 2Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China; 3The Second Hospital of Anhui Medical University, Hefei, China; 4Department of General Surgery of FenXian Hospital, Shanghai, China; 5Cancer Science Institute of Singapore, National University of Singapore, Singapore; 6Division of Hematology/Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA.

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their receptors, negative regulators, and RAS mutations. The aberrant RAS/RAF/mitogen-activated protein kinase signaling is an important therapeutic target in HCC.

Sorafenib is a potent inhibitor of RAF kinase both in vitro and in vivo. This inhibitor is also the first agent that demonstrated a benefit on overall survival (OS) of patients with advanced HCC and is currently the standard treatment of this disease. Nevertheless, sorafenib improved only a minor population as observed in the Sorafenib HCC Assessment Randomized Protocol, and no significant difference of the median time to symptomatic progression occurred in either the Sorafenib HCC Assessment Randomized Protocol or the Asia-Pacific Sorafenib trials. Moreover, only rarely do sorafenib-treated tumors regress completely, and the therapeutic effects of the drug are often temporary. Notably, the mechanism of resistance in HCC to sorafenib is unknown.

HCCs are inherently chemotherapy-resistant tumors. Recently, evidence has indicated that tumor initiating cells (TICs) play a pivotal role in recurrence, drug resistance, and patient survival in a variety of tumors. However, the ability of sorafenib to overcome the resistance of TICs is unclear.

In this study, we developed xenograft models using either patient tissues (patient-derived xenograft [PDX]) or cancer cell lines (cell line–derived xenograft [CDX]) to evaluate the therapeutic effect of sorafenib. Surprisingly, sorafenib enhanced progression of HCC associated with enrichment of the epithelial cell adhesion molecule (EpCAM)–positive TICs in a subtype of HCC through the TSC-AKT cascade. Moreover, all-trans retinoic acid (ATRA) reversed this resistance by inducing differentiation of hepatic TICs. Therefore, our study revealed an adverse effect of sorafenib in a subtype of HCC and that perhaps TSC2 can be a biomarker of response to this kinase inhibitor.

Materials and Methods

Establishment of Patient-Derived Xenograft (PDX) and Orthotopic Cell Line–Derived Xenograft (CDX) HCC Model. We improved the PDX modeling from our previous protocol. Fresh HCC samples were used in the establishment of our orthotopic PDX HCC model in 5–6 week old nude mice. Specimens from clinical hepatectomies were orthotopically implanted into the livers of nude mice. Several samples were successfully grown and passes as transplantable tumor lines. For CDX, the liver cancer cell lines (MHCC97H and Huh7) were injected subcutaneously into 4-week-old nude mice. Three weeks after the injection, these cells were orthotopically grafted into the livers of nude mice to establish CDX orthotopic HCC model.

Tumorigenicity Assay. Nude mice were housed under standard conditions. HCC cells (Huh7 or MHCC97H) were trypsinized, washed with D-Hank’s, suspended in DMEM without serum, pretreated either with or without sorafenib and injected subcutaneously into the flanks of 5-week-old male nude mice. Tumors were harvested after 4 weeks. All procedures were performed in agreement with SIBS Guide for the Care and Use of Laboratory Animals and approved by Animal Care and Use Committee, Shanghai Institutes for Biological Sciences.

Subcutaneous Xenograft Growth Model. Xenografts derived from Huh7, were subcutaneous implanted in nude mice and were randomly separated into four treatment groups (DMSO, ATRA, Sorafenib and combined treatment, n = 7 in each group). ATRA was injected intraperitoneally (once every 3 days). Tumor growth was measured every 4 days, and tumor volume was estimated using the formula $0.52 \times \text{length} \times \text{width}^2$. Tumors were harvested, photographed and weighted after 4 weeks. All procedures were in agreement with SIBS Guide for the Care and Use of Laboratory Animals and were approved by Animal Care and Use Committee, Shanghai Institutes for Biological Sciences.

For details of materials and methods, see the Supporting Information.

Results

Effect of Sorafenib on HCC Xenografts. Sorafenib is the first agent demonstrated to have a survival benefit for patients with advanced HCC and is currently the standard treatment of this malignancy. Nevertheless, the responses are variable, reflecting...
heterogeneity of this disease. To evaluate the effect of sorafenib on HCC, we developed PDX murine models. PDX models can reflect the morphology and retain some biomarkers of the primary cancer cells (Supporting Figs. S1-S3). PDX mice bearing different HCC xenografts were divided into two groups, sorafenib treatment or solvent control, and the survival curves plotted (Fig. 1A). The sorafenib-treated group with #064472 PDXs had a significantly longer OS time than the control group. No significant difference between the experimental and control groups was noted with #098203 PDXs. To our surprise, the sorafenib-treated group carrying #064687 and #137646 PDXs had a shorter OS compared to the corresponding control groups. Likewise, sorafenib administration shortened the OS of one (Huh7) of our CDX murine models (Fig. 1B). The third HCC murine model (intrahepatic injection) also showed that one group (Huh7) of two demonstrated shortened survival in sorafenib-treated mice (Fig. 1C). Taken together, these results showed that HCC cells
exhibited a heterogeneous response to sorafenib, indicating that a subset of HCC patients will probably benefit from sorafenib treatment while others will not benefit or may even do worse from the sorafenib therapy.

**Different Effects of Sorafenib on Hepatic TICs.** TICs are thought to be responsible for drug resistance. Compared to the traditional chemotherapy drugs, data are unclear on whether sorafenib can kill TICs. Increasing evidence supports the expression of EpCAM as a marker of TICs in HCC,\textsuperscript{22-25} and we confirmed it both in vitro and in vivo (Supporting Fig. S3A,B).\textsuperscript{26} Patient-derived HCC cells were treated with sorafenib and their hepatic TICs examined by fluorescence-activated cell sorting (FACS). The percentage of EpCAM-positive HCC cells (#064472) decreased from 8.89% to 3.83% with increasing concentrations of sorafenib (Fig. 2A). Paradoxically, the opposite situation occurred in the patient-derived cancer cell (#064687) tumor, with sorafenib treatment increasing the percentage of EpCAM-positive cells from 38.5% to 58.7% (Fig. 2B). Moreover, a similar phenomenon occurred in the HCC cell lines: the percentage of EpCAM-positive cells decreased from

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**Fig. 2. Different responses of the EpCAM-positive cell population to sorafenib treatment.** (A,B) Ratio of EpCAM-positive cell population changes in response to sorafenib treatment of patient-derived cancer cells. (C,D) The ratio of EpCAM-positive cell population alters in response to sorafenib in HCC cell lines. Both primary HCC cells (#064472 and #064687) and HCC cell lines (MHCC97-H and Huh7) were treated with sorafenib for 48 hours. The ratio of EpCAM-positive cell population was analyzed by FACS. The normalized fold change of the ratio of EpCAM-positive cells is displayed in the right panels. All data are the mean ± standard deviation of three independent experiments. Abbreviations: DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; ISO, isotype; SORA, sorafenib; SSC-A, side scatter-area.
1.22% to 0.56% in MCHH97-H and increased from 16.28% to 28.69% in Huh7 cells in the presence of sorafenib (Fig. 2C,D). To explore further, a number of other patient-derived cancer and HCC cell lines were examined. Again, sorafenib exerted different effects on HCC TICs (EpCAM-positive cells) (Supporting Fig. S3D,E), either increasing or decreasing the ratio of EpCAM-positive cells.
TICs are thought to play an important role in tumorigenesis, metastasis, and drug resistance, which are closely related with poor clinical prognosis. We analyzed the percentage of EpCAM-positive cells and the survival curves with sorafenib administration and found that the percentage of EpCAM-positive cells inversely covaried with survival time. This is illustrated in Fig. 1A versus Fig. 2A and in either Fig. 1B (MHCC97H) or C (MHCC97H) versus Fig. 2C (MHCC97H), where the sorafenib group benefited accompanied by a decrease of EpCAM-positive cells. In contrast, the sorafenib group had a worse prognosis in Fig. 4.

Phosphorylation of AKT mediates diverse response to sorafenib. (A,B) Change of Akt phosphorylation in response to sorafenib in Huh7 and MHCC97H cells. (A) Dose response and (B) time response of sorafenib (5 μM). All cells were serum-starved for 12 hours before treatment with sorafenib, then analyzed by western blot. (C) Immunohistochemical assay for pAKT after sorafenib treatment of PDX and CDX models. Tumor-bearing nude mice were established, and tumors were harvested after 4 weeks of sorafenib treatment, fixed in 4% formaldehyde solution in phosphate-buffered saline, embedded in paraffin for immunohistochemical assay, and stained for p-Ser473-AKT. (D) Ratio of EpCAM-positive cells after exposing Huh7 cells to sorafenib and/or LY294002. Cells (1 × 10⁵) were seeded in six-well plates and treated with 5 μM sorafenib or 5 μM LY294002 for 48 hours, followed by FACS analysis. (E) In vitro Huh7 cell colony formation in the presence of sorafenib and/or LY294002. Cells were pretreated as described in Fig. 3A,C. (F) Apoptotic morphology of Huh7 cells after exposure to sorafenib and/or LY294002. Cells (2 × 10⁵) were seeded in six-well plates, treated for 72 hours with sorafenib and/or LY294002, and then photographed. Abbreviations: DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ISO, isotype; NC, negative control; SORA, sorafenib; SSC-A, side scatter-area.
parallel with their increased EpCAM-positive cells (Fig. 1A versus Fig. 2B [Huh7]). We further checked the alterations of TICs by interrogating clonal growth *in vitro* and tumor formation *in vivo* in response to sorafenib treatment. Consistently, sorafenib treatment decreased the colony and tumor formation capability of MHCC97H cells, while it exerted the opposite effects on Huh7 cells (Fig. 3A,B). Moreover, a similar tumor progression and diverse numbers of tumor foci in liver were noted within the MHCC97H-derived and Huh7-derived CDX model (Fig. 3C,D; Supporting Fig. S4A,B). Therefore, our data indicated that a subpopulation of HCC patients may not be appropriate for single-agent sorafenib treatment, as determined by the response of HCC TICs to the drug.

**Activated AKT Blunts the Effects of Sorafenib on HCC TICs.** The mechanism underlying the diverse therapeutic effects of sorafenib on HCC was explored, focusing especially on the subtype of HCCs that had a paradoxical response to the drug. Several signaling pathways involved in the regulation of TICs and EpCAM expression were examined including Wnt/β-catenin signaling.24
Sorafenib did not activate this pathway, as shown by the TOP/FOP reporter assay, β-catenin expression, and its nuclear translocation (Supporting Fig. S5A-C).

AKT is involved in drug resistance in cancer cells and the maintenance of stem cells, leading us to examine whether AKT activity reflected the different responses.
Levels of activated (phosphorylated [p]) Akt changed with sorafenib treatment in a dose-dependent and time-dependent manner. pAKT levels decreased in drug-sensitive MHCC97H cells but increased in drug-resistant Huh7 cells (Fig. 4A,B). The response of pAKT to sorafenib was also examined in the PDX mouse model. pAKT levels did not change in #098203 PDX tumors but increased in xenografts derived from the drug-resistant Huh7 cells and the PDX #064687 and PDX #137646 tumors following sorafenib treatment (Fig. 4C). Taken together, the ability of sorafenib to either increase or decrease pAKT inversely correlated with the ability of the drugs to have a therapeutic effect. To clarify the contribution of AKT to TIC enrichment in selected HCC by sorafenib, we treated Huh7 cells with the phosphoinositide 3-kinase (PI3K)/AKT pathway inhibitor LY294002. As expected, sorafenib increased and LY294002 decreased the percentage of EpCAM-positive Huh7 cells (Fig. 4D). LY294002 also modulated clonal growth in sorafenib-treated cells (Fig. 4E). Also, LY294002 greatly enhanced sorafenib-induced cell death (Fig. 4F).

To explore further the EpCAM-positive cell population in response to sorafenib, we sorted EpCAM-positive and EpCAM-negative Huh7 cells by FACS and treated each with sorafenib. The EpCAM-positive cells were more resistant to sorafenib-induced cell death than the EpCAM-negative cells (Supporting Fig. S6A), but the cell cycle showed consistent changes when comparing the two populations (Supporting Fig. S6B). pAKT levels increased in both the EpCAM-positive and EpCAM-negative populations after sorafenib treatment (Fig. 5A). Sorafenib elevated expression of some “stemness”-related genes, including Sox2, Oct4, Nanog, and EpCAM, in the EpCAM-positive cells but had little effect on expression of these genes in the EpCAM-negative cells (Fig. 5B). Expression of these genes in EpCAM-positive cells decreased in the presence of the PI3K/AKT pathway inhibitor LY294002 (Fig. 5C). Furthermore, EpCAM-positive cells had increased clonal growth in vitro and tumorigenesis in vivo in the presence of sorafenib (Fig. 5D,E). These results suggested that sorafenib stimulation enhanced the stemness and tumorigenicity of EpCAM-positive cells, which was dependent on AKT activation.

**TSC-Mammalian Target of Rapamycin Complex Pathway Mediates Different Responses of AKT Signaling to Sorafenib.** Because sorafenib is a multi-kinase inhibitor, we wondered whether AKT...
phosphorylation was influenced by the RAF/mitogen-activated protein kinase kinase (MEK)/ERK pathway or other targets. Huh7 cells were treated with the MEK1 inhibitor PD98059 and the MEK1 activator phorbol 12-myristate 13-acetate. Both PD98059 and sorafenib increased AKT phosphorylation but phorbol 12-myristate 13-acetate suppressed it (Supporting Fig. S7A), indicating that the sorafenib-induced change of AKT phosphorylation was affected by RAF/MEK/ERK signaling. A prior study reported that stimulation of the insulin-like growth factor receptor/PI3K pathway mediated resistance to an ERK inhibitor in lung cancer.30 We also checked the

Fig. 8. ATRA can potentiate the therapeutic effect of sorafenib. (A) Ratio of EpCAM-positive cells in response to sorafenib and/or ATRA. Cells were treated with ATRA for 4 days and sorafenib for 48 hours and then analyzed by FACS. (B) In vitro cell clonal growth assay of Huh7 cells cultured with sorafenib and/or ATRA. (C) Effect of sorafenib and/or ATRA on phosphorylation of AKT. Cells were pretreated with ATRA (24 hours), followed by sorafenib treatment (1 hour), then analyzed by western blot. Combination sorafenib and ATRA treatment potentiated the therapeutic effect of sorafenib in the ectopic subcutaneous tumor model as measured by tumor weight (D) and growth rate (E). Equal-size xenografts were subcutaneously implanted in nude mice and randomly separated into four groups. ATRA (1.6 mg/kg) was injected intraperitoneally once every 3 days. Tumor growth was measured every 4 days, and tumor volume was estimated using the formula $0.52 \times \text{length} \times \text{width}^2$. Tumors were harvested and weighed after 4 weeks. (F) Survival curves of mice bearing HCC xenografts. Equal-size xenografts were orthotopically implanted in livers of nude mice, and then the tumor-bearing mice were randomly divided into four groups (n = 5 in each group) and orally given either the solvent control or sorafenib (40 mg/kg) every day for 4 weeks. ATRA (1.6 mg/kg)-treated mice received intraperitoneal injections once every 3 days. Abbreviations: DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SORA, sorafenib; SSC-A, side scatter-area.
insulin-like growth factor receptor/P13K pathway but did not find any response following sorafenib treatment (Supporting Fig. S7B). ERK has been reported to regulate phosphatase and tensin homolog (PTEN) translocation by phosphorylating MEK1 at T292, which promoted MEK1-PTEN complex formation.31 However, the phosphorylation of MEK1 (T292) decreased in both MHCC97H and Huh7 cells cultured with sorafenib, and no enhanced PTEN membrane translocation was noted in either cell line (Supporting Fig. S7B,C,D). Thus, the MEK1/PTEN pathway did not participate in the regulation of AKT in response to sorafenib.

As mammalian target of rapamycin (mTOR) complex 1 (mTORC1) is regulated by ERK by changing guanosine triphosphatase–activating protein activity of TSC2 and feedback regulation of AKT(T308) phosphorylation.32,33 mTOR activity was monitored by examining P70S6K phosphorylation. P70S6K phosphorylation was greater in Huh7 cells than in MHCC97H cells at basal conditions, while sorafenib greatly suppressed P70S6K phosphorylation in Huh7 cells but not in MHCC97H cells within a suitable time window (Fig. 6A; Supporting Fig. S8A). Also, the mTORC1 inhibitor rapamycin increased the percentage of EpCAM-positive cells (Fig. 6B) and activated AKT (Supporting Fig. S8B). But the percent of EpCAM-positive Huh7 cells was greater in response to sorafenib than rapamycin (Fig. 6B), indicating that the response to sorafenib should not be attributed to mTORC1 only. AKT phosphorylation at Ser473 is directly caused by mTORC2 kinase, which could be regulated by the TSC1/2 complex.34,35 Endogenous communoprecipitation assay showed that the interaction of TSC1/2 complex did not change in MHCC97H cells but was obviously enhanced in Huh7 cells after sorafenib treatment (Fig. 6C). In order to investigate whether the TSC1/2 complex plays a pivotal role in response to sorafenib, TSC2 expression was silenced by short hairpin RNA in Huh7 cells. This markedly decreased pAKT levels after sorafenib treatment (Fig. 6D), and the percentage of EpCAM-positive cells also did not increase as before (Fig. 6E,F).

In addition, expression levels of TSC2 were significantly lower in MHCC97H cells compared to Huh7 cells (Fig. 6C), suggesting that TSC2 expression level may closely parallel the cellular response to sorafenib. The basal level of TSC2 expression was examined in a variety of HCC cell lines. Hep3B and plc8024 cells weakly expressed TSC2, while 7404 and portal vein tumor thrombus cells displayed high expression of TSC2 (Supporting Fig. S8C). Consistently, sorafenib treatment decreased pAKT in Hep3B and plc8024 cells and increased pAKT in 7404 and portal vein tumor thrombus cells (Supporting Fig. S8D). Also, sorafenib treatment decreased AKT phosphorylation in TSC2 knockout MEF cells (Supporting Fig. S8E). Moreover, the expression level of TSC2 was examined in a set of clinical patients before sorafenib therapy. TSC2 exhibited diverse expression levels by immunohistochemical staining (Fig. 7A), and the level of expression negatively correlated to poor prognosis (Fig. 7B). The average survival time of our set (9.68 months) paralleled that of the Sorafenib HCC Assessment Randomized Protocol study (10.7 months). To our surprise, the mean survival time decreased to 5.58 months in those with a high expression level of TSC2 but increased to 13.67 months in the low TSC2 expression group (Fig. 7C). The difference between the two groups was statistically significant (Fig. 7D). Likewise, a significant difference in HCC relapse was noted between the highest and lowest expression levels of the TSC2 group (Fig. 7E). Thus, the expression level of TSC2 appears to be a predictor of response to sorafenib treatment in HCC.

**ATRA Potentiates the Therapeutic Effect of Sorafenib by Inducing Differentiation of TICs in Sorafenib-Resistant HCCs.** Previously, we demonstrated that ATRA could potentiate cisplatin by inducing differentiation of TICs in liver cancer.26 Because the resistance to sorafenib may be attributed to enrichment of TICs in specific HCCs, we explored if ATRA could eliminate resistance to sorafenib in a similar manner. ATRA treatment inhibited the ability of sorafenib to enrich for EpCAM-positive cells (Fig. 8A), to enhance clonal growth (Fig. 8B), to mediate AKT phosphorylation of Huh7 cells (Fig. 8C), and to increase tumor growth in #064687 PDX (Fig. 8D,E). Most relevant clinically, the combined treatment group had longer survival times than either drug alone in the orthotopic PDX model (Fig. 8F).

**Discussion**

HCC is frequently drug-resistant and usually has multiple molecular pathogenesis pathways.2 Sorafenib has been shown to prolong OS at the late stages of HCC.16-19 Therefore, this multikinase inhibitor was established as the standard of care for patients with advanced HCC. Nevertheless, sorafenib had limited survival benefits (OS, only 2-3 months longer than placebo). Likewise, sorafenib provided a low partial response rate and no increase in median time to symptomatic progression due to drug resistance.18,19 In our study, we identified a potential adverse effect of sorafenib on HCC using several different model systems. The drug enhanced the progression of HCC. This prompted us to explore the mechanism...
by which this occurred and to develop biomarkers to determine which patients might have either a beneficial or an adverse response to this therapy.

TICs (CD24-positive, CD133-positive, EpCAM-positive, and CD90-positive) play an important role in tumorigenesis, metastasis, and drug resistance.20,36 A previous study compared different TIC markers and concluded that EpCAM was a good candidate for HCC.22 We previously demonstrated that EpCAM shared some common characteristics with other cell surface markers, such as CD133, CD24, and CD90.26 In addition, EpCAM expression is regulated by the Wnt/β-catenin signaling pathway and participates in the regulation of several reprogramming genes, including c-MYC, OCT-4, NANO2, SOX2, and KLF4. Thus, EpCAM plays a critical role in the maintenance of "stemness" of HCC cells.24,25 In the present study, we demonstrated distinct effects of sorafenib on TICs in two types of PDX models as well as in HCC cell lines. Furthermore, we found a correlation between the therapeutic effect of sorafenib and the percentage of EpCAM-positive cells.

In our study, the TSC2-AKT pathway was responsible for the different responses to sorafenib in HCC cells. Sorafenib is an inhibitor of multiple kinases,15 including vascular endothelial growth factor receptor and platelet-derived growth factor receptor, which act upstream of Akt. Therefore, sorafenib can decrease the level of active AKT through suppression of the receptor tyrosine kinase. Ras/Raf/MEK/ERK signaling is another major target of sorafenib. Inhibition of Raf by sorafenib suppressed ERK activity, which relieved the suppression of TSC2 by ERK and facilitated formation of the TSC1/2 complex. This complex exerts distinct effects on the mTOR complex: it suppresses the activity of mTORC1, while strengthens the activity of mTORC2. mTOR complexes exert different effects on AKT: mTORC1 inhibits AKT, while mTORC2 promotes AKT activity. Therefore, enhancement of TSC1/2 by inhibition of ERK activity leads to activation of AKT. Thus, the level of active AKT is probably determined by the balance between sorafenib inhibition of receptor tyrosine kinases and signaling by the RAF/Ras/MEK/ERK pathway. We found that phosphorylation of AKT decreased upon sorafenib treatment of HCC cells with low TSC2 level, indicating that suppression of AKT by inhibition on receptor tyrosine kinases may be the major pathway. In contrast, AKT phosphorylation was increased in HCC cells that had relatively high TSC2 levels, suggesting a bias to activation of AKT through inhibition of ERK and subsequent enhancement of the TSC1/2 complex. Furthermore, knockdown of TSC2 increased the sensitivity and rescued the AKT response to sorafenib.

Moreover, the expression of TSC2 successfully predicted sorafenib response in HCC cell lines as well as correlated well to patients’ survival and relapse by examining the expression level of TSC2 in the original human tissue samples. Taken together, our studies suggest that the level of TSC2 might help to predict the prognosis of clinical therapy of HCC.

The retinoid ATRA is a potent mediator of differentiation of selected cancers.37 It can induce differentiation of a variety of tumor cells, including stem cells; and it is one of the treatments of choice for acute promyelocytic leukemia.38 Previously, we demonstrated that ATRA could induce differentiation of esophageal squamous carcinoma cells as well as potentiate cisplatin by inducing differentiation of TICs in HCC cells.26,39 In the present study, we demonstrated that ATRA decreased the percentage of EpCAM-positive cells even after sorafenib treatment and that the combination of sorafenib with ATRA potentiated the therapeutic effect using an HCC xenograft murine model.

Taken together, our results indicate the potential adverse effect of sorafenib and the underlying mechanisms of this effect. The expression level of TSC2 may be a good biomarker to predict the efficiency of sorafenib therapy. Moreover, ATRA plus sorafenib can have a combined anti-HCC activity. This is, to our knowledge, also one of the first demonstrations that a PDX model can mimic and reflect the clinical drug response and prognosis in HCC.

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