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"*Fibrous nests*" in human hepatocellular carcinoma express a Wnt-induced gene signature associated with poor clinical outcome

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ABSTRACT

Hepatocellular carcinoma (HCC) is the 3rd cause of cancer-related death worldwide. Most cases arise in a background of chronic inflammation, extracellular matrix (ECM) remodeling, severe fibrosis and stem/progenitor cell amplification. Although HCCs are soft cellular tumors, they may contain *fibrous nests* within the tumor mass. Thus, the aim of this study was to explore cancer cell phenotypes in fibrous nests. Combined anatomic pathology, tissue microarray and real-time PCR analyses revealed that HCCs (n = 82) containing fibrous nests were poorly differentiated, expressed Wnt pathway components and target genes, as well as markers of stem/progenitor cells, such as CD44, LGR5 and SOX9. Consistently, in severe liver fibroses (n = 66) and in HCCs containing *fibrous nests*, weighted correlation analysis revealed a gene network including the myofibroblast marker ACTA2, the basement membrane components COL4A1 and LAMC1, the Wnt pathway members FZD1; FZD7; WNT2; LEF1; DKK1 and the Secreted Frizzled Related Proteins (SFRPs) 1; 2 and 5. Moreover, unbiased random survival forest analysis of a transcriptomic dataset of 247 HCC patients revealed high DKK1, COL4A1, SFRP1 and LAMC1 to be associated with advanced tumor staging as well as with bad overall and disease-free survival. In vitro, these genes were upregulated in liver cancer stem/progenitor cells upon Wnt-induced mesenchymal commitment and myofibroblast differentiation. In conclusion, fibrous nests express Wnt target genes, as well as markers of cancer stem cells and mesenchymal commitment. Fibrous nests embody the specific microenvironment of the cancer stem cell niche and can be detected by routine anatomic pathology analyses.

1. Introduction

The incidence of human hepatocellular carcinoma (HCC) has doubled over the past 20 years and projections anticipate a further increase despite recent breakthroughs leading to virological cure of chronic hepatitis (Singal and El-Serag, 2015). More than 80% of HCCs arise in fibrotic livers as a result of HCV or HBV infections, alcohol, hemochromatosis, metabolic syndrome or genotoxins (Bruix et al., 2014). Recurrent bouts of liver cell damage and chronic inflammation result in progressive fibrosis and amplification of the stem cell niche (Gouw et al., 2011). Severe liver fibrosis is thus a pre-neoplastic condition at high risk of developing HCC (Hoshida et al., 2013).

Molecular analysis of liver tissues in search for stem cells reveals a diversity of phenotypes (Gouw et al., 2011) because the cells com-

Abbreviations: α-SMA, alpha smooth muscle actin; DKK, Dickkopf; ECM, extracellular matrix; FNH, focal nodular hyperplasia; FZD, frizzled; GSE, gene expression series; GSK3B, glycogen synthase kinase 3 beta; HCC, hepatocellular carcinoma; iPS cells, induced pluripotent stem cells LEF1 lymphoid enhancer-binding factor 1; NT, non-tumor; SFRPs, secreted frizzled-related proteins; TGFB, transforming growth factor beta; TNC, tenascin C.

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monly observed under the microscope are "transit-amplifying stem cells" (Gouw et al., 2011). They are the progeny of stem cells that combine stem, hepatocyte and biliary cell markers. Some of the current molecular markers that help their identification are CD44, which is a hyaluronic acid receptor (Orian-Rousseau, 2010); LGR5, which is an R-spondin receptor that amplifies Wnt signaling (Huch et al., 2015); SOX9, which contributes to the Wnt-dependent maintenance of progenitor cells (Carpentier et al., 2011; Blache et al., 2004) and, like EPCAM and KRT19, is expressed by multipotent ductal stem cells in normal liver (Gouw et al., 2011). Except for KRT19, all of them are Wnt/β-catenin target genes. This further highlights the importance of Wnt signaling in stem cell control (Clevers et al., 2014). EPCAM and KRT19 label bipotent progenitor cells with an intermediate phenotype between hepatocytes and bile duct cells and identify malignant bile duct cell components in combined hepatocellular-cholangiocarcinoma, which is a particularly aggressive form of liver cancer (Yamashita et al., 2009; Govaere et al., 2014). Other molecules highlight mesenchyme-committed progenitor cells, such as IGFBP5, which is a Wnt/β-catenin pathway target gene involved in mesenchymal commitment of stem cells (Renger et al., 2013). Along these lines, we recently showed that Wnt signals reprogram tumorigenic liver progenitor cells to replicating fibrogenic myofibroblast-like cells displaying stem and invasive features and expressing the stem cell markers IGFBP5, LEF1, CD44 and LGR5 (Mebarki et al., 2016).

The activation of the Wnt/β-catenin pathway involves interaction of Wnt ligands with cell surface Frizzled (FZD) receptors and LRP5/6 co-receptors, followed by disruption of the *Adenomatous polyposis coli* (APC)-axin platform, which blocks GSK3B-dependent phosphorylation of β-catenin. Non-phosphorylated β-catenin is thus stabilized, accumulates in the cytoplasm and nucleus and interacts with the family of T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors of target gene expression. As LEF1 is a β-catenin target gene, it leads to cell autonomous pathway activation (Filali et al., 2002). At the opposite, in the absence of interaction of Wnt ligands with their cell surface receptors, phosphorylated β-catenin undergoes proteasomal degradation (Clevers and Nusse, 2012).

Wnt signaling is regulated at the cell surface by extracellular modulators, including the Secreted Frizzled Related Protein (SFRPs) and Dickkopf (DKK) families, Wnt Inhibitory Factor-1 (WIF-1) and the ZNRF3 and RNF43 transmembrane E3 ubiquitin ligases (Malinauskas and Jones, 2014). SFRPs are soluble molecules that possess a Frizzled Cysteine-Rich Domain (FZD_CRD), structurally homologous to the Wnt-binding domain of the FZD receptors (Bovolenta et al., 2008). They also possess a netrin domain (Bovolenta et al., 2008). Through their FZD_CRD, not only can SFRPs bind Wnts, but also they may directly signal by forming heterodimers with the CRD of the FZD receptors (Bovolenta et al., 2008). Through their netrin domain, they bind heparan sulfates (Bovolenta et al., 2008; Xavier et al., 2014). SFRPs and SFRP-like proteins are short-range modulators of Wnt signals, i.e., secreted at low levels at the cell surface, they exert their effects upon nearest-neighbor cells, as we previously showed by co-culture experiments (Hendaoui et al., 2012; Lavergne et al., 2011). At low concentrations, SFRPs mask the hydrophobic domains of Wnts, increasing their diffusion at the cell surface, thereby promoting Wnt signaling. At high concentration, they block Wnt-FZD receptor interactions (Xavier et al., 2014). Thus, SFRPs may either promote or inhibit Wnt signaling depending on concentration, FZD receptor context and the composition of the cell surface microenvironment (Xavier et al., 2014). DKKs bind the Wnt co-receptors LRP5/6, blocking the formation of ternary LRP-FZD-Wnt complexes to inhibit Wnt signaling (Malinauskas and Jones, 2014). Last, the transmembrane ubiquitin ligases ZNRF3 and RNF43 promote endocytosis and degradation of FZD receptors from the cell surface. In turn, these ubiquitin ligases are inhibited by R-spondin binding to LGR5 receptors (Malinauskas and Jones, 2014).

Among the ECM of components of HCCs, COL4A1 and LAMC1 are major basement membrane proteins upregulated in angiogenesis and cancer extracellular matrix remodeling (Mouw et al., 2014; Liétard et al., 1997; Musso et al., 2001). Of note, LAMC1 expression is modulated by Wnt signaling (Nagendran et al., 2015). Although HCCs are soft cellular tumors with scant intratumor fibrosis (Goodman, 2007), they may contain fibrous hotspots within the tumor mass, which we called *fibrous nests*. The aim of this work was to study the cancer cell phenotypes in fibrous nests in HCC. Tumors containing fibrous nests revealed a gene expression network composed of cancer stem cell markers, cell surface Wnt components and Wnt/β-catenin target genes that correlated with myofibroblast and basement membrane markers. Unbiased validation of our data in an external 247 HCC patient cohort revealed a minimal bad outcome signature in HCCs. This minimal signature was in turn upregulated upon reprogramming of liver cancer stem/progenitor cells into fibrogenic, myofibroblast-like cells by soluble Wnt ligands in vitro. The findings suggest that a Wnt-enriched cancer cell microenvironment contributes to tumor dedifferentiation and aggressiveness.

2. Materials and methods

2.1. Real-time PCR primers and antibodies

See Supplementary Table 1 for real-time PCR primers and TaqMan probes and Supplementary Table 2 for antibodies.

2.2. Patients and tissue samples

Seventy HCC patients undergoing curative liver surgery at Rennes University Hospital between January 1992 and December 2007 were included. The microscopic features of tumors diagnosed as HCC by staff pathologists were reviewed by a senior pathologist (BT). Mixed hepatocellular-cholangiocarcinoma or fibrolamellar HCC were excluded. Demographic, clinical and biological data were retrieved from hospital charts by two experienced liver surgeons (LS & DB). Eighty-two frozen tumors from 70 patients were analyzed. Demographical and clinical data are described in Supplementary Table 3. We also included 66 matching non-tumor livers, 23 histologically normal livers and 11 focal nodular hyperplasias. Non-tumor and normal liver samples were obtained at >2 cm from the tumors to minimize non-specific events, as described (Musso et al., 1997). Frozen samples were from the Biological Resources Center (BRC) at Rennes University Hospital (BB-0033-00056). Fresh tissues were frozen at −80 °C in N_2-cooled isopentane and stored at −80 °C under quality-controlled conditions. Formalin-fixed, paraffin-embedded tissue blocks were obtained from the Anatomic Pathology laboratory. The study protocol complied with French laws and regulations and fulfilled the requirements of the local institutional ethics committee. Sample collection was reported to the Ministry of Education and Research (No. DC-2008-338).

2.3. Functional genomics analyses

Gene networks were generated by Weighted Correlation Network Analysis (WGCNA, WGCNA package) (Langfelder and Horvath, 2008) using real-time PCR mRNA expression data from the indicated patient groups, after removing the experimental batch effect with the *SVA* package (Leek et al., 2012). Gene networks were visually integrated with Cytoscape, with a correlation coefficient threshold >0.30 (Shannon et al., 2003).

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