Investigation of the Therapeutic Potential of Momelotinib on Hepatitis Virus-Associated Liver Cancer Is Through Suppressing Oncogenic/Stemnes IFNGR-JAK-STAT Pathway

Jiann Ruey Ong  
Taipei Medical University

Chi-Tai Yeh  
Taipei Medical University Hospital

Ting-Yi Huang  
Taipei Medical University Hospital

Ming-Shou Hsieh  
Taipei Medical University Hospital

Wei-Hwa Lee  
Taipei Medical University Hospital

Yih-Giun Cheng (✉ stainless@s.tmu.edu.tw)  
Taipei Medical University Hospital  https://orcid.org/0000-0002-0201-4380

Research

Keywords: Hepatocellular carcinoma, IFN gamma-Jak axis pathway, Momelotinib, EMT, Stemness

DOI: https://doi.org/10.21203/rs.3.rs-106308/v1

License: ©  This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background:** Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, accounting for more than 700,000 deaths annually. In this study, we hypothesized that momelotinib could regulate the progression of HCC by targeting Jak family related protein. The proteins of IFN gamma-related pathways are obviously activated after hepatitis virus infection. This result suggests the difference in signal transmission between viral liver cancer and general liver cancer. Based on this observation, we are linked to the relevant targets of the JAK family and the potential applications of targeted therapy inhibitors.

**Methods:** We analyzed possible synergism between Momelotinib and Sorafenib in hepatitis virus-associated liver cancer. Immunostaining, colony formation Assay, cell invasion, migration, and tumorsphere-formation assay was used drug cytotoxicity, cell viability and possible molecular mechanism.

**Result:** We found that Jak2 downstream gene STAT1 expression was correlated with poor prognosis and poor overall survival of patients with HCC. Treatment of momelotinib significantly inhibits Jak2, resultant in the reduction of the migratory, invasive property of vHCC cells. Interestingly, cell cycle arrest and inhibition of the stem cell-like phenotype of vHCC cells were also observed after the momelotinib treatment. Furthermore, the combined effect of momelotinib and sorafenib both at in-vitro and in-vivo synergistically suppresses the proliferation of vHCC cells and effectively reduces the tumor burden.

**Conclusion:** Our results showed that momelotinib effectively suppressed the expression of Jak2, which results in the downregulation of cancer stem cell genes and enhances the antitumor efficacy of sorafenib by initiating the expression of apoptosis-related genes in vHCC cells, thus maximizing its therapeutic potential for patients with HCC.

**Highlights**

(i) JAK2 Overexpression in viral infection-associated HCC.

(ii) Therapeutic potential of Momelotinib on hepatitis B virus-associated liver cancer.

(iii) A combination of momelotinib and sorafenib (a multikinase inhibitor) increased the sensitivity of vHCC cells.

1. Background

Hepatocellular carcinoma (HCC) is one of the most common cancer-associated diseases worldwide [1], and it accounts for more than 80% of primary liver cancers [2]. HCC poses a heavy disease burden, and the total number of liver cancer cases has been increasing with aging [2]. HCC is associated with liver cirrhosis in approximately 80–90% of cases [3]; patients with chronic viral hepatitis B and C infection
have a high risk of HCC [4, 5]. The risk of HCC increases after the development of cirrhosis, increasing the likelihood of death due to liver failure or HCC [6]. More than half a million individuals worldwide are annually diagnosed with HCC [7]. Currently, there are limited treatment options for HCC. For the past two decades, the median survival time for patients with advanced HCC is less than one year, and the 5-year relative survival rate is below 9% [8]. Patients with well-preserved and good liver function typically opt for surgical resection of the liver. However, the most effective treatment method to improve the survival of patients with HCC is liver transplantation [9]. Unfortunately, this treatment often results in poor prognosis, including a high risk of postoperative complications and tumor recurrence. Several strategies are available to extend the survival time of patients with liver cancer, such as transplantation; surgical resection; target drugs, such as sorafenib, lenvatinib, and regorafenib; and immunotherapy (nivolumab) [10].

Janus kinases (Jaks) are nonreceptor tyrosine kinases encoded by the Jak gene. The Jaks family comprises four members, namely Jak1, Jak2, Jak3, and Tyk2. The Jak2 gene is a major component of the Jak-Stat signaling pathway, which is linked by interferon-responsive genes to signal transduction [11, 12]. Jak2 was also found to participate in several different types of cancers and myeloproliferative diseases [13–16]. Currently, several important Jak2 inhibitors, such as ruxolitinib, pacritinib, fedratinib, and momelotinib, are available, and they differ from each other with respect to the inhibition of other kinases [17]. Interestingly, in vitro treatment with a combination of chemotherapy and momelotinib is a potent inhibitor of Jak2; it suppressed CSCs-like cells and reduced tumor burden in a mouse model of human ovarian cancer [18]. Epidemiological studies report that the hepatitis virus B and C (HBV and HCV) often were considered as the most common etiology of HCC, and these two viruses together were often known as one risk factor for HCC [19]. Viral infection-associated HCC (vHCC) often develops resistance to sorafenib (a multi-kinase inhibitor) therapy [20]. Thus, an important target that targets vHCC and overcomes drug resistance is required.

In this study, we hypothesized that momelotinib could regulate the progression of HCC by targeting Jak2. Our study suggests that Jak2 expressed higher in vHCC than in non-viral infection-associated HCC (nvHCC) and the normal liver. Jak2 expression was correlated with poor prognosis and poor overall survival of patients with HCC. Treatment of momelotinib significantly inhibits Jak2, resultant in the reduction of the migratory, invasive property of vHCC cells. Interestingly, cell cycle arrest and inhibition of the stem cell-like phenotype of vHCC cells were also observed after the momelotinib treatment. Furthermore, the combined effect of momelotinib and sorafenib both at in-vitro and in-vivo synergistically suppresses the proliferation of vHCC cells and effectively reduces the tumor burden.

2. Materials And Methods

2.1. Ethics Approval and Consent to Participate

We obtained 30 matched nvHCC, and vHCC samples as kind gift from Dr. Wei-Hwa Lee, from the Department of Pathology, Taipei Medical University-Shuang Ho Hospital HCC tissue bank, following
ethical approval for their use from the Institutional Review Board of the Taipei Medical University-Shuang Ho Hospital. Requirement for patients signed informed consent was waived because tissue samples were obtained retrospectively from the Taipei Medical University-Shuang Ho Hospital HCC archive. This study was conducted in a cohort of patients with HCC cancer at Taipei Medical University Shuang-Ho Hospital, Taipei, Taiwan. The study was reviewed and approved by the institutional review board (TMU-JIRB: 201302016).

2.2. Cell Lines and Reagents

The human SNU-387 and SNU-475 HCC cell lines were purchased from American Tissue Culture Collection. The cells were maintained under conditions recommended by the vendor, cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Thermo Fisher Scientific Inc, Waltham, MA, USA.) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Life Technologies) at 37 °C, in a 5% humidified CO2 incubator. Cells were sub-cultured at 80–90% confluency. The Jak inhibitor momelotinib (CYT387) [21] and sorafenib (Catalog No. S7397), purchased from Selleck Chemicals, were dissolved in DMSO.

2.3. Microarray and RNAseq Pre-Processing and Analysis

Gene expression profiles, GSE14323, GSE14520, and GSE6764, were downloaded from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/), and the data of RNA-seq expression results containing samples of vHCC and nHCC from patients with liver hepatocellular carcinoma (LIHC) were downloaded from TCGA portal Xena browser (http://www.xena browser.net/) used for survival analysis. GEO dataset GSE62813 was used to identify sorafenib resistance in HCC.

2.4. Differential Expression Analysis

To determine differentially expressed genes between tumor and adjacent normal liver tissues in the GSE14520 cohort, the Bayes method, and linear model were employed using the R package limma. P values were adjusted using the false discovery rate controlling the Benjamini–Hochberg procedure. Genes with log2 fold change > 1 and adjusted P values < 0.05 were considered significant.

2.5. Human Specimens and Immunohistochemistry (IHC)

Tumor tissues were harvested from patients with HCC. Samples were collected from the Department of pathology, Shuang Ho Hospital, after obtaining written informed consent from patients (TMU-JIRB: 201302016). Clinical details of patients from the Taipei Medical University Shuang-Ho Hospital HCC cancer cohort shown in Supplementary Table S1. The collected samples were fixed in 4% paraformaldehyde and embedded in paraffin, with 5-µm sections cut from paraffin blocks. Staining was performed using anti-Jak2 (1:500; cat. ab39636, Abcam), followed by staining using the secondary antibody and H&E. The percentage of stained area to the selected field was recorded in a 5% interval, ranging from 0–100%. The staining intensity was graded into 3 categories (absent or weak, 1; moderate, 2; strong, 3). Q-score was derived from the product of percentage (P) of tumor cells with characteristic IHC staining (0-100%) and the intensity (I) of IHC staining (1–3) (Q =P × I; maximum = 300).
2.6. Cell Viability Test and Calculation of the Combination Index

The stock of momelotinib and sorafenib was prepared by dissolving 20 mg/mL of the mixture in DMSO. The stocks of each drug were stored at -20 °C until use. Using the CompuSyn software to calculate the half-maximal inhibitory concentration (IC50) values of difference cell line as previously reported by Chou TC and Martin N. The calculation method of IC50 is as described in the PC Software and User’s Guide on the ComboSyn Inc. website (http://www.combosyn.com). The effects of momelotinib and sorafenib on cell proliferation were detected using the sulforhodamine B (SRB) assay. The synergistic effect of these two drugs was analyzed using isobolograms of the drug combination, as previously reported by Chou and Talalay [22, 23]. The interaction between the two drugs was also analyzed by the median-effect principle proposed by them. The combination index (CI) was calculated using CompuSyn software. A CI value less than 1 represented synergism [23]. Briefly, HCC cells or tumorspheres were seeded in 96-well plates (8 × 10^5 cells/well) and treated with the drugs (momelotinib or sorafenib alone or in combination) at the 5µM and 2.5µM concentration for 48 h, respectively. After respective drug treatments, the relative cell number was estimated by the SRB reagent according to the manufacturer’s protocol (Sigma, USA).

2.7. Transient Transfection and Dual-Luciferase Assay

SNU-387 and SNU-475 cells were seeded at a density of 1 × 10^6 cells in 100-mm2 culture plates. On the following day, cells were treated with TransFectin (Bio-Rad Laboratories) and transfected with 2 µg of pJAK2-TA-Luc and 2 µg of pRL-TK (Renilla luciferase control reporter plasmid [Promega]). After 5 hours of transfection, cells were trypsinized and seeded onto sterile, black-bottomed 96-well plates at a density of 1 × 10^4 cells per well and then incubated with the complete medium for 24 hours. Cells were treated with either test compounds or 0.1% DMSO for 24 hours. After treatment, cells were harvested in 20 µL of passive lysis buffer, and luciferase activity was evaluated using the dual-luciferase reporter assay kit (Promega) on Wallac 1420 VICTOR2 (PerkinElmer, Inc.). The experiments were performed in triplicate and repeated thrice. Relative luciferase activity was calculated according to the following formula: relative luciferase activity (%) = ([normalized luciferase activity of sample treated with a test compound] / [normalized luciferase activity of sample treated with 0.1% DMSO]) × 100.

2.8. Apoptosis and Cell Cycle Analysis

Apoptosis and cell cycle were analyzed through flow cytometry (Beckman, Fullerton, CA, USA) using the Annexin V/7AAD (FITC-conjugated) apoptosis kit (F-6012; US Everbright Inc.) or propidium iodide (PI) according to manufacturer’s protocol. To determine the effect on the cell cycle, HCC cells were exposed to momelotinib for 48 hours. Thereafter, cells were washed and fixed with 70% ethanol. The cells were washed, re-suspended, and stained with 10 µg/mL of PI in PBS for 30 minutes at room temperature in the dark. The cells were analyzed through flow cytometry (Becton Dickinson, Mountain View, CA, USA), and the population of cells in each phase was counted.

2.9. TUNEL Assay
Cell death (apoptosis) in the tumor tissue was detected by staining HCC tissues using an in situ cell death detection kit from Roche according to the manufacturer’s protocol. Quantification was performed by calculating the percentage of TUNEL-positive cells by using a fluorescence microscope. The results are expressed as the mean number of TUNEL-positive apoptotic HCC cells in each group.

2.10. ALDEFLUOR Assay and ALDH1 + Cell Sorting by FACS

ALDH activity in HCC cells was assayed using the ALDEFLUOR kit according to manufacturer’s instructions (STEMCELL Technologies, Durham, NC, USA). The cells that showed positive activity of aldehyde dehydrogenase 1 (ALDH1) were isolated and analyzed. Briefly, the human HCC cell lines SNU-387 was suspended at a concentration of $1 \times 10^6$ cells/mL in ALDEFLUOR assay buffer containing the ALDH substrate (BAAA, 1 µmol/L per $1 \times 10^6$ cells) and incubated for 40 minutes at 37 °C. Cells incubated with ALDEFLUOR substrate and treated with 50 mmol/L of diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, were used as a reference control. To avoid contamination of cells of mouse origin from xenotransplanted tumors, they were stained with the anti-H2Kd antibody (BD Biosciences, 1/200, 20 min on ice) and then with a secondary antibody labeled with phycoerythrin (Jackson Labs, 1/250, 20 min on ice). Cells stained with PI alone were used as negative controls, and ALDEFLUOR-stained cells treated with DEAB and those stained with the secondary antibody alone were considered viable.

2.11. Western Blotting and qRT-PCR

Cells were washed with PBS and then lysed in RIPA lysis buffer; cellular protein lysates were isolated using the protein extraction kit (QIAGEN, USA) and quantified using the Bradford protein assay kit (Beyotime, Beijing, China). Approximately 20 µg of the sample from different experiments was loaded and subjected to SDS-PAGE by using the Mini-PROTEAN III system (Bio-Rad, Taiwan). Separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane by using the Trans-Blot Turbo Transfer System (Bio-Rad), followed by blocking with Tris-buffered saline plus skimmed milk. These PVDF membranes were then probed with respective primary antibodies, followed by the secondary antibody. The Commercial antibodies are shown in Supplementary Table S2. The enhanced chemiluminescence detection kit was used to detect the proteins of interest. Images were captured and analyzed using the UVP BioDoc-It system (Upland, CA, USA). qRT-PCR was performed by using isolating total RNA using TRIzol-based protocol (Life Technologies) provided by the manufacturer. In brief, one µg of total RNA was reverse transcribed using QIAGEN OneStep RT-PCR kit (QIAGEN, Taiwan), and PCR was performed using a Rotor-Gene SYBR Green PCR kit (400; QIAGEN, Taiwan). The primer sequences are shown in Supplementary Table S3.

2.12. Colony Formation Assay

The colony formation assay was performed according to a previously explained protocol [24] with modifications. Briefly, a total of 500 colon cancer cells were seeded in six-well plates and treated with
momelotinib. The cells were allowed to grow for another week and then harvested, fixed, and counted.

### 2.13. Wound Healing Migration Assay

Cells were seeded in six-well plates (Corning, Corning, NY, USA) with RPMI 1640 medium containing 10% FBS and cultured to 95–100% confluence. A scratch along the median axis was then made with a sterile yellow pipette tip across cells. Cell migration pictures were captured at 0 and 48 hours after the medium scratch, under a microscope, and analyzed using the NIH Image J software. (https://imagej.nih.gov/ij/download.html).

### 2.14. Matrigel Invasion Assay

Cells (2 × 10^5) were seeded in 24-transwell chambers with an 8-µm pore membrane coated with Matrigel in the upper chamber of the transwell system containing serum-free RPMI 1640 medium. The lower chamber of the transwell system contained the medium with 20% FBS. After incubation at 37 °C for 6 hours, non-invaded HCC cells on the upper side of the membrane were carefully removed with a cotton swab, and the invaded cells were stained with crystal violet dye, air-dried, and photographed under a microscope. Images were analyzed using the NIH Image J software. (https://imagej.nih.gov/ij/download.html).

### 2.15. Sphere Formation Assay

Cells (5 × 10^3/well) were plated in ultra-low-attachment six-well plates (Corning) containing stem cell medium consisting of serum-free RPMI 1640 medium supplemented with 10 ng/mL of human basic fibroblast growth factor (bFGF; Invitrogen, Grand Island, NY, USA), 1 × B27 supplement, and 20 ng/mL epidermal growth factor (EGF; Invitrogen). The medium was changed every 72 hours. After 14 days of incubation, the formed spheres were counted and photographed.

### 2.16. Animal Studies

All animal experiments and maintenance were in strict compliance with the Animal Use Protocol Taipei Medical University (protocol LAC-2014-0170). The anti-proliferative effect of momelotinib and sorafenib in combination with vHCC cells in vivo investigated, athymic nude mouse models bearing HCC cell xenografts were established. Five-week-old male athymic nude mice were used for this study. The mice were maintained under pathogen-free conditions and were provided with sterilized food and water. First, 1 × 10^6 SNU-387 cells were subcutaneously injected into the right flank near the hind leg of each nude mouse. When the mice had palpable tumors (tumor volume of approximately 100 mm3), they were randomly divided into control (100 µL of normal saline [NS] by intraperitoneal injection plus 100 µL of 1%
DMSO) and 0.5% carboxymethyl cellulose ([CMC]-Na sterile water), momelotinib (200 mg/kg/day by intraperitoneal injection plus 100 µL of 1% DMSO and 0.5% CMC-Na sterile water), sorafenib (30 µg/kg/day by intragastric administration plus 100 µL NS by intraperitoneal injection), and combination (momelotinib, 30 mg/kg/day by intraperitoneal injection plus sorafenib 30 mg/kg/day by intragastric administration) groups (n = 6 animals/group). The treatments were performed 5 times/week for 4 weeks. The tumor volume was detected every week and was calculated using the following formula: volume (V) = π/6 × length × width × height. After 4 weeks, mice were humanely euthanized, and the tumors were isolated for further analyses.

2.17. Statistical Analysis

All assays were performed at least thrice in triplicate. Values are expressed as the mean ± standard deviation (SD). Comparisons between groups were estimated using Student's t-test for cell line experiments or the Mann-Whitney U-test for clinical data, Spearman's rank correlation between variables, and the Kruskal-Wallis test for comparison of three or more groups. The Kaplan-Meier method was used for the survival analysis, and the difference between survival curves was tested by a log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 20 (IBM, Armonk, NY, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. IFNGR-JAK-STAT Signaling Pathway Is Activated in vHCC

We investigated the expression of IFNGR-JAK-STAT in hepatitis C virus (HCV)-associated HCC (vHCC), non-HCV-associated HCC (nvHCC), and normal liver samples. The robust microarray analysis expression data for liver samples from GEO datasets GSE14323 were explored, and 10 patients containing both normal liver and vHCC sample pairs were obtained after gene expression profiling within this sample group by using a heatmap. Interestingly, the expression of Jak2 in HCC cells was higher than that in normal liver cells (Fig. 1A). The differential expression of IFNGR1, IFNGR2, Jak1, Jak2 and STAT1 from the entire series was analyzed; as expected, the normal cohort (n = 19) showed lower Jak2 mRNA expression than did the HCC (HCV-infected) cohort (n = 38), and the GSE121248 cohort showed the similar result that Jak1 mRNA expression is higher in HCC (HBV-infected, n = 70) than in normal liver (n = 37; Fig. 1B). Protein-protein interaction (PPI) network of IFNGR-JAK-STAT signaling proteins by STRING, clustering into 3 groups with blue, green and red, respectively (Fig. 1C). Intriguingly, the expression of Jak2 in nvHCC groups from TCGA LIHC data remained similar compared with the normal liver group (Fig. 1D), suggesting that Jak2 appears to be overexpressed only in vHCC. The Fig. 1E summarizes the heat map of different Hepatitis Virus-Associated Liver cancer samples, and shows the connection between the JAK2, STAT1 expression and virus infection. From the results in Fig. 1E, we analyzed the overall survival of HCC
patients with viral and non-viral infections. A higher expression of Jak2 is also associated with lower overall survival of patients with HCC (Fig. 1F). Further analyzing the relationship between the STAT1 gene expression and the overall survival of patients. We were surprised to find that the patients with high STAT1 expression have a lower overall survival rate in the vHCC group and it is statistically significant. In addition, this phenomenon was not found in the overall survival of HCC patients. Figure 1G shows the high correlation between JAK2 and STAT1. The preliminary results show that both seems to be related. Clinical details of HBV- and HCV-infected patients from the Taipei Medical University Shuang-Ho Hospital HCC cancer cohort shown in Supplementary Table S1. The results showed that patients related to hepatitis virus accounted for one-half of the primary tumor. Among patients with viral hepatitis, two patients were found to be infected with HBV and HCV. In order to further understand the connection between the JAK2 expression and virus infection, we conducted follow-up IHC experiments of different liver samples. Furthermore, by applying data from the cohort of patients with Taipei Medical University Shuang-Ho Hospital HCC cancer to corroborate this finding, we consistently observed that although Jak2 immunostaining in nvHCC (n = 10) samples remains unchanged compared with normal liver samples (n = 10), vHCC (n = 10) samples showed elevated Jak2 levels (Fig. 1H). The Fig. 1I showed the Q-score of JAK2 expression in Shuang Ho Hospital patients with Hepatocellular Carcinoma, JAK2 expression after vHCC infection is higher. The Fig. 1J showed the Q-score of JAK2 expression in Shuang Ho Hospital patients with Hepatocellular Carcinoma, JAK2 expression after vHCC infection is higher. The scoring matrix for the 30 samples analyzed in the Supplementary Table S4. These results indicate that Jak2 and downstream gene STAT1 overexpression is distinctively associated with vHCC, whereas Jak2 expression in nvHCC is similar to that in normal liver.

3.2. Momelotinib Inhibits Jak2 Expression, leading to Decreased Cell Viability

We investigated the potential of the Jak inhibitor momelotinib (CYT387; Fig. 2A) to inhibit Jak2. As expected, the expression of Jak2 in vHCC cell lines SNU-475 and SNU-387 was higher than that in nvHCC (HepG2) and normal liver (THLE-2) cell lines (Fig. 2B). Then, using the CompuSyn software to calculate the IC50 values at 48 h for all four cell lines are as follows: SNU-475 = 6.3236, SNU-387 = 5.59423, HepG2 = 6.89772 and THLE-2 = 12.4946. According to this result, we set 5µM as the subsequent experimental condition of SNU cell line. The cell proliferation, as well as Jak2 expression of both SNU-475 and SNU-387 cell lines, significantly decreased under treatment with 5µM momelotinib in a dose-dependent manner (Fig. 2C and 2D). Treatment with momelotinib inhibited the expression of Jak2 protein and Jak2 gene (Fig. 2E). These results suggest that vHCC is sensitive to the inhibition of Jak2 by momelotinib.

3.3. Momelotinib Inhibits Migration and Invasion and Triggers Cell Cycle Arrest of vHCC Cells

After 24 hours, we observed that a higher concentration of momelotinib led to a significantly increased proportion of cells at the G0/G1 phase and decreased proportion of cells at the S phase (Fig. 3A). The result indicated that momelotinib might inhibit the cell growth and DNA replication. Subsequently, the effect of momelotinib on the migration and invasion of vHCC cells (SNU-475 and SNU-387) was investigated. According to the IC50 results in Fig. 2C, treatment with 5 µM momelotinib for 48 hours
strongly inhibited the migration (Fig. 3B) and invasion (Fig. 3C) abilities of cells, indicating that the Jak2 inhibitor effectively reduced the mobility and invasiveness of vHCC cells when compared with their untreated control counterparts. EMT plays an important role in the invasion and metastasis of HCC cells [25]. Furthermore, the effect of momelotinib on EMT was evaluated. EMT biomarkers (E-cadherin and vimentin) and transcription factor (Slug) were determined through Western blotting. The results showed that the expression of Slug, N-cadherin and vimentin was significantly decreased and that of E-cadherin, a transmembrane protein with a tumor-suppressive effect, was significantly increased (Fig. 3D). These results suggest that momelotinib is effective in preventing the migration and invasion of vHCC cells.

3.4. Momelotinib Remarkably Suppresses Colony and Tumorsphere Formation of vHCC Cells

To further examine the effect of momelotinib in tumorigenesis, we assayed the colony and tumorsphere formation of the vHCC cell lines SNU-475 and SNU-387. Colony and tumorsphere formation assays are important for identification of stemness [26, 27]. Jak2 inhibition considerably suppressed tumorsphere and colony formation of cells (Fig. 4A and 4B). Momelotinib effectively suppressed tumorsphere and colony formation of SNU-475 and SNU-387 cells. Tumorsphere and stemness markers, such as CD133, KLF4, and SOX2, were also significantly decreased both at the protein and mRNA level (Fig. 4C) after treatment with momelotinib. Decreased generation of ALDH1+ cells were observed in the momelotinib-treated group than in the dimethyl sulfoxide (DMSO)-treated group (Fig. 4D). These results indicate that the stem cell-like phenotype of vHCC is modulated by the inhibition of Jak2.

3.5. Momelotinib Treatment Increased Sorafenib Sensitivity of vHCC Cells

Momelotinib is a potent ATP-competitive inhibitor of JAK2, it has been used for targeted therapies of myeloproliferative tumors. A previous study showed that treatment with sorafenib significantly improved the survival of patients with solid tumors (Abou-Alfa et al. 2006). In our results, combination of momelotinib and sorafenib suppressed proliferation of SNU-387 cells (Fig. 5A). In order to inhibit the effect of JAK2 pathway activation on drug resistance, we tested whether the combination of momelotinib and sorafenib can remarkably suppress vHCC cell proliferation and colony formation. We observed that the combination treatment with momelotinib and sorafenib synergistically inhibited vHCC cell proliferation and colony formation by inducing apoptosis. Figure 5B shows the momelotinib-sorafenib combination testing on SNU-387 and effect of treatment on colony forming capacity of SNU-387 cells and treated with the drugs (sorafenib and momelotinib in combination) at the 2.5µM and 5µM concentration for 48 hours. In addition, we also analyzed the drug sensitivity of the HCC (HepG2) cell line, showed in the Supplementary Figure S1. The results indicated that HepG2 cells had a the less obvious impacts on on JAK2 inhibitor (Momelotinib) compared with SNU-387. Momelotinib is a selective inhibitor of JAK1 and JAK2, we speculated that the hepatoma cell lines (SNU-387) infected by the virus might induce JAK2 expression through the IFN gamma pathway. This data might be supporting our proposed model of JAK2 expression in vHCC. According to these results, we found the optimized parameter of
combination. In Fig. 5C, we did the apoptosis analysis by flow cytometer for Annexin-V + and 7-AAD stained cells. The results indicated the elevated apoptosis in the combo treatment. Determining the expression of apoptosis marker is important to understand the functions of molecular mechanism. Hence, the Fig. 5D showed the representative Western blot images of the apoptosis markers in SNU-387. The expression levels of apoptosis markers including p-JaK2 and Bcl-x1 were decline, the cleaved-PARP, cleaved-Caspase 7 and 9 were rise in the combination-treated group. This result shows the therapeutic potential of oncogenic/Stemnes JAK2 pathway inhibitors on viral hepatitis. As stated above, these results demonstrated the potential inhibition of vHCC cells by treatment with momelotinib–sorafenib combination.

3.6. Momelotinib in Combination with Sorafenib Efficiently Suppressed Tumor-Initiating Ability in Xenograft Models

The effect of momelotinib on the inhibition of the tumor-initiating ability in xenograft models, HCC (SNU-387) cancer cells (1 × 10⁶ cells/injection) were subcutaneously injected into male athymic nude mice to establish a xenograft model. After 4 weeks of follow-up, mice were divided into four groups (vehicle control, momelotinib, sorafenib, and combination) when the tumor became palpable (approximately 100 mm³). The group that received combination treatment showed the best tumor growth inhibition. The tumor size in each treatment group was measured; a significantly smaller tumor size was found in the group that received combined treatment with momelotinib and sorafenib, indicating that the combination treatment suppressed tumorigenesis (Fig. 6A). Consistent with in vitro data, immunohistochemistry and TUNEL analyses (Fig. 6B and 6C) of xenograft tumors revealed that the momelotinib and sorafenib combination effectively inhibited the expression of Ki-67, a marker for representing tumor proliferation. In order to show relationship between Jak2 signaling and tumor progression. The IHC result indicated Ibutinib (JAK2 inhibitor) could reduce the expression of JAK2 in vivo. Moreover, momelotinib facilitated sorafenib-induced apoptosis in xenograft tumors, as evaluated by the cleaved caspase-3 staining and TUNEL assay.

4. Discussion

The growing HCC is one of the leading causes of cancer-associated deaths worldwide [1]. In more than 80–90% of cases, HCC is directly or indirectly associated with liver cirrhosis [3]; chronic viral hepatitis B and C infections are mainly responsible for the development of HCC [1, 4, 5]. Often this viral infection-associated with development of resistance to sorafenib (a multi-kinase inhibitor) therapy [20]. The overall 5 years survival rate of HCC is below than 9% [8]. Janus kinases (Jaks) are nonreceptor tyrosine kinases encoded by the Jak gene. The Jaks family comprises four members, namely Jak1, Jak2, Jak3, and Tyk2 [28]. Jak2 has a role in several different types of cancers and myeloproliferative diseases [13–16]. Significantly increased expressions of phosphorylated Jak2 and STAT3 are observed in vHCC [29]. Patients with this disease develop resistance to sorafenib, a multi-kinase inhibitor [20]. Currently, several important Jak2 inhibitors are available, such as ruxolitinib, pacritinib, fedratinib, and momelotinib [17], each of which has a different mode of action. Interestingly, the combination of chemotherapy and
Momelotinib is a potent inhibitor of Jak2, and it suppresses CSC-like cells and reduces tumor burden in vHCC [18]. Thus, finding an important target that re-sensitizes and overcomes the drug resistance of vHCC is required.

In the present study, we first demonstrated that the expression of Jak1 and 2 is significantly upregulated in vHCC than in nvHCC/normal liver tissues (Fig. 1). In addition, the proteins of IFN gamma-related pathways are obviously activated after virus infection (Fig. 1B). This result suggests the difference in signal transmission between viral liver cancer and general liver cancer. Based on this observation, we are linked to the relevant targets of the JAK family and the potential applications of targeted therapy inhibitors. To further confirm our hypothesis that the JAK family and virus infection cause cancer. We also analyzed the overall survival between vHCC and HCC. Among them, STAT1 expression in both are significant differences. In virus infection, the performance of IFN-gamma is an important indicator. IFN-gamma binds to nearby uninfected cell membrane receptors, stimulating signaling pathways to interfere with virus replication; stimulating cells to hydrolyze pathogenic proteins to prevent cells from being infected by the same or different viruses. In addition, STAT1 is a downstream gene of JAK2 in IFN-gamma signaling pathways, which can infer the significance of JAK2 in viral infection. Previous research indicated that overall Jak2 expression related to the overall survival in HCC patients [30]. Thus, Jak family gene might be playing a key role in patients with HCC. Verstovsek et al. [31] demonstrated the efficacy of momelotinib as a potent inhibitor of Jak1 and Jak2 in patients with primary and secondary myelofibrosis. Furthermore, we found that momelotinib significantly inhibited the growth of HCC cells (Fig. 2) and reduces the expression of Jak2, which verified the importance of momelotinib in targeting Jak2 and reducing tumorigenesis in HCC. EMT plays an important role in invasion and metastasis of HCC cells [25]. In vitro motility, such as migratory and invasive properties, is associated with the metastatic potential of HCC cells, rendering their treatment and measurement of their metastatic potential difficult [32]. Our data demonstrated that momelotinib effectively inhibited Jak2 and reduced the migratory/invasive ability of vHCC cells through affecting EMT biomarkers (E-cadherin and vimentin) and transcription factor (Slug). It also inhibits the cell cycle progression of vHCC cells (Fig. 3).

Often HCC patients show sorafenib therapy resistant [33]. Drug resistance is a major challenge in anticancer therapy. CSCs provide an alternative explanation for the aforementioned therapeutic challenges of several cancers [34]. This small population of cancer cells have stem cell-like features such as tumorigenicity, self-renewal, and more resistance to chemotherapeutic agents than that shown by cancer cells [35, 36]. Treatment of vHCC with momelotinib reduced the expression of cancer stemness markers, such as CD133, KLF4, and SOX2, and decreased ALDH1 activity (Fig. 4), suppressing tumorsphere and colony formation of HCC cells.

The combination therapy, a treatment modality that combines two drugs for cancer therapy, has gained wide acceptance and popularity. Combining two drug targets in multiple pathways involved in cancer, utilizing different mechanisms in order to reduce the development of drug resistance in tumors [37]. Combinations of two therapeutic agents into a biological system may produce a synergistic effect, or, sometimes, antagonistic, or identical effects, compared with their effects when acting separately.
Momelotinib, together with sorafenib, increased the sensitivity of HCC cells, resulting in decreased colony formation in cells treated with the combination, and also alleviated apoptotic markers (Annexin-V + and 7-AAD positive cells) PARP-1, PDCD4, and Bax molecules (Fig. 5).

Similarly, the combination of momelotinib and sorafenib efficiently suppressed the tumor-initiating ability in xenograft models (Fig. 6) and effectively inhibited the expression of Ki-67, a marker for representing tumor proliferation. Moreover, momelotinib facilitated sorafenib-induced apoptosis in xenograft tumors, as evaluated by the cleaved caspase-3 staining and TUNEL assay. Despite the fact that treatment with sorafenib is a useful therapeutic way for HCC, the survival rate of patients are still limited. This is because that HCC cells are heterogeneous cells with incongruous activation of several signaling pathways [38, 39]. The combination therapy strategies have been proposed to improve the efficacy of sorafenib based treatment of HCC patients in many research [40, 41]. HCC patients treated with a combination therapy strategies resulted in a more effective treatment, such as mTOR inhibitors and monoclonal antibody. The JAK/STAT is an important pathway for cellular functions, including cell proliferation and differentiation, it also participate the mechanism of liver regeneration and gluconeogenesis [42]. Many research indicated that the JAK/STAT pathway is often deregulated signaling in HCC and other cancer [43, 44]. The common JAK inhibitors including pacritinib, cryptotanishinone and ruxolitinib have been used for cancer and human relevant diseases studies. Recently, Justin Jit Hin Tang et al.(2020) discussed the JAK/STAT signaling in hepatocellular carcinoma and organised different JAK/STAT inhibitors used in targeting the JAK/STAT pathway for HCC treatment, including small molecule inhibitors and siRNAs [45]. In our report, the overexpression of Jak2 was suppressed by momelotinib, a Jak inhibitor, leading to a sharp reduction in the cell viability of vHCC cell lines. It has a wider significance and this model may lead to a new therapeutic strategy for vHCC and non-vHCC.

5. Conclusion

As shown in schema abstract, our results showed that momelotinib effectively suppressed the expression of Jak2 (Fig. 7), which results in the downregulation of cancer stem cell genes and enhances the antitumor efficacy of sorafenib by initiating the expression of apoptosis-related genes in vHCC cells, thus maximizing its therapeutic potential for patients with HCC.

Declarations

Ethics approval and consent to participate

This study was conducted in a cohort of patients with HCC cancer at Taipei Medical University Shuang-Ho Hospital, Taipei, Taiwan. The study was reviewed and approved by the institutional review board (TMU-JIRB: 201302016).

Consent for publication
The authors declare that they have no potential financial competing interests that may in any way, gain or lose financially from the publication of this manuscript at present or in the future. Additionally, no non-financial competing interests are involved in the manuscript.

**Authors’ contributions:** Jiann Ruey Ong: Study conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing. Chi-Tai Yeh, Ting-Yi Huang, Ming-Shou Hsieh, Wei-Hwa Lee: Data analysis and interpretation. Yih-Giun Cherng: Study conception and design, data analysis and interpretation, final manuscript approval. All authors read and approved the final manuscript.

**Funding:** This study was also supported by grants from and Taipei Medical University (102TMU-SHH-02) to Wei-Hwa Lee.

**Availability of data and materials**

The datasets used and analyzed in the current study are publicly-accessible as indicated in the manuscript.

**Conflict of Interests:** The authors declare that they have no potential financial competing interests that may in any way, gain or lose financially from the publication of this manuscript at present or in the future. Additionally, no non-financial competing interests are involved in the manuscript.

**Acknowledgments:** The authors thank all research assistants of the Cancer Translational Research Laboratory and Core Facility Center, Taipei Medical University - Shuang Ho Hospital, for their assistance with the flow cytometry, molecular and cell-based assays.

**References**

1. R.X. Zhu, W.-K. Seto, C.-L. Lai, M.-F. Yuen, Epidemiology of Hepatocellular Carcinoma in the Asia-Pacific Region, *Gut Liver*, 10 (2016) 332-339.

2. H.B. El-Serag, K.L. Rudolph, Hepatocellular Carcinoma: Epidemiology and Molecular Carcinogenesis, *Gastroenterology*, 132 (2007) 2557-2576.

3. G.L. Davis, J. Dempster, J.D. Meler, D.W. Orr, M.W. Walberg, B. Brown, B.D. Berger, J.K. O’Connor, R.M. Goldstein, Hepatocellular carcinoma: management of an increasingly common problem, *Proc (Bayl Univ Med Cent)*, 21 (2008) 266-280.

4. R.X. Zhu, W.K. Seto, C.L. Lai, M.F. Yuen, Epidemiology of Hepatocellular Carcinoma in the Asia-Pacific Region, Gut Liver, 10 (2016) 332-339.

5. H.B. El-Serag, Epidemiology of viral hepatitis and hepatocellular carcinoma, *Gastroenterology*, 142 (2012) 1264-1273.e1261.

6. M.J. Tong, L.M. Blatt, K.B. Tyson, V.W.C. Kao, Death from Liver Disease and Development of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Infection: A Prospective Study,
7. M.A. Gomes, D.G. Priolli, J.G. Tralhao, M.F. Botelho, Hepatocellular carcinoma: epidemiology, biology, diagnosis, and therapies, *Revista da Associacao Medica Brasileira* (1992), 59 (2013) 514-524.

8. H. Chen, Hu, M., Xu, F., Xu, H., She, J., & Xia, H., Understanding the inflammation-cancer transformation in the development of primary liver cancer, *Hepatoma Res* (2018) 4:29.

9. J.M. Llovet, J. Zucman-Rossi, E. Pikarsky, B. Sangro, M. Schwartz, M. Sherman, G. Gores, Hepatocellular carcinoma, *Nature reviews. Disease primers*, 2 (2016) 16018.

10. M. Kudo, Targeted and immune therapies for hepatocellular carcinoma: Predictions for 2019 and beyond, *World journal of gastroenterology*, 25 (2019) 789-807.

11. A.F. Wilks, A.G. Harpur, R.R. Kurban, S.J. Ralph, G. Zurcher, A. Ziemiecki, Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase, *Molecular and cellular biology*, 11 (1991) 2057-2065.

12. L. Velazquez, M. Fellous, G.R. Stark, S. Pellegrini, A protein tyrosine kinase in the interferon alpha/beta signaling pathway, *Cell*, 70 (1992) 313-322.

13. G. Genovese, A.K. Kahler, R.E. Handsaker, J. Lindberg, S.A. Rose, S.F. Bakhoum, K. Chambert, E. Mick, B.M. Neale, M. Fromer, S.M. Purcell, O. Svantesson, M. Landen, M. Hoglund, S. Lehmann, S.B. Gabriel, J.L. Moran, E.S. Lander, P.F. Sullivan, P. Sklar, H. Gronberg, C.M. Hultman, S.A. McCarroll, Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence, *The New England journal of medicine*, 371 (2014) 2477-2487.

14. S. Jaiswal, P. Fontanillas, J. Flannick, A. Manning, P.V. Grauman, B.G. Mar, R.C. Lindsley, C.H. Meruelo, N. Burtt, A. Chavez, J.M. Higgins, V. Moltchanov, F.C. Kuo, M.J. Kluk, B. Henderson, L. Kinnunen, H.A. Koistinen, C. Ladenvall, G. Getz, A. Correa, B.F. Banahan, S. Gabriel, S. Kathiresan, H.M. Stringham, M.L. McCarthy, M. Boehnke, J. Tuomilehto, C. Haiman, L. Groop, G. Atzmon, J.G. Wilson, D. Neuberg, D. Altshuler, B.L. Ebert, Age-related clonal hematopoiesis associated with adverse outcomes, *The New England journal of medicine*, 371 (2014) 2488-2498.

15. P. Sidon, H. El Housni, B. Dessars, P. Heimann, The JAK2V617F mutation is detectable at very low level in peripheral blood of healthy donors, *Leukemia*, 20 (2006) 1622.

16. X. Xu, Q. Zhang, J. Luo, S. Xing, Q. Li, S.B. Krantz, X. Fu, Z.J. Zhao, JAK2(V617F): Prevalence in a large Chinese hospital population, *Blood*, 109 (2007) 339-342.

17. J.W. Singer, S. Al-Fayoumi, J. Taylor, S. Velichko, A. O'Mahony, Comparative phenotypic profiling of the JAK2 inhibitors ruxolitinib, fedratinib, momelotinib, and pacritinib reveals distinct mechanistic signatures, *PLOS ONE*, 14 (2019) e0222944.

18. E. Chan, R. Luwor, C. Burns, G. Kannourakis, J.K. Findlay, N. Ahmed, Momelotinib decreased cancer stem cell associated tumor burden and prolonged disease-free remission period in a mouse model of human ovarian cancer, *Oncotarget*, 9 (2018) 16599-16618.

19. J. Ng, J. Wu, Hepatitis B- and hepatitis C-related hepatocellular carcinomas in the United States: similarities and differences, *Hepat Mon*, 12 (2012) e7635-e7635.
20. D. Witt-Kehati, A. Fridkin, M.B. Alaluf, R. Zemel, A. Shlomai, Inhibition of pMAPK14 Overcomes Resistance to Sorafenib in Hepatoma Cells with Hepatitis B Virus, *Transl Oncol*, 11 (2018) 511-517.

21. C.J. Burns, D.G. Bourke, L. Andrau, X. Bu, S.A. Charman, A.C. Donohue, E. Fantino, M. Farrugia, J.T. Feutriol, M. Joffe, M.R. Kling, M. Kurek, T.L. Nero, T. Nguyen, J.T. Palmer, I. Phillips, D.M. Shackleford, H. Sikanyika, M. Styles, S. Su, H. Treutlein, J. Zeng, A.F. Wilks, Phenylaminopyrimidines as inhibitors of Janus kinases (JAKs), *Bioorg Med Chem Lett*, 19 (2009) 5887-5892.

22. T.C. Chou, Q.H. Tan, F.M. Sirotnak, Quantitation of the synergistic interaction of edatrexate and cisplatin in vitro, *Cancer chemotherapy and pharmacology*, 31 (1993) 259-264.

23. T.C. Chou, Drug combination studies and their synergy quantification using the Chou-Talalay method, *Cancer research*, 70 (2010) 440-446.

24. N.A. Franken, H.M. Rodermond, J. Stap, J. Haveman, C. van Bree, Clonogenic assay of cells in vitro, *Nature protocols*, 1 (2006) 2315-2319.

25. A. Suetsugu, M. Nagaki, H. Aoki, T. Motohashi, T. Kunisada, H. Moriwaki, Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells, *Biochemical and biophysical research communications*, 351 (2006) 820-824.

26. S.F. Chen, Y.C. Chang, S. Nieh, C.L. Liu, C.Y. Yang, Y.S. Lin, Nonadhesive culture system as a model of rapid sphere formation with cancer stem cell properties, *PLOS ONE*, 7 (2012) e31864.

27. M.J. Jung, J.K. Rho, Y.M. Kim, J.E. Jung, Y.B. Jin, Y.G. Ko, J.S. Lee, S.J. Lee, J.C. Lee, M.J. Park, Upregulation of CXCR4 is functionally crucial for maintenance of stemness in drug-resistant non-small cell lung cancer cells, *Oncogene*, 32 (2013) 209-221.

28. K. Yamaoka, P. Saharinen, M. Pesu, V.E. Holt, 3rd, O. Silvennoinen, J.J. O'Shea, The Janus kinases (Jaks), *Genome biology*, 5 (2004) 253.

29. P. He, D. Zhang, H. Li, X. Yang, D. Li, Y. Zhai, L. Ma, G. Feng, Hepatitis B virus X protein modulates apoptosis in human renal proximal tubular epithelial cells by activating the JAK2/STAT3 signaling pathway, *Int J Mol Med*, 31 (2013) 1017-1029.

30. F. Sonohara, S. Nomoto, Y. Inokawa, M. Hishida, N. Takano, M. Kanda, Y. Nishikawa, T. Fujii, M. Koike, H. Sugimoto, Y. Kodera, High expression of Janus kinase 2 in background normal liver tissue of resected hepatocellular carcinoma is associated with worse prognosis, *Oncology reports*, 33 (2015) 767-773.

31. S. Verstovsek, S. Courby, M. Griesshammer, R.A. Mesa, C.B. Brachmann, J. Kawashima, J.D. Maltzman, L. Shao, Y. Xin, D. Huang, A. Bajel, A phase 2 study of momelotinib, a potent JAK1 and JAK2 inhibitor, in patients with polycythemia vera or essential thrombocytremia, *Leukemia Research*, 60 (2017) 11-17.

32. Y. Li, B. Tian, J. Yang, L. Zhao, X. Wu, S.-L. Ye, Y.-K. Liu, Z.-Y. Tang, Stepwise metastatic human hepatocellular carcinoma cell model system with multiple metastatic potentials established through consecutive in vivo selection and studies on metastatic characteristics, *J Cancer Res Clin Oncol*, 130 (2004) 460-468.
33. H. Huynh, R. Ong, K.Y. Goh, L.Y. Lee, F. Puehler, A. Scholz, O. Politz, D. Mumberg, K. Ziegelbauer, Sorafenib/MEK inhibitor combination inhibits tumor growth and the Wnt/β-catenin pathway in xenograft models of hepatocellular carcinoma, *International journal of oncology*, 54 (2019) 1123-1133.

34. V.S. Donnenberg, A.D. Donnenberg, Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis, *Journal of clinical pharmacology*, 45 (2005) 872-877.

35. J. Neuzil, M. Stantic, R. Zobalova, J. Chladova, X. Wang, L. Prochazka, L. Dong, L. Andera, S.J. Ralph, Tumour-initiating cells vs. cancer 'stem' cells and CD133: what's in the name?, *Biochemical and biophysical research communications*, 355 (2007) 855-859.

36. A. Scopelliti, P. Cammareri, V. Catalano, V. Saladino, M. Todaro, G. Stassi, Therapeutic implications of Cancer Initiating Cells, *Expert opinion on biological therapy*, 9 (2009) 1005-1016.

37. G. Housman, S. Byler, S. Heerboth, K. Lapinska, M. Longacre, N. Snyder, S. Sarkar, Drug resistance in cancer: an overview, *Cancers*, 6 (2014) 1769-1792.

38. Llovet JM, Bruix J., Molecular targeted therapies in hepatocellular carcinoma, *Hepatology*, 48(4) (2008) 1312–1327.

39. Cervello M, McCubrey JA, Cusimano A, Lampiasi N, Azzolina A, Montalto G., Targeted therapy for hepatocellular carcinoma: novel agents on the horizon, *Oncotarget*, 3(3) (2012) 236–260.

40. Chao Y, Chung YH, Han G, Yoon JH, Yang J, Wang J, et al., The combination of transcatheter arterial chemoembolization and sorafenib is well tolerated and effective in Asian patients with hepatocellular carcinoma: final results of the START trial, *Int J Cancer*. 136(6) (2015) 1458–1467.

41. Liu L, Chen H, Wang M, Zhao Y, Cai G, Qi X, et al., Combination therapy of sorafenib and TACE for unresectable HCC: a systematic review and meta-analysis, *PLOS ONE*. 9(3) (2014) e91124.

42. Svinka J, Mikulits W, Eferl R., STAT3 in hepatocellular carcinoma: new perspectives. *Hepat Oncol*. 1(1) (2014) 107–120.

43. Diego F Calvisi, Sara Ladu, Alexis Gorden, Miriam Farina, Elizabeth A Conner, Ju Seog Lee, Valentina M Factor, Snorri S Thorgeirsson, Ubiquitous activation of Ras and Jak/Stat pathways in human HCC, *Gastroenterology*. 130(4) (2006) 1117-28.

44. He G, Karin M. NF-κB and STAT3 – key players in liver inflammation and cancer. *Cell Res*. 21(1) (2011) 159–168.

45. Justin Jit Hin Tang, Dexter Kai Hao Thng, Jhin Jieh Lim and Tan Boon Toh, JAK/STAT signaling in hepatocellular carcinoma, *Hepat Oncol*. 7(1) (2020) HEP18.

**Supplementary Data**

**Supplementary Table S1.** Clinical details of HBV- and HCV-infected patients from the Taipei Medical University Shuang-Ho Hospital HCC cancer cohort. **Supplementary Table S2.** Commercial Antibodies list. **Supplementary Table S3.** RT-PCR Primer sequences. **Supplementary Table S4.** Figure 1H IHC scoring matrix. **Supplementary Figure S1.** Combination of momelotinib and sorafenib suppressed proliferation of
nvHCC cells, HepG2. **Supplementary Figure S2.** Full-size blots of **Figure 2B, Supplementary Figure S3.** Full-size blots of **Figure 2E, Supplementary Figure S4.** Full-size blots of **Figure 3D, Supplementary Figure S5.** Full-size blots of **Figure 4C, Supplementary Figure S6.** Full-size blots of **Figure 5D.**