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RESEARCH

Molecular Characteristics of Fibrolamellar Hepatocellular Carcinoma

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Abstract Fibrolamellar hepatocellular carcinoma (FLC) occurs in non-cirrhotic liver and the etiopathogenesis is still obscure. Both hepatocellular and cholangiocellular markers are expressed in the tumor, however, molecular alterations and altered pathways playing role in the tumor pathogenesis are not clearly identified. The purpose of the present study was to compare the expression level of EGFR, syndecan-1 and ß-catenin in FLC, conventional hepatocellular carcinoma (cHCC) and cholangiocellular carcinoma (CCC) and to investigate the possibility of mutation both in EGFR and K-RAS. Eight FLCs were compared with 7 cHCCs, 7 CCCs and 5 normal liver samples. Cytokeratins 7, 8, 18, 19, HepPar1 (HSA), EGFR, syndecan-1 (CD138) and ß-catenin were detected by immunohistochemistry. In addition EGFR, ß-catenin and syndecan-1 were evaluated by digital morphometry and K-RAS, EGFR mutations in FLC cases using paraffin-embedded samples. All FLCs were positive for HepPar1 (HSA) and cytokeratins 7, 8, 18, but negative for cytokeratin 19 by immunohistochemistry. EGFR was significantly overexpressed in all three tumor types, being highest in FLCs (p=0,0001). EGFR, K-RAS mutation analyses revealed no mutations in exons studied in FLCs. Our findings proved that expression of EGFR is higher in FLC than in other types of primary malignant hepatic tumors and no K-RAS mutation can be detected, so FLC is a good candidate for anti-EGFR treatment.

Keywords Fibrolamellar hepatocellular carcinoma · Hepatocellular carcinoma · Cholangiocellular carcinoma · Epidermal growth factor receptor · K-RAS mutation

Introduction

Fibrolamellar hepatocellular carcinoma (FLC) is a rare variant of hepatocellular carcinoma with distinct clinical and pathological characteristics found in younger individuals in non-cirrhotic liver [1–5]. The etiology of FLC is not clear. Contrarily, in the "conventional" types of hepatocellular carcinoma (cHCC), no viral infection, toxic effect, metabolic alterations or cirrhosis were detected in patients with FLC [4, 6].

The histopathological features of FLC are well defined, however little information is available on the molecular background and pathogenesis of FLC. The tumor expresses characteristics of cHCC, as hepatocyte specific antigen (HSA, HepPar1), cytokeratins (CK) 8, 18, however, alpha fetoprotein (AFP) is not expressed in FLC. Contrarily, cytokeratin 7 (CK7), a cholangiocellular marker, is positive in FLC (1, 4, 6).

Several newly detected tumor markers are available in the diagnostics of cHCC with varying value and prognostic significance [1, 2, 7–9]. More recently our group described alterations in tight junction protein claudin pattern in FLC [6]. Others suggested that FLC is chromosomally stable because it has fewer chromosomal alterations [7, 10] and a lower level of epigenetic instability [11] than cHCCs. Epidermal growth factor receptor (EGFR) expression [12] and genes involved in the RAS, MAPK, PIK3 pathways [2] were found to be overexpressed.

Our knowledge on the molecular pathomechanism of cHCC is still limited, however accumulating data might establish the basis of more successful target-oriented therapy in the near future [13].

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The aim of the present study was to investigate the expression of EGFR, syndecan-1 and ß-catenin in FLC and to compare it with other primary malignant liver tumors as cHCC and CCC. Further, we aimed to study the possibility of EGFR and K-RAS mutations in FLC with the idea to analyse the potency of target-oriented therapy with anti-EGFR treatment.

Material and Methods

Patients, Tissue Specimens

Eight cases of FLC were studied with the permission of the Regional Ethical Committee (#192). The patients were between 11 and 21 years of age (mean 16.2 years), the male/female ratio was 3/5 (Table 1). The cases were from the archives of the 1st and 2nd Departments of Pathology of the Semmelweis University, Budapest. Out of the 8 cases, 5 were surgically obtained resection specimens, while in 3 cases liver core needle biopsy samples were obtained for purpose of diagnosis and further studies.

Surrounding non-tumorous liver samples were available in the 5 surgically removed resection specimens. For comparisons, 5 "normal" liver samples obtained from surrounding normal liver tissue of patients with benign liver lesions (2 cases), one liver sample from an autopsy case (tissue samples taken within 12 h of a sudden death due to car accident) and 2 surrounding non-tumorous liver tissue samples from FLC cases were applied. Seven "conventional" (not fibrolamellar, trabecular-acinar type) HCC (cHCC) cases, grades II–III and 7 intrahepatic cholangiocellular carcinoma (CCC) cases, grades II–III were selected from the archives for comparison. There was no possibility for age matching, since the involved cHCC and CCC cases were of a substantially higher age group (average of 66 and 60 yrs, resp.) than the FLC cases (Tables 2 and 3). Three cHCCs case developed on cirrhotic background, whereas in 4 cases no cirrhotic architectural changes were notable.

Histology

The tissue samples were fixed immediately after removal in 10 % neutral buffered formalin (in PBS, pH 7.0) for 24 hrs at room temperature, dehydrated in a series of ethanol and xylene and embedded in paraffin (FFPE). The 3–4 μm thick sections were routinely stained with hematoxylin and eosin (HE) and picrosyrius red for detection of fibrous tissue.

Immunohistochemistry

FFPE sections 3–5 μm thick were cut. Primary antibodies and dilutions used are listed in Table 4. The primary antibodies were obtained from the following manufacturers; Ventana Medical Systems Inc. (Tucson, AZ, USA), BD Biosciences (Mississauga, ON, Canada), Dako Cytomation (Glostrup, Denmark A/S), Vision Biosystems/Novocastra (Newcastle upon Tyne, UK).

Reactions were carried out using Ventana Benchmark XT automated immunohistochemical staining system (Ventana) with HRP Multimer based, biotin-free detection technique. Reagents, secondary antibodies were obtained from Ventana and the reactions were visualized by UltraView™ Universal DAB Detection Kit (Ventana).

Morphometric Analysis

EGFR and syndecan-1 immunoreactions were evaluated by digital morphometrical analysis. All slides were digitalized by scanning with Mirax Panoramic MIDI and Mirax Panoramic SCAN digital slide scanners (3D Histotech Ltd., Budapest, Hungary). Fifteen non-overlapping fields of view were selected from each slide at 40x virtual objective magnification. The extension of immunostained areas was measured by Leica QWin Software (Leica Microsystems Imagine Solutions, Cambridge, UK). To compensate for discrepancies among samples in background noises, staining intensity and prevalence of non-specific reactions, minor adjustments in the color treshold levels of positive immunostaining were made.

Table 1 Patients with fibrolamellar hepatocellular carcinoma (FLC)

Patient	Gender	Age (yr)	Cirrhosis	Viral infection	Tumor size (mm)	Metastasis
1.	F	11	no	no	80×80×35	no
2.	M	15	no	no	66×96×66	lymph node
3.	F	14	no	no	100×80×80	no
4.	F	21	no	no	100×60×90	lymph node, lung
5.	M	19	no	no	110×70×100	lymph node, lung, liver
6.	M	16	no	no	30×40	no
7.	F	16	no	no	110×90×80	no
8.	F	18	no	no	60×36	retroperitoneal lymph node

Table 2 Patients with "conventional" hepatocellular carcinoma (cHCC)

Patient	Gender	Age (yr)	Cirrhosis	Viral infection	Tumor size (mm)	Metastasis
1.	F	82	yes	no	50×60	no
2.	M	78	no	no	65×55	no
3.	M	70	yes	no	90×75	no
4.	M	60	yes	HCV, EBV	55×50	multiplex HCC
5.	M	75	no	no	33×30	no
6.	M	67	no	no	42×30	no
7.	M	30	no	CMV	80×100	no

Statistical Analysis

Statistical analysis was carried out using STATISTICA software version 8.0 (Tulsa, OK, USA). The non-parametric Kruskal-Wallis test was used to analyse the differences. P-values <0.05 were considered statistically significant.

K-RAS Mutation Analysis by Restriction Fragment Microfluidic Based Detection (RFMD)

Total DNA was isolated from six or eight 3–5 μm thick sections of paraffin embedded tissues using QIAmp DNA FFPE Tissue Kit (QIAGEN) according to manufacturer instructions in 5 FLC cases. Absorption levels of DNA extracts were obtained using NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA). DNA amplifications were performed with AmpliTaq Gold and Mastercycler® gradient thermal cycler supplied by Eppendorf (Eppendorf AG, Hamburg, Germany). The reaction mixture of reagents for samples was prepared as follows: 2.5 μl 10x PCR puffer + $Mg2^{+}$, 200 μM/each dNTP, 1.00 pM/reaction of each primer, 0.8 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) per reaction. The sense primer was a mismatch primer and the product of PCR contained the recognition site of BstNI or BglI restriction endonuclease in the wild type of K-RAS gene. Primer pairs are shown in Table 5. Both reactions were carried out in 38 cycles: denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, chain elongation at 72 °C for 2 min. The amplified products were digested with 80U BstNI (New England BioLabs, Ipswich, MA, USA) at codon 12 and 80U BglI at codon 13. Enzymatic digestions were performed at 60 °C (codon 12) and 37 °C (codon 13) for 4 h in a total volume of 30 μL. Digested PCR product was analyzed by microfluidic based Experion Gel Electrophoresis System (BioRad Experion™ DNA 1 K Analysis Kit, BioRad Laboratories Inc., Hercules, CA, USA). Density ratio of alternative band to wt band was calculated and samples containing >5 % of alternative band were considered mutation positive due to the sensitivity threshold. Positive samples were verified through sequencing on ABI 3130 Genetic Analyser System (Applied Biosystems, Foster City) with BigDye® Terminator v1.1 Sequencing Kit (Applied Biosystems, Foster City).

EGFR Mutation Analysis

DNA was extracted from FFPE using the MasterPureTM DNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA) according to manufacturer instructions. Primers for EGFR exons 18–21 were designed by Array Designer: Primers for EGFR EXONS 18–21 are listed in Table 5. Amplicons of exons 18 and 21 were analyzed by high resolution melting using Roche Light Cycler 480 Real Time PCR machine (Roche Diagnostics Corp., Indianapolis, IN, USA) with High Resolution Melting Master mix (Roche Diagnostic GmbH, Mannheim, Germany). The method was validated by direct sequencing. Direct sequencing analysis was performed based on melting differences detected between exons 18 or 21 and the wild type one. Exons 19 and

Table 3 Patients with cholangiocellular carcinoma (CCC)

Patient	Gender	Age (yr)	Cirrhosis	Viral infection	Tumor size (mm)	Metastasis
1.	F	78	no	no	6×25	lymph node
2.	F	49	yes	no	85×70	lymph node
3.	M	65	no	no	40×8	lymph node
4.	F	61	no	no	50×30	no
5.	F	36	no	no	35×22	no
6.	F	74	no	no	50×30	no
7.	M	57	fibrosis	no	5×25	lymph node

Table 4 Primary antibodies used in the study

Primary antibodies	Dilution	Species/clonality	Positive control	Manufacturer
CK7	1: 1000	Monoclonal Mouse	Urinary bladder transitional cc	DAKO
CK 8	1: 100	Monoclonal Mouse	Normal liver	DAKO
CK 18	1: 400	Monoclonal Mouse	Normal skin	Vision/Novocastra
CK 19	1: 100	Monoclonal Mouse	Normal skin	DAKO
HepPar1 (HSA)	1: 50	Monoclonal Mouse	Normal adult liver	Vision/Novocastra
ß-catenin	1: 200	Monoclonal Mouse	Pancreas, neuroendcrine tumor	BD Biosciences
EGFR (ConfirmTM-3 C6)	1 μg/ml	Monoclonal Mouse	Lung squamous carcinoma	Ventana
Syndecan-1 (CD138)	1: 60	Monoclonal Mouse	Diffuse large B cell lymphoma	DAKO

20 were direct sequenced with ABI 3130 Genetic Analyser System (Applied Biosystems, Foster City) using BigDye® Terminator v1.1 Sequencing Kit (Applied Biosystems, Foster City).

Direct Sequencing Analysis

Primer pairs were identical with those used for the aforementioned EGFR melting analysis. Samples were verified through sequencing on ABI 3130 Genetic Analyser System (Applied Biosystems) using BigDye® Terminator v1.1 Sequencing Kit (Applied Biosystems, Foster City).

Results

Macroscopy of FLC

The 5 surgically removed FLC cases showed a small central scar, with soft to firm cut surface of yellowish-white color**.** The average tumor sizes were 86×67 mm in diameter (Table 1). The surrounding livers were not cirrhotic, the architecture was normal.

Histology

The FLC cases showed typical histopathology. The tumor cells presented large, polygonal form with eosinophilic cytoplasm, arranged in columns or forming glandular structures. The groups of tumor cells were separated by lamellar fibrotic bands (Fig. 1a, b). The surrounding liver tissues available in 5 cases were normal, with mild aspecific portal inflammation detectable in a few areas.

Immunohistochemistry

All FLC cases were positive for HepPar1 (HSA) and cytokeratin 7 (Fig. 1c, d) 8, 18, but were negative for cytokeratin 19 by immunohistochemistry. EGFR presented an intense membranous-cytoplasmic reaction and was higher in all tumor groups than in normal liver (Figs. 2a, b, c, d and 3). The β-catenin immunoreaction was membranous, no nuclear reaction was detected in FLC and cHCC cases using hepatoblastoma tissue as positive control for nuclear translocation (*not shown*). Syndecan-1 (CD138) showed membranous-submembranous reactions on all tumor groups (Fig. 4), being the strongest in the cHCC, however, FLC expressed syndecan-1 higher than CCC (Fig. 5).

Table 5 Primers used for RT-PCR

	Gene Name	Primer sequence (5′–3′)	
		Sense	Antisense
Housekeeping (reference) gene	36b4	AGATGCAGCAGATCCGCAT	ATATGAGGCAGCAGTTTCTCCAG
Target genes			
	KRAS codon 12	GAATATAAACTTGTGGTAGTTGGACCT	GGTCCTGCACCAGTAATATG
	KRAS codon 13	TATAAACTTGTGGTAGTTGGCCCTGGT	GGTCCTGCACCAGTAATATG
	EGFR codon 18	GTGACCCTTGTCTCTGTGTT	CCAGGGACCTTACCTTATACA
	EGFR codon 19	CACAATTGCCAGTTAACGTCTTC	TTAGGATGTGGAGATGAGCAGG
	EGFR codon 20	CAGGAAGCCTACGTGATG	TGATTACCTTTGCGATCTG
	EGFR codon 21	CAG GGT CTT CTC TGT TTC AG	CCTAAAGCCACCTCCTTACT

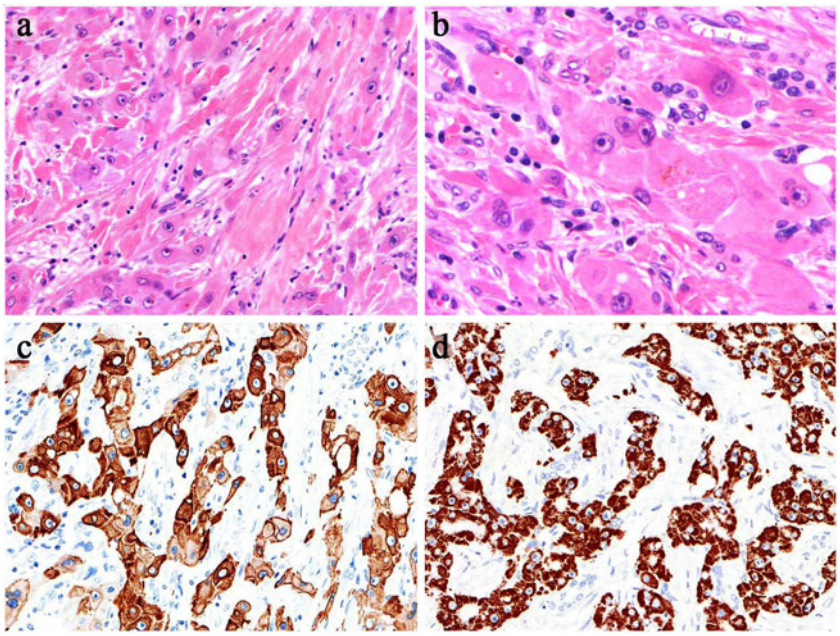

Fig. 1 Histological appearance and immunohistochemical reactions in fibrolamellar hepatocellular carcinoma (FLC). **a**, **b** FLC tumor cells with prominent nucleoli separated by lamellar fibrotic bands (hematoxylin and eosin, x200, x400). **c** Intensive HepPar1 (HSA) and (**d**) CK7 positivity by immunohistochemistry in FLC (x 200) *FLC* fibrolamellar hepatocellular carcinoma, *HSA* hepatocyte specific antigen

Statistical Analysis

Based on above presented data statistical analysis showed FLC to express significantly higher EGFR ($p=0.0001$) proteins than cHCC and the normal liver (Fig. 3) equally high as CCC. Syndecan-1 was significantly higher in FLC than in CCC, however there was no difference between FLC and cHCC (Fig. 3).

EGFR and KRAS Mutation Analyses

K-RAS mutation analysis did not reveal mutations in exons (codons) 12 and 13, respectively in any of the FLC cases. EGFR combined high resolution melting analysis and direct sequencing found single nucleotid polymorphism in exon 20 in two FLC cases. However, no known EGFR mutation in exons 18–21 was detected in any of the FLC cases.

Discussion

FLC is a variant of HCC, with unknown etiology and pathogenesis. Besides clinical characteristics, the expression or lack of expression of several markers differentiates FLC from cHCC, CCC and hepatoblastomas [14]. The cytokeratin pattern of FLCs presents both hepatocytic (CK8, 18)

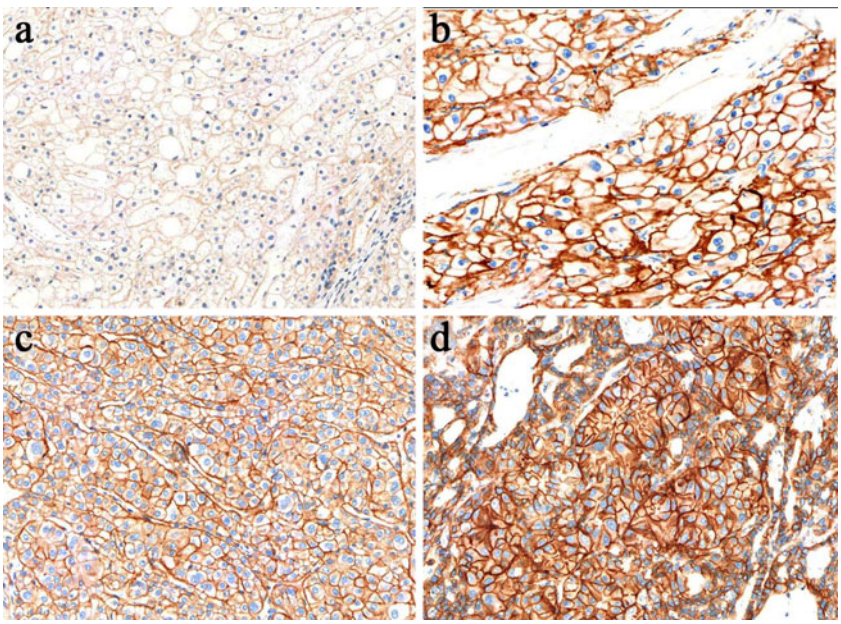

Fig. 2 Immunohistochemical reactions in (**a**) EGFR in normal liver (x200), (**b**) intensive EGFR immunostaining in FLC (x200), (**c**) cHCC and (x200) (**d**) cholangiocellular carcinoma (x200) *FLC* fibrolamellar hepatocellular carcinoma, *cHCC* "conventional" hepatocellular carcinoma, *CCC* cholangiocellular carcinoma, *NL* normal liver, *EGFR* epidermal growth factor receptor

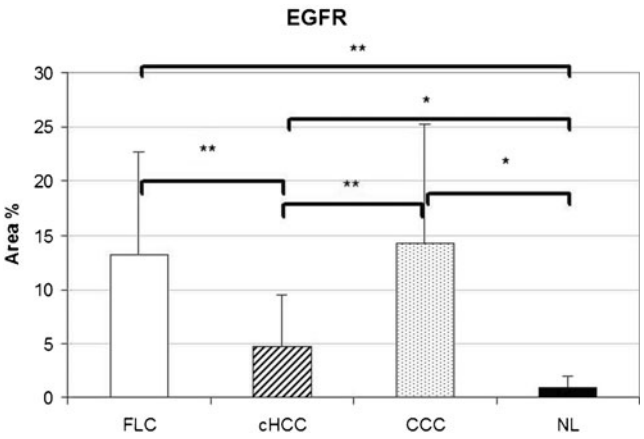

Fig. 3 Morphometric analysis of EGFR immunohistochemical reactions. Significant correlations: * $p<0.05$ ** $p<0.001$. *FLC* fibrolamellar hepatocellular carcinoma, *cHCC* "conventional" hepatocellular carcinoma, *CCC* cholangiocellular carcinoma, *NL* normal liver, *EGFR* epidermal growth factor receptor

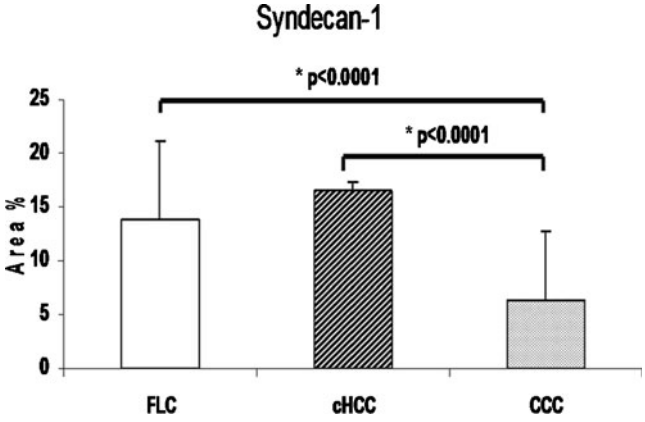

Fig. 5 Morphometric analysis of Syndecan-1 (CD138) immunohistochemical reactions. Significant correlations: * $p<0.05$ ** $p<0.001$. *FLC* fibrolamellar hepatocellular carcinoma, *cHCC* 'conventional" hepatocellular carcinoma, *CCC* cholangiocellular carcinoma

and biliary characteristics (CK7), as found in our study and by others [1, 5, 6]. Recent data proved expression of glypican3 in FLCs with variable percentage [1, 5, 9].

In our previous study we proved that the expression pattern of tight junction transmembrane protein claudins in FLCs is altered and is more similar to cHCCs than to CCCs, however, FLCs differ from both in quantitative and qualitative tight junction protein characteristics [6]

It has been suggested that the composition of TJs in hepatocytes is regulated by several cytokines and growth factors produced by non-parenchymal liver cells [15, 16]. Buckley et al. [12] previously showed that high expression of EGFR was associated with extra EGFR gene copy numbers, which is due to polysomy rather than gene amplification in FLCs. Our data are in agreement with the findings of high expression of EGFR, with the addition that overexpression of EGFR is the highest in FLC as compared with cHCC and CCC. We further analysed the possibilty of EGFR mutation in exons 18–21 with the finding that no mutations were detected in any of our FLCs.

Gene transcripts involved in RAS, MAPK and PIK3 signaling pathways and transcripts involved in xenobiotic degradation were found to be overexpressed in FLC [2]. Our data showed that no K-RAS mutations in exons 12 and 13 were detectable in any of the FLC cases. We and Buckley et al. [12] both found increased EGFR protein expression as well as lack of K-RAS mutation, further implying that FLC is a good candidate for anti-EGFR therapies.

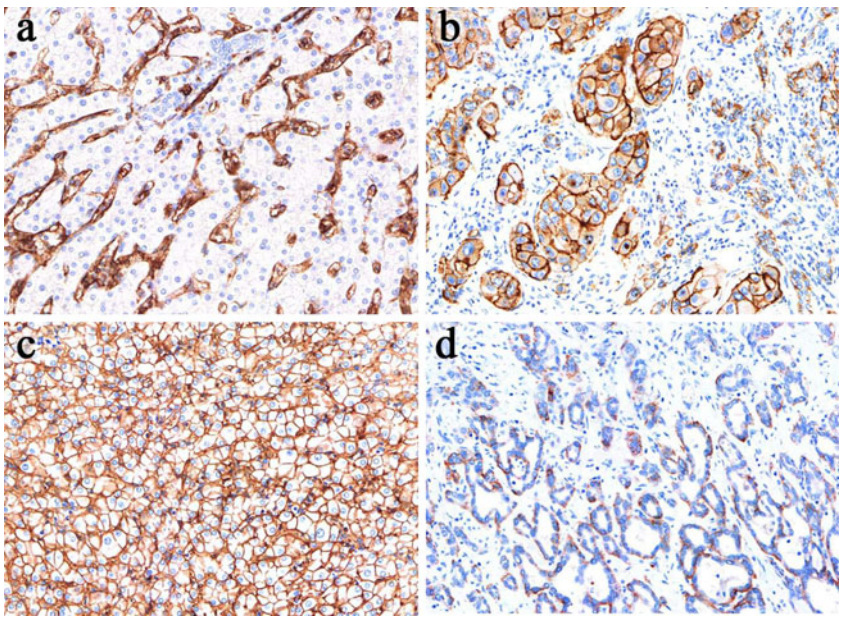

Fig. 4 Syndecan-1 immunohistochemical reactions in (**a**) normal liver (NL), (**b**) fibrolamellar carcinoma FLC and (**c**) hepatocellular carcinoma (cHCC), (**d**) cholangiocellular carcinoma (CCC)

No β-catenin nuclear translocation was demonstrated by immunohistochemistry in our FLC cases, which is in accordance with data that no mutation was detected in exon 3 of the β-catenin gene (CTNNB1) [17]. It has been demonstrated however that FLCs display significantly higher levels of total β-catenin, tyrosine-654-phosphorylated β-catenin (Y654-β-catenin) and cyclin-D1 as compared with cHCCs, which might be responsible for the invasive characteristics and could possibly be associated with the EGFR overexpression [17].

A striking characteristic of FLCs was found to be that the increased presence of mitochondria and metastatic FLCs is associated with increased mitochondrial DNA levels [18]. Several somatic mutations were found in the mitochondrial genome in FLC, however, no consistent pattern of mutation was noticeable [18]. Our data on EGFR and K-RAS mutation analyses further suggest that no consistent mutation is known in FLC so far.

FLCs show fewer chromosomal abnormalities compared with cHCCs, however the cases presenting chromosomal changes behave more aggressively [7]. Epigenetic instability is rare in FLCs in contrast to the virus-associated cHCCs [11]. Activation of nuclear factor-kappa B(NF-KB) signaling pathway was found in FLC, suggesting that it may represent novel targets for therapy [19]. It is not clear whether treatment schedule for cHCC with sorafenib can be generalized to FLC, however, the first case on treatment has been published [20].

Syndecans are transmembrane proteoglycans which interact with several growth factors and matrix components, present in normal liver and liver tumors [21, 22]. It has been shown that syndecan-1 negatively correlates with tumor differentiation and progression [22]. Interestingly, we found higher expression of syndecan-1 in FLC and cHCC compared with CCC, however, there was no difference in syndecan-1 expression between FLC and cHCC. No further data related to tumor grading were included in this study because the analysed cHCC and CCC cases were all of grades II and III. The obtained data are suggestive of the fact that the lower expression of syndecan-1 in CCC is associated with a more agressive behaviour of this tumor. This, however, needs further investigation.

Our study provides further data to the specific characteristics of FLC, especially the overexpression of EGFR. In addition to previous observations (12), our study revealed that the expression of EGFR is highest in FLC, compared with cHCC and CCC. Besides, no EGFR or K-RAS mutations were detected, giving further preference to FLC for anti-EGFR therapy.

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