YAP/TAZ expression in hepatocellular carcinoma (HCC) and effects of YAP/TAZ inhibition on HCC

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ABSTRACT

YAP/TAZ expression in hepatocellular carcinoma (HCC) and effects of YAP/TAZ inhibition on HCC

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Hepatocellular carcinoma is the most common malignancy in liver cancer and is the second leading cause of death worldwide. Despite surgical resection of early stage HCC, recurrence rate is high and prognosis is poor. Hippo signaling is a tumor suppressive pathway and its inactivation leads to tissue overgrowth and tumorigenesis via YAP- and TAZ- mediated transcriptional activation. Here, I investigated the expression levels of YAP and TAZ in patient-derived HCC tissue and identified the effects of YAP/TAZ inhibition depending on the baseline YAP/TAZ expression when combined with sorafenib using patient-derived multicellular tumor spheroid (MCTS) model.

HCC tissue was obtained from surgical resection of HCCs and YAP/TAZ expression was analyzed using western blot. Primary HCC cell lines were established from patient-derived tissue. I selected six patient-derived HCC cell lines according to YAP/TAZ expression and they were classified into three groups depending on the YAP/TAZ expression on western blot: high, medium, low. Then, I generated multicellular tumor spheroid (MCTS) by mixing patient-derived HCC cells and stroma cells (LX2, WI38, and HUVECs) at a 3:1:1:1 ratio

in ultra-low attachment plates. I assessed the YAP/TAZ expression from protein extracted from MCTS. TAZ expression was major in monolayer HCCs (2D culture) and YAP expression shown from tissue western blot reappeared in MCTS. I analyzed the cell viability upon 48hours of following drug treatment: sorafenib, sorafenib with CA3 0.1μM, and CA3 (novel YAP1 inhibitor). I confirmed that out of six patient-derived HCC cell lines, cell lines with high YAP/TAZ expression at MCTS level responded more sensitively to the combination therapy (Sorafenib + CA3 0.1μM) despite potent cytotoxic effect of CA3 exhibited in all of the patient-derived HCCs. MCTS with medium or low YAP/TAZ expression did not show difference in drug sensitivity: sorafenib vs. sorafenib combined with CA3 0.1μM.

In conclusion, targeting YAP/TAZ inhibition using novel YAP1 inhibitor CA3 could be a promising therapeutic strategy to enhance sensitivity to sorafenib especially in HCCs with high YAP/TAZ expression in MCTS.

Key words : hepatocellular carcinoma (HCC), multicellular tumor spheroids (MCTS), YAP, TAZ, CA3

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I. INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and second leading cause of cancer-related deaths worldwide[1,2]. Primary resection is recommended as the first line of treatment for patients with small solitary HCCs with well-preserved liver function[3,4]. Despite surgical resection of early stage tumors, less than 2 cm and without vascular invasion, recurrence rate reaches approximately 60% at 5 years[5]. Long term survival is significantly reduced in patients with HCC recurrence after primary resection (5-year survival: 58% vs. 34%) [6]. Interestingly, it is reported that the characteristics of the original tumor determine the outcome after development of a recurrent tumor within 24 months after resection[6]. Therefore, elucidating the character of the original tumor is imperative for the treatment of a recurrent tumor.

The Hippo signaling pathway is an important regulator of cell proliferation, organ development, and cell survival[7-9]. Yes-associated protein (YAP) and its paralog, transcriptional co-activator with PDZ-binding motif (TAZ) are the major effectors of the Hippo signaling pathway, and they are inactivated in Hippo signaling[10]. When Hippo signaling is inactive, YAP and TAZ co-activators translocate into the nucleus and interplay with TEA domain family members (TEAD) to form YAP/TAZ-TEAD complex which activates target genes associated with cell proliferation[11]. In recent years, functional studies have

elucidated that YAP is an important oncogene and YAP/TAZ is pervasively activated in human tumors[12]. YAP is overexpressed in 62% of patients with HCC, and YAP levels correlated with decreased survival after resection [13,14]. In addition, YAP is associated with stem cell-like behaviors and epithelial-mesenchymal transition (EMT), both of which are key factors to multi-drug resistance (MDR) to chemotherapy[15,16]. Previous study by Zhou demonstrated that HCC cell lines with chemoresistance exhibited overexpression of YAP, and inhibition of YAP endowed HCC with sensitivity to chemotherapeutic agents *in vitro* and *in vivo*[17]. Thus, YAP/TAZ has emerged as a potential therapeutic target in HCC playing important role in regulating chemotherapeutic sensitivity.

Previous study has proven that combining YAP inhibitor (Verteporfin) with chemotherapeutic agents (5-fluorouracil, doxorubicin) has effectively overcome chemoresistance in HCC cell lines (BEL/FU) and *in vivo*[17]. However, in the clinical setting, sorafenib is introduced as the first line systemic therapy in HCC and the therapeutic effect of YAP/TAZ inhibition when combined with sorafenib is not known. In real-world, YAP/TAZ expression levels differ among patients, and the effect of YAP/TAZ inhibition on the different level of YAP/TAZ expression is not studied in HCC.

Tumor microenvironment (TME) is known to correlate with poor response to chemotherapeutic agents in tumors[18,19]. The TME in HCC consists of cancer and stromal cells, including cancer-associated fibroblasts (CAFs), hepatic stellate cells (HSCs), immune and inflammatory cells, and endothelial cells. When HSCs are activated, they secrete growth factors such as, transforming growth factor ß1 (TGF-ß1), connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF) into the TME, resulting in activation of cancer-stroma leading to enhanced cancer cell proliferation, excessive ECM synthesis, EMT and invasion, as well as drug resistance[20]. In vitro two-dimensional (2D) co-culture models show tumor-CAF interactions but lack the potential to accurately mimic *in vivo* TME, and animal models lack the availability of human

fibroblasts limiting their usefulness as preclinical models. Therefore, multicellular tumor spheroid model (MCTS) has emerged as a promising tool for better mimicking TME interplaying with HCC. These 3D cultures have potential to improve cell-based drug screening. Considering the heterogeneity of HCC among patients, investigating drug response using patient-derived 3D culture may give us valuable information in terms of drug sensitivity.

Here, I investigated the YAP/TAZ expression of patient-derived HCCs and demonstrated the therapeutic effect of the combination of YAP inhibitor (CA3) and sorafenib using patient-derived 3D multicellular tumor spheroid model.

II. MATERIALS AND METHODS

1. Patients and tissue sample

Clinical study protocols were approved by the Institutional Review Board (project number IRB 4-2018-1087) at the College of Medicine, Yonsei University of Korea. Tissue samples of human HCC were obtained after curative surgical resection during 2020 January and 2020 September and processed at Department of Pathology, Severance Hospital, Yonsei University of Korea. Tissue was immediately fixed in 10% neutral buffered formalin for 4 to 8 hours depending on the sample size and was subjected to routine paraffin-embedding protocols.

2. Cell lines and culture conditions.

LX2 cells (human hepatic stellate cells; HSCs) were provided by Dr. Haeng Ran Seo (Institute Pasteur Korea). HUVEC cells (Human umbilical vein endothelial cells) were obtained from Lonza (Basel, Switzerland). WI38 cells (human fibroblast cell line) were obtained from the Korean Cell Line Bank. The cells were maintained at 37˚C in a humidified atmosphere (5% CO2/95% air). LX2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco,

Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1X penicillin (Welgene, Gyeongsan, Korea) (Complete media). HUVEC cells were maintained in Medium 200 (Gibco, USA) supplemented with 1X penicillin (Welgene, Gyeongsan, Korea), 1X LSGS (Gibco, USA) and heat-inactivated 10% FBS. For WI38 cells, Roswell Park Memorial Institute medium (RPMI 1640; Gibco) supplemented with 1X penicillin (Welgene, Gyeongsan, Korea), and 10% heat-inactivated FBS was used. Primary HCC cells were maintained in DMEM/F12 (Gibco, USA) supplemented with 1X penicillin (Welgene, Gyeongsan, Korea), 1X GlutaMmax (Gibco, USA), 1X Primocin (Invitrogen, Carlsbad, CA, USA), and 10% heat-inactivated FBS.

3. Primary culture of HCCs.

Immediately after surgery, a portion of the tumor was immersed in Hanks balanced salt solution (HBSS; Gibco) and transported from the operating room at 0˚C to the laboratory. The specimens were collected under sterile conditions and rinsed 2-3 times with HBSS free of calcium and magnesium to remove blood. After removal of blood, single-cell suspensions were prepared from liver tissue specimens using a GentleMACS™ Dissociator (Miltenyi Biotec, Germany). The resultant was filtered through a 100μm-nylon filter (BD Falcon, Franklin Lakes, NJ, USA) and centrifuged at 50x g for 2 minutes at 4˚C to obtain hepatocytes. The pellet was washed twice in HBSS containing 0.005% DNase. The final cell suspensions were cultured onto collagen-coated T25 flasks (BD Falcon) in F12/DMEM (Gibco), supplemented with 20% FBS, 1% NEAA, 1% glutamine, and 1% P/S at 37˚C in a humidified 5% CO2 incubator. The medium was changed 24 hours after seeding to remove dead cells and debris. When confluence reached 70-80%, the cells were re-plated using a 1:1 mixture of DMEM medium and F12/DMEM with supplements. After five passages, the cells

were grown on DMEM medium supplemented with 10% FBS and 1% P/S. Confluent cells were trypsinized, counted and split 1:3-1:5 at every passage. Once cell lines were maintained over 30 passages, they were collected and stored in liquid nitrogen.

4. Generation of Multicellular Tumor Spheroids (MCTS).

To generate MCTS, cells suspended in complete medium were seeded at a density of 6 x 10^3 cells/well in 96-well ultra-low-attachment (ULA) round-bottom microplates (Sumitomo Bakelite Co., Tokyo, Japan, PrimeSurface, MS-9096UZ). Mixed-cell spheroids were generated by seeding LX2 cells, HUVEC cells, WI38 cells, and patient-derived primary HCCs at a 1:1:1:3 ratio (1000: 1000: 1000: 3000) in ULA plates. The cells were cultured for 3 days at 37 ˚C in a humidified atmosphere (5% CO2/95% air) without daily media changes for drug treatment.

Once MCTS were generated, images of generated MCTS were captured in a 10μm stack using an inverted microscope (IX71; Olympus, Tokyo, Japan) with a 10X objective. Then, MCTS were seeded with drugs for 48hours. Images of MCTS with drug treatment were captured using an inverted microscope (IX71; Olympus, Tokyo, Japan) with a 10X objective.

For preparation of histological sections, generated MCTS were fixed in 4% paraformaldehyde (Biosesang, Korea) and cut into 5μm thick sections using a microtome and mounted on glass slides. The slides were stained with hematoxylin and eosin (H&E). Immunohistochemical staining of paraffin slides of MCTS was performed in order to validate the origin of consisting cells of MCTS: α-SMA (marker for hepatic stellate cells, LX2), fibronectin (marker for fibroblasts, WI 38), CD34 (marker for endothelial cells, HUVEC). Representative images were taken under a microscopy with a 200X objective.

5. Drug Response Assay

Sorafenib (Nexavar®) and CA3 were purchased from Selleckchem (Houston, TX, USA). Stock solutions of sorafenib and CA3 were prepared in 100% DMSO and stored at -20 ˚C. All drugs were diluted to different concentrations in culture media. For drug exposure, MCTS were incubated in drug-containing media for 48hours without media change. For drug combination, agents were given simultaneously at predetermined concentrations. After drug treatment, MCTS in each well was pipetted smoothly until being dissociated to single cells and was transferred to a standard white assay plate for cell viability assay. Equivalent volume of CellTiter-Glo® 3D Reagent (Promega, Germany) was added. The plates were shaken for 5 minutes and luminescence was recorded 30 minutes after reagent addition. Microplate luminometer (Berthold Technologies, Bad Wildbad, Germany, Centro XS3 LB960) was used.

6. Protein extraction and Western blot analysis

In order to detect various proteins from cells and spheroids, proper amount of samples were homogenized and digested in 1X RIPA buffer (Cell Signaling, Denver, MA, USA) which includes 1mM PMSF (Fluka, Switzerland), 2 g/ml Aprotinin (Sigama, Steinheim, Germany), 1mM DTT(Invitrogen, Carlsbad, CA, USA) and phosphatase inhibitor cocktail solution (GenDEPOT, Barker, TX, USA) on ice for 1hr. After the digestion, samples were centrifuged for 25 min at 14,000 RPM in cold microcentrifuge. Then supernatants were collected for use. Western blot experiments were performed following the standard protocol. The following primary antibodies were purchased: anti-YAP/TAZ (8418, Cell Signaling) and anti-EpCAM (ab71916, Abcam). For internal control, an anti-GAPDH antibody (2118, Cell Signaling) was used. Anti-rabbit IgG-HRP (A0545, Sigma) was used as the secondary antibody. Specific

bands were detected using the enhanced chemiluminiscence (ECL) Western blot detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

7. Immunohistochemistry

Paraffin sections were deparaffinized in xylene and rehydrated through gradual decrease in ethanol concentration. The antigen epitopes were then unmasked using sodium citrate buffer (pH 6.0). Subsequently, the sections were incubated overnight at 4˚C using primary antibodies. The following primary antibodies were used: anti-YAP/TAZ (1:200, 8418, Cell Signaling), anti-α-SMA (1:200, ab5694, Abcam), anti-fibronectin (1:100, ab2413, Abcam), CD34(1:100, ab81289, Abcam). After primary incubation, sections were incubated with the appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) followed by treatment with freshly prepared DAB substrates (Vector Laboratories). Sections were lightly counter-stained with hematoxylin and mounted.

8. Immunocytochemistry

To validate the primary cells, cells were fixed with 4% paraformaldehyde (Biosesang, Korea) for 10 min at room temperature, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's phosphate-buffered saline (DPBS; Welgene) for 30min at room temperature, and then washed three times with DPBS. The following primary antibodies were used: goat monoclonal anti-human serum albumin (A80-229A, 1:250, Bethyl laboratories, TX, USA), mouse monoclonal anti-human Hep-Par 1(OCH1E5, 1:250, Cell Marque, Darmstadt, Germany), and mouse monoclonal anti-alpha fetoprotein (ab3980, 1:250, abcam, MA, USA). Samples were incubated with the primary antibodies for 16h at 4℃ and then washed for 10 min

three times with DPBS. The secondary antibodies used for staining were donkey anti-mouse IgG conjugated with Alexa® Fluor 488 (A-21202, Invitrogen, Eugene, OR, USA) and donkey anti-goat IgG conjugated with Alexa® Fluor 647 (A32849, Invitrogen). Samples were then incubated with secondary antibodies for 1h at room temperature in the dark and washed for 10min five times with DPBS. For nuclei staining, cells were incubated with DAPI (D9542-10MG, Sigma-Aldrich, St.Louis, MO, USA) for 10min at room temperature in the dark and washed with PBS twice quickly. All fluorescence images were obtained using the LSM 700 (Zeiss, Oberkochen, Germany).

9. Statistical analysis

Statistical analyses were mainly performed using GraphPad Prism Software (GraphPad, La Jolla, CA, USA), using unpaired two-tailed Student's t-test and Fisher exact test (for immunohistochemistry). P values less than 0.05 were considered statistically significant, and all tests were two-sided.

III. RESULTS

1. Clinical, pathological features of HCC

In order to develop patient-derived MCTS for anti-cancer drug treatment, primary HCC tumors were isolated from liver resection specimens of liver cancer patients. The baseline characteristics of the ten patients with HCC are summarized in Table 1. The median age was 63.2 years, and there was a male predominance (8/10, 80%). Most of the cases were related to HBV infection (6/10, 60%) and rarely to HCV infection (1/10, 10%). Half of the cases (5/10, 50%) had cirrhosis. Major differentiation was most common (8/10, 80%), and both tumor marker levels (AFP, PIVKA-II) were relatively low except one case (Patient No. #8) which showed poor differentiation. The median tumor size was 3.5cm (1.6-

6.2), half of the cases (50%, 5/10) showed vascular invasion, and majority of the cases had single tumor (9/10, 90%).

2. Different YAP/TAZ expression among HCC tissue

Based on previous study of YAP/TAZ in HCC, I analyzed YAP/TAZ expression between HCC tissue and paired para-tumor liver tissue using immunohistochemistry (IHC). YAP/TAZ expression was observed in primary HCC tissue but not in paired para-tumor liver tissue (Fig. 1).

The expression of YAP/TAZ among 10 different patient-derived primary HCC tissue was observed using Western blot (Fig. 2). Patient No. #7, #8 showed the highest YAP/TAZ expression, whereas patient no. #1, #3, #6 showed the lowest YAP/TAZ expression. From these, I selected six patient-derived HCC cell lines which are representative of YAP/TAZ expression level as high, medium, and low. These six cell lines were classified into three subgroups according to YAP/TAZ expression level in HCC tissue in order to investigate drug response according to the degree of YAP/TAZ expression; High YAP/TAZ group (Patient No. #7, #8), Medium YAP/TAZ group (Patient No. #4, #5), and Low YAP/TAZ group (Patient No. #3, #6).

Immunohistochemical staining of YAP/TAZ of HCC tissue was conducted in order to compare YAP/TAZ expression among different HCC tissue. High YAP/TAZ expression was observed in high YAP/TAZ group, whereas medium and low YAP/TAZ expression was observed in medium and low YAP/TAZ group accordingly (Fig. 3). YAP/TAZ expression level assessed by western blot correlates with YAP/TAZ IHC staining in HCC tissue.

Table 1. Baseline characteristics of patients related to HCC

Patient No.	Age/sex	Etiology	Cirrhosis	Major differentiation	Worst Pathology	Tumor size (cm)	Number of tumor	Vascular invasion	AFP (ng/ml)	PIVKA-II (mAU/ml)
#1	65/M	non-viral	None	Well	Moderate	3.3	1	Present	2.5	42
#2	50/M	HBV	None	Moderate	Poor	3.2	1	not present	8.5	19
#3	71/M	non-viral	Cirrhosis	Moderate	Moderate	1.6	1	not present	4	31
#4	62/F	HBV	None	Moderate	Moderate	6.2	1	not present	2.2	73
#5	78/M	HCV	Cirrhosis	Moderate	Poor	3.7	1	Present	18.2	21
#6	53/M	HBV	Cirrhosis	Moderate	Poor	3.8	2	not present	7.4	49
#7	57/M	HBV	None	Moderate	Moderate	3.1	1	Present	1.9	77
#8	59/M	HBV	Cirrhosis	Poor	Poor	6	1	Present	31.3	445
#9	69/F	HBV	Cirrhosis	Moderate	Poor	4.4	1	Present	1.6	18
#10	68/M	non-viral	None	Moderate	Moderate	1.8	1	not present	5	71

*AFP : alpha feto protein; PIVKA-II : Protein induced by vitamin K absence or antagonistry-II

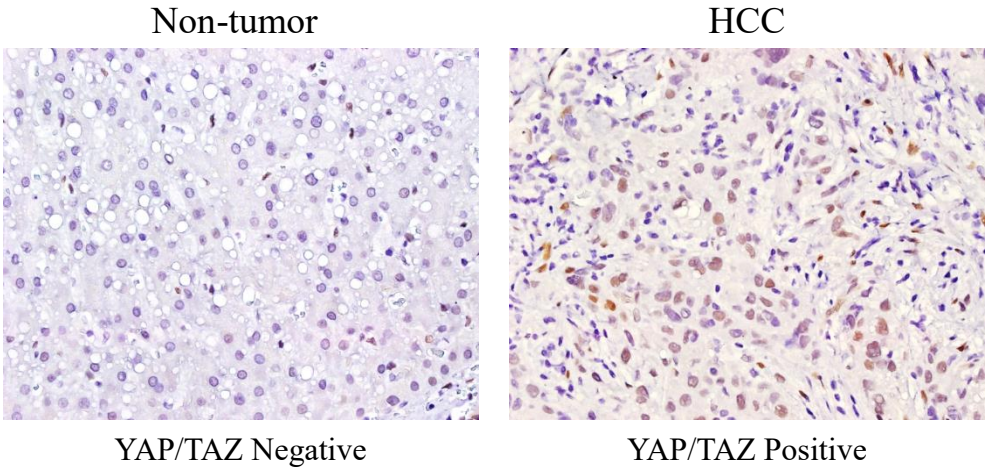

Figure 1. Comparison of YAP/TAZ expression between HCC and non-tumor using immunohistochemistry. Immunohistochemical expression of YAP/TAZ in tissues from HCC patients and adjacent nontumor tissues. Yellowish staining of nucleus demonstrates YAP/TAZ immunostaining, and this was shown positive in tumor tissue. Adjacent nontumor tissue did not show YAP/TAZ immunostaining. Representative images were taken under a microscope (X400).

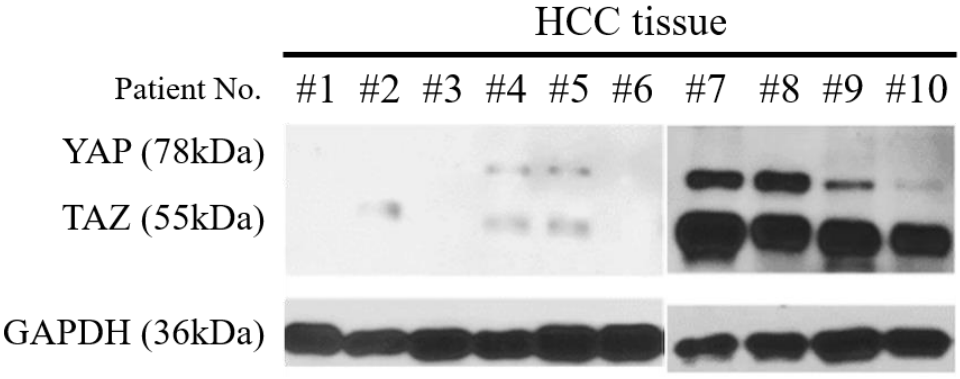

Figure 2. Western blot showing YAP/TAZ expression among HCC tissue. Lysates of HCC tissue were analyzed by western blotting with anti-YAP/TAZ and anti-GAPDH (control) antibodies.

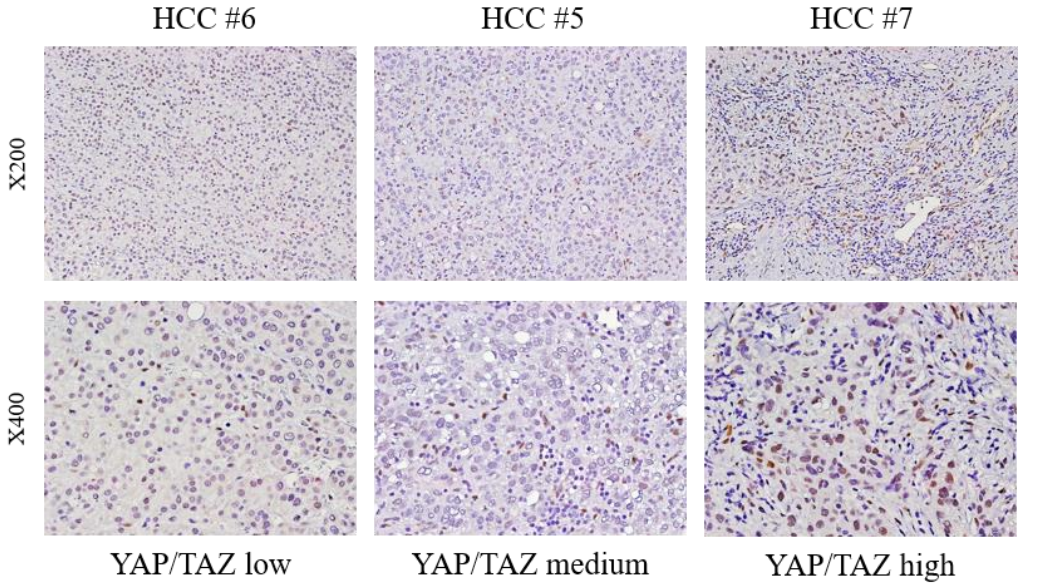

Figure 3. Comparison of YAP/TAZ expression among different primary HCC tissue. Immunohistochemical expression of YAP/TAZ in tissues from HCC patients. Yellowish staining of nucleus demonstrates positive immunostaining of YAP/TAZ. YAP/TAZ positivity was assessed according to the number of positively stained cells: score 0 (<5%), score 1 (6-25%), score 2 (26-50%), score 3 (51-75%), score 4(>75%). HCC #7 presented with YAP/TAZ score 3 (high), whereas HCC #5 and HCC #6 showed YAP/TAZ score 1 and score 0 respectively. Representative images were taken under a microscope (X200: first row; X400: second row).

3. Characterization of primary cultured HCC cells as original HCC

To define whether primary HCC cell lines maintained the original characteristics of HCC, I performed immunostaining with the hepatocyte-specific markers Hepatocyte Specific Antigen (Hep Par-1), alpha fetoprotein (AFP), and albumin (ALB) (Fig. 4). Hep par-1, AFP, and albumin were all expressed in all of the HCC cell lines and expression did not show much difference among all of the patient-derived cell lines.

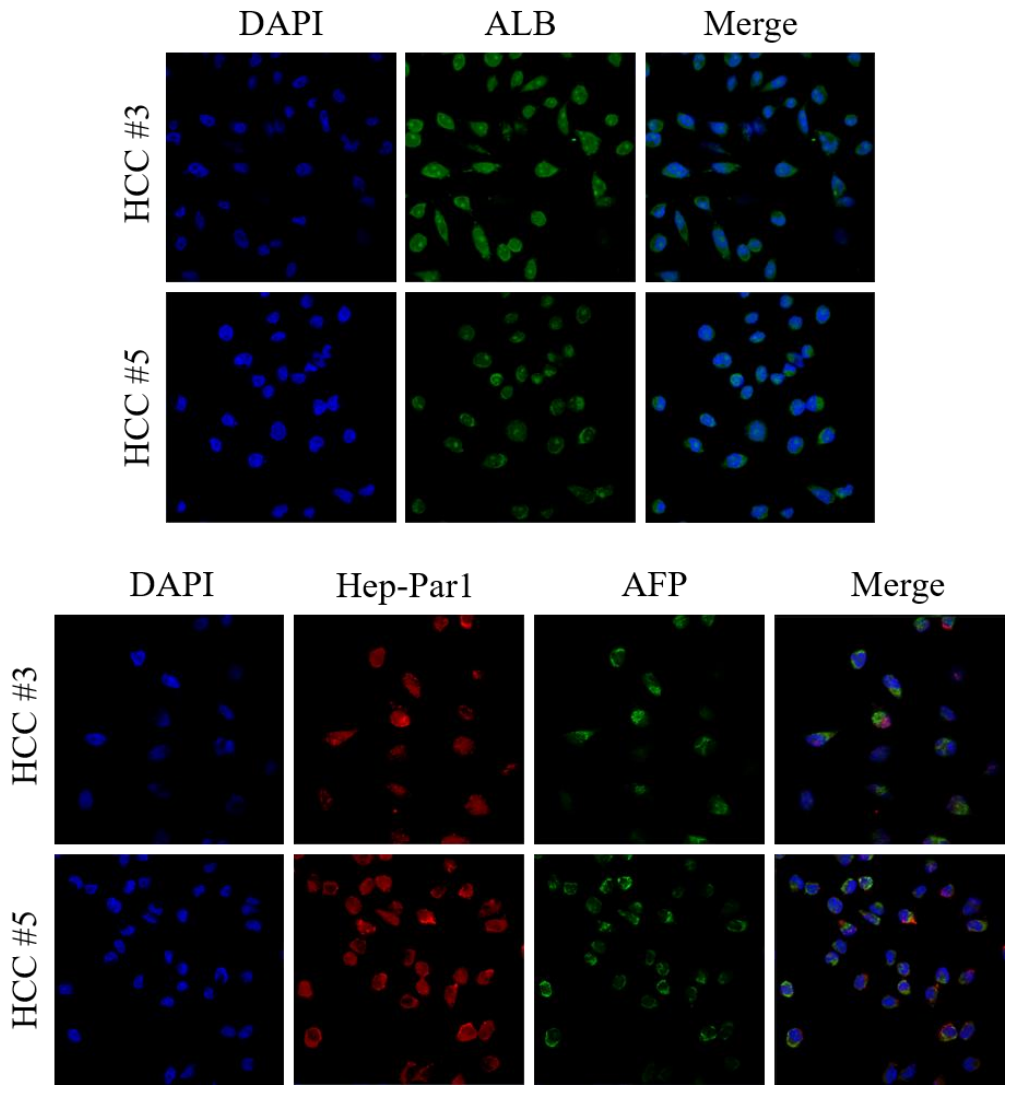

Figure 4. AFP, albumin, and Hep-Par1 immunocytochemical staining of primary HCC cells. AFP, albumin, and Hep Par-1 immunostaining of HCC cell lines were done to examine the cellular origin of primary HCC cells. Representative images were taken under confocal microscopy with a 200X objective.

4. Generation of MCTS model using patient derived HCC cell lines.

The origin of primary HCC cell lines obtained from patients was proven as HCC as mentioned previously. In order to study anti-cancer treatment using culture method which mimics characteristics of HCC *in vivo*, I generated multicellular tumor spheroid (3D). For stromal cells in MCTS, LX2 (human hepatic stellate cells, HSCs), WI38(human fibroblasts), and HUVECs (Human Umbilical Vein Endothelial Cells) were used.

MCTS paraffin-sections were stained with Hematoxylin & Eosin stain. (Figure 5A). Immunohistochemical analysis of MCTS paraffin-sections was performed in order to identify the origin of stromal cells used in MCTS (Figure 5B).

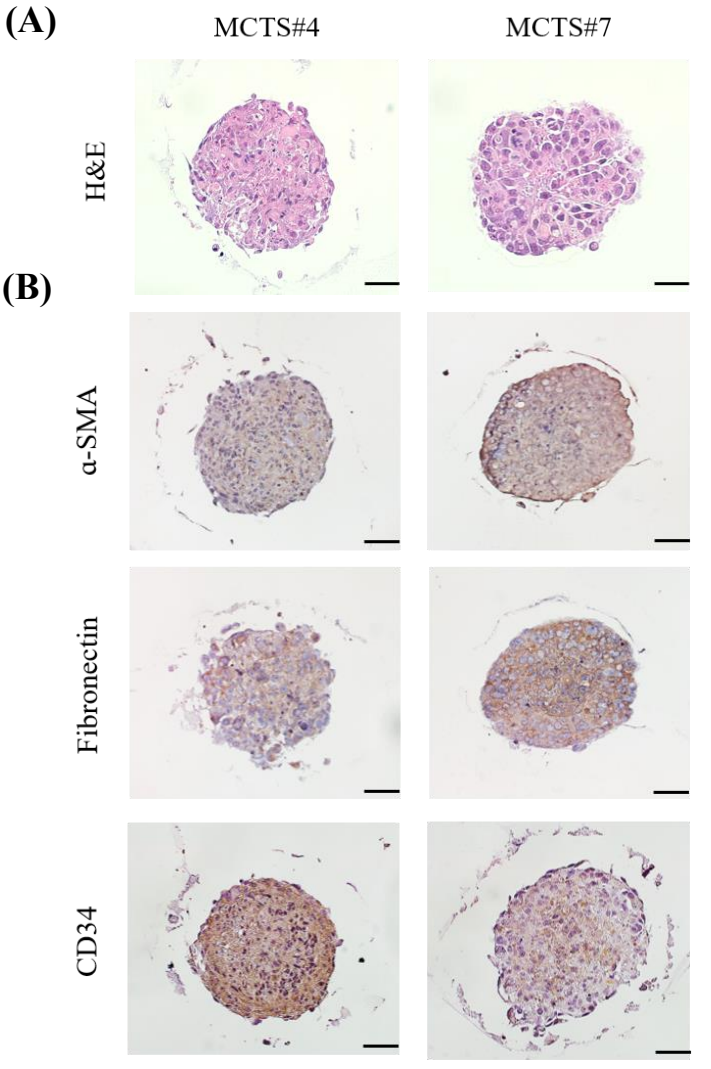

Figure 5. Establishment of MCTS model using patient-derived HCCs and stromal cells. (A) Hematoxylin & eosin staining of MCTS. **(B)** Immunohistochemical analysis of α-SMA **(**marker for hepatic stellate cells, LX2), fibronectin (marker for fibroblasts, WI38), and CD34 (marker for endothelial cells, HUVEC) of consecutive sections of the MCTS model generated from patient-derived primary HCC cells cocultured with human stromal cells (LX2, WI38, and HUVEC). Representative images taken under a microscope (X200). Scale bar, 50μm.

5. Comparison of YAP/TAZ expression between two different culture system: monolayer (2D) and MCTS (3D) using patient-derived primary HCC cells

In order to compare drug sensitivity according to different YAP/TAZ levels in HCCs, I selected six primary HCC cell lines with different YAP/TAZ levels as mentioned previously. I have generated MCTS using patient-derived HCC cell lines by co-culturing primary HCC cell lines and three types of stromal cells: LX2 (human hepatic stellate cells, HSCs), WI38 (human fibroblasts), and HUVECs (Human Umbilical Vein Endothelial Cells). Western blot was done to compare the differences of YAP/TAZ expression depending on the culture method; monolayer (2D) and MCTS (3D) (Fig. 6). Monolayer HCCs showed TAZ expression on western blot, whereas MCTS (3D) of patient-derived HCCs showed YAP expression which was also shown in HCC tissue.

EpCAM (epithelial cell adhesion molecule) expression was assessed using western blot in MCTS to look for stemness associated with high YAP/TAZ expression (Fig. 7). High YAP/TAZ expressed MCTS No. #7, #8 showed strong EpCAM expression whereas no expression was observed from MCTS with medium or low YAP/TAZ expression.

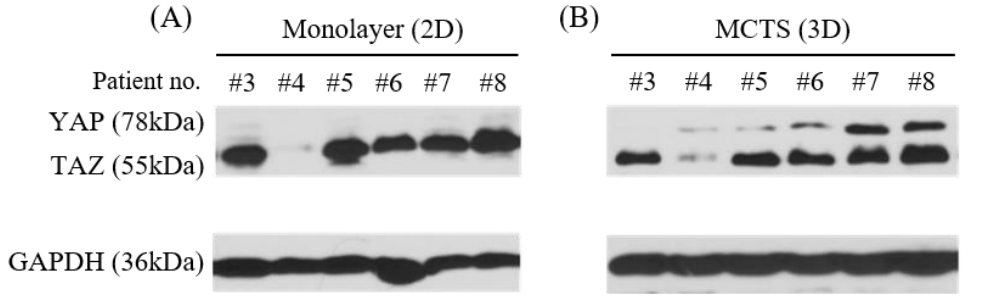

Figure 6. Comparison of YAP/TAZ expression using western blot between monolayer HCCs (2D) and MCTS (3D). (A) Different expression of YAP/TAZ were assessed by western blot analysis using monolayer patient-derived HCCs (2D). **(B)** Lysates of MCTS derived from patient-derived HCCs were analyzed by western blotting with anti-YAP/TAZ and anti-GAPDH (control) antibodies.

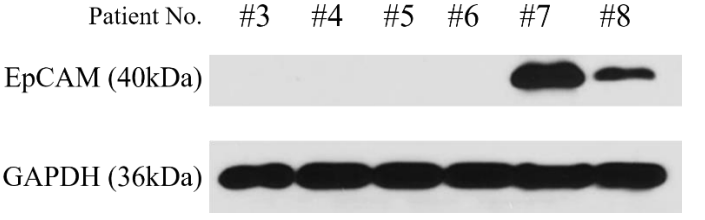

Figure 7. EpCAM expression using western blot in MCTS. Different expression of EpCAM was assessed by western blot analysis in MCTS

6. Comparison of sensitivity to anti-cancer drugs (Sorafenib, CA3, combined therapy) in MCTS

In this study, I sought to investigate the effect of YAP/TAZ inhibitor (CA3) when combined with sorafenib among different YAP/TAZ expressing patient-derived HCC using MCTS. I compared the half maximal inhibitory concentrations (IC_{50}) of sorafenib monotherapy, CA3 monotherapy, and combination therapy (sorafenib combined with CA3 0.1μM) in MCTS. I have classified MCTS into three subgroups according to different YAP/TAZ expression level according to western blot: YAP/TAZ high, YAP/TAZ medium, and YAP/TAZ low. Table 2. summarizes IC_{50} of anti-cancer drugs in MCTS and Figure 8 shows corresponding dose response curve. All MCTS showed sensitivity to CA3 monotherapy. With regard to combination therapy, YAP/TAZ high MCTS was more sensitive to combination of sorafenib and CA3 0.1μM than rest of the other MCTS (YAP/TAZ medium, YAP/TAZ low). YAP/TAZ medium MCTS and YAP/TAZ low MCTS did not show much difference between combination therapy (sorafenib and CA3 0.1μM) and sorafenib monotherapy.

Table 2. Half maximal inhibitory concentrations (IC_{50}) of anti-cancer drugs in patient-derived MCTS.

MCTS	CA3	Sorafenib+CA3 0.1μM	Sorafenib
YAP/TAZ high	0.45 μM	9.4 μM	16.4 μM
YAP/TAZ medium	0.46 μM	27.4 μM	28.7 μM
YAP/TAZ low	0.38 μM	16.7 μM	19.4 μM

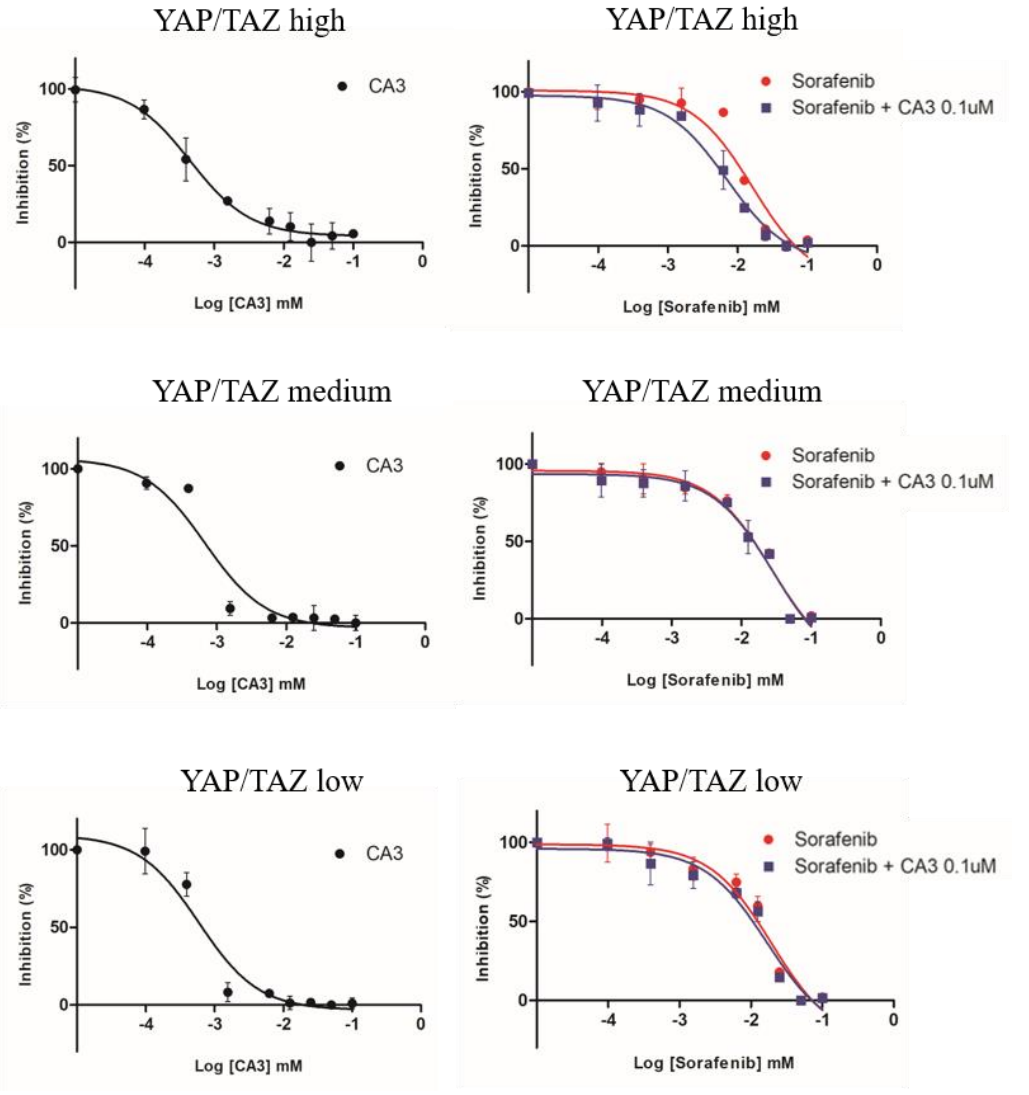

Figure 8. Comparative dose response curves using MCTS. MCTS were treated with CA3 at the indicated concentrations for 48hour. The dose response curve of CA3 monotherapy was presented as black full circles. MCTS were treated with sorafenib alone or sorafenib combined with CA3 0.1μM at the indicated concentrations for 48hour. Dose response curves of sorafenib (red full circles) and sorafenib combined with CA3 0.1μM (blue full squares) are presented.

Figure 9. shows the morphology of the generated patient-derived MCTS after 48hours of co-culture and interval change of the morphology of MCTS following drug treatment. Lysis of cells surrounding the surface border of MCTS was shown upon drug treatment. Morphologic changes between sorafenib monotherapy and combination treatment (Sorafenib + CA3 0.1 μM) did not show much difference among different YAP/TAZ level expressed MCTS.

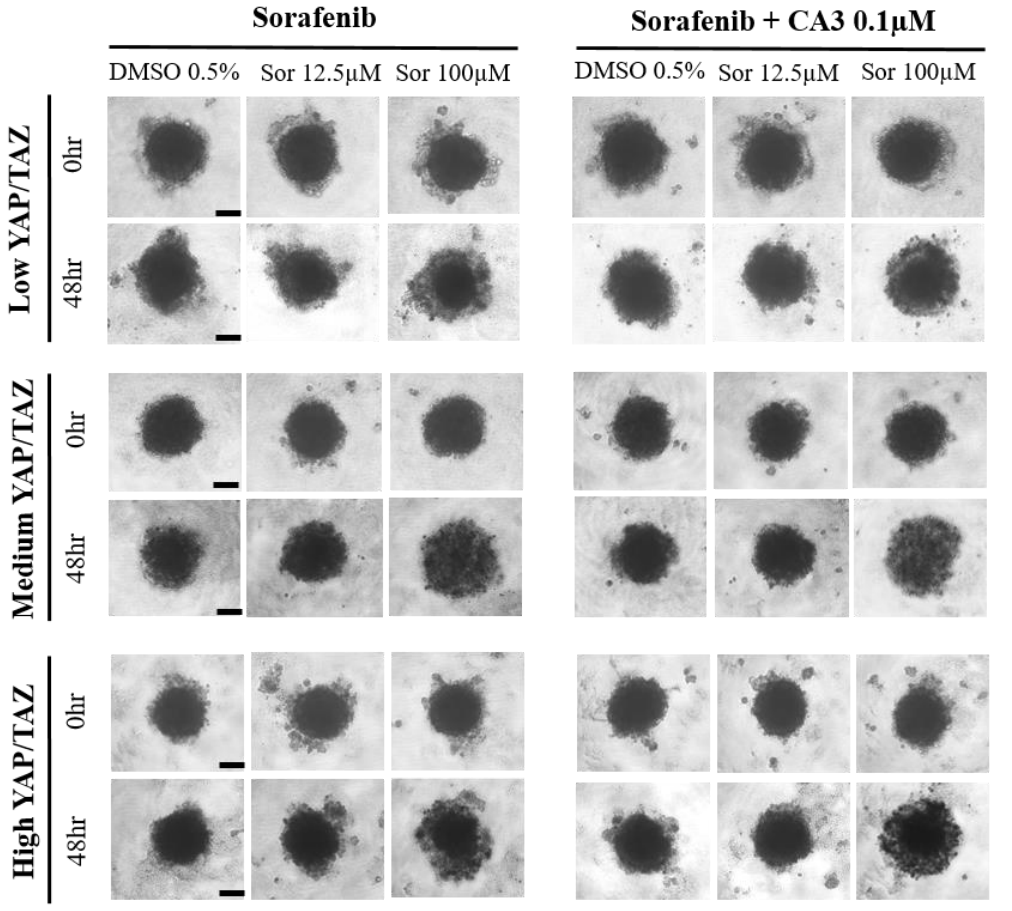

Figure 9. Morphology of generated patient-derived MCTS and serial morphologic changes upon drug treatment. Images of spheroids of MCTS were obtained using an inverted microscope (IX71; Olympus, Tokyo, Japan) with a 10X objective. Scale bar, 20μm.

IV. DISCUSSION

HCC takes up 90% of hepatic malignancy and is the second leading cause of cancer-related deaths worldwide. Treatment at an early stage includes surgical resection or ablative therapy but recurrence rate is high up to 70% within 5 years. Early recurred tumor has poor prognosis and is associated with the original primary tumor. First-line systemic therapeutic treatment is multi-tyrosine kinase inhibitor, sorafenib. Although several systemic therapeutic agents other than sorafenib has been introduced, therapeutic efficacy is still suboptimal. Therefore, novel therapeutic strategies are needed to improve survival in patients with HCC.

YAP/TAZ has emerged as therapeutic target in many types of cancer including HCC, as aberrant activation of YAP/TAZ has been implicated in cancer progression and development. Mounting evidence suggests that deregulation of Hippo signaling and activation of its co-activators YAP/TAZ leads to multi-drug resistance in many types of cancer, including HCC[21-23]. There have been attempts to overcome multi-drug resistance by combining with YAP/TAZ inhibitor in HCC. Song *et al* showed that BEL/FU HCC cell lines resistant to 5-Fluorouracil regain sensitivity when combined with Verteporfin (YAP inhibitor). A novel YAP inhibitor CA3 has shown potent inhibitory effects on YAP/TEAD transcriptional activity and synergistic inhibition when combined with 5-FU especially in high YAP and resistant cells in Esophageal Adenocarcinoma[24]. However, YAP/TAZ inhibition combined with sorafenib in HCCs with different YAP/TAZ expression has yet been studied. Given that sorafenib is the first-line systemic therapy in HCC in a later stage, it is imperative to investigate whether the sensitivity of sorafenib could be enhanced by YAP/TAZ inhibition depending on YAP/TAZ expression levels.

Besides YAP/TAZ signaling, tumor microenvironment (TME) plays an important role in cancer progression, cellular differentiation, and therapeutic efficacy[18,25,26]. Recent studies revealed that tumorigenesis relies on the interactions

between tumor cells and the surrounding stroma in HCC.[26-28]. MCTS model (3D) has emerged as a useful tool for anti-cancer research to implement tumor heterogeneity and complex TME *in vitro*. In this study, we generated MCTS using patient-derived HCC cells to mimic the characteristics of the HCCs *in vivo*. To better understand the effect of YAP/TAZ inhibition when combined with sorafenib depending on different YAP/TAZ expressed HCCs, we conducted drug response test of sorafenib and sorafenib combined with CA3 (YAP 1 inhibitor) using patient-derived MCTS with different YAP/TAZ levels.

Results of this study showing high YAP/TAZ expression in HCC tissue assessed by IHC staining correlate with previous study presented with YAP expression in HCC but not in normal liver tissue[29]. IHC staining of YAP/TAZ was localized in the nucleus of HCC tissue suggesting activated YAP/TAZ and suppressed Hippo signaling. Western blot analysis of YAP/TAZ expression in HCC tissue showed higher expression of TAZ than YAP expression and expression level does not correlate with the degree of differentiation which is similar to previous study[15].

YAP/TAZ expression levels from patient-derived HCC cell lines (2D culture) also showed higher expression of TAZ than YAP expression and this is confirmed by Hayashi *et al*[15]. Monolayer HCC cells did not express YAP expression which was shown in both HCC tissue and MCTS model. While only HCC cells are cultured in monolayer 2D culture system, HCC cells and stromal cells are co-cultured in MCTS model. Therefore, discrepancy in YAP expression according to culture methods may be attributable to the interaction between cancer cells and stromal cells. This stresses the importance of MCTS model which mimics actual tumor microenvironment leading to different protein expression pattern in YAP/TAZ. Song *et al* reported the role of hepatic stellate cells (HSCs) in MCTS as chemoresistance and migration, which exerts its effect by interaction between HSCs and HCC cells[30]. Cancer-associated fibroblasts (CAFs) are the major component of TME and YAP function is critical for the establishment and maintenance of CAFs. Growing body of evidence suggests that YAP-driven ECM

stiffening creates a positive feedback loop and sustains YAP activation [31-33]. This explains the increased expression of YAP levels in MCTS compared to original HCCs in monolayer (2D) with low YAP expression.

YAP is known to promote multi-drug resistance in HCC, and blockade of YAP using verteporfin conferred the sensitivity of chemoresistant HCC cell lines to chemotherapeutic agent[17]. This study chose novel YAP inhibitor, CA3, as this is a potent YAP inhibitor demonstrated in cancer stem cell (CSC)-enriched esophageal cancer[24]. My results presented that MCTS with high YAP/TAZ expression endowed sensitivity to sorafenib when CA3 was added. Interestingly, MCTS with low to medium YAP/TAZ expression did not show much difference to combination effect of CA3 despite its sensitivity to CA3 monotherapy.

Targeting YAP and TAZ is presumed to be safe, as deletion of YAP and TAZ did not present deleterious effect on liver homeostasis. Therefore, YAP inhibition using CA3 could be a feasible solution to treat HCC in real-practice. Expression level of YAP/TAZ in HCC in patients could be assessed using tissue microarray. Patients presenting with high YAP/TAZ expression could be selected for combined CA3 treatment with conventional sorafenib. Generation of patient-derived MCTS and drug treatment may provide informative results for drug application using CA3. This could possibly lead to anti-cancer therapy tailored to individual patient. Further studies are warranted for the correlation of YAP/TAZ expression level among different modalities: western blot analysis, tissue microarray.

This study demonstrated that CA3 was effective in reducing chemoresistance to sorafenib in MCTS which did not show a shift to predominant YAP expression upon TAZ depletion. This suggests that YAP inhibitor was effective when YAP and TAZ expression were well balanced. Therefore, CA3 might be a possible potential therapeutic agent to reduce chemoresistance of sorafenib in patients presenting with balanced YAP and TAZ expression in MCTS (3D).

V. CONCLUSION

In this study, different degrees of YAP/TAZ expressed MCTS models have been established using patient-derived HCC cell lines. MCTS with high YAP/TAZ expression showed increased response to sorafenib when combined with CA3, whereas MCTS with low or medium YAP/TAZ expression did not show any differences in drug sensitivity to sorafenib when combined with CA3.

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ABSTRACT(IN KOREAN)

간세포암에서의 YAP/TAZ 발현정도와 간세포암에 YAP/TAZ 저해가 미치는 효과

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한 소 정

간세포암은 전세계에서 두번째로 흔한 암 사망원인이다. 조기 간세포암의 치료는 국소적 치료나 수술적 간절제이며, 간절제에도 재발의 위험은 높으며 예후는 불량하다. Hippo signaling은 종양억제 신호기전으로 이 신호기전이 억제될 때 YAP과 TAZ 활성화를 통한 암발생에 기여한다고 알려져 있다. YAP/TAZ를 표적으로 암치료제에 대한 연구가 이루어지고 있으며, 효과가 있다고 알려져 있다. YAP 발현정도가 불량한 예후 및 약물저항성과도 연관이 있어, 환자들의 간암조직의 YAP/TAZ 발현 정도가 약물저항성에 영향을 미칠 것이란 가정하 YAP/TAZ 저해제인 CA3를 기존 간암치료제인 소라페닙과 병합하여 약물처리를 하여 YAP/TAZ 저해제가 YAP/TAZ발현정도에 따라 약물저항성 극복에 미치는 영향을 연구하였다.

수술적으로 절제하여 얻은 환자들의 간암조직을 이용하여 간암조직에서의 YAP/TAZ 단백질 발현정도를 확인하였다. 간암조직으로부터 간암세포를 분리하여 환자유래 간암세포와 간주위 기질세포(LX2, WI38, HUVEC)를 동시에 배양하여 실제 간암의 환경을 잘 구현하는 환자유래 multicellular tumor spheroid model (MCTS)를 구축하였다. 2D culture 간암세포와 MCTS(3D culture)에서 2D에서는

발현되지 않은 YAP이 MCTS(3D)에서는 발현되는 것을 확인하였다. 이는 2D에서의 YAP/TAZ발현이 실제 간암에서 발현되는 것과의 차이가 있음을 확인시키고 간암세포뿐 아니라 주위기질세포와의 상호작용이 실제 종양환경에 중요함을 말해준다. YAP/TAZ 발현정도를 YAP/TAZ 단백질 발현의 높고 낮음에 따라 고발현, 중간발현, 저발현군으로 구분하여 소라페닙 단독, CA3 단독요법의 약물반응을 확인하였고, 그 결과 CA3단독요법에는 YAP/TAZ 발현정도와 무관하게 매우 잘 반응하는 것을 확인하였다. YAP/TAZ 고발현군에서 소라페닙과 CA3 0.1 병합요법이 소라페닙 단독치료에 비해 약물반응성이 증가한데 반해 YAP/TAZ 저발현군이나 중간발현군에서는 차이가 없었다.

환자유래 간암세포를 이용하여 MCTS를 구축하였고, 이를 이용하여 CA3를 이용한 YAP/TAZ 저해가 YAP/TAZ 고발현 MCTS에서 소라페닙의 약물반응성을 증가시켰다.

핵심되는 말 : 간세포암, MCTS, YAP, TAZ, CA3