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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Serotonin Activated Hepatic Stellate Cells Contribute to Sex Disparity in Hepatocellular Carcinoma

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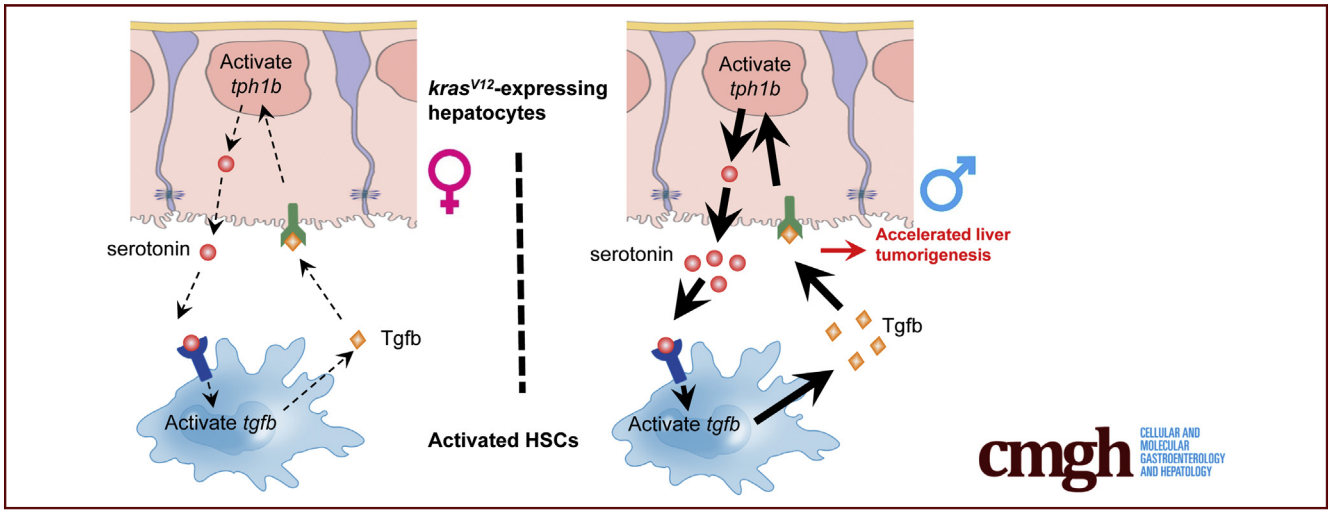

SUMMARY

A higher serotonin synthesis and accumulation in male zebrafish resulted in increased activation of hepatic stellate cells and transforming growth factor B1 expression and this has contributed to the accelerated progression of hepatocellular carcinoma in male zebrafish with activated *kras* oncogene expression.

BACKGROUND & AIMS: Hepatocellular carcinoma (HCC) occurs more frequently and aggressively in men than in women. Although sex hormones are believed to play a critical role in this disparity, the possible contribution of other factors largely is unknown. We aimed to investigate the role of serotonin on its contribution of sex discrepancy during HCC.

METHODS: By using an inducible zebrafish HCC model through hepatocyte-specific transgenic $kras^{V12}$ expression, differential rates of HCC in male and female fish were characterized by both pharmaceutical and genetic interventions. The findings were validated further in human liver disease samples.

RESULTS: Accelerated HCC progression was observed in $kras^{V12}$-expressing male zebrafish and male fish liver tumors were found to have higher hepatic stellate cell (HSC) density and activation. Serotonin, which is essential for HSC survival and activation, similarly were found to be synthesized and accumulated more robustly in males than in females. Serotonin-activated HSCs could promote HCC carcinogenesis and concurrently increase serotonin synthesis via transforming growth factor (Tgf)b1 expression, hence contributing to sex disparity in HCC. Analysis of liver disease patient samples showed similar male predominant serotonin accumulation and Tgfb1 expression.

CONCLUSIONS: In both zebrafish HCC models and human liver disease samples, a predominant serotonin synthesis and accumulation in males resulted in higher HSC density and activation as well as Tgfb1 expression, thus accelerating HCC carcinogenesis in males. *(Cell Mol Gastroenterol Hepatol 2017;3:484–499; http://dx.doi.org/10.1016/j.jcmgh.2017.01.002)*

Keywords: Liver Cancer; TGFB1; Kras; Zebrafish.

*Authors share co-first authorship.

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; cDNA, complementary DNA; dox, doxycycline; EGFP, enhanced green fluorescence protein; Gfap, glial fibrillary acidic protein; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; Htr2b, 5-hydoxytryptamine receptor 2B; IF, immunofluorescence; IHC, immunohistochemistry; PCR, polymerase chain reaction; P-Tph1, phosphorylated tryptophan hydroxylase 1; TGF, transforming growth factor; Tph1, tryptophan hydroxylase 1; WT, wild type.

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Hepatocellular carcinoma (HCC) is notably more prevalent in men than in women, with a male-to-female ratio of 4.5:1.[1] The sex disparity appears to be mediated by a sex hormone–regulated mechanism. After menopause, women showed increased occurrence of HCC, which could be reduced with estrogen treatment.[2,3] In a carcinogen-induced mouse HCC model, administration of estrogen to male mice inhibited HCC development.[4] It is interesting to note that effects elicited by sex hormones are associated commonly with tumor microenvironment-mediated inflammatory response. For example, estrogen inhibits interleukin 6 expression in Kupffer cells (liver resident macrophages) and confers resistance to HCC in female mice.[5] Androgen signaling polarizes tumor-associated macrophages to a protumor gene expression profile during early hepatocarcinogenesis.[6] Interestingly, numerous HCC studies have shown a more robust and active HCC tumor microenvironment in males than in females. For example, infiltrating tumor-associated macrophage density has been found to be higher in males than in females in a mouse HCC model.[7] In human HCC patients, men have considerably higher numbers of intratumoral infiltrated CD66b+ neutrophils and CD8+ T cells; both are indicators of poor disease prognosis.[8]

Hepatic stellate cells (HSCs), the main fibrinogenic cell type in the liver, primarily are responsible for the production of extracellular matrix materials.[9–11] Recent studies have shown a tumor-promoting role of HSCs.[12] Tumor cell–secreted signals promote HSCs to transdifferentiate into highly proliferating myofibroblast-like cells, also known as activated HSCs.[13] Activated HSCs can induce phenotypic changes in cancer cells, primarily by secretion of protumor growth factors and cytokines such as hepatocyte growth factor and transforming growth factor (TGF)β1.[14] An increased HSC-specific gene expression signature in HCC patients indicates poor prognosis.[15] Notably, HSCs are found to be of higher density in male than in female HCC patients.[16] Because only activated HSCs rapidly proliferate, a higher HSC density in male HCC patients implies that a sex-dependent mechanism could contribute to activation of HSCs.[13]

Our laboratory previously generated several inducible HCC models by transgenic expression of an oncogene in hepatocytes in zebrafish.[17–21] The inducible nature of these zebrafish HCC models allows the oncogene to be activated at a given and controlled timing in both sexes, providing an excellent platform to study sex disparity in HCC initiation and progression. In this study, we attempted to investigate the mechanism of the sex disparity observed in HCC patients. In our *kras*^{V12}-expressing HCC zebrafish model, male fish showed accelerated carcinogenesis as compared with females.[17] Interestingly, we found an increased level of serotonin production in male *kras*^{V12}-expressing livers as compared with female counterparts and showed that serotonin was necessary for maintaining HSC survival and activation during HCC carcinogenesis. The activated HSCs in turn promoted HCC progression and at the same time increased serotonin synthesis in hepatocytes via Tgfb1, hence maintaining and promoting the sex disparity observed in *kras*^{V12}-expressing zebrafish. The findings in zebrafish were confirmed by our analyses of human liver disease patient samples, suggesting that serotonin is involved in the sex disparity of human HCC.

Materials and Methods

Zebrafish Husbandry

Zebrafish were maintained in compliance with Institutional Animal Care and Use Committee guidelines from the National University of Singapore. Five transgenic lines, *Tg(fabp10:rtTA2s-M2; TRE2:EGFP-kras*^{G12V}*)* (gz32Tg) in a tetracycline-controlled transcription activation (Tet-On) system for inducible hepatocyte-specific expression of oncogenic *kras*^{G12V},[17] *Tg(hand2:EGFP)* (pd24Tg) with EGFP-labeled HSCs under the hand2 promoter,[22] *Tg(tp1:EGFP)* (um14Tg) with EGFP-labeled cholangiocytes under a notch-responsive element,[23] *Tg(lyz:DsRed2)* (nz50Tg) with DsRed-labeled neutrophils under the lysozyme C (*lyz*) promoter,[24] *Tg(mpeg1:mCherry)* (gl22Tg), with mCherry-labeled macrophages under the *mpeg1* promoter,[25] and *Tg(fabp10:DsRed; ela3l:EGFP)* (gz15Tg) with DsRed-labeled hepatocytes under the *fabp10* promoter[26] were used in this study and referred to as *kras*+, *hand2*+, *lyz*+, *mpeg*+, and *fabp10*+, respectively, in the present report.

Chemical Treatment

All chemical/reagent treatments were conducted in 3-month-old adult fish for 7 days. The chemicals/reagents used included doxycycline (dox) (D9891; Sigma-Aldrich, St. Louis, MO), serotonin (14927; Sigma), PCPA (4-chloro-DL-phenylalanine) (C6506; Sigma), BW723C86 (B175; Sigma), SB204741 (S0693; Sigma), and SB431542 (1614; Tocris, Minneapolis, MN). Serotonin, PCPA, BW723C86, SB204741, SB431542, and dox were used for adult exposure at 10 μmol/L, 25 μmol/L, 15 μmol/L, 15 μmol/L, 2.5 μmol/L, and 30 μg/mL, respectively. The dosages were selected based on the highest all-survival concentrations in preliminary experiments.

Photography and Image Analysis

At each time point of chemical treatment experiments, more than 10 adult fish in each group were used for imaging analyses. These zebrafish were anesthetized in 0.08% tricaine (E10521; Sigma) and immobilized in 3% methylcellulose (M0521; Sigma) before imaging. Each zebrafish was photographed individually with an Olympus microscope (Olympus, Tokyo, Japan).

Isolation of Hepatocytes, Neutrophils, Macrophages, Cholangiocytes, and HSCs by Fluorescence-Activated Cell Sorting

Fabp10+, *lyz*+, *mpeg*+, *tp1*+, and *hand2*+ transgenic zebrafish in wild-type background were used for fluorescence-activated cell sorting isolation of hepatocytes, neutrophils, macrophages, cholangiocytes, and HSCs,