An Imbalance in TAZ and YAP Expression in Hepatocellular Carcinoma Confers Cancer Stem Cell-like Behaviors Contributing to Disease Progression

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Abstract

Transcriptional coactivator with PDZ-binding motif (TAZ) and yes-associated protein (YAP) are equivalently placed downstream effectors of the Hippo pathway with oncogenic roles in human cancers. However, the expression profiles of TAZ/YAP differ depending on the cancer cell type, suggesting that these proteins have different roles during cancer progression, yet no studies have examined the biologic significance of the balance between TAZ and YAP expression levels. Here we examined the functional roles of TAZ/YAP in hepatocellular carcinoma progression. We found that TAZ, but not YAP, was predominantly expressed in HCC. TAZ knockdown under normal conditions attenuated cell growth in HCC cells; however, TAZ knockdown combined with 5-fluorouracil treatment significantly increased chemoresistance compared with control cells. Notably, TAZ knockdown induced compensatory YAP expression and was accompanied by upregulation of CD90, a HCC-specific cancer stem cell marker. Continuous treatment with 5-fluorouracil also induced YAP expression and promoted tumor formation in vivo. Conversely, double knockdown of TAZ/YAP reduced chemoresistance and tumorigenicity. Moreover, YAP knockdown aggravated HCC cell growth to a greater degree than TAZ knockdown, and YAP overexpression was strongly associated with poor prognoses in patients with HCC. Collectively, these studies demonstrate that TAZ and YAP exhibit different functional roles in cancer progression, and a shift to predominant YAP expression upon TAZ depletion conferred cancer stem cell-like properties including chemoresistance and tumorigenicity in HCC. Therefore, targeting of both TAZ/YAP will be required for a complete antitumor response in HCC.

Introduction

Contact inhibition of proliferation is crucial for well-controlled organogenesis, and disruption of this process results in sustained cell proliferation, which is a hallmark of solid tumors. The Hippo pathway is a highly conserved cascade from yeast to man, and many studies have linked the regulation of organ size, cell contact inhibition, and stem cell function to the Hippo pathway (1–4). Indeed, defects in the Hippo pathway, such as double knockout of mammalian Ste20-like serine/threonine kinase (MST) 1/2 in mice, induce the hyperactivation of Hippo downstream effectors, such as transcriptional coactivator with PDZ-binding motif (TAZ) and yes-associated protein (YAP), and result in liver outgrowth and the development of hepatocellular carcinoma (HCC; refs. 5–7). This is consistent with results obtained in YAP overexpression transgenic mice (8). Several studies have demonstrated the overexpression of TAZ or YAP in human cancers, including HCC (9–17). Thus, accumulating evidence has suggested that TAZ/YAP have oncogenic roles in human cancers, supporting the potential applications of pharmacologic inhibitors of TAZ or YAP activity in anticancer therapy. However, although TAZ and YAP are equivalently placed as downstream effectors in the Hippo pathway, it is unclear whether they have similar or different functional roles in cancer progression.

In the current study, we found that the expression profiles of TAZ and YAP differed among various types of gastrointestinal cancer, suggesting that these proteins may have different functional roles during cancer progression. However, the relevance of the balance between TAZ and YAP expression and the biologic significance of this balance during cancer progression are unknown.

In this work, we aimed to investigate the functional roles of TAZ and YAP and their interaction during cancer progression in HCC, which typically develop following breakdown of the Hippo pathway.
Materials and Methods

Cell culture
Human HCC cancer cell lines (HepG2, HLE, HLF, Huh1, Huh7, PLC/PRF/5, and SK-Hep1), colon cancer cell lines (HCT116, HT29, and SW620), gastric cancer cell lines (AGS, Kato III, and NUGC4), and esophageal cancer cell lines (TE-1 and TE-11) were routinely maintained in medium supplemented with 10% FBS (HyClone Laboratories, Inc.). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂ and passed for less than 3 months after receptor resuscitation. The cell lines were tested and authenticated by means of GenePrint 10 System (Promega) in June 2015.

RNA extraction and quantitative reverse transcription PCR
Total RNA extraction, cDNA synthesis, and quantitative reverse transcription PCR (qRT-PCR) were carried out as previously described (18–20). Further details and the primers used in this study were summarized in Supplementary Materials and Methods.

Protein sample preparation and Western blot analysis
Protein extraction from cultivated cells or human liver tissue and subsequent Western blot analyses were carried out as previously described (18–20). Further details were summarized in Supplementary Materials and Methods.

Extraction of cytoplasmic and nuclear protein proteins
The cells were washed by PBS and lysed by hypotonic buffer on ice for 10 minutes. The lysate were centrifuged at 1,500 g for 5 minutes at 4°C, and the supernatant were collected and used as the cytoplasmic fraction. The cytoplasmic protein was extracted by hypotonic buffer [20 mmol/L HEPES (pH 8.0), 10 mmol/L KCl, 1 mmol/L MgCl₂, 0.1% Triton X-100, 20% glycerol]. The pellets were washed twice in hypotonic buffer, centrifuged at 5,000 g for 5 minutes at 4°C, and lysed by RIPA buffer on ice for 15 minutes. The lysate were centrifuged at 21,000 g for 10 minutes at 4°C, and the supernatant were collected and used as the nuclear fraction. All buffers contained protease phosphatase inhibitor cocktail (Thermo Scientific).

Immunohistochemistry and immunocytofluorescence
Sample processing and immunohistochemical procedures were carried out as described previously (18–21). Further details were summarized in Supplementary Materials and Methods.

Antibodies and reagents
The primary antibodies used in this study were summarized in Supplementary Materials and Methods. Fluorouracil (5-FU) was purchased from Kyowa Hakko. Hydrogen peroxidase (H₂O₂) was purchased from Wako. The negative control siRNA was Stealth RNAi Negative Control siRNA; Invitrogen), and the most effective siRNA was used in subsequent experiments (Supplementary Fig S1). The most effective sequences were: siTAZ (WWTR1), 5'-CCCAAGCAUUGAGGCCAUCAUCUAU-3', siYAP 5'-CGAGGAGGAAUGAAUGAA-3', and the complementary sequences of each oligo. The negative control siRNA was Stealth RNAi Negative Control Duplexes (Medium GC Duplex; Invitrogen).

TAZ and YAP overexpression with plasmid vector
The CDNA corresponding to human TAZ or YAP1 was introduced into the pIREspuro3 Vector (631619; Takara). TAZ or YAP was transiently overexpressed by transfection with the resulting plasmid vectors into PLC/PRF/5 or HuH1 cells, which exhibit low TAZ/YAP expressions, using Lipofectamine 3000 (Invitrogen). Cells subjected to mock transfection were used as a control.

Chromatin immunoprecipitation assay
To elucidate whether the correlations between TAZ and YAP are direct or indirect using SimpleChIP Plus Enzymatic Chromatin IP Kit (Magnetic Beads; #9005; Cell Signaling Technology) and TAZ antibody (V386; #4883; Cell Signaling Technology) for chromatin immunoprecipitation (ChIP) assay according to the manufacturer’s instructions, SK-Hep1 cells were subjected to immunoprecipitation assay with anti-Histone 3 antibody (positive control), rabbit anti-TAZ antibody, or normal rabbit IgG (negative control), and thereafter the immunoprecipitated chromatin were decross-linked. According to the manufacturer’s instructions, recruited DNA was subjected to real-time PCR reaction using ChIP primer sets, which were designed to include the ortholog, promotor, exon1, or intron1 for YAP1. Human RPL30 primer was used for positive control in real-time PCR reaction.

Normoxia and hypoxia in monocultures
SK-Hep1 and HLF cells were cultured in 6-well plates under normoxic conditions for 24 hours to allow attachment to the dishes and then switched to hypoxic conditions (1% O₂, 5% CO₂) for 48 hours. Total RNA or whole cell lysate from cell lines cultured under normoxia or hypoxia were extracted for semiquantitative RT-PCR and Western blot analyses. Cobalt chloride (CoCl₂), a chemical hypoxia-inducing agent purchased from Sigma, was also used to induce hypoxia in SK-Hep1 cells. SK-Hep1 cells were cultured in 6-well plates in a normoxic environment for 24 hours to allow attachment to the dish and were then treated with CoCl₂ (50–200 μmol/L) for 48 hours before collection and preparation of cell lysates.

Cell proliferation assay
Cell proliferation assays were carried out in 96-well plates, and living cells were counted at each time point using WST-8 assays with a Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer’s instructions. Absorbance was measured at 450 nm. HepG2, HLF, SK-Hep1, and SW620 cells were transfected using Lipofectamine RNAiMAX (Invitrogen). TAZ and/or YAP expression were transiently downregulated using a predesigned siRNA duplex directed against TAZ/YAP. A nontargeting siRNA was used as a negative control. Various doses of 5-FU (KYOWA) and curcumin (cat. no. 038-04921; Wako) were added to the cells 48 hours after seeding. The medium was changed daily. The final concentration of DMSO did not exceed 0.1% (v/v) in each experiment.
Animal studies
All animal studies were conducted according to the guidelines of the Animal Care and Use Committee of Kumamoto University (approval number D26-087). Six-week-old male nude mice (BALB/cSlc-nu/nu; n = 6 for each group) were subcutaneously inoculated in the right and left flanks with $1 \times 10^5$ to $1 \times 10^6$ SK-HeP1 cells 48 hours after transfection with siControl, siTAZ, siYAP, or siTAZ/YAP in 50 μL medium containing 50% Matrigel (BD Biosciences). Tumor formation was evaluated 8 weeks after inoculation. For in vivo analysis of the shift from TAZ expression to YAP expression, 6-week-old male nude mice (BALB/cSlc-nu/nu) were subcutaneously inoculated in the flanks with $2 \times 10^6$ SK-HeP1 cells in 50 μL medium. Mice were then treated with 5-FU (35 mg/kg/d) or control (saline) intraperitoneally for 7 days.

Patients and tissue samples
Paraffin-embedded sections and frozen tissues were obtained from patients with HCC who underwent hepatic resection at the Department of Gastroenterological Surgery, Kumamoto University Hospital from January 2004 to December 2008. The study was approved by the medical ethics committee of Kumamoto University (Project No. 770), and written informed consent was obtained from the human subjects.

Curcumin
To investigate whether curcumin affected TAZ/YAP expression and cell proliferation, HepG2, SK-HeP1, and SW620 cells were plated in 96-well plates at 5,000 to 10,000 cells per well and allowed to attach overnight. Cells were then treated with curcumin at concentrations ranging from 20 to 40 μmol/L under standard culture conditions.

Statistical analysis
Data are expressed as mean ± SD. Data analysis was performed with SPSS 12.0.1 for Windows (SPSS Inc.). Statistical analyses were performed using the χ² test or Student t tests. Overall survival was calculated using the Kaplan–Meier method and compared using the log-rank test. Differences with P values of 0.05 or less were considered significant.

Results
Expression profiles of TAZ and YAP in gastrointestinal cancers and predominant expression of TAZ as a prognostic factor in HCC
In HCC tissues, overexpressed TAZ/YAP proteins was detectable in 54% of 100 samples, with 9% exhibiting strong positive expression as measured by immunohistochemical staining (Fig. 1A). Positive expression of TAZ/YAP was significantly associated with high proliferative activity, as assessed by Ki67 staining (P < 0.01; Fig. 1B). Interestingly, gastrointestinal cancer cell lines exhibited different TAZ and YAP expression profiles (TAZ > YAP, TAZ = YAP, or TAZ < YAP; Fig. 1C). These findings suggested that certain characteristics of gastrointestinal cancer cells were dependent on the balance between TAZ and YAP expression. Furthermore, these data implied that TAZ and YAP may have different roles in cancer progression. In contrast to other gastrointestinal cancer cells, TAZ was expressed at higher levels than YAP in all seven HCC cell lines and human HCC samples (Fig. 1D), indicating that TAZ was a predominant profile while YAP was a minor protein in these cells under normal culture conditions.

To assess the clinical relevance of TAZ expression in HCC, we examined the expression levels of TAZ mRNA in HCC specimens by real-time PCR (characteristics of patients from which the samples were collected are shown in Supplementary Table S1). These analyses showed that high TAZ mRNA expression was significantly associated with large tumor size and shorter overall survival (Fig. 1E and Supplementary Table S2). These findings indicated the applicability of TAZ mRNA level as a prognostic factor in HCC and suggested that TAZ played a crucial role as a pro-growth factor in HCC progression. Interestingly, although YAP was expressed at low levels under normal conditions, high YAP mRNA expression was also significantly associated with shorter overall survival (Fig. 1E). However, YAP expression was not significantly associated with clinical factors such as tumor size, multiple tumor, and tumor marker expression (Supplementary Table S2).

TAZ knockdown attenuated cancer cell growth by inactivating the PI3K/Akt/mTOR pathway
We further assessed the biologic significance of TAZ expression in HCC cell lines by silencing of TAZ expression. In HepG2 cells, which exhibit high endogenous TAZ expression, TAZ knockdown using siRNA depleted TAZ protein expression, but not YAP protein expression, at 24 hours after transfection (Fig. 2A). To validate TAZ knockdown, we confirmed the decreased expression levels of cysteine-rich angiogenic inducer 61 (CYR61) and connective tissue growth factor (CTGF) mRNA, which are both well-known downstream targets of TAZ/YAP (Fig. 2A). In HCC cell lines (HILF and HepG2, which exhibit high endogenous TAZ expression), TAZ knockdown decreased cell growth (Fig. 2B), which was further confirmed by decreased c-myc and cyclin D1 mRNA expressions (Fig. 2C and Supplementary Fig. S2). In contrast, TAZ knockdown in HCC cell lines with low endogenous TAZ expression (HuH1 and HuH7) did not show such inhibitory effects on cell growth and cyclin D1 mRNA expression (Fig. 2B and Supplementary Fig. S2).

We next attempted to identify the intracellular signaling pathways regulated by TAZ. TAZ knockdown attenuated the phosphorylation of Akt and p70S6 but not that of Erk1/2 in HCC cells (Fig. 2D). Conversely, TAZ overexpression upregulated the phosphorylation of Akt and p70S6 in PLC/PRF/5 cell (Fig. 2E). These findings showed that TAZ promoted cancer cell growth by activating the PI3K/Akt/mTOR pathway, and suggested that TAZ may be a useful therapeutic target in HCC cells exhibiting with high TAZ expression.

Functional roles of TAZ and YAP in chemoresistance
While several previous studies have reported that YAP plays an important role in cancer chemoresistance (22, 23), the role of TAZ in chemoresistance remains unclear. In addition, the PI3K/Akt/mTOR pathway has been reported to play a crucial role in chemoresistance (24). 5-FU is a key drug used in chemotherapy in various cancers including HCC. Treatment with 5-FU reduced TAZ protein and phosphorylated p70S6 expressions in a concentration-dependent manner at 48 hours in SK-HeP1 cells (Fig. 3A). Next, we examined whether TAZ played a role in chemoresistance during HCC progression and whether 5-FU treatment plus TAZ knockdown could accelerate the anticancer effects in HCC. Unexpectedly, TAZ knockdown plus 5-FU treatment (500 μmol/L)
failed to enhance the anticancer effects of either treatment alone and instead resulted in increased chemoresistance compared with that of the control at 48 hours or more in SK-Hep1 cells (Fig. 3B). HLF cells with TAZ knockdown also exhibited enhanced chemoresistance for 5-FU treatment compared with that of the control (Fig. 3B). To explore the mechanism underlying the increased chemoresistance in TAZ knockdown HCC cells treated with 5-FU, Western
blot analysis was performed. Intriguingly, while TAZ protein expression was completely diminished by 5-FU plus TAZ knockdown at 48 hours, a shift to YAP expression was observed, which resulted in increased phosphorylation of Erk1/2 (Fig. 3C). Moreover, double knockdown of TAZ and YAP blocked the enhanced chemoresistance induced by TAZ knockdown under 5-FU treatment in HLF and Sk-Hep1 cells (Fig. 3D). Furthermore, YAP overexpression suppressed the expression of cleaved caspase-3 and cleaved PARP induced by treatment with 5-FU (500 μmol/L) and resulted in enhanced chemoresistance in HuH1 cells (Fig. 3E). These findings suggested that HCC cells led to a shift to YAP expression in response to downregulation of TAZ and promoted chemoresistance to anticancer drugs. In HepG2 cells, treatment with 5-FU reduced TAZ/YAP protein expressions in a concentration-dependent manner at 48 hours (Fig. 3F). However, the shift to YAP expression was not observed in HepG2 cells in response to TAZ knockdown and 5-FU treatment (Fig. 3G). In HepG2 cells, TAZ knockdown plus 5-FU treatment (500 μmol/L) successfully enhanced the anticancer effects of 5-FU treatment alone (Fig. 3G). These findings suggested that TAZ inhibition enhanced anticancer effects under certain conditions, without stimulating a shift to YAP expression in response to TAZ inhibition. However, in certain HCC cell lines, TAZ and YAP could coordinately promote chemoresistance by switching an expression profile dominated by YAP expression. Under such conditions, a monotherapy targeting TAZ during chemotherapy paradoxically caused chemoresistance by compensatory YAP upregulation. Thus, this shift to YAP expression played a major role in chemoresistance in HCC, whereas TAZ played a role in chemoresistance under certain conditions without a shift to YAP expression. Therefore, a multitarget strategy against TAZ and YAP is required for effective HCC treatment during chemotherapy.
A shift to increased YAP expression in response to TAZ downregulation could also be induced under normal culture conditions

We further investigated whether this switching mechanism between TAZ and YAP expressions occurs specifically under chemotherapy treatment. Interestingly, under normal conditions in the absence of chemotherapy, TAZ knockdown also led to compensatory YAP upregulation at a later phase (48 hours) in HLF and SK-Hep1 cells (Fig. 4A). Nuclear localization of YAP was increased by TAZ knockdown compared with that in the control (Fig. 4A), and resulted in increased phosphorylation of Erk1/2 (Fig. 4B). YAP overexpression also increased phosphorylation of Erk1/2 in HuH11 cells (Supplementary Fig. S3). Indeed, in HCC cell lines (SK-Hep1 and HLF) exhibiting a shift to YAP expression in response to TAZ downregulation, the antiproliferative effects of TAZ knockdown was modest (Fig. 4C). In contrast, YAP knockdown resulted in dramatic anticancer effects equivalent to those of double knockdown of TAZ/YAP, despite the low expression of YAP compared with that of TAZ (Fig. 4C). Interestingly, the compensatory YAP upregulation in response to TAZ knockdown also occurred in a normal fibroblast cell line (Hs68) and SW620 colon cancer cell line (Fig. 4D), suggesting that the switching between TAZ and YAP may be an adaptive process in normal cells and other types of cancer cells. In contrast to TAZ knockdown, YAP knockdown did not induce compensatory TAZ upregulation in HCC cell lines (HLF and SK-Hep1), and additionally attenuated TAZ expression (Fig. 4E). In other gastrointestinal cell lines (SW620 and TE-1 cells) exhibiting high YAP expression, YAP knockdown did not induce compensatory TAZ upregulation, and attenuated the phosphorylation of Erk1/2 at 48 hours (Supplementary Fig. S4). Thus, YAP could rescue the antiproliferative effects induced by TAZ downregulation, whereas TAZ could not compensate for the loss of YAP expression. Collectively, these findings suggested that YAP may be more critical and stand on upper level of hierarchy than TAZ does in HCC progression, despite the lower expression of YAP compared with that of TAZ under normal conditions. On the other hand, such a shift into YAP expression in response to TAZ knockdown was undetectable in HuH7 and HepG2 cells, and the antiproliferative effects by TAZ knockdown and YAP knockdown were additive, supporting that a multi-target strategy against TAZ and YAP was required for effective HCC treatment (Fig. 4F). To further analyze the interaction between TAZ and YAP, ChIP assay was performed (Fig. 4G). Then, the immunoprecipitated chromatin by antibody against TAZ revealed low YAP expression levels in similar level to the negative control (Rabbit IgG), suggesting the indirect interaction between TAZ and YAP.

A shift into YAP expression resulted in upregulation of cancer stem cell markers and promotion of tumorigenicity

Chemoresistance is one of the cancer stem cell–like features. In HCC cells with mesenchymal features (i.e., HLF and SK-Hep1 cells), in contrast to HCC cells with epithelial features (i.e., HuH7 and HepG2 cells; refs. 20, 25), a shift into YAP expression in response to TAZ inhibition resulted in upregulation of CD90, a HCC-specific cancer stem cell marker (26, 27), at 48 hours (Fig. 5A). In SK-Hep1 cells, CD13 mRNA expression was also significantly upregulated at 48 hours in response to TAZ inhibition (Fig. 5A). On the other hand, in HuH7 and HepG2 cells, which did not exhibit the ability to shift into YAP expression in response to TAZ inhibition, CD13 and CD90 mRNA expression levels were not changed (Fig. 5A). Other stem cell markers, such as EpCAM, CD133, and CD44, did not show significant changes in response to TAZ knockdown at 48 hours in HuH7, HepG2, HLF, and SK-Hep1 cells (Supplementary Fig. S5). High YAP mRNA expression in HCC tissue was significantly associated with high CD90 mRNA expression in the clinical samples (Fig. 5B). In vivo, TAZ knockdown conferred increased capacity for tumor formation compared with the control, whereas double knockdown of TAZ and YAP reduced tumor formation capacity (Fig. 5C). Thus, the shift from TAZ into YAP expression upregulated cancer stem cell markers, such as CD90 and CD13, and conferred cancer stem cell–like properties represented by tumorigenicity and chemoresistance in HCC.

Next, we investigated whether chemotherapy could induce a shift into YAP expression in HCC cell, which was shown to play an important role in chemoresistance. Continuous 5-FU treatment (500 μmol/L) for 96 hours decreased TAZ protein expression and induced a shift to YAP expression accompanied by elevated CD90 mRNA expression (Fig. 5D). Furthermore, in xenograft model, HCC cell–derived tumors in mice treated daily with 5-FU displayed high YAP expression compared with control mice treated with saline for 7 days (Fig. 5E). These findings provided evidence that cancer cells exhibited a shift from TAZ into YAP expression to escape and survive from cellular stress, such as chemotherapy. The tumor microenvironment is also a crucial factor responsible for maintaining or obtaining cancer stem cell–like properties, and hypoxic conditions have been reported to induce cancer.
Figure A: Immunoblot analysis of YAP and TAZ in HLF and SK-Hep1 cells treated with siRNA for 24 and 48 hours. The blots show the expression levels of YAP, TAZ, α-Tubulin, Histone H3, YAP (#4192), and DAPI in the cytoplasm and nucleus.

Figure B: Immunofluorescence microscopy images of SK-Hep1 cells stained with YAP, α-Tubulin, DAPI, andMerge images. The images show the localization of YAP and α-Tubulin in the cytoplasm and nucleus.

Figure C: Graphs showing the optimal density of SK-Hep1 cells treated with siRNA for 0, 24, 48, and 96 hours. The graphs compare the effects of siControl, siTAZ, siYAP, and siTAZ/siYAP on the cell density.

Figure D: Immunoblot analysis of YAP and TAZ in Hs68 fibroblasts and SW620 colon cancer cells treated with siRNA for 24 hours. The blots show the expression levels of YAP, TAZ, pYAP, and β-Actin.

Figure E: Immunoblot analysis of YAP and TAZ in HLF and SK-Hep1 cells treated with YAP siRNA for 24 hours. The blots show the expression levels of YAP, TAZ, pYAP, and β-Actin.

Figure F: Immunoblot analysis of YAP and TAZ in HuH7 and HepG2 cells treated with siRNA for 24 and 48 hours. The blots show the expression levels of YAP, TAZ, pYAP, and β-Actin.

Figure G: Graph showing the % of total input chromatin for Histone H3, TAZ, and Rabbit IgG. The graph compares the effects of YAP1 gene silencing on the chromatin distribution.
stemness (28). Thus, we examined whether hypoxic conditions could induce a shift into YAP expression and CD90 mRNA upregulation. Interestingly, when cultured under hypoxic conditions or following treatment with a chemical hypoxia inducer (CoCL2), cells exhibited YAP upregulation and enhanced nuclear localization, although TAZ expression was also upregulated (Fig. 5F). While CD90 mRNA level was increased in response to TAZ knockdown under normoxic or hypoxic culture conditions, CD90 was not affected by hypoxic conditions in both HLF and SK-Hep1 cells (Fig. 5G). CD13 mRNA level was also not affected by hypoxic conditions in SK-Hep1 cells (Fig. 5G). These findings suggested that a shift from TAZ into YAP expression, rather than TAZ/YAP coexpression, played a crucial role in the upregulation of cancer stem cell markers, such as CD90 and CD13, in HCC. In addition, hypoxic conditions alone were not sufficient to induce such a shift.

Curcumin suppressed TAZ/YAP expression and exerted anticancer effects

As shown above, TAZ and YAP coordinately promotes cell growth, chemoresistance, and tumorigenicity during cancer progression by switching the predominant coactivator (TAZ or YAP). These findings indicated that TAZ/YAP coinhibition may be a promising therapeutic strategy for the treatment of HCC. We then found that curcumin, a major component of turmeric, suppressed TAZ and YAP expressions and exerted anticancer effects in HCC cell lines (HepG2 and HLF, which exhibited high TAZ expression) and colon cancer cell line (SW620, which exhibited high YAP expression; Fig. 6A and B). Interestingly, 20 μmol/L curcumin suppressed YAP expression and induced compensatory upregulation of TAZ in SW620 colon cancer cell lines, but not in HCC cells, although cell growth was markedly suppressed in SW620 cells (Fig. 6A and B). This finding suggested that TAZ could be upregulated in response to YAP downregulation under selective conditions in SW620 cells, which exhibited high YAP expression, but the rescue effect on cell survival was less than that of YAP. Treatment with a high concentration of curcumin (40 μmol/L) abolished TAZ/YAP expressions and blocked cell growth (Fig. 6A and B). Curcumin also induced a shift into YAP expression in HLF cells, which was suppressed by treatment with a high concentration of curcumin (40 μmol/L). Furthermore, curcumin treatment could attenuate the shift into YAP expression in response to TAZ knockdown in HCC cells (Fig. 6C). Thus, curcumin may exert powerful anticancer effects through suppression of TAZ/YAP expressions in HCC.

Discussion

Although TAZ and YAP have often been described to equivalent factors downstream of the Hippo pathway, TAZ initially exhibited higher expression than YAP in HCC, and TAZ overexpression at the mRNA level was a prognostic factor predicting poor outcomes in the current study. The inhibition of TAZ could reduce cell growth by deactivating the PI3K/Akt/mTOR signaling pathway and could enhance anticancer effects induced by chemotherapy in select HCC cells. Thus, although TAZ appeared to be a therapeutic target in HCC, YAP could compensate for loss of TAZ expression and confer cancer stem–like properties (such as chemoresistance and tumorigenicity) with elevated expression of cancer stem cell markers, such as CD90 and CD13, in certain HCC cells. Thus, a shift from TAZ into YAP expression may play a crucial role in the acquisition of cancer stem–like properties to escape from cell death during cancer progression. Indeed, in microarray analyses using embryonic, neural, and hematopoietic stem cells, YAP has been reported to be enriched in all three stem cells (29). In the current study, continuous treatment with 5-FU induced a shift into YAP expression with elevated CD90 expression in HCC cells. Hashimoto and colleagues reported that cancer stem–like sphere cells displayed chemoresistance to 5-FU in HCC cell lines (i.e., SK-Hep1 and HLF cells; ref. 30). In terms of cancer stem cell markers in HCC, Yamashita and colleagues (25) reported that EpCAM–positive cells (i.e., HuH1 and HuH7 cells), which exhibit epithelial features, and CD90–positive cells (i.e., HLF and SK-Hep1 cells), which exhibit mesenchymal features, were independently and distinctively expressed in different cellular lineages. In addition, the existence of CD90–positive cells in HCC tissues has been shown to be associated with a high incidence of distant organ metastasis within 2 years after surgery. CD13 has also been reported to be a marker of tumor-initiating and potentially dormant cancer stem cells in HCC cell lines (26). In the current study, a shift into YAP expression upregulated CD90 expression in HCC cells with mesenchymal features (i.e., HLF and SK-Hep1 cells). In SK-Hep1 cell, CD13 was also induced in response to TAZ inhibition. In contrast to HCC cells with mesenchymal features (i.e., HLF and SK-Hep1 cells), the shift into YAP expression in response to TAZ inhibition was not detectable in HCC cells with...
epithelial features (i.e., HuH7 and HepG2 cells). Furthermore, in HCC cells with epithelial features, cancer stem cell markers, such as EpCAM, CD13, CD44, CD90, and CD133, did not show any changes. Collectively, our data supported that the shift into YAP expression was a key step conferring cancer stem–like properties, including chemoresistance and tumorigenicity, in certain HCC cells with mesenchymal features. The current study provides important insights into the complementary molecular dynamics between TAZ and YAP in cancer progression and into when and how cancer cells acquire cancer stem–like properties to escape cell death.

Intriguingly, YAP could be induced to compensate for decreased TAZ expression, whereas TAZ did not display such compensatory expression following silencing of YAP in HCC cells. Such a shift into YAP expression was thought to be the cause of the modest antiproliferative effects achieved by targeting TAZ/YAP expression in HCC cells.
TAZ alone because double knockdown of YAP and TAZ enhanced the anticancer effects of single knockdown in the current study. In HCC cells with the ability to shift into YAP expression in response to TAZ inhibition, YAP knockdown dramatically exerted anticancer effects equivalently to those in double knockdown of TAZ/YAP, despite the low expression of YAP relative to that of TAZ. These findings suggested that YAP was more critical and stood on upper level of hierarchy than TAZ in HCC cancer progression. Indeed, in Tet-On inducible YAP transgenic mice under control of the apolipoprotein E promoter, YAP overexpression in hepatocytes drives cell proliferation and confers potent resistance to apoptosis, resulting in widespread development of HCC throughout the liver, even at only 3 months [8]. Hence, both TAZ and YAP are key therapeutic targets, despite the differences in their expression levels in HCC.

In the current study, we found that continuous 5-FU treatment could induce a shift into YAP expression with upregulated CD90 expression in HCC. However, one limitation of our current study was that it was unclear whether the tumor microenvironment (niche) induced such a shift into YAP-dominant expression under conditions without chemotherapy. As a clue, although hypoxic conditions could increase YAP and TAZ expressions, such conditions failed to induce CD90 upregulation. Thus, hypoxic conditions alone may be insufficient to induce a shift into YAP expression in HCC. Future studies are required to characterize the complicated tumor microenvironment that induces a shift into YAP-dominant profile in HCC.

In the current study, curcumin, a major component of turmeric and an old Indian spice, successfully suppressed TAZ/YAP expressions and exerted anticancer effects in HCC cell lines. Curcumin and its analogs have been previously shown to exhibit anticancer effects in HCC via a variety of biologic mechanisms in vitro and in vivo [31, 32]. The inhibition of TAZ and YAP may be one of the biologic anticancer mechanisms of curcumin in HCC. Indeed, in an epidemiological survey, the mortality rates in patients with HCC in India are very low (4%) and less than those in the United States or Europe (33), despite the relatively high carrier rate (3%) of hepatitis B virus in India, accounting for nearly 10% of hepatitis B virus carriers worldwide (34). Further studies (i.e., epidemiological surveys or randomized control trials) are needed to elucidate the biological and clinical roles of curcumin in HCC.

Collectively, TAZ and YAP coordinate in cancer progression, affecting tumor growth and cancer stem–like properties (chemoresistance and tumorigenicity). HCC exhibited a TAZ-dominant expression profile rather than a YAP-dominant expression profile under normal conditions, and a switch into YAP-dominant expression may be a key step to behave a cancer stem–like property in HCC. Therefore, a multitarget strategy against TAZ and YAP is required for effective HCC treatment. Curcumin may be an effective anticancer agent, as it can suppress TAZ/YAP expressions and a shift into YAP expression in HCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: H. Hayashi, T. Higashi, T. Beppu
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