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Activation of Hepatic Stellate Cells During Liver Carcinogenesis Requires Fibrinogen/Integrin αvβ5 in Zebrafish

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers and it usually develops from a background of liver fibrosis or inflammation. The crosstalk between tumor cells and stromal cells plays an important and stimulating role during tumor progression. Previously we found in a *kras*^{V12}–induced zebrafish HCC model that oncogenic hepatocytes activate hepatic stellate cells (HSCs) by up-regulation of serotonin and activate neutrophils and macrophages by up-regulation of cortisol. In the present study, we found a novel signaling transduction mechanism between oncogenic hepatocytes and HSCs. After *kras*^{V12} induction, fibrinogen was up-regulated in oncogenic hepatocytes. We reasoned that fibrinogen may bind to integrin αvβ5 on HSCs to activate HSCs. Consistent with this notion, pharmaceutical treatment using an antagonist of integrin αvβ5, cilengitide, significantly blocked HSC activation and function, accompanied by attenuated proliferation of oncogenic hepatocytes and progression of liver fibrosis. On the contrary, adenosine 5′-diphosphate, an agonist of αvβ5, activated HSCs significantly that further stimulated the tumor progression and liver fibrosis. Interestingly, in human liver disease samples, we detected an increased level of fibrinogen during tumor progression which indicated the potential role of fibrinogen signaling in HCC progression. Thus, we concluded a novel interaction between oncogenic hepatocytes and HSCs through the fibrinogen related pathway in both the zebrafish HCC model and human liver disease samples.

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Introduction

Hepatic stellate cell (HSCs) are liver specific mesenchymal cells. While quiescent HSCs are dormant, activated HSCs are involved in liver disease progression [1]. In a normal liver, HSCs as a vitamin A storing cells are in the quiescent and non-proliferative stage [2]. Upon liver injury, various cytokines, such as TGFB, IL1B and TNF, are produced to instigate the transformation of HSCs into disease-promoting state [3]. Activated HSCs are an important component in the liver tumor microenvironment (TME) and a key regulator of hepatic fibrosis and cirrhosis [4]. In human HCC patients, liver fibrosis shows a close relationship with HCC incidence and recurrence [5]. As we reported previously, serotonin activates HSCs through activation of serotonin receptor 2B and stimulates liver fibrosis, as characterized by deposition of collagen and laminin to the TME. Moreover, accelerated progression of liver fibrosis promotes tumor progression in our zebrafish HCC model [6].

Recently, it has been found that integrin, a surface receptor on HSCs could receive signal from TME and is critical to liver fibrosis [7]. Integrin signaling has been shown to be crucial for HSC survival and function. Disruption of integrin on HSCs inhibits HSC proliferation and induces HSC apoptosis through increase of Caspase 3 activity [8]. Pharmacological blockage of integrin attenuated liver fibrosis in carbon tetrachloride-induced hepatic fibrosis in mice [9].

Abbreviations: a-Sma, alpha-smooth muscle actin; dox, doxycycline; dpf, day post fertilization; dpi, day post induction; FACS, fluorescence-activated cell sorting; Gfap, glial fibrillary acidic protein; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; IF, immunofluorescence; IHC, immunohistochemistry; OH, oncogenic hepatocyte; TME, tumor microenvironment; WT, wild type

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We have previously generated several inducible HCC models in zebrafish by overexpression of an oncogene specifically in hepatocytes [9–13]. In our *kras*^{V12}-expressing zebrafish HCC model, we have observed a significant liver enlargement in 6-dpf (day post fertilization) larvae after 3 days of induction of *kras*^{V12} expression [14]. In the current study, we found that after 4 days of doxycycline exposure, both HSC density and ratio of activation were increased significantly. A blood glycoprotein, fibrinogen, was up-regulated after *kras*^{V12} induction in oncogenic hepatocytes (OHs). Fibrinogen has a high affinity to integrin and could activate it [15]. Pharmacological manipulation of integrin by its agonist, stimulated HSC proliferation and secretion of alpha-smooth muscle actin (a-Sma) and Tgfb1; meanwhile, we observed an accelerated tumor progression and a higher fibrosis level. In contrast, the antagonist treatment suppressed the secretion of a-Sma, Tgfb1 and attenuated both tumor and fibrosis progression. Furthermore, we confirmed that there was a progressively increased fibrinogen level in human patient samples with liver diseases, suggesting that fibrinogen signal is also involved in HCC progression in human patients.

Materials and Methods

Zebrafish Husbandry

All zebrafish were maintained according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore (Protocol Number: R17-0868). *Tg(fabp10:rtTA2s-M2; TRE2:EGFP-kras*^{G12V}*)* in a Tet-on system for induction of oncogenic *kras*^{G12V} expression in hepatocytes [10], *Tg(fabp10:DsRed; ela3l:EGFP)* for isolation of hepatocytes [16], and *Tg(hand2:EGFP)* for visualization of EGFP-labeled HSCs were used in this study and they are referred to as *kras*+, *fabp10*+, and *hand2*+ respectively.

Induction of Oncogene Expression and Chemical Treatments

Both *kras*^{V12} induction and chemical treatments were conducted in larvae from 3 dpf to 7 dpf. The chemicals used included doxycycline (dox) (20 μg/ml; D9891; Sigma), cilengitide (10 μm; SML1594; Sigma) and ADP (Adenosine 5′-diphosphate) (10 μm; A2754; Sigma). The dosages were selected based on the highest all-survival concentrations as determined in preliminary experiments [17,18].

Photography and Image Analyses

At the end of the chemical treatments, more than 20 larvae from each treatment group were selected for imaging analyses. All the larvae were anesthetized in tricaine (0.08%; E10521; Sigma) and immobilized in methylcellulose (3%; M0521; Sigma) before imaging. Each larva was photographed individually from the left lateral side using an Olympus microscope. 2D liver size were measured using ImageJ as previous described [19].

Histological and Immunocytological Analyses

All of the fish larvae were fixed in 4% paraformaldehyde (P6148; Sigma) in phosphate buffered saline, embedded in 1.5% Bacto-agar (A5306; Sigma), immersed in 30% sucrose (S7903; Sigma) in phosphate buffered saline and cryo-sectioned at 8 μm thickness using a Leica Cryostats CM1950, followed by immunofluorescence (IF) staining. The primary antibodies used included anti-proliferating cell nuclear antigen (PCNA) (FL-261; Santa Cruz Biotechnology, Dallas, TX), anti–Caspase 3 (C92-065; BD Biosciences, Singapore), anti–collagen I (ab23730; Abcam), anti-laminin (L9393; Sigma), anti-glial fibrillary acidic protein (Gfap) (154474; Abcam), anti-a-Sma (ab15734; Abcam, Singapore), anti-fibrinogen (sc69775; Santa Cruze) and anti-Tgfb1 (04-953; EMD Millipore, Billerica, MA). Anti-rabbit or anti-mouse secondary antibodies were purchased from Thermo Fisher Scientific. At least 10 fish larvae from each group were examined and at least one image was taken for each larva.

Fluorescence-activated Cell Sorting (FACS), RNA Extraction and RT-qPCR

7-dpf larvae (after removal of the tail from mid-intestine) were used for FACS using a cell sorter (BD Aria). Hepatocytes and HSCs were isolated from *fabo10+ (or kras+)* and *hand2*+ larvae respectively based on their dsRed or GFP expression. Total RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany). QuantiTect Whole Transcriptome Kit (Qiagen) was used to synthesize and amplify cDNA, as we described previously [20]. The sequences of PCR primers used are *β-actin* (forward: CTCTGGGTCACCGCTTCTTT; reverse: CAGATGCTCACGAAACCCCT), *fgα* (forward: GGGCAGAGAACGATGGTCAA; reverse: AGTCGAAGTTGGCGGTCAAT), *fgβ* (forward: CTCCATCCACGGCTATGTCC; reverse: CCAACGCCTGCCAAAATCAA); *fgγ* (forward: ACGACTTTGGTGACGATCCC; reverse: GACCATCGGGACTTGCGTAT); *itgav* (forward: GTACGAGGCTGACCTGATCG; reverse: AAGATCACACACGACCACCC; *itgb5* (forward: AACCCACTCAACAGGACAGC; reverse: ACATTCACAGAACGGACCCC). RT-qPCR was performed with cDNA as the template using the LightCycler ® 480 SYBR Green I Master system (Roche). Reactions were conducted in triplicate for each sample. Gene expression levels in each control or transgenic liver samples were normalized with the expression level of *β-actin* mRNA as the internal control. The $\log_2$ fold changes in expression in the transgenic samples as compared with the control samples were then calculated using the CT method [21] according to the formula: $\log_2$ fold changes = -ΔΔCT = -[(CT gene of interest–CT *β-actin*) transgenic sample–(CT gene of interest–CT β-actin) control sample].

Human Patient Samples

Paraffin-embedded human liver disease progression tissue microarray slides were purchased from Biomax, Inc (Derwood, MD) (LV8011a). Samples were classified into 4 groups: normal, inflammation, cirrhosis, and HCC. Information of histopathology of all patients was provided by Biomax. Patient sample slides were performed with H&E staining and IHC staining for fibrinogen.

Statistical Analyses

For statistical significance between two groups, 2-tailed unpaired Student t test was performed using GraphPad Prism version 7.00 for Windows. Statistical data are presented as means ± SEM.

Results

Kras^{V12}*-expression Induced Liver Proliferation and Fibrosis in Zebrafish Larvae*

In previous study, we observed a robust tumor progression with rapid increases of hepatocyte proliferation and fibrosis after *kras*^{V12} induction in zebrafish adult [6,20]. To examine *kras*^{V12}-induced tumorigenesis in zebrafish larva, heterozygous *kras*+ transgenic zebrafish were crossed with WT zebrafish to get *kras*+ and WT