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Anti-tumor Activity of the Small Molecule Inhibitor PRI-724 Against β-Catenin-activated Hepatocellular Carcinoma

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Abstract. *Background/Aim: CBP is a transcriptional coactivator in the Wnt/β-catenin pathway that is related to cell kinetics and differentiation. This study aimed to characterize β-catenin-activated hepatocellular carcinoma (HCC) and evaluate the direct effects of PRI-724 (a selective inhibitor of Wnt/β-catenin/CBP signaling) on HCC. Materials and Methods: Immunohistochemistry for β-catenin was performed in 199 HCC resected samples. Moreover, using cultured HCC cell lines, cell kinetics and its related proteins were analyzed after treatment of cells with C-82 (active form of PRI-724). Results: Nuclear β-catenin expression was found in 18% of HCC cases and the tumor sizes in these positive samples were larger. In HCC cell lines with a constitutively activated β-catenin, C-82 inhibited cell proliferation. C-82 led to an increase in the percentage of cells in the G_0/G_1 phase of the cell cycle. The percentage of cells in the sub-G_1 phase also increased. Moreover, C-82 treatment significantly decreased the expression of cell proliferating markers and increased the expression of apoptosis-related proteins. Conclusion: PRI-724(C-82) may be a novel drug for β-catenin-activated HCC therapy.*

Hepatocellular carcinoma (HCC) is the 6th most common cancer and the 4th leading cause of cancer-related deaths worldwide. Localized HCC at a relatively early stage is often effectively cured by the established standard surgery, radiofrequency ablation, and trans-arterial chemoembolization. In contrast, the treatment of advanced HCC is limited to systemic therapy. Following the development of sorafenib, a molecularly targeted drug for unresectable advanced HCC, most clinical trials for new therapeutic agents failed to achieve sufficient treatment efficacy (1, 2). Although the development of regorafenib and lenvatinib expanded the selection of treatments in recent years, there is an urgent need to provide alternative treatment options for advanced HCCs.

HCCs are well-known to occur in patients with chronic hepatitis or liver cirrhosis, mostly hepatitis virus-related. In addition, even in localized lesions, local recurrence and intrahepatic metastasis are very often, because of the high malignant grade of primary lesions and/or multicentric carcinogenesis. Moreover, it is suggested that HCC results from multilevel gene mutations along with a chronic persistent inflammation. Recently, similarly to other tumors, HCC was characterised at the molecular level; the most frequently mutated genes in HCC include the telomerase reverse transcriptase (*TERT*), catenin beta 1 (*CTNNB1*), tumor protein 53 (*TP53*), and AT-rich interaction domain 1A (*ARID1A*) (3). These genomic profiles were correlated with tumor stages, clinical features, and survival and helped to identify biomarkers of patients' response to targeted therapies.

β-catenin is a key component of the Wnt signaling pathway. In the absence of Wnt ligands, most β-catenin binds to E-cadherin in adherent junctions at the plasma membrane. In the presence of Wnt ligands, stabilized β-catenin translocates into the nucleus and forms a complex with TCF/LEF transcription factors. Then, this complex binds to the coactivators cAMP-responsive element-binding (CREB)-binding protein (CBP) or p300 (adenovirus early region 1A (E1A)-binding protein; ~300

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kDa) to promote the expression of target genes and regulate cell proliferation or differentiation, respectively (4). That is, the expression of genes regulating cell proliferation is activated by the interaction between β-catenin and the TCF/LEF complex with CBP. In some cancer cells, it has been demonstrated that following its excessive cytoplasmic accumulation and nuclear translocation, β-catenin preferentially interacts with CBP and that this binding is associated with the uncontrolled cell proliferation of cancers (4). This suggests the possibility that the abnormal cell proliferation of cancer cells results from the binding of CBP to β-catenin, caused by several mechanisms, such as gene mutations.

The objective of this study was to characterize β-catenin-activated HCC cases using human specimens and investigate the antitumor effect of inhibiting the interaction between CBP and β-catenin *in vitro* using HCC cell lines. PRI-724 (an active form, C-82) is a selective inhibitor of the CBP/β-catenin interaction developed by PRISM BioLab that competitively binds to the unstructured N-terminal region of CBP and blocks the binding of CBP with β-catenin (5, 6). However, the direct effects and mechanisms of PRI-724 in HCC remain unclear. To the best of our knowledge, our study is the first to demonstrate the anti-cancer effect of PRI-724 on HCC cell lines with activated β-catenin.

Materials and Methods

Liver tissue samples. A total of 199 surgically resected HCC specimens were included in this study. They were specimens obtained from partial hepatectomies, clinicopathologically diagnosed as HCC. Sections (4 μm-thick) were prepared from neutral formalin-fixed paraffin-embedded tissues for routine histologic observation and immunohistochemistry. All tissue specimens were collected from the hepatobiliary files of our Department. This study was approved by the Kanazawa University Ethics Committee [No.2016-072(305)].

Immunohistochemistry. Immunohistochemical analysis was performed using primary antibodies against human β-catenin (diluted 1:200, BD Biosciences, San Diego, CA, USA), CBP (ab50702, diluted 1:50, Abcam Cambridge, UK), and p300 (diluted 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After deparaffinization of the sections, antigen retrieval was performed by microwave treatment in 10 mmol/l citrate buffer (pH 6.0) or heating in Tris-ethylenediaminetetraacetic acid buffer (Tris-EDTA) (pH 9.0). After blocking endogenous peroxidase activity, the sections were incubated overnight at 4˚C and then, at room temperature for 1 h with goat anti-mouse immunoglobulin conjugated to a peroxidase-labeled dextran polymer (Simple stain, neat, Nichirei, Tokyo, Japan). Color development was performed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.03% hydrogen peroxide. The sections were lightly counterstained with hematoxylin or methyl green. No positive staining was observed when the primary antibodies were replaced with an isotype-matched, non-immunized mouse IgG as a negative control.

Cell lines and cell culture. Six human HCC cell lines, including HepG2 (JCRB1592), Huh7 (JCRB1600), Huh6 (JCRB0401), Huh28 (JCRB0462), JHH2 (JCRB1028), and JHH5 (JCRB1638), were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank and cultured according to the manufacturer's protocol. HepG2 and Huh28 cells were cultured in C-82 (activated form of prodrug PRI-724) to evaluate the inhibition of CBP/β-catenin interaction, because these two cell lines showed constitutive activation of β-catenin, as shown below.

Preparation of cell blocks. Cultured cells were harvested and fixed with 3 ml of formalin. After formalin-fixing for 24 h, cells were collected by centrifugation at 3,000 rpm for 5 min. Following aspiration of the supernatant, the pellet was resuspended in 1 ml of 1% sodium alginate. After centrifugation and aspiration of the supernatant, 100 μl of 1 M calcium chloride was gently added to the cell pellet. To prepare cell blocks, the gelatinous pellet was embedded in paraffin.

Cell proliferation assay. To assess proliferation, the total number of living cells was determined using a water-soluble tetrazolium salt (WST)-1 assay according to the manufacturer's instructions (Roche, Mannheim, Germany). Cells were seeded in quadruplicate in 96-well microplates at a density of 2.5×10^3 cells per well and incubated for 24 h at 37˚C with atmospheric CO_2 in the dark. After incubation, the supernatant was discarded and replaced with fresh serum-free medium. C-82 was dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture medium at various concentrations (0, 1, 5, 10, and 20 μM). After a 24 h exposure to C-82, WST-1 assay solution (10 μl/well) was added to each well followed by incubation for 3 h at 37˚C with 5% atmospheric CO_2 in the dark. The optical density was measured at 450 nm using a microplate reader. Four independent experiments were performed to obtain statisticalpower.

Cell cycle analysis. Cells were treated with 10 μM of C-82 for 24 h. Cells were stained with 500 μg/ml propidium iodide (SIGMA P-4170), 0.25 ng/ml RNase (SIGMA R-4875), 1 mg/ml sodium citrate, and 0.3% Triton X-100. Flow cytometry analysis was performed using a Beckman Coulter Gallios flow cytometer. The concentration of C-82 (10 μM) was determined according to our preliminary studies and a previously published paper.

Western blotting. Approximately 2×10^6 cells were lysed in RIPA buffer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing a 1% protease inhibitor cocktail (Sigma-Aldrich Co.). Proteins from each sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5%-20% gels (Bio-Rad Laboratories, Hercules, CA, USA). The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) and blocked with commercial gradient buffer (PVDF Blocking Reagent for Can Get Signal, Toyobo, Tokyo, Japan) at room temperature for 30 min. The membranes were incubated with the indicated primary antibody overnight at 4˚C. After washing, the membranes were incubated with a horseradish peroxidase (HRP)-labeled anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Antibody-antigen complexes were detected using an ECL Plus reagent (GE Healthcare Japan Co. Ltd.) and ChemiDoc XRS Plus system (Bio-Rad Laboratories, Hercules, CA, USA). Quantification was performed with Image Lab Software (Bio-Rad

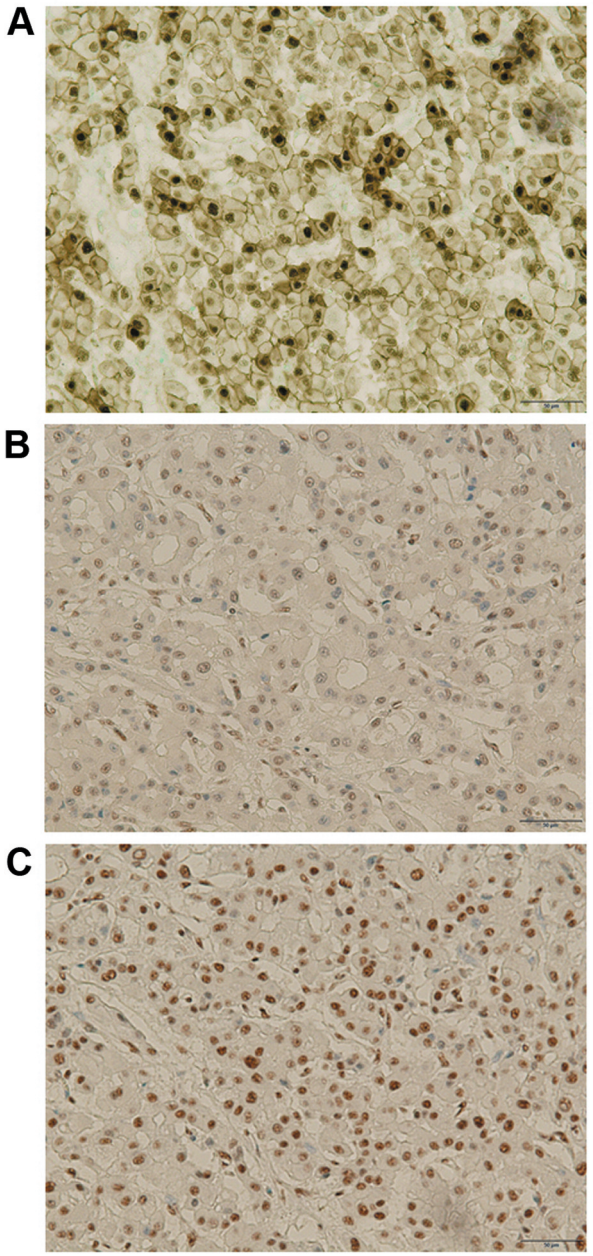

Figure 1. *Immunohistochemical expression of β-catenin (A), CBP (B), and p300 (C) in human hepatocellular carcinoma. Nuclear expression of β-catenin was found in 18% of HCC cases, whereas the nuclear expression of CBP and p300 was consistently observed in all HCC cases.*

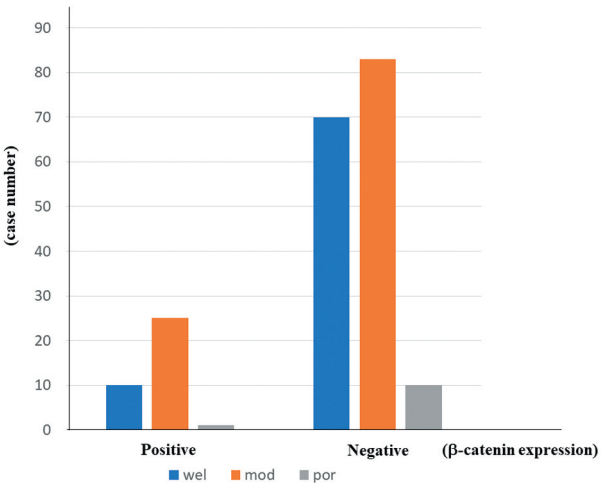

Figure 2. *Histological differentiation grade of hepatocellular carcinoma according to β-catenin expression. wel: Well-differentiated; mod: moderately differentiated; por: poorly differentiated.*

Laboratories). We used primary antibodies against the following proteins: PARP (CST9542, diluted 1:1,000, Cell Signaling Technology, Danvers, MA, USA), survivin (CST 2808, diluted 1:1,000, Cell Signaling Technology), c-Myc (ab32072, diluted 1:10000, Abcam), Skp2 (CST 2652, diluted 1:1,000, Cell Signaling Technology), p21 Waf1/Cip1 (CST 2947, diluted 1:1,000, Cell Signaling Technology), cleaved caspase-3 (CST 9664, diluted 1:1,000, Cell Signaling Technology), cleaved caspase-7 (CST 9491, diluted 1:1,000, Cell Signaling Technology), or tubulin (ab18251, diluted 1:2,000, Abcam) as the internal control.

Statistical analysis. The mean±SD was calculated for all parameters. Statistical analysis was performed using the JMP 10.0 software package (SAS Institute, Inc., Cary, NC, USA). Statistical differences were determined using Student's t-test and analysis of variance. A p-value<0.05 was accepted as the level of statistical significance.

Results

In situ detection of β-catenin in human HCC specimens. The immunohistochemical analysis results clearly demonstrated that β-catenin was expressed at high levels in the nucleus of tumor cells in some HCC cases (Figure 1A). Among 199 cases, there were 36 (18%) β-catenin-positive cases. The diameters of β-catenin-positive tumors were significantly larger than those of negative cases (average 4.2 cm *vs*. 3.1 cm; median 2.9 cm *vs*. 2.5 cm in positive and negative cases, respectively). As for the histological differentiation of HCC, moderately differentiated HCCs were prominent in the positive cases, whereas the proportion of well- and moderately differentiated HCCs was similar in the negative cases (Figure 2). Histological observation using HE staining was retrospectively performed by referring to the β-catenin expression data, but it was not possible to speculate the pattern of β-catenin expression based solely on morphology. In contrast to β-catenin, the expression of CBP and p300 was consistently found in the nucleus in all cases (Figure 1B and C) regardless of whether β-catenin was expressed or not in the nucleus.

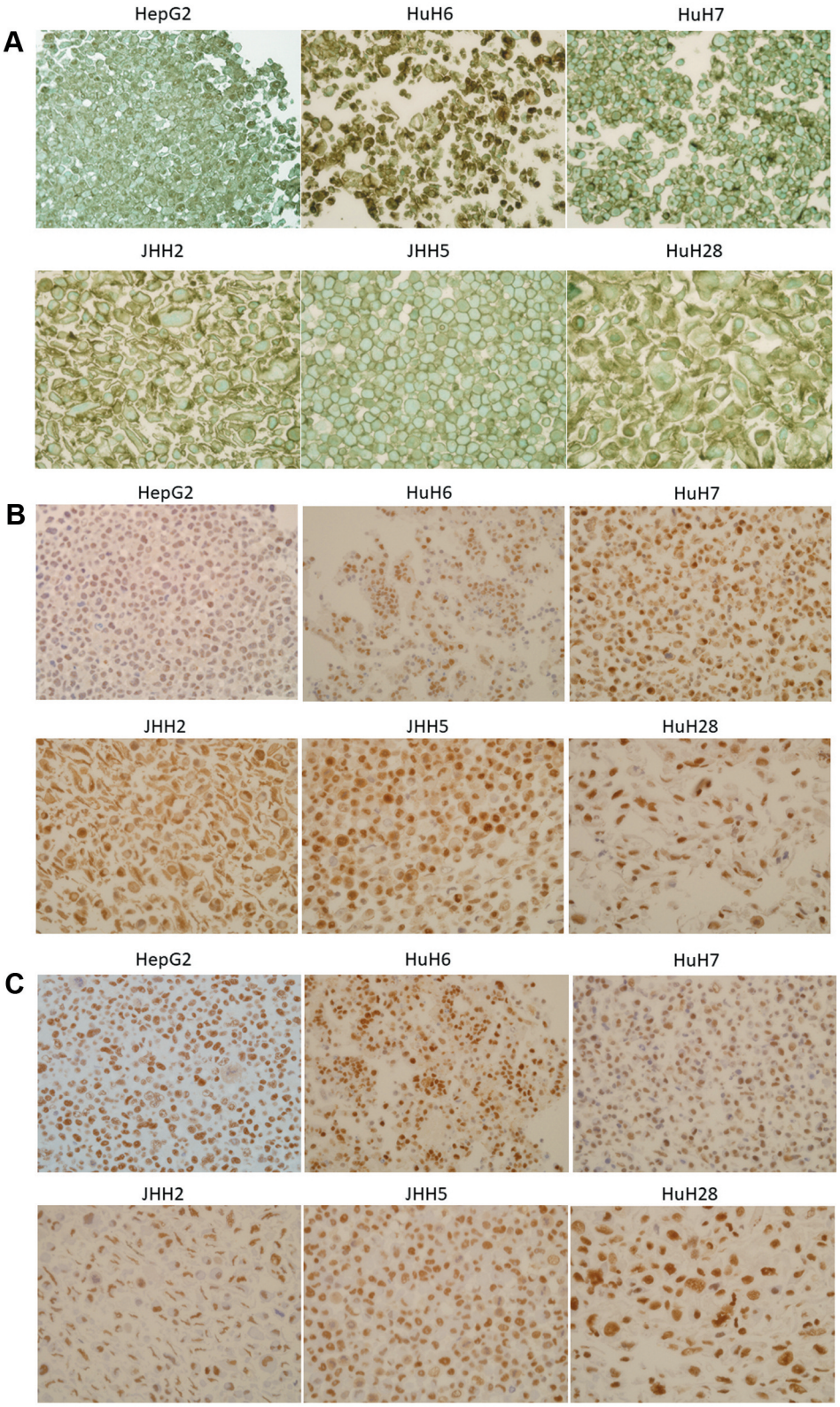

Figure 3. *Representative images of β-catenin, CBP, and p300 expression in cultured human hepatocellular carcinoma cells. A: β-catenin. HepG2 and Huh6 were estimated as positive cell lines because of the nuclear expression of β-catenin. B: CBP. All cell lines show a nuclear pattern. C: p300. All cell lines show a nuclear pattern.*

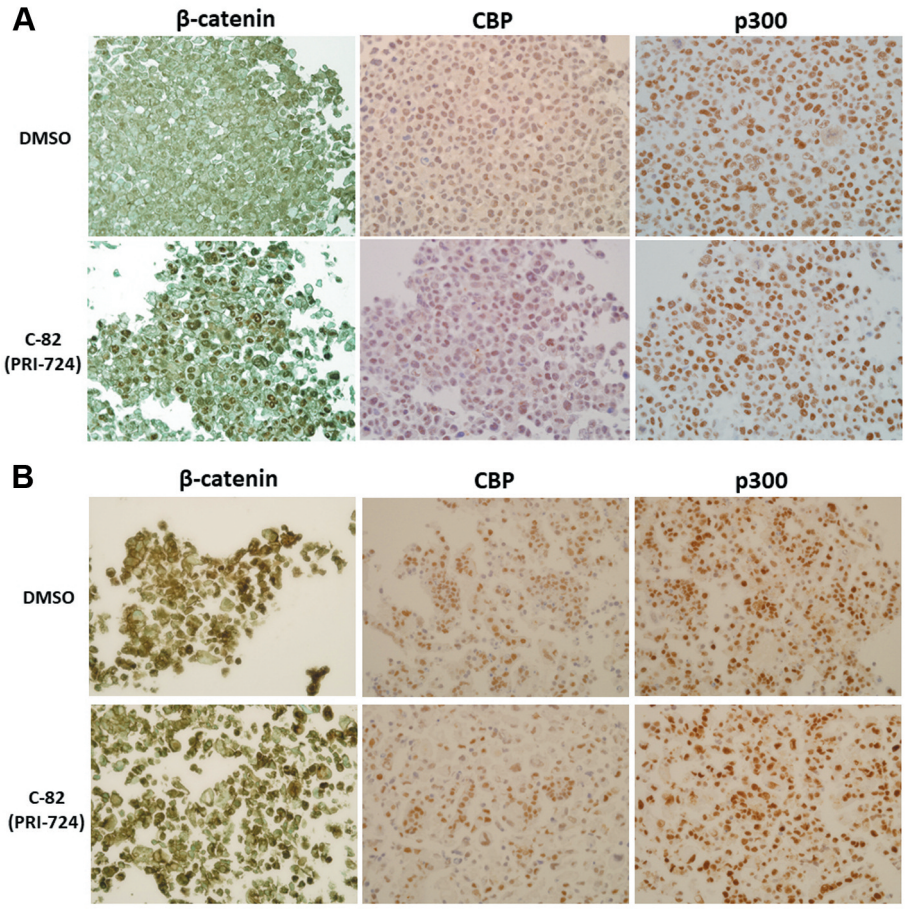

Figure 4. *Alterations in β-catenin, CBP, and p300 expression in HepG2 (A) and Huh6 (B) cells following treatment with C-82 (10 μM, PRI-724). Although the nuclear expression in C-82-treated HepG2 cells appears to be slightly enhanced, the nuclear pattern is preserved. CBP and p300 expressions of C-82-treated cells are similar to vehicle controls (DMSO).*

β-catenin, CBP, and p300 expression levels in cultured human HCC cells. β-catenin, CBP, and p300 expression levels were examined by immunohistochemistry using prepared cell blocks of all cultured human HCC cell lines. β-catenin expression was generally localized in the cytoplasm or at the cell membrane. However, distinct nuclear accumulation was observed in over 90% of HepG2 and Huh6 cells, but not in Huh7, Huh28, JHH2, or JHH5 cells (Figure 3A). According to these results, the following studies were conducted using HepG2 and Huh6 cell lines, because these were thought to be β-catenin-activated cells. In contrast to β-catenin, CBP and p300 were consistently found in the nucleus of all human HCC cells (Figure 3B and C). Because both CBP and p300 were expressed in and originally located in the nucleus irrespective of whether they were active or inactive, it was not possible to immunohistochemically estimate the activation state of CBP or p300 according to their expression levels and alterations in intracellular localization.

C-82 does not affect the nuclear expression of β-catenin, CBP, or p300 in cultured HCC cells. After pretreatment with 10 μM C-82 for 24 h, nuclear β-catenin, CBP, and p300 expression levels in HepG2 and Huh6 cells were verified by immunohistochemistry. The nuclear expression of β-catenin was observed in over 90% of both HepG2 (Figure 4A) and Huh6 (Figure 4B) cells, and pretreatment with C-82 did not affect the intensity and localization of β-catenin. The expression levels and patterns of CBP and p300 were also not affected by C-82 treatment.

C-82 inhibits the proliferation of cultured HCC cells. Based on the mechanism of action of C-82 against the Wnt/β-catenin signaling pathway, it was predicted that cellular proliferation would be inhibited by C-82 treatment. According to the WST-1 assay results, C-82 (5-20 μM) significantly impaired cell growth in a concentration-dependent manner (Figure 5). Reduced cell proliferation was

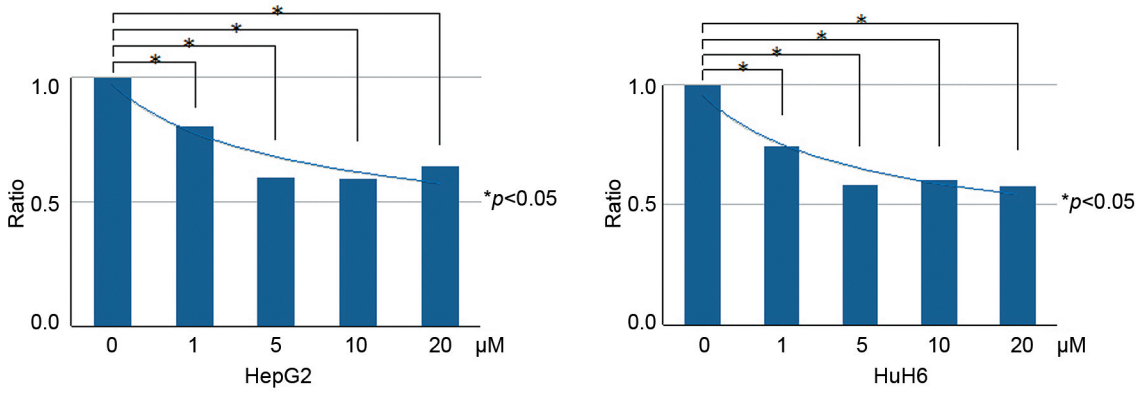

Figure 5. *C-82 (PRI-724) inhibited proliferation of HepG2 and Huh6 cells in a concentration-dependent manner as analysed by the WST-1 assay. Data are shown as the relative ratio to vehicle controls (0 μM).*

observed in both HepG2 and Huh6 cells, even in the presence of 1 μM C-82.

C-82 induces G_0/G_1 arrest in cultured HCC cells. To analyze the cell cycle distribution, vehicle- or C-82 (10 μM)-treated cells stained with propidium iodide 24 h after treatment were analyzed by flow cytometry. As shown in Figure 6, C-82 treatment significantly increased the percentage of G_0/G_1 phase HepG2 and HuH6 cells. Furthermore, we analyzed the expression of cell cycle-related proteins. Cells were incubated with vehicle or C-82 (10 μM) for 24 h, and extracted proteins were analyzed by western blotting for p21 Waf1/Cip1, c-Myc, Skp2, and α-tubulin (internal control). The results showed that C-82 treatment increased the expression of p21 Waf1/Cip1, which is an important mediator of cell cycle arrest. In contrast, the expression levels of the cell cycle accelerators c-Myc and Skp2 were decreased by C-82 treatment (Figure 7).

C-82 affects the expression of anti- and pro-apoptotic proteins in cultured HCC cells. We analyzed the expression of pro- and anti-apoptotic markers in HepG2 and Huh6 cells. Cells were incubated with vehicle or C-82 (10 μM) for 24 h, and extracted proteins were analyzed by western blotting for PARP, cleaved caspase-3, cleaved caspase-7, survivin, and α-tubulin (internal control). As shown in Figure 8, C-82 treatment increased the expression of cleaved caspase-3 and cleaved caspase-7, thereby inducing apoptosis of HepG2 and Huh6 cells. In contrast, C-82 treatment decreased the expression of survivin, which belongs to the inhibition of apoptosis (IAP) family (Figure 8).

Discussion

In the last decade, the molecular mechanisms associated with HCC carcinogenesis and development have been a topic of great interest. Several studies have previously clarified that multiple signaling pathways are dysregulated in HCC. In particular, alterations in molecular signaling pathways, such as Wnt/β-catenin, receptor tyrosine pathways, phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), Ras mitogen-activated protein kinase (Ras/Raf/MAPK), Janus kinase (Jak)-signal transducer activator of transcription factor (Stat) (JAK/STAT), Hedgehog (HH), and Hippo, contribute to HCC carcinogenesis (3). With advances in the elucidation of these molecular pathways, there is a growing interest in the development of novel therapeutic targets against the critical genes and signaling molecules involved in regulating multiple pathways (2).

Although the Wnt/β-catenin pathway was originally identified as a key regulator of embryonic development and differentiation, it is now well-known that this pathway is deeply involved in the carcinogenesis or tumorigenesis of several organs, including the liver (1). In the liver, the presence of mutant β-catenin is found in 11%–37% of human HCCs (1, 7) and characterizes a subtype of hepatocellular adenoma (8). In the current study, we initially analyzed the expression and pattern of β-catenin in human HCC samples, in which the activated form was immunohistochemically identified by its intense nuclear expression. As a result, 18% (36/199 cases) of HCC cases showed this distinct nuclear expression pattern of β-catenin, which is similar to the frequency reported in previous studies (1, 7). Moreover, the diameters of tumors in β-catenin-positive HCC cases were significantly larger than those in negative cases. This may reflect the high cell proliferative activity associated with enhanced CBP/β-catenin signaling.

In addition to CBP, the coactivator p300 is also recruited by β-catenin. CBP and p300 independently play definitive and unique roles as master regulators of transcription (9). CBP/β-catenin-mediated transcription is critical for proliferation/non-

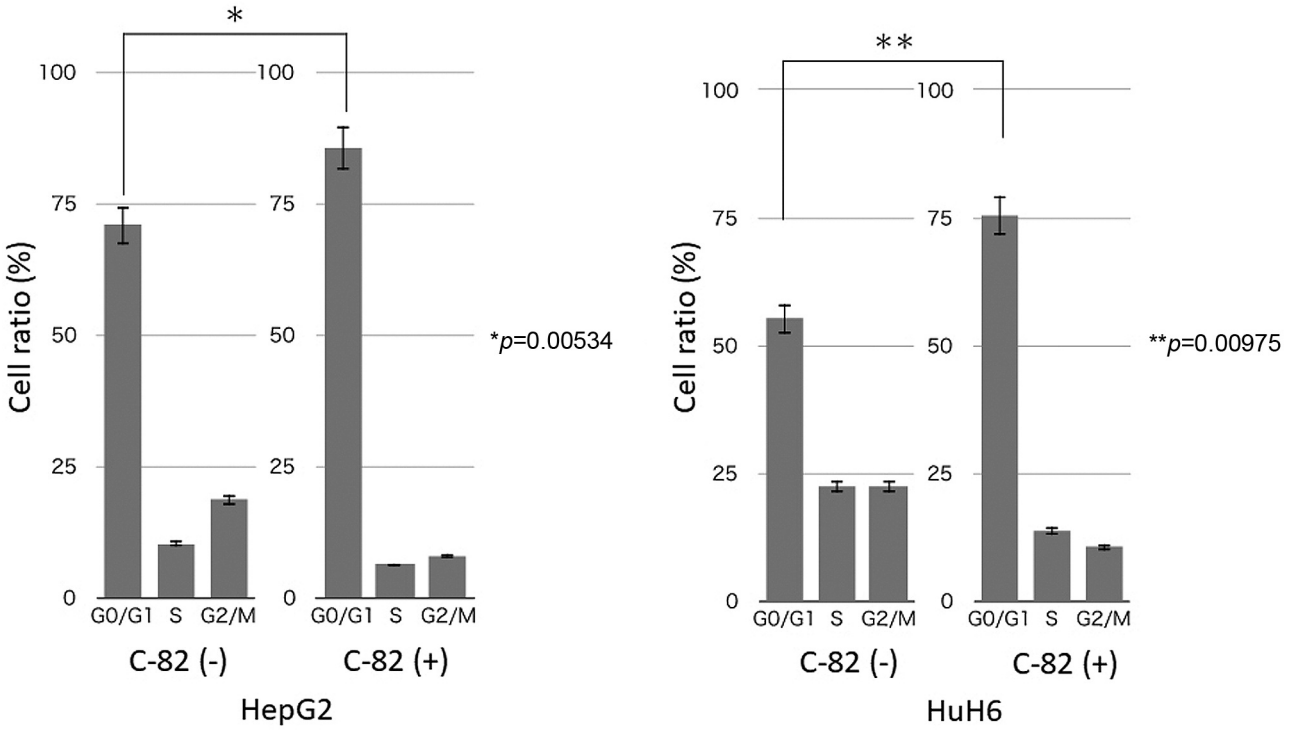

Figure 6. *Cell cycle analysis by flow cytometry by using propidium iodide staining. Treatment with C-82 (PRI-724) significantly increases the proportion of G_0/G_1 phase cells. Data are shown as the ratio (percentage) of whole cell counts.*

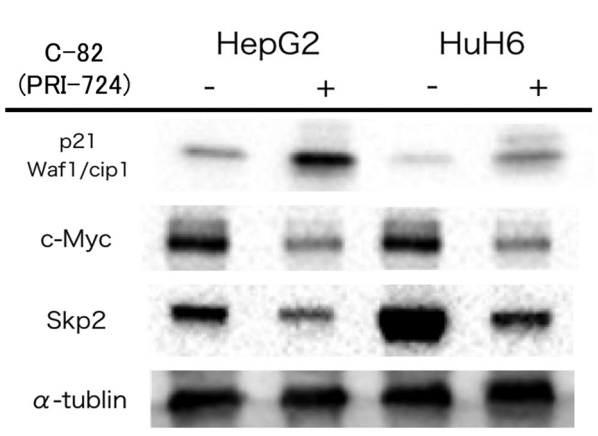

Figure 7. *Western blotting for p21 Waf1/Cip1, c-Myc, Skp2, and α-tubulin (internal control). C-82 (PRI-724) treatment increased the expression of p21 Waf1/Cip1 but decreased the levels of c-Myc and Skp2.*

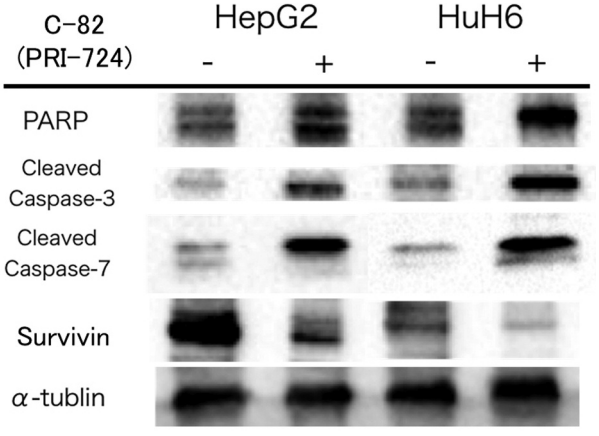

Figure 8. *Western blotting for PARP, cleaved caspase-3, cleaved caspase-7, survivin, and α-tubulin (internal control). C-82 (PRI-724) treatment increased the expression of PARP, cleaved caspase-3, and cleaved caspase-7, but decreased the levels of survivin.*

differentiation, whereas p300/β-catenin-mediated transcription initiates differentiation (4). The present study revealed that CBP and p300 are consistently expressed in the nucleus of all HCC cell lines, indicating that both CBP/β-catenin and p300/β-catenin signaling pathways are present in HCC cells, although it was not possible to directly evaluate their activation state by immunohistochemistry. Moreover, the *in vitro* study using human HCC samples revealed that there is no difference in the degree of histologic differentiation between β-catenin positive and negative cases. Therefore, the

activation of β-catenin may not directly indicate the progressive differentiation of HCC associated with p300/β-catenin signaling.

PRI-724 (C-82) is a specific inhibitor of CBP/β-catenin interactions in the Wnt-β-catenin signaling pathway (5, 6). There are a few reports regarding this inhibitory effect on CBP/β-catenin signaling (10-13). In particular, *in vitro* and *in vivo* studies using HCC cell lines have previously revealed that ICG-001, a predecessor compound of PRI-724, could enhance the anti-tumor effects of molecularly targeted drugs, such as sorafenib, against HCC (11). Although it has been shown that PRI-724 inhibits the interactions between β-catenin and CBP, the direct effects and mechanisms of PRI-724 in HCC remain unclear. In the present study, we confirmed the anti-tumor effects of PRI-724 (C-82) against constitutively β-catenin-activated HepG2 and Huh6 cell lines, as demonstrated by their decreased cell proliferative activity. Mechanistically, treatment with PRI-724 (C-82) significantly reduced the expression levels of the well-known β-catenin-induced cell proliferative proteins c-Myc and cyclin D1 in β-catenin-activated HCC cell lines. This suggests that PRI-724 may improve the neoplastic/uncontrolled cell proliferation by inhibiting the interactions between β-catenin and CBP. Moreover, the present data suggested that PRI-724 (C-82) could inhibit cell growth by inducing G_0/G_1 cell cycle arrest and enhance the apoptosis of abnormally proliferating cells. Although it was not possible to evaluate the degree of differentiation in these HCC cell lines, PRI-724 (C-82) possibly promotes cell differentiation *via* enhanced p300/β-catenin interactions as a reciprocal action of the inhibition of CBP/β-catenin interactions. If this induction of cell differentiation occurs in moderately or poorly differentiated HCCs *in vivo*, the degree of malignancy and uncontrolled cell proliferation could be ameliorated.

In addition to carcinogenesis, Wnt/β-catenin signaling has been implicated in the fibrogenesis of diverse organs. For example, the abnormal activation of Wnt/β-catenin signaling is a major factor that regulates fibrogenesis in several diseases, such as liver cirrhosis, idiopathic pulmonary fibrosis, chronic kidney disease, skin diseases, and eye diseases (14-16). Liver cirrhosis is the advanced terminal stage of most liver diseases, irrespective of their etiologies, and a well-known predisposing condition for the development of HCC. Therefore, the development of anti-fibrogenic treatments for liver cirrhosis is also important for the prevention of the occurrence of HCC. According to published experimental reports, PRI-724 could inhibit fibrosis progression and promote fibrinolytic action (17-19). As for the livers, PRI-724 treatment showed anti-fibrotic effects on liver fibrosis in experimental mice induced by CCl4 or bile duct ligation (18). In addition, these studies revealed that the inhibition of hepatic stellate cell activation and induction of fibrinolytic cells expressing matrix metalloproteinase (MMP)-8 improved liver fibrosis in HCV transgenic mice (19). Currently, in Japan, clinical trials for patients with cirrhosis caused by HCV and HBV, and treated with PRI-724 are ongoing, and future developments are expected (5, 6). Considering these reports and our present study, PRI-724 is speculated to show both anti-fibrosis effects against liver fibrosis and anti-tumorigenesis effects on HCC. Therefore, it may become a novel strategy for the treatment of HCC associated with liver cirrhosis, irrespective of the etiology.

In conclusion, we examined the expression of β-catenin, CBP, and p300 in human HCC specimens and the anti-tumorigenic effect of PRI-724 using sporadic β-catenin-activated HCC cell lines. The Wnt/β-catenin signaling pathway plays a key role in a subgroup of human HCCs, and the development of effective therapies would be beneficial to treat HCC. PRI-724 directly inhibits cell kinetics by inducing cell cycle arrest and enhancing apoptosis. PRI-724 is noted as an anti-fibrotic and anti-tumor agent against HCC, particularly β-catenin-activated HCC.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

Concept and design: K. Harada; Development of methodology: K. Harada, R. Gabata; Acquisition of data (acquired and managed patients, provided facilities, *etc*.): K. Harada, R. Gabata, A. Kitao, K. Yoshimura, K. Kimura, H. Kouji,; Review of slides, analysis and interpretation of data: K. Harada, R. Gabata, Y. Sato; Immunohistochemistry and cell culture analysis: R. Gabata, Y. Mizutani, H. Ouchi, Writing the manuscript: K. Harada; H. C. Nguyen; Review, and/or revision of the manuscript: R. Gabata, K. Harada, Y. Sato, K. Kimura, H. Kouji, T. Miyashita, H. Tajima, T. Ohta; Study supervision: K. Harada.

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