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Cancer stem cells generated by alcohol, diabetes, and HCV

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Abstract

Cancer stem cells (Tumor-initiating stem-like cells: TISCs) are resistant to chemotherapy and are associated with metastatic hepatocellular carcinoma (HCC), which is commonly observed in hepatitis C virus (HCV)-infected patients with obesity or alcohol abuse. However, it is unknown whether the TLR4-NANOG pathway serves as a universal oncogenic signaling in the genesis of TISCs and HCC. We aimed to determine whether *Tlr4* is a putative proto-oncogene for TISCs in liver oncogenesis due to different etiologies and how *Tlr4* is regulated at the transcriptional and epigenetic levels. CD133+/CD49f+ TISCs were isolated using FACS from HCC developed in HCV Core Tg mice fed alcohol, diethylnitrosamine-treated mice, and alcoholic patients with or without HCV infection. CD133+/CD49f+ cells isolated from the animal models and patients are tumorigenic both in vitro and in a xenograft model, and *Tlr4* or *Nanog* silencing with shRNA attenuates their tumor initiating property. Functional oncogene screening of a cDNA library identified the organ size control pathway targets *Yap1* and AKT activator *Igf2bp3* as NANOG-dependent genes that inhibit TGF- β signaling in TISCs. *Tlr4* expression is higher in TISCs compared to CD133-/CD49f+ cells, and DNA hypomethylation, histone acetylation and demethylation drive *Tlr4* induction through NOTCH signaling or *Hif-1 α* . Taken together, *Tlr4* is induced by hypoxia and NOTCH signaling and is a universal proto-oncogene responsible for the genesis of TLR4-NANOG dependent TISCs, and this pathway serves as a novel therapeutic target for HCC.

Keywords

Alcohol; HCV; HCC; Tumor-initiating stem-like cells; Obesity

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Conflict of interest

The authors declare no competing financial interests.

HCV, diabetes, alcohol, and HCC

Chronic liver damage caused by viral infection, alcohol, or metabolic syndrome can result in increased risk for HCC which is the third most common cancer in the world ¹. Clearly, understanding the molecular mechanisms of HCV-induced hepatocarcinogenesis is required for the eventual development of improved therapeutic modalities for this disease ². In particular, chronic infection with HBV or HCV represents a major risk factor for HCC ¹. HCV affects more than 170 million people worldwide ^{1,3,4}.

Ample epidemiological evidence suggests that there is a strong connection between HCV and obesity/alcoholic liver diseases (ALD). First, the prevalence of HCV is significantly higher among obesity/alcoholics than in the general population; for example, while the HCV positive rate in the general population of the U.S. is roughly 1%, it is 16% for alcoholics and nearly 30% for alcoholics with liver diseases ⁵. Second, the presence of HCV infection correlates with the severity of the disease in alcoholic subjects, i.e. HCV-infected patients with obesity and ALD develop liver cirrhosis and HCC at a significantly younger age than uninfected ALD patients, suggesting that alcohol and HCV work synergistically to cause liver damage ⁶. Many studies also support synergistic interactions between HCV and alcoholism in hepatocarcinogenesis ⁷⁻¹¹. Heavy alcohol consumption/obesity and viral hepatitis synergistically increase the risk for HCC among blacks and whites in the U.S. ¹⁰. HCC odds ratio increases to 48.3-fold or 47.8 from 8.1 or 8.6 by having concomitant alcohol abuse or obesity in HBV/HCV infected patients, respectively ^{9,10}. Indeed, our recent result demonstrates that the incidence of spontaneous HCC induction in the HCV core transgenic mice is increased 2-fold by chronic alcohol intake.

Recent studies with mice expressing HCV proteins have shed pivotal insights into the mechanisms underlying this synergism. The HCV core protein causes overproduction of reactive oxygen species which appears to be responsible for mitochondrial DNA damage ^{3,12,13}. Thus, these core-induced perturbations such as oxidant stress and insulin resistance, which are also known risk factors for ALD, may underlie the synergism reproduced in alcohol-fed core transgenic mice ¹⁴. TLR2 and TLR4 are markedly upregulated in hepatocytes, Kupffer cells, and peripheral monocytes of patients with chronic hepatitis C. TLR-mediated signals result in liver disease associated with hepatitis B, hepatitis C, alcoholic non-alcoholic steatohepatitis, and hepatic fibrosis ¹⁵. The most devastating consequence of the synergism between viral hepatitis and alcohol is HCC ⁷⁻¹¹.

TLRs signaling in HCC

The TLR signaling pathway is upregulated in chronic liver diseases. Many different cell types in the liver express TLRs ¹⁵. Chronic alcohol consumption activates other TLRs, such as TLR1, 2 and 6-9, which further increases the TNF- α response to LPS in mice ¹⁵. Human monocytes exposed to ethanol for a week develop hypersensitivity to LPS through decreased IRAK-M expression, which activates mitogen-activated protein kinase (MAPK) and NF- κ B through TLR4 signaling, leading to activation of NF- κ B, AP-1, and ERK ¹⁶. The direct mechanistic evidence has recently been attained by our research using mice expressing the HCV non-structural protein NS5A in a hepatocyte-specific manner. These mice when fed alcohol for 12 months, develop liver tumors in a manner dependent on TLR4 induced by NS5A ¹⁷. This NS5A-induced TLR4 is activated by endotoxemia associated with alcohol intake, leading to accentuated TLR4 signaling which in turn upregulates the stem cell marker Nanog required for TLR4-dependent liver oncogenesis. This finding on the NS5A-TLR4-Nanog axis in synergistic oncogenesis, is beginning to shed a novel insight into molecular mechanisms for HCC in alcoholic HCV patients.

Tumor-initiating stem-like cells and HCC

Stem cells have three major characteristics, self renewal, asymmetric and multiple cell division (clonality), and plasticity. The liver has a high regenerative potential, and hepatic small oval progenitor cells around the peripheral branches of the bile ducts, the canals of Hering, can differentiate into biliary epithelial cells and hepatocytes¹⁸. These oval liver progenitor cells share molecular markers with adult hepatocytes (albumin, cytokeratin 7 [CK7], CK19, oval cell markers [OV-6, A6, and OV-1], chromogranin-A, NCAM [neural cell adhesion molecule]) and fetal hepatocytes (α -fetoprotein)^{18,19}. They are also positive for more common stem cell markers such as CD34+, Thy-1+, c-Kit+, and Flt-3+ (FMS-like tyrosine kinase 3)²⁰. Thus, it currently remains unclear whether these stem cells are derived from the bone marrow and just migrate to this periportal niche or whether they represent true resident liver stem/progenitor cells. Binding of stroma-derived factor-1 α (SDF-1 α) to its surface receptor CXCR4 activates oval hepatic cells²¹. Forty percent of HCC have clonality, and thus are considered to originate from progenitor/stem cells^{19,22–24}. Recent studies of HCC have centered on TISCs, including detection of TISCs in cancer, identification of TISCs markers, and isolation of TISCs from human HCC cell lines. TISCs were identified as a CD117+/CD133+ hepatic precursors in regenerating liver tissue²⁵ and a CD45–/CD90+ subpopulation of tumor cells in HCC²⁶. The CD90+ cells are not present in the normal liver and, when injected into immunodeficient mice, create tumors repeatedly. In human HCC and HCC cell lines, specifically CD133+ cells, not CD133– cells, had the ability to self-renew, create differentiated progenies, and form tumors²⁷. One potential reason for this chemoresistance may lie in the plasticity of cancer stem cells with dysregulated signaling and gene expression.

Nanog is one of the core transcription factors found in pluripotent embryonic stem cells (ESCs)²⁸. It is essential for maintaining self-renewal and pluripotency of both human and mouse embryonic stem cells^{29–32}. Overexpression of Nanog induces and maintains the pluripotency and self-renewing characteristics of ESCs under what normally would be differentiation-inducing culture conditions³³. Recently, Nanog expression has been reported in human neoplasms, including germ cell tumors^{34–37}, breast carcinomas³⁷, osteosarcoma³⁸, and HCC³⁹. Ectopic expression of Nanog induces an oncogenic potential in NIH3T3⁴⁰.

Nanog-positive cancer stem cells induced by HCV and alcohol

Alcohol synergistically enhances the progression of liver disease and the risk for liver cancer caused by HCV. TLR4 is induced by hepatocyte-specific transgenic (Tg) expression of the HCV nonstructural protein NS5A, and this induction mediates synergistic liver damage and tumor development by alcohol-induced endotoxemia¹⁷. The stem/progenitor cell marker, Nanog, is up-regulated as a novel downstream gene by TLR4 activation and the presence of CD133/Nanog-positive cells in liver tumors of alcohol-fed NS5A Tg mice¹⁷. Transplantation of p53-deficient hepatic progenitor cells transduced with TLR4 results in liver tumor development in mice following repetitive lipopolysaccharide (LPS) injection, but concomitant transduction of Nanog short-hairpin RNA abrogates this outcome¹⁷. Despite the common understanding that TLR4 is one of the pattern recognition receptors expressed predominantly by innate immune cells such as macrophages and lymphocytes, our study demonstrates that hepatocytes can be the primary cellular site of both TLR4 upregulation and its pathologic consequences in the context of HCV infection. Therefore, the TLR4-dependent mechanism synergizes liver disease by HCV and alcohol and is partly dependent on Nanog, a TLR4 downstream gene.

Nanog transduction alone is not as effective as TLR4 activation in liver tumorigenesis, as shown by our cell transplantation experiment¹⁷. We believe that TLR4 activation induces

other tumor-driver genes which cooperatively work with Nanog to cause liver oncogenesis. Thus, Nanog is still essential for TLR4-dependent oncogenesis, but it alone is poorly oncogenic. In our previous work using a cell line, we demonstrated that TLR4 promoter up-regulation by NS5A is mediated by PU.1, Oct-1, and AP-1 elements⁴¹. The similar transcriptional mechanism may underlie TLR4 induction in primary hepatocytes.

Epigenetic regulation of tumor-initiating stem-like cells

Cellular memory mechanisms enable cells to remember their chosen fate over many cell divisions. The dynamic interactions between epigenetic regulators and DNA components orchestrate transcriptional memory system for several hundred genes. DNA methylation is a major epigenetic modification of the genome particularly as it relates to pluripotency and differentiation of stem cells^{42–44}. Genomic methylation patterns in somatic differentiated cells are generally stable and heritable. In contrast, aberrant genome-wide DNA hypomethylation links to tumorigenesis^{45,46}. In TISCs, DNA methylation patterns and histone modifications are suspected to be reprogrammed genome-wide, leading to generation of cells with altered gene expression profile for stem cell-like characters⁴⁷. However, epigenetic mechanism and implications in HCC remain to be elucidated, and the role of epigenetic reprogramming in generation of cancer stem cells is yet to be investigated.

In summary, alcohol, obesity, and HCV synergistically induce liver tumor development via induction and activation of TLR4 in mice. Pharmacologic inhibition of TLR4 signaling may become a novel therapeutic strategy for HCV-associated liver tumors.

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Fig. 1. These diagrams depict histone lysine methyltransferases, target lysine methylation sites in histones, methyl-lysine binding protein modular domains and the consequent epigenetic regulation (right), as well as the hypothetical epigenetic mechanisms of cancer stem cell generation (left). Modified from original figures shown in references ^{47,48}.