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How to cite

ROUGEMONT-PIDOUX, Anne-Laure et al. Extensive biliary intraepithelial neoplasia (BilIN) and multifocal early intrahepatic cholangiocarcinoma in non-biliary cirrhosis. In: Virchows Archiv, 2010, vol. 456, n° 6, p. 711–717. doi: 10.1007/s00428-010-0899-3

This publication URL: https://archive-ouverte.unige.ch/unige:20704
Publication DOI: 10.1007/s00428-010-0899-3

Virchows Arch (2010) 456:711–717
DOI 10.1007/s00428-010-0899-3

CASE REPORT

Extensive biliary intraepithelial neoplasia (BilIN) and multifocal early intrahepatic cholangiocarcinoma in non-biliary cirrhosis

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Received: 23 September 2009 / Revised: 14 January 2010 / Accepted: 20 February 2010 / Published online: 29 April 2010

Abstract Biliary intraepithelial neoplasia (BilIN), a preneoplastic condition that may precede invasive intrahepatic cholangiocarcinoma (ICC), has been compared to pancreatic intraepithelial neoplasia (PanIN), a precursor lesion of pancreatic carcinoma. Biliary tract carcinoma development and progression is associated with several gene alterations, but BilIN lesions have yet to be studied in detail by molecular techniques. We describe a case of extensive intrahepatic biliary dysplasia, with lesions ranging from BilIN-1 to BilIN-3 lesions, and multifocal microscopic ICC in hepatitis C virus (HCV)- and alcohol-related cirrhosis. The small ICC foci had remained undetected prior to transplantation. Fluorescence in situ hybridization (FISH) analysis was performed on three foci of BilIN-3 lesions and on three microinvasive ICC foci with a combination of three FISH probes directed against genes frequently altered in pancreatic and biliary tract carcinomas. FISH analysis revealed a *CDKNA2* heterozygous deletion in one BilIN-3 focus, and in one non-contiguous ICC focus, although the deletion was just above the chosen threshold. No deletions were detected in the genomic regions encoding *TP53* and *SMAD4*. This report documents for the first time the development of multifocal ICC in the setting of extensive biliary dysplasia in a patient with three risk factors, HCV infection, alcohol abuse, and cirrhosis, and suggests heterogeneous carcinogenesis in ICC and possible involvement of the *CDKNA2* gene.

Keywords Biliary dysplasia · Multifocal intrahepatic cholangiocarcinoma · FISH

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Introduction

Intra- and extrahepatic cholangiocarcinoma can arise from two main premalignant conditions, flat or low-papillary biliary dysplasia (biliary intraepithelial neoplasia, BilIN) and intraductal papillary neoplasm (IPN) [1, 2]. IPN, a macroscopically visible papillary proliferation, is considered to be a counterpart of pancreatic intraductal papillary mucinous neoplasm (IPMN) [2], while morphological and immunophenotypic similarities suggest that BilIN could be related to pancreatic intraepithelial neoplasia (PanIN) [3]. BilIN may further progress to cholangiocarcinoma with features of tubular adenocarcinoma [3], mirroring the multistep carcinogenesis progression of PanIN. The three-tiered grading system (BilIN-1 to BilIN-3) depends mainly on the degree of cytological atypia and corresponds respectively to mild, moderate, and severe dysplasia [2, 4]. Invasive adenocarcinoma defines intrahepatic cholangiocarcinoma (ICC).

Risk factors for ICC include chronic biliary disease, such as hepatolithiasis or primary sclerosing cholangitis (PSC), liver fluke infestation, biliary cysts, and bile duct dilatation (such as Caroli's disease or choledochal cyst) or Thorotrast deposition [5, 6]. A "hyperplasia–dysplasia–carcinoma" sequence has been reported in such conditions [6]. BilIN lesions have been initially described mainly in the setting of

PSC [7–9], with biliary dysplasia representing a potential marker for concomitant or later-occurring CC [10]. Several arguments point to a major role for hepatitis C virus (HCV) infection in ICC pathogenesis, including epidemiological correlations [11]; the recent identification of biliary dysplasia in HCV-related cirrhotic livers [12, 13]; the detection of HCV RNA in ICC [14]; and the detection of HCV core proteins by immunohistochemistry in ductular cells in HCV-positive patients [15]. HCV-positive patients have a 2.55-fold increased risk of ICC development, while hepatocellular carcinoma (HCC) risk is increased 15-fold [16]. HCV-positive patients are also at risk for large B cell lymphoma, albeit rarely [17]. ICC in the setting of viral hepatitis or cirrhosis mostly originates from the periphery of the liver [11, 18].

The high sensitivity of a combination of three fluorescence in situ hybridization (FISH) probes in the diagnosis of pancreatic adenocarcinoma has been recently described by our group [19]. The rationale for the choice of the three FISH probes was the frequent occurrence of mutations in the *TP53* (encoding p53), *CDKN2A* (encoding p16(INK4a) and p14(ARF)) and *DPC4*/*SMAD4* (encoding Dpc4) tumor suppressor genes in both pancreatic and biliary tract malignancy [20–23]. It is noteworthy that these gene alterations have also been described in PanIN lesions [24]. Analysis using these probes was intended to clarify the relationship between BilIN with ICC, and PanIN with pancreatic adenocarcinoma.

We describe here a case of mild to severe dysplasia BilIN lesions (BilIN-1 to BilIN-3) with microinvasive ICC foci, in the setting of HCV- and alcohol-related cirrhosis. We report on the results of a FISH analysis of this dysplasia to invasive neoplasia sequence using the *TP53*, *CDK2NA*, and *MALT1* (used as a surrogate for *SMAD4*) probes.

Clinical report

A 42-year-old man underwent liver transplantation for Child C cirrhosis. HCV conversion followed a 5-year drug abuse period, between ages 24 and 29. He had a past history of alcohol abuse. Cirrhosis complications included ascites, esophageal varices, and hepatic encephalopathy while on the waiting list for liver transplantation. Imaging was consistent with cirrhosis, but revealed no focal lesions. Liver tests during pre-transplantation evaluation were nearly within normal ranges (aspartate aminotransferase 97 U/L, alanine transaminase 57 U/L, alkaline phosphatase 85 U/L; γ-glutamyltransferase 20 U/L, total bilirubin 184 μmol/L, conjugated bilirubin 75 μmol/L). Evolution was unfavorable, with hypovolemic shock and septicemia, and the patient died 16 days after transplantation from central pontine myelinolysis, despite normal liver function.

Materials and methods

Representative sections from the liver explant were routinely processed, fixed in 10% buffered formalin and paraffin embedded. For routine histological examination, 3-μm-thick sections were stained with hematoxylin–eosin (H&E), and with the following special stains: Masson trichrome, reticulin stain, PAS with prior diastase digestion, and a Pearl's blue stain. H&E slides allowed selection of three different BilIN-3 foci and of three different ICC foci. One of each of these two lesions was selected on three different representative slides. We chose to initially focus this study on lesions with a higher degree of dysplasia, with further analysis of BilIN-1 and BilIN-2 lesions as a second step, should the initial FISH evaluation reveal major alterations.

The FISH probes were ordered from Vysis, Inc® (Downers Grove, IL). The protocol applied was the same as provided in the study by Genevay et al. [19]. Four micrometer thick tissue sections were placed on Superfrost® Plus (Menzel Glasser®) coated slides, dewaxed, rehydrated, and then immersed successively in two baths of 100% ethanol (5 min at room temperature). The slides were then dried and incubated in Vysis "pre-treatment solution" at 80°C for 15 min, washed in distilled water for 2 min, and dried at 45°C. Tissue sections were then digested with protease at 37°C 60 min for LSI-p16 (CDKN2A); 90 min for LSI-MALT1 (18q21); and 105 min for LSI p53 (17p13) and washed in distilled water for 2 min. Subsequently, they were dehydrated by successive immersions in 70%, 80%, and 100% ethanol (2 min each at room temperature). Probe mixtures were then separately prepared by mixing 1-μl probe with 7 μl hybridization buffer and 2 μl purified water. The LSI p16/CEP9 Dual Color Probe is a mixture of the LSI p16 probe labeled with SpectrumOrange and the CEP9 probe labeled with SpectrumGreen. The chromosome nine centromeric region probe (CEP9) served as an internal control. Accordingly, labeled probes specific to the pericentromeric region of chromosomes 17 (CEP17) and 18 (CEP18) were used as internal controls for the SpectrumOrange TP53 probe (CEP17) and for the SpectrumOrange MALT1 probe (CEP18), respectively. One microliter of CEP17 was placed in 10 μl of LSI-p53 probe mixture, and 1 μl of CEP18 was placed in LSI-MALT1 probe mixture. The *MALT1* gene, located at 18q21, is separated from the *SMAD4* gene by 10 Mb. The use of the commercially available MALT probe seemed reasonable, given this relatively small separation between the two genes, and since a loss of heterozygosity at 18q is described in PanIN lesions [24]. Seven microliters of probe mixture was placed onto the denatured slides and covered with glass coverslips. Hybridization was carried out in a humidified chamber at 72°C for 15 min. The

slides were then washed in post-hybridization wash buffer and incubated in wash buffer for 3 min at 75°C, then washed in distilled water. Subsequently, 7 µl of DAPI counterstain was placed on the slides, which were then mounted with glass coverslips. After hybridization, all slides were stored in the dark at −20°C. As final procedure, the slides were counterstained with DAPI.

Analysis was performed with a Zeiss® Axioplan two fluorescence microscope equipped with 10×, 20×, 60×, and 100× objectives and specific filters (Yellow simple band, Aqua simple band, DAPI simple band, and green/red double band). Image capture was provided by the IP Lab for Macintosh software (Scanalytics® Inc; Rockville, MD). FISH signal counts were repeated on three different BilIN-3 foci, and on three different ICC foci, one of each from three different representative slides. The different foci were located with the DAPI counterstaining and comparison to the original H&E slides. Hybridization signals were counted in at least 200 BilIN-3 dysplastic cell nuclei and 200 adenocarcinoma cells per focus. Genes were considered deleted or amplified when ≤1 or ≥3 nuclear signals were detected, respectively. The cutoff values defined by our group with a receiver operating characteristic (ROC) curves analysis (AUC), and providing a specificity >90% for all three probes, were applied [19]. In this previous study, the ROC curves were calculated using Medcalc® v9.6.4.0. The value for the area under the curve (AUC) had to be at least 0.5 for the test to be considered as efficient. The cutoff value (the minimum percentage of cells exhibiting deletions or amplifications required to consider the test result as positive) was chosen to maximize the test specificity (at least 90%).

Accordingly, from the results of this previous study, the following thresholds were chosen: 7% for the LSI-MALT1 and LSI-p16 probes and 17% for the LSI-p53 probe. With these criteria, a FISH sensitivity of 82% and specificity of 100% had been obtained in the diagnosis of pancreatic adenocarcinoma when ≥2 genetic modifications were observed [19]. Although no FISH data exists to date in PanIN lesions, alterations have been shown in the three selected regions in PanIN lesions [23, 25–27].

Results

Upon gross examination, the liver weighed 1,053 g and showed a micronodular cirrhosis, with no overtly malignant macronodules on cut section. Multiple white nodules measuring up to 0.5 cm in greatest diameter were observed in both lobes, mostly in peripheral locations (Fig. 1). No papillary proliferation was grossly visible in bile ducts. Upon histological evaluation, the cirrhosis was confirmed using Masson trichrome and reticulin stains, and a mainly

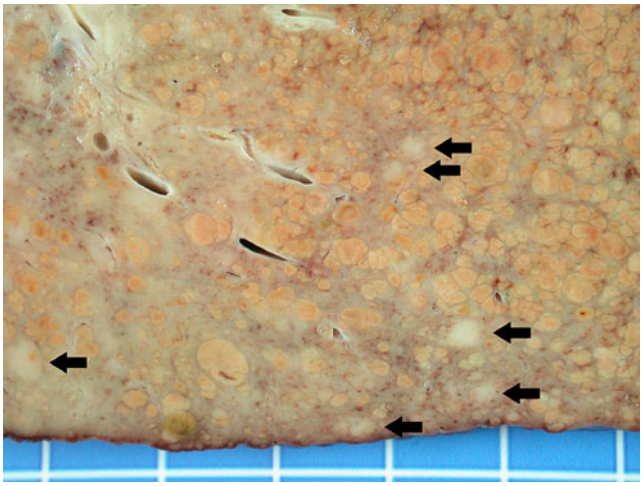

Fig. 1 Gross findings of liver cirrhosis and multiple white nodules, measuring up to 0.5 cm in greatest diameter and mostly located at the periphery (*black arrows*)

lymphocytic portal tract infiltrate with no hepatitis activity was observed (METAVIR score A0-F4) [28] with H&E. There was mild iron deposition in hepatocytes, owing to cirrhosis, and no hyaline globules. A strikingly diffuse flat or pseudo-papillary proliferation of the biliary epithelium with mucosecretion in a minority of cells involved larger intrahepatic biliary tracts and extended to the interlobular ducts (Fig. 2a). These features were observed both in peripheral and central locations, with sparing of the large perihilar bile ducts. Cytological changes ranged from mild, with basally located slightly elongated nuclei (BilIN-1), to severe, with hyperchromatic and large nuclei and loss of cell polarity (BilIN-3) (Fig. 2b–c). Additional small foci consisted of a proliferation of angular and irregular glands, surrounded by a stromal reaction consistent with stromal invasion (Fig. 2d). These ICC foci were distinguished from ductular reaction by: 1) the presence of irregular and angulated glands, with an open lumen; 2) a higher degree of cytonuclear atypia; and 3) the identification of a desmoplastic reaction. Fibrosis in such areas was prominent, and correlated with microscopic zones in some of the grossly identified fibrotic white nodules. There was no evidence of alcohol-related lesions.

FISH analysis in the 200 or more cell nuclei showed *CDKN2A* heterozygous deletion above the 7% threshold in one of the three chosen BilIN-3 foci (10% of cell nuclei) and in one of the three ICC foci (8.5% of cells) (Fig. 3). Loss of one *TP53* signal was observed in 13.8 and 15% of the nuclei analyzed in 2 ICC foci, and in 15.9% of one BilIN-3 focus, but failed to attain the previously defined cutoff value of 17%. *MALT1* showed a normal FISH signal in all the BilIN-3 and ICC foci. In view of these results, shown in Table 1, no further analysis of BilIN-1 and BilIN-2 lesions was performed.

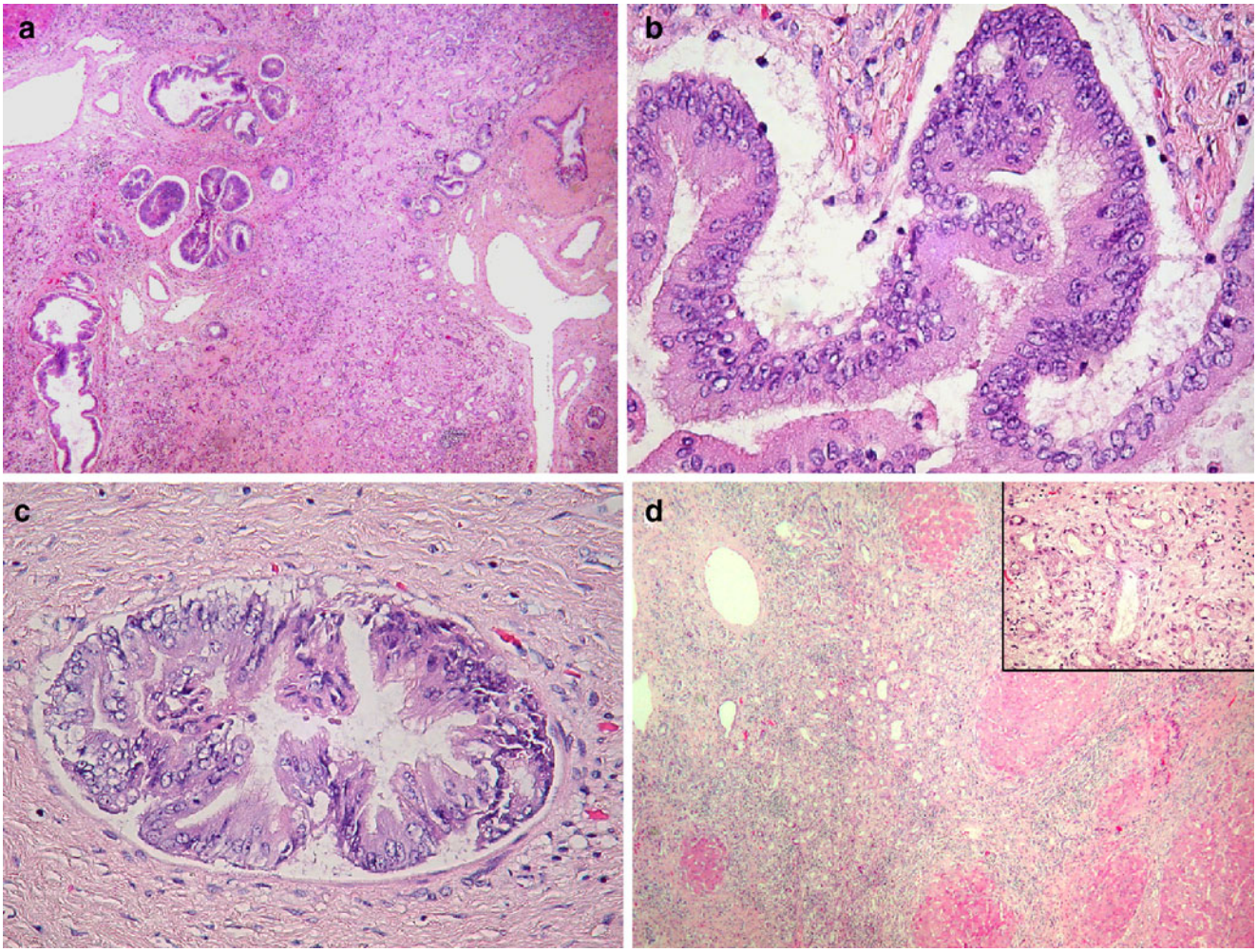

Fig. 2 Histological progression from BilIN lesions to ICC, according to the criteria by Zen et al. [2] hematoxylin & eosin (H&E). **a** Multifocal flat or pseudo-papillary proliferation of the biliary epithelium, extending from the segmental bile ducts to the interlobular ducts (original magnification 100×). **b** BilIN-2 (400×). **c** BilIN-3 (200×). **d** Microinvasive ICC focus, identified at low power (100×) and confirmed at higher power (*inset*—400×). *Inset* illustrates the distorted, angulated and branching glands, with an open lumen, and the stromal invasion

Discussion

We report here the first case of extensive BilIN lesions with multiple microscopic ICC foci, in the setting of HCV- and alcohol-related cirrhosis. BilIN lesions extended from septal-sized to interlobular bile ducts. Multiple ICC foci were located in the two lobes. A *CDKN2A* heterozygous deletion was observed in one BilIN-3 focus and in one non-contiguous ICC focus. No *TP53* or *MALT1* deletion was observed.

BilIN is a rare finding in cirrhotic livers. In a large retrospective study of 1,058 liver explants by Torbenson et al., dysplasia was observed in 1.8% of cases, and only in cirrhotic livers [12]. Addition of alcohol consumption to HCV infection increased the incidence of bile duct dysplasia from 2% (HCV infection alone) to 5% (HCV and alcohol) [12]. Multifocal dysplastic changes involved only septal-sized bile ducts [12]. In a series of 468 cases of liver cirrhosis, in patients lacking a history of cholangitis or hepatolithiasis, Aishima et al. identified two cases of high-grade biliary dysplasia with a micropapillary growth pattern and extensive spread [13]. The lesions involved the intra-hepatic septal ducts, the interlobular ducts, and the ductules of the ductular reaction, and had developed in one case in a HCV-related cirrhotic liver, with two HCC, and in the second case, in a liver with alcohol-related cirrhosis [13]. No ICC foci were described. The case presented here thus represents the third description of multifocal BilIN lesion in non-biliary cirrhotic liver, involving not only septal-sized bile ducts, but also interlobular bile ducts, and the first with co-existent ICC foci.

ICC microinvasive foci are difficult to diagnose on morphological aspects solely, and we wished to determine whether FISH techniques might be of diagnostic value. The

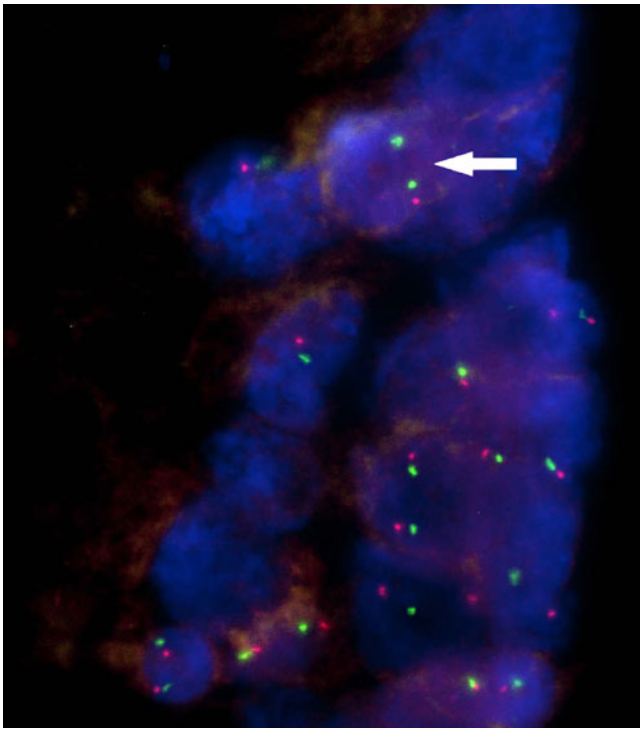

Fig. 3 FISH results, with the LSI p16 (*CDKN2A*) probe showing heterozygous deletion in a subset of the ICC cells in a BilIN-3 focus, with loss of one of the SpectrumOrange signals in a nucleus and preservation of the 2 centromeric SpectrumGreen signals (*white arrow*)

proposed relationship between these lesions and PanIN/pancreatic carcinoma justified the use of probes directed against genes involved both in pancreatic and biliary tract oncogenetic pathways [20–23]. Several studies have confirmed the importance of allelic losses at 9p21 (*CDKN2A*), 17p13 (*TP53*), and 18q21 (*SMAD4*) in pancreatic carcinoma, with loss of one or more of these loci in a vast majority of cases [29–33]. Early loss of *CDKN2A* has been shown in the PanIN to pancreatic adenocarcinoma sequence, with *TP53* and *SMAD4* being deleted later [23–27]. In invasive biliary tract tumors, or in their metastases, losses of regions harboring each of the three genes studied have been demonstrated in 64% (9p), 45% (17p), and 55% (18q) of cases [22]. This, along with the absence of deletions in the majority of lesions studied in this case, suggests molecular differences in pancreatic and biliary tract oncogenesis with chromosomal deletion, possibly occurring later in the pathway.

CDKN2A gene alterations are described in many cancer types [34]. Homozygous 9p21 deletion, encompassing the *CDKN2A* gene, was observed in 11 of 22 ICC by FISH analysis, of which 16 were sporadic and the remaining six were PSC related [35]. Homozygous 9p21 deletion has also been described in three of five PSC-related biliary dysplasia specimens [35]. We observed a *CDKN2A* heterozygous deletion in a subset of the BilIN-3 and ICC foci. These findings evoke the possibility of involvement of different molecular pathways from one ICC focus to another, as has been described in cases of synchronous multicentric HCC [36].

A progressive decrease in immunohistochemical reactivity of Dpc4 expression, the *SMAD4* gene protein product, has been observed from non-neoplastic biliary epithelium to invasive CC [37]. Despite this previous description, we observed no alteration in the *MALT1* gene, used as a surrogate for *SMAD4*, suggesting that at least part of the reduction of protein expression might result from point mutations or epigenetic inactivation.

Inactivating *TP53* mutations represent the most frequent gene alteration in human cancers [38]. *TP53* mutations have been detected particularly in the mass-forming ICC type in the setting of viral hepatitis, suggesting a relationship to ICC arising from small peripheral bile ducts [5, 39]. However, the alterations observed were missense or, rarely, nonsense mutations [39]. We observed no *TP53* alteration in the case reported here.

We described here a third case of extensive BilIN lesions in the setting of HCV- and alcohol-related cirrhosis. Multiple microscopic ICC foci were too small to allow radiological detection. BilIN has recently been described in non-biliary liver cirrhosis, and as described in PSC patients, dysplasia in liver biopsy specimens might be a marker of synchronous or metachronous ICC. However, BilIN may affect only the rarely biopsied segmental bile ducts. FISH techniques revealed a *CDKN2A* heterozygous deletion in

Table 1 FISH analysis results, in the cell nuclei of three BilIN-3 and three ICC foci, from three representative slides (foci one to three)

Significance threshold		LSI-p16 (*CDKN2A*)		LSI-MALT1 (*SMAD4* surrogate)		LSI-p53	
		7%		7%		17%	
BilIN-3	Focus 1	21/201	10%	0/202		16/155	10%
	Focus 2	3/272	1.1%	4/201	1.9%	42/264	15.9%
	Focus 3	2/213	0.9%	0/240		9/212	4%
ICC	Focus 1	8/227	3.5%	0/300		26/188	13.8%
	Focus 2	12/266	4.5%	2/201	0.9%	6/111	5.4%
	Focus 3	13/152	8.5%	0/200		38/252	15%

one BilIN-3 and in one ICC focus. We hypothesize that these *CDKN2A* alterations could represent a late event and would have been more pronounced had the lesions progressed to a more overt mass-forming ICC, in contrast to observations in PanIN, where *CDKN2A* alterations appear to be an early event. Heterogeneous findings were observed in the different BilIN-3 and ICC foci studied, evoking the possibility of involvement of different molecular pathways from one ICC focus to another, as has been described in cases of synchronous multicentric HCC. BilIN and PanIN share close phenotypic and immunohistochemical aspects, and seem to share some oncogenic mechanisms. However, the observation of heterogeneous *CDKN2A* alterations and the absence of *TP53* and *MALT1* (surrogate for *SMAD4*) deletions in the case presented here point towards different oncogenetic pathways. The study of large numbers of lesions is warranted.

Conflict of interest statement We declare that we have no conflict of interest.

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