Sorafenib/MEK inhibitor combination inhibits tumor growth and the Wnt/β-catenin pathway in xenograft models of hepatocellular carcinoma

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Abstract. Mutations affecting the Wnt/β-catenin pathway have been identified in 26-40% of hepatocellular carcinoma (HCC) cases. Aberrant activation of this pathway leads to uncontrolled cell proliferation and survival. Thus, identifying Wnt/β-catenin pathway inhibitors may benefit a subset of patients with HCC. In the present study, the effects of sorafenib and a MEK inhibitor on tumor growth and Wnt/β-catenin signaling in HCC models were evaluated. A β-catenin mutant and β-catenin wild-type HCC models were treated once daily with i) 10 mg/kg sorafenib, ii) 15 mg/kg refametinib (or 25 mg/kg selumetinib), or iii) sorafenib/refametinib. Western blotting was employed to determine changes in biomarkers relevant to Wnt/β-catenin signaling. Apoptosis, cell proliferation and β-catenin localization were analyzed by immunohistochemistry. Sorafenib/refametinib markedly inhibited tumor growth and cell proliferation, and caused cell death in naïve and sorafenib-resistant HCC models. Despite similar total β-catenin levels, significant reductions in phosphorylated (p)-RanBP3 Ser58, p-β-catenin Tyr142, active β-catenin and β-catenin target genes were observed in sorafenib/refametinib-treated tumors. Greater levels of β-catenin in sorafenib/refametinib-treated tumors were accumulated at the membrane, as compared with in the control. *In vitro*, sorafenib/refametinib inhibited the Wnt/β-catenin pathway and suppressed Wnt-3A-induced p-low-density lipoprotein receptor-related protein 6 Ser1490, p-RanBP3 Ser58 and p-β-catenin Tyr142 in HCC cells. Combination of sorafenib and refametinib inhibits the growth of naïve and sorafenib resistant HCC tumors in association with active suppression of β-catenin signaling regardless of β-catenin mutational status. Thus, the sorafenib/MEK inhibitor combination may represent an alternative treatment for patients with HCC whose tumors develop resistance to sorafenib therapy.

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Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer mortality worldwide, and accounts for 500,000 to 1 million deaths annually worldwide (1). More than 80% of HCCs are discovered at a late stage when surgery is not an option (2). The 5-year-survival rate for resectable HCC ranges between 15 and 39% (3), largely due to tumor recurrence. Two randomized controlled trials of sorafenib in patients with HCC demonstrated an improvement in median overall survival of ~3 months, and established sorafenib as a standard of care in advanced HCC (4,5). Although sorafenib improves overall survival, the benefit is at best modest and confers a rather transient clinical benefit (4,5). Recently, FDA has approved lenvatinib and regorafenib as first line and second line treatments, respectively, for HCC (6). Thus, effective novel therapies to combat this deadly disease are urgently required.

Aberrant activation of the Wnt/β-catenin signaling pathway is found in up to 67% of HCC cases, underlining its importance in hepatocarcinogenesis (7). Tumors with nuclear and/or cellular accumulation of β-catenin had, generally, poorly differentiated morphology (8), high proliferative activity (9), high vascular invasion and a dismal prognosis (7-9). Gene expression studies have revealed distinct molecular subclasses in HCC, each associated with unique clinicopathological features. CTNNB1 (β-catenin exon 3 mutated) class signature, which accounts for 20-30% of all HCC cases, a subset of S3, is enriched in hepatitis C virus (HCV)-related HCC and portends a better prognosis. Notably, the S1 subclass, which is characterized by non-mutated Wnt/β-catenin activation signaling, is associated with non-HCV-related HCC, a larger tumor with moderately/poorly differentiated histology, propensity for vascular invasion

and the development of satellite lesions, which is translated to early recurrence compared with other subclasses (10,11). Several non-mutational mechanisms, including mutations in Axin 1 and Axin 2, have been put forward to explain the increased Wnt/β-catenin signaling (12).

Besides cooperation with transforming growth factor-β, promoter methylation of secreted frizzled-related protein members and the overexpression of frizzled (FZD) receptors have been implicated (11). The accumulation of Wnt/FZD-signaling dysregulation was also revealed to be associated with increased activation of downstream effectors, β-catenin, protein kinase C and c-Jun N-terminal kinase further implicating the importance of Wnt/β-catenin signaling in hepatocarcinogenesis (13). These observations lend support to its functional relevance and therapeutic value.

Selumetinib (AZD6244; AstraZeneca, Cambridge, UK) is an allosteric adenosine triphosphate-uncompetitive inhibitor of MEK1/2 (14). Refametinib (BAY 86-9766; Bayer AG, Leverkusen, Germany) is a nonadenosine triphosphate competitive inhibitor targeting MEK 1/2 (15). Refametinib and selumetinib were selected for the present study as both have similar mechanisms in HCC. Although lack of activity was observed with selumetinib (14) and refametinib (16) monotherapies, they exhibited significant antitumor activity when combined with sorafenib in clinical trials (16,17).

The aim of the present study was to investigate the effects of sorafenib and MEK inhibitor on tumor growth and the β-catenin signaling pathway in HCC.

Materials and methods

Reagents. Antibodies against disheveled segment polarity protein (DVL)2 (#3224), DVL3 (#3218), protein kinase B (AKT; #9272), β-catenin (#8480), Axin2 (#5863), Cyclin D1 (#2978), c-Myc (#5605), c-Jun (#9165), low-density lipoprotein receptor-related protein (LRP)6 (#2560), survivin (#2803), E-cadherin (#3195), cleaved caspase 3 (#9661), cleaved poly (ADP-ribose) polymerase (PARP; #5625), and phosphorylation-specific antibodies against AKT Ser473 (#9271), glycogen synthase kinase (GSK)-3αβ (Ser21/9; #9331), RanBP3 Ser58 (#9380), non-p (Active) β-catenin Ser33/Ser37/Thr41 (#8814), phosphorylated (p)-histone 3 Ser10 (#9701), LRP6 Ser1490 (#2568), α-tubulin (#2144) and extracellular signal-regulated kinase (ERK)1/2 Thr202/Tyr204 (#4370) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies against ERK1/2 (sc-94), glutamine synthetase (SC-74430) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-mouse cluster of differentiation (CD)31 antibody (#102502) was from BioLegend, Inc. (San Diego, CA, USA). p-β-catenin Tyr142 (#CP1081) was purchased from ECM Biosciences LLC (Versailles, KY, USA).

Conditioned medium. L-cells (ATCC CRL-2648) and L-Wnt-3A cells (ATCC CRL-2647) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Control-conditioned medium from L-cells and Wnt-3A-conditioned medium from L-Wnt-3A cells was prepared according to the protocols provided by the ATCC. The conditioned medium was concentrated 2X and sterile-filtered.

Cell culture. Between January 2004 and September 2018, 280 primary HCCs were obtained during surgery at Department of General Surgery, Singapore General Hospital (Singapore) and used for establishment of HCC PDX models. Written informed consent was obtained from all patients prior to tissue collection and the present study received Ethics Committee approval from National Cancer Centre Singapore (NCCS; Singapore) as well as Singapore General Hospital. HCC PDX models were established as described previously (18). Primary HCC13-0109, HCC26-0808A, and HCC06-0606 cells were freshly isolated from PDX tumors as follows: HCC tumors were finely minced and washed 3 times with modified Eagle's medium (MEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The minced tissue was incubated with MEM containing 5% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA) and 5 mg/ml collagenase (Roche Diagnostics, Indianapolis, IN, USA) at 37˚C for 12 h. Cells were harvested by centrifuging at 4˚C at 800 x g for 10 min. The cell pellets were washed 3 times with serum-free MEM, and primary HCC cells were plated at a density of $5.0x10^6$ cells per well in MEM containing 10% FBS and 1% penicillin-streptomycin (growth medium), and incubated at 37˚C and 5% CO_2 for 48 h. The primary cells were then used for subsequent experiments.

Primary HCC06-0606 or HCC07-0409 cells were treated with the vehicle, 0.5 μM refametinib, 2.5 μM sorafenib or 2.5 μM sorafenib plus 0.5 μM refametinib in growth medium at 37˚C for 24 h then stimulated with concentrated conditioned medium prepared either from the control L-cells or L-Wnt-3A cells at 37˚C for 2 h. The cells were harvested, and changes in the levels of proteins of interest were determined by western blotting.

Antisense β-catenin transfection. Phosphorothioate oligonucleotides (ODN) directed against β-catenin antisense (5'-TAAGAGCTTAACCACAACTG-3') and scrambled control (5'-CAGTAATCGAATAGCTACCA-3') were purchased from TriLink Biotechnologies (San Diego, CA, USA). A further $5x10^6$ HCC06-0606 cells were transfected with 150 nM scrambled control or 150 nM ODN directed against β-catenin using Plus™ Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C for 48 h. The cells were subsequently assayed for β-catenin protein and its downstream targets by western blotting.

Combined treatment with sorafenib plus MEK inhibitor (refametinib or selumetinib). The present study was approved by the Ethics Board at NCCS (Singapore). Mice were maintained at NCCS according to the 'Guide for the Care and Use of Laboratory Animals' (19). All animal experiments were performed at NCCS.

Sorafenib (Nexavar™; Bayer AG) and selumetinib (14) (Selleck Chemicals, Houston, TX, USA) were suspended in vehicle [30% captisol in water: 5% glucose (50% v:v)] at 1.25 and 3.125 mg/ml, respectively. Refametinib (Bayer AG) (15) was diluted in dimethyl sulfoxide at a concentration of 100 mg/ml as stock. Stock refametinib (0.150 ml) was

further diluted in 3.925 ml 30% captisol in water followed by 3.925 ml 5% glucose to obtain an appropriate concentration.

Establishment of HCC patient-derived xenograft (PDX) model. Primary HCCs have previously been used to establish HCC PDX xenograft lines (18), of which the following 9 HCC lines (HCC01-0207; HCC25-0705A; HCC10-0505; HCC09-0913; HCC01-0708; HCC29-0909A; HCC06-0606; HCC26-0808A, and HCC07-0409), as well as one sorafenib-resistant HCC26-0808ASora54, were used to establish tumors in 450 male C.B-17 SCID mice aged 9-10 weeks and weighed 23-25 g (InVivos Pte. Ltd., Singapore) as described previously (18,20). Mice were provided with sterilized food and water *ad libitum*, and housed in negative pressure isolators, which were set at 23˚C and 43% humidity, with 12-h light/dark cycles.

Development of a sorafenib-resistant HCC model. C.B-17 SCID mice bearing HCC26-0808A tumors (18) were treated once daily with 10 mg/kg sorafenib for 80 to 90 days. Following the initial response to sorafenib, HCC26-0808A mice gradually acquired resistance, leading to further tumor growth. Sorafenib-resistant tumors were harvested for serial transplantation when they reached the size of 1,500 mm^3. The cycle was repeated until sorafenib had minimal impact on the growth of treated tumors.

Mice bearing the indicated tumors (8-10 mice per group) were orally administered either 200 μl vehicle, 10 mg/kg sorafenib, 15 mg/kg refametinib (or 25 mg/kg selumetinib) or 10 mg/kg sorafenib plus refametinib daily for 12-14 days. Treatment commenced when the tumors reached 150-175 mm^3 in size. Bi-dimensional measurements were performed once every 2-3 days and tumor volumes were calculated based on the following formula: Tumor volume = [(length) x ($width^2$) x (π/6)], and plotted as the means ± standard error of the mean for each treatment group vs. time, as previously described (18). Body and tumor weights were recorded at the time of sacrifice. The tumors were harvested 2 h following the final treatment and stored at -80˚C for later biochemical analysis. The efficacy of each treatment was determined using the T/C ratio, where T and C are the median weight of drug-treated and vehicle-treated tumors, respectively, at the end of treatment.

Western blot analysis. Tumors from vehicle- and drug-treated mice were homogenized in buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 25 mM NaF, supplemented with proteinase inhibitors and 10 mM Na_3VO_4. Protein concentration was determined by Bradford assay. In total, 80 μg protein/lane was resolved by either 8% or 14% SDS-PAGE, and western blotting was performed as previously described (21). All primary antibodies were diluted in 1% low-fat skimmed milk powder dissolved at a ratio of 1:1,000 to a final concentration of 1 μg/ml. Blots were incubated with primary antibodies for 14-16 h at 4˚C. After 3 washes in TBST, blots were incubated with a secondary antibody [horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G (H+L; #31460; 1:5,000; Thermo Fisher Scientific, Inc.] for 60 min at room temperature. All primary antibodies were then visualized using WesternBight™ ECL chemiluminescent detection reagents (Advansta, Inc., Menlo Park, CA, USA). For quantification analysis, total density of the band corresponding to protein blotting with the indicated antibody was quantified using the GS-900 Calibrated Densitometer and Image Lab™ Software 6.0.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), normalized to both the tubulin loading control and the appropriate phosphorylated/total protein where applicable, and expressed as the fold-change.

Immunohistochemistry. Tumor tissues were fixed in PBS buffer containing 4% formaldehyde (ICM Pharma, Pte. Ltd, Singapore) for 24 h at room temperature and embedded in paraffin. Tissue sections (5-μm) were blocked with 5% low-fat skimmed milk powder dissolved in TBS containing 0.1% Tween (TBST) for 1 h at room temperature, followed by 3 washes in TBS. Sections were then incubated with CD31 (1:100), p-histone 3 Ser10 (1:300), cleaved PARP (1:100), and total β-catenin (1:150) antibodies for 14-16 h at 4˚C to assess micro-vessel density, cell proliferation, and apoptosis, cytoplasmic and nuclear β-catenin, respectively, as described (20). Following 3 washes in TBST (5 min each), sections were incubated with biotin-conjugated goat anti-rabbit IgG secondary antibody (#31820; 1:150; Thermo Fisher Scientific, Inc.) for 45 min at room temperature, followed by 3 washes in TBS and then incubated with streptavidin HRP-conjugated antibody (#21126; 1:500; Thermo Fisher Scientific, Inc.). Slides were counterstained with hematoxylin (Sigma Diagnostics, Inc., Livonia, MI, USA) for 10 sec at room temperature. Images were captured on an Olympus BX60 light microscope (Olympus Corporation, Tokyo, Japan). Five random 0.159-mm^2 fields at x100 magnification were captured for each tumor. The number of p-histone 3 Ser10 and cleaved PARP-positive cells among at least 500 cells per field was counted and expressed as the number of positive cells per 1,000 cells. For the quantification of mean microvessel density in sections stained for CD31, 5 random fields at a magnification of x100 were selected for each tumor.

Whole exome sequencing (WES). The CTNNB1 (β-catenin) mutational status of 9 HCC models was determined via WES as described previously (22).

Statistical analysis. Data were presented as the mean ± standard error of the mean. Differences in the protein level, tumor weight at sacrifice, p-histone 3 Ser10 index, mean micro-vessel density, and the number of cleaved PARP-positive cells were compared. Student's t-test was used for comparisons between two groups. One-way analysis of variance followed by the Tukey-Kramer method post-hoc test was used when comparing more than two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Sorafenib/refametinib treatment inhibits tumor growth independent of β-catenin mutational status. WES analysis revealed that HCC29-0909A harbored a T41A β-catenin mutation, Y126 p53 mutation and EZH2 intergenic deletion; HCC10-0505 harbored a S45P β-catenin mutation, p53 deletion, and 2108G>T JAK1 mutation. HCC01-0708 harbored a T41A β-catenin mutation, Y126 p53 mutation, and N-RAS amplification; HCC07-0409 harbored a T41A

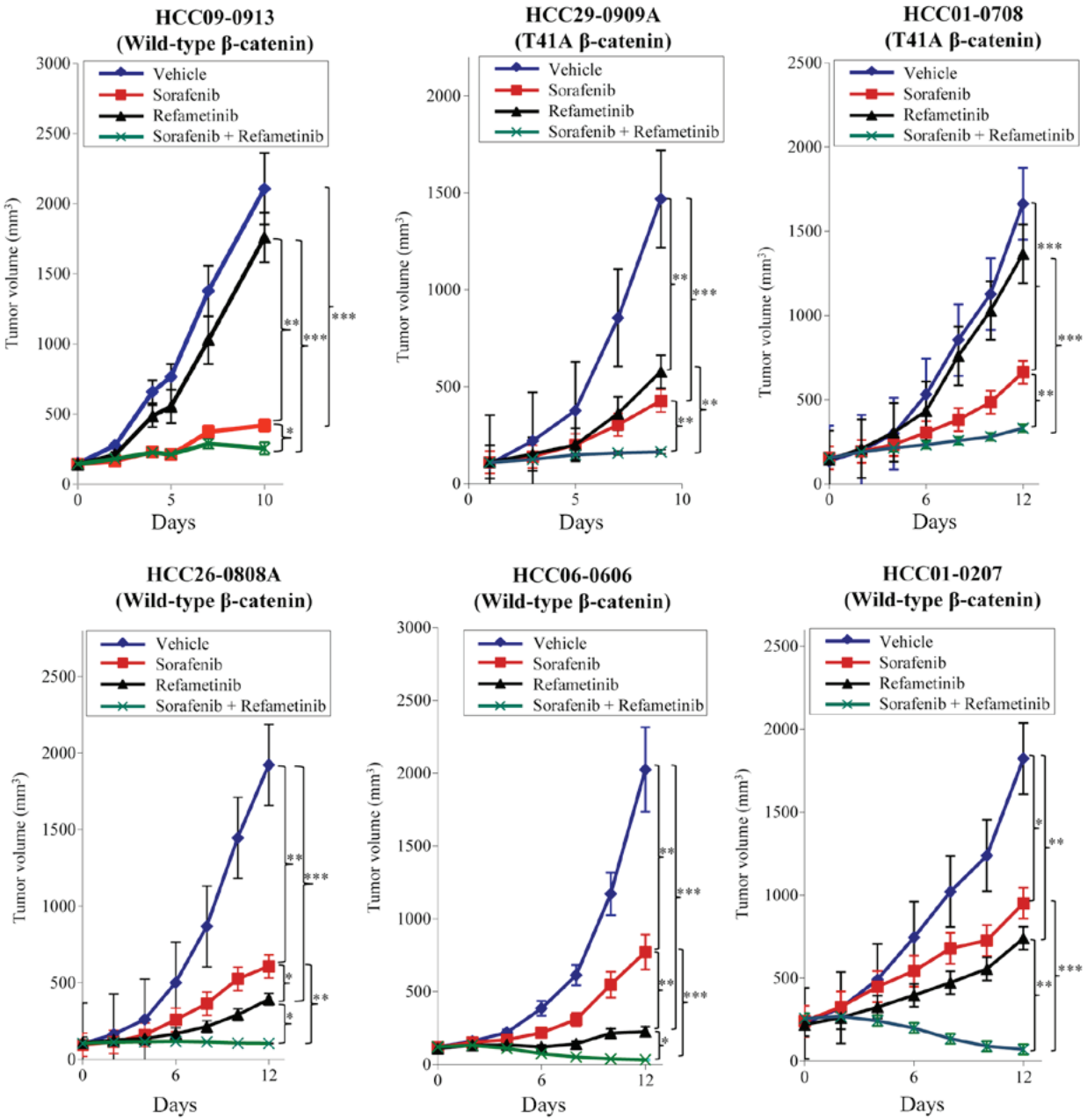

Figure 1. Effects of sorafenib, refametinib and sorafenib plus refametinib on tumor growth in wild-type and mutant β-catenin hepatocellular carcinoma models. Mice bearing the indicated tumors were orally administered 200 μl vehicle, 10 mg/kg sorafenib, 15 mg/kg refametinib or sorafenib plus refametinib for the indicated number of days. Sorafenib and refametinib were given once daily. Each treatment arm comprised 8-10 independent tumor-bearing mice. Tumor volumes were calculated and plotted as the means ± standard error of the means. One-way analysis of variance followed by the Tukey-Kramer method post-hoc test was used to establish significant differences among groups. *P<0.05, **P<0.01 and ***P<0.001.

β-catenin mutation, Y126 p53 mutation, and CCND1 and N-RAS amplification; HCC26-0808A had a NF1 deletion, H168R p53 mutation, 3G>T VHL mutation, and loss of CDKN2A and CDKN2B; HCC06-0606 harbored a 407A>C p53 mutation and a MCL1 amplification; HCC01-0207 had a 341G>A RET mutation, R249S p53 mutation and LRP1B truncation; HCC25-0705A had a 341G>A RET mutation and R249S p53 mutation; HCC09-0913 harbored a R249S p53 mutation. HCC01-0708, HCC07-0409 and HCC29-0909A harbored a T41A β-catenin mutation, and HCC10-0505 had S45P β-catenin mutations. These observations are consistent with those of previous studies, indicating that mutations in the β-catenin gene account for 20-30% of all HCC cases (10,11).

As treatment with sorafenib led to the upregulation of p-ERK1/2 (21) and ERK kinase activated Wnt/β-catenin signaling (23), the combined effects of sorafenib/MEK inhibitor on tumor growth and the Wnt/β-catenin signaling pathway were evaluated. Consistent with our previous study (21), oral delivery of sorafenib led to significant inhibition of tumor growth as compared with the vehicle group (P<0.01). Addition of refametinib to sorafenib resulted in significantly improved anti-tumor activity in all HCC models tested when compared with either agent alone (P<0.01; Fig. 1). As compared with the vehicle group, refametinib significantly inhibited tumor growth of HCC29-0909A, HCC26-0808A, HCC06-0606 and HCC01-0207 (P<0.01; Fig. 1) but not HCC09-0913 and

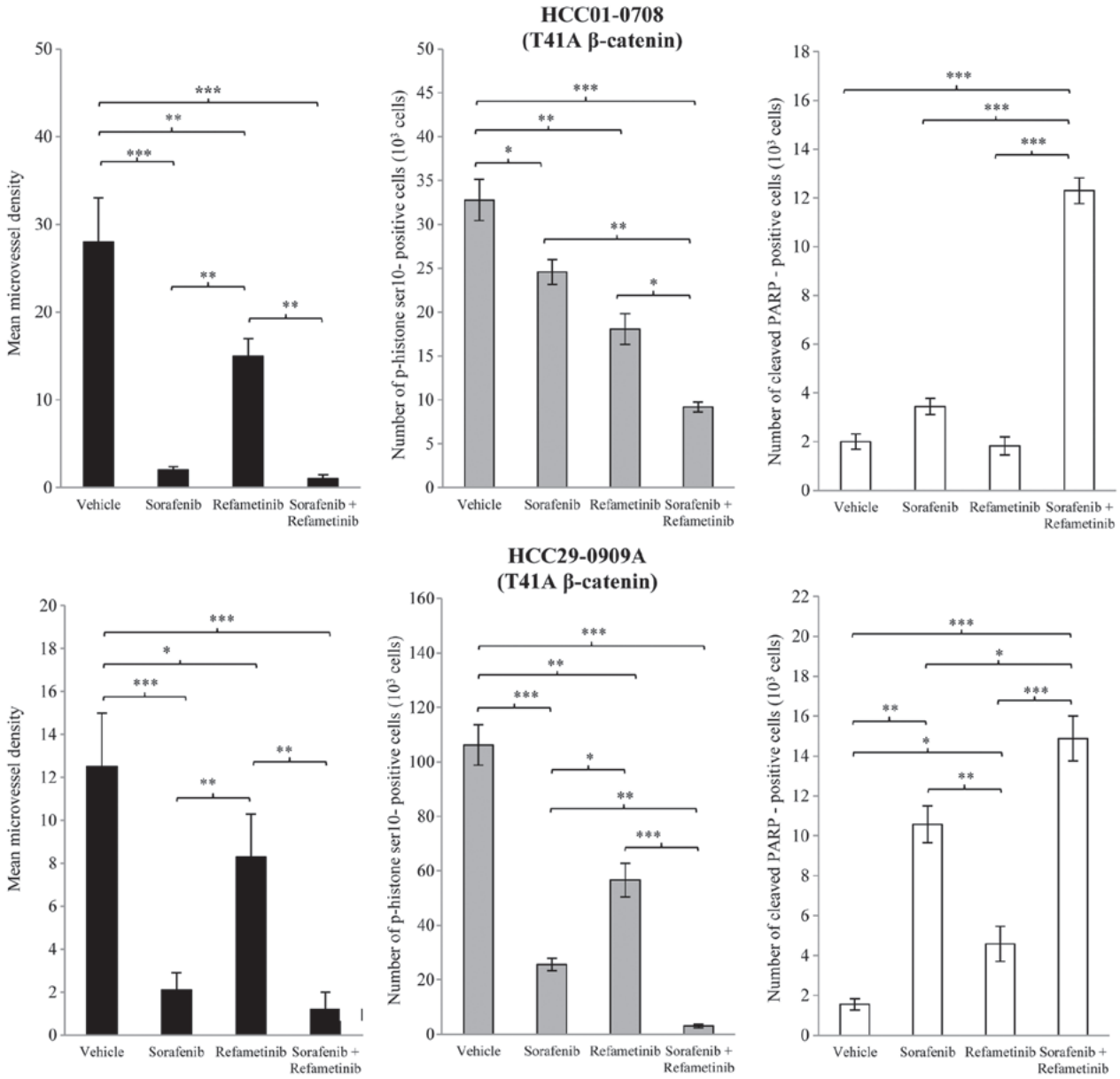

Figure 2. Effects of sorafenib/refametinib on angiogenesis, cell proliferation and apoptosis in T41A mutant β-catenin HCC01-0708 and HCC29-0909A models. Mice bearing the indicated tumors were treated with 200 μl vehicle, 10 mg/kg sorafenib, 15 mg/kg refametinib or sorafenib/refametinib for 10-15 days. Sorafenib and refametinib were given once daily. Tumor tissues were collected 2 h following the final treatment. Each treatment arm comprised 8 independent tumor-bearing mice. Sections (5-μm) were immunostained with cluster of differentiation 31, p-histone 3 Ser10 and cleaved PARP antibodies to assess microvessel density, cell proliferation and apoptosis, respectively. The number of p-histone 3 Ser10 and cleaved PARP-positive cells, among at least 500 cells counted per region, was determined and plotted as the mean number of positive cells per 1,000 cells ± SE. For the quantification of the mean microvessel density ± SE in each section, 5 random 0.159 mm^2 fields at a magnification of x 100 were observed for each tumor. Images were captured via light microscopy. Differences in mean microvessel density, p-histone 3 Ser10 and cleaved PARP-positive cells were compared using one-way analysis of variance followed by the Tukey-Kramer method post-hoc test. *P<0.05, **P<0.01 and ***P<0.001. p, phosphorylated; PARP, poly (ADP-ribose) polymerase; SE, standard error of the mean.

HCC01-0708. Tumor regression was observed in wild-type β-catenin HCC26-0808A, HCC06-0606, HCC01-0207 and HCC09-0913 models following sorafenib/refametinib treatment. The antitumor activity of sorafenib/refametinib was independent of β-catenin mutational status as both mutant and wild-type β-catenin xenografts were inhibited by this combination (Fig. 1). Refametinib monotherapy was not considered to be active in HCC01-0708 (mutant β-catenin) and HCC09-0913 (wild-type β-catenin) models (Fig. 1), suggesting that factors other than β-catenin were responsible for the responsiveness. As predicted, sorafenib/refametinib inhibited cell proliferation and caused increased apoptosis, as compared with sorafenib or refametinib alone (P<0.05; Fig. 2). Similar results were obtained when selumetinib was combined with sorafenib (data not shown).

Sorafenib/refametinib combination treatment suppresses the β-catenin signaling pathway independent of its mutational status. The effects of sorafenib/refametinib combination on β-catenin and its downstream targets in mutant β-catenin HCC29-0909A and wild-type β-catenin HCC06-0606 xenografts were then investigated. As presented in Fig. 3, sorafenib alone had minimal effect on p-LRP6 Ser1490

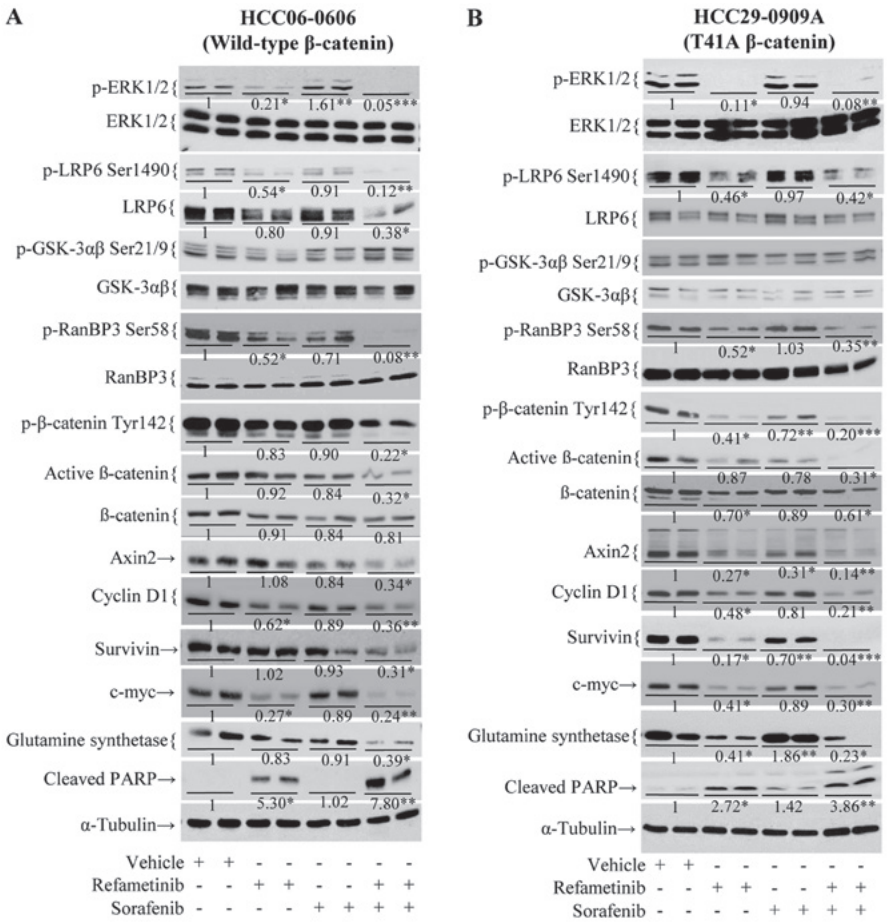

Figure 3. Effects of sorafenib/refametinib on Wnt/β-catenin signaling in (A) wild-type β-catenin HCC06-0606 (B) and T41A mutant β-catenin HCC29-0909A models. Mice bearing the indicated tumors were treated with 200 μl vehicle, 10 mg/kg sorafenib, 15 mg/kg refametinib and sorafenib/refametinib once daily for 5 days. Each treatment arm comprised 3-4 independent tumor-bearing mice. Tumors were collected 2 h following the final dose for marker analysis. Tumor lysates were analyzed by western blotting. Blot membranes were incubated with the indicated antibodies. Representative blots are presented. Total density of the band corresponding to protein blotting with the indicated antibody was quantified, normalized to both the tubulin loading control and the phosphorylated/total protein (e.g., ERK and LRP6) and expressed as the fold-change. *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle. p, phosphorylated; ERK, extracellular signal-regulated kinase; LRP, low-density lipoprotein receptor-related protein 6; GSK, glycogen synthetase kinase; PARP, poly (ADP-ribose) polymerase.

and p-RanBP3 Ser58. Quantification of the immunoblots revealed that a decrease in these proteins was observed in refametinib-treated HCC06-0606 (Fig. 3A) and HCC29-0909A xenografts (Fig. 3B). Notably, sorafenib/refametinib resulted in a significant decrease in p-LRP6 Ser1490 (P<0.05; Fig. 3). As presented in Fig. 3, there was no significant difference in p-GSK-3αβ (Ser21/9) levels between the treatment groups. Although total β-catenin was modestly reduced, p-β-catenin Tyr142, p-RanBP-3 Ser58 and active β-catenin in sorafenib/refametinib-treated tumors were significantly reduced (P<0.05; Fig. 3). Significant reduction of β-catenin downstream targets (Axin-2, survivin, c-Myc, cyclin D1 and glutamine synthetase) in sorafenib/refametinib-treated tumors was observed (P<0.05; Fig. 3). Similar results were observed when wild-type HCC09-0913 and mutant β-catenin HCC01-0708 were treated with sorafenib/selumetinib (data not shown).

Sorafenib/refametinib combination treatment causes export of β-catenin to the membrane. The tyrosine kinases have been demonstrated to induce phosphorylation of β-catenin at tyrosine 142 (24,25). As p-β-catenin Tyr142 (24) and p-RanBP3 (26) have been reported to be involved in β-catenin export, whether sorafenib/refametinib treatment caused nuclear export was examined. Tumor sections among different treatment groups were stained with total β-catenin antibody. Intense nuclear and cytoplasmic β-catenin staining was observed in vehicle-treated mutant β-catenin tumors (HCC29-0909A and HCC01-0708) (Fig. 4). The majority of β-catenin in sorafenib-treated tumors was located in the cytoplasm and nucleus (Fig. 4). Refametinib markedly reduced nuclear and cytoplasmic β-catenin concomitant with a marked increase in membranous β-catenin (Fig. 4). There was no clear difference in β-catenin relocalization between sorafenib- and refametinib-treated tumors. Notably, nuclear β-catenin staining was barely detectable in HCC29-0909A and HCC01-0708 tumors treated with sorafenib/refametinib (Fig. 4). The nuclear staining was less pronounced in the HCC10-0505 model with the S45P mutant of β-catenin, suggesting that there may be functional differences amongst the various mutations (data not shown). In HCC29-0909A and HCC01-0708 models, a membranous signal accumulated in areas of intercellular contact, likely representing β-catenin associated with membranous cadherins (24,27). These observations suggest that β-catenin export from the nucleus and/or the cytoplasm to the membrane following sorafenib/refametinib treatment likely occurs via

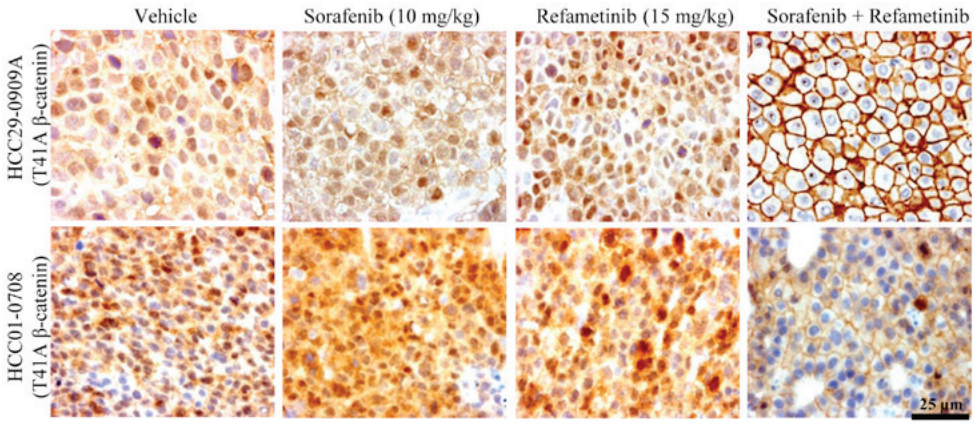

Figure 4. Membrane localization of β-catenin following sorafenib/refametinib treatment in T41A mutant β-catenin HCC29-0909A and T41A mutant β-catenin HCC01-0708 patient-derived xenograft models. Mice bearing the indicated tumors were treated with 200 μl vehicle, 10 mg/kg sorafenib, 15 mg/kg refametinib or sorafenib/refametinib for 10-12 days. Each treatment arm comprised 8-10 independent tumor-bearing mice. Tumor tissue samples were collected 2 h following the last dose and paraffin-embedded. Sections (5-μm) were immunostained with total β-catenin antibody to assess cytoplasmic, membranous and nuclear β-catenin. Representative images of tumor sections from vehicle- and drug-treated tumors stained for β-catenin are presented. Scale bar, 25 μm.

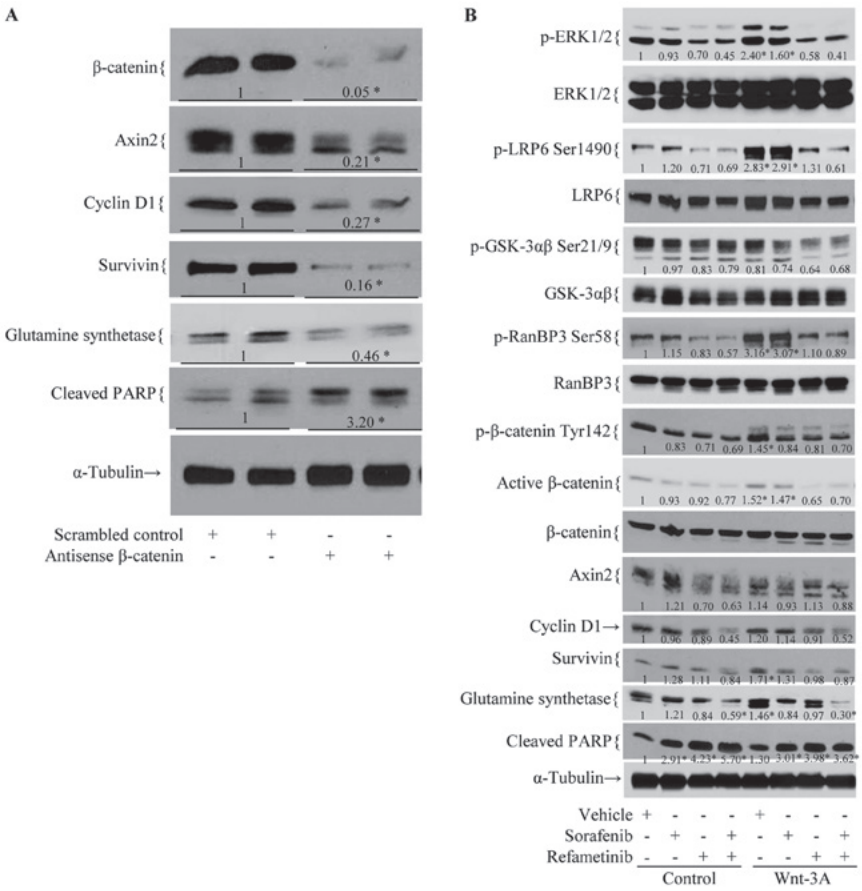

Figure 5. Effect of antisense β-catenin on β-catenin signaling, and the effect of sorafenib/refametinib on Wnt-3A-stimulated activation of Wnt/β-catenin signaling in wild-type β-catenin HCC06-0606 cells. (A) Primary HCC06-0606 cells were transfected with scrambled control or antisense β-catenin for 48 h. The cells were subsequently assayed for β-catenin protein and its downstream targets by western blotting. *P<0.05 vs. scrambled control. (B) Primary HCC06-0606 cells were treated with 1 μl of vehicle, 0.5 μM refametinib, 2.5 μM sorafenib or 2.5 μM sorafenib plus 0.5 μM refametinib in modified Eagle's medium containing 2% fetal bovine serum for 24 h then stimulated with concentrated conditioned medium prepared either from the control L-cells or L-Wnt-3A cells for 2 h. Western blot analysis was performed. Representative blots are presented. Quantification of proteins involved in the β-catenin-signaling pathway was performed. *P<0.05 vs. vehicle control. PARP, poly (ADP-ribose) polymerase; p, phosphorylated; ERK, extracellular signal-regulated kinase; LRP6, low-density lipoprotein receptor-related protein 6; GSK, glycogen synthase kinase.

the inhibition of p-β-catenin Tyr142 (25) and p-RanBP3 (26). β-catenin relocalization also occurred when HCC09-0913 tumors were treated with sorafenib/selumetinib (data not shown).

Sorafenib/refametinib inhibits Wnt-3A-induced activation of the β-catenin signaling pathway. As presented in Fig. 5A, treatment with antisense, but not scrambled control, β-catenin

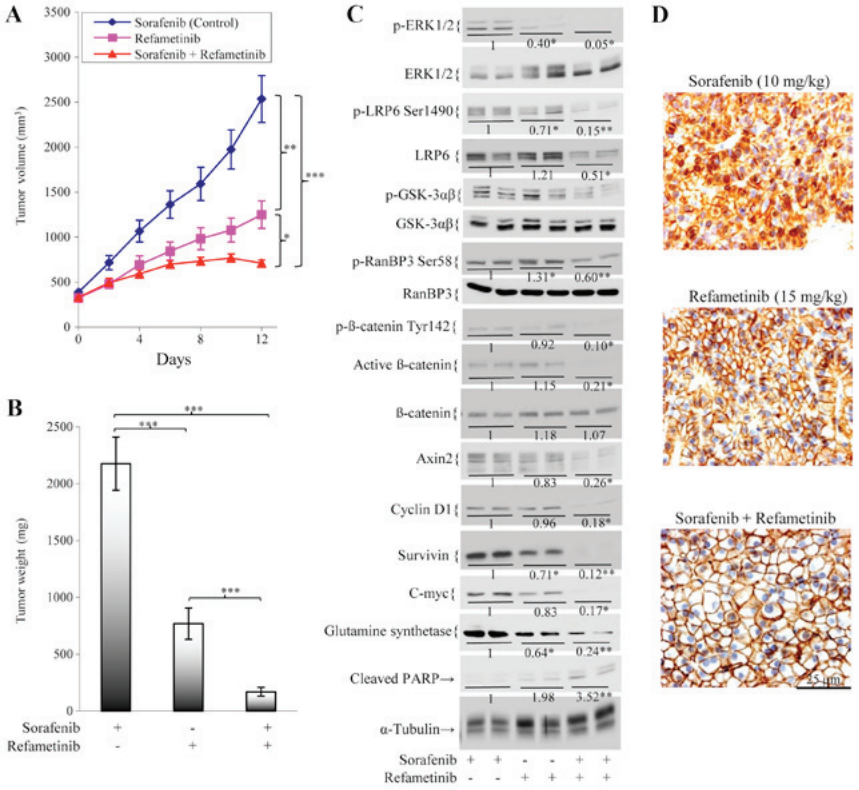

Figure 6. Effects of sorafenib/refametinib on tumor growth and the β-catenin-signaling pathway in sorafenib-resistant HCC26-0808ASora54 xenografts. Mice bearing HCC26-0808ASora54 tumors were treated with 10 mg/kg sorafenib (control treatment), 15 mg/kg refametinib or sorafenib/refametinib once daily for 12 days. Each treatment arm comprised 10 independent tumor-bearing mice. Treatments began when the tumors reached ~350 mm^3 in size. (A) Tumor volumes at given time points and (B) the corresponding tumor weights are presented. Data are presented as the mean ± standard error of the mean. One-way analysis of variance followed by the Tukey-Kramer method post-hoc test was used to establish significant differences among groups. *P<0.05, **P<0.01 and ***P<0.001. (C) Western blot analysis of β-catenin signaling pathway proteins and quantification of proteins involved in the β-catenin-signaling pathway are presented. *P<0.05 and **P<0.01 vs. sorafenib. (D) Representative pictures of β-catenin stained with an anti-β-catenin antibody are presented. Scale bars, 25 μm. p, phosphorylated; ERK, extracellular signal-regulated kinase; LRP6, low-density lipoprotein receptor-related protein 6; GSK, glycogen synthase kinase; PARP, poly (ADP-ribose) polymerase.

resulted in decreased cell viability, β-catenin levels and those of its known downstream targets. Treatment of wild-type β-catenin HCC06-0606 cells with Wnt-3A-conditioned medium led to the significant upregulation of p-LRP6 Ser1490, p-ERK1/2 Thr202/Tyr204, p-RanBP3 Ser58 and p-β-catenin Tyr142 (P<0.01; Fig. 5B) as compared with control-conditioned medium. Marked increases in survivin, cyclin D1 and glutamine synthetase were also noted. Pre-treatment of HCC06-0606 cells with refametinib or sorafenib/refametinib, but not with sorafenib, for 24 h abolished Wnt-3A-induced upregulation of p-LRP6 Ser1490, p-β-catenin Tyr142, p-RanBP3 Ser58 and p-ERK1/2 Thr202/Tyr204 (P<0.05; Fig. 5B). These findings suggest that sorafenib/refametinib profoundly suppressed β-catenin activity, resulting in decreased proliferation and elevated levels of apoptosis. Similar observations were obtained when mutant β-catenin HCC07-0409 cells were treated with Wnt-3A-conditioned medium (data not shown).

Sorafenib/refametinib treatment has anti-tumor effects in a sorafenib-resistant HCC model. While growth of the parental HCC26-0808A line was significantly reduced by sorafenib, growth of the HCC26-0808ASora54 line was unaffected (data not shown). This indicates that HCC26-0808ASora54 is resistant to sorafenib treatment. As presented in Fig. 6A, sorafenib-resistant HCC26-0808ASora54 tumors that continued to grow in the presence of sorafenib experienced a significant reduction in tumor growth when ERK activity was blocked by refametinib (P<0.001). The addition of refametinib to sorafenib abolished tumor growth (Fig. 6A) and the final tumor weight of sorafenib/refametinib group was significantly smaller than that of the refametinib or sorafenib groups (P<0.001; Fig. 6B). This was associated with inhibition of the β-catenin signaling pathway, as evidenced by a significant reduction in p-LRP6 Ser1490, p-β-catenin Tyr142, p-RanBP-3 Ser58, and active non-phospho-β-catenin, Axin2, survivin, cyclin D1, c-Myc, and glutamine synthetase levels (P<0.05; Fig. 6C). Nuclear exportation of β-catenin was also observed when HCC26-0808ASora54 was treated with sorafenib/refametinib (Fig. 6D).

Discussion

HCC is the second leading cause of cancer mortality worldwide, often presenting in the advanced stage when cure is no longer possible. Although sorafenib improves the overall survival of patients with HCC, the benefit is modest and treatment confers only a transient clinical benefit (4,5). As such, there is an urgent need for more efficacious systemic therapies. Mutations affecting the Wnt/β-catenin pathway have been found in 26-40% of HCC cases (28-30). Activation of the Wnt/β-catenin

pathway leads to upregulation of downstream target genes that regulate cell proliferation and tumor progression (31,32). As such, agents targeting the Wnt/β-catenin pathway may be an alternative therapy for patients with β-catenin-dependent tumors. Targeting Wnt/β-catenin has been comprehensively discussed (33,34). Wnt/β-catenin pathway inhibitor ICG001, FH535 and other small inhibitors have been tested in HCC models (35-37). Combinations of sorafenib/ICG001 or sorafenib/FH535 had better treatment outcomes in their experimental models (38,39). These studies have demonstrated that the promoted downregulation or degradation of wild-type β-catenin is the mechanism of β-catenin pathway inhibition in HCC.

In the present study, combinations of sorafenib/refametinib or sorafenib/selumetinib inhibited cell proliferation and induced apoptosis, both of which were associated with the downregulation of p-ERK1/2, p-RanBP3 Ser58, p-LRP6 Ser1490, p-β-catenin Tyr142, and β-catenin target genes. In addition, the sorafenib/refametinib combination suppressed Wnt-3A-induced activation of the Wnt/β-catenin signaling pathway in HCC cells. Although the overall levels of β-catenin were not significantly changed, those of non-p- (active) β-catenin in sorafenib/refametinib-treated tumors were significantly reduced. Whereas vehicle-treated and sorafenib-treated mutant β-catenin tumors exhibited strong cytoplasmic and nuclear β-catenin localization, the majority of the β-catenin in sorafenib/refametinib-treated tumors was accumulated at the membrane. The precise mechanisms by which sorafenib/refametinib or sorafenib/selumetinib inactivates the Wnt/β-catenin pathway remain unclear. It is possible that sorafenib/refametinib or sorafenib/selumetinib inactivates the Wnt/β-catenin pathways by inhibiting p-β-catenin Tyr142 (40) and p-RanBP3 Ser58 (26). This leads to a reduction in nuclear β-catenin and β-catenin-dependent transcription (40-43). As phosphorylation of β-catenin at tyrosine 142 is also essential for binding to E-cadherin (41,43) and α-catenin (27,40,42,44), respectively, inhibition of p-β-catenin Tyr142 by sorafenib/MEK inhibitor may facilitate the interaction of E-cadherin and α-catenin with β-catenin at the membrane, leading to a decrease of the cytosolic pool without reducing total β-catenin levels (45). In the present study, suppressing β-catenin by sorafenib/refametinib reduces p-histone 3 Ser10, cyclin B1, cyclin D1, c-Myc, and survivin protein expression, providing the link between β-catenin signaling pathway and cell cycle. In addition, sorafenib/refametinib also reduced glutamine synthetase, which in turn may deplete cellular glutamine. As activation of Wnt/β-catenin is implicated in maintenance of tumor initiating cells, drug resistance, tumor progression and metastasis (31), it remains to be determined if sorafenib/refametinib inhibits the proliferation of liver cancer stem cells.

Sorafenib/refametinib or sorafenib/selumetinib targets wild-type as well as T41A- and S45P-mutated β-catenin, which have been identified in 2-18.8% of analyzed HCC cases (7,46-50). These mutations were revealed to activate the β-catenin pathway by preventing GSK3-mediated phosphorylation at Ser33/37, further avoiding β-TrCP recognition (51-53). In the present study, it was observed that the levels of p-GSK-3αβ (Ser21/9) and total β-catenin were not significantly altered by sorafenib/refametinib or sorafenib/selumetinib treatment. It is unlikely that downregulation or degradation of β-catenin by GSK3 is the mechanism by which sorafenib/refametinib or sorafenib/selumetinib inhibits the β-catenin pathway. Enhanced nuclear β-catenin export due to the inhibition of p-β-catenin Tyr142 (40,42) and p-RanBP3 Ser58 (26) may have a key role in sorafenib/MEK inhibitor mediated inactivation of β-catenin signaling pathway.

Treatment with sorafenib, although associated with inhibition of tumor growth and angiogenesis in *in vivo* studies, led to the upregulation of p-ERK1/2 (21), which is one of the most critical cellular signaling pathways supporting hepatocarcinogenesis. The addition of a MEK inhibitor (selumetinib or refametinib) to sorafenib resulted in the attenuation of p-ERK1/2 activity and greater antitumor effects (17,21). In the present study, 'adaptive' tumor growth in the presence of sorafenib experienced a marked reduction when MEK/ERK activity was blocked. These findings suggest that resistance to sorafenib may be overcome by adding refametinib (or selumetinib) to sorafenib.

We recently reported that a phase Ib study of selumetinib (AZD6244) in combination with sorafenib in advanced HCC demonstrated encouraging anti-tumor activity with acceptable adverse events (17). In the SHARP trial, sorafenib monotherapy was associated with an median progression-free survival (mPFS) and median overall survival (mOS) of 5.5 and 10.7 months, respectively (5). A phase III study of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma reported an mPFS and mOS of 2.8 and 6.5 months, respectively (4). mPFS and mOS of 5.6 and 14.4 months, respectively, achieved with combination sorafenib and selumetinib is encouraging. Lack of activity observed with selumetinib monotherapy in a previous study suggests synergistic anti-tumor effect are possible with this combination (17). The robust anti-tumor activity of sorafenib/selumetinib presented in HCC PDX models and in phase 1b clinical trial warrants the development of phase II clinical trials. In a phase 1b study of selumetinib in combination with sorafenib in HCC, a high incidence of grade 3/4 diarrhea (44%) and the finding that 66% of SAEs were GI-related (vomiting/diarrhea) are testament of overlapping toxicities between sorafenib and selumetinib (17). In the present study, dose reductions were required for sorafenib. Patient education and prompt aggressive management strategies may potentially mitigate some of the toxicities. A phase II study of refametinib and sorafenib also demonstrated these agents to be clinically active in patients with HCC and mutant KRAS tumors (16). These results also suggest that a therapy regimen involving the combination of sorafenib and a MEK inhibitor could possibly reverse sorafenib-resistance in patients with HCC.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

HH, LL and RO were responsible for collection and assembly of data. FP, AS, OP, DM, KZ and HH were responsible for financial support, provision of study materials and study design. HH and KG were responsible for data analysis, interpretation and writing the manuscript. All authors provided final approval of the article.

Ethics approval and consent to participate

Written informed consent was obtained from all patients prior to tissue collection. The present study was approved by the Ethics Board at National Cancer Centre Singapore (Singapore) and at Singapore General Hospital (Singapore). All mice were maintained according to the 'Guide for the Care and Use of Laboratory Animals'.

Patient consent for publication

Not applicable.

Competing interests

FP, AS, OP, DM and KZ are employees of Bayer AG (Leverkusen, Germany).

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