



INVESTIGATING THE ROLE OF SEROTONIN SIGNALING IN LIVER CANCER USING THE CRISPR/CAS9 SYSTEM

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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer related deaths in the world¹. But despite its high mortality rate, there is a lack of drug treatments available partly due to the various subsets of HCC which have different genetic and clinical features. Previous studies have found that the use of antidepressant drugs have decreased the risk of an HCC subset in a veteran patient cohort, and reduced oncogene-induced hepatic hyperproliferation and tumor burden in zebrafish and mice, respectively^{1,2}. Therefore, we hypothesize that serotonin-related proteins in the liver contribute to HCC progression. To gain further insight into the mechanism by which serotonin signaling can drive HCC, we created a transgenic line of zebrafish using the CRISPR/Cas9 system to knockout liver-specific serotonin-related proteins and compare liver cancer endpoints - such as liver size - to their non-transgenic control siblings. Once germ-line mutations are established, we aim to screen for antidepressants that reduce HCC risk and research the antidepressant's mechanism of action to lay down the foundation to not only repurpose FDA-approved antidepressants as potential treatment alternatives, but to also allow for the development of targeted drugs that inhibit these serotonin-specific pathways.

Overview and background

The high mortality rate of hepatocellular carcinoma (HCC) is partially due to the lack of drug treatments available to treat the various subsets of HCC which have different genetic and clinical features. Additionally, the lack of relevant animal models with these diverse subsets makes it difficult to determine targeted treatment. The zebrafish was chosen as the animal model as fertilization occurs externally and embryogenesis is translucent allowing internal organs such as the liver to be easily visualized. Additionally, zebrafish tumor morphology is also similar to human tumors both genetically and histologically. β -catenin is an example of an oncogene causing a subgroup of liver cancer that does not have specific treatment and is highly similar to human and zebrafish β -catenin (Fig. 1). In previous drug screens, β -catenin transgenic zebrafish showed suppressed liver growth by c-Jun N-terminal kinase (JNK) inhibitor and 2 types of antidepressants (amitriptyline and a selective serotonin reuptake inhibitor)¹. Thus, we believe that researching specific pathways that serotonin acts upon can represent a worthy method of targeted treatment.

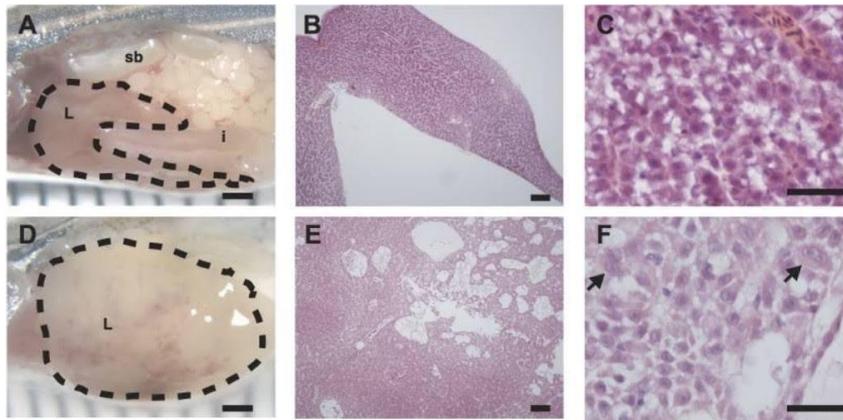


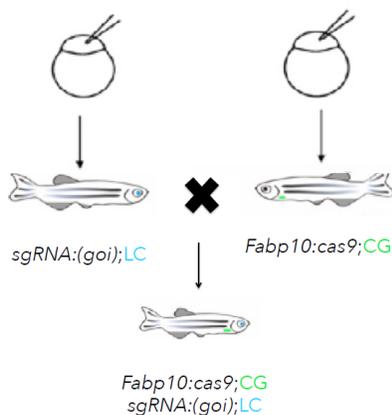
Fig. 1 Shown here (A-C) is the liver with images of the normal hepatocyte architecture in a 4-month-old zebrafish. (B-C). There are also images of a transgenic (β -catenin) zebrafish (D-F) with an enlarged liver (D) with abnormal hepatocytes¹.

Serotonin is most commonly known as a neurotransmitter; however, it also has extra-neuronal activities in various cell types such as hepatocytes. Numerous studies have found mitogenic properties of serotonin in hepatocyte growth and regeneration. Serotonin is able to bypass the mammalian target of Rapamycin (mTOR) pathway and directly affect its downstream targets p70s6K and 4E-BP1³. mTOR is a signaling pathway that promotes cellular growth in high levels - potentially simulating cancer - and can simulate autophagy in low levels. As serotonin acts through an mTOR independent pathway, this allows for the continued activation of p70s6K and 4E-BP1 to promote hepatocyte growth. Additionally, human HCC biopsies showed the expression of serotonin receptor *HTR2A* which is associated with mTOR downstream signals³.

Increased expression of *HTR2A* and *HTR2B* also increased after hepatectomy⁴. The liver is the only organ that has the capacity to regenerate due to platelets, which are major contributors to hemostasis and function as carriers of serotonin. The rate of liver regeneration was reduced in several groups of mice: thrombocytopenic mice, mice containing serotonin antagonists to serotonin receptors *HTR2A* and *HTR2B*, and mice lacking the enzyme tryptophan hydroxylase which is an enzyme that catalyzes the synthesis of serotonin.

These studies show the role of serotonin is in the process of liver proliferation. Therefore, targeting a serotonin-specific pathway is a viable treatment option for HCC. My goal in this project is to understand the mechanism of serotonin signaling in liver cancer by achieving the knockdown of various serotonin-related genes, such as the enzyme that degrades serotonin (*mao*), the serotonin transporters (*slc6a4a* and *slc6a4b*), and a serotonin receptor (*HTR2B*).

Methods



In order to create transgenic fish with gene knockdowns as described above, I utilized a CRISPR/Cas9 system that allowed for multiplex conditional mutagenesis in a single generation⁵. This method of mutagenesis is modular with easy addition of any desired gRNA. I first injected zebrafish embryos with constructs containing sgRNA (*sgRNA:(goi):LC*) targeting serotonin-related genes. The construct also includes cerulean fluorescent protein (CFP) eye markers to allow for non-invasive identification of embryos containing the injected DNA. Parents of embryos that exhibit blue eyes at 3 days post fertilization are deemed as founders with germ-line transmission. Once they are 3-months-old, the founders are crossed with zebrafish containing liver specific promoter *fabp10a* with a cardiac

specific enhanced green fluorescent protein (EGFP) (*fabp10a:cas9:CG*).

Results

3 lines of zebrafish expressing gRNA targeting *mao*, and 2 lines of zebrafish expressing gRNA targeting *slc6a4a* and *slc6a4b* were established: *Tg(u6:sgRNA_{mao})*, *Tg(u6:sgRNA_{slc6a4a/slc6a4b})*, and *Tg(u6:sgRNA_{htr2b})*. Confirmed founders of these lines were then crossed to transgenic zebrafish expressing Cas9 in a liver-specific manner (*Tg(fabp10a:Cas9)*)⁴. So far, we have been able to confirm that progeny resulting from a cross of *Tg(u6:sgRNA_{slc6a4a/slc6a4b})* and (*Tg(fabp10a:Cas9)*) possess genomic aberrations at the desired CRISPR sites in both genes *slc6a4a* and *slc6a4b*. These alterations are also liver-specific. The genomic aberrations were identified using High Resolution Melt Curve Analysis.

Future experiments

Transgenic lines expressing gRNAs targeting *HTR2B* remain to be identified and characterized. Founders of all lines will be crossed with zebrafish with ubiquitous expression of Cas9 to generate whole-body mutants of the aforementioned genes (*mao*, *slc6a4a*, *slc6a4b*, and *HTR2B*). Then, the founders will be crossed with *Tg(fabp10a:Cas9)* fish to determine the effect of hepatocyte-specific loss of these genes. Upon mating, the progeny will have activated CRISPR/Cas9 system with the liver specific knockdown of serotonin proteins creating our desired transgenic line. The fish of interest will be studied for liver size, proliferation, hepatic apoptosis, and incidence of liver tumors.

References

1. Evason KJ, Francisco MT, Juric V, et al. Identification of Chemical Inhibitors of β -Catenin-Driven Liver Tumorigenesis in Zebrafish. *PLoS Genetics*. 2015;11(7).
2. Chang CM, Hsieh MS, Yang TC, et al. Selective Serotonin Reuptake Inhibitors and the Risk of Hepatocellular Carcinoma in Hepatitis B Virus-Infected Patients. *Cancer Management and Research*. 2017;9.
3. Soll C, Jang JH, Riener MC, et al. Serotonin Promotes Tumor Growth in Human Hepatocellular Cancer. *Hepatology*. 2010;51(4).
4. Lesurtel M, Graf R, Aleil B, et al. Platelet-Derived Serotonin Mediates Liver Regeneration. *Science*. 2006;312(5770).
5. Yin L, Maddison LA, Li M, et al. Multiplex Conditional Mutagenesis Using Transgenic Expression of Cas9 and sgRNA. *Genetics*. 2015;200(2).