A Zebrafish Model of Intrahepatic Cholangiocarcinoma by Dual Expression of Hepatitis B Virus X and Hepatitis C Virus Core Protein in Liver

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The mechanisms that mediate the initiation and development of intrahepatic cholangiocarcinoma (ICC) associated with hepatitis B and C virus (HBV and HCV, respectively) infection remain largely unclear. In this study we conditionally coexpressed hepatitis B virus X (HBx) and hepatitis C virus core (HCP) proteins in zebrafish livers, which caused fibrosis and consequently contributed to ICC formation at the age of 3 months. Suppressing the transgene expression by doxycycline (Dox) treatment resulted in the loss of ICC formation. The biomarker networks of zebrafish ICC identified by transcriptome sequencing and analysis were also frequently involved in the development of human neoplasms. The profiles of potential biomarker genes of zebrafish ICC were similar to those of human cholangiocarcinoma. Our data also showed that the pSmad3L oncogenic pathway was activated in HBx and HCP-induced ICC and included phosphorylation of p38 mitogen-activated protein kinase (MAPK) and p44/42 mitogen-activated protein kinase (ERK1/2), indicating the association with transforming growth factor beta 1 (TGF-β1) signaling pathway in ICC. Bile duct proliferation, fibrosis, and ICC were markedly reduced by knockdown of TGF-β1 by in vivo morpholinos injections. Conclusion: These results reveal that TGF-β1 plays an important role in HBx- and HCP-induced ICC development. This in vivo model is a potential approach to study the molecular events of fibrosis and ICC occurring in HBV and HCV infection. (HEPATOLOGY 2012;56:2268-2276)

Intrahepatic cholangiocarcinoma (ICC), a highly malignant neoplasm originating from the intrahepatic bile ducts, is the second most common liver cancer worldwide. ICC is difficult to diagnose at an early stage, and effective nonsurgical adjuvant therapy has not been developed.

Hepatitis C virus (HCV) is a causative agent of liver diseases. Approximately 3% of the world population is infected with HCV. Although hepatocytes are the major site of HCV replication, several reports have demonstrated that HCV- and HCV-related cirrhosis may be risk factors for ICC. However, the...
mechanism by which HCV induces ICC formation is largely unknown. The HCV core protein (HCP) consists of 192 amino acids and modulates multiple cellular processes, including oncogenesis.\(^5\)

Hepatitis B virus (HBV) infection is a global health problem that affects more than 350 million people. Although HBV specifically infects hepatocytes,\(^6\) HBV has recently been considered a risk factor for ICC.\(^7\) HBV X (HBx) is a 16-kDa protein that has been implicated in the enhancement of viral replication, cell death, lipid metabolism, cell cycle regulation, DNA repair, and epigenetic modification.\(^8-13\) HBx is frequently expressed in the tissues surrounding the tumor and may be involved in ICC formation.\(^14\) It remains unclear whether the expression of HBx plays a functional role in the pathogenesis of ICC.

Fibrosis, a sign of liver damage, is a predominant feature of hepatobiliary disorder.\(^15\) Although several reports have shown that HBx and HCP contribute to fibrogenesis by way of the up-regulation of TGF-\(\beta\),\(^16,17\) no animal model has been developed to study the relationship between HBV and HCV and ICC. Previous results have demonstrated that HCP facilitates tumorigenesis in the zebrafish liver.\(^18\) Therefore, we investigated whether the coexpression of the HBx and HCP proteins can induce fibrosis and consequently contribute to ICC formation in zebrafish. We established single- and double-transgenic zebrafish lines (expressing one or two viral genes, respectively) and analyzed the histology and gene expression profiles of ICC in these zebrafish.

Materials and Methods

Vector Construction. The liver-specific tetracycline transactivator (tTA) construct pT2-LF-tTA was generated by modifying the T2KXIGD in plasmid,\(^19\) in which L-FABP promoter was used to control the liver specificity.\(^20\) (Supporting Experimental Procedures).

Microinjection of Zebrafish Embryos and Production of Transgenic Zebrafish Lines. The transposase messenger RNA (mRNA) and vector were coinjected into embryos and excision efficiency assays were performed as described\(^21\) (Supporting Experimental Procedures).

Green Fluorescent Protein (GFP) Induction and Detection. The embryos derived from the HBx+HCP transgenic lines were maintained in water until 7 days postfertilization (dpf). The larvae were treated with 30 mg/mL Dox for GFP ablation. After induction or ablation, GFP expression was observed under a Zeiss Stemi SV 11 fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with fluorescence modules (Leica 216 MacroFluo, Heerbrugg, Switzerland) and enhanced filter cubes (Kramer Scientific, Hampton, NJ). Images were taken using a SPOTRTK E color 3-Shot CCD camera photographic system (Diagnostic Instruments, Sterling Heights, MI).

Identification and Mapping of Integration Sites. The genomic DNA was isolated from F5 transgenic line. The 3’ flanking fragment of expression cassettes were cloned and sequenced (Supporting Experimental Procedures).

Transcriptome Sequencing and Analysis. Total RNA was purified from a pool of partial liver of three fish from each transgenic zebrafish line (WT, HBx, HCP, and HBx+HCP) and sequenced using the SOLiD System 3.0 (Applied Biosystems, Foster City, CA). The primary analysis was performed using the SOLiD Analysis Tool Pipeline (Applied Biosystems). The data were expressed as log2-fold changes of gene expression. The gene ontology was categorized using MetaCore software (GeneGo). The log2-fold change of gene expression was determined by comparing the RPKM values of the HBx+HCP and HCP transgenic zebrafish.

Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Total RNA from fish liver tissue was purified and reverse transcribed to complementary DNA (cDNA). Quantitative RT-PCR was performed using Roche LightCycler 480 Real-Time PCR System (Supporting Experimental Procedures). The primer sequences are listed in Supporting Table S1.

Whole-Mount In Situ Hybridization. Sense and antisense digoxigenin probes for HBx and HCP were generated by in vitro transcription using T7 or SP6 RNA polymerase as described.\(^22\)

Histologic and Immunohistochemical Studies. Liver tissues were fixed with 10% formalin and embedded in paraffin. The 4-5-μm thick sections were cut and

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DOI 10.1002/hep.25914

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.
stained with hematoxylin and eosin (H&E) for histologic examinations. For immunohistochemical study, the antibodies included anti-TGF-β1 (AnaSpec, Fremont, CA), anti-p-38 (1:50), anti-p-ERK1/2 (Thr202/Tyr204, 1:100) (Cell Signaling Technology, Danvers, MA), anti-pSmad3L (1:200, Immuno-Biological Laboratories), anti-CK18 (cytokeratin 18; 1 μg/mL, LifeSpan Biosciences), anti-α-SMA (alpha smooth muscle actin; 1:200), and anti-PCNA (proliferating cell nuclear antigen; 1:1,000, Abcam, Cambridge, MA).

Terminal Transferase dUTP Nick End-Labeling (TUNEL) Assay. The 4.5-μm thick liver sections were labeled with fluorescein-dUTP and apoptotic cells were detected using in situ cell death detection kit (Roche Applied Science).

Immunofluorescence Assay. Fresh liver tissues were embedded in OCT medium and stored at −20°C until use. Ten-μm thick frozen sections were labeled with anti-zebrafish gut secretory cell epitopes antibody (1:200, FIS2F11/2, Abcam, Cambridge, MA) and counterstained with DAPI (4′, 6-diamidino-2-phenylindole) solution. Images were taken using a Leica TCS SP5 MP microscope system.

Mason’s Trichrome and Sirius Red Staining. Five-mm-thick formalin-fixed, paraffin-embedded sections were used for Mason’s trichrome and Sirius red stains using commercial kits (Sigma Aldrich, St. Louis, MO).

In Vivo Morpholino (MO) Knockdown. Twenty 2-month-old F4 HBx+HCP transgenic zebrafish were intraperitoneally injected with Vivo-MO twice per week for 1 month.23 Partial liver samples were obtained for further gene expression analysis (Supporting Experimental Procedures).

Western Blotting. Protein was isolated from liver tissues obtained from 3-month-old morphants. Western blotting was performed using a standard protocol (Supplementary Experimental Procedures).

Results

Generation of Liver-Specific Tet-Off Transgenic Zebrafish Lines. To establish inducible viral transgenes in zebrafish lines, we microinjected wildtype (WT) zebrafish embryos with four sets of two separate plasmids as following: HBx line, pT2-LF-tTA and pT2-GFP-BI-HBx; HCP line, pT2-LF-tTA and pT2-GFP-BI-HCP; HBx+HCP transgenic line, pT2-LF-tTA, pT2-HcRed-BI-HBx, and pT2-GFP-BI-HCP; and vector control (CT), pT2-LF-tTA and pT2-GFP-BI (Supporting Fig. S1A). We generated CT (Tg(LFABP,tTA; TRE-CMV:GFP)), HBx alone (Tg(LFABP,tTA; TRE-CMV:GFP; TRE-CMV:Hb)), HCP alone (Tg(LFABP,tTA; TRE-CMV:GFP; TRE-CMV:Hcp)), and HBx+HCP (Tg(LFABP,tTA; TRE-CMV:GFP; TRE-CMV:HcRed; TRE-CMV:HBx; TRE-CMV:HCP)) transgenic zebrafish lines. The stable expression of transgenes in each line was confirmed by fluorescent imaging (Fig. 1A; Supporting Fig. S1B) and RT-PCR (Supporting Fig. S1C). We performed whole-mount in situ hybridization to further confirm the expression of HBx and HCP in transgenic zebrafish liver. In Fig. 1B, HBx was specifically expressed in HBx and HBx+HCP fish livers (left panel). The HCP and HBx+HCP fish livers were labeled with the HCP probe (Fig. 1B, right panels). No signals were detected either in HBx+HCP line with sense probes or in HCP line with HBx-AS probe and HBx line with HCP-AS probe (Supporting Fig. S2). These results revealed that HBx and HCP are specifically expressed in transgenic fish livers. We then performed immunofluorescent assays to examine the specificity of the L-FABP
promoter. In the liver of CT zebrafish, L-FABP-driven GFP was not localized in the bile duct cells expressing 2F11/2 protein (red fluorescence) (Supporting Fig. S3). To identify the integration locus, we sequenced the 3' flanking fragment of expression cassettes in HBx, HCP, and HBx+HCP lines. The results showed that only tTA cassette inserted in the exon of a predicted gene (LOC100150696 similar to Zinc finger protein 264) in HBx line (Table S2). The other 10 integration loci of cassettes are not tagged genes or intron of genes.

**Induction of ICC in the Livers of HBx+HCP Transgenic Zebrafish.** Histologically, the livers of F3 HBx+HCP transgenic zebrafish exhibited several morphologic alterations including cytoplasmic vacuolation (Fig. 2A, left panel), bile duct dilation (Fig. 2A, middle panel), and fibrosis (Fig. 2A, right panel) as early as 1 month of age. In 3-month-old HBx+HCP zebrafish, some of the fluorescent areas in the liver were no longer present (Supporting Fig. S4). The liver of HBx+HCP zebrafish lines was almost replaced by ICC composed of proliferation and infiltration of irregularly shaped and variably sized neoplastic glands surrounded by a dense fibrous stroma (Fig. 2B). In contrast, none of the zebrafish carrying a single viral gene or the vector had ICC formation (Supporting Fig. S5). At 1 month, 25% of the HBx+HCP zebrafish exhibited fibrosis. This percentage increased to 45% at 3 months and included 35% of zebrafish with early and severe ICC (Table 1). Both Masson’s trichrome and Sirius red stains revealed fibrosis in HBx+HCP transgenic zebrafish (Fig. 3A, B), whereas no fibrosis was observed in the liver of HBx, HCP, CT, and WT zebrafish lines (Fig. 3C-F, respectively).

**Table 1. Histological Subject Characteristics**

<table>
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<tr>
<th>Age (month)</th>
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<th>HBx</th>
<th>HCP</th>
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<th>WT</th>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>65%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>25%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Bile duct dilation</td>
<td>15%</td>
<td>20%</td>
<td>30%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Bile duct proliferation</td>
<td>30%</td>
<td>15%</td>
<td>45%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Bile duct fibrosis</td>
<td>25%</td>
<td>20%</td>
<td>45%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>ICC</td>
<td>10%*</td>
<td>0%</td>
<td>0%</td>
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<td>0%</td>
</tr>
</tbody>
</table>

The sample size of each group is 20.

The extent of involved area is less than 50% of total areas (early ICC). In most severe cases, the entire liver was nearly replaced by proliferating neoplastic bile ducts (severe ICC).

*Early ICC; †20% early ICC and 15% severe ICC.
To confirm that these phenotypes were induced by the transgenes, we turned off transgenes expression using 30 μg/mL Dox treatment. No significant phenotype was observed in the liver of Dox-treated HBx+HCP transgenic zebrafish at 1.5 or 3 months (Supporting Figs. S3B, S6A). Furthermore, fibrosis and ICC were induced in the liver of 4.5-month-old HBx+HCP zebrafish after inducing transgene expression at 1.5 months of age (Supporting Fig. S6C). We then performed immunohistochemistry (IHC) and TUNEL assays to examine the fate of the tumor cells. Using an antibody against PCNA, we found that liver of HBx+HCP zebrafish showing prominent fibrosis by Masson’s trichrome (A) and Sirius red (B) stains. Absence of fibrosis in the liver of (C) HBx, (D) HCP, (E) CT, and (F) WT zebrafish.

![Immunohistochemistry and TUNEL images](image)

**Fig. 3.** Fibrosis demonstrated by Mason’s trichrome (blue) and Sirius red (red) stains in all zebrafish lines. Liver of HBx+HCP zebrafish showing prominent fibrosis by Masson’s trichrome (A) and Sirius red (B) stains. Absence of fibrosis in the liver of (C) HBx, (D) HCP, (E) CT, and (F) WT zebrafish.

**Gene Expression Analysis of ICC.** To analyze the biological significance of regulatory pathways involved in viral protein-induced ICC, we investigated the global gene expression in liver samples from each zebrafish line to define common and differential gene expression signatures and dysregulated genes. We sequenced mRNA purified from the livers of WT, single-transgenic zebrafish, and tumor samples of double-transgenic zebrafish. We identified 75-102 million 50-bp sequence reads per sample (GEO database; accession number GSE34493). Approximately 34-44 million reads could be mapped onto the zebrafish genome (Supporting Table S4), and these reads were analyzed to obtain the differential expression data for the transgenic zebrafish liver samples using enrichment analysis with MetaCore. The data for the ICC samples were compared with those for the HCP samples (Supporting Table S5). The results show that the different gene expression profiles in the ICC include genes involved in cytoskeletal remodeling, cell adhesion, development, and cell cycle regulation among the top 10 GeneGo pathways (Fig. 4A). The different expression profiles of genes are related to cell cycle processes, development, proteolysis, immune responses, and transcription among the top 10 GeneGo process networks (Fig. 4B). Furthermore, the expression profiles were consistent with genes involved in regulating several types of neoplasms among the top 10 GeneGo biomarker networks (Fig. 4C). The quantitative features of the potential cholangiocarcinoma marker genes in zebrafish were consistent with those in humans (Table 2).

**JNK/pSmad3L Oncogenic Pathway Activation in ICC.** TGF-β-dependent JNK/pSmad3L oncogenic pathway activation has been observed in rat hepatic stellate cells and myofibroblasts after liver injury.24,25 The status of the TGF-β-dependent JNK/pSmad3L oncogenic pathway can be used to evaluate the risk for developing hepatocellular carcinoma (HCC) in HBV-infected patients.26 Based on the results of the gene expression analysis, TGF-β-dependent epithelial-mesenchymal transition (EMT) and cell proliferation occurred among the top 10 rankings (Fig. 4A). To identify the TGF-β-dependent JNK/pSmad3L oncogenic pathway that might be responsible for cholangiocarcinogenesis, we evaluated the expression of TGF-β1- and pSmad3L-related genes using qRT-PCR and compared these data to the transcriptome sequencing data. The results demonstrated that all of the selected genes (tgfb1, smad2, smad3, p38, erk1, kras, csgf, ccnd1, and vegfa) were up-regulated in ICC samples (Supporting Fig. S9). The analysis of the TGF-β1 expression revealed that the liver exhibited a robust...
TGF-β1 signal in HBx+HCP zebrafish liver (Fig. 5). In Fig. 5, fibrosis was further confirmed by evaluating the expression for α-SMA (marker for activated stellate cells), HBx+HCP liver revealed higher levels of expression in ICC when compared with HBx fish (HBx+HCP, Fig. 5; HBx, Supporting Fig. S10). No α-SMA was detected in HCP fish liver (Supporting Fig. S10). We then investigated the phosphorylation level and localizations of pp38, pERK1/2, and pSmad3L, which are downstream of TGF-β1, during HBx- and HCP-induced cholangiocarcinogenesis in HBx+HCP zebrafish livers. As shown in Fig. 5, high level of pp38, pERK1/2, and pSmad3L were detected in the nuclei of cholangiocytes, which are neoplastic bile duct epithelial cells. In contrast, less of the phosphorylated proteins were detected in the livers isolated from HBx or HCP transgenic zebrafish (pp38, Supporting Fig. S10; pERK1/2 and pSmad3L, Supporting Fig. S11). ICC isolated from HBx+HCP zebrafish livers were strongly positive for CK18 (bile duct cell marker in zebrafish) labeling using IHC compared with HBx or HCP zebrafish livers (HBx+HCP, Fig. 5; HBx or HCP, Supporting Fig. S11).

Reduced Fibrosis and ICC by TGF-β1 Knockdown. To determine whether TGF-β1 plays a role in bile duct neoplasms, we intraperitoneally injected tgfb1-MO into 2-month-old zebrafish twice per week. The correlation between tgfb1-MO administration and phenotypes was evaluated using the chi-squared test. The results showed that the percentage of zebrafish with bile duct proliferation, fibrosis, and ICC was significantly reduced after injection for 1 month (Table 3). The suppressed expression of tgfb1 in 3-month-old morphants was further confirmed using RT-PCR (Supporting Fig. S12A). The TGF-β1 downstream proteins, including phosphorylated p38, ERK1/2, and Smad3L were decreased in MO-injected zebrafish livers (Supporting Fig. S12B). At the RNA level, only tgfb1 and mmp9 were significantly decreased in tgfb1 morphants (Supporting Fig. S12C). These results indicate the down-regulation of protein phosphorylation by TGF-β1 knockdown.

Discussion

Zebrafish are an ideal model for studying embryonic development because their transparency allows one to observe their dynamic physiology in a noninvasive manner. Additionally, zebrafish are a good animal model for studying human diseases due to their ability to produce pathological phenotypes comparable to those in humans. For instance, a zebrafish carcinogen-induced liver tumor displays a transcriptome profile comparable to that of human liver cancer. This conservation emphasizes the potential of zebrafish as a...
model for liver cancer research.\textsuperscript{31} Although evidence linking HBV and HCV infection to ICC development is based on statistical analyses of patient samples,\textsuperscript{2,7} the mechanisms by which HBV and HCV mediate ICC development remain largely unknown. In this study, our transgenic zebrafish model is the first to provide correlative evidence that ICC can be induced by the coexpression of HBx and HCP. We substantiated this conclusion by repressing or reinducing transgenes in the adult liver using Dox treatment. ICC of this model displays the histopathologic characteristics that are highly identical to human ICC as well as the molecular features of human diseases, including the neoplasm networks and potential biomarkers.

Tumor stroma provides the microenvironment necessary for cancer growth.\textsuperscript{45} TGF-\(\beta\) is one of the cancer-derived cytokines that drive the conversion of normal fibroblasts into cancer-associated fibroblasts. A previous study demonstrated that HBx induces TGF-\(\beta\) secretion from hepatocytes and activates hepatic stellate cells (HSCs) to promote collagen secretion. These data suggest a role for HBx in HBV-related liver fibrosis.\textsuperscript{17} In this model, TGF-\(\beta1\) and connective tissue growth

### Table 2. Conserved Quantitative Features of Potential Cholangiocarcinoma Marker Genes Between Zebrafish and Human

<table>
<thead>
<tr>
<th>EntrezGene IDs from Zebrafish</th>
<th>EntrezGene IDs from Human†</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Gene Synonyms</th>
<th>RPKM Value in Zebrafish ICC</th>
<th>Expression Change in Zebrafish ICC (log2 ratio)</th>
<th>(qRT-PCR) (log2 ratio)</th>
<th>Reference (PubMed ID)</th>
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<td>565373</td>
<td>375790</td>
<td>AGRN</td>
<td>agrin</td>
<td>agrin proteoglycan</td>
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<td>up (2.21)</td>
<td>4.39</td>
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<td>30222</td>
<td>595</td>
<td>CCND1</td>
<td>cyclin D1</td>
<td>G1/S-specific cyclin D1</td>
<td>0.064</td>
<td>up (4.0)</td>
<td>6.16</td>
<td>16142313</td>
</tr>
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<td>393775</td>
<td>1191</td>
<td>CLU*</td>
<td>clusterin</td>
<td>testosteron-repressed prostate message 2</td>
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<td>up (2.63)</td>
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<td>7852</td>
<td>CXCR4*</td>
<td>chemokine (C-X-C motif) receptor 4</td>
<td>leukocyte-derived seven transmembrane domain receptor 4-receptor tyrosine-protein kinase erbB-1</td>
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<td>up (2.28)</td>
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\*It is uniquely up-regulated in zebrafish ICC.
†Those genes are up-regulated in human cholangiocarcinoma samples.
factor (CTGF) were highly expressed in the HBx-HCP zebrafish livers. In addition, we detected collagen fiber and \( \alpha \)-SMA accumulation around abnormal bile ducts. Several TGF-\( \beta \)-dependent pathways were ranked among the top 10 pathways using MetaCore software. In the HBx-HCP zebrafish livers, TGF-\( \beta \)1 was detected in the tissues surrounding the tumor that indicate the involvement of TGF-\( \beta \)1 in HBx- and HCP-induced stroma. To elucidate the molecular mechanisms involved in HBx- and HCP-induced ICC, we suppressed \( tgf \beta 1 \) expression. We showed that fibrosis and ICC formation as well as decreased pSmad3L signaling were markedly reduced by knockdown of TGF-\( \beta \)1 in HBx- and HCP-induced ICC.

More severe liver diseases have been identified in patients with coinfections of HBV and HCV, although another study reported interference between HBV and HCV. In contrast, a previous study using an in vitro system has shown that the replication of both viruses proceeded without interference. More severe liver diseases have been identified in patients with coinfections of HBV and HCV, although another study reported interference between HBV and HCV. In contrast, a previous study using an in vitro system has shown that the replication of both viruses proceeded without interference. Moreover, HBx increased liver pathogenesis in HCV transgenic mice by causing an imbalance between hepatocyte death and regeneration within the context of severe steatosis. HBx and HCP significantly reduced the sensitivity to reactive oxygen species (ROS)-induced cell death and induced survival benefits in HepG2 cells. Our results show that the ICC induced by coexpression of HBx and HCP are histologically and genetically similar to their human counterparts, thus supporting the synergistic role of both viruses in the development of liver disease. Collectively, this study demonstrates the potential of this zebrafish model to study HBV- and HCV-induced fibrosis and bile duct neoplasms.

Acknowledgment: The authors thank the Core Facility of the ICOB, Academia Sinica, and the Taiwan Mouse Clinic for technical support. The authors also thank the Taiwan Zebrafish Core Facility at Academia Sinica (ZCAS) for providing AB wildtype fish.

Table 3. Histological Subject Characteristics of Morphants

<table>
<thead>
<tr>
<th>Control MO (n = 17)</th>
<th>TGF-( \beta )1 MO (n = 19)</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unremarkable</td>
<td>35%</td>
<td>58%</td>
</tr>
<tr>
<td>Bile duct dilation</td>
<td>12%</td>
<td>16%</td>
</tr>
<tr>
<td>Bile duct proliferation</td>
<td>53%</td>
<td>32%</td>
</tr>
<tr>
<td>Bile duct fibrosis</td>
<td>53%</td>
<td>32%</td>
</tr>
<tr>
<td>ICC</td>
<td>29%*</td>
<td>11%†</td>
</tr>
</tbody>
</table>

Chi-square was used to determine the relationship between TGF-\( \beta \)1 and each phenotype with unremarkable.

The criteria of early and severe ICC are the same as defined in Table 1.

*12% early ICC and 17% severe ICC; †early ICC.

References


