Activated TAZ Induces Liver Cancer in Collaboration with EGFR/HER2 Signaling Pathways

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Research Article

Keywords: Hepatocellular Carcinoma, Cholangiocarcinoma, TAZ, Hydrodynamic Transfection, EGFR/HER2

Posted Date: August 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-796723/v1>

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Version of Record: A version of this preprint was published at BMC Cancer on April 19th, 2022. See the published version at <https://doi.org/10.1186/s12885-022-09516-1>.

Abstract

Liver cancer is a major global health concern due to the steady increases in its incidence and mortality. Transcription factors, yes-associated protein (YAP) and WW domain-containing transcription regulator protein 1 (WWTR1, also known as TAZ) have emerged as critical regulators in human hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC), the two major types of primary liver cancer. However, our study as well as other previous reports have shown that activation of YAP and TAZ (YAP/TAZ) in adult murine livers is insufficient for the development of liver cancer, suggesting a requirement for an additional oncogenic collaborator for liver carcinogenesis in adulthood. Therefore, we sought to identify the oncogenic partners of YAP/TAZ that promote hepatocarcinogenesis in adults. Through database analyses, we identified EGFR/HER2 signaling to be essential in human cancers with high TAZ activity. Furthermore, immunohistochemical analyses showed that human HCC and CC tissues with high YAP/TAZ activities exhibited concomitant activation of EGFR/HER2 downstream signaling pathways. To demonstrate that EGFR/HER2 signaling promotes YAP/TAZ-mediated hepatocarcinogenesis, a constitutively active form of TAZ was simultaneously expressed in murine adult livers with effector molecules downstream of EGFR/HER2 signaling pathways. Interestingly, activated TAZ and BRAF induced HCC, whereas activated TAZ and PI3K led to the development of CC-like cancer, demonstrating that TAZ collaborates with EGFR/HER2 signaling pathways to induce both HCC and CC.

Introduction

Liver cancer is the fourth most common cancer worldwide, and its incidence and mortality rates have been increasing steadily (Jung et al. 2011; Villanueva 2019). Liver cancer typically exhibits heterogeneous histological features and poor prognosis (D. Y. Kim & Han 2018; Llovet et al. 2016). The most common type of primary liver cancer is hepatocellular carcinoma (HCC), which accounts for about 80% of the cases, followed by cholangiocarcinoma (CC), which contributes to 10–20% of primary liver cancers (Hyder et al. 2014; Villanueva 2019). Although the two types of liver cancers have fundamentally different molecular and clinical characteristics, they share overlapping risk factors and oncogenic signaling pathways. Moreover, adult hepatocytes have been identified as the origin of both HCC and CC in recent studies (Y. Chen et al. 2012; Sia et al. 2017; Tanimizu et al. 2013).

The Hippo signaling pathway consists of a kinase cascade that regulates a variety of cellular processes (K. Harvey & Tapon 2007; K. F. Harvey et al. 2013; Pan 2007). The core kinases include the mammalian STE20-like protein kinase 1 and 2 (MST1 and MST2, also known as STK4 and STK3, respectively), that phosphorylate the large tumor suppressor 1 (LATS1) and 2 (LATS2). When Hippo signaling is activated, LATS1 and LATS2 (LATS1/2) phosphorylate yes-associated protein (YAP) and WW domain-containing transcription regulator protein 1 (WWTR1, also known as TAZ), thereby inhibiting the nuclear import of YAP and TAZ (YAP/TAZ). In contrast, when Hippo signaling is inactivated, dephosphorylated YAP/TAZ is transported into the nucleus resulting in the transcriptional activation of a plethora of genes involved in cell proliferation and survival through interaction with the TEA domain family members (TEADs) (Mo et al. 2014; Moroishi et al. 2015; Zancanato et al. 2016). Recent years have witnessed significant advances in

our understanding of the roles of YAP/TAZ in HCC and CC (Sugiura et al. 2019; Xu et al. 2009; Zender et al. 2006). Knockouts of the key mediators of the Hippo signaling pathway in embryos developed HCC and CC in adult mice (Hong et al. 2019; Lu et al. 2010; Song et al. 2010; N. Zhang et al. 2010). Likewise, prolonged overexpression of YAP/TAZ from neonatal stages induced HCC later in adulthood (Dong et al. 2007; Liu-Chittenden et al. 2012). In contrast to the prolonged activation of YAP/TAZ from embryonic stages or starting at birth, activation of YAP in the adult liver generally failed to induce cancer, but caused hepatomegaly or preneoplastic lesions (Dong et al. 2007; Yimlamai et al. 2014). This suggests that activation of YAP or TAZ alone, is insufficient to induce cancer in adult livers, and requires additional oncogenic partners. Liver cancers often show dysregulation of multiple signaling pathways such as RAS-RAF-MEK-ERK, PI3K-AKT-mTOR, Wnt/ β -catenin, and SHH signaling pathways (C. Chen & Wang 2015; Yim & Lee 2019). Concurrent alterations in multiple signaling pathways in liver cancer suggests that oncogenic collaborations may be required to initiate or promote tumor development in the liver. In this study, we used database analyses and a murine liver-specific transgenic model to identify the oncogenic partners of TAZ that promote tumorigenesis in the liver.

Materials And Methods

Genomic data analyses from publicly available databases

Data analysis of the transcriptome of patients with liver cancer was performed using the following publicly available databases: National center for biotechnology information (NCBI) gene expression omnibus (GEO) database (Accession Nos. GSE36376, GSE26566, GSE64041, GSE32958, GSE54236, and GSE32225) and the cancer genome atlas (TCGA) projects TCGA-LIHC (liver hepatocellular carcinoma) and TCGA-CHOL (cholangiocarcinoma).

Statistical analysis

Statistical analyses were carried out with two-tailed unpaired *t*-tests using GraphPad Prism Software (GraphPad, La Jolla, CA, USA). All values are expressed as means. Significant differences between two groups are denoted by asterisks (***, $P < 0.001$).

Gsea

Gene sets were downloaded from the molecular signatures database (MSigDB) (<http://software.broadinstitute.org/gsea/>) of the Broad Institute (Cambridge, MA, USA). Using the gene set permutation, the signal-to-noise ratio of the genes was used to determine the statistical enrichment of the gene sets associated with YAP/TAZ, MAPK, and AKT signaling pathways.

Animal experiments

Wild-type (C57BL6/N) male mice were purchased from Orientbio (Seongnam, Korea), maintained in a specific pathogen free (SPF) facility under a 12 h light/dark cycle, and provided food and water ad libitum. All experiments using mice were approved by the Institutional Animal Policy and Welfare Committee. Mice were randomly assigned to a hydrodynamic tail vein injection. For hydrodynamic tail vein injection, DNA mixtures containing transposons and transposase-encoding plasmids were prepared using the Endo-Free Maxi Kit (Qiagen, Hilden, Germany). The DNA plasmids were diluted in lactated Ringer's solution, and then injected into the lateral tail veins of male mice (5–6 weeks old; 0.1 mL/g body weight) within 7 s, as previously described (Ju et al. 2013).

Recombinant DNA

Transposons encoding the constitutively activated forms of YAP (pT3/EF5a YAPS127A) and TAZ (pT3/EF5a TAZS89A) were kind gifts from Dr. Xin Chen at the University of California, San Francisco. The plasmids pT2/EGFP and pPGK-SB13 were described previously (Moon et al. 2017). Open reading frames (ORFs) encoding the activated forms of BRAF (BRAF^{V600E}) and PIK3CA (PI3K^{E545K}) were polymerase chain reaction (PCR)-amplified from pBabe-B-RAF-V600E (#17544; Addgene, Watertown, MA, USA) and pBabe-puro-HA-PIK3CA-E545K plasmids (#12525; Addgene), respectively. These amplified PCR products were cloned into pT2/BH transposon vectors (#26556; Addgene) to generate the plasmids, pT2/BRAF^{V600E}, and pT2/PI3K^{E545K}, respectively.

CCL sensitivity analysis for compounds

CTRP (v2) (<http://www.broadinstitute.org/ctrp.v2.2>) was used to investigate the correlation between sensitivity to chemicals and the copy number of TAZ in the CCLs. The CTRP v2 contains 860 CCLs, of which 827 are characterized as part of the Cancer Cell Line Encyclopedia, and represent 25 different lineages. The data included a 72 h, 16-point dose-response screen to assess the sensitivity of these cell lines to a total of 481 compounds, including 70 FDA-approved drugs, 100 candidate compounds, and 311 small molecules (Rees et al. 2016; Seashore-Ludlow et al. 2015).

Histology and IHC

Liver tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. The paraffin sections were deparaffinized in xylene and rehydrated by passing through gradually decreasing strengths of ethanol. The sections were then stained with hematoxylin and eosin (H&E) and standard IHC for histopathological analysis. IHC staining for YAP/TAZ, phospho-MEK, phospho-AKT, and CK19 was conducted using antibodies against anti-YAP/TAZ (1:100; #8418; Cell Signaling Technology, Danvers, MA, USA), anti-MEK1/2 (phospho-Ser217/221; 1:200; #9154; Cell Signaling Technology), anti-AKT1 (Phospho-Ser473; 1:400; ab81283; Abcam, Cambridge, UK), and anti-cytokeratin 19 (1:1000; ab133496; Abcam). After incubation with the primary antibodies, the sections were incubated with the appropriate biotinylated secondary antibodies, followed by treatment with freshly prepared DAB substrates (Vector Laboratories, Burlingame, CA, USA).

Protein extraction and western blotting

Frozen mouse liver samples were homogenized and digested in 1× RIPA lysis buffer containing complete protease and phosphatase inhibitor cocktails (P3200; GenDEPOT, Barker, TX, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were immunoblotted with antibodies against phospho-AKT (#4060; Cell Signaling Technology), phospho-MEK1/2 (#9154; Cell Signaling Technology), NFκB p65 (sc-372; Santa Cruz Biotechnology, Dallas, TX, USA), Notch1 (ab8925; Abcam), and GAPDH (#2118; Cell Signaling Technology). Finally, the immunoreactive proteins were detected using the West-Q Pico Dura ECL Solution (W3653; GenDEPOT, Barker, TX, USA).

Human liver tissue specimens

Human primary HCC and CC tissues were obtained from the biobank at the Severance Hospital, Seoul, Korea. Tissues were collected immediately following surgery and stored at -80°C until processing and use. This study was approved by the Independent Institutional Review Board of Severance Hospital (IRB number: 4-2018-1087) and conformed to the ethical guidelines of the Declaration of Helsinki (1975).

Results

Activation of YAP/TAZ signaling in human liver cancer

First, we investigated whether YAP/TAZ activity was elevated in human liver cancer using publicly available databases. Expression levels of YAP and TAZ were significantly higher both in HCC and CC when compared to that in matching non-tumor hepatic tissues ($P < 0.001$, Fig. 1A and B). Further, gene set enrichment analysis (GSEA) showed that YAP-conserved signature gene sets were highly enriched in both HCC and CC (Fig. 1C and Supplementary Table 1), confirming that YAP/TAZ activity is significantly upregulated in both types of human liver cancers.

TAZ induces proliferation and de-differentiation of hepatocytes

Given that YAP/TAZ activity was significantly upregulated in liver cancers, we tested whether YAP or TAZ was capable of inducing liver cancer in mice using a liver-specific transgenic approach (Ju et al. 2013). Transposons expressing a constitutively active form of human YAP (YAP^{S127A}) or TAZ (TAZ^{S89A}) were hydrodynamically delivered to the liver together with plasmids encoding the *Sleeping Beauty* transposase (Fig. 2A). Expression of either YAP^{S127A} or TAZ^{S89A} in the liver did not induce visible tumors when examined at 16 weeks following the injection (Fig. 2B). Notably, microscopic examination of liver tissues from TAZ^{S89A} mice revealed small hyperplastic lesions, which are much less frequently found in livers from mice expressing YAP^{S127A} (Fig. 2C). Cells in the preneoplastic lesions expressing TAZ^{S89A} stained positive for CK19, a molecular marker for liver progenitor cells (Fig. 2C). As TAZ induced proliferation and presumably de-differentiation of hepatic cells more efficiently than YAP, we focused on TAZ for the rest of the study.

EGFR/HER2 signaling as a putative oncogenic collaborator of TAZ in liver

Because TAZ^{S89A} alone was insufficient to induce liver cancer *in vivo*, we speculated that an oncogenic collaborator is required for TAZ-mediated liver carcinogenesis in adult livers. To identify signaling pathways that are closely associated with TAZ in human carcinogenesis, we took advantage of the sensitivity analyses of compounds in human cancer cell lines (CCLs) available online through the Broad Institute (<http://www.broadinstitute.org>). We conducted a correlation-based analysis of 860 human CCLs with 481 compounds through the cancer therapeutic response portal (CTRP v2). The analysis identified lapatinib, erlotinib, and afatinib as the most lethal compounds for cell lines with a high copy number of the TAZ gene (Fig. 3A). All three chemical compounds are inhibitors of receptor tyrosine kinases (RTKs), mainly targeting EGFR and HER2. As the data showed that cancer cells with a high copy number of the TAZ gene were sensitive to EGFR/HER2 blockade, we speculated that the EGFR/HER2 signaling pathways may be required for the survival of cancer cells with high TAZ activity, or possibly that activation of EGFR/HER2 signaling may be required for TAZ-mediated carcinogenesis.

To confirm the association of EGFR/HER2 signaling with TAZ, we performed both database and immunohistochemistry (IHC) analyses. Analysis of the gene expression omnibus (GEO) database revealed that human HCC and CC with high expression levels of TAZ showed enrichment of the RAS-RAF-MEK-ERK and/or PI3K-AKT-mTOR signaling pathways, both of which are downstream signaling cascades of EGFR/HER2 (Fig. 3B and Supplementary Table 2). Furthermore, IHC analysis of human HCC and CC with a high YAP/TAZ activity showed a concomitant activation of the RAS-RAF-MEK-ERK and/or PI3K-AKT-mTOR signaling pathways (Fig. 3C and Table 1). About 87% of human liver cancer with high YAP/TAZ activity (26 out of 30 cancer samples) showed concomitant activation of the downstream signaling pathways of EGFR/HER2 (Table 1). In contrast, 11% of liver cancer samples with minimal YAP/TAZ activity (one out of nine cancer samples) revealed activation of the EGFR/HER2 signaling pathways. Thus, both the database and IHC analyses indicated a strong association between TAZ and EGFR/HER2 downstream signaling pathways in human hepatocarcinogenesis.

Table 1
IHC positivity in human HCC and CC tissues

IHC results	HCC	CC
YAP/TAZ+	13/19 (68%)	17/20 (85%)
pMEK1/2 + in YAP/TAZ+	11/13 (85%)	12/17 (71%)
pAKT + in YAP/TAZ+	3/13 (23%)	15/17 (88%)
pMEK1/2- & pAKT- in YAP/TAZ+	2/13 (15%)	2/17 (12%)
YAP/TAZ-	6/19 (32%)	3/20 (15%)
pMEK1/2- & pAKT- in YAP/TAZ-	6/6 (100%)	2/3 (67%)

TAZ induces cancer through the cooperation with RAS and PI3K signaling pathways

Based on these data, we tested whether the EGFR/HER2 downstream signaling pathways (RAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways) collaborated with TAZ to induce cancer in adult livers. Transposons encoding a constitutively active form of BRAF (BRAF^{V600E}) were delivered to the liver together with those encoding TAZ^{S89A} via hydrodynamics-based *in vivo* transfection (Fig. 4A). Simultaneous expression of TAZ^{S89A} and BRAF^{V600E} in the liver induced liver cancer with an incidence rate of 100% at 6 weeks following the hydrodynamic injection (Fig. 4B). Likewise, co-expression of a constitutively active form of PIK3CA (PI3K^{E545K}) with active TAZ induced liver cancer in all the mice tested, confirming that the PI3K-AKT-mTOR signaling pathway also efficiently cooperates with TAZ to induce liver cancer. Microscopic examination of the liver cancers revealed that the TAZ^{S89A} plus BRAF^{V600E} tumors were HCC-like, while the TAZ^{S89A} plus PI3K^{E545K} tumors resembled CC (Fig. 4C). Western blotting of protein extracts from the tumor tissues revealed elevated expression levels of Notch1 and NF-κB in tumors induced by TAZ^{S89A} plus PI3K^{E545K} (Supplementary Fig. 1), compared to that in normal control livers or TAZ^{S89A} plus BRAF^{V600E}. As expected, both groups of liver cancers showed overexpression of CK19, a putative progenitor cell marker, likely due to TAZ-mediated de-differentiation of liver cells (Fig. 4C).

Discussion

YAP/TAZ signaling is involved in multiple processes during carcinogenesis, including promotion of cell proliferation, induction of tissue invasion of tumor cells, and maintenance of cancer stem cells (Zanconato et al. 2016). Several oncogenic signaling pathways have been reported to crosstalk with YAP/TAZ in carcinogenesis, including the Wnt/β-catenin and LKB1 signaling pathways (Azzolin et al. 2014; M. Kim & Jho 2014; Mohseni et al. 2014). Notably, the involvement of YAP in KRAS-mediated neoplastic progression in pancreatic ductal adenocarcinoma (PDAC), underscores the potential crosstalk between YAP/TAZ and RAS signaling pathways in PDAC (W. Zhang et al. 2014).

The RAS-RAF-MEK-ERK signaling pathway is activated via cell surface receptors such as EGFR, HER2, and platelet-derived growth factor receptor (PDGFR). Binding of ligands to these receptors leads to the activation of the cytoplasmic tyrosine kinases that phosphorylate tyrosine residues in the cytoplasmic tails of the receptors. This event recruits the Grb2/Shc/SOS adapter complex to the plasma membrane and subsequently converts membrane-tethered GDP-bound RAS to active GTP-bound RAS (Margolis & Skolnik 1994; Rajalingam et al. 2007). Activated RAS triggers the mitogen-activated protein kinase signaling cascade through the RAF-MEK-ERK axis. The ligand-bound receptor tyrosine kinases also trigger the PI3K-AKT-mTOR signaling axis via phosphorylation of tyrosine residues in the cytoplasmic tails and thus recruit PI3K to the phosphorylated tyrosine residues proximal to the plasma membrane (Lemmon & Schlessinger 2010; Porter & Vaillancourt 1998). The RAS-RAF-MEK-ERK and PI3K-AKT-mTOR signaling pathways are activated in approximately 50% of human liver cancers, implying their significant

roles in hepatocarcinogenesis (Dimri & Satyanarayana 2020; Javle et al. 2006; Llovet et al. 2015; Neuzillet et al. 2014; Schmitz et al. 2007).

In this study, IHC analysis showed that RAS-RAF-MEK-ERK signaling was activated in 85% of human HCC with high YAP/TAZ activity, whereas PI3K-AKT-mTOR signaling was active only in 23% of human HCC with high YAP/TAZ activity, suggesting that the RAS-RAF-MEK-ERK pathway is the major contributor to HCC development with YAP/TAZ activation (Table 1). In line with the IHC results, co-expression of BRAF^{V600E} and TAZ^{S89A} induced HCC in the liver (Fig. 4). Of note, activation of PI3K signaling (through the expression of PI3K^{E545K}) together with TAZ activation led to CC-like liver cancer, suggesting a role for PI3K-AKT-mTOR signaling in the development of cholangiocarcinoma. Tumors induced by TAZ^{S89A} plus PI3K^{E545K} showed elevated expression of Notch1, a critical player in CC (Banales et al. 2020; Rizvi & Gores 2013). Further studies are needed to verify the identity of the cancer. In summary, our study identified RAS-RAF-MEK-ERK and PI3K-AKT-mTOR signaling pathways as oncogenic collaborators of TAZ during liver carcinogenesis.

Declarations

AUTHOR CONTRIBUTION

SHM and SWR designed experiments, analyzed data, and wrote the manuscript. HM performed experiments. HJC contributed to data analyses. DYK and SWR supervised the research. All authors read and approved the final manuscript.

FUNDING

The research was supported by a grant from Kyung Hee University in 2020 (KHU-20201758 awarded to SWR), and also by the National Research Foundation of Korea (NRF) grants 2019R111A1A01055805 (awarded to HM), 2017R1C1B2007770 (awarded to DYK), and 2019R1A2C2009518 (awarded to SWR) which were funded by the Korea government (MSIT).

DATA AVAILABILITY

The data that support the findings of this study are available on request from the corresponding author.

CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experiments using mice were approved by the Animal Policy and Welfare Committee of the Yonsei University College of Medicine (Permit Numbers: 2013-0268 and 2013-0293). Human primary HCC and CC tissues were obtained from the biobank at the Severance Hospital, Seoul, Korea. This study was approved

by the Independent Institutional Review Board of Severance Hospital (IRB number: 4-2018-1087) and conformed to the ethical guidelines of the Declaration of Helsinki (1975).

CONSENT FOR PUBLICATION

Not applicable.

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Figures

Figure 1

Activation of YAP/TAZ signaling in human liver cancers.(A, B)Expression levels of YAP and TAZ (WWTR1) in tumor and non-tumor counterparts were compared using gene expression omnibus (GEO) database for HCC (A)and CC (B). Mean expression level of each group is indicated with horizontal lines. (***, $P < 0.001$). (C)Enrichment plots from gene set enrichment analysis (GSEA). YAP/TAZsignalsare highly enriched both in HCC and CC ($P < 0.001$). Barcode indicates gene positions and the y-axis indicates the extent of enrichment.

A **B** **C** **Figure 2**

YAP or TAZ alone does not induce tumors. (A) Schematic illustration of the experimental procedure. Transposons encoding each oncogene were mixed with plasmids expressing the Sleeping Beauty transposase and injected hydrodynamically (B) Gross morphology of representative liver expressing YAP S127 and TAZ S89A, respectively, are shown. Livers were harvested at 16 weeks following the hydrodynamic injection (n= 5). (C) Microscopic images showing H&E and IHC staining for

CK19 in sections of livers shown in (B). In sections of livers expressing YAPS127, ductal cells positively stained for CK19 are shown. Scale bar, 50 μ m.

Figure 3

EGFR/HER2 signaling pathways are associated with TAZ in human cancer.(A)Correlation between chemical sensitivity and copynumber ofTAZ gene. Y-axis indicates Z-scored Pearson correlations. Box plot represents Tukey outliers.(B)GSEA was performed withregard to MAPK and mTOR signaling

pathways using HCC (GSE54236) and CC (GSE32225) with high and low levels of TAZ expression. (C) IHC analyses of YAP/TAZ, pMEK, and pAKT in human HCC and CC. Scale bar, 100 μ m.

Figure 4

TAZS89A induces liver cancer in collaboration with either BRAFV600E or PI3KE545K. (A) Schematic illustration of the experimental procedure. (B) Gross morphology of representative livers expressing EGFP (control), BRAFV600E, and PI3KE545K, respectively, together with TAZS89A. Livers were harvested at 6

weeks following the hydrodynamic injection (n = 5 mice/group). (C) H&E and IHC staining of tumor sections from mice expressing EGFP, BRAFV600E, and PI3KE545K, respectively, together with TAZS89A. Scale bar, 100 μ m.

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