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Comparative protein expression profiles of hilar and peripheral hepatic cholangiocarcinomas☆

Nathalie Guedj[1,4], Qian Zhan[2], Martine Perigny[1], Pierre-Emmanuel Rautou[2], Françoise Degos[2], Jacques Belghiti[3], Olivier Farges[3], Pierre Bedossa[1,4], Valérie Paradis[1,4,*]

[1]*Department of Pathology, Beaujon Hospital, 100 boulevard du Général Leclerc, 92118 Clichy, France*
[2]*Department of Hepatology, Beaujon Hospital, Clichy, France*
[3]*Department of Hepato-biliary Surgery, Beaujon Hospital, Clichy, France*
[4]*INSERM U773, Paris, France*

***Background/Aims*: Hepatic cholangiocarcinomas are tumors with poor prognosis and with increasing incidence worldwide. The aim of the study was to compare morphological features and protein profiles of hilar and peripheral cholangiocarcinomas.**

***Methods*: Clinicopathological data were collected from 111 cholangiocarcinomas (59 peripheral and 52 hilar). Protein expression, assessed on tissue samples using tissue microarray and protein array technologies, was compared between both types of tumors and with extrahepatic cholangiocarcinoma and hepatocholangiocarcinoma.**

***Results*: Hilar cholangiocarcinomas were smaller in size (mean: 2.7 vs. 8 cm, $p < 0.001$), were more often well differentiated adenocarcinomas (65% vs. 36% well differentiated, $p < 0.01$) and carried out stronger perineural invasion (83% vs. 42%, $p < 0.001$) than peripheral cholangiocarcinomas. Regarding protein expression, hilar cholangiocarcinomas more often expressed MUC5AC (62% vs. 22%, $p < 0.0001$), Akt2 (54% vs. 27%, $p < 0.001$), CK8 (98% vs. 81%, $p < 0.005$) and annexin II (92% vs. 66%, $p < 0.001$). Interestingly, VEGF A expression was more frequently encountered in peripheral cholangiocarcinoma (69% vs. 25%, $p < 0.0001$) and correlated with increased vascular density. Using protein array antibody, we identified filamin A as significantly overexpressed (>2-fold) in peripheral cholangiocarcinomas.**

***Conclusions*: Our results show that hilar and peripheral cholangiocarcinomas display specific protein profiles, especially regarding VEGF expression. This suggests a potential benefit for anti-angiogenic therapies in peripheral hepatic CCs.**

***Keywords*: Hepatic cholangiocarcinoma; Biliary carcinogenesis; Tissue microarray; Protein array; Vascular endothelial growth factor; Filamin**

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* Corresponding author. Tel.: +33 1 40875463; fax: +33 1 40870077.
E-mail address: vparadis@teaser.fr (V. Paradis).

Abbreviations: CC, cholangiocarcinoma; CK, cytokeratin; HCC, hepatocellular carcinoma; TMA, tissue microarray; HCV, hepatitis C virus; HBV, hepatitis B virus; H&E, hematein and eosin; MVD, microvessel density; SD, standard deviation; MUC, mucin; VEGF, vascular endothelial growth factor.

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1. Introduction

Cholangiocarcinomas (CCs) are the second most frequent primary malignant tumor of the liver, accounting for 10–15% of all primary liver cancers. Interestingly, recent studies have reported that the incidence and mortality rates of CCs are rising worldwide, with approximately 3500 new cases diagnosed annually in the United States. Up to now, CCs have been associated with poor prognosis, characterized by overall survival of less than 5% at 5 years, which has not changed significantly over the past 30 years [1–3]. Such poor survival is mainly due to late diagnosis and the absence of effective non-surgical therapeutic modalities for these tumors.

CCs are malignant tumors that develop from epithelial cells of the biliary tract, either within the liver or from the extrahepatic bile ducts; therefore, current classification of CC distinguishes intra- and extra- hepatic CCs. In fact, the group of intrahepatic CCs defined by Okuda et al., is very heterogeneous and includes both tumors which develop in the hilum and peripheral CCs that develop at a distance from the hilum. However, the two groups of tumor strongly differ in terms of clinical presentation. Whereas hilar CCs display symptoms of biliary obstruction, peripheral CCs are diagnosed as mass lesions within the liver. In addition to their differing growth patterns and symptoms, they may also diverge in etiopathogenesis and risk factors [4,5].

Biliary carcinogenesis remains poorly understood. A number of molecular events have already been described in the course of CC, with some of them pointing to environmental factors and host genetics [6,7]. In addition, it has recently been pointed out that patients with cirrhosis carry a 10-fold-increased risk of developing CC [8,9]. Because hilar and peripheral CCs display such differences in symptoms, evolution and epidemiology, we hypothesized that the two tumors may also differ in associated molecular alterations, which have not been investigated so far.

Therefore, the aim of the present study was to compare clinicopathologic characteristics and molecular signatures of intrahepatic hilar and peripheral CCs at the level of protein expression. To address this issue, we assessed protein profiles of CCs by antibody array analysis and immunohistochemistry using tissue microarrays (TMAs) and compared these profiles with extrahepatic CCs, hepatoCCs and cytokeratin (CK) 19 positive hepatocellular carcinomas. These complementary procedures enabled us to obtain an overview of expression of a large number of proteins potentially involved in carcinogenesis, in a large series of intrahepatic CCs. Our results support the notion that hilar and peripheral CCs are phenotypically different. Interestingly, they also suggest that specific targeted anti-cancer strategies should be efficient in peripheral CC.

2. Materials and methods

2.1. Patients

We retrospectively retrieved, from the files of surgical and pathology departments, cases of patients who had undergone liver resection for intrahepatic CCs between 1995 and 2006 at Beaujon Hospital, Clichy, France. Patients who had only undergone biopsy or who had received neoadjuvant therapy before surgery were ruled out. We also included cases of extrahepatic CCs, hepatoCCs, CK 19 positive HCC. When available, any potential cause of chronic liver disease, including hepatitis C virus (HCV) and hepatitis B virus (HBV) infection (serological tests), genetic hemochromatosis (positive genetic testing or hepatic iron index >1.9), autoimmune liver diseases (serum autoantibodies) or excessive alcoholic consumption (defined by consumption higher than 40 g/day) were retrieved. All clinicopathological and follow-up data of patients were collected and registered in a database. Informed consent was obtained in all cases before surgery and the study protocol conformed to the ethical guidelines of the 1975 declaration of Helsinki as reflected in *a priori* approval by the institution's human research committee. After surgery, each surgical sample was immediately transferred fresh and conventionally processed in the Department of Pathology.

2.2. Tissue samples, histologic classification and tissue microarray (TMA) construction

For each case, gross examination was performed on the fresh surgical sample and the liver resection specimen was largely sampled. Histopathologic analysis was performed on formalin-fixed, paraffin-embedded tissue sections stained with H&E. Tumor characteristics, including size, number of nodules and location, satellite nodules, vascular and perineural invasion, grade of differentiation, presence of necrosis or hemorrhage and mucus secretion (Alcian blue staining) were reported. Histologically, the tumors were categorized into three groups from well to poorly differentiated adenocarcinoma according to their degree of papillary and/or tubular formation. Pathologic analysis of adjacent peritumoral liver was also systematically performed using H&E, Masson's trichrome and Perl's staining.

Based on the criteria of World Health Organization Classification of tumors, a tumor that arises peripheral to the secondary bifurcation of the left or right hepatic duct was considered as peripheral CC. Inversely, CC arising from the right or left hepatic ducts at or near their junction was classified as hilar CC. We also determined among peripheral and hilar CCs the macroscopic type according to the classification of intrahepatic CC established by the Liver Cancer Study Group of Japan [10].

Paraffin-embedded tissue blocks were used for tissue microarray construction. The slides were reviewed to identify and mark representative areas of viable tumor tissue. Taking tumor heterogeneity into account, five tissue cores of 1 mm each were punched from selected tumor areas of any donor tissue block and brought into a recipient paraffin block at defined array coordinates using a tissue-arraying instrument (MTA-1, Beecher Instruments, Inc., Sun Prairie, WI, USA). A total of 11 TMA blocks containing all tumors and normal liver, were built. Sections from these arrays were then stained with H&E to assess adequacy of the sampling.

2.3. Immunohistochemistry on TMA

For this procedure, we selected, according to published data, a set of 35 antibodies against proteins that are potentially deregulated in CC [11–15]. Selected proteins included cytokeratins, apomucins, adhesion molecules, cell cycle proteins, cell signalization proteins and angiogenesis (Table 1). Immunohistochemical study was performed on TMA using an automated immunohistochemical stainer according to the manufacturer's guidelines (streptavidin-peroxidase protocol; Benchmark, Ventana, Tucson, AZ). All sections were evaluated independently by two pathologists (NG, MP) using semi-quantitative analysis for each antibody. The percentage of stained cells was assessed

Table 1
List of primary antibodies used on TMA (M, monoclonal; P, polyclonal).

Antibody	M/P	Manufacturer	Clone	Dilution
CK 5/6	M	Dako	D5/16B4	1/500
CK 7	M	Dako	OVTL12/30	1/500
CK19	M	Dako	RCK108	1/500
CK 8	M	Dako	35Bh11	1/200
CK 20	M	Dako	Ks20/8	1/200
MUC 2	M	NovoCastra	Ccp58	1/500
MUC 5AC	M	NovoCastra	CLH2	1/500
MUC 1	M	NovoCastra	Ma695	1/200
Akt2	P	Cell Signaling		1/500
pAkt	P	Cell Signaling		1/10
MAPK p38	M	Cell Signaling	12F8	1/500
p42/44	M	Cell Signaling	197G2	1/500
PTEN	M	Santa Cruz	A2B1	1/50
pPTEN	P	Santa Cruz		1/50
cERB2	M	NovoCastra	CB11	1/200
EGFR	M	Zymed	31G7	1/20
cKit	P	Dako		1/200
VEGF A	P	Santa Cruz		1/300
FGF-R3	M	R&D Systems	136312	1/50
E-cadherin	P	Santa Cruz		1/50
B catenin	M	BD Biosciences	14	1/200
Annexin II	P	Santa Cruz		1/50
Glypican 3	M	Biomosaics	1G12	1/100
Smad 4	M	Santa Cruz	B-8	1/50
Estrogen	M	NovoCastra	6F11	1/200
Progesteron receptors	M	NovoCastra	16	1/200
Mdr 1	M	Santa Cruz	G1	1/200
Hep Par 1	M	Dako	OCH1E5	1/400
Bcl2	M	Dako	124	1/100
Bax	P	Dako		1/250
Filamin A	M	Thermo Scientific	PM6/317	1/500

for each core and a mean was calculated between the five cores for each case. A case was considered positive when at least 20% of tumor cells were immunostained. We did not take into account the intensity and the type of staining because there was a good concordance between each spot from each case. Three cases of normal liver were also evaluated for all antibodies.

2.4. Antibody array

In order to increase the number of potential targets investigated, we completed the study with an additional antibody array procedure. In brief, three cases of hilar and peripheral CCs, whose clinicopathologic characteristics are summarized in Table 2, were selected and proteins extracted from each frozen tissue blocks. Proteins from hilar and peripheral CCs were, respectively, randomly labeled with two different fluorescent dyes by conjugation to amino acid residues (Alexa Fluor 680 and 800, Molecular Probes, France). One hilar was paired with one peripheral CC and differentially labelled proteins were pooled and hybridized against an antibody array containing 124 different antibodies spotted on a glass slide and directed against cell cycle, cytoskeleton and transcriptional factor proteins (MAGYarray, Protneteomix®). Bound antigens on the antibody arrays were detected using a fluorescence scanner. Quantitation and differential analysis of fluorescence intensity was performed using in-house-developed software (Protneteomix®). Each experiment was performed in duplicate. Data were normalized according to cytokeratin 19 expression levels in order to correct for tumor heterogeneity. Proteins whose levels of expression significantly differed ($2 \times N$) in all three paired hilar and peripheral CC samples studied were selected for further analysis and assessed on TMA slides for validation.

Table 2
Main clinicopatholgical characteristics of the three pairs of hilar and peripheral cholangiocarcinomas used for the antibody array analysis.

	Hilar type ($n = 3$)	Peripheral type ($n = 3$)
Age (years)	57.3	62.7
Gender: male/female	2/1	2/1
Size (cm)	3.3	8.1
Grade: well differentiated	2	2
Vascular invasion	3	1
Perineural invasion	3	0
Satellite nodules	1	1
Lymph node metastasis	1	0

2.5. Microvessel density (MVD)

In order to compare MVD in hilar and peripheral CCs, we randomly selected from the overall series of CC representative paraffin blocks of 10 hilar and 10 peripheral tumors. Clinicopathologic characteristics of these selected cases were not significantly different from the overall series. For each case, one 3-μm thick section was immunoassayed with CD31 antibody (Monoclonal antibody, Dako, 1/200). MVD was assessed in three areas of the tumors containing the highest number of capillaries expressing CD31 (hot spot) at 250× magnification using a Mertz eyepiece graticule containing 100 points. MVD was calculated as the mean value of three graticule counts.

2.6. Statistical analysis

Continuous variables were summarized as mean (±SD) or median with range and categoric variables as frequency and percentage. Comparisons between groups of quantitative variables were performed using Student's *t*-test when variable distributions were normal and using Mann–Whitney test in other cases. Comparisons between groups of qualitative variables were performed using chi-square test and the Fisher exact tests, as appropriate. All tests were two-sided and used a significance level of 0.05. Overall survival curves were estimated with the Kaplan-Meier method and were statistically analyzed with the Logrank- test. In the survival analysis, patients with curative and non-curative resections were pooled. All analyses were performed with Statview software 0 version 5.0 (SAS Institute Inc.).

3. Results

3.1. Clinicopathologic characteristics of intrahepatic CCs

The overall series included 111 CCs. Sixty-six patients were male and 55 were female with a mean age of 59.5 ± 11 years (33–83 years). Fifty-nine CCs (53%) were peripheral CCs and 52 (47%) hilar intrahepatic CCs. In 104 patients, CC was removed by liver resection and liver transplantation was indicated for 7. Risk factors for chronic liver disease were noted in 63 patients (57%) (primary sclerosing cholangitis, $n = 3$; excessive alcohol consumption, $n = 45$; HCV or HBV positive serological markers, $n = 11$; and hemochromatosis, $n = 4$).

Mean size of the tumor was 5.5 ± 4.1 cm (0.5–22 cm). Macroscopically, all peripheral CC were of a mass-forming type except for one periductal-infiltrating type. Most hilar CC were of a periductal infiltrating type with only two cases of intraductal growth pattern. All tumors were adenocarcinomas. Fifty-five cases (50%) were well differentiated, 45 (40%) moderately differentiated and 11 (10%) poorly differentiated adenocarcinomas. Peritumoral liver was available in 110 cases for histopathological analysis. This was normal in 46 patients (42%), whereas a significant degree of fibrosis consistent with biliary fibrosis was noted in 55 patients (50%), including six cases with biliary cirrhosis, significant fibrosis unrelated to biliary origin in nine cases (8%), extensive fibrosis in three patients and cirrhosis related to chronic viral infection in six patients. The mean duration of follow-up was 14 months (range 2–90 months) with 31 patients lost to follow-up. The overall five-year survival rate was 65%, 54% and 20% at 1, 2 and 5 years, respectively.

When hilar and peripheral CCs were compared according to clinicopathologic criteria, no significant differences were found in terms of gender, age or risk factors for CC. However, hilar CCs, as compared to peripheral CCs, were significantly smaller in size (2.7 vs. 8 cm, $p < 0.001$), and more often well-differentiated adenocarcinomas (65% vs. 36%, $p < 0.01$) with mucus secretion (91% vs. 68%, $p < 0.01$) and perineural invasion (83% vs. 42%, $p < 0.001$). In contrast, satellite nodules around the tumor were more often observed in peripheral than in hilar CCs (51% vs. 6%, $p < 0.001$). The aspect of non-tumoral liver was not significantly different in the two groups of patients. Finally, cumulative survival of patients was no different in the two groups of tumors (Fig. 1).

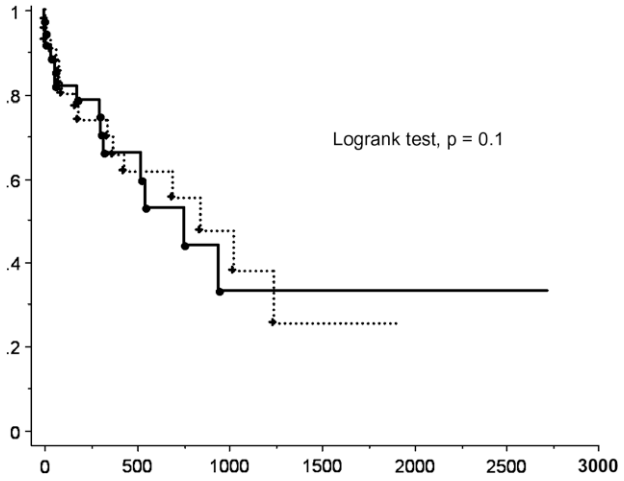

Fig. 1. Cumulative survival in cholangiocarcinomas (dotted line, peripheral; full line, hilar; *x*-axis, days after surgery).

3.2. Clinicopathologic characteristics of extrahepatic CCs, hepatoCCs and CK19 positive HCCs

In order to assess tumoral progression of intrahepatic CCs, we included tumors developed either in the extrahepatic bile duct or in peripheral liver. This group comprised 11 extrahepatic CCs (9 gallbladder carcinoma and 2 common bile duct adenocarcinoma), 12 hepatoCCs and 6 CK19 positive HCCs. Twenty patients were male and nine were female with a mean age of 56 ± 13 years (23–78 years). Risk factors for chronic liver were noted in six patients (excessive alcohol consumption, $n = 1$; HCV or HBV positive serological markers, $n = 5$). Mean size of the tumor was 6.5 ± 4.2 cm (1.1–14.5 cm). Morphologically, extrahepatic CCs were well differentiated ($n = 7$) or moderately differentiated ($n = 4$) adenocarcinomas. HepatoCCs were all single tumors with mixed unequivocal hepatocellular and cholangiocarcinomas components of varying degree of differentiation. Peritumoral liver was normal in 14 patients whereas fibrosis secondary to biliary obstruction was noted in eight patients and in seven, cirrhosis was observed.

Comparing hilar CCs and extrahepatic CCs, there were no significant differences in terms of main clinicopathological features, except for tumor size which was larger in extra-hepatic CC (4.4 vs. 2.7 cm, $p = 0.05$).

In the same way, comparing peripheral CCs and hepatoCCs and CK19 positive HCC, there were no significant differences in terms of main clinicopathological

Table 3
Clinicopatholgical features of hilar, peripheral, extrahepatic cholangiocarcinomas (CCs), hepatocholangiocarcinomas and CK19 positive hepatocellular carcinoma (HCC CK19).

	Hilar CC ($n = 52$)	Peripheral CC ($n = 59$)	p	Extra-hepatic CC ($n = 11$)	HepatoCC ($n = 12$)	HCC CK19 ($n = 6$)
Age (years)	59 ± 10	60 ± 11	0.5	62 ± 11	54 ± 11	48 ± 18
Gender: male/female	32/20	34/25	0.2	7/4	8/4	5/1
Size (cm)	2.7 ± 1.6	8 ± 4.3	<0.001	4.5 ± 2.8*	7.3 ± 4	9 ± 4.8
Grade: well differentiated	34 (65%)	21 (36%)	<0.01	7 (64%)	5 (42%)	0**
Mucus secretion	47 (91%)	40 (68%)	<0.01	10 (91%)	9 (75%)	0
Vascular invasion	30 (57%)	44 (75%)	0.1	8 (73%)	10 (83%)	5 (83%)
Perineural invasion	43 (83%)	25 (42%)	<0.001	7 (63%)	4 (33%)	0
Satellite nodules	3 (6%)	30 (51%)	<0.001	1 (9%)	4 (33%)	3 (50%)
Lymph node metastasis	17 (33%)	19 (32%)	0.6	3 (27%)	1 (17%)	1 (50%)

* Significant difference between extrahepatic CC and hilar CC ($p = 0.05$).
** Significant difference between peripheral CC and CK19 HCC ($p = 0.004$).

criteria. The main clinicopathological features of all tumors are summarized in Table 3.

3.3. Protein profiles of intrahepatic CCs, extrahepatic CCs, hepatoCCs and CK19 positive HCCs

3.3.1. Immunohistochemistry on TMA

As expected, a large majority of CCs displayed an excreto-biliary phenotype [CK7-positive in 104 cases (94%); CK19- or MDR1-positive in 109 cases (98%)]. Only 10% and 2% of CCs also expressed CK 20 or Hep-par 1, respectively.

Among selected antibodies evaluated on TMA, five proteins were significantly differentially expressed in hilar and peripheral CCs (Table 4). Hilar CCs more often expressed MUC 5AC (62% vs. 22%, $p < 0.0001$), Akt2 (54% vs. 27%, $p < 0.001$), CK8 (98% vs. 81%, $p < 0.005$) and annexin II (92% vs. 66%, $p < 0.001$). MUC 5AC, Akt2 and CK8 expression were cytoplasmic, and annexin II expression was cytoplasmic with

Table 4
Protein expression comparison of hilar and peripheral cholangiocarcinomas on Tissue MicroArray.

Antibody	Hilar type ($n = 52$)	Peripheral type ($n = 59$)	p	Normal liver
Muc5AC	32 (62%)	13 (22%)	<0.0001	−
Akt 2	28 (54%)	16 (27%)	<0.001	−
Annexin II	48 (92%)	39 (66%)	<0.001	+
CK8	51 (98%)	48 (81%)	<0.005	Biliary cells+
VEGF A	13 (25%)	41 (69%)	<0.0001	Hepatocytes+
Filamin A	15 (29%)	37 (63%)	<0.001	−
CK20	5 (10%)	6 (10%)	1.00	−
CK5/6	1 (2%)	3 (5%)	0.62	−
CK19	52 (100%)	57 (97%)	0.49	Biliary cells+
Muc 2	5 (10%)	2 (3%)	0.25	−
Muc 1	23 (44%)	23 (39%)	0.58	−
EGFR	26 (50%)	34 (58%)	0.42	Hepatocytes+
CD117	1 (2%)	0	0.47	−
B catenin	51 (98%)	54 (92%)	0.21	+
E cadherin	51 (98%)	55 (93%)	0.37	+
FGF	0	1 (2%)	1.00	−
c-Erb 2	2 (4%)	0	0.21	−
PTEN	0	1 (2%)	1.00	−
p38	0	0	1.00	−
Bcl 2	4 (8%)	3 (5%)	0.70	Hepatocytes+
P53	17 (33%)	16 (27%)	0.52	−
Mib 1	12 (23%)	12 (20%)	0.76	Hepatocytes+
Bax	11 (21%)	9 (15%)	0.42	−
Smad 4	3 (6%)	4 (7%)	1.00	−
P44/42	0	1 (2%)	1.00	−
Estrogen	0	0	1.00	−
Progesterone	0	0	1.00	−

+ Expression in biliary cells and hepatocytes.
− Negative in both.

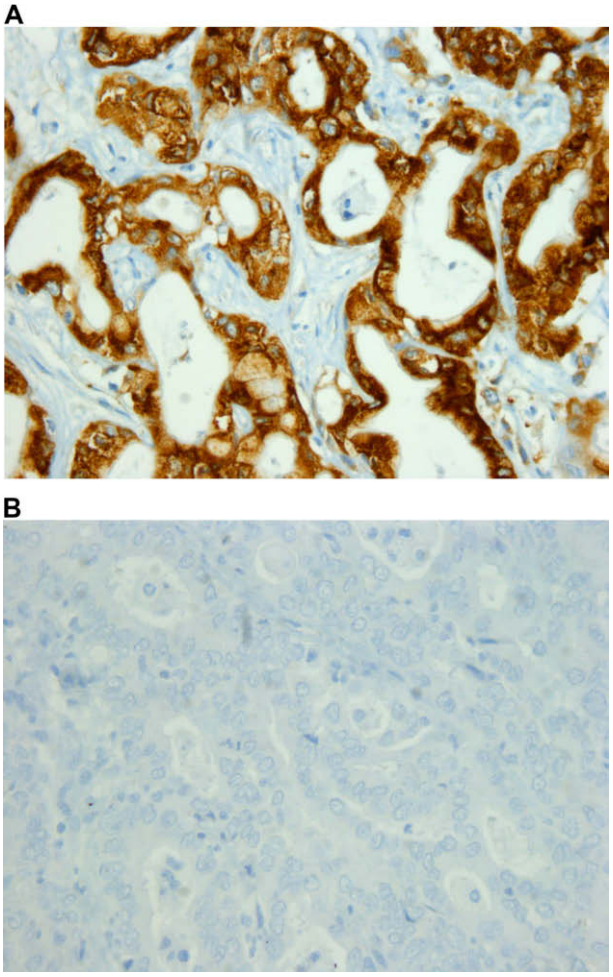

Fig. 2. MUC 5AC immunostaining: (A) strong cytoplasmic expression in hilar cholangiocarcinoma compared to (B) negative staining in peripheral cholangiocarcinoma (original magnification 400×). [This figure appears in colour on the web.]

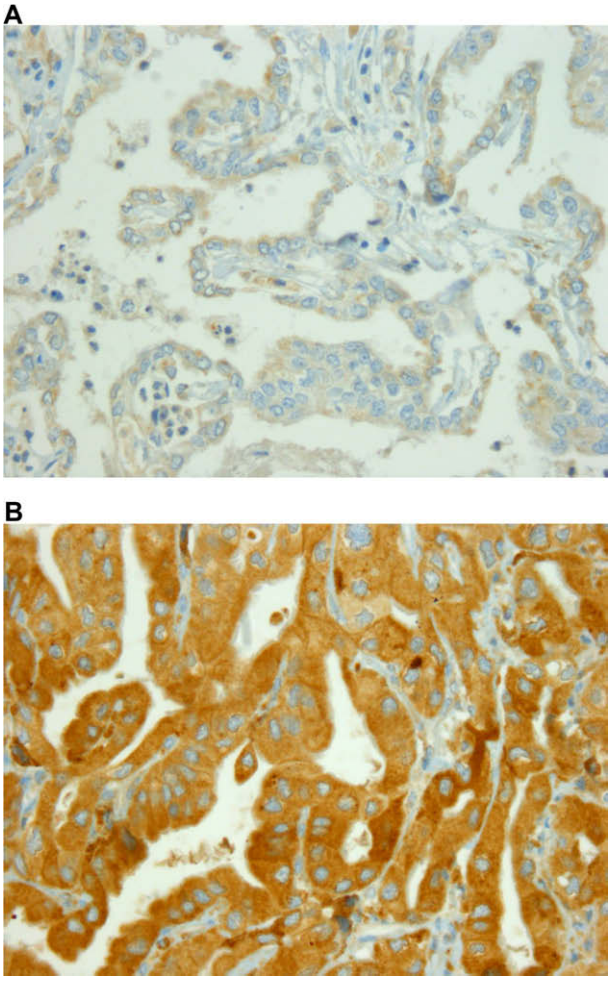

Fig. 3. VEGF A immunostaining: (A) weak cytoplasmic staining in hilar cholangiocarcinoma compared to (B) strong cytoplasmic expression in peripheral cholangiocarcinoma (original magnification 400×). [This figure appears in colour on the web.]

membrane strengthening. Fig. 2 illustrates MUC 5AC immunostaining in typical cases of peripheral and hilar types of CC. Interestingly, VEGF A expression was strongly and significantly more frequently expressed in peripheral than in hilar CCs (69% vs. 25%, $p < 0.0001$) (Fig. 3).

Among the control group, a large majority of tumors expressed CK7 (79%), CK 19 (90%) and MDR 1 (93%). All hepatoCCs and CK19 HCC strongly expressed Hep-Par and only one case of extrahepatic CCs also expressed Hep-Par.

We did not find any significant difference in terms of immunophenotype comparing hilar and extrahepatic CCs or comparing peripheral with hepatoCCs or with CK 19 positive HCCs (data not shown).

3.3.2. Protein array

Screening of differentially expressed antigen using MAGYarray antibody array confirmed significant overexpression of VEGF A in peripheral CCs. Interestingly, filamin A was constantly and significantly overexpressed (>2-fold) in peripheral CCs in each pair of hilar and peripheral CC tested (Fig. 4). Immunostaining of filamin A on TMA confirmed that filamin A was significantly more often expressed in peripheral than in hilar CCs (63% vs. 29%, $p < 0.001$) (Fig. 5). No correlation was observed between filamin A expression, size of tumor, grade of differentiation or survival.

3.4. MVD in hepatic CCs

In order to evaluate the potential relevance of VEGF A overexpression in peripheral CCs, we quantitated MVD in hilar and peripheral types. We noted that the vascular hot spots were located at the invasive edge of the tumor for peripheral CCs, and were haphazardly distributed in hilar CCs. Mean MVD was significantly higher in peripheral CCs than in hilar CCs (6.5 ± 1 vs.

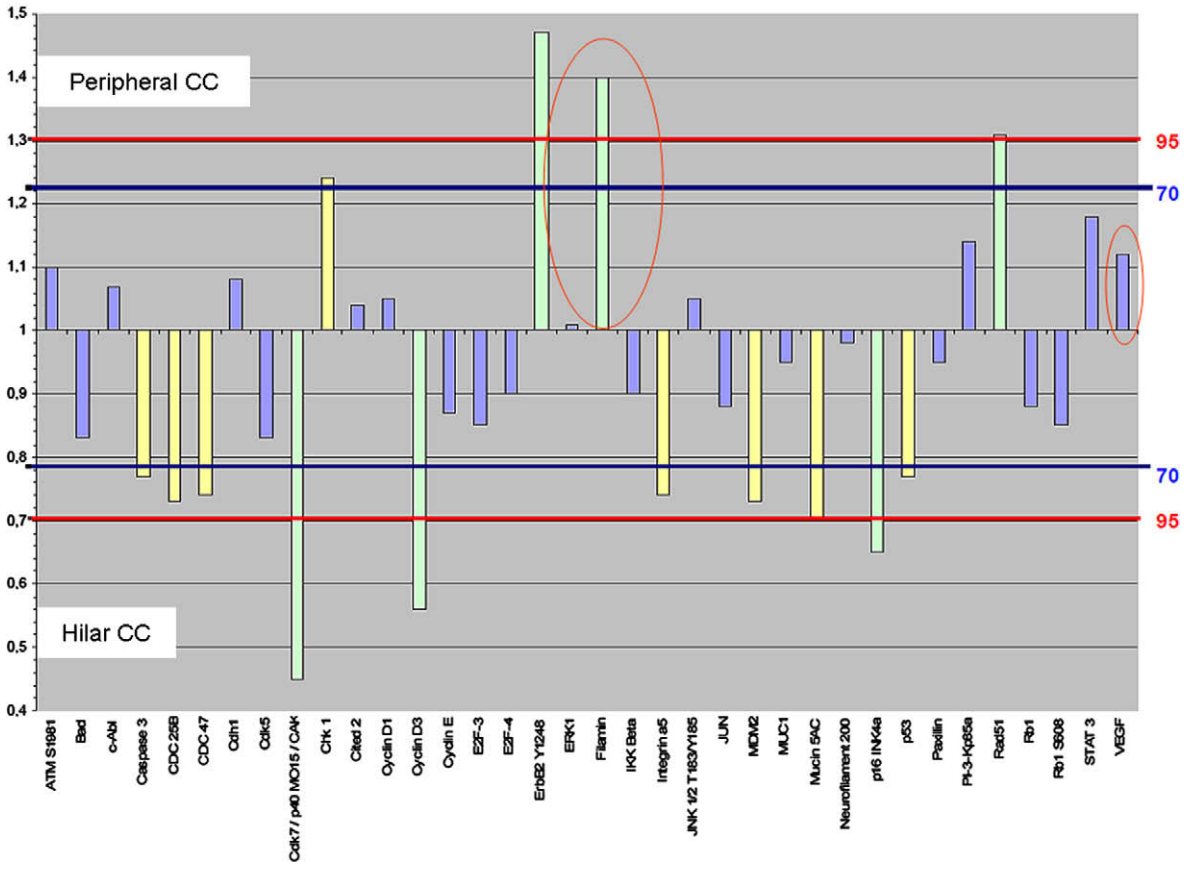

Fig. 4. Protein profiling comparison by protein array of one pair of hilar/peripheral cholangiocarcinoma. We observed an up-regulation of filamin A and VEGF (red circle) in peripheral CC (up) compared to hilar CC (down). *X*-axis, antibodies. [This figure appears in colour on the web.]

3.6 ± 1, $p < 0.01$). Furthermore, MVD was significantly correlated with VEGF A expression evaluated in the same tumors ($r = 0.8$) (Fig. 6).

4. Discussion

CCs are adenocarcinomas arising from bile duct epithelium; they are usually categorized according to their location as either intrahepatic or extrahepatic CCs. Although peripheral and hilar CCs are both considered to be intrahepatic CCs, they significantly differ in terms of clinical symptoms and histopathological characteristics despite a common biliary phenotype [5,16–18]. As previously reported, our results clearly demonstrate that hilar and peripheral CCs differed in the incidence of perineural invasion, differentiation grade and satellites nodules. However, we failed to find a significant difference in term of survival between hilar and peripheral CCs probably because we included larger peripheral CC [19]. Interestingly, we did not find any significant differences in terms of clinicopathological and immunophenotypical features comparing hilar and extrahepatic CCs, except for tumor size. Such differences could be explained by the inclusion of tumors developed in gallbladder that were larger. According to these data, we can support that hilar CCs resembled more extrahepatic bile duct carcinomas than peripheral CCs, as it was initially proposed by Okuda et al. [16]. Furthermore, our data showing a similar phenotype between CK19 positive HCCs, hepatoCCs and peripheral CCs, support the concept developed by Komuta et al. and led to consider some types of HCC as tumors close to peripheral CCs [20]. Regarding hepatoCCs, it has been shown, using molecular tools, that these tumors display similar characteristics to peripheral CCs [21].

In addition to these clinicopathological features, hilar and peripheral CCs also display specific protein profiles that suggest differences in the mechanisms of carcinogenesis and provide further evidence for developing new therapeutic strategies.

Using immunohistochemistry and TMA, several proteins were significantly differentially expressed in hilar and peripheral CCs. Among them, VEGFA expression was upregulated in peripheral compared to hilar CCs, a finding confirmed by the quantitative protein array approach. In parallel with increased expression of VEGFA by tumor cells in peripheral CCs, MVD was

also increased, with a strong correlation with VEGFA expression, suggesting a functional role for this growth factor in tumor angiogenesis. Such an association is reinforced by the study by Benckert et al., which reported expression of VEGFA in tumor cells of CC samples, whereas VEGF receptors were restricted to endothelial cells [22].

In addition to immunohistochemistry performed on TMA, we used an antibody array approach that investigates more than 100 different relevant antibodies spotted on a glass slide, which is then hybridized with 2 paired tissue samples labeled with different fluorophores. Such antibody array technology provides a relevant platform for quantitatively analyzing, in a single experiment, differential expression of a large set of proteins in paired samples [23,24]. Since this approach is designed to screen only two samples at a time, results should be confirmed on a larger scale on TMA with the potential target. Using this combined technology, and by comparing several paired hilar and peripheral CC, we observed that several proteins were differentially expressed in each of the pairs tested. Interestingly, we showed that filamin A was markedly up-regulated in peripheral CCs compared to hilar CCs. Filamin A, a member of the non-muscle actin-binding protein family, is a scaffolding protein that regulates signaling events involved in cell shape change and motility by interacting with integrins, transmembrane receptor complexes and second messengers [25,26]. Very few data have been reported in human malignancies. Filamin A seems to be involved in melanoma carcinogenesis by upregulating Ras/ERK pathways [27]. Recently, silencing of the filamin C gene in human gastric carcinoma and downregulation of the endogenous fragment of filamin A in prostate carcinoma led to speculation that modulation of filamin expression could be involved in the cancer phenotypes [28–30]. To our knowledge, this is the first study that demonstrates filamin A expression in liver tumors. We did not observe significant expression in normal liver, including normal biliary cells, and the mechanism by which filamin A is up-regulated in CCs, and especially in peripheral types, is still unknown. Due to the role of this protein in cell spreading and apoptosis [31], overexpression of filamin A in CCs may reflect aggressive behavior. However, we failed to observe any significant correlation between filamin A expression and histoprognostic factors for CCs in our series. This result may be explained by the high number of peripheral CCs that were positive for filamin A by immunohistochemistry.

Finally, our aim was to provide further insight into clinicopathologic and molecular characteristics of hepatic CCs from a single European institution. Although no significant difference was observed between hilar and peripheral CCs in terms of risk factors and actuarial five-year disease survival, our protein expression results, associated with several morphological characteristics strongly support the hypothesis that hilar and peripheral hepatic CCs can be considered distinct tumors that fol-

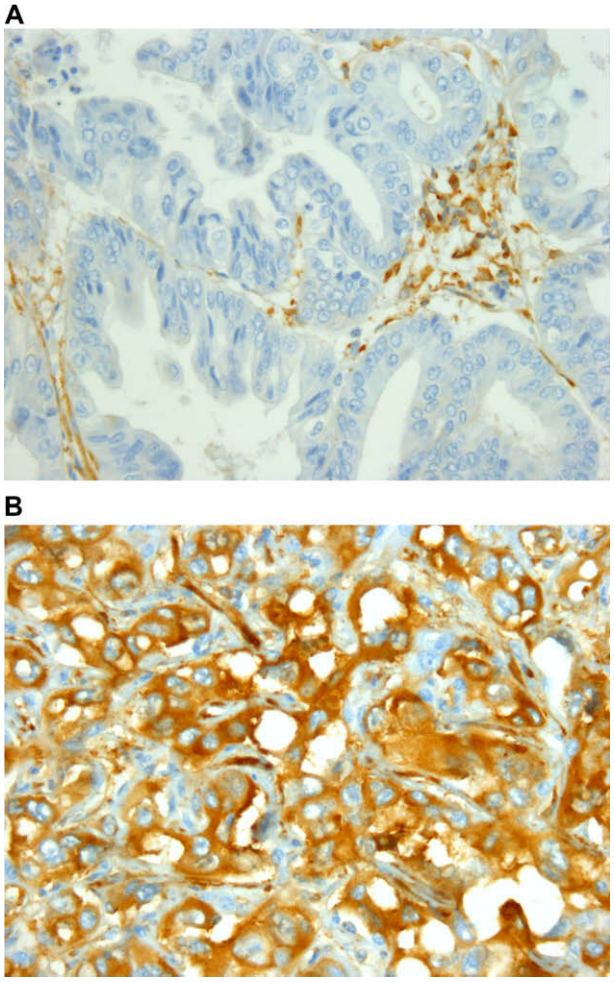

Fig. 5. Filamin A immunostaining: (A) negative staining in hilar cholangiocarcinoma compared to (B) strong cytoplasmic expression in peripheral cholangiocarcinoma. We can note in figure A an internal control with the cytoplasmic staining of stroma cells. (original magnification 400×). [This figure appears in colour on the web.]

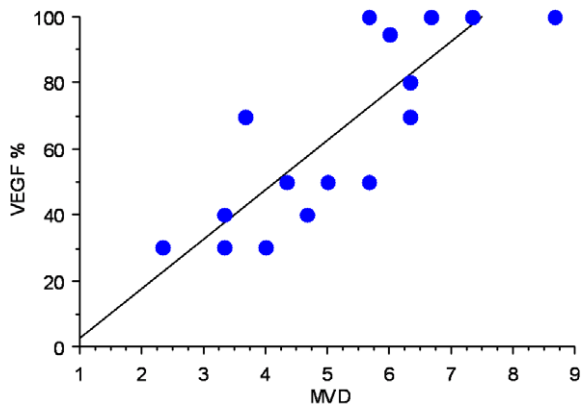

Fig. 6. Correlation between microvascular density and VEGF A expression (percentage of positively immunostained cells) in cholangiocarcinomas. [This figure appears in colour on the web.]

low specific molecular pathways of carcinogenesis one reflecting extrahepatic bile duct carcinoma and one close to subtypes of HCC.

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