Synergistic Inhibition of HCC and Liver Cancer Stem Cell Proliferation by Targeting RAS/RAF/MAPK and WNT/β-Catenin Pathways

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Abstract. Background/Aim: The aim of this study is to find synergistic effect using FH535 and sorafenib by targeting the RAS/RAF/MAPK and WNT/β-catenin pathways. Materials and Methods: 3H-Thymidine incorporation assays were performed to address Huh7 and liver cancer stem cell (LCSC) inhibition using FH535 and sorafenib, alone and in combination. CalcuSyn analysis was used to calculate the combination index (CI). A western blot assay was performed to check for potential targets. Results: FH535 and sorafenib caused inhibition of Huh7 and LCSC. Combination therapy was significantly better than monotherapy in inhibition of Huh7. Combination with sorafenib and FH535 was found to be synergistic in inhibition of LCSC with a CI of less than 1. The western blot assay demonstrated enhanced cleaved poly (ADP-ribose) polymerase (PARP) and inhibition of cyclin D1, B-cell lymphoma 2 (Bcl2), survivin and cellular myelocytomatosis oncogene (c-MYC). Conclusion: FH535 and sorafenib combination produced synergistic effect on inhibition of HCC and LCSC. Our study demonstrated that FH535 can induce apoptosis in these two different hepatocellular carcinoma (HCC) cell lines.

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and the third most common cause of cancer-related death worldwide, representing a significant health care problem (1). The prevalence of HCC differs greatly by geographical location, reflecting variations in the main risk factors. Most cases of HCC (80%) arise in the Asia-Pacific and sub-Saharan African regions, where the dominant risk factor is chronic infection with hepatitis B virus (HBV). In Western countries, the incidence of HCC has been rising rapidly in recent decades due to infection with hepatitis C virus (HCV) and alcohol use (2).

Several signaling pathways have been found to be disregulated in HCC including the RAS/RAF/MAPK, PI3K/AKT/mTOR, HGF/c-MET, IGF, VEGF, PDGF and WNT/β-catenin pathways. Among them, de-regulation of the WNT/β-catenin pathway by far the most difficult to treat (3). The WNT/β-catenin pathway involves three complexes: the ligand/receptor cell membrane complex, the cytosol β-catenin destruction complex, and the nuclear β-catenin/ T cell factor (TCF)/Lymphoid enhancer factor (LEF) transcription complex (4, 5). Once the membrane complex is activated the cytosol destruction complex is de-activated, resulting in increased accumulation of β-catenin in the cytoplasm which enters the nucleus and interacts with the TCF/LEF family of transcription factors, activating TCF/LEF target genes including cyclin D1, cellular myelocytomatosis oncogene (c-MYC) and survivin (6).

Aberrant activation of the WNT/β-catenin pathway has been observed in roughly 1/3 of HCCs. In HCC, nuclear and cellular β-catenin accumulation, a hallmark of activated canonical WNT/FZ signaling has been observed in 33-67% of tumors. Roughly 20% of HCCs have mutations in the β-catenin gene (7, 8). The chemical agents used to target WNT/β-catenin pathway are at the membrane, cytosol and nuclear complexes. Our group recently investigated the ability of FH-535, a dual inhibitor of the peroxisome proliferator-activated receptor (PPAR) and β-catenin/TCF/LEF to inhibit HCC and liver cancer stem cell growth in vitro. This drug was shown to inhibit growth of hepatoblastoma cell lines HepG2 and Huh7 (9). Sorafenib, a multi-kinase inhibitor, has been found to be active against HCC in several pre-clinical and clinical studies. Sorafenib is considered the drug-of-choice to treat patients with advanced HCC that are deemed unsuitable for other types of surgical, ablative and embolization interventions (10).

The aim of this study was to determine the sensitivity of HCC and liver cancer stem cell (LCSC) lines using sorafenib, FH-535 alone and in combination.
Materials and Methods

Cell culture. The human HCC cell line Huh7 was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) medium (Invitrogen, Carlsbad, CA, USA) with 10% heat-inactivated fetal bovine serum (FBS) at 37°C with 5% CO2. LCSC (CelProgen, San Pedro, CA, USA) were cultured in CelProgen Liver Cancer Stem Cell Growth Media with 10% FBS.

Mouse tumorigenesis models. Female NOD/SCID mice (n=3) (NOD.CB17-prkdc^SCID/NCrSD, 4-5 weeks old) were purchased from Harlan Animal Research Laboratory (Indianapolis, IN, USA) and maintained in the Division of Laboratory Animal Resources facility at our Institution. Mice received filtered air, sterile water and irradiated food ad libitum. Tumors were generated by harvesting first passage LCSC that were trypsinized, washed and resuspended in a 50% mixture of Matrigel (BD Bioscience, San Diego, CA, USA) in CelProgen Liver Cancer Stem Cell Growth Media (serum-free) to a final cell count of 20,000 cells/ml. A volume of 0.1 ml of the cell suspension (2000 cells) was injected subcutaneously in the right flank of each mouse (10). The mice were weighed and checked for tumor growth every other week. Tumors were initially observed 28 days after inoculation, at which time the tumor was measured twice weekly using an optical caliper and tumor size was calculated using the following formula: length x (width)^2 x 0.4 according to a published method (11). When the tumor size reached 940-1,020 mm^3, the mice were euthanized with CO2. The tumors were isolated and fixed in 10% formalin for 48 h and then transferred to 70% ethanol. The tumors were then embedded in paraffin, cut to 5 μm sections and stained with hematoxylin and eosin for histological analysis.

Chemicals and antibodies. FH535 and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methyl-3H-thymidine (two Ci/mM) was from MP Biomedicals (Costa Mesa, CA, USA). Antibodies from Cell Signaling(Boston, MA) were used for Western Blot assay.

3H-Thymidine incorporation assay. Huh7 cells were plated in 96-well plates at 2,500 cells/well in 0.2 ml DMEM with 10% FBS and treated with different concentrations of FH535 and sorafenib, as single agents or in combination, and cultured for 72 h. 3H-Thymidine incorporation assays were performed as described (13).

Western blot analysis. Huh7 cells were cultured in DMEM + 10%FBS in 100x20 mm tissue culture dishes until about 70% confluence. The cells were treated with FH535 from 0 to 10 μM for 38h. Dimethyl sulfoxide (DMSO) (<0.1%) were used as vehicle control. All the other procedures were performed as previously described (12).

Statistical analysis. All analyses were performed using SPSS software version 19 (Chicago, IL, USA). Data are presented as mean±SE. For nominal data, ANOVA followed by Tukey multiple range test was used; for two groups of continuous data, paired t-test was used. The level of statistical significance was set at p<0.05. Analysis of synergism was performed using the software CalcuSyn version 2.0 from Biosoft® (Great Shelford, Cambrigde, UK).

Results

LCSC profile. Flow cytometric analysis indicated that the LCSC cells comprised 64.4% CD133-positive, 83.2% CD44-positive, 96.4% CD24-positive and 96.9% aldehyde A1-positive cells.

Tumorigenesis of LCSC in animal model. Female NOD/SCID mice (4-5 weeks old) were inoculated with 0.1 ml of the cell suspension (2,000 cells) subcutaneously at the right flank of each mouse. All mice developed tumors within 4-6 weeks after inoculation. Tumor size reached 940-1020 mm^3 at day 60 and all mice were euthanized. Stained tumor sections were analyzed by a trained liver pathologist at our Institution to determine cancer development and characteristics. All mice developed poorly-differentiated lesions after inoculation of these liver cancer stem cells (CD133-, CD44-, and CD24-negative) seen on microscopic examination.

The combination of FH535 and sorafenib synergistically inhibits LCSC proliferation. We found that FH535 in combination with sorafenib was significantly better than monotherapy on inhibition of LCSC proliferation (Figure 1). CalcuSyn analysis demonstrated that combination therapy caused synergistic inhibition of LCSC proliferation. We also found that FH535 and sorafenib in combination significantly inhibited the Huh7 HCC cell line (Figure 2).
Western blot assay after FH-535 treatment demonstrated inhibition of survivin and BCL2 and an enhanced cleavage of PARP. Western blot analysis demonstrated a dose-dependent inhibition of cyclin D1, survivin and BCL2 expression at the protein level. These data also showed that levels of cleaved PARP are increased in Huh7 human HCC cells after FH-535 treatment (Figure 3). Expression of c-MYC, encoded by an oncogene that regulates numerous genes important for cell proliferation, was inhibited by FH-535 (Figure 4).

Discussion

In recent years, numerous signaling pathways such as RAS/RAF/MAPK, WNT/β-catenin, EGFR, insulin-like growth factor receptor, AKT-mTOR (mammalian target of rapamycin), notch and hedgehog have been implicated in hepatic carcinogenesis (3). The WNT/β-catenin pathway has been associated with cellular processes such as development, growth, survival, regeneration, and self-renewal. Disruption of this balance results from both genetic and epigenetic changes found in many types of cancer, including colon cancer and HCC. Moreover, recent studies indicate that the WNT/β-catenin signaling pathway has an important role in the maintenance of CSCs (14). Failure of cytotoxic chemotherapy for advanced HCC and the development of sorafenib have significantly stimulated interest in different cell signaling pathways involved in hepatic carcinogenesis. Our group and others have focused on inhibition of LCSCs and differences in resistance patterns with non-liver CSC lines both in vitro and in vivo (15). LCSCs are thought to be responsible for tumor development, progression, recurrence and metastasis, and targeting signaling pathways required for CSCs activation and proliferation should bring important and revolutionary advances in cancer therapeutics.

Despite numerous efforts, the etiology of HCC tumorigenesis, whether originating from mature hepatocytes or stem/progenitor cells, remain unclear. Stem cells are defined by their potential for self-renewal and their ability to proliferate and differentiate into diverse cell types. This suggests that a tumor is comprised of a heterogeneous population of cells that form a distinct hierarchy. Although the existence of CSCs was first proposed over 40 years ago, it has only been slightly over a decade since Dick et al. first demonstrated a role for stem cells in hematological malignancies (16). More recently, studies have provided convincing evidence that these cells do exist in solid tumors of many types including those of brain, breast, colorectal, liver, pancreas and prostate (17). The liver tumor microenvironment is a complex mixture of tumor cells within the extracellular matrix combined with an intricate mix of stromal cells and the proteins they secrete. Together, these elements contribute to the carcinogenic process (18) by sustaining proliferation, evasion of growth suppressors,
resistance to cell death, induction of angiogenesis, activation of invasion and metastasis, reprogramming of energy metabolism and evasion of immune destruction (19). We have recently been working on tumorigenesis and patterns of resistance to drugs in liver cancer.

In the present study, we initially focused on LCSC profiling by measuring CSC markers and tumorigenesis experiments using low cell densities. We then examined responses to therapy with single agents and drug combination in two different HCC cell lines including LCSCs. Lastly, we performed a western blot assay to analyze for significant potential targets such as cyclin D1, survivin, BCL2 and oncogene c-MYC. Our main focus was to determine patterns of response to therapy and the possibility of finding a synergistic effect using combination drugs targeting RAS/RAF/MAPK and WNT/β-catenin pathways.

We have confirmed by flow cytometry that these cells were positive for CD133 (64.4%), CD44 (83.2%), CD24 (96.4%) and aldehyde A1 (96.9%). All SCID mice inoculated subcutaneously with a low dose of LCSCs (2,000 cells) developed tumors, whereas our previous experience and published data from other groups indicated that 100,000 HCC cells were needed to develop tumors in SCID mice. Tumors from these LCSCs demonstrated features of poorly-differentiated cancer with a significant proportion of pleomorphism and mitosis.

The WNT/β-catenin pathway has been extensively studied and known to be important in HCC proliferation. In normal cells, extracellular WNT ligands can interact with a host of secreted antagonists, including secreted frizzled-related protein and dickkopf family members, preventing activation of the pathway. The best-studied mutations in this pathway are the inherited and sporadic mutations in the tumor suppressor Adenomatous polyposis coli (APC), which reduce β-catenin degradation, causing increased β-catenin levels and activation of target genes such as the oncogenes cyclin D1 and c-MYC (20). Mutations of β-catenin in HCC are often located in the CTNNB1 gene exon 3, which encodes the phosphorylation site for glycogen synthase kinase 3 (GSK-3) (21). Numerous other signaling pathways have been involved in HCC carcinogenesis. The WNT/β-catenin pathway plays an important role in stem cell self-renewal and differentiation. Pro-angiogenic factors such as VEGF, angiopoietin, EGF, PDGF, hepatocyte growth factor (HGF) induce angiogenic signaling via RAS/RAF/MEK/ERK, mTOR and WNT signal transduction pathways can contribute to HCC progression. The WNT/β-catenin pathway has been described as one of most difficult pathways to target in HCC. We studied Huh7 proliferation and the response to sorafenib and FH-535 alone and in combination. We demonstrated that the sorafenib-and-FH-535 combination is significantly better than monotherapy in inhibition of HCC proliferation as shown in 3H-thymidine incorporation assay. We previously demonstrated an additive effect of targeting PI3K/mTOR and RAS/RAF/MAPK pathways with several different compounds in vitro and in vivo (12). Other investigators have tried combinations targeting different pathways to induce HCC inhibition in vitro and in vivo.

In the present study, using the CalcuSyn software, we found that FH-535 and sorafenib synergistically inhibit...
LCSC with a combination index (CI) of less than 1. To the best of our knowledge, this is the first report of synergistic inhibition of HCC and LCSCs targeting RAS/RAF/MAPK and WNT/β-catenin pathway in combination.

We also analyzed levels of the apoptosis inhibitors survivin and BCL2. Survivin inhibits caspase activation, leading to inhibition of apoptosis. Survivin, whose expression is highly regulated by the cell cycle and is only found in the G2-M phase of the cell cycle is regulated by β-catenin. Cleaved PARP, another marker of apoptosis, was enhanced in Huh7 after FH-535 treatment. We also demonstrated that FH-535 inhibits cyclin D1 in Huh7 cells.

In conclusion, HCC has proven to be a very heterogeneous disease. Regardless of the recent advances in the understanding HCC pathophysiology, it remains a complex and poorly-understood disease. Numerous signaling pathways such as RAS/RAF/MAPK, WNT/β-catenin, EGFR, insulin-like growth factor receptor, AKT-mTOR, notch, hedgehog have been implicated in hepatic carcinogenesis and their components represent molecular targets for therapy in HCC. Interest in the CSC hypothesis is increasing, and according to it, cancer initiation, progression, recurrence, metastasis and therapy resistance are unique properties implicitly dependent on CSC subsets. Our LCSC (positive for CD133, CD44 and CD24) were able to develop HCC with very low cell dose. LCSC-derived tumors demonstrated typical characteristics of poorly differentiated HCC. FH-535 and sorafenib combined synergistically inhibit LCSC proliferation. Apoptosis was enhanced in Huh7 cells after inhibiting the WNT/β-catenin pathway with FH-535. However, understanding the role of hepatic CSCs remains limited and its role in tumorigenesis, tumor progression and resistance to treatment deserves further investigation.

References


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