Minireview

A new, effective and high-yield approach for identifying liver tumor suppressors

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Abstract

Despite recent advances in research in hepatocarcinogenesis, we still lack a comprehensive view of the major pathways involved in liver carcinogenesis. Current concepts suggest that a limited number of molecular alterations involving oncogene activation and tumor suppressor inhibition are responsible for initiation of cancer. A recent publication by Zender *et al.* utilizes a combination of high-resolution comparative genomic hybridization, short hairpin RNA inhibition of target genes at the locations of focal genomic deletions, and a primed cell mosaic mouse model to identify novel tumor suppressors in hepatocellular carcinoma. This exciting new model promises to provide additional insights into the mechanisms of carcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of death from cancer [1]. The major risk factors for development of HCC include chronic hepatitis B (HBV) and hepatitis C virus infection, high dietary exposure to fungal aflatoxins, and other disorders causing cirrhosis, such as hereditary hemochromatosis, α_1-antityripsin deficiency, primary biliary cirrhosis, non-alcoholic steatohepatitis and alcoholic cirrhosis [2]. Despite significant improvements in our understanding of the pathogenesis of HCC over the past few decades, we still do not have a comprehensive understanding of the major molecular pathogenic processes involved in liver carcinogenesis.

Cancer biology

Current conceptions of carcinogenesis posit that a limited number of molecular alterations involving oncogene activation and tumor suppressor inhibition are responsible for the initiation of the cancer phenotype [3]. The 'cancer platform' concept proposes that most oncogenic molecules also have the inherent ability to activate tumor suppressor genes or pathways through oncogene-induced apoptosis or senescence, thus limiting their oncogenic effects in a homeostatic fashion. Oncogene activation is therefore generally only tumorigenic when it is coupled with inactivation or inhibition of oncogene-induced pro-apoptotic or senescence pathways [4].

The specific effects that lead to inactivation of these pro-apoptotic or senescence pathways may impinge on major known tumor suppressors such as p53, but may also be due to effects on other genes and molecules within the p53 or other tumor suppressor pathways [5]. These effects may be mediated through epigenetic or genetic mechanisms. The known risk factors for HCC are associated with chronic inflammation or hepatocellular injury. These insults result in repeated cycles of hepatocyte injury, death and repair that eventually lead to the premature senescence of the liver [6]. Senescent hepatocytes lose the telomeric repeats that protect chromosomal ends from inter- and intra-chromosomal fusion, deletion, rearrangement, and transposition events that contribute to genomic instability.

Loss of telomeres typically triggers cellular apoptosis in the process referred to as telomeric crisis. Within the population of senescent hepatocytes, subpopulations may arise that reactivate telomerase, escape telomeric crisis and become immortalized [7,8]. These subpopulations, present within the highly genotoxic inflammatory environment typical of chronic liver disease, are then at especial risk of acquiring additional genetic and epigenetic alterations that predispose them to carcinogenesis. Genomic instability is a hallmark of the cancer phenotype, and cancer is considered to be a disease of the cell's disordered genome.

Mechanisms and pathways in the process of liver carcinogenesis

Over the past few decades, multiple approaches have been used to explore the mechanisms of liver carcinogenesis. These include: (1) the use of chemical tumor initiators and promoters in animal models, (2) studies of oncogenic growth factors, such as insulin-like growth factors and fibroblast growth factors, (3) the use of transgenic mouse models overexpressing cytokines, growth factors or oncogenes, such as transforming growth factor-α (TGF-α), c-Myc, TGF-β, and platelet-derived growth factor C, either in isolation or in combination, (4) studies investigating immune-mediated mechanisms of hepatocellular injury, (5) analyses of molecular genetic and genomic changes occurring in HCCs, including allelic imbalance/loss of heterozygosity, gene copy number, gene mutation, gene methylation and messenger RNA (mRNA) and microRNA expression, (6) analyses of proteomic changes and post-translational phosphorylation, glycomic or sulfation status and (7) studies of the gene alterations induced by HBV integration and the oncogenic effects of the protein products of the hepatitis B and C viruses [9].

These studies have revealed multiple mechanisms and pathways in the carcinogenic process. Pathways identified as important in hepatocarcinogenesis include the Wnt/β-catenin signaling pathway, insulin/insulin-like growth factor pathway, multiple receptor tyrosine kinases activating downstream of PI3K/Akt and MEK/ERK signaling, and the TGF-β, interferon-inducible, mTOR and hedgehog signaling pathways [10-13]. Key tumor suppressor genes identified thus far include *p53*, *CDKN2A*, which encodes $p16^{INK4A}$ and p14ARF, *E cadherin*, *AXIN1* and *AXIN2* [14,15].

A novel approach to identifying liver tumor suppressors

Recent technological improvements permit the study of cancer cell genomes at a higher resolution than ever before. High-resolution array-based comparative genomic hybridization allows detailed characterization of the regions of copy number alterations in tumors [16,17]. This allows the identification of local regions of copy number gain or loss that may represent the locations of oncogenes or tumor suppressors. In this review, we discuss an exciting new model that promises to provide additional unique insights into the mechanisms of liver carcinogenesis.

In a recent publication, Zender *et al.* [18] have combined an integrated cancer genomic analysis, RNA interference (RNAi) technology and cancer-susceptible mouse models to discover and validate tumor suppressor genes contributing to hepatocellular carcinoma. The authors analyzed 98 human hepatocellular carcinomas of different etiologies. They investigated the DNA copy number alterations by using representational oligonucleotide microarray analysis (ROMA) [18], which allowed them to detect many small and larger deletions. However, they hypothesized that genomic regions containing focal deletions were more likely to encode tumor suppressor genes. Consequently, they selected 58 regions containing small (less than 5Mb) and recurrent deletions for further analysis. Within these regions, 362 genes were selected, for which 301 mouse orthologs were identified. From the Cold Spring Harbor RNAi Codex library, 631 mir-30-based short hairpin RNAs (shRNAs) targeting the 301 mouse genes (an average of two shRNAs per gene) were selected. They then performed an *in vivo* RNAi screen using a mosaic mouse model, which was formed by subcutaneous transplantation of immortalized embryonic hepatocytes lacking p53 and overexpressing Myc. These immortalized cells are primed for carcinogenesis. The cells were used as a substrate for screening pools of shRNAs targeting the genes of interest for development of liver carcinomas. The shRNA pools were cloned into a plasmid construct co-expressing green fluorescent protein (GFP), thus facilitating the identification and characterization of tumors resulting from shRNA transduction.

Remarkably, most mice transplanted with hepatocytes transfected with shRNA pools targeting the recurrently deleted genes developed GFP-positive tumors within a few weeks of transplantation, compared with control mice transduced with randomly selected shRNAs, which did not develop an increased frequency of tumors. The integrated shRNAs present in the most aggressive tumors were then amplified by polymerase chain reaction and cloned for subsequent validation. Tumor suppressor genes identified in this manner included the well-known tumor suppressor *PTEN*, the histone chaperone or protein phosphatase inhibitor gene *SET*, which is associated with a rare translocation in acute myeloid leukemia, as well as a number of genes not previously identified as tumor suppressors, including *Xpo4*, *Ddx20*, *Gjd4*, *Fstl5* and *Nrsn2*. The most enriched shRNA in this study targets exportin 4 (Xpo4), which belongs to the importin-β family of nuclear transporters. The two known substrates for exportin 4 are the TGF-β effector SMAD3 and the eukaryotic translation initiation factors EIF5A1 and EIF5A2. Mouse hepatoma cells expressing shRNAs targeting Xpo4 showed increased total and phospho-Smad3 in the nucleus, with associated increases in

the levels of several TGF-β target genes. Similar to Smad3, the Xpo4 targets Eif5a1 and Eif5a2 also accumulated in the nucleus after knockdown of Xpo4 in murine hepatoma cells [18]. Consistent with the putative oncogenic effect of nuclear EIF5A2, Zender *et al.* show that EIF5A2 is amplified in human tumors, stimulates proliferation of XPO4-deficient tumor cells, and promotes hepatocarcinogenesis in mice.

Historical and biological context and clinical implications

There are currently very limited therapeutic options for advanced or metastatic HCC. It is therefore critical to understand the genetic background and molecular pathways contributing to intiation and progression of HCC, to aid the development of rational, targeted therapies [19,20]. The completion of the human and mouse genome sequences and the development of methods for cloning integrated sequences and targeted downregulation of specific mRNAs provide unique opportunities for screening efforts to identify novel pathway molecules that contribute to oncogene activation or tumor suppressor inactivation. Zender *et al.* [18] used an elegant primed cell mosaic model to identify novel tumor suppressors in HCC. While the majority of the newly identified genes require validation, the careful characterization of *Xpo4*, the most profoundly suppressing gene, provides novel insight into the gene's potential roles in carcinogenesis.

The approach used by Zender *et al.* is particularly advantageous because it first uses ROMA to narrow down the field of potential candidate genes, then uses screening with pools of shRNAs co-expressed with GFP, rather than the individual shRNAs, thus substantially reducing the cost and enhancing the efficiency with which tumors induced by shRNA transduction can be identified.

The variety of genes identified in this study, most of which have not previously been characterized as tumor suppressors, calls to mind previous debate about the multiplicity of genes identified at the sites of HBV integration in HBV-induced HCCs. HBV integrations disrupt the genome, induce genome instability, and place strong HBV promoter and enhancer elements adjacent to human genes. These integrations may occur preferentially at genomic sites that are prone to breakage and rearrangement. The changes induced by HBV integration may lead to the development and selection of clones of cells with an increased propensity to carcinogenesis. Consequently, HBV DNA genomic integrations may represent the natural biological equivalent of the experimental strategy pursued by Zender *et al.* The initial conventional wisdom was that since there were multiple genes identified at or adjacent to HBV integration sites in humans, the HBV integration process must be random and of no significance for hepatocarcinogenesis. More recent results have identified multiple integrations at the human telomerase reverse transcriptase (*TERT*) gene locus, and suggest that many of the somewhat disparate genes belong to pathways important for telomerase activation, cellular calcium signaling or apoptosis [21-24].

The results reported by Zender *et al.* may, therefore, lead to the identification of new pathways of significance in the hepatocarcinogenic process. The complete characterization and validation of the additional genes identified in this screen, as well as those from additional screens incorporating different combinations of predisposing oncogenes and inactivated tumor suppressors, will be eagerly awaited.

Abbreviations

GFP, green fluorescent protein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; mRNA, messenger ribonucleic acid; RNAi, RNA interference; ROMA, representational oligonucleotide microarray analysis; shRNA, short hairpin RNA; TERT, telomerase reverse transcriptase; TGF-β, transforming growth factor beta; Xpo4, exportin 4.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EO drafted the manuscript. LRR revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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