Thioacetamide accelerates steatohepatitis, cirrhosis and HCC by expressing HCV core protein in transgenic zebrafish *Danio rerio*

Ravikumar Deepa Rekha a,1, Aseervatham Anusha Amali a,1, Gour Mour Her b, Yang Hui Yeh a, Hong-Yi Gong a, Shao-Yang Hu a, Gen-Hwa Lin a, Jen-Leih Wu a,c,*

a Laboratory of Marine Molecular Biology and Biotechnology, Institute of Cellular and Organismic Biology, Academia Sinica, NanKang, Taipei 11529, Taiwan
b Graduate Institute of Biotechnology, National Taiwan Ocean University, Keelung, Taiwan
c Institute of Fisheries Sciences, National Taiwan University, Taipei, Taiwan

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**Abstract**

Hepatocellular carcinoma (HCC) is one of the common cancers worldwide, caused by Hepatitis C virus (HCV) and hepatotoxins. Here we report the development of HCC in wild type as well as HCV core protein (HCP)-transgenic zebrafish upon treatment with a hepatotoxin, thioacetamide (TAA). Two-fold accelerated HCC development could be achieved in the TAA-treated transgenic fish, that is, the progression of the disease in TAA-treated wild type zebrafish developed HCC in 12 weeks whereas that of HCP-transgenic zebrafish shortened the HCC progression to 6 weeks. Histopathological observation showed the specific pathological features of HCC. The HCC progression was confirmed through RT-PCR that revealed an up and down regulation of different marker genes at various stages of HCC progression such as, steatohepatitis, fibrosis and HCC. Moreover, HCV core protein expressed in the HCP-transgenic zebrafish and TAA synergistically accelerate the HCC development. It must be mentioned that, this is the first report revealing HCV core protein along with TAA to induce HCC in zebrafish, particularly, in a short period of time comparing to mice model. As zebrafish has already been considered as a good human disease model and in this context, this HCC-zebrafish model may serve as a powerful preclinical platform to study the molecular events in hepatocarcinogenesis, therapeutic strategies and for evaluating chemoprevention strategies in HCC.

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**Keywords:** Hepatocellular carcinoma; Thioacetamide; Zebrafish

**Abbreviations:** HCC, hepatocellular carcinoma; HCP, hepatitis C virus core protein; HCV, hepatitis C virus; TAA, thioacetamide.

* Corresponding author at: Laboratory of Marine Molecular Biology and Biotechnology, Institute of Cellular and Organismic Biology, Academia Sinica, 128, Academia Road, Section 2, NanKang, Taipei 11529, Taiwan. Tel.: +886 2 27899568; fax: +886 2 27824595.

E-mail address: jlwu@gate.sinica.edu.tw (J.-L. Wu).

† Both authors contributed equally.

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, being the fourth leading cause of cancer death worldwide (Parkin, 2000). Development of HCC involves multiple steps including, steatosis, fibrosis, chorirosis, adenoma and carcinoma (Tarantino et al., 2007). Hepatitis B and hepatitis C are the major viruses causing HCC however, hepatitis C virus (HCV) infection is the main cause of chronic hepatitis. The rise in the incidence as well as the mortality due to HCC recently observed in most industrialized countries likely reflects the increased prevalence of HCV infection (El-Serag et al., 2003). HCV core protein (HCP) modulates gene transcription, cell proliferation, cell death, oxidative stress, and immunomodulation in host cells, including hepatocytes, and is involved in the pathogenesis of hepatocellular carcinoma (HCC) (Lai, 2002).

Thioacetamide (TAA), a well-known hepatotoxin, has been considered to be an inducer of liver cirrhosis (Nozu et al., 1992). The bioactive metabolites of TAA namely TAA-sulfoxide and TAA-sulfidioxide, are well known hepatotoxins (Novosyadlyy et al., 2005) which causes hepatocellular necrosis in perivenous areas of the liver acinus. Prolonged administration of TAA leads to hyperplastic liver nodules, liver cell adenomas and hepatocarcinomas (Yeh et al., 2004). It has been demonstrated in rats that regenerative nodules and liver fibrosis are more prominent in the cirrhotic model induced by TAA than in the model induced by carbon tetrachloride and that the histology of the TAA model more closely resembles that of human cirrhosis (Zimmermann et al., 1987). The present study explores the acceleration of HCC development using TAA in HCP-transgenic zebrafish. While changes in expression of treatment options for HCC patients. Therefore, it is extremely important to identify new therapeutic targets for treatment of this malignancy. The zebrafish is a good human disease model, so the HCC-zebrafish model may serve as a valuable platform to study the molecular events in hepatocarcinogenesis, and to evaluate preventive and therapeutic strategies. Furthermore, the results of this study have given the confidence of using the zebrafish as a cancer model. When coupled with carcinogen treatment, the susceptible zebrafish strains can serve as powerful models for understanding the mechanism of carcinogenesis and are also excellent in vivo systems for rapid screening of genetic or chemical modifiers that can suppress certain cancer phenotypes. Thus, by using the zebrafish model, carcinogenesis, rapid screening of modifiers and high-throughput genomic analyses, can be performed in vivo using the same organism.

2. Materials and methods

2.1. HCP-transgenic zebrafish

A transgenic line of zebrafish, expressing liver-specific HCP was generated by microinjecting the Not I- and Sf I-digested dual-expression vector pLF2.8-HCV-core (Fig. 1A) into the blastomere of early one-cell-stage embryos. RFP-positive larvae were picked out for examining HCP expression in hepatocytes. Adult zebrafish (Danio rerio) were obtained from a local aquarium, and a transgenic LF2.8-TG1 line was generated as described elsewhere. The zebrafish were maintained in a controlled environment with 14-h light/10-h dark cycle at 28°C. For experiment, we used 2 months old female zebrafish and injected 300 mg/kg TAA intraperitoneally, three times in a week.

2.2. RT-PCR analysis

First strand cDNA was synthesized from 5 μg of total RNA using Thermoscript™ RT-PCR system (Invitrogen). After the reverse transcription reaction, the cDNA template was amplified by polymerase chain reaction with Taq polymerase (Invitrogen). PCR was performed with 2 μl cDNA using a programme comprising, 1 cycle 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. After 35 cycles, the reaction mixtures were incubated at 72°C for an additional 7 min to allow complete synthesis. The RT-PCR products were subjected to 2% agarose gel electrophoresis. Max was used as an internal control. The primers used were given in Table 1.

2.3. Western blotting

Total protein extract from the adult control and HCP over-expressed liver was prepared using lysis buffer and separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked by net buffer at 4°C overnight and the bands were detected using the anti-hepatitis C virus monoclonal antibody.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<tr>
<td>ACC</td>
<td>5'-TTA GAC CTG GAT CAA CGG CG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CAT GAT CTG TCC TGT ACG GG-3'</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>5'-AGG CTT AGA CTG TGA ACG GTG GGA C-3'</td>
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<tr>
<td></td>
<td>5'-AGC AGG TGT GTC CAG ATG TTT CCA G-3'</td>
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<tr>
<td>C/EBPα</td>
<td>5'-ACC ATG GAG CAA GCA AAC TTC TAC GAG G-3'</td>
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<td>5'-AGC GCA GTT GCC CAT GGC TTT GAC G-3'</td>
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<tr>
<td>PTL</td>
<td>5'-TCC GTG TGC ACA TTC TCA AC-3'</td>
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<td></td>
<td>5'-CGG GGA ACG TAG AGA CAT GC-3'</td>
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<td>5'-TCAT GTC CAC TGA GGC CTC CCA GAA CAT TAC-3'</td>
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<tr>
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<tr>
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<td>5'-GGT TGC ATT CCA GGA GTG TGC -3'</td>
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<td>5'-GCT AAA GAC CTC TAT TAC CTC GAG ATG ACC-3'</td>
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<td>5'-CCA CAG TCA CCA CAT CAA TTT CCT CTC CC-3'</td>
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<tr>
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<td>5'-CGA AAG GAA AAG AGC GAG GTC G-3'</td>
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<tr>
<td>Cyclin D1</td>
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<tr>
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<td>5'-CTT GTG GCT AAC GTA GTT TTC C-3'</td>
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Fig. 1. Expression of HCP in transgenic zebrafish. (A) Map of dual-expression transgenic vector, pLF2.8-HCV-core, containing two expression cassettes. The HCP transgene and RFP marker are driven by L-FABP and CMV promoters, respectively. (B) RT-PCR for HCP in WT and HCP-transgenic zebrafish; max was used as an internal control. (C) Expression of HCP in WT and HCP-transgenic zebrafish by Western blotting.

2.4. Histological analysis

Liver from WT and HCP-transgenic zebrafish was fixed in 4% paraformaldehyde and embedded in agarose, and cryosectioned (10 μm). Section was stained with haematoxylin and eosin. Liver fibrosis was determined using Sirius red staining. Direct red 80 and fast green FCF (color index 42053) were obtained from Sigma–Aldrich Diagnostics (St. Louis, MO). The liver was diced into 5 mm × 5 mm sections, immersion fixed in PBS containing 4% paraformaldehyde for 24 h at 4 °C, 10 μm sections were mounted on glass slides. Sections were deparaffinized and the slides were dehydrated as follows, with a wash for each 5 min step: xylene (×2), 100% ETOH, 95% ETOH, 70% ETOH, 30% ETOH, 1 × PBS, and distilled water (×2). The sections were incubated for 30 min in room temperature with an aqueous solution of saturated picric acid containing 0.1% fast–green FCF and 0.1% direct red 80. The sections were washed slowly under running distilled water for 6 min, dehydrated (for each step 3 min), mounted, and examined by light microscopy.

3. Results

3.1. Expression of HCP in the zebrafish transgenic liver

To examine the expression of HCP transgene in the zebrafish liver, we performed RT-PCR and Western blotting and observed the over expression of HCV core protein in transgenic male and female and no expression in the control. The results obviously show the HCP expression in the transgenic zebrafish (Fig. 1B and C). However, no pathologic changes were evident in the transgenic fish indicating the inability of HCP alone to induce HCC in zebrafish.

3.2. Pathogenesis of HCC in WT and HCP transgenic fish treated with TAA

The gross morphology of the liver of WT and HCP transgenic fish was normal, whereas with TAA treatment, WT fish showed unevenness on the liver surface after 12 weeks, and HCP-transgenic fish exhibited pale color, multiple cystic structures, and adenoma on the surface after 6 weeks (Fig. 2).

Histological analysis of liver from WT (n = 25) and HCP-transgenic fish (n = 23) showed normal cell structure with well-preserved cytoplasm and prominent nuclei and nucleoli (Figs. 3A and 4A). After 1 week of TAA treatment, we observed mild steatosis in WT fish (Fig. 3B), whereas HCP-transgenic fish treated with TAA showed higher steatosis (Fig. 4B). However, after 2 weeks of TAA treatment, the liver cells of WT (n = 19) (Fig. 3C) and HCP-transgenic fish (n = 23) (Fig. 4C) showed severe steatosis. Many hepatocytes are balloononed with an occasional focus of necrosis and the cell membrane of many swollen and balloononed hepatocytes are indistinct and lysed. The TAA-treated livers from both WT and HCP-transgenic fish exhibited severe lipid changes (Figs. 3D and 4C), vacuoles that coalesced to form larger vacuoles displacing the nucleus to the periphery, and mild sinusoidal fibrotic changes. Sirius red staining showed positive signal both in wild type (n = 16) and HCP fish (n = 20) treated with TAA. TAA-induced fibrosis exhibited characteristic fibrous connective tissue and proliferation of bile duct cells was observed in (Figs. 3E and 4D). The damage in bile duct was also observed as early as 4 weeks (Fig. 4E and F) in
the transgenic fish when compared to the WT which showed the bile duct damage at 10 weeks (Fig. 3F) of post treatment. The fibrogenesis was observed at 2 weeks in the HCP-transgenic model, much earlier than that of WT.

The WT fish showed hepatic nodules after 12 weeks of TAA treatment (Fig. 3G). The transgenic model showed nodules, arising as highly differentiated HCC in trabecular structures and also nodule in nodule formation, within 6 weeks (Fig. 4G). Most hepatic nodules exhibited a pathology characterized by nodule-in-nodule formation and HCC at low degrees of differentiation developed adenomas whereas in higher stages those cancer cells pressed the adjacent non-tumorous hepatocytes. This was evident in our observation, as the HCP-transgenic model ($n=17$) (Fig. 4H) produced more trabecular structures at high levels of differentiation when compared to the WT ($n=12$) (Fig. 3H). Figs. 3I and 4I are the 40× magnification.

3.3. TAA accelerates the progression of HCC in HCP transgenic line

To investigate and confirm the progression of the HCC, we analyzed the expression of marker genes of various stages of liver disease (Figs. 5 and 6). The first stage of disease development was steatohepatitis, for which we used the lipogenic genes, acetyl-CoA (ACC); the adipocyte specific marker, adiponectin; CCAAT/enhancer binding protein (C/EBPα), the transcriptional activators which are important for adipocyte differentiation; and peroxisomal 3-ketoacyl-CoA thiolase (PTL), one of the PPARα regulated peroxisomal fatty acid β-oxidation system enzymes. The ACC expression was very low at around 2 weeks in WT TAA-treated fish, while its expression was significantly higher in HCP-transgenic fish, showing the severity of steatohepatitis. Similarly, other indicators such as PTL, C/EBPα, and adiponectin were also significantly increased during the steatohepatitis stage, perhaps due
to accumulation of lipids in the hepatocytes resulting in the appearance of adipocyte markers and fat droplets (Matteoni et al., 1999).

The next stage, fibrosis, was assessed by analyzing major fibrosis markers, collagen α1, transforming growth factor β1 (TGF β1), connective tissue growth factor (CTGF), tissue inhibitors of metalloproteinases (TIMP), matrix metalloproteinase (MMP2), heparanase, and leptin receptor. There was significant increase in these markers at 4–8 weeks in WT livers and from 2 to 4 weeks in HCP-transgenic livers. Collagen α1 is regarded as the most prevalent extracellular matrix (ECM) protein in hepatic fibrosis (Lee et al., 1995). TGF β1 is increased in experimental and human hepatic fibrosis (Gressner and Bachem, 1995). In our model also, the level of expression of this gene increased, reflecting the initiation of fibrosis. CTGF upregulates several ECM components, including collagen in fibroblasts, and is one of the downstream effectors of TGF β1 (Paradis et al., 2001). Both TGF β1 and CTGF are reported to induce connective tissue cell proliferation in vitro and in vivo and to stimulate extracellular matrix synthesis (Tamatani
et al., 1998). The decrease in collagenolytic activity observed in chronic liver disease can be attributed largely to increased TIMP expression (Arthur et al., 1999). TAA induced liver fibrosis is associated with increased levels of MMP-2 and heparanase (Goldshmidt et al., 2004). Increased expression of MMP-2 has also been reported in HCV-induced cirrhosis (Lichtinghagen et al., 2003). In our study, heparanase levels increased markedly after 2 weeks TAA treatment. With increased fibrosis there has been a reported decrease in the levels of heparanase, which suggests a different regulatory mechanism for heparanase. This correlates with our results and implies that heparanase expression depends on the architecture and proper function of the liver tissue.

The expression profile of the tumor markers showed consistent alterations in the tumor suppressor genes p53 and RB, oncogenes c-myc and survivin, cell cycle related gene cyclin D1, and the insulin-like growth factor
2 (IGF-2). The tumor suppressor gene p53 and RB (Figs. 5c and 6c) were down regulated in the wild type treated with TAA and HCP treated with TAA. The other genes like c-myc, cyclin-D, IGF-2, survivin were upregulated evident that HCC formation in zebrafish.

Fig. 7 shows the confocal data obtained from hepatocytes of the TAA treated in liver specific GFP transgenic fish (Her et al., 2003). It shows the damage in the liver with fat droplets and after 12 weeks of treatment it caused severe disruption of the cells and also the intensity of the fluorescence decreased significantly. In the control fish (Fig. 7a) hepatocytes are densely packed and distinct. We can see vacuoles in (Fig. 7b) after 1 week of injection and in the second and 8 week the hepatocytes are dispersed and in the 12th week no distinct hepatocytes can be observed.

4. Discussion

Here we tried to figure out the characteristics of a HCV core protein (HCP) expressing transgenic zebrafish
that accelerates the development of HCC upon treatment with a hepatotoxin. To begin with, we assessed the quality of transgenic fish for HCP expression in liver. Our data showed that HCP expressed well without producing any pathologic changes in the fish. This suggests that HCP cannot induce HCC, but may function as a cofactor. Hepatitis C virus gene products have been expressed either alone or in combination in the liver of transgenic mice by using different liver-specific promoters. As already mentioned, three different HCV core transgenic lines develop liver steatosis and HCCs (Lerat et al., 2002); but other animals show only steatosis (Perlemuter et al., 2002) or different phenotypes (Okuda et al., 2002), depending on the factors such as, the promoter used, the context of expression and the mouse strain background. The NS5A transgenic mice, in spite of the pleiotropic functions of the protein in vitro, do not have any significant phenotype (Majumder et al., 2003).

The liver as seen in the histopathology showed severed deposition of lipid droplets, which can be anticipated that the free fatty acids in the liver may be oxidized to triglycerides and cholesterol by mitochondria, and if the free fatty acids exceed the capacity of mitochondrial oxidation, fatty acid accumulation in the liver occurs (Reid, 2001). These histologic findings are strikingly similar to that reported for non-alcoholic steatohepatitis (NASH) in humans. Increased connective tissue was observed in the hyperplastic bile ductular cells and the activated stellate (Ito) cells. TAA induces fibrosis and it was also reported that HCV proteins stimulate the fibrogenesis by interacting with hepatic stellate cells (HSCs) (Bataller et al., 2004). HCV infects hepatocytes that release profibrogenic substances like ROS, cytokines which in turn activate the neighboring HSCs (Schuppan et al., 2003).

The significant changes in the steatosis marker genes like ACC, adiponectin, C/EBPα and PTL may be due to the tremendous accumulation of the lipids in the hepatocytes, because of an up regulation of PPAR which ultimately resulted in the appearance of adipocyte markers and fat droplets (Horie et al., 2004). The PTL is one of the H₂O₂-generating enzymes belonging to the peroxisomal β-oxidation system (Reddy and Hashimoto, 2001). The reduction in the H₂O₂ degrading enzymes, catalase and glutathione peroxidase with that of disproportionate increase in H₂O₂ generating enzymes leads to sustained oxidative stress in the liver (Gonzalez et al., 1998).

Similarly, the major changes in the fibrotic markers is also seen. The fibrogenic stimulation leads to the transdifferentiation of hepatic stellate cells (HSCs) to myofibroblastic cells to produce excessive extracellular matrix (ECM) (Yavrom et al., 2005). During this pro-
cess we observed the induction of the fibrogenic ECM gene namely Collagen α1, TGF β one of the autocrine cytokines, changes in the expression of ECM proteases MMPs and their inhibitors TIMP. It has been previously reported that MMP2 among several other MMPs degrades the basement membrane collagen so that denatured fibril collagens replace normal as ECM during fibrogenesis (Schuppan et al., 2001). During fibrosis TIMP levels are seen to be increased significantly while MMP levels increase modestly or remain relatively static (Hung et al., 2005). Cytokines are normally involved in matrix remodeling and TGF β enhances the collagen production. Upregulation of TGF β and Collagen α1 gene occurs in HSC during CCl4 induced fibrosis. It has been reported that serum leptin levels are increased in patients of alcoholic cirrhosis (McCullough et al., 1998) and also HSCs have been shown to produce leptin when they get activated (Potter et al., 1998). Leptin and its functional receptors play a crucial role in hepatic fibrogenesis through the upregulation of TGF β expression in the liver (Ikejima et al., 2005). The CTGF is highly profibrogenic molecule overexpressed in the fibrotic liver and it stimulates proliferation of the fibroblasts and ECM synthesis (Rachfal and Brigstock, 2003). HSCs are major producers of this factor and they are regulated by cytokines in particular TGF β (Ozaki et al., 2005). Heparanase activity has been reported to correlate with the metastatic potential of tumor cells in animal model (Ikuta et al., 2001). In human cancers it has been reported with a high heparanase expression in oral cancer (Koliopanos et al., 2001) and in pancreatic cancer (Ikeguchi et al., 2003). But in our results we find a decrease in this gene expression in HCC suggesting that it may not correlate with tumor progression. It has been reported recently that the hep mRNA may be lost during the malignant transformation of hepatocytes and it may result in an abnormal cell growth and may correlate with hepatocarcinogenesis (Edamoto et al., 2003).

Pathways dominated by two tumor suppressor genes, RB and p53, are the most frequently disrupted in cancer cells (Wu et al., 2002). One of the most common oncogenes associated with the pathogenesis of liver tumor is the MYC oncogene. Overexpression of MYC in animal models can induce HCC (Shachaf et al., 2004), and its inactivation is reported to effect sustained regression of invasive liver cancers (Kannangai et al., 2005). Our results show a significantly high expression of the gene, a clear indication for the onset of tumor. Survivin has been described as an anti-apoptotic protein which is suppressed by p53 and is overexpressed in HCCs. Cyclin D1 is a known oncogene and a key regulator of cell cycle progression. Amplification of the cyclin D1 gene and its overexpression has been associated with aggressive forms of human HCC (Kannangai et al., 2005). The expression pattern of these genes in our model increased, confirming the development of HCC. It was seen much earlier in the HCP-transgenic fish that were treated with TAA. ROS also plays an important role in the pathogenesis of liver tumor. The poorly differentiated HCC cells are more likely to proliferate and metastasize due to the much lower activities or expression of specific antioxidant enzymes that fail to scavange the ROS produced in the poorly differentiated HCC cells (Chen et al., 2003). In addition, it should be emphasized that alcohol is synergistic with HCV core protein in the induction of ROS (Moriya et al., 2001). In our model, we assumed that the HCV core protein may acts synergistically with TAA and shortens the time of the formation of HCC and increase the ROS production.

The HCV core transgenic animal model provides a molecular basis for studying the modification of clinical manifestations produced in viral liver disease by chemical or environmental factors. In our present study we demonstrate that HCP fishes treated with TAA involves early lipid accumulation and lipid peroxidation in hepatocytes which is followed by liver cell injury and inflammation, upregulation of profibrotic genes Colα1 and TIMP and eventually hepatic fibrosis and finally to adenoma and HCC at a much earlier time when compared to that of the wild type fish.

The recent report reveals the molecular similarities between zebrafish and a human liver tumor which extends to tumor progression (Lam et al., 2006). HCP induces HCC in transgenic mice (Moriya et al., 1998). These mice developed hepatic nodules at 16 and 19 months which progressed to well-differentiated HCC with trabecular features and cells containing cytoplasmic fat droplets. Our model produced similar results in the WT with TAA as early as 3 months and in the transgenic fish treated with TAA 1.5 months. Although the mechanism of the cofactor role of HCP remains unknown, the ability of HCV to accelerate HCC development may be due to the synergistic effect of TAA with HCV. Previous reports reveal that TAA, as well as such hepatotoxins as CCl4, and CHCl3, mediate their toxicity via the formation of free radicals, especially ROS, which interact with membrane unsaturated lipids, consequently promoting lipid peroxidation (Fadhel and Amran, 2002). HCP has been reported to interact directly with the mitochondria, impairing electron transport and thus increasing ROS production. Core protein is associated with the endoplasmic reticulum (Moradpour et al., 1996) and intracellular lipid droplets, and this amplifying effect of core protein on mitochondria makes the cells more sensitive to other
oxidative insults. Human hepatocellular carcinoma is a disease that is prevalent world-wide with limited treatment options available. Many factors may contribute to the poor prognosis of HCC, but lack of understanding of the molecular pathways involved before and during tumor progression has limited our aptitude to design effective treatments.

We conclude that HCP alone is not sufficient for carcinogenesis. Although there are many small animal models of chemical hepatocarcinogenesis, no useful small animal models of HCV-related hepatocarcinogenesis exist. Our zebrafish model is also unique in that it dramatically shortens the time of HCC development as compared to others. It will prove to be a useful tool for further study of HCC using new technologies of genomic analysis which may identify genetic defects that activate or suppress specific molecular pathways leading to HCC and also for high throughput drug screening.

References

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