STAT3 mediates regorafenib-induced apoptosis in hepatocellular carcinoma

Wei-Tien Tai ¹,²,*, Pei-Yi Chu ³,*, Chung-Wai Shiau ⁴,*, Yao-Li Chen ⁵, Yong-Shi Li ¹,², Man-Hsin Hung ⁶,⁷, Li-Ju Chen ¹,², Pei-Lung Chen ⁸, Jung-Chen Su ⁹, Ping-Yi Lin ⁵, Hui-Chuan Yu ¹,², and Kuen-Feng Chen ¹,²

¹Department of Medical Research, National Taiwan University Hospital, Taipei, Taiwan

²National Center of Excellence for Clinical Trial and Research, National Taiwan University Hospital, Taipei, Taiwan

³Department of Pathology, St. Martin De Porres Hospital, Chiayi, Taiwan

⁴Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taipei, Taiwan.

⁵Department of Surgery, Changhua Christian Hospital, Changhua, Taiwan

⁶Division of Hematology and Oncology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan

⁷Program in Molecular Medicine, School of Life Sciences, National Yang-Ming University, Taipei, Taiwan

⁸Graduate Institute of Medical Genomics and Proteomics, National Taiwan University College of Medicine, Taipei, Taiwan

* W.-T. T., P.-Y. C., and C.-W. S. contributed equally to this work.

Corresponding authors: Kuen-Feng Chen, Department of Medical Research, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, Taiwan, R.O.C.,
Conflict of interest

All authors have nothing relevant to this manuscript to disclose.

Translational Relevance

The use of tyrosine kinase inhibitors (TKI) as HCC targeted therapies is an actively researched area. But, in light of several failed clinical trials of VEGFR inhibitors such as sunitinib and brivanib, there are still open questions about what exactly drives drug efficiency. Consequently, further study of determinant factors in addition to their kinase activity, is necessary. Here, we put forward a detailed molecular mechanism to explain the biological effect of regorafenib, and present a critical transcription factor, STAT3, which involves in the clinical stages of HCC and determines the effect of regorafenib. Also, we identified SHP-1, a tyrosine phosphatase of STAT3, as a target of regorafenib and explored the mechanism by which regorafenib increases the activity of SHP-1 through direct interactions. Our findings thus provide new mechanistic insights into the effect of regorafenib in HCC and indicate that STAT3 is a druggable target for HCC.
Abstract

Purpose: Here, we aim to investigate the molecular mechanism of regorafenib and verify the potential druggable target for the treatment of HCC.

Experimental Design: HCC cell lines (PLC5, HepG2, Hep3B, SK-Hep1, and HA59T) were used to investigate the in vitro effect of regorafenib. Phosphatase activity was analyzed in HCC cells and purified SHP-1 proteins. PLC5-bearing mice were tested the therapeutic efficiency with 20 and 40 mg/kg/day treatment of regorafenib (n ≥ 8 mice). The clinical relevance of STAT3 signaling was investigated with 142 tumor samples from different patients with HCC. Descriptive statistical analysis was used to compare the baseline characteristics of patients and the expression of p-STAT3

Results: Regorafenib inhibited STAT3-related signaling in a dose-dependent manner and was a more potent inhibitor of STAT3 than sorafenib. Regorafenib increased SHP-1 phosphatase activity in purified SHP-1 protein directly. N-SH2 domain deletion and D61A mutants mimicking open-form SHP-1 partially abolished regorafenib-induced STAT3 inhibition and apoptosis. Importantly, a higher level of expression of STAT3 was found in patients with advanced clinical stages (p=0.009) and poor differentiated tumors (p=0.035).

Conclusions: Regorafenib induced significant tumor inhibition by relieving auto-inhibited N-SH2 domain of SHP-1 directly and inhibiting p-STAT3 signals. STAT3 may be suitable as a prognostic marker of HCC development, and may be a druggable target for HCC targeted therapy using regorafenib.
**Introduction**

Regorafenib (Stivarga), a novel oral multiple kinase inhibitor, potentially inhibits tumor growth through anti-angiogenesis (1). Several angiokinases such as VEGFR1/2, PDGFR-β, and FGFR1 are believed to be major targets of regorafenib for cancer treatment (1, 2). Regorafenib was approved for the treatment of patients with metastatic colorectal cancer and advanced gastrointestinal stromal tumors by the US Food and Drug Administration in 2012. As the chemical structure of regorafenib is very similar to that of sorafenib, the first and only small molecule targeted therapy approved for HCC treatment, several *in vitro* studies and clinical trials have been initiated to evaluate the efficiency of regorafenib for HCC. The safety of regorafenib at a dose of 160 mg once daily has been validated in HCC patients following first-line sorafenib (3). Participants are currently being recruited for a randomized, double blind, phase III study of regorafenib in patients with HCC who have progressed on sorafenib treatment (NCT01774344).

Protein tyrosine phosphatases (PTPs) are also vital regulators involved in many cellular signaling processes, which make PTPs potential prognostic markers and druggable targets. Src homology region 2 (SH2) domain-containing phosphatase 1, SHP-1, acts as a negative regulator of STAT3. The 3D structure of SHP-1 shows that the ligand-free SHP-1 has an auto-inhibited conformation through blocking catalytic PTPase via the N-SH2 domain (4-6). However, the underlying molecular mechanism by which SHP-1 is involved in tumorigenesis is still unknown. In this study, we first clarified the molecular mechanism by which regorafenib inhibits STAT3 signaling, and then disclosed the direct effect of regorafenib on SHP-1. We demonstrate that regorafenib has potent anti-HCC potency via targeting STAT3, and provide evidence to suggest that STAT3 may be a useful prognostic marker and druggable target for the...
treatment of HCC.

Materials and Methods

Cell culture and antibodies. The HA59T HCC cell line was obtained from the Bioresources Collection and Research Center (BCRC) (Food Industry Research and Development Institute, Hsin-Chu, Taiwan). The PLC/PRF/5 (PLC5), Sk-Hep-1, HepG2, and Hep3B cell lines were obtained from American Type Culture Collection (Manassas, VA). All cells obtained from BCRC or ATCC were immediately expanded and frozen down such that all cell lines could be restarted every 3 months from a frozen vial of the same batch of cells. No further authentication was conducted in our laboratory. Antibodies for immunoblotting such as cyclin D1 and PARP were purchased from Santa Cruz Biotechnology (San Diego, CA). Other antibodies such as p-STAT3, STAT3, survivin, Mcl-1, caspase-9, and myc-tag were from Cell Signaling (Danvers, MA). SHP-1 antibody was purchased from Abcam (Cambridge, MA).

Reagents. Regorafenib (BAY 73-4506) was purchased from Selleck Chemicals (Houston, TX). For cell-based studies, regorafenib at various concentrations was dissolved in DMSO and then added to the cells in serum-free DMEM. SHP-1 inhibitor (PTP III) was purchased from Calbiochem (San Diego, CA). The DNA fragmentation was demonstrated in cell death ELISA assay kit purchased from Roche Molecular Biochemicals (Mannheim, Germany).

Plasmids, siRNA and transfection. Plasmids encoding the human STAT3 and SHP-1 (PTPN6) mutant, in which the SH2/PTP domain was truncated or one aspartic acid at 61 was changed into an alanine residue were cloned into pCMV6-Entry vector with the myc-tag. All of the truncated mutants were confirmed by DNA sequence and their expression level in HCC cells was assayed. SMARTpool siRNA, including control (D-001810-10) and SHP-1 (PTPN6, L-009778-00-0005) were all purchased from
Dharmacon (Lafayette, CO). For transient transfection, plasmids or siRNA were pre-transfected with lipofetamine 2000 (Invitrogen, CA) for 24 h and then processed with the indicated treatment for another 24 h as described previously (7).

**Expression of recombinant protein.** The human full-length SHP-1-GST fusion protein was inserted into the pGEX-4T-1 vector (Amersham Biosciences, NJ). The GST fusion protein of wild-type SHP-1 was expressed in *Escherichia coli* and affinity purified using glutathione-sepharose 4B beads (Amersham Biosciences, NJ).

**SHP-1 phosphatase activity.** HCC protein extracts pretreated with regorafenib were incubated with anti-SHP-1 antibody in immunoprecipitation buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 1% sodium deoxycholate) overnight. Protein G-Sepharose 4 Fast flow (GE Healthcare Bio-Science, NJ) was added to each sample, followed by incubation for 3 h at 4°C with rotation. A RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit (R-22067) was used to assay SHP-1 activity (Molecular Probes, Invitrogen, CA). For in vitro assay, SHP-1-containing extract or purified recombinant SHP-1 protein was incubated with regorafenib at the indicated dose for 30 min at 4°C and the phosphatase activity of SHP-1 was assessed.

**Xenograft tumor growth.** For the subcutaneous xenograft tumor model (n = 10), each mouse was inoculated s.c. in the dorsal flank with 1 × 10⁶ PLC5 cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA). When tumors reached 100–200 mm³, mice received vehicle or regorafenib (20 mg/kg) p.o. once daily. Tumors were measured twice weekly using calipers and their volumes calculated using the following standard formula: width × length × height × 0.523.

**Immunohistochemistry.** Tumors from hepatocellular carcinoma patients who

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received surgical resection, post-operative treatment and follow-up in Changhua Christian Hospital from June 2012 to June 2013 were enrolled for analysis (CCH IRB No. 120504). Paraffin-embedded hepatocellular carcinoma tissue sections (4-μm) on poly-1-lysine-coated slides were deparaffinized and rinsed with 10 mM Tris-HCl (pH 7.4) and 150 mM sodium chloride. Paroxidase was quenched with methanol and 3% hydrogen peroxide. Slides were then placed in 10 mM citrate buffer (pH 6.0) at 100°C for 20 minutes in a pressurized heating chamber. After incubation with 1:50 dilution of p-STAT antibody (Rabbit monoclonal [EP2147Y] to STAT3 (phospho Y705) (AB76315), Abcam, Cambridge, UK) for 1 hour at room temperature, slides were thoroughly washed three times with phosphate buffered saline. Bound antibodies were detected using the EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse kit (Dako, Glostrup, Denmark). The slides were then counterstained with hematoxylin.

Paraffin-embedded sections of mouse kidney tissue and human colon carcinoma were used as positive controls for p-STAT3, respectively, as described in the datasheet from the manufacturer. Negative controls had the primary antibody replaced by phosphate buffered saline. The expression of p-STAT3 was assessed semiquantitatively based on the intensity of staining and percentage of cell involved by a board certified pathologist. The intensity of staining was scored as negative, low and high. This study was approved by the ethics committee of the Institutional Review Board of Changhua Christian Hospital. All informed consents from sample donors were in accordance with the Declaration of Helsinki and were obtained at the time of their donation.

Statistical analysis. Descriptive statistical analysis was used to compare the baseline characteristics of patients and the expression of p-STAT3. A p value < 0.05 was regarded as statistically significant on the two-tailed tests. All statistical analysis was computed using SPSS for Windows software (version 18.0; SPSS, Inc., Chicago, IL) (8).
Results

Regorafenib, a multi-target angiokinase inhibitor, exhibited significant apoptosis in HCC cells. Angiogenesis plays a central role in cancer progression and metastasis including hepatogenesis. First, we assessed the new anti-angiogenesis drug, regorafenib, for treatment of HCC. As shown in Fig. 1A, regorafenib treatment significantly inhibited cell growth in a panel of five HCC cell lines. Regorafenib was also found to induce apoptosis in a dose-dependent manner (Fig. 1B). In addition, regorafenib exhibited significant cell death at clinically relevant doses as measured by DNA fragmentation assay (Fig. 1C). Induction of apoptosis was further confirmed by the activation of caspase-9 and PARP cleavage in regorafenib-treated cells (Fig. 1D). These results suggest that regorafenib has significant anti-HCC activity.

Inhibition of p-STAT3 signaling by regorafenib mediates HCC apoptosis. To investigate the potential mechanism of action by which regorafenib induces apoptosis of HCC cells, we first examined the STAT3-related signal pathway which has been reported to be ubiquitously activated in HCC progression. As shown in Fig. 2A, regorafenib inhibited p-STAT3 and its related proteins including Mcl-1, cyclin D1, and survivin in a dose-dependent manner. Regorafenib exhibited significant p-STAT3 inhibition after an extremely short treatment span (Fig. 2B). Regorafenib inhibited p-STAT3 signaling in dose- and time-dependent manners. In addition, regorafenib exhibited strong STAT3 inhibition as determined by p-STAT3 ELISA (Fig. 2C, left). Regorafenib demonstrated more potent inhibition of STAT3 than sorafenib, an approved clinical angiokinase inhibitor which also shows p-STAT3 inhibitory activity (Fig. 2C, right). To clarify whether STAT3 inhibition induced by regorafenib sensitizes HCC cells to apoptosis, we analyzed the effect of regorafenib in PLC5 cell overexpressing STAT3. As shown in Fig. 2D, overexpression of STAT3 almost
abolished the apoptotic effect of regorafenib, indicating that inhibition of p-STA3-related signaling determines the sensitizing effect of regorafenib on HCC.

**Regorafenib directly activates SHP-1 by targeting the autoinhibited SH2 domain to inhibit p-STAT3 signaling.**

To explore the underlying mechanism by which regorafenib inhibits p-STAT3 signaling in-HCC, next, we examined potential inhibitors of p-STAT3 such as SHP-1, a known SH2 domain-containing protein tyrosine phosphatase (PTP) involved in dephosphorylation of STAT3, to clarify its anti-HCC effect. As shown in the results presented in Fig. 3A (*left*), we found that regorafenib increased SHP-1 activity at the same doses that inhibited p-STAT3 in PLC5 cells. Moreover, regorafenib also activated SHP-1 in other tested HCC cell lines at 10 μM (Fig. 3A, *right*). Importantly, both specific SHP-1 inhibitor and siRNA against SHP-1 (*PTPN6*) reversed the regorafenib-induced STAT3 inhibition and apoptosis, suggesting that SHP-1 may be involved in the effect of regorafenib on HCC (Fig. 3B). Further, we found that regorafenib increased SHP-1 activity in purified recombinant SHP-1 proteins and SHP-1-containing IP lysate *in vitro* (Fig. 3C), indicating that regorafenib activates SHP-1 by direct interaction. As the activity of SHP-1 was strongly regulated by the autoinhibited 3D structure, we further investigated the effect of the autoinhibited N-SH2 domain responsible for autoinhibition with catalytic PTPase, on regorafenib potency. We constructed wild-type, deletion of N-SH2 (dN1), and D61 single mutant (D61A) of SHP-1 to investigate the effect of regorafenib on different SHP-1 statuses (Fig. 4A). As demonstrated in Fig. 4B, regorafenib induced significantly less inhibition of STAT3 and apoptosis in PLC5 cells expressing the dN1 and D61A mutants than in the vector, implying that regorafenib may directly affect the autoinhibition by N-SH2 and PTPase. Furthermore, dN1 decreased
regorafenib-induced apoptosis in a dose-dependent manner (Fig. 4C). These results suggest that relieved SHP-1 counteracts regorafenib-induced anti-HCC effect. Regorafenib, therefore, potentially relieves autoinhibition of SHP-1 and further inhibits p-STAT3 signals directly.

**Regorafenib exhibited significant tumor inhibition in an HCC subcutaneous tumor model.** To examine whether regorafenib displays anti-HCC potency *in vivo*, we tested regorafenib orally in a HCC-bearing subcutaneous xenograft mouse model. Regorafenib-treated mice displayed significant tumor inhibition compared with the mice treated with vehicle (Fig. 5A, left). The significant difference between regorafenib-treated (20 mg/kg/day) and vehicle-treated mice was found after 14 days of treatment (** P<0.05). In the end of treatment, regorafenib induced 34.72% of tumor volume in this PLC5-bearing animal model. Also, the average tumor weight of mice treated with regorafenib was lower than that of the vehicle-delivered mice in the end of treatment (Fig. 5A, right). Notably, mice treated with 20 mg/kg/day regorafenib did not lose body weight until the end of the treatment (Fig. 5B). To verify the detailed molecular mechanism determining regorafenib-induced anti-HCC potency, we collected the tumor lysate and investigated the SHP-1/p-STAT3-related signaling pathway. As shown in Fig. 5C, regorafenib inhibited p-STAT3 expression and increased the SHP-1 activity in vivo. Moreover, regorafenib showed better anti-tumor effect at the dose of 40 mg/kg/day treatment. Both the average tumor size and tumor weight of animals were less than 50% of those of control mice at the end of treatment (Fig. 5D). Taken together, we found that regorafenib, a multi-target anti-angiokinase inhibitor, exhibited potent anti-HCC effect in vitro and in vivo through direct targeting of SHP-1/STAT3 signaling.
**STAT3 determines the progression and differentiation of HCC.**

To investigate the clinical relevance of STAT3 signaling, we analyzed the correlation between the expression status of p-STAT3 and important clinical features of the patients, such as clinical stage, tumor differentiation and cirrhosis severity. There were 142 patients with HCC enrolled for this study and their general characteristics were summarized as Table 1. Surgical resection was the primary tumor-specific treatment for all our patients. Within a median follow-up of 11.34 months (interquartile range 8.07- 14.7), 16 patients (11.3%) had progression and 14 of them died (9.9%).

Among all the tumor samples examined, highly positive p-STAT3 staining was identified in 92 tumor samples (64.8%). The intensity of p-STAT3 was highly correlated with the aggressiveness of tumors; tumors with high p-STAT3 expressions were more likely to be found in patients with advanced clinical stages (p= 0.009) and poor differentiated tumors (p= 0.035). (Fig. 6) The expression of p-STAT3 was not significantly correlated with patient’s gender (p=0.459), severity of fibrosis (p=0.658) and cirrhosis (p=0.066), and chronic viral infection (p=0.085 for HBV infection and p=0.147 for HCV infection).

**Discussion**

Although one receptor tyrosine kinase (RTK) inhibitor has been approved and several are currently being tested in clinical trials for the treatment of HCC, the unsatisfactory response rate of kinase inhibitors still highlights the urgent need for a precise understanding of which molecule(s) determine HCC progression and may be druggable. In this study, we disclosed that SHP-1 is a potential tumor suppressor, and a direct target of regorafenib for anti-HCC. Previously, SHP-1 was found to be...
responsible for several immune responses in hematopoietic cells, such as trapping by inhibitory ITAM signaling to form inhibisome clusters (9), negative regulation of T-cell receptor engagement by inactivation of Lck (10), and downregulation of T cell antigen receptor signaling by direct dephosphorylation of 3BP2 (11).

Recently, several studies have verified the role of SHP-1 in cancer progression. In breast cancer, SHP-1 expression was demonstrated to define a subset of high-grade tumors and correlated with HER-2 (12). SHP-1 also suppressed growth and increased apoptosis in prostate cancer, even under IL-6 growth advantage (13). Importantly, promoter hypermethylation of SHP-1 induces ubiquitous activation of the Jak/STAT pathways in human liver cancer (14) suggesting that SHP-1 may be a useful prognostic marker and therapeutic target for cancer treatment. We proved that regorafenib directly targets SHP-1 to inactivate the STAT3-mediated signaling pathway and induce anti-HCC activity in vitro and in vivo, suggesting that SHP-1 may be a druggable protein for the treatment of HCC. Similarly, Nicola et al. reported that SHP-1 expression determines the resistance to imatinib for CML treatment and lower levels of SHP-1 were also found in patients with imatinib treatment failure (15). In light of these findings and the current study, we propose that HCC patients with lower expression of SHP-1 may be more resistant to regorafenib than those patients with higher SHP-1 expression. Further HCC clinical study is warranted to explore the role of SHP-1 as a prognostic and druggable marker.

Currently, several STAT3 inhibitors are being tested in clinical trials, such as OPB-31121 (NCT00955812) and WP1066 (NCT01904123). Our findings show that P-STAT3 expression is strongly associated with advanced clinical stages and poor differentiation in clinical HCC tissue, further suggesting that STAT3 may be a suitable
candidate for prediction of HCC progression (Fig. 6). Notably, STAT3 was believed to be a critical factor involved in microenvironment and cancer development. In our present HCC subcutaneous animal model, it is difficult for us to assay the effect of regorafenib on the tumor microenvironment and angiogenesis. As anti-angiogenesis has been considered as the main mechanism of action of TKIs for the treatment of HCC, the further investigation of regorafenib on angiogenesis, tumor microenvironment, and cancer metabolism will be needed to analyze the overall anti-HCC effect of regorafenib.

Here, we reported that regorafenib inhibited p-STAT3-mediated signaling by targeting autoinhibited SHP-1 directly to induce potent anti-HCC activity in vitro and in vivo. The correlation between P-STAT3 expression and HCC prognosis further provided a mechanism-based rationale to target STAT3 by regorafenib. In conclusion, we suggest that STAT3 may be a useful prognostic marker for HCC progression and determine the sensitivity of regorafenib as a druggable target for patients with HCC.

Acknowledgments

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References


Figure Legends

Figure 1. Regorafenib has a significant apoptotic effect in HCC cell lines. A. Dose-escalation effects of regorafenib on cell viability in HCC cell lines. PLC5, HepG2, Hep3B, SK-Hep1, and HA59T cells were exposed to regorafenib at the indicated doses for 48 h and cell viability was assessed by MTT assay. Columns, mean; bars, SE (n = 8). B. Effects of regorafenib on apoptosis in HCC cells. Cells were exposed to regorafenib at the indicated doses for 48 h and apoptotic cells were determined by flow cytometry (sub-G1). Columns, mean; bars, SE (n ≥ 3). C. The effect of regorafenib on DNA fragmentation was determined by cell death ELISA. Columns, mean; bars, SE (n = 6), *P < 0.05, **P < 0.01. D. Regorafenib induced apoptosis-related signals in HCC cell lines.

Figure 2. Inhibition of p-STAT3 determines the sensitizing effects of regorafenib in HCC cells. A. Dose-dependent effects of regorafenib on STAT3-related proteins. Cells were treated with regorafenib at the indicated doses for 24 h. B. Regorafenib induced p-STAT3 inactivation in a time-dependent manner. C. Left, dose-dependent effects of regorafenib on p-STAT3 activity. PLC5 cells were exposed to the indicated doses of regorafenib for 24 h and assayed in p-STAT3 ELISA. Right, regorafenib exhibited more potent p-STAT3 inhibition than sorafenib in PLC5 and HepG2 cells. D. STAT3 reverses the apoptotic effect of regorafenib. PLC5 cells transiently expressing STAT3 with Myc-tag were treated with regorafenib at 7.5 μM for 24 h and the percentage of apoptosis was measured by sub-G1 analysis. Columns, mean; bars, SE (n = 3) *P < 0.05, **P < 0.01.

Figure 3. SHP-1 plays a vital role in regorafenib-induced STAT3 inhibition and apoptosis. A. Left panel, regorafenib increased SHP-1 activity in PLC5 cells in a
dose-dependent manner. Right panel, regorafenib activated SHP-1 in several HCC cell lines, including HA59T, Hep3B, and HepG2. Cells were treated with regorafenib at 10 μM for 24 h. Columns, mean; bars, SE (n = 3). B. Left, PLC5 cells were pretreated with specific SHP-1 inhibitor (PTPIII) for 30 min before regorafenib treatment. Right, knockdown of SHP-1 reduces the effects of regorafenib on p-STAT3 and apoptosis. PLC5 cells were transfected with control siRNA (scramble) or SHP-1 siRNA for 24 h then treated with regorafenib for another 24 h. Columns, mean; bars, SE (n ≥ 3), *P < 0.05, **P < 0.01. C. Regorafenib activates SHP-1 directly. Left panel, regorafenib increases the phosphatase activity of SHP-1 in IP-SHP-1 cell lysate from PLC5 cells. Right panel, regorafenib increased SHP-1 activity in cell-free purified SHP-1 proteins. Columns, mean; bars, SE (n ≥ 3), *P < 0.05, **P < 0.01.

Figure 4. Regorafenib directly activates SHP-1 by relieving the autoinhibition of the SH2 domain. A. Schematic representation of deletion and single mutants of SHP-1. B. Regorafenib potentially interferes with the N-SH2 domain and further activates the catalytic activity of SHP-1. Deletion of the N-SH2 domain (dN1) and single-point mutant of D61 (D61A) reversed the effect of regorafenib on p-STAT3 inhibition and apoptosis. C. Dose-dependent N-SH2 (dN1) gradually restored the effect of regorafenib on p-STAT3 and cell death. The apoptotic effect was determined by sub-G1 analysis. Columns, mean; bars, SE (n ≥ 3), *P < 0.05, **P < 0.01.

Figure 5. Regorafenib induced significant tumor growth inhibition in vivo by targeting SHP-1-dependent STAT3 inhibition. A. Regorafenib exhibited significant tumor growth inhibition in a PLC5-bearing HCC subcutaneous model. Left, mice received regorafenib at 20 mg/kg/day and tumor growth was measured twice weekly. Points, mean; bars, SE (n = 10), *P < 0.05, **P < 0.01. Right, tumor weight at the
end of treatment. B. Body weight of mice with regorafenib treatment. C. Left, Regorafenib induced potent STAT3 inhibition in tumor lysate. Right, Activated SHP-1 in regorafenib-treated tumor sample. Columns, mean; bars, SE (n = 8), *P < 0.05. D. Left, tumor growth curve of mice treated regorafenib at 40 mg/kg/day. Points, mean; bars, SE (n = 8), *P < 0.05, ** P < 0.01. Right, tumor weight at the end of treatment.

Figure 6. Level of P-STAT3 expression in clinical HCC samples. The expression of p-STAT3 in HCC is significantly correlated with disease aggressiveness. Within the 142 clinical HCC tumors, positive p-STAT3 staining was observed in 92 tumor samples. A and B were the representative cases showing low p-STAT3 expression within well-differentiated tumors. C and D were the representative immunohistochemical patterns of p-STAT3 in clinical HCC tissues. In line with the results of statistical analysis showing below, high expressions of p-STAT3 were significantly associated with poor tumor differentiation (p= 0.009).
Fig. 2

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Apoptotic cells (%)

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Fig. 3

A

PLC5

HCCs

SHP-1 activity (% of control)

Regorafenib 0 1 5 10

SHP-1 activity (% of control)

Regorafenib 0 10

SHP-1 IP extract

Purified SHP-1

SHP-1 activity (% of control)

Regorafenib 0 0.01 0.1 1

SHP-1 activity (% of control)

Regorafenib 0 0.01 0.1 1 10

B

Apoptotic cells (%)

Regorafenib - + - +

SHP-1 inhibitor - - + +

p-STAT3

STAT3

Actin

Apoptotic cells (%)

Regorafenib - + - +

siSHP-1 - - + +

p-STAT3

STAT3

SHP-1

Actin
Fig. 4

A) Schematic representation of SHP-1 and its mutants.

B) Bar graph showing the percentage of apoptotic cells for different treatments.

C) Western blot analysis of p-STAT3, STAT3, SHP-1, Myc tag, and Actin under different conditions.

Legend:
- Vector
- wtSHP-1
- dN1
- D61A

Regorafenib:
- -
- +

Significance:
- * p < 0.05
- ** p < 0.01
Fig. 6

<table>
<thead>
<tr>
<th></th>
<th>Low to non-STAT3 expression (n = 72)</th>
<th>High STAT3 expression (n = 70)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced stage (%)</td>
<td>40 (55.6)</td>
<td>53 (75.7)</td>
<td>0.009</td>
</tr>
<tr>
<td>Poor differentiation (%)</td>
<td>30 (41.7)</td>
<td>39 (55.7)</td>
<td>0.035</td>
</tr>
<tr>
<td>Characteristics</td>
<td>N</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>----</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Male gender</td>
<td>103</td>
<td>72.5</td>
<td></td>
</tr>
<tr>
<td>Median age (IQR)</td>
<td>64 (57-72)</td>
<td></td>
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</tr>
<tr>
<td>Initial BCLC stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stage 0-A</td>
<td>49</td>
<td>34.5</td>
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</tr>
<tr>
<td>stage B</td>
<td>76</td>
<td>53.5</td>
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<tr>
<td>Stage C-D</td>
<td>17</td>
<td>11.9</td>
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<tr>
<td>Poor differentiation</td>
<td>69</td>
<td>48.6</td>
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<tr>
<td>Child-Pugh score</td>
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<tr>
<td>A</td>
<td>127</td>
<td>89.4</td>
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</tr>
<tr>
<td>B</td>
<td>15</td>
<td>10.6</td>
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<tr>
<td>Presence of ascites</td>
<td>4</td>
<td>2.8</td>
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<tr>
<td>Elevated AFP</td>
<td>87</td>
<td>61.3</td>
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<tr>
<td>HBV infection +</td>
<td>61</td>
<td>43.0</td>
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<tr>
<td>HCV infection +</td>
<td>42</td>
<td>29.6</td>
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<tr>
<td>Metavir score F3-F4</td>
<td>79</td>
<td>55.6</td>
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<tr>
<td>ISHAK score ≥ 3</td>
<td>115</td>
<td>81.0</td>
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</tbody>
</table>

Abbreviation: IQR, interquartile range. BCLC stage, Barcelona Clinic Liver Cancer stage. AFP, alpha-fetoprotein. HBV, Hepatitis B Virus. HCV, Hepatitis C Virus.
Clinical Cancer Research

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Kuen-Feng Chen, Wei-Tien Tai, Pei-Yi Chu, et al.

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