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▶ **To cite this version:**

Romain Désert, Sihem Mebarki, Mireille Desille, Marie Sicard, Elise Lavergne, et al.. “Fibrous nests” in human hepatocellular carcinoma express a Wnt-induced gene signature associated with poor clinical outcome. International Journal of Biochemistry and Cell Biology, 2016, 81 (Part A), pp.195-207. 10.1016/j.biocel.2016.08.017 . hal-01357500

HAL Id: hal-01357500

https://univ-rennes.hal.science/hal-01357500

Submitted on 29 Aug 2016

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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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Keywords: Wnt; β-catenin; fibrogenesis; LGR5; CD44; SFRP; extracellular matrix remodeling; tumor microenvironment; transit-amplifying liver progenitor cells; stem cells; hepatocellular carcinoma

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List of 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.

Author contributions:

Study design, OM, RD, SM, BC, CP; Real-time PCR, SM, MS, SR, EL; Functional genomics; RD, OM; Statistical analyses, meta-analyses from mRNA expression datasets, RD, OM; Tissue microarray design OM, MD; Tissue microarray scoring, OM, RD; Frozen tissue bank management, nucleic acid extraction, quality controls, BT, MD, OM, SM; Clinical, biological and follow-up data collection, LS, DB; Anatomic Pathology annotations, BT; Tumor fibrosis scoring: BT, OM; Manuscript preparation, OM, RD.

Financial support: Inserm, Université de Rennes 1, Région Bretagne, Agence Nationale de la Recherche, Institut National du Cancer, Fondation Recherche Médicale, Ligue Nationale Contre le Cancer (Comité des Côtes d'Armor), Feder, Contrat de Plan Etat Région 2007-2013 projet Cancéropôle.

Acknowledgements: We thank the following core facilities: *« Structure Fédérative de Recherche en Biologie et Santé de Rennes »* UMS CNRS 3480/US INSERM 018 (Health Genomics, Jean Moser; High Precision Histopathology, Alain Fautrel ; Pascale Bellaud, Roselyne Viel). We are indebted to Patricia Jouas and Michèle Le Guennec for secretarial support, to Gaelle Angenard and Catherine Ribault for tissue management and slide scanning. We thank Ms Ellen Giai Gianetto for artwork. We thank Prof. Ruth Chiquet-Ehrismann and Dr Ismaïl Hendaoui for helpful discussions.

This manuscript is dedicated to the memory of Prof. Ruth Chiquet-Ehrismann.

Title: 110 characters

Abstract: 245 words

Manuscript: 4665 words

References: 71

Figures: 7

Supplementary Figures: 5

Supplementary Tables: 8

Highlights: 62 words

Graphical Abstract: 1 figure (caption to be found with Figure Legends)

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.

Highlights

- *Fibrous nests* contain myofibroblast activation and cancer stem/progenitor cell markers.
- Cancers carrying *fibrous nests* express a minimal signature associated with advanced tumor stage and bad clinical outcome.
- Wnt signals induce the minimal *"fibrous nest signature"* in liver cancer stem/progenitor cells *in vitro.*
- Wnt signals lead to mesenchymal commitment of liver cancer stem/progenitor cells *in vitro*.

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(1). More than 80% of HCCs arise in fibrotic livers as a result of HCV or HBV infections, alcohol, hemochromatosis, metabolic syndrome or genotoxins(2). Recurrent bouts of liver cell damage and chronic inflammation result in progressive fibrosis and amplification of the stem cell niche(3). Severe liver fibrosis is thus a pre-neoplastic condition at high risk of developing HCC(4).

Molecular analysis of liver tissues in search for stem cells reveals a diversity of phenotypes(3) because the cells commonly observed under the microscope are “transit-amplifying stem cells”(3). 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(5); LGR5, which is an R-spondin receptor that amplifies Wnt signaling(6); SOX9, which contributes to the Wnt-dependent maintenance of progenitor cells(7, 8) and, like EPCAM and KRT19, is expressed by multipotent ductal stem cells in normal liver(3). Except for KRT19, all of them are Wnt/β-catenin target genes. This further highlights the importance of Wnt signaling in stem cell control(9). 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(10, 11). 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 (12). 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(13).

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(14). At the opposite, in the absence of interaction of Wnt ligands with their cell surface receptors, phosphorylated β-catenin undergoes proteasomal degradation(15).

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(16). SFRPs are soluble molecules that possess a Frizzled Cysteine-Rich Domain (FZD_CRD), structurally homologous to the Wnt-binding domain of the FZD receptors(17). They also possess a netrin domain(17). 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(17). Through their netrin domain, they bind heparan sulfates(17, 18). 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 (19, 20). 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(18). Thus, SFRPs may either promote or inhibit Wnt signaling depending on concentration, FZD receptor context and the composition of the cell surface microenvironment(18). DKKs bind the Wnt co-receptors LRP5/6, blocking the formation of ternary LRP-FZD-Wnt complexes to inhibit Wnt signaling(16). 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(16).

Among the ECM of components of HCCs, COL4A1 and LAMC1 are major basement membrane proteins upregulated in angiogenesis and cancer extracellular matrix remodeling(21-23). Of note, LAMC1 expression is modulated by Wnt signaling(24). Although HCCs are soft cellular tumors with scant intratumor fibrosis(25), 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.

Materials and Methods

Real-time PCR primers and antibodies

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

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(26). 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).

Functional Genomics Analyses

Gene networks were generated by Weighted Correlation Network Analysis (WGCNA, WGCNA package)(27) using real-time PCR mRNA expression data from the indicated patient groups, after removing the experimental batch effect with the *SVA* package(28). Gene networks were visually integrated with Cytoscape, with a correlation coefficient threshold >0.30(29). Genomics analysis using microarray-based raw gene expression data downloaded from Gene Expression Omnibus were background corrected and $\log_2$ intensity summary values for each probe set were calculated using Robust Multi Array Average (*Affy* package). Probes were further pre-filtered using a p-value detection threshold ($p<0.05$) in all samples. When multiple probe sets mapped a unique gene, we used the probe with a p-value detection <0.05 in most samples. Finally, data were quantile normalized (*preprocessCore* package) and hierarchical clustering was performed with the *hclust* function in R software, applying the Ward method to correlation-based distances. Gene set enrichment analysis (GSEA) was performed with the web-based tool developed at the Broad Institute (30).

Statistical Analyses

Raw feature data from Gene Expression Omnibus were background corrected and log2 intensity expression summary values for each probe set were calculated using the Robust Multiarray Average package (RMA, Affymetrix). Probes were further pre-filtered using P-value (<5% in all samples). When multiple probe sets mapped a unique gene, we used the probe with a p-value detection<0.05 in most samples. For

survival analyses, non-tumor and normal tissue samples were excluded from the analysis. We then generated a transcriptomic signature associated with disease-free survival measured by a Recurrence Index (RI). To this end, we applied the Random Survival Forest (RSF) method (RandomForestSRC package). RSF is a method for prediction and variable selection for right-censored survival by growing a high number of decision trees after bootstrap subsampling(31). A Cumulative hazard function (CHF) was calculated for each individual and used to estimate the RI. Input variables were the 17 genes analyzed throughout this study. Serum alpha-fetoprotein (with a cutoff over 300ng/ml) was also integrated for variable selection. The events to predict by RSF were the disease-free survival status at each time. Variable importance (VIMP) scores were computed for all the variables used to grow the trees, and the most informative from this model were selected to fit a new RSF model. Once the model was built, we tested whether selected variables were redundant or "noisy" by generating incremental RSF models adding one more variable, ranked by the VIMP calculated in the previous model. The final model was used to generate a RI score for each individual. RI scores were computed as the sum of the CHF for each patient weighted by the number of individuals at risk at distinct time points. Survival analyses were performed using the Log Rank test and Kaplan-Meier curves. Correlation analyses between gene expression levels were performed with the Spearman rank order correlation test (continuous variables, non-parametric distribution). Association analyses between binary or ordinal variables (expression scores, clinical, biological or anatomic pathology data) were performed with Pearson's Chi2 or Fisher exact test. Group comparisons were done with non-parametric ANOVA and the Kruskal Wallis post hoc tests, unless otherwise indicated. Heatmaps showing associations between binary patient data were constructed with

Excel (Microsoft Office 2010). Statistical calculations were performed with the R software (version 3.1.1) and the packages *survival, colorspace, made4* and *marray* or with Statistica 10 (Statsoft, Maisons-Alfort, France).

For cell culture and clonogenesis assay, nucleic acid extraction, real-time PCR, tissue microarray (TMA) and immunohistochemistry scoring of human tissues, see Supplementary Materials and Methods.

3. RESULTS

3.1. Tumors containing fibrous nests are less differentiated, enriched in Wnt/β-catenin pathway components and stem/progenitor cell markers

To study the molecular features of HCCs containing fibrous nests, we reviewed archival hematoxylin-eosin-safran-stained tissue sections of 82 HCCs from 70 patients. We carried out systematic microscopic examination in search for fibrous hotspots and we drew a ~1 cm diameter circle around the most fibrotic area on each slide. Circled areas were blindly scored for intensity of fibrosis by two observers (BT, OM) on a four-point scale (Fig. 1A). Tumor differentiation was assessed by the Edmondson's score on a four-point scale, where score 1 indicates well-differentiated tumors and score 4 corresponds to poorly differentiated ones(32). High-grade intratumor fibrous hotspots (Fig. 1B) were associated with poor tumor differentiation and high expression of the tumor progenitor cell marker mRNAs CD44, IGFBP5, SOX9 and LEF1, the soluble modulators of Wnt signals SFRP2 and SFRP5, as well as the cell surface anchored heparan sulfate proteoglycan GPC3 (Fig. 1B). Tissue microarray-based immunohistochemistry in mirror-image formalin-fixed, paraffin-embedded sections from the same tumors showed that high-grade intraturmor fibrous hotspots contained cancer cell clusters expressing GPC3, CD44, LGR5 and SOX9 (Figs. 1C and D). By contrast, KRT19 and EPCAM, which label liver progenitor cells having an intermediate phenotype between hepatocytes and biliary cells(10, 11), were not associated with intratumor fibrosis (Fig. 1B). Fibrous hotspots expressed alpha smooth muscle actin (ACTA2), indicating that fibrous nests were enriched in myofibroblasts (Fig. 1D).

We then studied the relationships between the liver progenitor cell markers KRT19, EPCAM, LGR5, SOX9 and GPC3. We chose GPC3 because it is a well-characterized tumor hepatocyte marker and a cell surface heparan sulfate proteoglycan(33, 34) that binds Wnts and FZD receptors, amplifying Wnt signals(34). As expected(7, 10), KRT19 was highly correlated with EPCAM (Fig. 2A), and both were weakly correlated with SOX9. CD44 was correlated with LGR5, as well as with SOX9 and GPC3. Associations between these markers were more easily pinpointed by in situ protein analysis of fibrous hotspots, rather than by solution-based mRNA analysis of whole frozen tissue blocks. These data are consistent with the notion that “fibrous nests” are localized entities, where tumor cells expressing stem/progenitor markers may be found at higher densities.

Then, we split the HCC population in two subsets: low and high tumor fibrosis, using a cutoff score ≥2. HCCs with high tumor fibrosis showed higher mRNA and protein levels of CD44; GPC3 and SOX9 (Fig. 2, B-D). LEF1 (analyzed at the mRNA level only) was also higher in HCCs with high fibrosis scores (Fig. 2E). By contrast, LGR5 was only associated with high fibrosis scores by immunohistochemistry (Fig. 2F). Taken together, these findings suggested that HCCs containing fibrous nests were enriched in Wnt/β-catenin pathway target genes and such as CD44, SOX9, LEF1, SFRP2 and LGR5 (Fig. 1B, C and 2B, D-F). Some of them are also stem/progenitor cell markers, such as CD44, IGFBP5, SOX9 and LGR5 (Fig. 1B, C and 2B, D-F).

3.2. Myofibroblast activation is associated with tumor fibrosis and with the expression of cell surface Wnt pathway and basement membrane components.

We were surprised to find an association of SFRP2 and 5 with tumor fibrosis (Fig. 1B), because SFRPs are frequently downregulated in cancers(35). To confirm this finding, we searched for correlations between SFRPs and smooth muscle actin (ACTA2), which is a myofibroblast activation marker. High mRNA levels of ACTA2 were associated with high scores of fibrosis in 78 HCCs (Fig. 3A). In non-tumor livers, fibrosis was graded by the METAVIR score(36). Out of 66 available non-tumor samples, 62 (92%) showed severe fibrosis, i.e., scores ≥3 (Supplementary Table 3). In these samples, SFRP1; 2 and 5 and, in HCCs with high tumor fibrosis, SFRP1 and 2 correlated with ACTA2 (Fig. 3B). Consistently, Fig. 3C shows that SFRP1; 2 and 5 were higher in HCCs expressing high ACTA2 levels. We thus asked whether other Wnt pathway components were associated with myofibroblast activation in HCCs. High levels of the basement membrane components COL4A1 and LAMC1 were associated with ACTA2. In addition, DKK1; FZD1; FZD7; WNT2 and LEF1 were increased in HCCs expressing high ACTA2 levels. By contrast, WNT3; KRT19 and EPCAM were not associated with ACTA2. Taken together, these data suggest that myofibroblast activation is associated with the expression of cell surface Wnt pathway components.

3.3. SFRP1; 2 and 5 are expressed by mesenchyme-committed cancer stem/progenitor cells.

Cancer-associated myofibroblast-like cells may either originate from stromal cell recruitment or from cancer cell dedifferentiation and epithelial-mesenchymal transition (EMT)(37, 38). We thus asked whether SFRP expression was a general theme in mesenchymal lineage commitment. To achieve a general overview, we

studied five cellular models involving mesenchymal commitment and/or myofibroblast differentiation. First, we analyzed the GSE49910 microarray dataset gathering 745 primary cell samples(39). To identify the cell lineages expressing the highest levels of SFRPs, we set the cutoff at >0.91 centile for SFRPs 1 and 2 and >0.5 centile for SFRP5 (Fig. 4A and Supplementary Fig. 1A). Consistently with our in vivo findings, SFRP1 was mainly expressed by smooth muscle cells, stem cell-derived neural precursors, stem cells, Schwann cells and fibroblasts (Fig. 4A). SFRP2 was mainly expressed by stem cells and NCAM(+)/EPCAM(+) mesodermal progenitors (Fig. 4A), which represent the first stages of mesoderm commitment of undifferentiated human embryonic stem cells(40). In turn, SFRP5 (Supplementary Fig. 1A) was predominantly expressed by cells of mesodermal origin, except for Schwann cells and keratinocytes (ectodermal origin). Particularly, the highest levels of SFRP5 were detected in mesenchymal stromal cells undergoing osteoblast differentiation.

Second, a convenient model to study cell plasticity is the generation of hepatocyte-like cells from fibroblast-derived undifferentiated iPS cells(41) (Fig. 4B). In this model, SFRP1 and 2 were preferentially expressed by undifferentiated iPS cells, their expression dramatically decreasing upon hepatocyte differentiation (Fig. 4B). By contrast, SFRP5 was detected at low levels in fibroblast-derived undifferentiated iPS cells (Supplementary Fig. 1B).

Third, we recently showed that soluble Wnt signals reprogram liver progenitor cells to replicating fibrogenic myofibroblast-like cells with stem and invasive features(13). Consistently, we confirm here that reprogramming liver progenitors with 200 ng/ml Wnt3a induces a dramatic fall in albumin expression by hepatocyte-like cells, shifting the cell phenotype toward myofibroblast-like cells expressing ACTA2, COL4A1,

LAMC1 (Fig. 4C), as well as SFRP1 and DKK1, with a considerable decrease in the epithelial cell marker E-cadherin (Fig. 4D).

Fourth, cancer cells can dedifferentiate to stem/progenitor cells through transient EMT, which fosters tissue invasion, cell proliferation and tumor aggressiveness(38). To study the involvement of SFRPs in this process, we used HepaRG progenitor cells(38, 42), which are derived from a human HCC(43). Like 60% HCCs, they contain a TERT mutation(44), can be tumorigenic in mice(45), but have wild-type β-catenin pathway components(42). HepaRG progenitor cells differentiate into hepatocyte-like and biliary-like cells when grown at confluency for 30 days(42). When these differentiated HepaRG cells are split and seeded at low density, they express TWIST1 and SNAI1 transcription factors, undergo EMT, dedifferentiate to liver progenitors displaying mesenchymal features (fusiform morphology, expression of mesenchymal markers such as vimentin and ECM proteins) and express an mRNA signature of high tumor aggressiveness four days after seeding (38). Thus, four days after seeding differentiated HepaGR cells at low density, we observed high expression of SFRP1 and 2, which gradually decreased over 30 days, along mesenchymal to epithelial transition (MET) and hepatocyte differentiation (Fig. 5A). Of note, SFRP5 was expressed at very low levels in HepaRG cells (Supplementary Fig. 3B) and not modulated along hepatocyte differentiation (not shown). SFRP1 and 2 expression kinetics matched those of LGR5(6) and the fibroblast marker COL1A1 (Fig. 5A). It was opposite to the kinetics of the hepatocyte differentiation marker ALDOB (Fig. 5A). These data indicate that SFRP1 and 2 are upregulated during EMT. Conversely, their expression falls during MET in liver cancer cells.

Fifth, to further explore cell plasticity, we used the 3P/3SP model of spontaneous EMT in human HCC(46). Both cell lines derive from the same HCC, 3SP resulting

from 3P cells that underwent spontaneous EMT(46). Thus, using publically available transcriptomic data from 3P and 3SP cell lines, we found that the genes associated with fibrous nests and/or myofibroblast activation were expressed at higher levels in mesenchymal 3SP than in epithelial 3P cells (Fig. 5B). Of note, epithelial 3P cells expressed higher levels of the tumor hepatocyte marker GPC3(33) and the epithelial stem cell marker LGR5(6) than 3SP cells. In addition, although GPC3 and LGR5 were associated with fibrous nests in HCCs, they were not associated with high ACTA2 expression. This is explained by the topography of LGR5 and GPC3 expressing cells. As shown in Fig. 1D, LGR5 and GPC3 were expressed by nodules of epithelial tumor cells. Moreover, we have previously shown that LGR5-expressing cells are seen both in epithelial tumor nodules and as single rows of cancer cells invading the tumor stroma, in an “Indian file” pattern, interspersed along the axis of ECM fibers(13). Further confirming our findings in HCCs, unbiased Gene Set Enrichment Analysis (GSEA) revealed that the top 20% of mRNA signatures matching the transcriptome of mesenchymal 3SP cells included Wnt/β-catenin signaling, stem cells, extracellular matrix remodeling, TGFB signaling, bad outcome in cancer and resistance to anti-cancer treatments. Importantly, these stem cell signatures matched those of aggressive non-hepatic cancers, indicating that mesenchyme-committed liver cancer cells are highly undifferentiated (Fig. 5C and Supplementary Table 4).

3.4. Cell surface Wnt pathway components network with the myofibroblast activation marker alpha smooth muscle actin (ACTA2) and with the basement membrane components collagen type IV (COL4A1) and laminin ɣ1 (LAMC1) in severe liver fibrosis and in HCCs with high tumor fibrosis.

SFRPs are soluble tissue morphogens that bind the cell surface matrix, thus creating short range concentration gradients spanning only a few cells(35). In normal liver, SFRPs 1 to 5 mRNAs were expressed at low levels (Supplementary Fig. 2). In eight HCC and two colorectal cancer cell lines, SFRP1 and 5 were dramatically lower than in normal liver (Supplementary Fig. 3, A and B) and restoring SFRP1 and SFRP5 in the Huh-7 HCC cell line decreased clonogenesis (Supplementary Fig. 3, C and D). The Huh-7 cell line carries wild-type Wnt/β-catenin pathway components(47), but shows active β-catenin as a result of an increase in Wnt ligands and SFRP promoter methylation(48, 49). Likewise, we(19, 20, 50) and others(51) have shown that SFRP1 blocks Wnt/β-catenin signaling and tumor growth in cells carrying β-catenin exon 3 mutations, including the hepatoblastoma cell line HepG2(50).

The increase in SFRPs 1; 2 and 5 in HCCs showing myofibroblast activation was difficult to reconcile with the dramatic downregulation of SFRPs in cancers(35). To understand this paradox, we analyzed 82 HCCs, 66 matching non-tumor livers, 23 histologically normal liver controls and 11 focal nodular hyperplasias (FNH). FNHs are benign liver tumors that display features of the stem cell niche, particularly rich in transit-amplifying liver cell progenitors and extracellular matrix remodeling, without the potential for tissue destruction of cancer(3, 52).

Supplementary Fig. 4 shows mRNA expression analysis confirming downregulation of SFRP1(48) and upregulation of WNT3(53) in HCCs. SFRP5 was downregulated as well. As SFRPs 1 and 5 were lower by several folds in HCCs than in normal liver controls, matching non-tumor livers and FNHs, this downregulation appears to be cancer-specific and could help the diagnosis of HCCs. By contrast, WNT3, FZD1 and FZD7 were higher in HCCs, non-tumor livers and FNHs than in controls. COL4A1 was expressed at higher levels in HCCs, non-tumor livers and FNHs than in controls.

Supplementary Fig. 5 shows that SFRP2, ACTA2, WNT2 and LAMC1 were not significantly different in HCCs from controls or non-tumor samples, but that DKK1 was expressed at higher levels in HCCs than in controls..

Ninety-two percent of non-tumor livers in this series showed high fibrosis (61 out of 66 with METAVIR score ≥3; Supplementary Table 3). In these samples, COL4A1, LAMC1 and ACTA2, were correlated with all the Wnt pathway components tested but Wnt3 (Supplementary Table 5A). In 82 HCCs, COL4A1, LAMC1 and ACTA2 were correlated with SFRPs 1 and 2. The correlation of ACTA2 with SFRPs 1 and 2 and of COL4A1 with SFRP1 was only seen in HCCs with high tumor fibrosis. We then applied Weighted Correlation Network Analysis(27, 29) that revealed associations of ECM remodeling and Wnt pathway components, particularly in liver fibrosis and in HCCs with high tumor fibrosis, but not in HCCs with low tumor fibrosis (Fig. 6). These data indicate that despite the dramatic decrease in SFRP1 and SFRP5 expression in HCCs, these SFRPs (within a low-level expression range), as well as other cell surface Wnt pathway components (i.e., WNT2, DKK1, FZD7 or FZD1) were associated with the basement membrane components COL4A1 and LAMC1 and with the myofibroblast activation marker ACTA2. We have previously shown that COL4A1 expression is correlated with the mRNA expression of the ECM remodeling enzymes MMP2 and MMP14 and with MMP2 enzymatic activity(54). Reanalysis of the available unpublished data from that report involving 47 human HCCs confirmed that this was also the case for LAMC1 (Supplementary Table 5B). Taken together, these findings suggest that the microenvironment of fibrous nests brings together myofibroblast activation, expression of basement membrane components and cell surface Wnt signals.

3.5. A minimal Wnt & extracellular matrix signature is associated with tumor recurrence and ominous clinical features in patients with HCC.

Using the 17 genes analyzed in this study, we applied the unbiased random survival forest (RSF) method to generate a minimal signature associated with patient outcome. To this end, we analyzed a public dataset including whole genome tumor tissue mRNA expression data (GSE14520) and clinical annotations (Supplementary Table 6) from 247 patients with HCC treated by surgical resection(55). Serum alpha-fetoprotein >300 ng/ml was also included in variable selection. The events to predict were the disease-free survival status at each time point. The final model yielded four variables: DKK1; COL4A1; SFRP1 and LAMC1 and was used to generate a recurrence risk index (RI; range, 17-132) for each individual. Using the centile 0.75 of this index (RI>62), we split the 247-patient dataset in high and low risk groups. Fig. 7 shows that the high risk group had lower disease-free and overall survival rates over a 5-year follow-up. Clinical features of patients with high RI were: advanced tumor staging, HCCs arising at a younger age, high serum alpha fetoprotein levels and high incidence of the *Metastasis Signature* (Fig. 7, C, D and Supplementary Table 7). The *Metastasis Signature*(55) is a 161-gene risk classifier enabling prediction of tumor relapse in early-stage HCC patients. Clinical features not associated with high RI are shown in Supplementary Table 7. In addition, high mRNA levels of FZD7; COL4A1; LAMC1; DKK1; SOX9; GPC3 and LEF1 were associated with clinical features announcing bad HCC outcome (Supplementary Table 8).

DISCUSSION

We found that tumors containing fibrous nests were less differentiated and expressed Wnt pathway components and target genes as well as stem/progenitor cell markers. Tissue microarray-based immunohistochemistry analysis of tissue carrots directly punched from fibrous nests in formalin-fixed paraffin-embedded HCC tissues revealed GPC3 (+) HCC cells expressing the tumor stem/progenitor cell markers CD44, LGR5 and SOX9. By contrast, neither KRT19 nor EPCAM were associated with fibrous nests, probably because combined hepatocellular-cholangiocarcinomas were excluded from our study. Similarly, a previous work found that the association of KRT19 with fibrous stroma was cohort-dependent(56). GPC3(34) and LGR5(57) are both cell surface enhancers of Wnt signaling. GPC3 is specific of tumor hepatocytes and is used in routine diagnosis of HCC, with a 77% sensitivity(33). LGR5 hallmarks Wnt-driven amplification of liver progenitors(57). In addition, we found other molecules associated with fibrous nests and/or myofibroblast activation, such as LAMC1, COL4A1, SFRP1; DKK1; FZDs 1 and 7; WNT2, IGFBP5 and LEF1.

Downregulation of SFRP expression by promoter methylation is a common theme in most human cancers, including HCCs(35, 49, 58). Conversely, ectopic expression of SFRP1 (48, 51) and SFRP5 (this work) decreases tumor cell growth. By contrast, a recent methylome profiling of 304 HCCs did not confirm SFRP promoter methylation among the top-100-ranked probes, the frequency of SFRP5 methylation in HCCs being of only 3 %(59). As SFRP1 and 5 were by several folds lower in HCCs than in non-tumor livers, other mechanisms of SFRP downregulation in HCC should be explored. Whatever the case, two lessons were drawn from our findings. First, in contrast with FZDs 1; 7 and WNT3, which were upregulated in both tumor and non-tumor samples, the fall in SFRPs 1 and 5 was HCC-specific. Second, in HCCs,

SFRP1 was associated with myofibroblast activation. We confirmed these findings by five different cell biology approaches, including Wnt-induced differentiation of liver progenitor cells into fibrogenic myofibroblast-like cells, which upregulated SFRP1 expression. Consistently, SFRP1 favors amplification of mesenchymal stem cells in muscular dystrophy(60) and induces matrix metalloproteinase expression and pulmonary fibrosis(61). In addition, direct interaction of SFRP1 with the FZD2 receptor modulates axon growth, the final output depending on the fibronectin/laminin balance in the ECM(17).

We recently showed that soluble Wnt signals reprogram liver cancer progenitor cells into myofibroblast-like cells(13). In this context, we confirmed that Wnt-induced myofibroblast-like cells upregulate the expression of DKK1, COL4A1, SFRP1 and LAMC1. In 247 HCC patients, these four genes were associated with bad disease-free and overall outcome as well as with advanced tumor staging, high alpha-fetoprotein levels and a 161-gene signature predicting tumor relapse. Furthermore, seven genes upregulated in fibrous nests and/or in HCCs with myofibroblast activation were associated with bad outcome predictors (Supplementary Table 8). These included FZD7 and DKK1, which are markers of Wnt/β-catenin activation in HCCs(62-64). DKK1 is a Wnt pathway inhibitor, which expression is induced by active β-catenin. It is thus involved in a negative feedback loop that controls Wnt pathway activation in normal cells(65). However, in some cancer types, high DKK1 reflects active Wnt/β-catenin signaling(64, 66). Particularly, DKK1 is a diagnostic biomarker of HCC(67). In turn, WNT2, which is secreted by fibroblasts, activates Wnt signaling and tumor progression in oesophageal cancer(68), and is associated with the differentiation of pancreatic stellate cells to myofibroblasts in chronic pancreatitis(69). In the liver, WNT2 is secreted by sinusoidal endothelial cells and,

like LGR5, contributes to the stem cell niche for hepatocyte regeneration after injury (6, 57, 70, 71). Altogether, these findings indicate that tumor fibrous hotspots are contingent with upregulated expression of cell surface Wnt components and target genes, myofibroblast activation and undifferentiated cancer stem cell markers. These elements come together within a specific, topographically defined tumor microenvironment that we called *fibrous nests.* This term conveys the notion of cell surface signals nurturing tumor growth and associates the cancer stem cell niche with tumor tissue structures detectable by routine anatomic pathology analyses.

Figure legends

Graphical Abstract: ***"Fibrous nests"*****:** HCCs containing fibrous nests are enriched in Wnt signals, i.e., cell surface Wnt pathway components and target genes. These are associated with markers of mesenchymal commitment, stem cells and extracellular matrix remodeling, indicating tumor aggressiveness. By random forest survival analysis, we show a minimal gene signature (DKK1, COL4A1, LAMC1, SFRP1) associated with poor clinical outcome. By in vitro experiments, we confirm that Wnt signals induce the expression of this minimal gene signature, as well as mesenchymal commitment of liver cancer stem/progenitor cells.

Fig. 1. Tumors containing *fibrous nests* are less differentiated, enriched in Wnt/β-catenin pathway components and stem/progenitor cell markers: (A) Hematoxylin-eosin-safran staining of formalin-fixed paraffin-embedded HCCs showing different grades of tumor fibrosis (TFi). Grade 0, no fibrosis; 1, mild fibrosis (i.e., slender fibrous strands in an otherwise sinusoidal stroma, *arrows);* 2, moderate fibrosis (i.e., compact fibrous tissue forming incomplete septa, *arrows);* 3, severe fibrosis (tumor cell clusters completely trapped within dense bridging fibrous tissue). (B and C) Data from 82 human HCCs. Tissues were collected and processed to create mirror image frozen *(B, FROZEN)* and formalin-fixed, paraffin-embedded *(C, PARAFFIN)* blocks. Each column corresponds to a tumor. Rows: TFi in 82 human HCCs (Low, 0-1; n=43; High, 2-3; n=34), Edmondson-Steiner's score (Low, 1-2; High, 3-4), real-time PCR mRNA expression analysis *(qPCR)*, immunochemistry scoring *(IHC)* on contiguous *FROZEN* sections and tissue microarray-based *IHC* on *PARAFFIN* sections. White cells, missing data. *P,* Fisher's exact test and Gamma correlation coefficient (R=) are shown on the right. (D) TMA-based

immunohistochemistry in HCC tissues punched from fibrous hotspots. Peroxidase-labelled antibodies *(brown);* hematoxylin counter-staining *(blue). Upper left corners,* gene symbols. *Lower left corners,* TFi scores. *Lower right corners,* magnification. Tissue slides were scanned at 20X. *T,* tumor; *TFi,* tumor fibrosis. Red arrowheads indicate isolated CD44, GPC3 or SOX9-staining cells within the tumor stroma. SOX9 (+) tumor cell clusters are embedded within the fibrous tissue, some of them lying within the subendothelial zone of capillary vessels *(V,* red arrowheads). LGR5 (+) tumor cords are interspersed within a loose fibrovascular stroma.

Fig. 2. **High expression of cancer stem/progenitor markers in HCCs with tumor fibrosis**: (A) Spearman's correlation coefficients between the indicated proteins (TMA, Tissue MicroArray-based immunohistochemistry detection) and mRNAs (QPCR). Asterisks denote statistical significance *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. The number of analyzed samples is indicated between parentheses. (B-E) Tumor fibrosis is associated with high CD44, GPC3, SOX9 (mRNA and protein), LGR5 (protein) and LEF1 (mRNA) levels. Mean±confidence intervals. *P,* Mann-Whitney U test.

Fig. 3. Myofibroblast activation is associated with tumor fibrosis and with the expression of cell surface Wnt pathway and basement membrane components. (A) High ACTA2 mRNA levels in tumors with high fibrosis scoring. (B) Spearman's correlation analysis of SFRP1; 2 and 5 with ACTA2 mRNA levels in fibrotic non-tumor livers and in HCCs with *High* and *Low* Tumor Fibrosis *(High TFi; Low TFi).* Statistically significant correlations are highlighted in red. (C) ACTA2 mRNA levels in HCCs were split into *High* (n=20) and *Low* (n=58) groups (cutoff = median). SFRP1;

2 and 5; the extracellular matrix components COL4A1 and LAMC1; the Wnt inhibitor DKK1; the Wnt cell surface receptors FZD1 and FZD7; the WNT2 ligand, the mesenchymal cell marker IGFBP5 and the β-catenin pathway transcription factor LEF1 are associated with high levels of ACTA2 expression.

Fig. 4. SFRP1 is expressed by mesenchyme-committed cancer stem/progenitor cells. (A) SFRP1 and SFRP2 mRNA expression data extracted from the GSE49910 microarray meta-analysis dataset consisting of 745 primary cell samples analyzed with Affymetrix Human Genome U133 Plus 2.0 expression arrays. Normalized background-corrected intensities(39) of mRNA expression were filtered to reveal the cell lines expressing the highest (>20xmedian; >centile 0.91) mRNA levels of SFRPs 1 and 2 in at least duplicate samples. Cell lines below the defined threshold but originally used as controls of at least one cell line showing >20x median mRNA levels, were included for comparative purposes. Threshold lines for each gene are indicated (-----). (B) Generation of hepatocyte-like cells from fibroblast-derived undifferentiated iPS cells, as described(41). SFRPs 1 and 2 are shown in human foreskin fibroblasts; foreskin fibroblast-derived iPS cells; iPS cells undergoing hepatocyte differentiation; human fetal liver and primary human hepatocytes. (C) Immunodetection of albumin (ALB), alpha smooth muscle actin (ACTA2), collagen type IV (COL4A1) and laminin ɣ1 (LAMC1). Red, FluoProbes 594; green, FITC; orange, TRITC; blue, DAPI (nuclei). Liver progenitors incubated with Wnt3a (200 ng/ml; for 13 days) show few cells containing albumin *(white arrow).* Instead, they differentiate into spindle ACTA2 (+) cells which develop stress fibers typical of myofibroblasts *(white arrows).* The extracellular matrix surrounding these cells contains COL4A1 and LAMC1 *(white arrows).* Control cells receiving control medium

(BSA) develop a hepatocyte-like, albumin (+) phenotype. Images were acquired with a Cellomics station at 20X. (D) Relative RNA expression of the indicated genes in HepaRG liver progenitor cells incubated with control medium (BSA) versus 200 ng/ml Wnt3a.

Fig. 5. Bidirectional **epithelial-mesenchymal cancer cell plasticity modulates the expression of Wnt pathway components, cancer stem cell and myofibroblast marker genes.**

(A) Kinetics of mRNA expression of the indicated genes along the differentiation of HepaRG progenitors into hepatocyte-like cells. HepaRG liver progenitors were plated at $1x10^4$ cells/cm^2 and incubated during 30 days, as described(38). Samples harvested at day 4 were used as calibrators. Histograms show mean±SD from triplicates. SFRP5 is not shown here because it is hardly detected in HepaRG cells (Ct>33, Supplementary Fig. 3B). (B and C) 3P/3SP model of spontaneous epithelial to mesenchymal transition (EMT) in human HCC(46). Both cell lines are derived from the same HCC, mesenchymal 3SP arose from 3P cells through EMT. (B) Hierarchical clustering of publically available (GSE26391) whole genome mRNA data from 3P and 3SP cell lines (at early and late passages). Genes of interest are indicated. (C) Unsupervised Gene Set Enrichment Analysis (GSEA) of the GSE26391 mRNA set shows that 3SP cells are enriched in Wnt pathway, stem cell and extracellular matrix remodeling signatures. The top 20% signatures matching the 3SP transcriptome are shown in Supplementary Table 4.

Fig. 6. A network involving cell surface Wnt pathway components, basement membrane gene expression and myofibroblast activation in severe liver

fibrosis and HCCs with high tumor fibrosis. Weighted Correlation Network Analysis (WGCNA)(27) of mRNA expression data from the indicated patient groups. Networks with a correlation coefficient threshold >0.30 were visually integrated with Cytoscape(29). Closeness between nodes is proportional to the number of connections. Thickness of links is proportional to the correlation coefficients between genes. Correlation coefficients are shown in Supplementary Table 5A.

Fig. 7. A minimal signature is associated with lower survival rates and ominous clinical features in patients with HCC. A minimal signature (DKK1; COL4A1; SFRP1 and LAMC1) was generated by the random survival forest (RSF) method from a public mRNA expression dataset (GSE14520) with clinical annotations (Supplementary Table 6) from 247 patients with HCC treated by surgical resection (55). This signature was used to generate a recurrence risk index (RI; range, 17-132) for each individual. Using the centile 0.75 of this index (RI=62), we split the 247-patient dataset in high (RI>62) and low (RI<62) risk groups. Kaplan-Meier curves and Log-Rank tests show significantly different (A) Disease-free and (B) overall survival rates. (C and D) Differential clinical features of patients with high and low RI. *Age* is only shown in *D*. NA, number of patients with missing data for the specified variable.

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