GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Oncogenic Determination of a Broad Spectrum of Phenotypes of Hepatocyte-Derived Mouse Liver Tumors

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Activation of the phosphoinositide 3-kinase—AKT, Yes-associated protein (YAP), and MYC pathways is involved in human liver cancers, including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). However, the nature of the interactions among these pathways has remained poorly understood. Herein, we demonstrate the coordination of these pathways during the formation of mouse liver tumors induced by hepatocyte-specific somatic integration of myristoylated AKT, mutant YAP, Myc, or their combinations. Although the introduction of YAP or Myc alone was inefficient in inducing tumors, these proteins accelerated tumorigenesis induced by AKT. The generated tumors demonstrated various histological features: low-grade HCC by AKT/Myc, CC by AKT/YAP, and high-grade HCC by AKT/Myc/YAP. CC induced by AKT/YAP was associated with activation of the Notch pathway. Interestingly, the combination of Myc and YAP generated tumors composed of hepatoblast/stem-like cells expressing mRNA for *Afp*, *Dlk1*, *Nanog*, and *Sox2* and occasionally forming immature ducts. Finally, immunohistochemical analysis revealed that human HCC and CC were predominantly associated with phosphorylation of S6 and glycogen synthase kinase-3 β , respectively, and >60% of CC cases were positive for both phosphorylated glycogen synthase kinase-3 β and YAP. Our study suggests that hepatocyte-derived tumors demonstrate a wide spectrum of tumor phenotypes, including HCC, CC, and hepatoblastoma-like, through the combinatory effects of the oncogenic pathways and that the state of the phosphoinositide 3-kinase—AKT pathway is a key determinant of differentiation. (*Am J Pathol* 2017, 187: 2711–2725; <https://doi.org/10.1016/j.ajpath.2017.07.022>)

As the third leading cause of cancer death in the world, primary liver cancer is refractory to various treatment modalities. This finding may be partly explained by its wide genetic variations, as reflected by diverse phenotypes and histological types.¹ Although hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (CC) are prototypical epithelial liver cancers, there are many intermediate variants [combined hepatocellular cholangiocarcinoma (cHCC-CC)] and tumors reminiscent of immature (fetal) liver [hepatoblastoma (HB)]. On morphological and immunohistochemical grounds, it has been hypothesized that HCC and CC are derived from hepatocytes and bile ducts/ductules (cholangiocytes), respectively. Some cHCC-CC subtypes and a fraction of HCCs have been regarded

as tumors of immature bipotential cells or liver stem/progenitor cells with hepatoblastic features, which have been postulated to present in the adult liver.^{2,3} However, the cells of origin and the mechanisms of phenotypic determination of primary liver cancer have remained obscure.

Mouse hepatocarcinogenesis models using diethylnitrosamine or chronic liver injuries (eg, carbon tetrachloride and thioacetamide) to induce liver tumors apparently exhibit hepatocytic differentiation (hepatocellular adenoma or HCC)

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with reactivation of a variety of fetal/neonatal liver genes.⁴ Various transgenic models of HCC have been generated through liver-specific overexpression of oncogenes, such as *Myc*, *Tgfa*, *E2f1*, *Ccnd1*, and *HRAS*^{G12V}, or genes that encode viral proteins, such as *HbsAg*, *HBX*, and *SV40 T-Ag*.⁵ In addition, various types of HCC have been induced in mice lacking *Mdr2*, *Lkb1*, or *AOX* genes.⁶ However, *Myc* overexpression also leads to tumors with mixed hepatocytic and cholangiocytic phenotypes⁷ and cancer stem cell–like features.⁸ Furthermore, the liver-specific knockout of phosphatase of tensin homolog, which activates the phosphoinositide 3-kinase (PI3K)–AKT pathway, induces both HCC and CC.^{9,10} Similarly, the activation of the Yes-associated protein (YAP) pathway by knockout of upstream negative regulators induces HCC, CC, and cHCC-CC.^{11–13} Finally, the deregulated enhanced green fluorescent signaling induced by liver-specific deletion of *Nf2/Merlin* generates both HCC and CC.¹⁴

The broad phenotypic spectrum of primary epithelial liver cancers might be explained by the variable cells of origin: hepatocytes, cholangiocytes, hepatoblasts, and liver stem/progenitor cells. Recent lineage tracing experiments in mice have demonstrated that HCC is derived from mature hepatocytes rather than from liver stem/progenitor cells.¹⁵ The presence of CC of intrahepatic bile duct origin has been proved experimentally by bile duct–specific activation of the PI3K-AKT pathway and YAP¹⁶ or KRAS.¹⁷ However, given that both hepatocytes and intrahepatic bile ductal/ductular cells originate from hepatoblasts during development¹⁸ and that several lines of evidence have demonstrated that mature hepatocytes retain phenotypic plasticity to differentiate into cholangiocytes *in vitro* and *in vivo*,^{19–22} it is also possible that phenotypes of primary liver cancers might be determined by the combination of transdifferentiation and possibly dedifferentiation. This might be induced by genetic and cellular alterations occurring during tumorigenesis. Furthermore, it has been shown that CC can be induced from mature hepatocytes when the PI3K-AKT pathway is activated together with the Notch or YAP pathway.^{23,24} Thus, liver tumors derived from mature hepatocytes demonstrate a wide spectrum of phenotypes from HCC to CC, possibly encompassing cHCC-CC and HB.

In this study, we explored the interactions of the PI3K-AKT, YAP, and *Myc* pathways in hepatocytic tumorigenesis and phenotypic determination, using a combination of Sleeping Beauty (SB) transposon-mediated oncogene integration and hydrodynamic tail vein injection (HTVi) in mice. Herein, we demonstrate that the activation of the PI3K-AKT pathway alone in hepatocytes induces HCC with bile ductular transdifferentiation and that the simultaneous activation of the *Myc* or YAP pathway with the PI3K-AKT pathway promotes hepatocarcinogenesis with unidirectional differentiation, thus resulting in HCC and CC, respectively. Furthermore, unless the PI3K-AKT pathway is active, coactivation of the *Myc* and YAP pathways generates dedifferentiated liver tumors reminiscent of HB composed of hepatoblast-like cells with a high nuclear/cytoplasmic ratio.

Materials and Methods

Animals

C57BL/6J mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). The mice were euthanized under deep anesthesia, and the livers were removed for further examination. The protocols used for animal experimentation were approved by the Animal Research Committee, Asahikawa Medical University (Asahikawa, Japan). All animal experiments adhered to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*.²⁵

Plasmids

An SB13 transposase-expressing vector [pT2/C-Luc/PGKSB13; Addgene plasmid #20207 deposited by Dr. John Ohlfest (University of Minnesota)], a myristoylated AKT-hemagglutinin (HA)-expressing transposon cassette vector [pT3-EF1 α -myrAKT-HA; Addgene plasmid #31789 deposited by Dr. Xin Chen (University of California, San Francisco)], and a mutant YAP-expressing transposon cassette vector (pT3-EF1 α -FLAG-YAPS127A; Addgene plasmid #46049 deposited by Dr. Xin Chen) were obtained from Addgene (Cambridge, MA). cDNA fragments of Notch1 intracellular domain (N1ICD), Notch2 intracellular domain (N2ICD), and enhanced green fluorescent protein were amplified from p3xFlag-N1ICD [Addgene plasmid #20183 deposited by Dr. Raphael Kopan (University of Cincinnati), Addgene], p3xFlag-N2ICD [Addgene plasmid #20184 deposited by Dr. Raphael Kopan (University of Cincinnati), Addgene], and pCMV-enhanced green fluorescent protein (Takara Bio, Kusatsu, Japan), respectively. A full-length *Myc* fragment was amplified from the cDNA of diethylnitrosamine-induced mouse liver tumors. These cDNA fragments were cloned into the pT3-EF1 α plasmid with the Gateway system (ThermoFisher Scientific, Waltham, MA). LoxP sites were removed from pT3-EF1 α -myrAKT-HA and pT3-EF1 α -FLAG-YAPS127A using the GENEART Site-Directed Mutagenesis System (ThermoFisher Scientific) (pT3-myrAkt- $\Delta\Delta$ LoxP and pT3-YAPS127A- $\Delta\Delta$ LoxP, respectively). All plasmids were amplified and purified using the EndoFree Plasmid Maxi kit (Qiagen, Hilden, Germany).

Transposon-Mediated Introduction of Oncogenes into Mouse Hepatocytes

The combination of the SB transposon system and HTVi was used to introduce genes into hepatocytes *in vivo*. The SB transposon system can introduce any gene within the transposon cassette into the host genome in a cut-and-paste manner in the presence of transposase activity, thus resulting in stable expression of exogenous genes. The transposon cassette plasmids were coinjected with an SB13

transposase-expression plasmid into male C57BL/6J mice (aged 8 to 12 weeks). For HTVi, plasmids were dissolved in 2.5 mL Ringer solution (Otsuka Pharmaceutical Co, Naruto, Japan) and rapidly injected via the lateral tail veins of mice within 8 seconds. The total amount of plasmid DNA was 16, 25, and 36 µg for mixtures of two, three, and four different plasmids (including the transposase-expressing vector), respectively. Equimolar amounts of each transposon cassette plasmid containing various genes were mixed, and the molar ratio of the transposase-expressing vector/each transposon cassette plasmid was 1:2.

To estimate copy numbers of the integrated oncogenes per genome, we extracted genomic DNA from tumor samples by DNeasy Blood & Tissue kit (Qiagen) and performed quantitative real-time PCR (qPCR) analyses using FastStart Universal SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany). The amounts of genomic DNA from tumor samples were normalized by estimation of the copy numbers of the endogenous *Notch2* gene. The crossing point values obtained in each tumor were interpolated on standard curves representing known copy numbers of each oncogene-containing plasmid versus their corresponding crossing point values. The sequences of the specific primers are listed in Table 1.

Quantitative RT-PCR

Total RNA was extracted from frozen liver tissues and tumors and subjected to quantitative RT-PCR analyses, which were performed using the $\Delta\Delta C_t$ method with FastStart Universal SYBR Green Master Mix. Each reaction was performed in duplicate, and the mRNA levels were normalized against *Gapdh*. The sequences of the specific primers are listed in Table 1.

Microscopic Examination and Immunohistochemistry

The livers were fixed in phosphate-buffered 4% paraformaldehyde for 24 hours at 4°C, and paraffin sections were prepared. Immunohistochemical staining was performed using an EnVision/HRP system (Dako, Carpinteria, CA) on deparaffinized sections treated with Target Retrieval Solution (Dako). The following antibodies were used: anti-Ki-67 (Nichirei, Tokyo, Japan), anti-phosphorylated AKT (pAKT; Ser473; 3787; Cell Signaling Technologies, Danvers, MA), anti-phosphorylated glycogen synthase kinase-3 (pGSK3) β (Ser9; 9336; Cell Signaling Technologies), anti-phosphorylated S6 (pS6; Ser235/236; 2211; Cell Signaling Technologies), anti-YAP (kindly provided by Dr. Hiroshi Nishina, Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan), anti-Myc (ab32072; Abcam, Cambridge, UK), anti-cytokeratin 19 (CK19; kindly provided by Dr. Atsushi Miyajima, Institute of Molecular and Cellular Biosciences, Tokyo University, Tokyo, Japan), anti-sex-determining region Y-box 9 (Sox9; Millipore, Billerica, MA), anti-grainy head-like 2 (Grhl2; Sigma, St. Louis, MO), anti-hepatocyte nuclear factor 4 α P1 (K9218; Perseus Proteomics, Tokyo, Japan), anti-major urinary protein (Nordic-MUBio, Susteren, the Netherlands), anti-HA (Roche Diagnostics), anti- α -fetoprotein (Proteintech, Chicago, IL), anti- δ -like protein (Medical and Biological Laboratories, Nagoya, Japan), anti-green fluorescent protein (A11122; ThermoFisher Scientific), anti- β -catenin (BD Biosciences, Franklin Lakes, NJ), and anti-vimentin (ab92547; Abcam). The chromogen 3,3'-diaminobenzidine tetrahydrochloride was used (Vector Laboratories, Burlingame, CA) in immunohistochemistry.

Table 1 Sequences of Primers for Real-Time Quantitative PCR

Gene	Forward primer	Reverse primer
<i>Afp</i>	5'-TGAAATTTGTCATGAGACGG-3'	5'-TGTCGTACTGAGCAGCCAAG-3'
<i>Cyr61</i>	5'-GAGACCCGGATCTGTGAAGTG-3'	5'-TGGGGATTTCTTGGTCTTGC-3'
<i>Ctgf</i>	5'-CTGCTGTGCATCCTCCTACC-3'	5'-CTGCAGTCTGGCCCATAG-3'
<i>Dlk1</i>	5'-ATGTCTGCAGGTGCCATGTTG-3'	5'-GGCTTGCACAGACACTCGAAG-3'
<i>Gapdh</i>	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'
<i>Hes1</i>	5'-TACCCAGCCAGTGTCAAC-3'	5'-CGCCTCTTCTCCATGATAGG-3'
<i>Hes2</i>	5'-TCATGGAGAAGAGGCGAAG-3'	5'-GCCTTCTCTAGCTTGGAATGC-3'
<i>Jag1</i>	5'-GACCAGAACGGCAACAAAAC-3'	5'-CCTGCAGTCACCTGGAAGTT-3'
<i>Nanog</i>	5'-AAAGGATGAAGTGCAAGCGG-3'	5'-CTTCCAGATGCGTTTACCAG-3'
<i>Notch1</i>	5'-CTGCTGTTGTGCTCCTGAAG-3'	5'-CAGTCTCATAGCTGCCCTCA-3'
<i>Notch2</i>	5'-GACAACATGGGCCGATGTC-3'	5'-GTTACACGGTTGCGGATCAG-3'
<i>Sox2</i>	5'-CTCTGCACATGAAGGAGCAC-3'	5'-CTCCGGGAAGCGTGTACTTA-3'
<i>Tgfb1</i>	5'-TGGAGCAACATGTGGAATC-3'	5'-CGCTCAAAGACAGCCACTCA-3'
<i>Tgfb2</i>	5'-GCTTTGGATGCTGCCTACTG-3'	5'-CCCGAGCACAGAAGTTAGCA-3'
<i>EF1A-AKT*</i>	5'-TCAAGCCTCAGACAGTGGTTC-3'	5'-TCACAATGGCTACGTCGTTTC-3'
<i>EF1A-Myc*</i>	5'-TCAAGCCTCAGACAGTGGTTC-3'	5'-GGAAATCCAGCCTTCAAACA-3'
<i>EF1A-YAP*</i>	5'-TCAAGCCTCAGACAGTGGTTC-3'	5'-GCCCCGGATCCATAGGTAC-3'
<i>Notch2*</i>	5'-AAGCTTGGGCAGGTTACATCC-3'	5'-AAGTCACGATGGGAGGCAAG-3'

*Primers used for quantification of copy number of the integrated genes in the tumors (Supplemental Figure S3).

For the detection of pAKT, pGSK3 β , and Myc, signal amplification was performed using a TSA Plus DIG Kit (PerkinElmer, Waltham, MA). To evaluate the extent of fibrosis, the sections were stained with Sirius Red F3B (Waldeck, Münster, Germany).

In Vivo Hepatocyte Lineage Tracing

To genetically label hepatocytes, *ROSA26R* mice (provided by Dr. Philippe Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA) were infected with adeno-associated virus serotype 8 (AAV8) expressing Cre recombinase under the control of a hepatocyte-specific thyroxine-binding globulin (TBG) promoter (AAV8-TBG-Cre). The promoter was prepared by the triple-transfection method with pENN-AAV-TBG-PI-Cre-rBG, pBS-E2A-VA-E4, and p5E18-VD2/8 plasmids into 293T cells. All of the plasmids were obtained from the Penn Vector Core, University of Pennsylvania (Philadelphia, PA). Virus purification was performed by ultracentrifugation, and the viral particles were titrated by qPCR with primers for Cre [5'-AACTAACTGGTCGAGC-GATG-3' (forward) and 5'-AGGGCGCGAGTTGATAGC-3' (reverse)]. To confirm the hepatocytic origin of the tumor cells with a ductular phenotype, *ROSA26R* mice were injected with 3×10^{10} copies of AAV8-TBG-Cre via the lateral tail vein. One week later, PGK-SB13, pT3-myrAKT- $\Delta\Delta$ LoxP, and pT3-YAPS127A- $\Delta\Delta$ LoxP plasmids were coinjected via the HTVi method. The liver was fixed with 4% paraformaldehyde and then soaked in 30% sucrose overnight at 4°C. Frozen sections were reacted with X-Gal solution [1 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; Sigma-Aldrich), 5 mmol/L potassium ferrocyanide, and 5 mmol/L potassium ferricyanide] at 37°C overnight. X-Gal-stained sections were then subjected to CK19 immunohistochemistry using an alkaline phosphatase method with detection of the enzyme reaction by Permanent Red (Dako). To precisely estimate double-positive tumor cells for β -galactosidase and CK19, we documented X-Gal-stained sections by a whole slide scanner (VS120; Olympus, Tokyo, Japan) before and after CK19 immunohistochemistry and examined the same areas.

Human Samples of Hepatocellular Carcinoma and Intrahepatic Cholangiocellular Carcinoma

The retrospective analysis of surgical specimens was approved by the internal review board of Asahikawa Medical University (approval number 14004). A total of 33 HCC and 18 CC samples from patients who underwent surgical resection were collected. Immunohistochemistry for pAKT, pGSK3 β , pS6, MYC, and YAP was performed, as described above. MYC and Ki-67 immunohistochemistry data for the HCC samples were also used in another study performed in our laboratory.²⁶

Statistical Analysis

Results from experiments are expressed as the means $\pm$ SEM. One-way analysis of variance with Tukey post hoc test, *t*-test, χ^2 test, or *U*-test was performed to compare differences. For comparison of tumor-free survival (Kaplan-Meier plot), a log-rank (Mantel-Cox) test was performed. We used Prism 6 (GraphPad Software, La Jolla, CA) for all statistical analyses.

Results

Hepatocarcinogenesis Is Enhanced by Various Combinations of Oncogenes Involved in the PI3K-AKT, YAP, and Myc Pathways

The introduction of Myc alone did not induce liver tumors, even after long periods of >1 year (Figure 1). In five mice in which YAP (YAP^{S127A}) was injected, we observed only three tumors in two animals (Figure 1 and Supplemental Figure S1A). Time course analyses revealed that hepatocytes transduced by Myc or YAP were initially present, but almost all were eliminated after 1 month, partly through an apoptotic mechanism (Supplemental Figure S2). The myristoylated and constitutively active AKT induced multiple

Figure 1 Synergistic effects of Sleeping Beauty-transposon-mediated integration of AKT, Myc, and Yes-associated protein (YAP) on liver tumor development. **A:** Tumor-free survival of the mice after hydrodynamic tail vein injection (HTVi) of myrAKT (AKT), YAP^{S127A} (YAP), Myc, AKT/YAP, AKT/Myc, Myc/YAP, and AKT/Myc/YAP. Number of mice is indicated in the figure. **B:** Gross appearance of the livers. Control: liver from a 17-week-old male C57BL/6J mouse without HTVi. Scale bar = 10 mm (**B**).

liver tumors after approximately 28 weeks (median, 48 weeks) (Figure 1). Its tumorigenic activity was greatly enhanced by either Myc or YAP, generating multiple tumors of various sizes with AKT/Myc and diffusely infiltrating tumors with a hard consistency with AKT/YAP within 10 weeks (Figure 1). The combination of the three genes (AKT/Myc/YAP) further enhanced tumorigenesis, and diffuse tumors replaced the entire liver tissue within 3 weeks (Figure 1). Furthermore, the cointroduction of Myc and YAP generated multiple tumors within 20 weeks (Figure 1). None of the tumors metastasized to the lungs, although small pulmonary tumor emboli were found in some of the animals harboring AKT-induced tumors (14.3%) and AKT/Myc-induced tumors (9.1%). AKT/Myc-induced tumors occasionally disseminated into the peritoneal cavity (27.3%). The copy numbers of the integrated exogenous genes in the tumors, which were estimated by qPCR, were 2.6 ± 0.5 , 2.5 ± 0.5 , and 3.8 ± 0.3 for AKT, Myc, and YAP, respectively (Supplemental Figure S3).

Microscopically, AKT-induced tumors were characterized by the proliferation of large hepatocyte-like tumor cells with abundant cytoplasmic lipid and intermingled small ductular structures (Figure 2A). However, a minority of tumors were composed solely of HCC or CC (Supplemental Figure S4). At earlier periods, wherein gross tumors were not evident, transduced hepatocytes containing abundant lipid formed clusters without apparent ductular structures (Supplemental Figure S5A). The three tumors induced by YAP alone were also heterogeneous and similar to those induced by AKT (Supplemental Figure S1B). Although

hepatocytes transduced by AKT/Myc initially contained abundant lipids in their cytoplasm (Supplemental Figure S5B), they gradually lost lipid and eventually formed well to moderately differentiated HCCs. These HCCs showed thick trabecular or nest-like patterns accompanied by sinusoidal vascular networks (Figure 2A). In contrast, the tumors induced by AKT/YAP were composed of irregular ductular structures exhibiting characteristic CC histology (Figure 2A). Although AKT/YAP-induced preneoplastic hepatocytes were large cells with abundant cytoplasmic lipid, which were similar to those induced by AKT or AKT/Myc, they subsequently lost lipid, became smaller, and gradually acquired the ductular phenotype (Supplemental Figure S6 and Figure 2B). In Myc/YAP-induced tumors, small tumor cells with a high nuclear/cytoplasmic ratio proliferated in mixed trabecular and ductular growth patterns, with the latter more evident at the periphery of the tumors (Figure 2, A and B). In the earlier period of tumorigenesis, hepatocytes transduced by Myc/YAP had ample basophilic cytoplasm devoid of lipid and ductular phenotype (Supplemental Figure S5C). Although AKT/Myc/YAP-induced tumors initially demonstrated a heterogeneous feature, with some cells containing abundant lipid (Supplemental Figure S5D), they later exhibited a solid sheet-like pattern. This pattern had no discernible ductular structures and was composed of tumor cells of intermediate size without cytoplasmic lipid, thus suggesting a poorly differentiated state (Figure 2 A and B). Nucleoli were particularly prominent in the tumors induced by AKT/Myc and AKT/Myc/YAP (Figure 2A). Ki-67

Figure 2 Histological features and proliferative activities of the oncogene-induced tumors. **A:** Histology of the intact liver (control) and tumors induced by AKT, AKT/Myc, AKT/Yes-associated protein (YAP), Myc/YAP, and AKT/Myc/YAP. Hematoxylin and eosin staining. **B:** Quantitative analyses of the sizes of the tumor cells. The sizes of 20 tumor cells were measured in a tumor nodule and averaged. Ten randomly selected, distinct tumor nodules were analyzed in each tumor type. **C:** Immunohistochemistry for Ki-67. **D:** Comparison of cell-proliferative activities among the tumors. Percentages of Ki-67-labeled cells were quantified in two high-power fields in 10 distinct tumor nodules in each tumor type. Statistical analysis: one-way analysis of variance with the Tukey post hoc test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.0001$ versus control. Scale bars = 50 μm (A and C).

immunohistochemistry revealed that the proliferative activity of the tumors induced by AKT alone was low but was much higher in tumors induced by multiple oncogenes. The most frequent Ki-67 nuclear labeling was observed in Myc/YAP- and AKT/Myc/YAP-induced tumors (Figure 2 C and D).

To examine whether the introduced AKT, Myc, and YAP were expressed or activated in tumor cells, we performed immunohistochemistry detecting pAKT, pGSK3 β , pS6, Myc, and YAP. All of the tumors that integrated myristoylated AKT (AKT-, AKT/Myc-, and AKT/Myc/YAP-induced tumors) demonstrated strong staining of pAKT and pGSK3 β , whereas Myc/YAP-induced tumors showed only weak phosphorylation of AKT and GSK3 β (Figure 3). pS6, which was readily detectable in hepatocytes in the control liver, was positive in AKT/Myc-, Myc/YAP-, and AKT/Myc/YAP-induced tumors but scarcely detected in AKT/YAP-induced tumors (Figure 3). High levels of YAP protein were detected in the tumors in which YAP was introduced, whereas weak YAP staining was observed in AKT/Myc-induced tumor cells (Figure 3). Myc was detected at high levels in the nuclei of tumor cells induced by AKT/Myc, Myc/YAP, and AKT/Myc/YAP (Figure 3). In addition, some tumor cells induced by AKT/YAP expressed Myc (Figure 3).

Malignant Tumors with a Cholangiocytic Phenotype Can Be Induced from Hepatocytes when the PI3K-AKT and YAP Pathways Are Simultaneously Activated

We next examined the hepatocytic and cholangiocytic differentiation of the oncogene-induced tumors by using immunohistochemistry. Hepatocyte nuclear factor 4 α P1 and

major urinary protein, markers for hepatocytic differentiation, were variously positive in AKT-, AKT/Myc-, Myc/YAP-, and AKT/Myc/YAP-induced tumors and virtually negative in AKT/YAP-induced tumors (Figure 4A). As markers for bile duct differentiation, we used CK19, Sox9, and Grhl2, a transcription factor specifically expressed in fully differentiated bile ductal/ductular cells.²⁷ The small ductular cells in the AKT-induced tumors were strongly positive for CK19 and Sox9 and faintly positive for Grhl2 (Figure 4A). Given that these ductules were positive for HA, they were considered to be derived from transduced hepatocytes expressing HA-tagged AKT (Figure 4B). HCC induced by AKT/Myc, which was composed entirely of HA-positive cells (Figure 4B), did not contain any CK19- or Grhl2-positive cells, whereas some tumor cells were positive for Sox9 (Figure 4A). CC-like tumors induced by AKT/YAP were composed entirely of CK19-, Sox9-, and Grhl2-positive (Figure 4A) and HA-positive tumor cells (Figure 4B). When CC was induced by AKT/YAP in the livers of ROSA26R mice infected with AAV8-TBG-Cre, all CK19-positive atypical ductular structures were positive for β -galactosidase, thus further confirming that they were of hepatocytic origin (Figure 4C). In Myc/YAP-induced tumors, cells forming ductular structures were positive for CK19 and Sox9, but only a few were Grhl2 positive (Figure 4A). In AKT/Myc/YAP tumors, all tumor cells were positive for Sox9, whereas only a few CK19-positive cells and no Grhl2-positive cells were observed (Figure 4A).

In liver development, differentiation of hepatoblasts into bile ducts/ductules is triggered by the deposition of periportal connective tissues.²⁸ We examined whether desmoplastic changes were associated with ductular differentiation of the tumors. In AKT/Myc- and AKT/Myc/YAP-induced

Figure 3 Immunohistochemical detection of the signaling molecules relevant to the phosphoinositide 3-kinase–AKT, Yes-associated protein (YAP), and Myc pathways in the oncogene-induced tumors. Immunohistochemistry for phosphorylated AKT (pAKT), phosphorylated glycogen synthase kinase-3 β (pGSK3 β), phosphorylated S6 (pS6), YAP, and Myc. Scale bars = 50 μ m.

Figure 4 Induction of tumors of cholangiocytic phenotype from mature hepatocytes by simultaneous activation of the phosphoinositide 3-kinase–AKT and Yes-associated protein (YAP) pathways. **A:** Immunohistochemistry for hepatocyte nuclear factor 4 α P1 (HNF4 α P1), major urinary protein (MUP1), cytokeratin 19 (CK19), sex-determining region Y–box 9 (Sox9), and grainy head–like 2 (Grhl2). **B:** Immunohistochemistry for hemagglutinin (HA). Cells transfected with the myrAKT plasmid (pT3-EF1 α -myrAKT-HA) also express HA. The biliary cells (arrows) intermingled with the hepatocellular carcinoma–like tumor cells in the AKT-induced tumor are also positive for HA. **C:** Combined X-Gal histochemistry (blue) and immunohistochemistry for CK19 (red) of the liver tissues of ROSA26R mice infected with adeno-associated virus serotype 8–thyroxine-binding globulin-Cre. Shown are the images documented by a whole slide scanner before and after CK19 immunohistochemistry. The CK19-positive cells were indicated in the control liver (arrowheads). Control, intact liver; AKT/YAP, AKT/YAP-induced tumor. Scale bars = 50 μ m (A–C).

tumors, which did not contain ductular structures, the tumor stroma was composed of delicate connective tissues stained by Sirius Red (Figure 5A). Dense connective tissue stroma surrounded the distinct ductular structures in AKT- and Myc/YAP-induced tumors (Figure 5A). In Myc/YAP tumors, peripherally located ductular structures were associated with a slight desmoplastic reaction (Figure 5A). We also examined the mRNA expression of several genes involved in fibrotic reactions. The mRNA levels of *Tgfb2*, but not *Tgfb1*, were markedly increased in AKT/YAP-induced tumors (Figure 5B). In addition, *Ctgf* and *Cyr61* mRNA expression was elevated in AKT/YAP-induced tumors and YAP/Myc-induced tumors, albeit to a lesser degree (Figure 5B).

The Notch signaling pathway, which is activated by the YAP pathway, is one of the most important determinants of bile duct differentiation in the developing liver.^{29,30} Thus, we examined the mRNA expression of major components of the Notch pathway: the ligand (*Jag1*), receptors (*Notch1* and

Notch2), and effectors (*Hes1* and *Hes2*). *Jag1*, *Notch2*, *Hes1*, and *Hes2* mRNA expression levels were significantly increased in AKT/YAP-induced tumors, but not in other tumors (Figure 5C). Although *Notch1* mRNA expression in the AKT/YAP-induced tumors was comparable to that in the normal liver, *Notch1* expression levels were significantly lower in the tumors induced by AKT/Myc and AKT/Myc/YAP, in which no ductular structures were present (Figure 5C).

Simultaneous Activation of the PI3K-AKT and Notch Pathways in Hepatocytes Induces Biliary Cystadenoma, and Coactivation of Myc Confers a Malignant Phenotype

To examine whether the activation of the Notch pathway was sufficient to induce CC when the PI3K-AKT pathway was concomitantly activated, we introduced N2ICD with

Figure 5 Cholangiocytic differentiation of AKT/Yes-associated protein (YAP)-induced tumors accompanied by stromal fibrosis and increases in mRNA expression of the genes relevant to fibrogenesis and Notch signaling. **A:** Sirius red staining. The most prominent increase of fibrous stroma was in AKT/YAP tumors. The bile duct-like structures in AKT- and Myc/YAP-induced tumors are also surrounded by a small amount of fibrous stroma (arrows). **B:** Quantitative RT-PCR (RT-qPCR) analyses of the expression of genes related to fibrogenesis (*Tgfb1*, *Tgfb2*, *Ctgf*, and *Cyr61*). **C:** RT-qPCR analyses of the Notch-related genes (*Jag1*, *Notch1*, *Notch2*, *Hes1*, and *Hes2*). Values were normalized against *Gapdh*. Statistical analyses: one-way analysis of variance with the Tukey post hoc test. $n = 8$ control (intact liver) and AKT/YAP-, AKT/Myc-, and AKT/Myc/YAP-induced tumors (**B** and **C**); $n = 6$ AKT-induced tumors (**B** and **C**); $n = 7$ Myc/YAP-induced tumors (**B** and **C**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$, and $^{\dagger\dagger\dagger\dagger}P < 0.0001$ versus control. Scale bars = 50 μm (**A**).

AKT into hepatocytes. After 5 to 6 weeks, the livers were markedly enlarged, and the normal tissue had been almost completely replaced with multiple cysts containing straw-colored serous fluid (Figure 6 A and B). Histologically, the cysts were well demarcated and were lined with cuboid epithelial cells. The cyst walls were composed of irregular ductular structures and fibrous tissues, without infiltration into the surrounding liver. The nuclei of the epithelial cells were bland, and the Ki-67-labeling index was $<20\%$ (Figure 6 C and D). These features suggested that a diagnosis of biliary cystadenoma was appropriate. We also examined the additional effect of Myc activation in the AKT/N2ICD-induced tumors. When AKT, N2ICD, and Myc were simultaneously introduced, gray-white tumors without cyst formation were induced and had replaced the entire liver tissue within 3 weeks (Figure 6 A and B). Histological examination revealed ductular proliferation of atypical cells with hyperchromatic nuclei, almost all of which were positive for Ki-67, a result compatible with the features of aggressive, high-grade CC (Figure 6C). Immunohistochemically, YAP was undetectable, but pGSK3 β and

pS6 were readily detected in tumor cells in either AKT/N2ICD- or AKT/N2ICD/Myc-induced tumors (Figure 6E). CK19 and Grhl2 were strongly positive in both AKT/N2ICD- and AKT/N2ICD/Myc-induced tumors (Figure 6E), whereas hepatocyte nuclear factor 4 α P1 and major urinary protein were negative (data not shown), confirming cholangiocytic differentiation.

mRNA expression of the Notch-related genes (*Jag1*, *Hes1*, and *Hes2*) was markedly elevated in AKT/N2ICD-induced tumors (Figure 6F). The introduction of Myc significantly suppressed *Jag1* but not the Notch effectors (*Hes1* and *Hes2*) (Figure 6F), in contrast to the findings in AKT/Myc/YAP-induced tumors (Figure 5C). However, the increased mRNA expression of *Ctgf* and *Tgfb2* in AKT/N2ICD-induced tumors was almost completely inhibited by concomitant Myc activation (Figure 6F).

Similar results were obtained when N1ICD was introduced instead of N2ICD (Supplemental Figure S7), suggesting that the activation of either Notch1 or Notch2 is almost identical in regard to the activation of bile ductular differentiation.

Figure 6 Induction of aggressive cholangiocarcinoma by simultaneous activation of the phosphoinositide 3-kinase–AKT, Notch, and Myc pathways. **A:** Tumor-free survival of mice after the introduction of AKT/Notch2 intracellular domain (N2ICD) or AKT/N2ICD/Myc. *P* value was calculated by the log-rank test and was significant versus AKT/N2ICD. **B:** Gross appearances of the tumors induced by AKT/N2ICD and AKT/N2ICD/Myc. The AKT/N2ICD tumors are composed of multiple cysts, whereas AKT/N2ICD/Myc tumors are diffusely infiltrating. **C:** Histology of the tumors (hematoxylin and eosin staining) and immunohistochemistry for Ki-67. **D:** Comparison of cell-proliferative activities between AKT/N2ICD- and AKT/N2ICD/Myc-induced tumors. Percentages of Ki-67–labeled cells were quantified in two high-power fields in 10 different areas in each tumor type. Statistical analyses: *t*-test. **E:** Immunohistochemistry for Yes-associated protein (YAP), phosphorylated glycogen synthase kinase (pGSK)-3β, phosphorylated S6 (pS6), cytokeratin 19 (CK19), and grainy head–like 2 (Grhl2). **F:** Quantitative RT-PCR analyses of mRNA expression of the Notch-related genes (*Jag1*, *Hes1*, and *Hes2*) and those involved in fibrogenesis (*Ctgf* and *Tgfb2*). Values were normalized against *Gapdh*. Statistical analyses: one-way analysis of variance with the Tukey post hoc test. *n* = 12 AKT/N2ICD-induced tumors (**D**); *n* = 9 AKT/N2ICD/Myc-induced tumors (**D**). *n* = 8 control (intact liver) and AKT/N2ICD-induced tumors (**F**); *n* = 10 AKT/N2ICD/Myc-induced tumors (**F**). ***P* < 0.01, *****P* < 0.0001; ††*P* < 0.01, †††*P* < 0.001, and ††††*P* < 0.0001 versus control. Scale bars: 10 mm (**B**); 50 μm (**C** and **E**). HTVi, hydrodynamic tail vein injection.

Combinatory Activation of YAP and Myc in the Absence of PI3K-AKT Pathway Activation Induces Hepatoblast/Stem Cell–Like Differentiation in Tumor Cells

The tumors induced by the combination of Myc and YAP were composed of tumor cells with several characteristic features reminiscent of immature hepatoblasts: small cells with high nuclear/cytoplasmic ratios, intermingled trabecular and ductular patterns, and a high Ki-67 labeling index. Therefore, we examined the mRNA expression levels of markers for hepatoblasts (*Afp* and *Dlk1*) and stem cells (*Nanog* and *Sox2*) in Myc/YAP-induced and other tumors. Although *Afp* mRNA expression was increased in both Myc/YAP- and AKT/Myc/YAP-induced tumors, the expression levels of *Dlk1*, *Nanog*, and *Sox2* were specifically increased in Myc/YAP-induced tumors (Figure 7A). In agreement with the quantitative RT-PCR data, immunohistochemistry for *Afp* and *Dlk1* proteins revealed that the former was expressed in Myc/YAP- and AKT/Myc/YAP-induced tumors, whereas the latter was expressed only in Myc/YAP-induced tumors (Figure 7B). These results suggested that simultaneous activation of the

Myc and YAP pathways induced dedifferentiated liver tumors in the absence of PI3K-AKT pathway activation. Intriguingly, Myc/YAP-induced tumors were strongly positive for β-catenin with a membranous staining pattern (Figure 7B), which has been known to be a characteristic feature of the fetal subtype of hepatoblastoma.³¹

The PI3K-AKT, MYC, and YAP Pathways Are Frequently Activated in Human Cholangiocarcinoma

Finally, to explore the status of the activation of the PI3K-AKT, MYC, and YAP pathways in human liver cancers, we performed immunohistochemistry for pAKT, pS6, pGSK3β, YAP, and Myc in 33 cases of HCC and 17 cases of intrahepatic CC. Although the staining intensity of pAKT was generally low and focal, it was more frequently positive in HCC than in CC (Figure 8A and Table 2). Phosphorylation of S6, an AKT substrate, was positive in most HCC cases (87.9%) and positive in approximately half of CC cases (52.9%) (Figure 8A and Table 2). In contrast, another AKT substrate, GSK3β, was more frequently and strongly

Figure 7 Specific expression of the markers for hepatoblasts and stem cells in tumors induced by Myc/Yes-associated protein (YAP). **A:** Quantitative RT-PCR analyses of mRNA expression of markers for hepatoblasts (*Afp* and *Dlk1*) and for stem cells (*Nanog* and *Sox2*). RNA samples were identical to those used in Figure 5. Values were normalized against *Gapdh*. Statistical analyses: one-way analysis of variance with the Tukey post hoc test. **B:** Immunohistochemistry for α -fetoprotein (AFP), δ -like protein (DLK1), and β -catenin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; † $P < 0.05$, †† $P < 0.001$, and ††† $P < 0.0001$ versus control. Scale bars = 50 μ m (**B**).

phosphorylated in CC compared with HCC (HCC, 45.5%; CC, 82.4%) (Figure 8A and Table 2). Nuclear MYC positivity was observed in some cases of either HCC or CC (Figure 8A and Table 2). YAP was positive in the nuclei of tumor cells in 33.3% of HCC cases and 94.1% of CC cases (Figure 8A and Table 2). We next examined the possible association of the combinatory activation of these molecules and tumor phenotypes. HCC cases with evidence of activation of the PI3K-AKT pathway (positive for pAKT, pS6, or pGSK3 β) were also variously positive for MYC and/or YAP (Figure 8B). In particular, 6 HCC cases (18.2%) were triple positive for pGSK3 β , MYC, and YAP and contained poorly differentiated histology with significantly increased Ki-67 nuclear labelling ($45.3\% \pm 9.3\%$) compared with other HCC cases ($10.7\% \pm 2.1\%$) ($P = 0.0004$, U -test) (Figure 8C). A substantial fraction (64.7%) of CC cases were both positive for pGSK3 β and YAP (Figure 8B), thus suggesting an important correlation between the pathways involving these molecules.

Discussion

The combination of SB transposon-mediated integration of various oncogenes and HTVi has been widely applied to

induce mouse liver tumors.³² The delivered DNA is introduced exclusively into hepatocytes, thus making this technique particularly applicable for the examination of phenotypes of hepatocyte-derived tumors. Using this system, we demonstrated that activation of the PI3K-AKT pathway by myrAKT alone generated HCC with intermingled, bland, ductular cells originating from transduced hepatocytes (HCC with biliary differentiation). Calvisi et al³³ have reported that the tumors induced by the introduction of myrAKT exhibit characteristics of HCC, but there is a small fraction of nonmalignant ductular tumors. In this study, we also found that cointroduction of YAP^{S127A} and myrAKT induced almost pure CC, which was derived from hepatocytes. Li et al²⁴ have reported that the introduction of YAP^{S127A} and PIK3CA^{H1047R}, a constitutively active mutant of PI3K, induces HCC, CC, and cHCC-CC. Despite slight differences in the histological features of the induced tumors, these results, including ours, are consistent with the notion that mature hepatocytes can be transformed to generate tumors with cholangiocytic differentiation, as first proposed by Fan et al,²³ through the simultaneous introduction of myrAKT and N1ICD. In agreement with these data obtained from mouse experiments, the incidence of CC is high in patients with chronic

Figure 8 Combinatory activation of the phosphoinositide 3-kinase (PI3K)—AKT, Yes-associated protein (YAP), and MYC pathways in human hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). Immunohistochemical analyses for phosphorylated AKT (pAKT), phosphorylated S6 (pS6), phosphorylated glycogen synthase kinase-3 β (pGSK3 β), MYC, and YAP in human HCC and CC. **A:** Representative immunohistochemical images. **B:** Percentages of cases positive for the phosphorylated substrates in the PI3K-AKT pathway (pAKT, pS6, and pGSK3 β) together with MYC and/or YAP. Combination of pAKT, MYC, and YAP (**top panel**); pS6, MYC, and YAP (**middle panel**); and pGSK3 β , MYC, and YAP (**bottom panel**). Statistical analysis: χ^2 test. **C:** Percentages of Ki-67-positive cells in tumors with various combinations of immunoreactivity for pGSK3 β , MYC, and YAP in HCC. * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 versus CC; ††† P < 0.001, †††† P < 0.0001 versus HCC. Scale bars = 50 μ m (**A**). —, all negative.

Table 2 Number of Cases Positive for pAKT, pS6, pGSK3 β , MYC, and YAP in HCC and CC

Variable	HCC (n = 33)	CC (n = 17)	P value (Fisher exact test)
pAKT	16 (48.4)	2 (11.8)	0.0133
pS6	29 (87.9)	9 (52.9)	0.0123
pGSK3 β	15 (45.5)	14 (82.4)	0.0164
MYC	9 (27.3)	4 (23.5)	1.000
YAP	11 (33.3)	16 (94.1)	<0.0001

Data are expressed as n (percentage) unless otherwise indicated.

CC, cholangiocarcinoma; HCC, hepatocellular carcinoma; pAKT, phosphorylated AKT; pGSK3 β , phosphorylated glycogen synthase kinase-3 β ; pS6, phosphorylated S6; YAP, Yes-associated protein.

hepatitis.³⁴ Recent whole exome analyses have revealed that CC associated with chronic hepatitis demonstrates gene mutation profiles similar to HCC,³⁵ thus suggesting that there might be a subclass of CC that shares a cellular origin and carcinogenic processes with HCC.

The cholangiocytic differentiation of hepatocytic tumors might reflect the phenotypic plasticity of mature hepatocytes. We have previously demonstrated that hepatocytes differentiate into bile ductular cells *in vitro* in response to collagenous extracellular matrices.¹⁹ Using hepatocyte lineage tracing techniques *in vivo*, we and other investigators have shown that hepatocytes can change their phenotype to that of bile ductules after chronic liver injury.^{21,22} The phenotypic transition of mature hepatocytes generates so-called intermediate hepatobiliary cells³⁶ or hybrid hepatocytes.³⁷ These cells do not express markers for hepatoblasts, such as α -fetoprotein and δ -like protein, thus suggesting that the process is transdifferentiation rather than dedifferentiation. Because the tumors induced by myrAKT or myrAKT/YAP did not express α -fetoprotein and δ -like protein, cholangiocytic differentiation in these tumors might also be interpreted as transdifferentiation of transformed hepatocytes.

Although the introduction of AKT or YAP alone was inefficient in inducing liver tumors, their combination markedly facilitated tumorigenesis with cholangiocytic differentiation. Activation of YAP by acute inactivation of Hippo *in vivo* induces aberrant cholangiocytic differentiation of mature hepatocytes through activation of the Notch signaling pathway,³⁰ which plays a critical role in the differentiation of immature hepatoblasts into bile ducts during development.^{29,38} In agreement with the notion that the cholangiocytic differentiation of hepatocytes might be determined by Notch activation, the introduction of N2ICD (or N1ICD) together with myrAKT induced cystic biliary tumors. These tumors lacked the definitive malignant morphological features demonstrated in a similar study performed by Fan et al.²³ Because activation of the Notch signaling pathway via stimulation by Jagged-1 has been reported in human HCC lacking ductular differentiation,³⁹ YAP-induced activation of the Notch signaling pathway

may not be sufficient to induce cholangiocytic differentiation in liver tumors. However, it is evident that the differentiation of liver tumors toward the cholangiocytic phenotype could be induced through the collaborative effects of the YAP or Notch signaling pathway and the PI3K-AKT pathway.

The MYC oncogene is critically involved in many tumors, including liver tumors.⁴⁰ Recently, we demonstrated that the activation of endogenous Myc is critical in AKT/HRAS-induced hepatocarcinogenesis, and forced Myc activation further facilitates tumorigenesis induced by AKT and/or HRAS.²⁶ Cointroduction of Myc also greatly facilitated tumorigenesis by YAP and conferred more aggressive features with higher proliferative activities in ductular tumors induced by AKT and N2ICD (or N1ICD). Interestingly, the typical CC-like ductular histology of AKT/YAP-induced tumors was lost in less-differentiated AKT/Myc/YAP-induced tumors, although some tumor cells were CK19 positive. This phenotypic change was accompanied by the suppression of the Notch signaling pathway and other known targets of the YAP pathway (*Cyr61*, *Ctgf*, and *Tgfb2*), thus suggesting that Myc might partly inhibit the YAP pathway, sparing its oncogenic capacity. In mammary epithelial cells and breast cancers, Myc suppresses YAP/transcriptional coactivator with PDZ-binding motif transcriptional function by altering mitochondrial activity and the subsequent activation of AMP-activated kinase.⁴¹ Distinct cholangiocytic differentiation in the AKT/N2ICD (or N1ICD)/Myc-induced tumors might be explained by Myc's inability to completely neutralize the effects of N2ICD (or N1ICD).

We showed that the combination of YAP and Myc specifically induced dedifferentiated tumors composed of highly proliferative hepatoblast-like cells that expressed markers for hepatoblasts (*Afp* and *Dlk1*) and stem cells (*Nanog* and *Sox2*). Overexpression of Myc induces liver cancers with bimodal and stem-like phenotypes.^{7,8} Pediatric liver cancers (HB) frequently exhibit a specific MYC signature of gene expression, which is closely related to poorer prognosis.⁴² Furthermore, in aggressive HB, MYC induces the mRNA-371-3 cluster, which is involved in the maintenance of stemness, while suppressing several mRNAs that are abundant in differentiated cells.⁴³ It has also been hypothesized that YAP may either stimulate cell proliferation or induce stem cell features, depending on the levels of expression.⁴⁴ YAP/Myc-induced tumors exhibited the formation of immature ducts/ductules with slight desmoplastic reactions, especially at the periphery of the tumors, a phenotype reminiscent of cholangioblastic HB.⁴⁵ Tao et al⁴⁶ have reported that the transposon-mediated cointroduction of YAP^{S127A} and Δ N90- β -catenin induces liver tumors with expression of *Afp* mRNA and δ -like protein, although their histology is apparently similar to that of HCC. Our data provide the first evidence that concomitant activation of YAP and MYC is a potent stimulus for transformation of mature hepatocytes into hepatoblast-like cells, thus suggesting the capacity of hepatocytes to dedifferentiate.

Figure 9 Generation of hepatocytic, cholangiocytic, and hepatoblastic tumors from hepatocytes through complex interactions of the phosphoinositide 3-kinase (PI3K)—AKT, MYC, Yes-associated protein (YAP), and Notch signaling pathways. The activation of the PI3K-AKT pathway in hepatocytes is important in both tumorigenesis and phenotypic determination, either hepatocytic or cholangiocytic. The combinatory activation of the Myc and YAP pathways induces tumors with dedifferentiated hepatoblastic features if the PI3K-AKT pathway is not activated. CC, cholangiocarcinoma; HB, hepatoblastoma; HCC, hepatocellular carcinoma; pGSK3 β , phosphorylated glycogen synthase kinase-3 β .

In contrast to YAP/Myc-induced tumors, AKT/Myc/YAP-induced tumors did not express mRNA for *Dlk1*, *Nanog*, and *Sox2*. Furthermore, although they were more aggressive and microscopically less differentiated, indicating that they were high-grade tumors, they did not demonstrate the hepatoblastic morphology that was noted in YAP/Myc-induced tumors. Our study has also shown that the tumors harboring activated AKT are either hepatocytic (AKT/Myc-induced tumors) or cholangiocytic (AKT/YAP-, AKT/N2ICD-, and AKT/N1ICD-induced tumors), suggesting that the PI3K-AKT pathway plays an important role in facilitation of differentiation toward a certain cell type. In mouse liver tumors, mechanistic target of rapamycin complex 1 and AKT are critical in the determination of hepatocytic and cholangiocytic phenotypes, respectively.¹⁰ The PI3K-AKT pathway phosphorylates and suppresses tuberous sclerosis 2, which inhibits mechanistic target of rapamycin complex 1, thereby activating S6K, resulting in phosphorylation of S6 and activation of eIF4E and its effector MYC.⁴⁷ Suppression of tuberous sclerosis 2 also stimulates the YAP pathway through the inhibition of the mechanistic target of rapamycin complex 2 pathway, which inhibits the YAP pathway via phosphorylation of angiotensin-like 2, an angiotensin family member.⁴⁸ Furthermore, prominent S6 phosphorylation was noted in YAP/Myc-induced tumors, probably through the direct suppressive effect of MYC on tuberous sclerosis 2,⁴⁹ suggesting that S6 phosphorylation per se might not be the phenotype determinant of liver tumors. The PI3K-AKT pathway also phosphorylates GSK3 and suppresses its function, resulting in the activation of Wnt/ β -catenin signaling.

GSK3 is known to play multiple physiological roles in a variety of cell types. GSK3 inhibition has been shown to be required for hepatocyte specification,⁵⁰ as well as proliferation of fetal and adult mouse hepatocytes with potential differentiation toward bile duct cells.^{51,52} These findings suggest that the PI3K-AKT pathway, especially by the regulation of the GSK3 pathway, might serve as an inducer of hepatocytic or cholangiocytic phenotypes of the tumors through interactions with other signaling pathways (Figure 9).

We demonstrated immunohistochemically that human liver cancer tissues were variably positive for phosphorylated forms of signaling molecules involved in the PI3K-AKT pathway. pS6 and pGSK3 β were more frequently detected in HCC and CC, respectively. It has been suggested that the interaction of the PI3K-AKT and YAP pathways might be involved in human liver cancers, including HCC, CC, and cHCC-CC.²⁴ Our study demonstrated that phosphorylation of GSK3 β was often associated with YAP positivity in human CC. Intriguingly, HCC positive for pGSK3 β , MYC, and YAP was highly proliferative and contained poorly differentiated and high-grade histology. However, further studies with a larger sample population are needed to confirm our findings and to determine the relationship between the immunohistochemical coincidence and patient prognosis. This finding in human HCC is compatible with the aggressive phenotype of the mouse liver tumors induced by simultaneous activation of the AKT, Myc, and YAP pathways. These results highlight that the elucidation of the intricate interactions of various oncogenic pathways should be important to understand the mechanisms for phenotypic determination of hepatocyte-derived liver tumors and to identify the most appropriate and effective therapeutic targets.

In conclusion, primary liver cancers of hepatocytic origin demonstrate a widely varying degree of phenotypic complexity, according to altered differentiation states of transformed hepatocytes via transdifferentiation and dedifferentiation. Our observations show that the state of the PI3K-AKT pathway is a key determinant of differentiation. If this pathway is activated, the interaction with the Myc and the YAP pathways promotes tumors with hepatocytic and cholangiocytic differentiation, respectively.

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Supplemental Data

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