Tumorigenesis and Neoplastic Progression

Keratin 19 Epithelial Patterns in Cirrhotic Stroma Parallel Hepatocarcinogenesis

Jochen K.M. Lennerz,^{*,†} William C. Chapman,[‡]
and Elizabeth M. Brunt[†]

From the Department of Pathology,* Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; and the Departments of Pathology and Immunology,[†] and Surgery,[‡] Washington University School of Medicine, St. Louis, Missouri

**Cirrhotic septa harbor vessels and inflammatory, fibrogenic, and ductular epithelial cells, collectively referred to as the ductular reaction (DR). Lack of the DR in the stromal compartment around hepatocellular carcinoma (HCC) has been documented; however, the relationship of epithelial keratin 19 (K19) structures to progression of intralesional carcinogenesis has not been explored. K19 immunoreactivity in the stromal compartment around 176 nodules in cirrhotic explants was examined. Quantitative differences ($P < 0.0001$) were manifested in three distinct histologically identifiable patterns: “complex” around cirrhotic nodules (CN), “attenuated” around dysplastic nodules (DN), and “absent” around HCC. Markers of necrosis or apoptosis could not explain the perinodular K19 epithelial loss; however, multicolor immunolabeling for K19, vimentin, E-Cadherin, SNAIL, and fibroblast-specific protein 1 (FSP-1) demonstrated discrepancies in immunophenotype and cytomorphic features. Variability of cellular features was accompanied by an overall decrease in epithelial markers and significantly increased fractions of SNAIL- and FSP-1-positive cells in the DR around DN when compared with CN ($P < 0.0001$). Immunolabeling of transforming growth factor- β signaling components (TGF β R1, SMAD3, and pSMAD2/3) demonstrated increased percentages of pSMAD2/3 around DN when compared with CN ($P < 0.0001$). These findings collectively suggest marked alterations in cellular identity as an underlying mechanism for the reproducible extral-
esional K19 pattern that parallels progressive stages of intranodular hepatocarcinogenesis. Paracrine signaling is proposed as a link that emphasizes the importance of the epithelial-stromal compartment in malignant progression of HCC in cirrhosis. (*Am J Pathol* 2011, 179:1015–1029; DOI: 10.1016/j.ajpath.2011.04.040)**

In advanced chronic liver disease (cirrhosis) from virtually any cause, cirrhotic nodules (CN) containing hepatocytes are surrounded by perinodular stroma, that is, cirrhotic septa, that consists of an expanded fibrous matrix harboring lymphovascular structures, mixed inflammatory cells, and epithelial cells with ductular phenotype, the “ductular-reaction” (DR)^{1–3} (Figure 1A). The presence of these biliary-type epithelial cells between mesenchymal elements makes perinodular cirrhotic septa by definition an epithelial-stromal compartment (ESC) (Figures 1B; see also Supplemental Figure S1 at <http://ajp.amjpathol.org>). Although this ESC is the primary site for adaptations to sustained injuries (eg, fibrogenesis in chronic inflammation),^{4,5} the number of investigations of the ESC in hepatocarcinogenesis is limited.^{6–8} This is surprising because stromal invasion is a key, if not challenging,⁶ histopathologic feature used to differentiate premalignant dysplastic nodules (DN) from malignant hepatocellular carcinoma (HCC).^{9–11} Furthermore, one of the proposed human stem cell niches resides in the ESC and includes epithelial cells that express type-1 keratin 19 (K19) (Figure 1A),^{1,12,13} a more specific biliary marker than is type-2 keratin 7.^{7,13–15}

Based on the relatively limited characterization of perinodular ESC surrounding hepatocellular nodules (HCN) in cirrhosis^{7,16} the present study initially correlated perinodular K19 patterns with the intranodular hepatocellular pathologic alterations and quantified a compelling relationship of intranodular and extranodular findings to demonstrate that the ESC demonstrates significant alterations in the progression from CN to DN to HCC that can func-

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Address reprint requests to Elizabeth M. Brunt, M.D., Department of Pathology and Immunology, Washington University School of Medicine, 660 Euclid Ave., St. Louis, MO. E-mail: EBrunt@path.wustl.edu.

Figure 1. Epithelial-stromal compartment in cirrhosis. **A:** Hepatocyte interface demonstrates brisk ductular reaction highlighted by K19 immunostaining within the cirrhotic stroma that also contains vessels and inflammatory and fibrogenic cells (hematoxylin counterstain). The K19-positive cells are interspersed between K19-negative hepatocytes, forming spoke-like extensions oriented perpendicular to the nodular perimeter. Note that some of these juxtahepatic K19-positive cells demonstrate weak immunoreactivity. Scale bar = 20 μ m. **B:** Schematic overview of the relationship of K19-positive ductular reaction (DR; red-brown) with adjacent hepatocellular nodules (HCN) and mesenchymal elements (see Supplemental Figure S1 at <http://ajp.amipathol.org>).

tion as a sensitive and specific surrogate for intralesional alterations. The implications of the vanishing epithelial K19 compartment in the progressive stages of hepatocarcinogenesis, however, go beyond routine diagnostics. From studies in other chronic inflammatory conditions of the liver, it is known that K19-positive cells can undergo a phenotype switch referred to as epithelial-to-mesenchymal transition (EMT).^{4,5} Thus, the presence and extent of implicated markers were assessed in the perinodular epithelial compartment surrounding HCN, and findings strongly suggest cellular identity alterations similar to reported phenotype switches as an underlying morphologic mechanism. These new findings suggest paracrine signaling involving the perinodular epithelial-mesenchymal compartment in the multistep process of hepatocarcinogenesis in cirrhosis. Although determination of the direction and exact driving forces will require reliable model systems, results of this comprehensive phenotype assessment demonstrate the extralesional ductular alteration as an indicator, if not a participant, in the peritumoral microenvironment.

Materials and Methods

Approval and Case Selection

The Human Studies Committee of Washington University School of Medicine approved the study. Patient samples were chosen as consecutive liver transplant explants from our files and were tested as anonymous samples.

Histologic Analysis

One of us (E.M.B.) selected nodules from liver explants and applied established diagnostic criteria: CN, DN, and HCC.^{9–11} Differentiation between low- and high-grade dysplasia was not performed. Each HCN was regarded as a separate lesion, outlined on whole-mount images generated via virtual microscopy (see Supplemental Figure S2, A and B, at <http://ajp.amipathol.org>), and another author (J.K.L.), blinded to the diagnosis (H&E-based), performed an independent review by noting the perinodular K19 pattern for each lesion. Necrosis was defined using established criteria including nuclear swelling, chromatin flocculation (Hoechst 33258 stain), loss of nuclear basophilia (H&E), and breakdown of cytoplasmic structures including the membrane.^{17–19} Apoptosis was assessed using morphologic features including cell shrinkage, chromatin cleavage, nuclear condensation and formation of pyknotic bodies of condensed chromatin,^{17,19} and abnormal pattern of incorporated Hoechst 33258,^{19–21} immunostaining for activated caspase 3, and detection of DNA fragmentation and chromatin cleavage using TUNEL.^{19,22} Cellular phenotype [epithelial (Ep) versus mesenchymal (Me)] was defined via a combination of topographic (Ep, location within confines of basement membrane, versus Me, cellular location within stroma), architectural (Ep, cohesive arrangement in compact configurations, versus Me, individual cells in extracellular matrix), cytomorphic (Ep, sharp cell borders with polygonal shape, versus Me, elongated ill-defined cytoplasmic outlines), nuclear (Ep, round to oval nucleus with peripheral chromatin condensation and nonprominent nucleolus, versus Me, elongated to spindle-shaped nucleus with isodense chromatin with minimal irregularity and no nucleolus), and immunophenotypic features adapted from proposed criteria.²³ Cellular identity alteration was defined as discrepancies in these features at the cellular level.

Immunolabeling

Details of antibodies, dilutions, and staining characteristics are given in Table 1. Immunohistochemistry (IHC) was performed automatically using a Benchmark XT automated slides stainer (Ventana Medical Systems, Inc., Tucson, AZ) using established protocols.²⁴ In brief, protocols consisted of pretreatment with CC1 (pH 8.0), incubation with primary antibodies, and detection using an IVIEW-DAB (diaminobenzidine) detection system (catalog No. 760-500; Ventana Medical Systems, Inc.) including ultraview inhibitor, horseradish peroxidase, multimer, chromogen, H₂O₂, and copper. For dual-color labeling,

we used the ultraview Universal Alkaline Phosphatase Red Detection kit (catalog No. 760-501; Ventana Medical Systems, Inc.) including enhancer, naphthol, red A, and fast red B. Immunofluorescence was performed manually according to established protocols.^{24,25} In brief, sections were washed for 5 minutes (xylene ×3, 100% ethanol ×2, 95% ethanol ×1, 70% ethanol ×1, and PBS ×1), boiled in Trilogy for antigen retrieval (Cell Marque, Hot Springs, AR), rinsed in deionized water for 15 minutes, and washed with PBS. Sections were blocked in 1% bovine serum albumin and 0.3% Triton X-100 in PBS and were incubated using combinations of the primary antibodies. Visualization used secondary antibodies conjugated to Alexa Fluor 488 (green), 596 (red), and 647 (magenta) (Molecular Probes, Eugene, OR) at 1:500. Nuclear counterstaining was optimized to differentiate epithelial from mesenchymal nuclei using titrated Hoechst 33258 stain (blue) at 1:20,000 for 5 minutes (dark) or hematoxylin counterstaining for 4 seconds, both at room temperature.

Controls

Staining properties and specificity have been determined previously (Table 1), which we additionally ascertained using i) preabsorption controls using the peptides against which the antibodies were raised at 1:10 and 1:20 dilution; ii) normal serum control in which the primary antibody was replaced with normal (rabbit) serum using 1:10 and 1:20 concentrations; iii) negative control (secondary antibody used without the primary antibody, which was replaced with Tris-buffered saline solution); and iv) comparison control on normal tissues ($\pm$) and comparison with expression data from publicly available sources (<http://www.proteinatlas.org>; last accessed March 19, 2011).²⁶

TUNEL Staining

TUNEL staining was performed using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore Corp., Billerica, MA). In brief, deparaffinized tissue sections 5 μ m thick were washed in PBS and incubated for 30 minutes at 37°C with 10 μ g/mL proteinase K (S3020; Dako Corp., Carpinteria, CA). After quenching of endogenous peroxidase with 3% H₂O₂ in PBS for 5 minutes, washes, and 15-minute transfer into equilibration buffer, sections were incubated with TDT (terminal deoxynucleotidyl transferase) enzyme at 10 U/50 pL, and digoxigenin-labeled nucleotides in a preheated humidified chamber at 37°C for 60 minutes. Subsequent incubation with anti-digoxigenin-peroxidase for 30 minutes and color development with H₂O₂-diaminobenzidine for 5 to 6 minutes was followed by counterstaining using methyl green (S1962; Dako Corp.). Evaluation included positive (intestinal mucosa) and negative (omission of TDT) controls according to established protocols.²²

Microscopy

For light microscopy, either slides were scanned using a ScanScope XT scanner (Aperio Technologies, Inc., Vista, CA) or images were captured using a 12 Mpixel Olympus DP70 camera attached to an Olympus BX51 light microscope (Olympus America, Center Valley, PA). An Axiovert 200 with a 1.4 Mpixel Axiocam MRM camera and Apotome optical sectioning filter was used for acquisition of immunofluorescence images (Carl Zeiss Imp Corp., Maple Grove, MN).

Quantification was performed in perinodular sectors defined using the following histologic landmarks: <25% of the perinodular circumference starting at a random point, determined using a random-number generator [RandBetween (1-361)]; and the interface between the hepatocellular nodule and the surrounding perinodular stroma formed the internal border of the perinodular sector; whereas the external border was defined by outward projection of the internal border to half of the septal thickness (see Supplemental Figure S2, C and D at <http://ajp.amjpathol.org>). K19 quantification (cell and profile counts) was determined covering the entire sector using whole-slide imaging. For high-power quantification, we determined the number of high-power fields necessary to capture at least 1000 biliary epithelial cells ($n = 27$ fields around CN); accordingly, at least 27 fields per marker were quantified. Cells were defined via marker co-localized with nuclear staining in the same plane, whereas the outline of at least one cell formed a profile. Computer-assisted quantification algorithms were applied,²⁴ and segmentation of cells and profiles was achieved using threshold filters in combination with circularity and size cutoffs using “cell counter” and “analyze particle” plug-ins in ImageJ software (National Institutes of Health, Bethesda, MD). A customized link²⁴ between ImageJ, Photoshop CS3 (Adobe Systems, Inc., San Jose, CA), and ImageScope (Aperio Technologies, Inc.) was generated using AutoIT (version 3.2.12.0 by Jonathan Bennett), a freeware scripting language for automating the graphic user interface.

Density Modeling

To test whether the density of K19 elements can be explained by dispersion over a larger area alone, we compared observed densities around DN and HCC using modeled densities calculated using the observed cell number around CN in combination with the mean areas of sectors around DN and HCC.

Statistical Analysis

Unpaired Student's *t*-tests, κ statistic, and intraclass coefficient were used, with $P < 0.05$ considered significant. Sample size estimation followed suggestions by Silcocks,²⁷ and assuming a $\kappa_{\min}$ of 0.75 and $P < 0.05$ for three diagnostic categories, we determined the minimal samples size of 86, which was doubled for this study. Data were analyzed using Prism 5.0b (GraphPad Software, Inc., San Diego, CA), Microsoft Excel 2008 (version 12.1.9; Microsoft Corp., Redmond WA), or the online statistical toolbox of the Chinese University of Hong Kong

Table 1. List of Antibodies

Name	Host	Antigen characteristics	Source catalog or order no.	Dilution	Pattern	Reference
Activated caspase 3	Rabbit (p)	Synthetic peptide corresponding to Ser29+ of human caspase 3 (N terminus of p17 subunit)	Abcam, Inc. ab-32042	1:50	C	28 29
E-cadherin (ECAD/CDH1)	Mouse (m)	Human transmembrane protein 36	Ventana Medical Systems, Inc. 790-4497	Prediluted by manufacturer	M/C	85
K7 (CK7/K2C7)	Mouse (m)	Clone OV-TL 12/30	Cell Marque, Corp. CMA538	Prediluted by manufacturer	C	13
K19 (CK19/K1CS)	Mouse (m)	Clone RCK108 (Immunogen: Human Cell line T24)	Dako Corp. M0888	1:100	C*	13 84
K19 (CK19/K1CS)	Mouse (m)	Clone A53-B/A2.26	Ventana Medical Systems, Inc. 760-4281	Prediluted by manufacturer	C*	13 84
pSMAD3 (Figure 5, A and B)	Rabbit (p)	Synthetic phosphopeptide corresponding to residues surrounding Ser 423/425 of SMAD3	Cell Signaling Technology, Inc. 9520S	1:100	N	85 87
pSMAD2/3 (Figure 5, C–H)	Goat (p)	Short amino acid sequence containing phosphorylated Ser423/425 of human SMAD3	Santa Cruz Biotechnology, Inc. sc11769 (peptide sc-11769P)	1:50	N	4
S100 (Figure S4B)	Rabbit (p)	Purified, full-length, native bovine S100 protein	Ventana Medical Systems, Inc. 760-2523	Prediluted by manufacturer	C	88
SMAD3 (Figure 5, D, E, G, H)	Rabbit (p)	Synthetic peptide corresponding to AA100-150 of human SMAD3	Thermo Fisher Scientific, Inc. PA1-38613	1:100	C*	89
SMAD3 (Figure S6A)	Rabbit (p)	Synthetic peptide corresponding to residues at the AA terminus of human SMAD3	Cell Signaling Technology, Inc. 9523S	1:100	C*	90
SMAD4 (Figure S6B)	Rabbit (p)	Recombinant protein epitope signature tag (PrEST) corresponding to AA118-240 of human SMAD4	Sigma-Aldrich Corp. HPA019154	1:150	C*	91 92
SMAD4	Rabbit (p)	Synthetic peptide corresponding to residues surrounding Pro278 of human SMAD4	Cell Signaling Technology, Inc. 9515	1:100	C*	93 94
pSMAD2 (Figure S6C)	Rabbit (p)	Synthetic phosphopeptide corresponding to residues surrounding Ser 465/467 of human SMAD2	Cell Signaling Technology, Inc. 3101S	1:100	N	95
pSMAD2 (Figure S6D)	Rabbit (p)	Synthetic phosphopeptide corresponding to residues surrounding Ser 245/250/255 of human SMAD2	Cell Signaling Technology, Inc. 3104S	1:100	N	96
SNAIL (SNAI1/SLUGH2)	Rabbit (p)	Synthetic KLH-conjugated peptide AA1-100 of human Snail	Abcam, Inc. ab-17732 (peptide ab19126)	1:100 150 (IF)	N	97

(table continues)

Table 1. *Continued*

Name	Host	Antigen characteristics	Source catalog or order no.	Dilution	Pattern	Reference
TGF β 1R (ALK-5)	Rabbit (p)	Synthetic peptide corresponding to AA158-179 of human TGF β 1R	Abcam, Inc. ab-31013	1:50	M/C	98
FSP-1 (S100-A4)	Rabbit (p)	Recombinant protein encoding full-length human FSP-1/S100-A4	Abcam, Inc. ab-27427	Prediluted by manufacturer	C/N	4 43 44
Vimentin	Mouse (m)	Clone V9	Ventana Medical Systems, Inc. 790-2917	Prediluted by manufacturer	C	99

*Staining pattern identical.

C, cytoplasmic staining; IF, immunofluorescence; K19, cytokeratin 19; m, monoclonal; M, membranous staining pattern; N, nuclear; p, polyclonal.

Antibody source locations were as follows: Abcam, Inc., Cambridge, MA; Ventana Medical Systems, Inc., Tucson, AZ; Cell Marque, Corp., Hot Springs, AR CA; Dako Corp., Carpinteria, CA; Cell Signaling Technology, Inc., Beverly, MA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; Thermo Fisher Scientific, Inc., Rockford, IL; Sigma-Aldrich Corp., St. Louis, MO; Abcam, Inc., Cambridge, MA.

by Prof. Allan Chang (<http://department.obg.cuhk.edu.hk/researchsupport/statmenu.asp>; last accessed March 19, 2011). Statistical performance measures and confidence intervals were determined using the Hutchon toolkit (<http://www.hutchon.net/EPRval.htm>; last accessed March 19, 2011).

Results

The study cohort consisted of 176 HCN [mean: $n = 5$ per liver (range $n = 3-7$)] from 32 cirrhotic liver explants.

Each HCN was assigned to one appropriate diagnosis (CN, DN, or HCC) according to established criteria.^{10,11,25} Based on these diagnoses, the cohort consisted of 170 nodules: 71 CN, 45 DN, and 54 HCC (Figure 2, A and B). Five HCN demonstrated a nodule-in-nodule configuration,⁹⁻¹¹ and one CN exhibited mixed features of DN; these six HCN were assigned to the DN category (subgroup with atypical or overlapping features). The representative nature of the cohort was ascertained by inclusion of a variety of underlying liver diseases and morphometric confirmation of size differences between

Figure 2. Perinodular K19 pattern. **A:** Number of hepatocellular nodules diagnosed using H&E staining as cirrhotic nodules (CN), dysplastic nodules (DN), or hepatocellular carcinoma (HCC) is provided. Representative nodules are outlined on an H&E-stained histotopogram. The total number of nodules assigned to the appropriate pattern of perinodular ductular reaction is provided along with a representative image. See *Results* for detailed descriptions of the three patterns: complex, attenuated, and absent. Scale bars: whole mount, 5 mm; insets, 50 μ m. **B:** Correlation matrix of H&E-based diagnostic categories (columns, diagnosis by H&E) with perinodular K19 pattern (rows). Each field of the matrix shows the absolute number of lesions per combination of category and pattern, and the background gray scale encodes the relative frequency. Thereby, correlation is visualized as a darker diagonal from the **top left** to the **bottom right** (see *Results*). **C:** Bar graph demonstrates prevalence of K19 pattern in each diagnostic category: CN, DN, and HCC. The highest prevalence of the complex pattern was observed around CN, the attenuated pattern around DN, and the absent pattern around HCC. The complex perinodular K19 pattern was not observed around HCC. Statistical performance measures are given in *Table 2*.

Table 2. Statistical Performance Measures of Perinodular K19 Pattern

Variable	Hepatocellular Nodules	
	Without atypical features (<i>n</i> = 170)	With atypical features (<i>n</i> = 176)
True positive	47	47
False positive	15	20
True negative	101	102
False negative	7	7
Sensitivity (95% CI)	87.04 (75.1–94.6)	87.04 (75.1–94.6)
Specificity (95% CI)	87.07 (79.6–92.6)	83.61 (75.8–89.7)
Positive predictive value (95% CI)	75.80 (63.3–85.8)	70.15 (57.7–80.7)
Negative predictive value	93.5 (87.1–97.4)	93.6 (87.2–97.4)
Accuracy	87.06	84.7
Likelihood ratio	6.731	5.309
Pretest odds positive	0.47	0.44
Posttest odds positive	3.13	2.35

Validity comparison of HCN without (*N* = 170) and with (*N* = 170 + 6) atypical features for K19 versus H&E and HCC (positive) versus non-HCC (negative). CI, confidence interval (calculated with binomial expansion); HCC, hepatocellular carcinoma; K19, cytokeratin 19.

CN, DN, and HCC (see Supplemental Tables S1 and S2 at <http://ajp.amjpathol.org>).

Perinodular Structure of K19 Patterns Are Complex, Attenuated, and Absent

Three patterns of DR surrounding CN, DN, and HCC, ie, complex, attenuated, and absent, respectively, were reproducibly documented. An overview is shown in Figure 2A. Specifically, the DR around CN demonstrated numerous ductules consisting of interlacing epithelial clusters that blended into the hepatocyte interface. Three arbitrary zones could be distinguished (see Supplemental Figure S2D at <http://ajp.amjpathol.org>): i) central regions of the internodular septa contained larger ductules, referred to as internodular ducts, often interspersed with thin-walled vessels; ii) the perinodular zone, in which a mixture of ring-shaped ductules and small circular epithelial groups, with or without a central lumen, predominates; and iii) an interface in which slender strings of biliary-type epithelial cells are located between hepatocytes and merge into HCN, resulting in a radial or spoke-like appearance with orientation of cells perpendicular to the perimeter of the nodule. These features are readily appreciated using K19 labeling, and the pattern of DR was termed “complex” (Figures 1A and 2A). The DR around DN demonstrated a well-demarcated interface zone characterized by absence of interspersed biliary-type elements (loss of radial spokes) (Figure 2A), and the remaining epithelial elements were fragmented and predominated as small accumulations of slender cells oriented parallel to the perimeter of the HCN (Figure 2A). Depletion of ductules with lumina left the remaining cellular aggregates as thin cores of ovoid to elongated cells rather than tubular formations (“attenuation”). Internodular ductules around DN were preserved but typically located closer to the surrounding CN. The stroma around well-differentiated HCC was depleted of biliary-type epithelial elements, and

the DR and K19 immunoreactivity was “absent” (Figure 2A).

Perinodular K19 Pattern as a Valid Surrogate for Intranodular Disease

Investigations were performed to determine whether perinodular patterns correlated with HCN (Figure 2). To ensure an unbiased review, nodules were outlined on whole-mount maps, and one K19 pattern was assigned to each nodule. The K19 patterns in the diagnostic groups CN, DN, and HCC included 67 complex, 41 attenuated, and 62 absent, respectively, resulting in substantial correlation with H&E-based diagnoses (Figure 2B). The intraclass coefficient for H&E versus K19 was 0.878 ($P < 0.02$), and differentiation of non-HCC (CN plus DN) versus HCC was almost perfect (0.899; $P < 0.0001$). The negative predictive value of the complex pattern for HCC was 94%. Some DN demonstrated the K19 pattern typically observed around CN (12 of 45; 26%) and individual DN demonstrating absence of perinodular K19 (11 of 45; 24%) (Figure 2, B and C); this overlap was compatible with separate diagnostic groups applied on a continuous biological spectrum. The six additional DN with atypical or overlapping features using H&E-based diagnosis demonstrated absence of DR in the five HCN with a nodule-in-nodule configuration and a complex pattern in the CN with mixed features. Comparison of statistical performance measures with and without these six additional nodules using a binary classification of HCC versus non-HCC (CN plus DN) is given in Table 2 and confirmed that the perinodular K19 pattern can function as an accurate surrogate for intranodular disease.

Quantification of Perinodular K19 Pattern

Next, a more objective quantitative approach was undertaken, and extraction of 56,378, 9008, and 63 cells and

Table 3. Quantification of Perinodular K19 Compartment

Variable	CN (N = 71)			DN (N = 45)			HCC (N = 54)		
DR H&E	+++			+			–		
Pattern	Complex			Attenuated			Absent		
K19	n ⁺	P _{CD}	%	n ⁺	P _{DH}	%CN	n ⁺	P _{CH}	%CN
Profiles (sectors)	408.9 ± 23 (36)	1.5e ^{−14}	Set 100	106.3 ± 14 (27)	4.1e ^{−10}	26	1.52 ± 0.2 (30)	1.7e ^{−23}	0.4
Cells (sectors)	2088 ± 262 (27)	2.9e ^{−9}	Set 100	300 ± 44 (30)	1.3e ^{−5}	14	2.4 ± 0.3 (27)	1.4e ^{−7}	0.1
Density, cells/mm ² (sectors)	16925 ± 2191 (27)	1.6e ^{−9}	Set 100	1610 ± 429 (30)	5.8e ^{−4}	5.8	9.6 ± 3.3 (27)	6.8e ^{−10}	0.02
Marker (HPF)	Σ ⁺ /Σ _t	x ⁺ /x _t	% _t	Σ ⁺ /Σ	x ⁺ /x _t	% _t	%		
FSP-1 (27)	140/1344	5.19 ± 0.4/49.8 ± 4.3	10*	106/175	3.9 ± 0.3/6 ± 0.6	65*	NA		
ECADH (54)	2777/3412	51.4 ± 4/63.2 ± 5	80	377/487	7 ± 0.6/9 ± 0.7	77	NA		
SNAIL (27)	696/2068	25.8 ± 2.3/76.6 ± 7.7	34*	161/312	5.96 ± 0.6/11.6 ± 1.2	52*	NA		
TGFβR1 (27)	1095/2293	40.6 ± 3.6/84.9 ± 7.6	48*	39/248	1.4 ± 0.2/9.2 ± 0.8	15*	NA		
SMAD3 (27)	1573/2293	58.3 ± 5/84.9 ± 7.6	68	184/248	6.8 ± 0.5/9.2 ± 0.8	74	NA		
pSMAD2/3 (27)	619/2293	22.9 ± 2/84.9 ± 7.6	27*	148/248	5.5 ± 0.5/9.2 ± 0.8	60*	NA		

Values (n⁺) are given as mean ± SEM over the number of examined sectors. P values were determined using the Student t-test (P_{CD}, CN versus DN; P_{DH}, DN versus HCC; P_{CH}, CN versus HCC).

*P < 0.0001 (Fisher's exact test, marker fraction CN versus marker fraction DN).

+, present; +++, numerous; –, absent; %_t, percentage of marker-positive elements normalized to the total number of perinodular epithelial cells; % CN, percentage of immunoreactive elements normalized to the number observed in the epithelial-stromal compartment surrounding CN; CN, cirrhotic nodule; DN, dysplastic nodule; DR, ductular reaction; HCC, hepatocellular carcinoma; N = number of hepatocellular nodules (CN, DN, or HCC); HPF, high-power fields within sectors; K19, cytokeratin 19 immunohistochemistry; n⁺, number of immunoreactive elements (profiles or cells); NA, not applicable; x⁺/x_t, average cell number per high-power field ± SEM over average total number of cells in perinodular epithelial compartment; Σ⁺/Σ_t, sum of all marker-positive cells over sum of all cells in perinodular epithelial compartment.

14,722, 2869, and 45 profiles in perinodular sectors of CN, DN, and HCC, respectively, was performed. Density of cells and profiles was expressed per sector μm², and statistical comparison demonstrated a highly significant reduction in K19-positive elements from CN to DN to HCC (Table 3). Of note, when cellular density around the larger DN and HCC was calculated (using numbers observed in CN sectors), the modeled sector densities around DN (5353 ± 671/mm²) and HCC (2784 ± 349/mm²) significantly exceeded the observed number (Table 3; P_{DN} < 0.0001 and P_{HCC} < 0.0001, unpaired Student's t-test). These mathematical considerations excluded K19 reduction around the DN and HCC based on dispersion over a larger area alone (DN observed 74% versus modeled 57%; HCC observed 99.6% versus modeled 78%; all normalized to CN). On the cellular level, the differences were even more pronounced, and an 86% reduction around DN was observed, whereas the reduction was 99.9% around HCC (Table 3). Based on the reductions in cell versus profile counts, the mean number of cells per profile was determined [CN, 5.13 ± 0.5 (range, 1 to 13); DN, 2.88 ± 0.15 (range, 1 to 4); and HCC, 1.69 ± 0.2 (range, 0.1 to 3)], and comparison demonstrated highly significant differences (P_{CN} versus P_{DN} = 1.23e^{−4}; P_{CN}

versus P_{HCC} = 1.43e^{−7}; and P_{DN} versus P_{HCC} = 1.34e^{−5}). These values confirmed that fewer cells contribute to perinodular profile counts in the progressive stages of hepatocarcinogenesis and support the morphologic rather than temporal descriptors complex, attenuated, and absent.

Necrosis and Apoptosis Are Not the Principal Morphologic Mechanisms for Perinodular K19 Loss

Because both apoptosis and necrosis are mechanisms of cell loss and are associated with distinct morphologic and nuclear hallmarks, H&E- and Hoechst-stained sections were evaluated. Neither cytologic nor nuclear features of epithelial necrosis were observed (Figure 3, A and F). Likewise, DR around HCN did not demonstrate cytomorphologic evidence of apoptosis (Figure 3B). However, in contrast to necrosis, during apoptosis the cell membrane and nuclear features remain intact for a relatively long time, and identification of early apoptosis may not be reliable.¹⁹ Therefore,

Figure 3. Pathologic mechanisms of perinodular K19 loss. **Top** of the panel provides an overview of the assessed morphologic mechanisms in ductular epithelium around cirrhotic nodules (CN) and dysplastic nodules (DN). Necrosis (A/F) was evaluated using routine H&E staining (A) and titrated DNA labeling with Hoechst 33258 staining (F). There was no evidence of loss of structural integrity, loss of nuclear basophilia, nuclear swelling, or chromatin flocculation within the ductules (F, outline). Compare open epithelial nuclear staining with uniform chromatin pattern of surrounding mesenchymal nuclei. Apoptosis was assessed via routine H&E staining (B), activated caspase 3 staining (D), TUNEL assay (G; inset, positive control, intestinal mucosa; counterstain methyl green), and Hoechst staining (F). Differences in chromatin condensation, activated caspase 3 labeling, dUTP incorporation, or nuclear staining pattern were not observed around DN when compared with CN. Screening for cellular phenotypes via immunolabeling demonstrated elongated fibroblast-like cells that stained weakly with the ductular-epithelial marker K19 (C and E, arrows; inset, K19 single channel; dotted line, hepatocellular interface) as well as epithelioid cells within the confines of the basement membrane (H, outline) that demonstrated strong staining with the mesenchymal marker vimentin (H, arrow). See *Discussion* for additional mechanisms that were not assessed (indicated by broken line and three dots on top of the panel). Scale bars = 100 μ m (A and G); 50 μ m (inset in G; 20 μ m (B–F, inset in E and H).

activated caspase 3 was evaluated using immunolabeling^{28,29} (Figure 3D). No differences in percentages between the perinodular epithelial compartments of CN (34 ± 2.2 activated caspase 3–positive of 2287 ± 85 , or 1 of 67 ductular epithelial cells in 10 sectors) versus DN (3.7 ± 0.51 of 244.3 ± 27.7 , or 1 of 65 cells in 10 sectors) versus HCC (0.04 ± 0.06 of 2.1 ± 0.57 cells, or 1 of 55 cells in 26 sectors) were noted (P_{CN} versus $P_{\text{DN}} = 0.85$; P_{CN} versus $P_{\text{HCC}} = 0.844$; and P_{DN} versus $P_{\text{HCC}} = 0.94$). Furthermore, DNA fragmentation using the TUNEL assay on formalin-fixed, paraffin-embedded sections was undertaken (Figure 3G, inset)^{19,22}; however, DR surrounding CN and DN did not demonstrate notable levels of TUNEL-positive nuclei (Figure 3G). Thus, assessment of features of both necrosis and apoptosis argue against cell death as the predominant morphologic mechanisms contributing to the documented perinodular K19 loss.

Cytomorphology and Immunolabeling in the Perinodular Compartment Are Indicative of Substantial Variability in Cellular Phenotypes

As an alternative possibility, cellular identity switch of biliary-type epithelial cells was assessed. Because relying on individual markers is insufficient for the study of cellular phenotype alterations,^{30,31} combined assessment of cytomorphologic features, localization, and immunophenotype was performed.²³ The possibility of increased biliary-to-hepatocytic transition^{32,33} was discounted because the perinodular and internodular zone in CN and DN demonstrated considerable numbers of weakly K19-positive

epithelial cells with an elongated fibroblast-like appearance. These cells were not present within the confines of the basement membrane but were frequently associated with clusters of strongly K19-positive epithelial cells (Figure 3, C and E; see also Supplemental Figure S3, A and B, at <http://ajp.amjpathol.org>). In contrast, isolated epithelial-like cells within the confines of the basement membrane around CN and DN demonstrated strong vimentin immunoreactivity (Figure 3F; see also Supplemental Figure S3, C and D at <http://ajp.amjpathol.org>). These findings suggested that perinodular K19 cell loss could be related to alterations in epithelial cellular phenotype. In addition to ascertaining staining patterns of additional markers, verification that antibodies against fibroblast-specific protein 1 (FSP-1), an S100-protein family member (also known as S100A4), label differentially when compared with S100 was performed (see Supplemental Figure S4 at <http://ajp.amjpathol.org>).

Absence of K19 positivity in the vicinity of HCC was associated with numerous FSP-1–positive cells (Figure 4A), and the few remaining K19-positive internodular ductules were in direct contact with spindle-shaped FSP-1–positive cells (Figure 4A, inset). The attenuated DR around DN frequently demonstrated numerous spindle-shaped K19-positive and spindle-shaped FSP-1–positive cells (Figure 4B). The complex DR around CN did not demonstrate FSP-1–positive cells; however, internodular ducts contained exceptionally rare FSP-1–positive or K19-negative columnar to pyramidal epithelioid cells (Figure 4C). Around DN, these FSP-1–positive epithelioid cells were more frequent (Figure 4D) and were observed as elongated

Figure 4. Epithelial phenotypes in perinodular sectors of cirrhotic nodules (CN), dysplastic nodules (DN), and hepatocellular carcinoma (HCC) using combinations of multicolor brightfield and immunofluorescence immunolabeling. **A:** Perinodular stroma of HCC demonstrated abundance of FSP-1 reactivity (red) and lack of K19 (green) at the hepatocellular interface (outlined in yellow). Few remaining internodular ductules demonstrated attenuated edges with lack of K19 staining and multifocal presence of FSP-1 labeling where elongated cells blend into the surrounding FSP-1-positive stroma (**inset**). **B:** Perinodular attenuated ductules around DN demonstrated numerous spindle-shaped K19-positive cells (green; **arrow**). Individual cells show diffuse cytoplasmic FSP-1 reactivity (red; **open arrow**) and nuclear features similar to nearby spindle-shaped K19-positive epithelioid cells. **C:** Brightfield multicolor staining of ductules around CN rarely demonstrated isolated epithelioid cells that were FSP-1-positive without K19 immunoreactivity (**arrowhead**). **D:** Numerous elongated or spindle-shaped fibroblast-like cells with weak K19 and FSP-1 immunoreactivity (**open arrows**) complemented the attenuated pattern of K19 immunoreactivity around DN (without extension between hepatocytes). Isolated or paired FSP-1-positive epithelioid cells without K19 immunoreactivity were also observed within the confines of the basement membrane (**arrow in D** and **inset in E**). Note the dense homogeneous chromatin of stromal FSP-1-positive cellular elements (**arrowhead**) in comparison with the open epithelial chromatin (**arrow in E**), demonstrating peripheral chromatin condensation and central clearing of FSP-1-positive and K19-negative epithelioid cells. **F:** Attenuated ductular epithelium at the interface around DN typically demonstrated several cells with weak K19 and weak FSP-1 positivity (**arrows**). Compare the open epithelioid chromatin configuration with peripheral condensation and central clearing (**arrows**) with dense homogeneous nuclear staining of stromal and inflammatory cells (**arrowhead**). **G:** A peculiar alternating pattern of weak (**arrows**) and stronger (**arrowheads**) K19-reactivity was noted around DN. Note that some of the faint or negative epithelioid cells exhibit cytoplasmic immunoreactivity for FSP-1 (**open arrowhead**). **H** and **I:** Additional assessment of phenotypes included tricolor immunofluorescence for ECADH (green) in combination with FSP-1 or SNAIL, which demonstrated rare single elongated FSP-1-positive cells (red) (**H**), ECADH-negative cells within the confines of the basement membrane around CN (**arrow in H**); nuclear counterstain in blue channel (see Supplemental Figure S5, A–C at <http://ajp.amjpatbol.org>). **I:** SNAIL positivity in attenuated ductules around DN was observed in ECADH-positive cells (green) and ECADH-negative epithelial cells (**arrow**); note the presence of ECADH-positive, SNAIL-negative epithelial cells (**open arrow**; see Supplemental Figure S5, D–I at <http://ajp.amjpatbol.org>). **J:** Substantial variability in cellular phenotypes in the perinodular epithelial compartment of DN was assorted based on immunophenotype (Ep, epithelial marker, brown; and Me, mesenchymal marker, magenta) and cytomorphologic features (tall columnar, cuboidal, or spindle shaped). At least two distinct cellular phenotypes were observed, characterized by discrepant morphologic or immunophenotypic features suggestive of cellular identity alterations (see also *Results* and *Discussion*). Scale bars: 200 μ m (**A**); 50 μ m (**D**) and **inset in A**; 20 μ m (**B**, **C**, and **E–I**).

cells at the hepatocellular interface zone (rim of attenuated DR; Figure 4D) or as flat cuboidal cells in internodular ducts (Figure 4D). These features of cellular identity alterations were found in both the internodular

ducts (Figure 4E) and the interface zone (Figure 4F). Around DN, individual polygonal or tall to slender columnar cells demonstrated alternating strong and weak K19 staining (Figure 4G), whereby negative or faintly

staining cells also exhibited faint cytoplasmic FSP-1 immunoreactivity (Figure 4G). Thus, K19- and FSP-1 labeling indicated substantial variability in cellular features in the perinodular compartment around DN that is morphologically and immunophenotypically compatible with cellular identity alteration.²³

Confirmation of variability in cellular phenotypes was achieved by using the epithelial marker ECADH in combination with FSP-1 (Figure 4H; see also Supplemental Figure S5, A–C, at <http://ajp.amjpathol.org>) or SNAIL (Figure 4I), which demonstrated spindle-shaped ECADH- or SNAIL-positive cells (Figure 4I; see also Supplemental Figure S5, D–F at <http://ajp.amjpathol.org>), as well as additional nonconventional phenotypes (see Supplemental Figure S5, G and H at <http://ajp.amjpathol.org>). The similarity to findings in K19 and FSP-1 labeling also included the alternating staining pattern, for example, demonstrating admixture of SNAIL-positive and SNAIL-negative cells (see Supplemental Figure S5C at <http://ajp.amjpathol.org>).

Of importance, all sectors contained ECADH-positive SNAIL-negative epithelial cells (Figure 4I), and these “normal” epithelial cells were observed at overall lower frequency around DN than around CN. In contrast, cells with discrepant features (ie, epithelioid with mesenchymal immunophenotype or fibroblast-like with epithelial immunophenotype and nuclear features; Figure 4J) were more frequent around DN than around CN. Quantification in perinodular sectors was performed; however, epithelial elements around HCC were too rare to enable meaningful random-field capture, and formal comparisons were restricted to perinodular fields in CN and DN (Table 3). Comparison of raw numbers confirmed the qualitative morphologic assessment and indicated that markers decrease in conjunction with loss of perinodular K19 structures and malignant potential of the nodule itself. Despite the overall reduced amount of FSP-1 and SNAIL around DN, marker percentages were altered. Specifically, the ECADH-positive fraction in DN was maintained, whereas the fraction of FSP-1- and SNAIL-positive epithelial cells was significantly increased (Table 3). Together these findings demonstrate increased phenotypic variability in epithelial cells around DN and support the notion that cellular phenotype alterations may contribute to perinodular K19 loss.

Perinodular Epithelial Alterations Are Associated with Quantitative Differences in TGF- β Signaling Components

SMAD and phosphorylated SMAD (pSMAD) antibodies were tested for labeling pattern in the perinodular compartment (Table 1; see also Supplemental Figure S6 at <http://ajp.amjpathol.org>), and co-localization studies of K19 (Figure 5A) demonstrated increased levels of nuclear pSMAD3 around DN and in particular in those epithelial cells with weak K19 positivity (Figure 5B; see also Supplemental Figure S7, A–F, at <http://ajp.amjpathol.org>).

Quantification demonstrated that the significantly increased levels of pSMAD2/3 around DN compared with CN were associated with reduced levels of transforming growth factor beta receptor type 1 (TGF β R1) and unaltered levels of nonactivated SMAD3 (Table 3). The alternating pattern noted in sections stained for K19 and FSP-1 or ECADH and SNAIL was also observed using pSMAD2/3 stains (Figure 5, D, E, G, and H; see also Supplemental Figure S7, G–N at <http://ajp.amjpathol.org>) and together suggest alterations of individual cells or small clusters rather than homogeneous labeling of entire ductules (see Supplemental Figure S8 at <http://ajp.amjpathol.org>). When marker percentages were compared (DN:ECADH>SMAD3>FSP-1>pSMAD2/3>SNAIL>TGF β R1 versus CN:ECADH>SMAD3>TGF β R1>SNAIL>pSMAD2/3>FSP-1), the partial reversal confirmed, at least by immunophenotype, a substantially different makeup of the perinodular compartment, which may reflect functional changes in signaling associated with malignant progression within the nodule.

Discussion

This study describes and further interrogates a compelling relationship of the extralesional K19 compartment that parallels intralesional malignant transformation in progressive stages of hepatocarcinogenesis in cirrhosis. In particular, K19 loss follows distinct morphologic patterns, with a complex pattern around CN, an attenuated configuration around DN, and absence around HCC (Figure 2).

To better understand the seemingly vanishing K19 compartment, we excluded necrosis and apoptosis as morphologic mechanisms of cell loss and documented highly assorted immunophenotype along with cytomorphic features to describe substantial variability in cellular phenotypes in the perinodular epithelial compartment. Despite existing controversies in the field of hepatocytic phenotype switch,^{30,34–37} alterations of ductular epithelial phenotype as it relates to progression of adjacent hepatocellular lesions have, to the best of our knowledge, not been reported. Several reports, however, have recently implicated the biliary compartment^{4,5,38,39} in addition to the hepatocellular compartment^{40,41} as a source of stromal and mesenchymal cells,³⁶ albeit in the non-neoplastic setting.⁴² For example, in EMT, expression of SNAIL leads to repression of ECADH and indirectly triggers expression of FSP-1.^{4,23,35,36,43–51} Similarly, our assortment of cytomorphic features with immunophenotype in conjunction with published criteria^{23,35} revealed such phenotypes (Figure 4J),^{4,23,44,51,52} and findings can be regarded as static morphologic indications compatible with reported stages of the various types of epithelial transition.^{35,36,53} Despite the compelling resemblance, the possibility of discerning dynamic processes in human tissue-based studies has important limitations. For example, differentiation of EMT from the reverse, mesenchymal-to-epithelial transformation,^{30,35}

Figure 5. Immunolabeling of TGF- β signaling components in the perinodular epithelial compartment of cirrhotic nodules (CN) and dysplastic nodules (DN). **A:** Small K19-positive (green) ductules around CN demonstrate absence or only focal spotty immunoreactivity for pSMAD3 (red). **B:** Ductal epithelial cells around DN demonstrated irregular K19 reactivity (green) with individual weakly staining cells (compare staining pattern with brightfield appearance in Figure 4G); some of these demonstrated nuclear pSMAD3 reactivity (red; **arrowheads**). **C:** Small ductules around CN (outlined in **C–E**) demonstrated TGF β R1 expression (magenta) in isolated cone- or pyramid-shaped cells (**arrows**). Note the lack of nuclear TGF β R1 staining (Hoechst, blue). **D:** Most ductal epithelial cells demonstrated cytoplasmic SMAD3 positivity (red), whereas only a small fraction of nuclei (blue) were pSMAD2/3-positive, resulting in the merged color turquoise (**filled arrowheads**). Note the alternating organization with interspersed pSMAD-negative nuclei (**open arrowhead**). **E:** Quadruple merge illustrated presence of TGF β R1 expression in cells with cytoplasmic SMAD3 (**arrows**), indicative of at least two components of the TGF- β signaling pathway in cells without immunoreactivity for activated components (pSMAD2/3-negative). **F:** Small ductules around DN (outlined in **F–H**) demonstrated TGF β R1-expressing epithelial cells (**arrows**). **G:** Cytoplasmic SMAD3 staining (red) was similar to that observed in ductules around CN (**D**); however, the number and staining intensity of pSMAD2/3 (green) in ductular nuclei (blue) was increased (merged color turquoise; **filled arrowheads**). Note individual pSMAD2/3-positive flat circumferentially oriented nuclei within the confines of the basement membrane (**open arrow**) and individual pSMAD2/3-negative interspersed nuclei (**open arrowhead**). **H:** Quadruple merge illustrates presence of TGF β R1 expression in cells with cytoplasmic SMAD3 (**arrows**), as well as focal nuclear pSMAD2/3 staining (**arrowheads**), and interspersed pSMAD2/3-negative nuclei (**open arrowhead**). Circumferentially oriented nucleus (**open arrow**) (**G**; see also Supplemental Figure S7 at <http://ajp.amjpathol.org>). Scale bars = 20 μ m.

or maintenance of a plastic phenotype (dynamic reversibility)^{51,54,55} is not possible, and we abstain from attributing directionality, time scale, reversibility, and bidirectionality or whether alterations represent the pivotal underlying structural mechanism of perinodular K19 loss. Furthermore, definitive proof that perinodular K19-positive cells are able to transdifferentiate into mesenchymal elements would require *in vivo* lineage tracing with

cholangiocyte-specific marker genes,⁵⁶ and even then, the lack of reliable animal models for HCC in the setting of cirrhosis⁵⁷ will make further experimental exploration of possible mechanisms challenging.

To further narrow a connection of perinodular epithelial cell loss and hepatocellular neoplasia at the tissue level, we interrogated putative upstream signaling pathways, and although a variety of neuroendocrine factors influ-

ence biliary epithelial function,^{58–60} we focused on TGF- β because of its major profibrogenic role,⁶¹ established implications in human biliary EMT,^{4,55,62} and signaling upstream of SNAIL via recruitment of SMAD molecules^{4,55,62–66} and because assessment of pSMAD2/3 via immunolabeling in formalin-fixed, paraffin-embedded samples has been reported.^{4,55,59,62,66} Our findings of increased levels of nuclear pSMAD were indicative of increased levels of activated TGF- β signaling around DN; however, in combination with reduced TGF β R1 levels, recruitment of alternative upstream activators in dysplasia remains possible.⁵⁵ Methodologic limitations of assessing signaling status or nucleotide incorporation in formalin-fixed, paraffin-embedded samples apply.^{34,67} Nonetheless, the reproducibility using different antibodies in a large number of nodules that includes alternating pSMAD staining patterns also observed using K19 or FSP-1 and ECADH or SNAIL labeling suggest morphologic and immunophenotypic alterations in the tight locoregional control of the functional heterogeneity in biliary epithelial cells⁶⁸ in progressive stages of hepatocarcinogenesis.

In conjunction, the observed structural alterations are associated with discrepancies regarding cellular identity and alterations in TGF- β signaling, and we propose paracrine signaling across the lesional interface as a possible underlying pathophysiologic link. This hypothesis of paracrine signaling does not exclude additional contributing mechanisms such as non-TGF- β -mediated triggers (eg, tyrosine-kinase-mediated signaling or hedgehog signaling^{39,41,50,56}); stromal and mesenchymal factors such as immunoregulatory, inflammatory, or stromal cells^{4,48,69,70}; extrahepatic so-called circulating stem cells^{71,72}; autophagy-associated (caspase-independent) cell death¹⁸; or changes in anoikis (apoptosis induced by detachment from anchorage).⁷³ Inasmuch as similar signaling has been demonstrated in the setting of biliary fibrosis,³⁹ our results implicate the K19 compartment as a morphologically distinct and significant stromal (epithelial) factor in HCC pathogenesis in cirrhosis. It remains to be determined whether perinodular phenomena also apply to the noncirrhotic liver; however, we examined perinodular sectors in a variety of underlying diseases, and DN occur predominantly, if not only, in the setting of cirrhosis.^{6,7,9,11,74–78} The observed progressive alterations in the ductular compartment of premalignant and malignant hepatocellular lesions also indicate a substantial change in the otherwise prototypic ductular response to chronic injury (ie, cirrhosis).^{1–3,79} In addition to exhaustion of the K19-based stem cell niche,^{32,33,56,79,80} our findings emphasize alterations of an intricate relationship between intralesional and extralesional compartments as hepatocarcinogenesis progresses. Notwithstanding the known relation of intranodular mesenchymal elements of HCC (eg, reticulin pattern, unpaired arteries, sinusoidal capillarization, and vascular and extranodular stromal invasion^{9,10,76,77}), as well as imaging applications that use altered vascular biology in HCC for detection via dynamic methods,⁸¹ changes in the nonhepatocellular epithelial compartment remain largely unexplored,^{7,8,16,45} and our findings provide a morphologic starting point to examine

whether loss of perinodular DR is a bystander or contributing factor in progressive stages of hepatocarcinogenesis.

In terms of application in routine diagnostic pathology, the present study demonstrates that perinodular K19 patterns can function as a highly reliable extralesional surrogate for intralesional disease, with sensitivity and specificity values well above 83%, even when atypical hepatocellular lesions were included (Table 2). However, it remains to be determined whether perinodular K19 assessment can overcome the recognized limitations associated with needle core biopsy sampling.^{78,82,83} Based on our preliminary case-based evidence,⁹ assessment of the perinodular K19 pattern in biopsy specimens is possible; however, it is questionable how regional variability and limited sampling will alter the overall good diagnostic performance (Table 2). Caution must be used in interpretation of immunohistochemical findings in the context of cancer diagnostics. The perinodular K19 pattern in the setting of cirrhosis and encapsulated HCC will likely demonstrate absence adjacent to HCC; however, this should not diminish careful examination of the lesion using traditional means. Nonetheless, the widespread availability of K19 staining and straightforward assessment (Figure 2) adds a pattern-based extralesional feature to existing strategies,^{74,75,84} which should encourage external validation as a diagnostic tool.⁸⁶

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References

1. Roskams T, Desmet V: Ductular reaction and its diagnostic significance. *Semin Diagn Pathol* 1998, 15:259–269
2. Desmet V, Roskams T, Van Eyken P: Ductular reaction in the liver. *Pathol Res Pract* 1995, 191:513–524
3. Popper H, Kent G, Stein R: Ductular cell reaction in the liver in hepatic injury. *J Mt Sinai Hosp NY* 1957, 24:551–556
4. Rygiel KA, Robertson H, Marshall HL, Pekalski M, Zhao L, Booth TA, Jones DE, Burt AD, Kirby JA: Epithelial-mesenchymal transition contributes to portal tract fibrogenesis during human chronic liver disease. *Lab Invest* 2008, 88:112–123
5. Kirby JA, Robertson H, Marshall HL, Rygiel KA, Hudson M, Jones DE, Burt AD: Epithelial to mesenchymal transition in primary sclerosing cholangitis. *Liver Int* 2008, 28:1176–1177
6. Kojima M: Pathological diagnosis at early stage: reaching international consensus. *Oncology* 78(Suppl 1):31–35

7. Park YN, Kojiro M, Di Tommaso L, Dhillon AP, Kondo F, Nakano M, Sakamoto M, Theise ND, Roncalli M: Ductular reaction is helpful in defining early stromal invasion, small hepatocellular carcinomas, and dysplastic nodules. *Cancer* 2007, 109:915–923
8. van Zijl F, Mair M, Csizsar A, Schneller D, Zulehner G, Huber H, Eferl R, Beug H, Dolznig H, Mikulits W: Hepatic tumor-stroma crosstalk guides epithelial to mesenchymal transition at the tumor edge. *Oncogene* 2009, 28:4022–4033
9. Lennerz JK, Crippin JS, Brunt EM: Diagnostic considerations of nodules in the cirrhotic liver: a case-based perspective. *Pathol Case Rev* 2009, 14:3–12
10. International Working Party: Terminology of nodular hepatocellular lesions. *Hepatology* 1995, 22:983–993
11. Hytioglou P, Park YN, Krinsky G, Theise ND: Hepatic precancerous lesions and small hepatocellular carcinoma. *Gastroenterol Clin North Am* 2007, 36:867–887, vii
12. Lefkowitz JH: Hepatobiliary pathology. *Curr Opin Gastroenterol* 2008, 24:269–277
13. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R: The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 1982, 31:11–24
14. Tan J, Hytioglou P, Wiecezorek R, Park YN, Thung SN, Arias B, Theise ND: Immunohistochemical evidence for hepatic progenitor cells in liver diseases. *Liver* 2002, 22:365–373
15. Van Eyken P, Desmet VJ: Cytokeratins and the liver. *Liver* 1993, 13:113–122
16. Terada T, Hosono M, Nakanuma Y: Distribution of cytokeratin 19–positive biliary cells in cirrhotic nodules, hepatic borderline nodules (atypical adenomatous hyperplasia), and small hepatocellular carcinomas. *Mod Pathol* 1995, 8:371–379
17. Malhi H, Gores GJ, Lemasters JJ: Apoptosis and necrosis in the liver: a tale of two deaths? *Hepatology* 2006, 43:S31–S44
18. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE: Cell death. *N Engl J Med* 2009, 361:1570–1583
19. Zhivotovsky B, Orrenius S: Assessment of apoptosis and necrosis by DNA fragmentation and Morphological Criteria. *Curr Protoc Cell Biol* 2001, Chapter 18: Unit 18.3
20. Bowen ID: Techniques for demonstrating cell death. *Cell Death in Biology and Pathology*. Edited by Bowen ID, Lockshin RA. London-New York, Chapman & Hall, 1981, pp. 379–444
21. Kerr JF, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972, 26:239–257
22. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992, 119:493–501
23. Zeisberg M, Neilson EG: Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest* 2009, 119:1429–1437
24. Lennerz JK, Kim SH, Oates EL, Huh WJ, Doherty JM, Tian X, Brede-meyer AJ, Goldenring JR, Lauwers GY, Shin YK, Mills JC: The transcription factor MIST1 is a novel human gastric chief cell marker whose expression is lost in metaplasia, dysplasia, and carcinoma. *Am J Pathol* 2010, 177:1514–1533
25. Lennerz JK, Ruhle V, Ceppa EP, Neuhuber WL, Bunnett NW, Grady EF, Messlinger K: Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. *J Comp Neurol* 2008, 507:1277–1299
26. Berglund L, Bjorling E, Oksvold P, Fagerberg L, Asplund A, Szigyarto CA, Persson A, Ottosson J, Wernerus H, Nilsson P, Lundberg E, Sivertsson A, Navani S, Wester K, Kampf C, Hober S, Ponten F, Uhlen M: A gene-centric human protein atlas for expression profiles based on antibodies. *Mol Cell Proteomics* 2008, 7:2019–2027
27. Silcocks PB: Measuring repeatability and validity of histological diagnosis: a brief review with some practical examples. *J Clin Pathol* 1983, 36:1269–1275
28. Pupure J, Isajevs S, Skapare E, Rumaks J, Svirskis S, Svirina D, Kalvins I, Klusa V: Neuroprotective properties of mildronate, a mitochondria-targeted small molecule. *Neurosci Lett* 2010, 470:100–105
29. Aguzzi MS, Fortugno P, Giampietri C, Ragone G, Capogrossi MC, Facchiano A: Intracellular targets of RGDS peptide in melanoma cells. *Mol Cancer* 2010, 9:84
30. Tarin D, Thompson EW, Newgreen DF: The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* 2005, 65:5996–6000; discussion 6000–5991
31. Zhang DW, Bian H: No contribution to liver fibrosis, but possible carcinogenesis? *Hepatology* 51:1468–1469; author reply 1469
32. Kuwahara R, Kofman AV, Landis CS, Swenson ES, Barendsward E, Theise ND: The hepatic stem cell niche: identification by label-retaining cell assay. *Hepatology* 2008, 47:1994–2002
33. Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhattacharya PS, Bioulac-Sage P, Brunt EM, Crawford JM, Crosby HA, Desmet V, Finegold MJ, Geller SA, Gouw AS, Hytioglou P, Knisely AS, Kojiro M, Lefkowitz JH, Nakanuma Y, Olynyk JK, Park YN, Portmann B, Saxena R, Scheuer PJ, Strain AJ, Thung SN, Wanless IR, West AB: Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology* 2004, 39:1739–1745
34. Taura K, Miura K, Iwaisako K, Osterreicher CH, Kodama Y, Penz-Osterreicher M, Brenner DA: Hepatocytes do not undergo epithelial-mesenchymal transition in liver fibrosis in mice. *Hepatology* 2010, 51:1027–1036
35. Giannelli G: The epithelial-mesenchymal transition: fact or fiction in cancer? *Hepatology* 2009, 50:1344–1346
36. Wells RG: The epithelial-to-mesenchymal transition in liver fibrosis: here today, gone tomorrow? *Hepatology* 2010, 51:737–740
37. Scholten D, Scholten A, Brenner DA, Kisseleva T: Epithelial-to-mesenchymal transition (EMT) in cholangiocytes does not contribute to liver fibrosis. *Hepatology* 2009, 50(Suppl):818A
38. Robertson H, Kirby JA, Yip WW, Jones DE, Burt AD: Biliary epithelial-mesenchymal transition in posttransplantation recurrence of primary biliary cirrhosis. *Hepatology* 2007, 45:977–981
39. Omenetti A, Porrello A, Jung Y, Yang L, Popov Y, Choi SS, Witek RP, Alpini G, Venter J, Vandongen HM, Syn WK, Baroni GS, Benedetti A, Schuppan D, Diehl AM: Hedgehog signaling regulates epithelial-mesenchymal transition during biliary fibrosis in rodents and humans. *J Clin Invest* 2008, 118:3331–3342
40. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R: Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J Biol Chem* 2007, 282:23337–23347
41. Choi SS, Omenetti A, Witek RP, Moylan CA, Syn WK, Jung Y, Yang L, Sudan DL, Sicklick JK, Michelotti GA, Rojkind M, Diehl AM: Hedgehog pathway activation and epithelial-to-mesenchymal transitions during myofibroblastic transformation of rat hepatic cells in culture and cirrhosis. *Am J Physiol Gastrointest Liver Physiol* 2009, 297: G1093–G1106
42. Fabris L, Strazzabosco M: Epithelial-mesenchymal interactions in biliary diseases. *Semin Liver Dis* 2011, 31:11–32
43. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG: Identification and characterization of a fibroblast marker: fSP1. *J Cell Biol* 1995, 130:393–405
44. Mazzucchelli L: Protein S100A4: too long overlooked by pathologists? *Am J Pathol* 2002, 160:7–13
45. Yang MH, Chen CL, Chau GY, Chiou SH, Su CW, Chou TY, Peng WL, Wu JC: Comprehensive analysis of the independent effect of Twist and Snail in promoting metastasis of hepatocellular carcinoma. *Hepatology* 2009, 50:1464–1474
46. Nieto MA: The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* 2002, 3:155–166
47. Le Hir M, Hegyi I, Cueni-Loffing D, Loffing J, Kaissling B: Characterization of renal interstitial fibroblast-specific protein 1/S100A4–positive cells in healthy and inflamed rodent kidneys. *Histochem Cell Biol* 2005, 123:335–346
48. Robertson H, Ali S, McDonnell BJ, Burt AD, Kirby JA: Chronic renal allograft dysfunction: the role of T cell-mediated tubular epithelial to mesenchymal cell transition. *J Am Soc Nephrol* 2004, 15:390–397
49. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA: The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2000, 2:76–83
50. Huber MA, Kraut N, Beug H: Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005, 17:548–558
51. Kalluri R, Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003, 112:1776–1784

52. Okada H, Danoff TM, Kalluri R, Neilson EG: Early role of Fsp1 in epithelial-mesenchymal transformation. *Am J Physiol* 1997, 273: F563–F574
53. Wasilenko S, Mason AL: New insights from recurrent primary biliary cirrhosis in liver transplantation: the paradox of becoming a fibroblast? *Hepatology* 2007, 45:837–840
54. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002, 110:341–350
55. Tyler JR, Robertson H, Booth TA, Burt AD, Kirby JA: Chronic allograft nephropathy: intraepithelial signals generated by transforming growth factor-beta and bone morphogenetic protein-7. *Am J Transplant* 2006, 6:1367–1376
56. Greenbaum LE: Hedgehog signaling in biliary fibrosis. *J Clin Invest* 2008, 118:3263–3265
57. Fausto N, Campbell JS: Mouse models of hepatocellular carcinoma. *Semin Liver Dis* 2010, 30:87–98
58. Glaser SS, Gaudio E, Miller T, Alvaro D, Alpini G: Cholangiocyte proliferation and liver fibrosis. *Exp Rev Mol Med* 2009, 11:e7
59. Kiss A, Schnur J, Szabo Z, Nagy P: Immunohistochemical analysis of atypical ductular reaction in the human liver, with special emphasis on the presence of growth factors and their receptors. *Liver* 2001, 21:237–246
60. Llovet JM, Bruix J: Molecular targeted therapies in hepatocellular carcinoma. *Hepatology* 2008, 48:1312–1327
61. Gressner AM, Weiskirchen R, Breitkopf K, Dooley S: Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 2002, 7:d793–d807
62. Tan CE, Chan VS, Yong RY, Vijayan V, Tan WL, Fook Chong SM, Ho JM, Cheng HH: Distortion in TGF beta 1 peptide immunolocalization in biliary atresia: comparison with the normal pattern in the developing human intrahepatic bile duct system. *Pathol Int* 1995, 45:815–824
63. ten Dijke P, Hill CS: New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004, 29:265–273
64. Dooley S, Hamzavi J, Ciuculan L, Godoy P, Ilkavets I, Ehner S, Ueberham E, Gebhardt R, Kanzler S, Geier A, Breitkopf K, Weng H, Mertens PR: Hepatocyte-specific Smad7 expression attenuates TGF-beta-mediated fibrogenesis and protects against liver damage. *Gastroenterology* 2008, 135:642–659
65. Stopa M, Anhof D, Terstegen L, Gatsios P, Gressner AM, Dooley S: Participation of Smad2, Smad3, and Smad4 in transforming growth factor beta (TGF-beta)-induced activation of Smad7 THE TGF-beta response element of the promoter requires functional Smad binding element and E-box sequences for transcriptional regulation. *J Biol Chem* 2000, 275:29308–29317
66. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R: BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 2003, 9:964–968
67. Grasl-Kraupp B, Ruttkay-Nedecy B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R: In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. *Hepatology* 1995, 21:1465–1468
68. Kanno N, LeSage G, Glaser S, Alvaro D, Alpini G: Functional heterogeneity of the intrahepatic biliary epithelium. *Hepatology* 2000, 31: 555–561
69. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB: Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature* 1994, 372:190–193
70. Fu S, Zhang N, Yopp AC, Chen D, Mao M, Zhang H, Ding Y, Bromberg JS: TGF-beta induces Foxp3 + T-regulatory cells from CD4+CD25- precursors. *Am J Transplant* 2004, 4:1614–1627
71. Kisseleva T, Uchinami H, Feirt N, Quintana-Bustamante O, Segovia JC, Schwabe RF, Brenner DA: Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis. *J Hepatol* 2006, 45:429–438
72. Forbes SJ, Russo FP, Rey V, Burra P, Rugge M, Wright NA, Alison MR: A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology* 2004, 126:955–963
73. Frisch SM, Screaton RA: Anokis mechanisms. *Curr Opin Cell Biol* 2001, 13:555–562
74. Di Tommaso L, Destro A, Seok JY, Balladore E, Terracciano L, Sangiovanni A, Iavarone M, Colombo M, Jang JJ, Yu E, Jin SY, Morenghi E, Park YN, Roncalli M: The application of markers (HSP70 GPC3 and GS) in liver biopsies is useful for detection of hepatocellular carcinoma. *J Hepatol* 2009, 50:746–754
75. Di Tommaso L, Franchi G, Park YN, Fiamengo B, Destro A, Morenghi E, Montorsi M, Torzilli G, Tommasini M, Terracciano L, Tornillo L, Vecchione R, Roncalli M: Diagnostic value of HSP70, glycican 3, and glutamine synthetase in hepatocellular nodules in cirrhosis. *Hepatology* 2007, 45:725–734
76. Park YN, Yang CP, Fernandez GJ, Cubukcu O, Thung SN, Theise ND: Neoangiogenesis and sinusoidal “capillarization” in dysplastic nodules of the liver. *Am J Surg Pathol* 1998, 22:656–662
77. Roncalli M, Borzio M, Di Tommaso L: Hepatocellular dysplastic nodules. *Hepatol Res* 2007, 37(Suppl 2):S125–S134
78. Wanless IR: Liver biopsy in the diagnosis of hepatocellular carcinoma. *Clin Liver Dis* 2005, 9:281–285, vii
79. Falkowski O, An HJ, Ianus IA, Chiriboga L, Yee H, West AB, Theise ND: Regeneration of hepatocyte “buds” in cirrhosis from intrabiliary stem cells. *J Hepatol* 2003, 39:357–364
80. Yoon SM, Gerasimidou D, Kuwahara R, Hytioglou P, Yoo JE, Park YN, Theise ND: Epithelial cell adhesion molecule (EpCAM) marks hepatocytes newly derived from stem/progenitor cells in humans. *Hepatology* 2011, 53:964–973
81. Bruix J, Sherman M: Management of hepatocellular carcinoma. *Hepatology* 2005, 42:1208–1236
82. Kalinski T, Roessner A: Hepatocellular carcinoma: pathology and liver biopsy. *Dig Dis* 2009, 27:102–108
83. Roncalli M: Hepatocellular nodules in cirrhosis: focus on diagnostic criteria on liver biopsy: a Western experience. *Liver Transpl* 2004, 10:S9–S15
84. Jain R, Fischer S, Serra S, Chetty R: The use of cytokeratin 19 (CK19) immunohistochemistry in lesions of the pancreas, gastrointestinal tract, and liver. *Appl Immunohistochem Mol Morphol* 2010, 18:9–15
85. Choi YJ, Pinto MM, Hao L, Riba AK: Interobserver variability and aberrant E-cadherin immunostaining of lobular neoplasia and infiltrating lobular carcinoma. *Mod Pathol* 2008, 21:1224–1237
86. Bergstrom R, Savary K, Moren A, Guibert S, Heldin CH, Ohlsson R, Moustakas A: Transforming growth factor beta promotes complexes between Smad proteins and the CCCTC-binding factor on the H19 imprinting control region chromatin. *J Biol Chem* 2010, 285:19727–19737
87. Baghy K, Dezso K, Laszlo V, Fullar A, Peterfia B, Paku S, Nagy P, Schaff Z, Iozzo RV, Kovalszky I: Ablation of the decorin gene enhances experimental hepatic fibrosis and impairs hepatic healing in mice. *Lab Invest* 2011, 91:439–451
88. Kahn HJ, Marks A, Thom H, Bauman R: Role of antibody to S100 protein in diagnostic pathology. *Am J Clin Pathol* 1983, 79:341–347
89. Vincent T, Neve EP, Johnson JR, Kukalev A, Rojo F, Albanell J, Pietras K, Virtanen I, Philipson L, Leopold PL, Crystal RG, de Herrerias AG, Moustakas A, Pettersson RF, Fuxe J: A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-beta mediated epithelial-mesenchymal transition. *Nat Cell Biol* 2009, 11:943–950
90. Li Q, Pangas SA, Jorgez CJ, Graff JM, Weinstein M, Matzuk MM: Redundant roles of SMAD2 and SMAD3 in ovarian granulosa cells in vivo. *Mol Cell Biol* 2008, 28:7001–7011
91. Bardeesy N, Cheng KH, Berger JH, Chu GC, Pahler J, Olson P, Hezel AF, Horner J, Lauwers GY, Hanahan D, DePinho RA: Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. *Genes Dev* 2006, 20: 3130–3146
92. Hornick JL, Lauwers GY, Odze RD: Immunohistochemistry can help distinguish metastatic pancreatic adenocarcinomas from bile duct adenomas and hamartomas of the liver. *Am J Surg Pathol* 2005, 29:381–389
93. Van Themsche C, Chaudhry P, Leblanc V, Parent S, Asselin E: XIAP gene expression and function is regulated by autocrine and paracrine TGF-beta signaling. *Mol Cancer* 2010, 9:216
94. Louafi F, Martinez-Nunez RT, Sanchez-Elsner T: MicroRNA-155 targets SMAD2 and modulates the response of macrophages to transforming growth factor-beta. *J Biol Chem* 2010, 285:41328–41336
95. Kondo M, Cubillo E, Tobiume K, Shirakihara T, Fukuda N, Suzuki H, Shimizu K, Takehara K, Cano A, Saitoh M, Miyazono K: A role for Id in the regulation of TGF-beta-induced epithelial-mesenchymal trans-differentiation. *Cell Death Differ* 2004, 11:1092–1101

96. Rachakonda G, Sekhar KR, Jowhar D, Samson PC, Wikswo JP, Beauchamp RD, Datta PK, Freeman ML: Increased cell migration and plasticity in Nrf2-deficient cancer cell lines. *Oncogene* 29: 3703–3714
97. Huang W, Zhang Y, Varambally S, Chinnaiyan AM, Banerjee M, Merajver SD, Kleer CG: Inhibition of CCN6 (Wnt-1-induced signaling protein 3) down-regulates E-cadherin in the breast epithelium through induction of snail and ZEB1. *Am J Pathol* 2008, 172:893–904
98. Tzur G, Israel A, Levy A, Benjamin H, Meiri E, Shufaro Y, Meir K, Khvalevsky E, Spector Y, Rojansky N, Bentwich Z, Reubinoff BE, Galun E: Comprehensive gene and microRNA expression profiling reveals a role for microRNA in human liver development. *PLoS One* 2009, 4:e7511
99. Azumi N, Battifora H: The distribution of vimentin and keratin in epithelial and nonepithelial neoplasms: a comprehensive immunohistochemical study on formalin- and alcohol-fixed tumors. *Am J Clin Pathol* 1987, 88:286–296