

Signal Transducer and Activator of Transcription 3-Mediated CD133 Up-Regulation Contributes to Promotion of Hepatocellular Carcinoma

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Enhanced expression of the cancer stem cell (CSC) marker, CD133, is closely associated with a higher rate of tumor formation and poor prognosis in hepatocellular carcinoma (HCC) patients. Despite its clinical significance, the molecular mechanism underlying the deregulation of CD133 during tumor progression remains to be clarified. Here, we report on a novel mechanism by which interleukin-6/signal transducer and activator of transcription 3 (IL-6/STAT3) signaling up-regulates expression of CD133 and promotes HCC progression. STAT3 activated by IL-6 rapidly bound to CD133 promoter and increased protein levels of CD133 in HCC cells. Reversely, in hypoxic conditions, RNA interference silencing of STAT3 resulted in decrease of CD133 levels, even in the presence of IL-6, with a concomitant decrease of hypoxia-inducible factor 1 alpha (HIF-1 α) expression. Active STAT3 interacted with nuclear factor kappa B (NF- κ B) p65 subunit to positively regulate the transcription of HIF-1 α providing a mechanistic explanation on how those three oncogenes work together to increase the activity of CD133 in a hypoxic liver microenvironment. Activation of STAT3 and its consequent induction of HIF-1 α and CD133 expression were not observed in Toll-like receptor 4/IL-6 double-knockout mice. Long-term silencing of CD133 by a lentiviral-based approach inhibited cancer cell-cycle progression and suppressed *in vivo* tumorigenicity by down-regulating expression of cytokinesis-related genes, such as TACC1, ACF7, and CKAP5. We also found that sorafenib and STAT3 inhibitor nifuroxazide inhibit HCC xenograft formation by blocking activation of STAT3 and expression of CD133 and HIF-1 α proteins. **Conclusion:** IL-6/STAT3 signaling induces expression of CD133 through functional cooperation with NF- κ B and HIF-1 α during liver carcinogenesis. Targeting STAT3-mediated CD133 up-regulation may represent a novel, effective treatment by eradicating the liver tumor microenvironment. (HEPATOLOGY 2015;62:1160-1173)

Hepatocellular carcinoma (HCC) is the sixth-most commonly diagnosed and the third-most lethal neoplasm causing an estimated 700,000 deaths worldwide annually.¹ In the United States, the incidence of HCC has doubled over the past two decades, with only 30%~40% of patients eligible for curative treatments, such as liver transplantation or surgical resection owing to late diagnosis, underlying liver dis-

ease, and lack of effective treatment options.² HCC is closely associated with chronic inflammation caused by viral hepatitis, alcoholic liver disease, and autoimmune hepatitis, in which continuous inflammation and hepatocyte regeneration occur.³ These long-term processes may include accumulation of genetic and/or epigenetic changes, alteration of liver tumor microenvironment, and generation of liver cancer stem cells (CSCs).⁴

Abbreviations: Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; ChIP, chromatin immunoprecipitation; CSC, cancer stem cell; DEN, diethylnitrosamine; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; FCM, flow cytometry; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HIF-1 α , hypoxia-inducible factor 1 alpha; IgG, immunoglobulin G; IHC, immunohistochemistry; IL-6, interleukin-6; JAK, janus kinase; KAIST, Korea Advanced Institute of Science and Technology; KO, knockout; Mcl-1, myeloid cell leukemia 1; MKI, multiple tyrosine kinase inhibitor; mRNA, messenger RNA; NF- κ B, nuclear factor kappa B; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RNAi, RNA interference; SC, subcutaneously; shRNA, short hairpin RNA; siRNA, small interfering RNA; shRNA, short hairpin RNA; STAT3, signal transducer and activator of transcription 3; TLR-4, Toll-like receptor 4; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor; WT, wild type.

Recently, growing evidences support the novel notion that tumor initiation can be driven by a CSC subset that is responsible for tumor persistence and relapse, metastasis, chemoresistance, and radioresistance. CD133 antigen, also known as prominin 1, has been identified as a putative CSC marker in various cancer types, including HCC.⁵ CD133⁺ cells possess greater colony-forming efficiency, higher proliferation rate, and higher incidence of tumorigenesis. Clinically, HCC patients with increased CD133 levels have shorter overall survival and higher recurrence rates than patients with low CD133 expression.⁶ Despite the immense clinical significance, molecular mechanisms underlying enhanced expression of CD133 remain to be defined.

Aberrant activation of signal transducer and activator of transcription 3 (STAT3) is frequently associated with various human malignancies, including liver, hematological, breast, head and neck, and prostate cancers.⁷ Many compelling evidences support that STAT3 is critical in inflammation-associated tumorigenesis by regulating numerous oncogenic and inflammatory genes.⁸ Interleukin-6 (IL-6), a potent STAT3 activator, is highly released in response to hepatitis viral infection and systemic inflammation in the liver.⁹ The IL-6/STAT3-signaling pathway has been previously reported to be involved in liver inflammation/regeneration.¹⁰ It maintains liver CSC population by interacting with the transforming growth factor beta–signaling pathway.¹¹ More

recently, it has been found that CD24⁺ liver CSCs, a subset of cells that shares a high overlapping expression as CD133⁺ liver CSCs, drive tumor initiation through STAT3-mediated NANOG regulation.¹²

The hypoxic microenvironment of a developing tumor has been shown to be correlated to regulation of immune response and inflammation promotion.¹³ In malignant tumors, macrophages rapidly up-regulate expression of IL-6 under hypoxic conditions by altering expression of a wide array of genes.¹⁴ Expression of hypoxia-responsible genes is shown to be almost doubled when stimulated with IL-6 in HCC cells.¹⁵ Of those, hypoxia inducible factor 1 alpha (HIF-1 α) is a key hypoxia-regulatory protein that not only contributes to restoration of oxygen homeostasis by promoting glycolysis, erythropoiesis, and angiogenesis, but also plays an important role in acquiring resistance against chemotherapeutic agents.¹⁶ Recent studies have shown that autocrine IL-6 induces a loss of methylation at the CD133 proximal promoter region in breast cancer cells,¹⁷ and that activated HIF-1 α enhances self-renewal activity of CD133⁺ cells and inhibits induction of CSC differentiation.¹⁸

Putting these evidences together, we hypothesized that CD133 might be an inducible gene in HCC progression, rather than just a CSC marker, which is consistently expressed in a particular subset of cells in the liver tumor microenvironment. Our results provided *in vitro*

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and *in vivo* evidences that IL-6-mediated activation of STAT3 induces expression of CD133 through transcriptional activation in liver carcinogenesis. This up-regulation is accompanied with functional activation of the key downstream players, nuclear factor-kappa B (NF- κ B) and HIF-1 α . Treatment of sorafenib and STAT3 inhibitor nifuroxazide effectively suppressed HCC xenograft formation by blocking activation and expression of STAT3, CD133, and HIF-1 α proteins. Our findings provide not only a novel mechanism of IL-6/STAT3 signaling-mediated CD133 up-regulation to drive HCC, but also an effective molecular pathway that can be targeted with the hope of eradicating the liver tumor microenvironment.

Materials and Methods

Human HCC Tissue Collection. Twenty pairs of liver tumor and adjacent noncancerous tissue specimens were collected from the Department of Pathology, Seoul National University College of Medicine (Seoul, Korea), with institutional review board approval. All patients participating in this study gave their informed consent before surgery.

Cell Lines. Human HCC cell lines, Hep3B, Huh7, PLC/PRF/5, SNU-398, SNU-423, SNU-449, SNU-761, and SNU-878, and a human hepatoblastoma cell line, HepG2, were purchased from ATCC (Manassas, VA) or Korean Cell Line Bank (Seoul, Korea). Normal liver epithelial cell line Chang was purchased from CLS Cell Line Service (Eppelheim, Germany).

Cloning Human CD133 Promoter and STAT3-Specific Short Hairpin RNA Construction. A 2.4-kilobase region of human CD133 promoter was amplified by polymerase chain reaction (PCR) from human U2OS genomic DNA using primers. PCR-amplified fragments were cloned into *Kpn*I and *Xho*I sites of pGL3-basic luciferase vector (Promega, Madison, WI). STAT3-specific short hairpin RNA (shRNA) was designed using BLOCK-iTTMRNAi Designer (Invitrogen, Carlsbad, CA) (Supporting Table 1) and annealed both strands to generate double-stranded oligos with 4-nucleotide overhangs. Double-stranded oligos were then directly inserted into BLOCK-iTTMpENTRTM/H1/TO RNA interference (RNAi) vector (Invitrogen).

shRNA Preparation and Plasmid Construction. pLKO plasmid (OriGene, Rockville, MD) was used for lentiviral DNA vector-based shRNA construction. Based on complementary DNA sequence of CD133 (GenBank accession no.: NM_001145847) and STAT3 (GenBank accession no.: NM_003150), an shRNA designing tool was provided freely by Invitrogen at its website (www.invitrogen.com/rnai). We synthesized DNA templates encoding one CD133-specific shRNA

and one STAT3-specific shRNA. The core sequences of oligonucleotides encoding CD133 and STAT3 shRNA were 5'-CATTGGCATCTTCTATGG-3' and 5'-GCGTCCAGT TCACTACTAAAG-3', respectively.

In Vivo Tumorigenicity Experiments. Viable Huh7 cells were injected subcutaneously (SC) into the dorsal flank of Balb/c nude mice (1×10^6 cells/mouse). Tumor-bearing mice were then randomly assigned to three groups when tumor sizes were reached to 10 mm in diameter. Each group (n = 6) was treated with vehicle (1% dimethyl sulfoxide [DMSO] diluted into phosphate-buffered saline [PBS]), 30 mg/kg of sorafenib, or 60 mg/kg of nifuroxazide with a 2-day interval during 2 weeks. All surgical and experimental procedures were approved by the institutional animal care and use committee at the Seoul National University College of Medicine (Seoul, Korea). Tumors were measured by a caliper ruler with a 2-day interval, and tumor volumes were calculated using $a \times b^2/2$ (where a is the width at the widest point of the tumor and b is the width perpendicular to a).

DEN-Induced Liver Cancer Model. Male C57BL/6J wild-type (WT), IL-6 knockout (KO; IL-6^{-/-}), and Toll-like receptor 4 (TLR-4) knockout (TLR-4^{-/-}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). To obtain IL-6^{-/-}/TLR-4^{-/-} double KO, IL-6^{-/-} mice were crossed with TLR-4^{-/-} mice. Mice were maintained in a specific pathogen-free facility at the Korea Advanced Institute of Science and Technology (KAIST; Daejeon, South Korea). All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The animal protocol was approved by the institutional animal care and use committee of KAIST. To induce HCC, 2-week-old male mice received an intraperitoneal injection of diethylnitrosamine (DEN; 20 mg/kg; Sigma-Aldrich, St. Louis, MO) and mice were sacrificed at 10 months after DEN treatment.

Microarray Analysis. Total RNA was extracted from Huh7 cells transduced with either lentiviral vector or shCD133 using TRIzol reagent, *in vitro* transcribed, and hybridized on Illumina Human HT-12 v4 Expression BeadChip (Illumina, San Diego, CA). All microarray data were submitted to the Gene Expression Omnibus database with the accession number GSE60194. Statistical analysis was performed using R and BioConductor software (<http://www.bioconductor.org/>). All data were normalized with robust spline normalization method after variance-stabilizing transformation provided by the LUMI package.

Statistical Analysis. All data were statistically analyzed using Microsoft Excel 2013 software (Microsoft, Redmond, WA) or Sigma Plot 12. Tumor volumes in

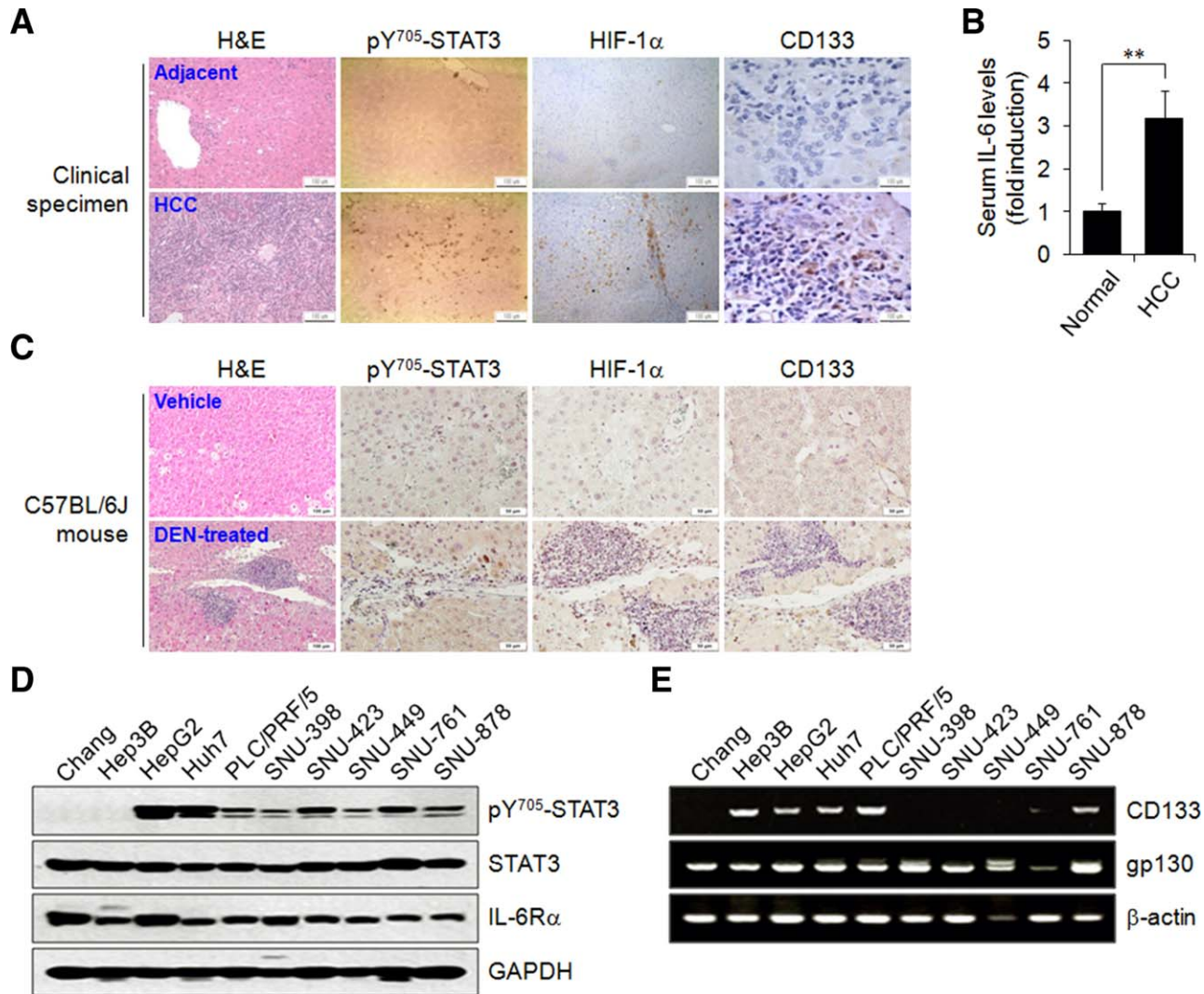


Fig. 1. Levels of active STAT3, HIF-1 α , CD133, and IL-6 were elevated in HCC progression. (A) H&E staining and IHC revealed activation of STAT3 and enhanced expression of HIF-1 α and CD133 in human HCC tissues, when compared to adjacent noncancerous tissues. Scale bar = 100 μ m. (B) Plasma IL-6 levels were determined in cohorts of healthy adults (n = 10) and HCC patients (n = 20). ** P < 0.005. (C) Confirmation of activation of STAT3 and enhanced expression of HIF-1 α and CD133 in the DEN-induced HCC model. Scale bar = 50 μ m. (D and E) Active endogenous STAT3 and IL-6R α (D) and CD133 and gp130 (E) levels were determined by western blotting or reverse-transcriptase PCR analysis in human HCC cell lines. GAPDH and β -actin were used as loading controls. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin.

vehicle-, sorafenib-, and nifuroxazide-treated groups were compared by analysis of variance followed by Duncan's multiple range test. Differences were considered statistically significant at the P < 0.05 level. All statistical tests were two-sided.

Results

Enhanced Levels of Active STAT3, HIF-1 α , and CD133 in Human HCC Clinical Specimen and DEN-Induced HCC Mouse Model. HCC is mainly caused by either hepatitis B- or C-type virus, both of which are accompanied with chronic inflammation and hypoxia.^{19,20} Therefore, as a first step, we examined lev-

els of active STAT3 (pY⁷⁰⁵-STAT3), HIF-1 α , CD133, and IL-6 in fresh tumor tissues and blood samples of HCC patients by immunohistochemistry (IHC) and enzyme-linked immunosorbent assay analyses. IHC demonstrated that levels of active STAT3, HIF-1 α , and CD133 were significantly elevated in HCC, tissues when compared to adjacent noncancerous tissues (Fig. 1A). In addition, serum levels of proinflammatory cytokine IL-6 were consistently higher in HCC patients than in control subjects (Fig. 1B). Importantly, active STAT3 was colocalized with CD133 as well as HIF-1 α (Supporting Fig. 1), indicating that their functions are positively correlated in tumorigenesis. Consistent with the observation in human HCC tissues, levels of active

STAT3, HIF-1 α , and CD133 were significantly increased in DEN-induced liver tumors, when compared to normal liver in a control C57BL/6J WT mouse (Fig. 1C). Before addressing the hypothesis of this study, we checked in advance the expression status of active STAT3 and CD133 as well as IL-6 receptors, such as IL-6R α and gp130, in normal liver Chang and various HCC cell lines (Hep3B, HepG2, Huh7, PLC/PRF/5, SNU-398, SNU-423, SNU-449, SNU-761, and SNU-878). Active STAT3 was detected in almost all cell lines, but not in Chang and Hep3B cells (Fig. 1D). CD133 messenger RNA (mRNA) was not detected in all cell lines, but expressed, albeit at a slightly different degree, in a subset of HCC cell lines, such as Hep3B, HepG2, Huh7, PLC/PRF/5, and SNU-878 (Fig. 1E). Detection of CD133 protein levels by fluorescence-activated cell sorting (FACS) analysis confirmed this phenomenon (Supporting Fig. 2). Overall, levels of active STAT3 were not coincided with the endogenous expression of CD133. This might be owing to the different genetic characterization of each cell line. However, IL-6R α and gp130 were ubiquitously expressed in all HCC cell lines tested (Fig. 1D,E). Thus, we used this different status of CD133 to experimentally prove the functional role of IL-6/STAT3 signaling to induce CD133 expression in HCC progression.

IL-6 and STAT3 Promote CD133 Transcription.

In light of increased expression of liver tumor microenvironmental factors in HCC, we then investigated whether elevated levels of IL-6 could affect CD133 transcriptional activity. In serum-free culture conditions, addition of IL-6 promoted transcriptional activity of CD133 by around 2-fold in Huh7 cells (Fig. 2A) and subsequently increased the portion of CD133⁺ cell population by approximately 2.4-fold (Fig. 2B). Next, we asked how exogenous ligand IL-6 could activate the function of CD133. Both janus kinase (JAK) and STAT3 have previously been shown to be critical in mediating IL-6 receptor-mediated signaling cascades to transcribe expression of a variety of downstream target genes, such as c-Myc and Mcl-1, which have been shown to be involved in cancer cell proliferation or inhibition of apoptosis.²¹ Thus, we performed various experimental approaches to confirm the involvement of STAT3 in activation of CD133 transcription. Addition of IL-6 induced expression of CD133 both in mRNA and protein levels through activation of STAT3 (Fig. 2C). However, functional inactivation of STAT3 by pan-JAK inhibitor AG-490 abolished the induction of CD133 expression and decreased the levels of active STAT3 (Fig. 2C). The reverse inhibition of CD133 expression by AG-490 was similarly observed in other

HCC cell lines of PLC/PRF/5, SNU-449, Hep3B, and SNU-398 (Fig. 2D and Supporting Fig. 3A). Interestingly, IL-6 stimulation was able to induce expression of CD133, even in the SNU-449 and SNU-398 cell lines in which endogenous CD133 mRNA levels were very low or not detected by PCR (Fig. 1E), indicating that CD133 is an IL-6-inducible gene. Furthermore, treatment of AG-490 decreased activities of JAK1, JAK2, and STAT3. Consequently, expression of CD133 was decreased both in Huh7 and PLC/PRF/5 cells, which highly express CD133 without IL-6 stimulation (Fig. 2E), implying that endogenous expression of CD133 is maintained by IL-6 signaling. STAT3 activated by IL-6 was found to be translocated into the nucleus (Fig. 2F), and then increased the levels of acetylated histone H3 by directly binding to CD133 promoter (Supporting Fig. 3B). This phenomenon was owing to corecruitment of acetyl transferase p300 and active STAT3 to the CD133 promoter, mainly leading to acetylation of histone H3 at lysine 9 and histone H4 at lysine 12 residues, whereas epigenetic regulators for histone deacetylation, such as histone deacetylase (HDAC)1, HDAC3, and MeCP2, were gradually dissociated from the CD133 promoter upon cytokine stimulation (Supporting Fig. 3C). Targeting of HDACs (HDAC1, HDAC2, and HDAC3) with pan-HDAC inhibitor SAHA blocked induction of CD133, even in the presence of IL-6 (Supporting Fig. 3D). These results suggest that histone modification functionally involves IL-6/STAT3-mediated transcriptional activation of CD133. To further confirm that IL-6 induces CD133 expression, CD133⁺ CSC population was sorted out from the CD133⁻ population of Huh7 cells by FACS using allophycocyanin-conjugated CD133 antibody. Both types of populations were incubated with IL-6 and then induction of CD133 expression and sphere forming capacity was observed. As a result, IL-6 stimulation increased expression of CD133 and stemness-related genes, such as Nanog, Sox-2, and Oct-4, both in CD133⁺ and CD133⁻ cells (Supporting Fig. 4A). These molecular changes led to the phenotypic changes in terms of increase in size of CD133⁺ cell population and sphere-forming capacity (Supporting Fig. 4B, 4C), providing that CD133 up-regulation reflects increase in cancer stemness. Noticeably, the extent of increase in CD133 expression was much more in CD133⁻ cancer cells than in CD133⁺ cancer stem cells, when compared to each control not stimulated with IL-6 (e.g., 8.4%-46.7% vs. 11.8%-20.0%). The increase of CD133 expression was accompanied with up-regulation of cell-cycle regulators, such as cyclin A1 and cyclin D1, but levels of p21 were not changed (Supporting Fig. 4D). Direct inhibition of

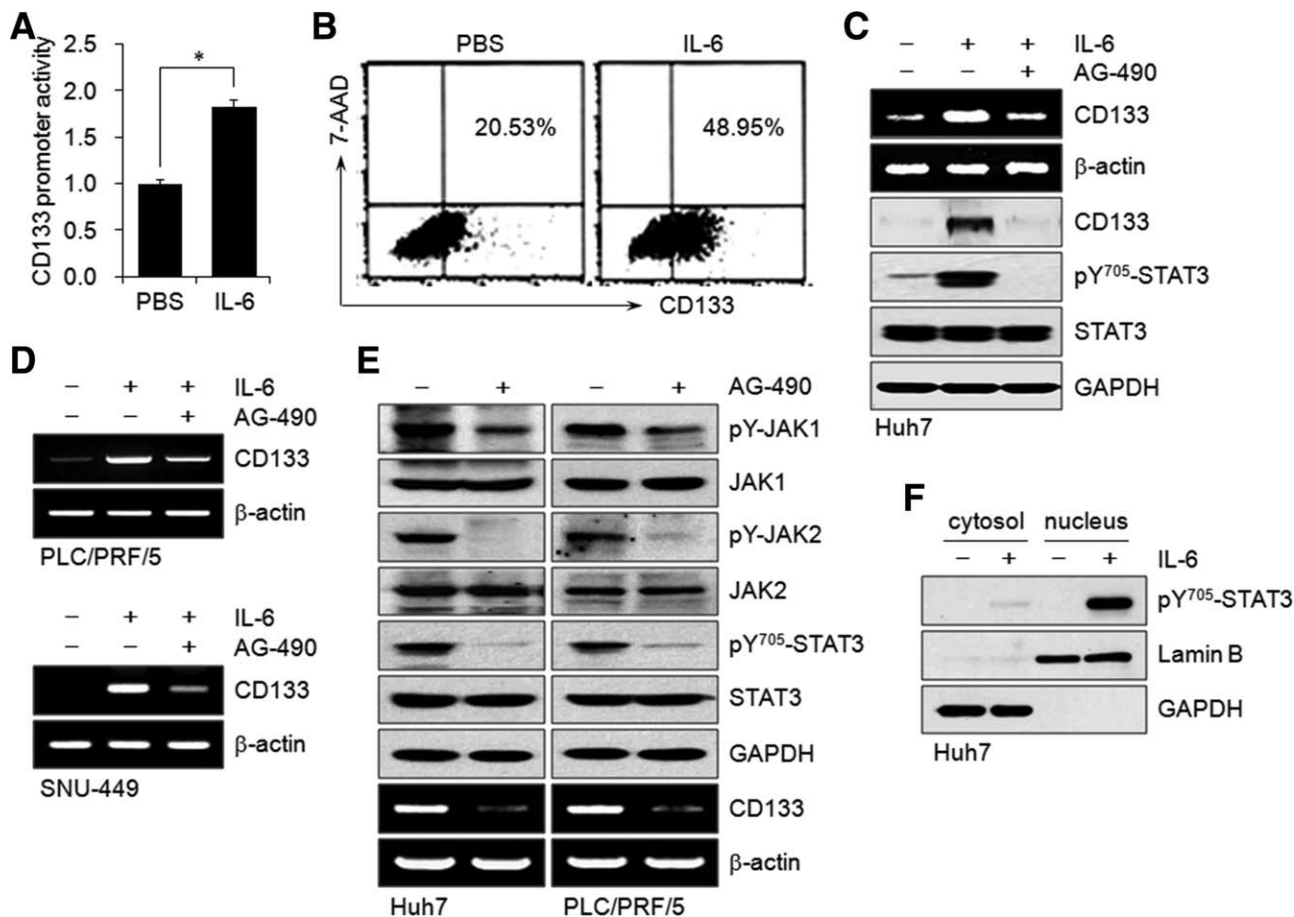


Fig. 2. IL-6 induces CD133 expression through activation of STAT3 signaling. (A) Huh7 cells were transfected with CD133-promoter-Luc and pRL-TK constructs and incubated for 16 hours with IL-6 (20 ng/mL). Luciferase activities were normalized to *Renilla* activities (mean \pm standard deviation). * $P < 0.05$. (B) Huh7 cells cultured in serum-free media were incubated with IL-6 (20 ng/mL) for 24 hours. CD133 expression was quantified by FCM. (C) Huh7 cells were pretreated with AG-490 (100 μ M) for 2 hours and then incubated with IL-6 (20 ng/mL) for 12 hours in serum-free conditions. CD133 and active STAT3 levels were determined by western blotting or reverse-transcriptase PCR (RT-PCR) analysis. (D) PLC/PRF/5 and SNU-449 cells pretreated with AG-490 (100 μ M) for 1 hour were incubated with IL-6 (20 ng/mL) for 12 hours in a serum-free condition. CD133 levels were determined by RT-PCR. (E) Huh7 and PLC/PRF/5 cells were pretreated with AG-490 (100 μ M) for 2 hours and then incubated for 12 hours. CD133, active JAK1, JAK2, and STAT3 levels were determined by western blotting or RT-PCR analysis. (F) Huh7 cells were incubated with IL-6 (20 ng/mL) for 30 minutes in a serum-free condition. Proteins were extracted from both cytosol and nuclei in order to examine localization of STAT3. Lamin B and GAPDH were used as nucleic and cytosolic markers, respectively. Abbreviations: 7-AAD, 7-aminoactinomycin D; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

STAT3 expression by STAT3-specific small interfering RNA (siRNA) showed a similar effect with AG-490, abolishing up-regulation of CD133 both in Huh7 and PLC/PRF/5 cells (Fig. 3A,B). Recently, it has been demonstrated that TLR-4 induces downstream IL-6 gene expression in tumorigenesis.²² Thus, we next observed the expression status of the three important regulators in the DEN-induced HCC mouse model with TLR-4/IL-6 double KO. As a result, blockade of IL-6 expression neither activated the function of STAT3 nor induced expressions of HIF-1 α and CD133 (Fig. 3C). Taken together, these data strongly suggest that IL-6-mediated STAT3 activation plays a pivotal role in the induction of CD133 expression in HCC progression.

IL-6/STAT3 Signaling Increases CD133 Expression by HIF-1 α in Hypoxia. Recent studies reported that hypoxia not only activates HIF-1 α to strengthen self-renewal activity and maintain the undifferentiated status of CD133-expressing cells,¹⁸ but also provokes endogenous c-Src/STAT3-signaling cascade.²³ Therefore, we examined whether hypoxia could trigger STAT3-mediated CD133 up-regulation. In a hypoxic condition, transient transfection of the active form of c-Src (c-Src^{Y527F}) significantly increased STAT3 activity and CD133 expression in Huh7 cells with a concomitant increase of HIF-1 α levels, and the effects were abolished by expressing the inactive form of c-Src (c-Src^{Y416F}; Fig. 4A). This negative effect was further

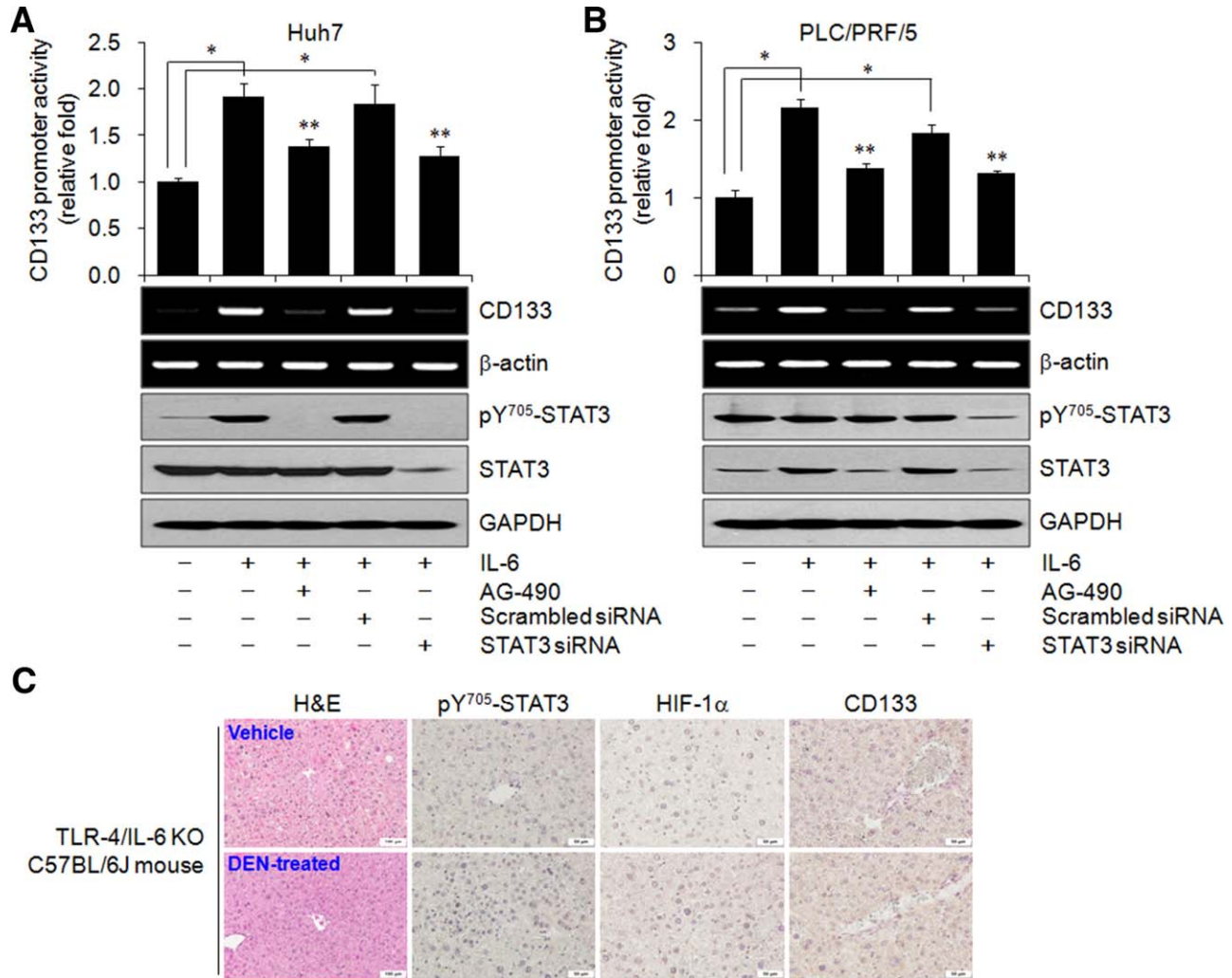


Fig. 3. IL-6 induces CD133 expression in a STAT3-dependent manner. Huh7 (A) and PLC/PRF/5 cells (B) were transfected with CD133-promoter-*Luc* and pRL-TK constructs with or without scrambled or STAT3 siRNA. The cells were incubated with IL-6 (20 ng/mL) in the presence or absence of either vehicle or AG-490 (100 μ M) for 24 hours. Luciferase activities were measured and normalized to *Renilla* activities (mean \pm standard deviationSD), and CD133 and active STAT3 levels were determined by western blot or reverse-transcriptase PCR analysis. * $P < 0.05$ versus vehicle-treated group; ** $P < 0.05$ versus IL-6-induced or scramble siRNA-transfected group ($n = 6$). GAPDH and β -actin were used as loading controls. (C) H&E staining and IHC confirmed that blockade of IL-6 expression did not induce activation of STAT3 and expression of HIF-1 α and CD133 in TLR-4/IL-6 double-KO mice. Scale bar = 50 μ m. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin.

validated by inhibiting expression of STAT3 by shRNA (Fig. 4B). Sorafenib, a multityrosine kinase inhibitor, is the first approved targeted therapeutic reagent for HCC. Mechanistically, it has been recently reported that sorafenib not only overcomes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance of HCC cells through inhibition of STAT3 activity,²⁴ but also suppresses angiogenesis by blocking synthesis of HIF-1 α in HCC.²⁵ Thus, we tested whether sorafenib could inhibit STAT3-mediated CD133 up-regulation upon hypoxia. As expected, treatment of sorafenib to hypoxic Huh7 cells showed a similar effect with shRNA silencing of STAT3, down-regulating expression of CD133 with a concomitant decrease in levels of STAT3 and HIF-1 α

(Fig. 4B). Phenotypically, sorafenib treatment significantly reduced the size of the IL-6-induced CD133⁺ Huh7 cell population (Fig. 4C). These data indicate that STAT3 up-regulates CD133 expression through activation of HIF-1 α signaling upon hypoxic stimulation. Our next question was whether endogenous HIF-1 α protein is essential for facilitation of CD133 up-regulation. Substantially, siRNA silencing of HIF-1 α significantly decreased levels of CD133 in hypoxic conditions (Fig. 4D). Next, we tested whether the combination of hypoxia with IL-6 stimulation could synergistically affect STAT3-mediated CD133 up-regulation. Surprisingly, the combinatory stimuli effect was almost doubled in terms of the increase of both

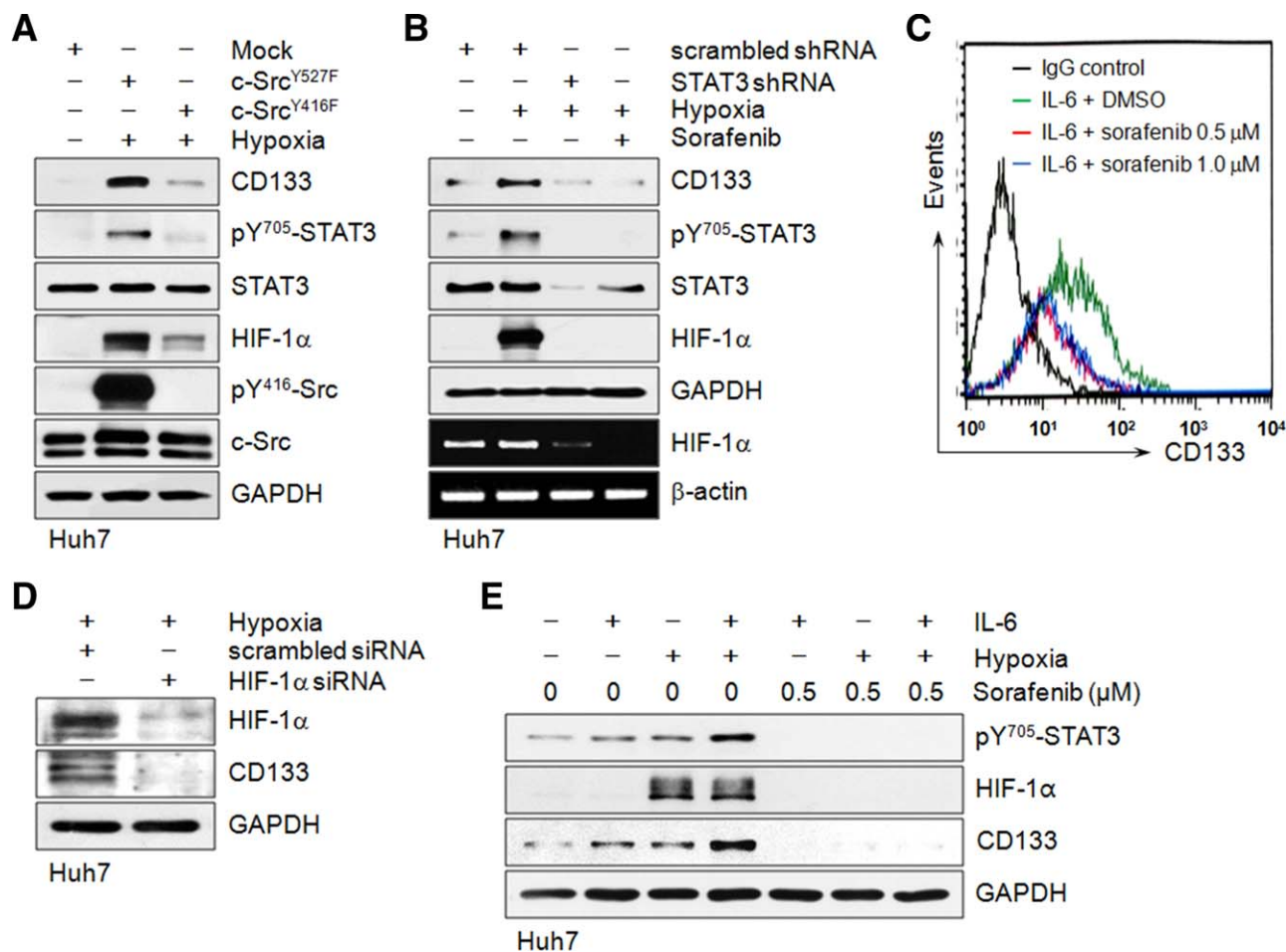


Fig. 4. STAT3-dependent HIF-1 α levels regulate CD133 expression in hypoxia. (A) Huh7 cells were transfected with either control vector, active c-Src (c-Src^{Y527F}), or inactive c-Src (c-Src^{Y416F}), and cells were incubated under hypoxic conditions for 40 hours. Western blotting analysis was then performed with the indicated antibodies. (B) Huh7 cells were transfected with either scrambled or STAT3 shRNA and incubated for 40 hours in the presence or absence of sorafenib (0.5 μ M) under hypoxic conditions. Western blotting or reverse-transcriptase PCR analysis was then performed. (C) Huh7 cells were incubated with IL-6 (20 ng/mL) for 24 hours in the presence or absence of sorafenib and the CD133 population was detected by FCM. (D) Huh7 cells were transfected with either scrambled or HIF-1 α siRNA and then incubated under hypoxic conditions for 40 hours. Proteins were analyzed by western blotting analysis. (E) Huh7 cells were pretreated with sorafenib (0.5 μ M) for 1 hour and then incubated with IL-6 (20 ng/mL) for 24 hours under hypoxic conditions. Western blotting analysis was performed. GAPDH was used as a loading control. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

STAT3 activity and CD133 levels, when compared with single stimulus, in Huh7 cells (Fig. 4E). The synergistic effect was reproduced in four additional HCC cell lines (PLC/PRF/5, SNU-398, Hep3B, and SNU-449), regardless of the difference in endogenous levels of CD133 expression (Supporting Fig. 5). Sorafenib treatment completely abolished STAT3-mediated CD133 up-regulation. These results strongly imply that proinflammatory signaling can boost HCC development under the hypoxic liver tumor microenvironment through STAT3-mediated CD133 up-regulation.

Active STAT3 Directly Interacts With NF- κ B p65 and Recruits It to HIF-1 α Promoter in Hypoxia. Hypoxia enhances activity of transcription factor NF- κ B, which is required for mRNA expression of HIF-1 α , by inhibiting prolyl hydroxylases that negatively regulate

I kappa B kinase beta catalytic activity.²⁶ Persistently activated STAT3 directly binds to released nuclear NF- κ B and maintains its activity by blocking NF- κ B/I kappa B complex from shuttling out of the nucleus.²⁷ Thus, we were determined to investigate whether the functional activity of NF- κ B could correlate to STAT3/HIF-1 α cooperation to exert CD133 up-regulation in the hypoxic tumor microenvironment. We have observed that both active STAT3 and NF- κ B are translocated into the nucleus upon hypoxia (Fig. 5A) and active STAT3 binds to endogenous NF- κ B p65 in HCC cells tested (Fig. 5B). To validate a predictive binding of STAT3/NF- κ B p65 dimer to the HIF-1 α promoter region, we performed a chromatin immunoprecipitation (ChIP) assay by using the antibodies to high-quality ChIP-grade active forms of STAT3, NF- κ B p65,

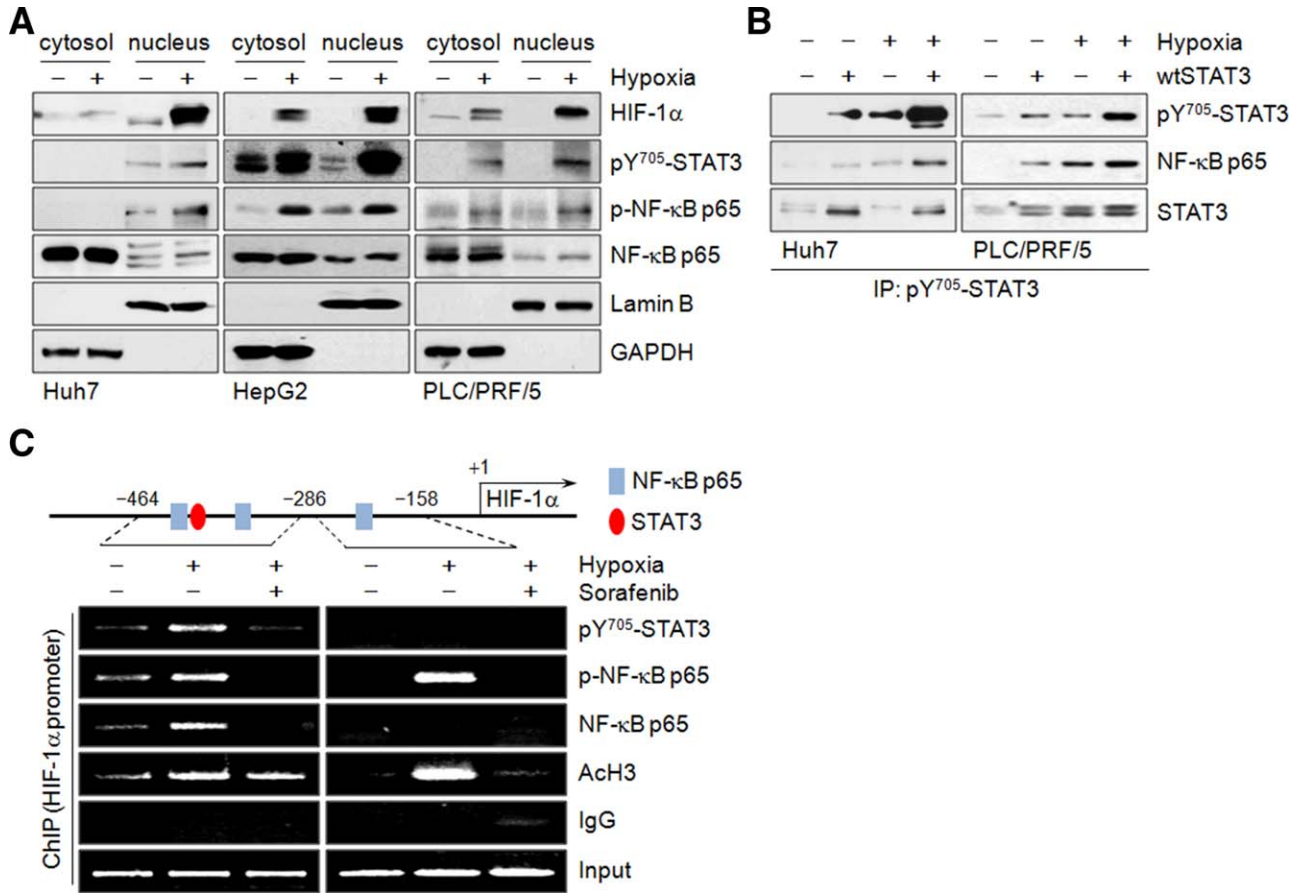


Fig. 5. Active STAT3 directly interacts with NF-κB p65 and recruits it to the HIF-1 α promoter region in hypoxia. (A) HCC cells were incubated under hypoxic conditions for 6 hours. Proteins were extracted from both cytosol and nuclei fractions in order to examine localization of HIF-1 α , active STAT3, and NF-κB p65. Lamin B and GAPDH were used as nucleic and cytosolic markers, respectively. (B) Huh7 and PLC/PRF/5 cells were transfected with either control vector or wt-STAT3 for 24 hours and further exposed to hypoxia for 6 hours. Cell lysates were immunoprecipitated with anti-pY⁷⁰⁵-STAT3 antibody and then incubated with the indicated antibodies. (C) Soluble chromatin was prepared from Huh7 cells and then immunoprecipitated with the indicated antibodies. DNA extracts were amplified using pairs of primers that cover putative consensus motif of the HIF-1 α promoter. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

acetylated histone H3, and rabbit immunoglobulin G (IgG) control. Importantly, high enrichment of active STAT3/NF-κB p65 to the HIF-1 α promoter was detected in hypoxia-stimulated Huh7 cells (Fig. 5C). Sorafenib treatment reverted hypoxia-induced STAT3 activation and inhibited binding of STAT3/NF-κB p65 dimer to the HIF-1 α promoter. These data clearly suggest that active STAT3 directly interacts with NF-κB p65 and recruits it to the HIF-1 α promoter under hypoxic conditions. This may represent a novel molecular mechanism by which STAT3, NF-κB, and HIF-1 α work together to induce expression of the CSC marker, CD133, during HCC development.

Functional Loss of CD133 Reduces Tumorigenic Potency In Vitro and In Vivo. To address the biological functions of CD133 in HCC tumor formation, Huh7 and PLC/PRF/5 were transfected with lenti-shRNA molecules and then observed for growth inhibition. Target gene knockdown was specific because trans-

fection with a negative control shRNA did not affect CD133 transcription (Fig. 6A). Targeting CD133 caused a significant reduction in growth rate of HCC cells in 72 hours, when compared to control shRNA treatment (Fig. 6B). shRNA silencing of CD133 gene expression did not affect the growth of SNU-449 cells in which endogenous CD133 level is very low (Supporting Fig. 6). Instead, the growth rate of cells was remarkably increased when CD133 was up-regulated by IL-6 stimulation, compared to control shRNA transduction (Supporting Fig. 6). This phenomenon was reversed by sorafenib treatment, indicating that suppression of CD133 expression is one of the molecular mechanisms underlying the tumor growth-inhibitory effect caused by sorafenib treatment. We next investigated whether shRNA silencing of CD133 could suppress liver tumor growth *in vivo*. To accomplish this, 1×10^6 cells with Huh7-lenti-vehicle or Huh7-lenti-shCD133 were SC injected into immunodeficient mice and then observed

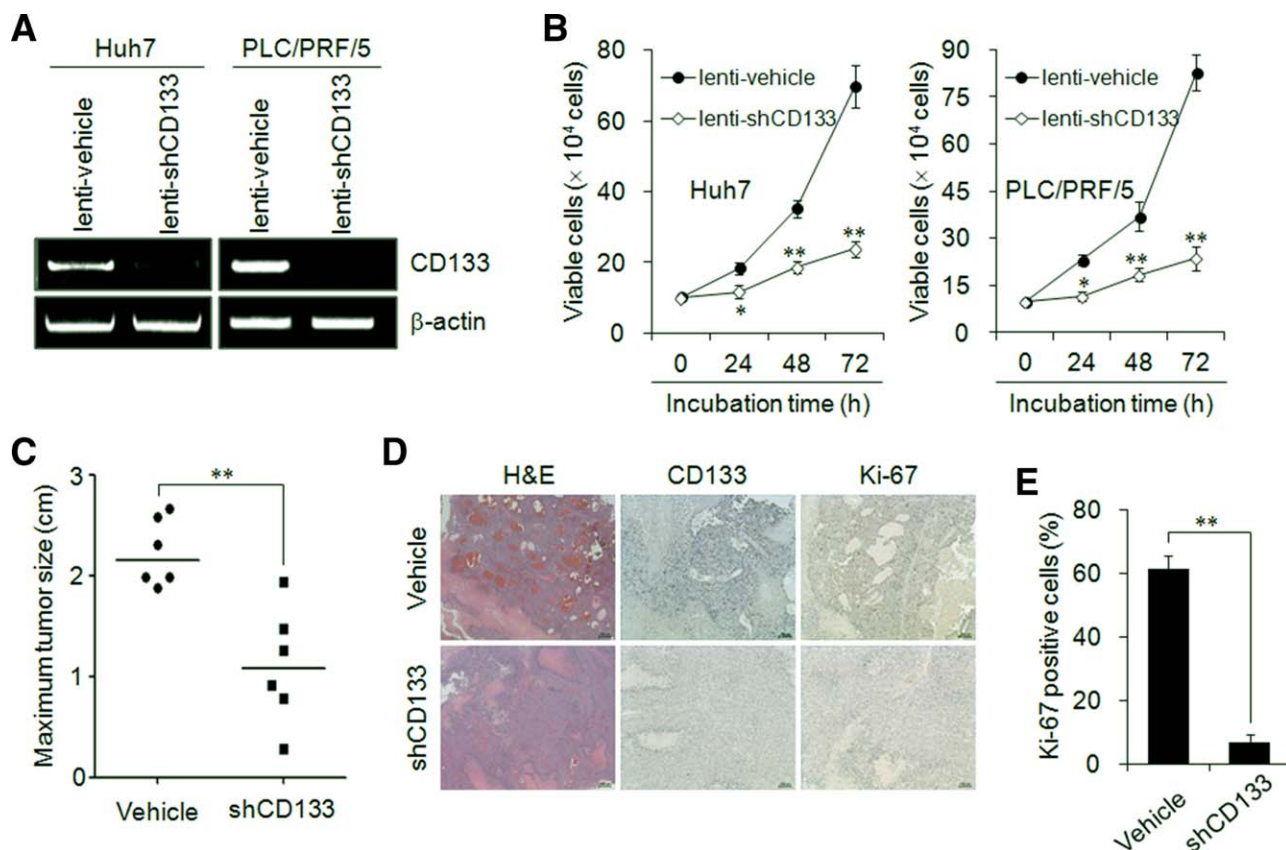


Fig. 6. CD133 silencing inhibits HCC cell proliferation *in vitro* and suppresses tumor formation *in vivo*. (A) Levels of CD133 mRNA were analyzed in Huh7 and PLC/PRF/5 cells transduced with lenti-vehicle or lenti-shCD133. (B) Lentiviral transduced Huh7 and PLC/PRF/5 cells were incubated for the indicated time points and viable cells were counted using trypan blue staining. Data represent the mean \pm standard deviation derived from three independent experiments. (C) Lentiviral-transduced Huh7 cells were SC injected into the dorsal flank of Balb/c nude mice. Tumor xenografts were isolated in 8 weeks of injection, and the size was measured. Maximum tumor size (diameter) was examined before sacrifice. ** $P < 0.005$. (D) H&E staining and IHC analysis were performed in xenograft tissues. (E) Quantification of Ki-67 expression. Two sections of each xenograft (5-10 fields/section) were subject to histological assessment ($n = 5$). ** $P < 0.005$. Abbreviation: H&E, hematoxylin and eosin.

for tumorigenicity. Tumors grew very rapidly in control mice that received Huh7-lenti-vehicle cells, whereas shRNA silencing significantly inhibited xenograft formation (Fig. 6C). Upon histopathological evaluation, mice receiving Huh7-lenti-shCD133 cells showed a better histology with the reduced tumor burden and lower size of cell populations that were expressing CSC marker CD133 and proliferation marker Ki-67 (Fig. 6D,E). Next, to study the molecular basis of growth inhibition caused by CD133 loss, we compared global gene expression patterns of CD133-deficient Huh7 cells to those of control cells with a control shRNA by using an Illumina microarray platform. We first observed that the growth inhibition in CD133-depleted Huh7 cells could be attributed, in part, to the defective cell-cycle progression. Flow cytometry (FCM) analysis revealed that shRNA silencing of CD133 had increased G_0/G_1 and decreased S-phase cell populations, suggesting a cell-cycle arrest in G_1 phase (Fig. 7A). Supporting this phenotypic change, CD133 silencing decreased levels of

cell-cycle regulators, such as cyclin A and D1, and increased levels of cdk inhibitor p21 (Fig. 7B). In this condition, a total of 1,540 genes were dysregulated (300 genes up-regulated and 1,240 down-regulated) by more than 2-fold in 48 hours of CD133 knockdown (Fig. 7C). Of those, in particular, eight genes (TACC1, ACF7, CKAP5, SKAP2, CLASP1, CLASP2, TRIP-Br2, and MAP4K4) that are functionally involved in mitotic progression, such as localization of centrosomes and regulation of microtubule dynamics at the kinetochore,^{28,29} were down-regulated (Fig. 7D). These results indicate that CSC marker CD133 may be an important regulator of HCC progression, and therapeutic targeting of CD133 can be a novel therapeutic modality for HCC.

Sorafenib and STAT3 Inhibitor Nifuroxazide Suppress CD133 Expression and Liver Tumor Growth In Vivo. We observed that CD133 levels were elevated in human HCC clinical specimens as well as in a DEN-induced HCC mouse model. Whereas sorafenib inhibited transcriptional activity and expression

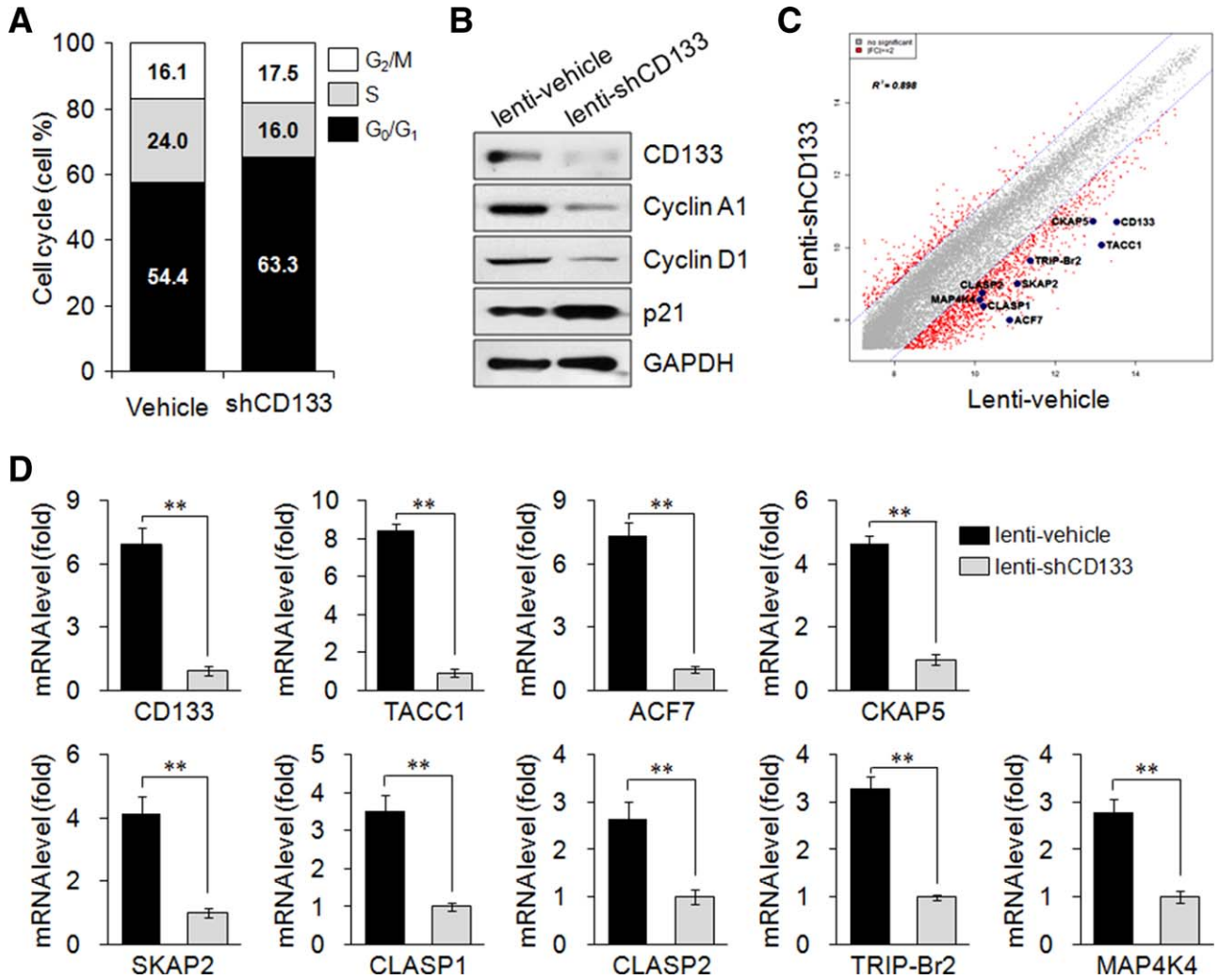


Fig. 7. CD133 silencing induces cell-cycle arrest by inhibiting expression of cytokinesis-related genes. (A) Cell-cycle profiles were evaluated using propidium iodide staining and FCM. (B) Proteins were prepared from Huh7 cells transduced with lenti-vehicle or lenti-shCD133 and analyzed by western blotting analysis. (C) Comparison of global gene expression in between Huh7-lenti-vehicle and Huh7-lenti-shCD133 cells. Plot locations of cytokinesis-related genes are highlighted. (D) Expression of cytokinesis-related genes was validated by quantitative real-time PCR analysis. $**P < 0.005$. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

of CD133 as well as disrupted STAT3-mediated CD133 up-regulation in either IL-6- or hypoxia-induced HCC cells, there was a concomitant decrease in levels of active STAT3 and HIF-1 α . Therefore, we investigated whether sorafenib and STAT3 inhibitor nifuroxazide³⁰ could suppress liver tumor growth *in vivo* by inhibiting the functional changes of tumor microenvironmental factors. HCC tumor growth and population were dramatically increased in control mice with SC injection of Huh7 cells upon 2 weeks, whereas treatment of either sorafenib or nifuroxazide remarkably suppressed growth and population (Fig. 8A). Upon histological evaluation, elevated tumor population and levels of active STAT3, HIF-1 α , and CD133 were observed in control mice. However, tumor growth-associated population and factors were dramatically decreased in sorafe-

nib- and nifuroxazide-treated mice (Fig. 8B). Coinciding with the microarray analysis *in vitro* (Fig. 7D), both sorafenib and nifuroxazide down-regulated expression of TACC1, ACF7, CKAP5, SKAP2, CLASP1, CLASP2, TRIP-Br2, and MAP4K4 genes by suppressing CD133 expression in Huh7-derived xenograft (Supporting Fig. 7). Western blotting also showed that down-regulation of CD133 consequently decreased levels of downstream targets, such as cyclin A, cyclin D1, B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), myeloid cell leukemia 1 (Mcl-1), and survivin, and increased cdk inhibitor p21 in tumor tissues (Fig. 8C). Meanwhile, owing to antiangiogenic activity of sorafenib by inhibiting levels of vascular endothelial growth factor (VEGF) and CD31 in HCC, we further evaluated molecular changes of these factors. Consistent

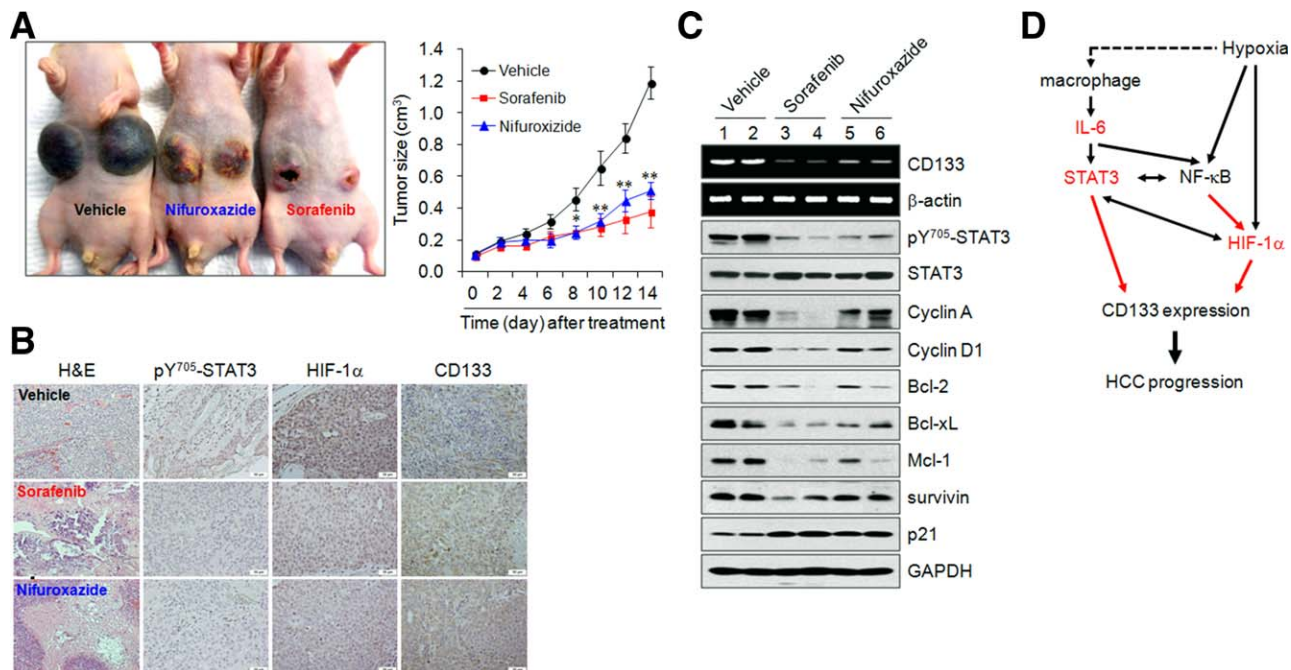


Fig. 8. Sorafenib and nifuroxazide suppress liver tumor growth in mice by suppressing activation and expression of STAT3, HIF-1 α , and CD133. (A-C) Viable Huh7 cells were injected SC into the dorsal flank of Balb/c nude mice and tumor-bearing mice were randomly assigned to three groups. Mice were treated with vehicle, sorafenib (30 mg/kg), or nifuroxazide (60 mg/kg) with a 2-day interval during 2 weeks. (A) Differences of tumor sizes in between vehicle- and agent-treated groups were compared using analysis of variance. Data represent the mean \pm standard error of the mean ($n = 6$); * $P < 0.05$; ** $P < 0.005$. (B) IHC analysis of active STAT3, HIF-1 α , and CD133 in xenograft tissues. (C) Reverse-transcriptase PCR or western blotting analysis of the indicated targets in xenograft tissues. β -actin and GAPDH were used as loading controls. (D) Schematic diagram of IL-6/STAT3 signaling-mediated CD133 up-regulation responsible for HCC tumorigenesis in the hypoxic micro-environment. Red arrows and words indicate novel mechanisms in HCC progression. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin.

with previously reported results,²⁵ sorafenib effectively reduced these levels in tissues of HCC tumor xenograft mice (Supporting Fig. 8). VEGF- and CD31-immunoreactive cells were distributed around microvessels in tumor tissues of control mice, but these cells were not observed in sorafenib-treated xenograft mice. Our findings suggest that IL-6/STAT3 signaling promotes expression of CD133 with a concomitant increase of HIF-1 α during liver carcinogenesis (Fig. 8D), and thus targeting IL-6/STAT3-mediated CD133 up-regulation may be a valuable therapy in eradication of this subset of cells.

Discussion

Identification of signaling pathways or biomarkers and correlation of their functions to CSC biology are priorities for development of novel cancer therapeutic strategies. There are accumulating evidences to show that several hepatic stem/progenitor markers, such as CD133, CD24, epithelial cell adhesion molecule, and CD90, are useful for isolating a subpopulation of HCC cells that have the preferential ability to induce self-

renewal, tumorigenicity, and chemoresistance.^{4-6,31,32} However, most studies have concentrated on the identification of surface markers to isolate this subset of CSCs. Studies focused on characterizing the molecular signaling pathways and functions of CSC marker in tumor microenvironment are lacking. There is accumulating evidences to show that CD133, a universal marker of organ-specific stem cells and CSCs, can be used to predict and diagnose development of various malignancies, including HCC. In addition to its role as a liver CSC marker, CD133 was also found to be functionally important in regulating tumorigenesis of liver CSCs through CD133 shRNA knockdown assays.³² This finding now clearly suggests that CD133⁺ liver CSCs represent an important subpopulation of cells responsible for driving and maintaining HCC.

Despite the significant relevance of enhanced expression of CD133 to HCC progression, fundamental mechanisms underlying deregulation of CD133 are not known. The improved understanding of the molecular pathway by which CD133 is deregulated in HCC will provide useful information for elucidation of liver CSC origin and development of novel treatment strategies

against the deadly disease. Here, we provide data to show that IL-6/STAT3 signaling is critical in the induction of CD133 expression through activation of HIF-1 α in a hypoxic liver tumor microenvironment. Therapeutic targeting of CD133 by shRNA knockdown induced cell-cycle arrest in HCC cells *in vitro* and led to retardation of xenograft-tumor progression *in vivo*. More important, our findings suggest that CD133 expression can be induced by IL-6 and hypoxic conditions in a STAT3-dependent manner. STAT3 promotes tumorigenesis and regulates numerous oncogenic and inflammatory genes.⁸ We have recently reported that STAT3 contributes to angiogenesis by enhancing stability of HIF-1 α protein under hypoxic conditions and induces aggressive proliferation by regulating expression of cyclin D1, an important cell-cycle-regulatory protein required for G₁/S-phase transition.³³

In this study, we demonstrated that IL-6 and the hypoxic tumor microenvironment are critical for induction of CD133 expression by STAT3 activation. We also found that STAT3 plays an important role in switching over transcriptionally active or inactive forms of CD133 promoter to accompany histone modifications. As demonstrated by ChIP assays, STAT3 activated by IL-6 rapidly translocated to the nucleus and bound to the CD133 promoter region. On the other hand, nonhistone chromosomal proteins, such as methyl-CpG binding protein MeCP2, HDAC1, and HDAC3, were gradually discharged from CD133 promoter by active STAT3. Another report in support of the epigenetic modification of CD133 promoter is that histone demethylase KDM5A was significantly elevated in drug-resistant cancer cells and exhibited higher levels of CD133 cells owing to demethylation of the CD133 promoter region.³⁴ In endometrial cancer cells, the regions of CD133 promoter were also hypomethylated in malignant endometrial tissue, relative to benign control endometrial tissue.³⁵ Taken together, these results suggest that STAT3 induces nonmutational chromatin-mediated modulation of the CD133 promoter, leading to aggressive tumor progression in HCC cells.

Sorafenib, originally developed as an inhibitor of Raf and receptor tyrosine kinase signaling, suppresses tumor propagation and angiogenesis by inhibiting Raf/mitogen activated protein kinase kinase/extracellular signal-regulated kinase as well as various receptor tyrosine kinases, such as VEGFR and platelet-derived growth factor receptor.²⁵ Recent studies have reported that sorafenib down-regulates the expressions of Bcl-xL and Mcl-1, the transcriptional targets of STAT3, and sensitizes cells to TRAIL-mediated apoptosis at micromolar concentrations.^{36,37} However, a multiple tyrosine kinase

inhibitor (MKI), sorafenib, causes “hand-foot skin reaction” owing to a potentially dose-limiting cutaneous toxicity in 21%-45% of advanced HCC patients. This condition is mainly determined by dosage, peak plasma level, total cumulative dose, and schedule of administration of MKIs.^{38,39} Given that subnanomolar concentrations of sorafenib would be effective against STAT3/HIF-1 α activation, as demonstrated in this study, patients with advanced HCC can be considered for administration of low-dose sorafenib with a combined therapy regimen.

Taken together, we report, for the first time, that IL-6 and hypoxia regulate expression of CD133 in a STAT3-dependent manner, resulting in HCC tumorigenesis. Our results indicate that targeting the tumor microenvironment can be considered as one of the therapeutic strategies to prevent patients with liver diseases from development of malignancies. In summary, CD133 is an important regulator of HCC growth, survival, and angiogenesis, and STAT3-mediated CD133 up-regulation may represent a promising target functional pathway for systemic therapy of a wide spectrum of human HCC.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.27968/supinfo.