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REVIEW

Preneoplastic lesions in the liver: Molecular insights and relevance for clinical practice

Elvire Desjonqueres[1,2,3,4] | Claudia Campani[2,3,4,5] | Fabio Marra[5] | Jessica Zucman-Rossi[3,4,6] | Jean-Charles Nault[1,2,3,4]

[1]Service d'hépatologie, Hôpital Avicenne, Hôpitaux Universitaires Paris-Seine-Saint-Denis, Assistance-Publique Hôpitaux de Paris, Bobigny, France

[2]Unité de Formation et de Recherche Santé Médecine et Biologie Humaine, Université Paris 13, Communauté d'Universités et Etablissements Sorbonne Paris Cité, Paris, France

[3]Centre de Recherche des Cordeliers, Sorbonne Université, Inserm, Université de Paris, team « Functional Genomics of Solid Tumors », Paris, France

[4]Equipe labellisée Ligue Nationale Contre le Cancer, Labex OncoImmunology, Paris, France

[5]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

[6]Hôpital Européen Georges Pompidou, APHP, Paris, France

Correspondence
Jean-Charles Nault MD, PhD, Service d'hépatologie, Hôpital Avicenne, 125 rue de Stalingrad, 93000 Bobigny, France.
Email: naultjc@gmail.com

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Abstract

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the most frequent primary liver cancers, accounting for approximately 80% and 15%, respectively. HCC carcinogenesis occurs mostly in cirrhosis and is a complex multi-step process, from pre-cancerous lesions (low-grade and high-grade dysplastic nodules) to progressed HCC. During the different stages of liver carcinogenesis, there is an accumulation of pathological, genetic and epigenetic changes leading to initiation, malignant transformation and finally tumour progression. In contrast, a small subset of HCC occurs in normal liver from the transformation of hepatocellular adenoma (HCA), a benign hepatocellular tumour. The recent molecular classification enables to stratify HCAs according to their risk of complication, in particular malignant transformation, associated with mutations in exon 3 of the catenin beta 1 *(CTNNB1)* gene. Cholangiocarcinoma (CCA) derives from the multistep malignant transformation of preneoplastic lesions, like biliary intraepithelial neoplasia (BilIN) and intraductal papillary neoplasm of the bile duct (IPNB), for which a pre-operative diagnosis remains difficult. Different genetic alterations are involved in BilIN and IPNB progression, leading to the development of tubular or intestinal adenocarcinoma. The aims of this review are to describe the main clinical and molecular features of preneoplastic lesions leading to the development of HCC and CCA, their implications in clinical practice and the perspectives for future research.

1 | INTRODUCTION

Primary liver cancer is the seventh most frequently occurring cancer in the world and the second most common cause of cancer mortality. Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the dominant type of liver cancer accounting for approximately 80% and 15%, respectively. Both HCC and CCA are often diagnosed when the disease is already in advanced stages, resulting in a dismal prognosis. Preneoplastic lesions of hepatocellular carcinoma are low grade-dysplastic nodules and high-grade dysplastic nodules developed in cirrhosis, and hepatocellular adenomas that occur mainly in the normal liver. These premalignant lesions are sometimes identified in clinical practice and questions related to their management are still unanswered. For cholangiocarcinoma, preneoplastic lesions are biliary intraepithelial neoplasia and intraductal papillary neoplasms of the bile duct that remains rarely identified in clinical practice. Despite the screening guidelines established for the early detection of HCC and CCA, the poor prognosis of these cancers is mainly associated with their late diagnosis, limiting access to curative treatment. A better understanding of the mechanisms of

carcinogenesis is necessary to improve the detection of preneoplastic lesions and to optimize screening for early initiation of treatment in these patients.

In this review, we aim to decipher the main molecular mechanisms leading to the occurrence of these premalignant lesions, dissect the mechanisms of malignant transformation and discuss the appropriate management of preneoplastic liver lesions in clinical practice.

Key points

- Low grade and high-grade dysplastic nodules are premalignant lesions in cirrhosis and mutation in the telomerase reverse transcriptase (*TERT*) promoter is the gatekeeper events leading to transformation on HCC.
- HCA with catenin beta 1 (*CTNNB1*) exon 3 mutations are at risk of malignant transformation in HCC and mutations in the *TERT* promoter is the second hit leading to HCC occurrence.
- Biliary intraepithelial neoplasia and intraductal papillary neoplasm of the bile duct are the main premalignant lesions leading to cholangiocarcinoma.

2 | PRENEOPLASTIC LESIONS OF HEPATOCELLULAR CARCINOMA ON CIRRHOSIS

2.1 | Histological definition

2.1.1 | Dysplastic nodules

Dysplastic nodules (DN) are macroscopic lesions usually measuring between 1 and 20 mm and are classified as low grade (LGDN) or high grade (HGDN), depending on their degree of cellular and architectural atypia.[1,2] These lesions mainly develop in the context of cirrhosis and there is a histological continuum between LGDN and HGDN, HGND and HCC,[2] respectively. LGDNs consist of hepatocytes of normal appearance or with minimal cellular atypia, rarely with increased cytonuclear ratio. Their vascularization is exclusively portal[3] and their risk of malignant transformation is considered low.[4] Furthermore, the histological distinction between LGDN and cirrhotic regenerative macronodules (RN) is difficult, and its clinical utility is questionable. For these reasons, this distinction was abolished by the International Consensus Group for Hepatocellular Neoplasia (ICGHN) in 2009.[1] HGDNs are characterized by the presence of moderate cellular and/or architectural atypia.[1] These lesions frequently exhibit an increased cytonuclear ratio, high cell density and a vascularization profile resembling HCC, including rarefaction of the portal network and presence of aberrant unpaired arteries.[3,5,6] Unlike LGDN, the risk of progression to HCC in HGDN is high, estimated around 30%-40% after 2 years of follow-up, although more data are required to better assess their risk of malignant transformation.[3,4]

2.1.2 | Early-HCC

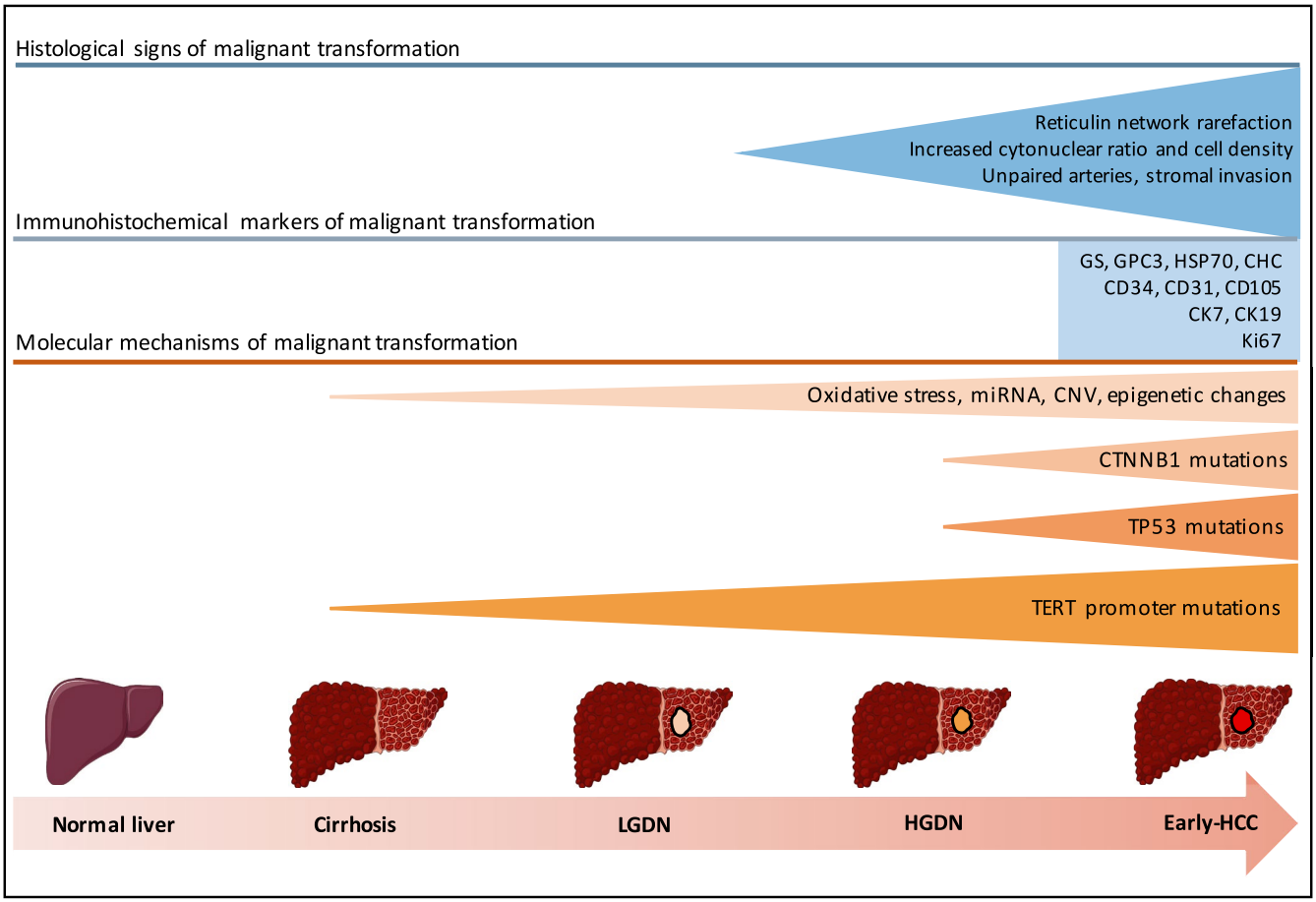
Early-HCC corresponds to the earliest stage of carcinoma observed in the liver (Figure 1). Macroscopically, these lesions appear vaguely nodular, without tumour capsule and measuring less than 2 cm.[1,7] Microscopically, they are usually well-differentiated tumours with increased cell density and small dysplastic cells with an increased cyto-nuclear ratio.[3,8] Thus, the microscopic distinction with LGDN or HGDN may be difficult, even for an expert pathologist. Unlike dysplastic nodules, rarefaction of the reticulin network and stromal invasion (tumour invasion in fibrous septa or portal tracts) help to identify early HCC.[1,9] Although there is no vascular invasion in early-HCC, signs of arterial neo-angiogenesis are frequently present (Figure 1), characterized by the development of isolated (unpaired) arteries.[10]

3 | MECHANISMS OF MALIGNANT TRANSFORMATION INTO HCC

The molecular process of HCC carcinogenesis is the consequence of an accumulation of genetic and epigenetic alterations[11] (Figure 1). The main somatic mutations identified target telomerase reactivation pathways, allowing maintenance of telomeres by increasing telomerase activity and leading to escape from telomere shortening and replicative senescence.[12] These alterations occur early during tumourigenesis. Hot spot mutations of the telomerase reverse transcriptase (*TERT*) promoter are found in dysplastic nodules developed in cirrhosis. Their prevalence increases with the degree of dysplasia, from 6% in LGDN to 19% in HGDN, reaching 60% in early-HCC.[13] Other mechanisms to activate *TERT* are identified in HCC, that is insertion of HBV viral DNA into the promoter, chromosome amplifications and translocations but they were not described in preneoplastic lesions. Overall, during the malignant transformation of cirrhotic hepatocytes, *TERT* activation is a key event to achieve and *TERT* promoter mutations are the most frequent and earliest recurrent genomic alteration.[13,14]

Activation of the Wnt/βcatenin signalling pathway plays a critical role in liver carcinogenesis and tumour progression.[15,16] This activation is mainly secondary to activating mutations of catenin beta 1 (*CTNNB1)* (37% of CHCs) but also to inactivating mutations of axin 1 (*AXIN1)* (15%) or adenomatous polyposis coli *APC* (2%).[17] However, the low prevalence of these mutations in dysplastic nodules suggests a contribution preferentially in tumour progression rather than initiation of carcinogenesis on cirrhosis.[18]

Inflammation observed in chronic liver diseases is associated with increased oxidative stress in the liver parenchyma. Activation of the nuclear factor erythroid 2-related factor 2 *(NFE2L2)* and



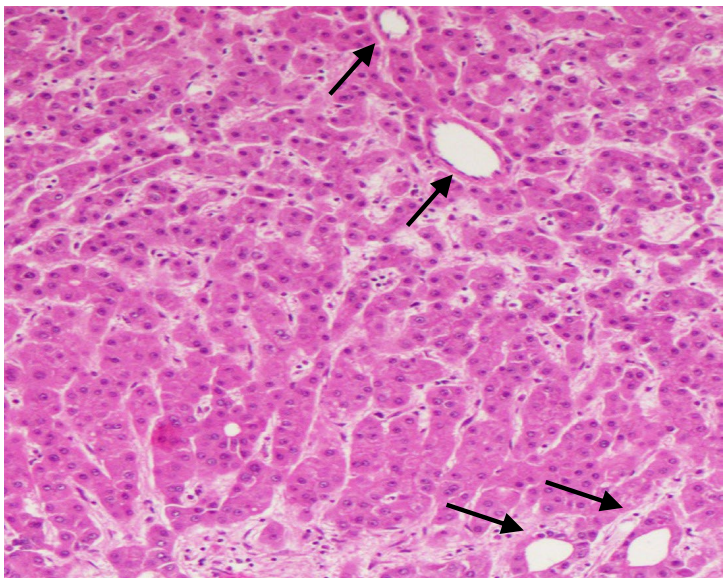


FIGURE 1 Multistep process of liver carcinogenesis on cirrhosis. On the upper part, we represented the main pathological, immunohistochemical and molecular alterations from preneoplastic lesions to HCC. On the lower part, we provided a picture of an early HCC with unpaired arteries (black arrow) (haematoxylin and eosin stain). LGDN, low-grade dysplastic nodule; HGDN, high-grade dysplastic nodule; GS, glutamine synthase; GPC3, Glypican 3; HSP70, Heat shock protein 70; CHC, Clathrin heavy chain; CK7/19, Keratin 7 and 19; CNV, copy number variation; TERT, telomerase reverse transcriptase

kelch-like ECH-associated protein 1 *(KEAP1)* signalling pathways play a protective role against oxidative stress at the cellular level. Inactivating mutations of *NFE2L2* and *KEAP1* are found in 5%-15% of HCCs, conferring an advantage of resistance to oxidative stress in cancer cells. These mutations mainly appear at an early stage of malignant transformation, in preneoplastic lesions or early-HCC, in animal models.[17,19,20] In humans, transcriptomic activation of *NFE2L2/KEAP1* pathway is also observed early in liver carcinogenesis.

Inactivating mutations of the tumour suppressor gene tumour protein 53 (*TP53*), which regulates the cell cycle, have been reported in 20%-50% of HCCs. These mutations are rarely found in dysplastic nodules[18] and this pathway seems to be particularly involved in tumour progression more than in its initiation.[21] More rarely, inactivating mutations of the retinoblastoma pathway (8% of retinoblastoma 1 – *RB1* – mutations and 12% of cyclin-dependent kinase inhibitor 2A – *CDKN2A* – deletions) are identified in HCC. This pathway is involved in transition from the G1 to S phase of the cell cycle, and its alteration in HCC seems to be associated with an unfavourable prognosis and is enriched in advanced HCC with portal invasion and metastasis.[22,23] Somatic mutations have also been linked to tumour immune escape in preclinical models. For example, activation of the β-catenin pathway promotes immune evasion in HCC, with lower tumour enrichment of T cells and downregulation of CCL-4 and CCL5 chemokines. In addition, c-myelocytomatosis (c-MYC) overexpression could play a role in immune evasion by upregulation of programmed cell death ligand 1 (PDL1) in neoplastic hepatocytes.[24,25] Furthermore, changes in the immune infiltration of the underlying liver have been described in the course of hepatic disease. In particular, an increase in cluster differentiation (CD) 8 + T cells was observed on cirrhosis in parallel to a decrease in CD4 + T cells, as well as an expansion of profibrogenic macrophages.[26] These deregulations could participate in the occurrence of HCC on cirrhosis.

Chromosomal alterations, such as copy number variation (CNV), have been described in hepatic premalignant lesions and mostly concern gains or deletions of the arms of chromosome 8 (8p or 8q) and gains of 1q. Their number increase in frequency during carcinogenesis.[18,27] In addition, focal amplification of oncogenes can also be observed at an early stage of carcinogenesis, and one study suggested that the *c-MYC* oncogene could be a central mediator of hepatic carcinogenesis and malignant transformation of preneoplastic lesions.[18,28]

Epigenetic dysregulations play an important role in hepatic carcinogenesis by modifying gene expression through various mechanisms, including chromatin remodelling, histone modifications and methylation. These alterations have been described in dysplastic lesions, early-HCC and progressed-HCC, and contribute to the sequential process of malignant transformation.[29] For example, aberrant changes in the methylation of several genes involved in hepatic carcinogenesis (*CDKN2A*, *APC*, suppressor of cytokine signalling protein 1- *SOCS1*, serine protease inhibitor Kazal type 1 gene-*SPINK1*, etc) are observed in the early stage of malignant transformation and are associated with an increased cell proliferation.[18,30,31] A recent genome-wide DNA methylation study[32] found a gradual increase in DNA methylation changes among cirrhosis, dysplasia and HCC and identified four gatekeeper genes (testis-specific Y-encoded-like protein 5-*TSPYL5;* Kcna3 potassium voltage-gated channel, shaker-related subfamily, member 3-*KCNA;* lactate dehydrogenase B-*LDHB* and serine peptidase inhibitor Kunitz Type 2-*SPINT2*) exhibiting a progressive increase in promoter methylation, leading to decreased expression of these genes during transition to eHCC. These data suggested a role of epigenetic alterations in the early stage of HCC development. In addition, the striatin 4 (*STRN4)* gene was identified as a potential epigenetically regulated oncogene. Its progressive hypomethylation would lead to an increase in its gene expression in the advanced stages of hepatocarcinogenesis and was associated with unfavourable prognostic implications for patients with HCC.[33] Furthermore, epigenetic methylation signature as well as microRNA dysregulations were associated with tumour progression, degree of differentiation as well as overall survival.[23,34-36]

3.1 | Implications in clinical practice

3.1.1 | Histological diagnostic challenge

The histopathological diagnosis of HCC is based on the criteria of the World Health Organization (WHO) and the International Consensus Group for Hepatocellular Neoplasia (ICGHN).[1,37] Obtaining histological evidence for HCC is essential in non-cirrhotic livers,[38] but it is also useful for atypical hepatic lesions at imaging in cirrhosis. When classical non-invasive criteria are not met at imaging, the histological proof is necessary to discriminate benign nodules and preneoplastic lesions from well-differentiated HCCs. The sensitivity of liver biopsy, around 90%,[39] depends on the size of the nodule (with a decreased sensitivity below <2 cm),[40] the location, the degree of differentiation of the tumour (challenging in very well differentiated and early-HCC), the operator of the biopsy and the pathologist.[38,41] Assessment of cellular criteria of malignancy (atypia, mitosis and cytonuclear ratio) and architectural abnormalities (isolated arteries, reticulin network, stromal and vascular invasion) is obviously more difficult in biopsy samples than in surgical specimen.[41,42] Thus, the use of immunohistochemical markers of malignant transformation is helpful for the histological diagnosis of benign nodules, precancerous lesions and early-HCC.[8,38,43]

An immunohistochemical panel (Figure 1) comprising glypican 3 (GPC3), heat shock protein 70 (HSP70) and glutamine synthetase (GS) has been validated to discriminate DNs from early-HCC in patients with cirrhosis. The overall sensitivity and specificity for the diagnosis of early-HCC of at least two markers being positive were 70% and 100%, respectively.[43,44] The addition of the fourth marker to this panel, that is clathrin heavy chain (CHC), was associated with a gain in sensitivity and specificity for the diagnosis of early-HCC from DNs in liver biopsy.[45,46]

The angiogenic markers CD34, CD31 and CD105 improve the assessment of tumour neovascularization, a surrogate marker of malignant transformation in HCC.[6,47] However, the gradual increase in their expression between HGDNs, early-HCCs and

well-differentiated HCCs, limits its power of discrimination.[8] Loss of reticulin network is a useful pathological finding to assess malignant transformation in HCC.[9] Additional information may be provided by immunostaining of keratin 7 and 19 (CK7, CK19), markers of ductular reaction, which is frequently positive in non-cancerous nodules and rarely found in HCC with stromal invasion.[8,48]

3.2 | Non-invasive tools for the identification of preneoplastic lesions

The distinction between dysplastic nodules and HCC also is a diagnostic challenge for radiologists, in particular regarding HGDN and early-HCC. Advances in the understanding of the vascular tumour pattern[49] have enabled the development and validation of numerous imaging techniques for the non-invasive detection of HCC. The main morphological changes observed during liver carcinogenesis are the development of new abnormal vessels (neovascularization) and the progressive decrease in hepatocellular function, reflected by the decline in the expression of organic anion transporting polypeptides (OATPs).[50] The assessment of these two criteria by imaging is helpful to discriminate HCC from dysplastic or regenerative nodules in cirrhosis.[51]

Liver ultrasonography (US) is frequently used for the early detection of HCC, due to its accessibility and favourable cost effectiveness-risk ratio, but is significantly exposed to inter-operator variability. Contrast-enhanced US (CEUS), dynamic CT and MRI allow the evaluation of the vascularization profile of hepatic lesions in order to provide a non-invasive diagnosis of HCC using the wash-in/wash-out criteria in cirrhosis. In CEUS, dysplastic nodules typically appear isovascular or hypovascular in the arterial phase, and isoechoic in the subsequent phases.[52] A nodule-in-nodule profile is more rarely observed in CEUS but is strongly suggestive of a focus of HCC within a DN.[53] The distinction between early-HCC and DN remains complex, with early-HCC (well-differentiated HCC) frequently appearing hypo or isovascular in the arterial phase.[54]

The diagnostic sensitivities of contrast-enhanced CT and MRI (iodinated contrast media and extracellular contrast agents, respectively) are greater than US, especially for the detection and characterisation of HCC < 20 mm, with a sensitivity of 48%-68% and 62%-71%, respectively for CT and MRI, depending on the series.[55-57] The use of specific hepatobiliary contrast agents in MRI, such as gadoxetic acid (Gd-EOB-DTPA) or gadobenate dimeglumine (Gd-BOPTA), preferentially absorbed by non-tumour hepatocytes and then excreted in the bile,[58] was proposed to increase the diagnostic performance for focal hepatic lesions, in particular, for distinguishing LGDN, HGDN and early-HCC in the cirrhotic liver[5] with a sensitivity of 86%.[59] However, the use of MRI-specific hepatobiliary contrast agent to assess preneoplastic lesions in clinical practice is still debated.

Fibrolamellar hepatocellular carcinoma (FLC) is a rare variant of HCC occurring in young patients without chronic liver disease.[60] It is characterized by specific histological features (well-differentiated hepatocytes surrounded by thick fibrous bands, abundant granular and eosinophilic cytoplasm due to abundant mitochondria), but shares features common to focal nodular hyperplasia (FNH) on contrast-enhanced MRI (extracellular contrast agents), including a central scar and hypervascularity at the arterial phase,[61] making radiologic discrimination between FNH and FLC sometimes challenging. Portal venous hypoenhancement and tumour heterogeneity appear to be useful criteria in distinguishing FLC from FNH using contrast-enhanced MRI, and the presence of hypointensity in the hepatobiliary phase of Gd-EOB-DTPA MRI could also be a discriminating factor.[62]

Overall, dynamic imaging techniques (CEUS, MRI, CT) play a central role in the diagnosis of HCC, with a superior diagnostic accuracy of MRI for lesions <2 cm.[56] However, the distinction between LGDN/HGDN and early HCC is sometimes not possible at dynamic imaging techniques in the presence of an atypical nodule.

3.2.1 | Monitoring and management of preneoplastic lesions

A tumour biopsy is indicated by international recommendations for ≥1 cm lesions which remain undetermined or atypical after two dynamic imaging methods.[38,63-65] A conservative approach has been proposed in the EASL and AASLD guidelines[38,55] for infra-centimetric nodules developed in cirrhosis, with imaging monitoring at 3-4 months. However, a change in size (more than 1 cm) during follow-up requires histological examination of the nodule if this remains atypical at imaging.[66] Thus, monitoring should be carried out by dynamic imaging rather than by simple US.

The treatment of preneoplastic lesions is still debated. EASL and AASLD do not currently recommend treatment for DN.[38,63] In Japan, close monitoring by MRI with hepatobiliary contrast agent of hypovascular nodules is recommended, in order to treat nodule-in-nodule lesions when they appear.[65] A retrospective study conducted by Kim et al in 2008[67] evaluating radiofrequency ablation for HGDN and small-HCC did not demonstrate an advantage of the procedure in overall survival or recurrence-free survival for preneoplastic lesions. Forty-eight per cent of patients treated for HGDN developed HCC distant from the ablation, during their follow-up. More data are warranted to accurately assess the risk of malignant transformation of premalignant lesions, the risk factors of malignant transformation and the need to treat these lesions in clinical practice.

4 | PRENEOPLASTIC LESIONS OF HEPATOCELLULAR CARCINOMA IN THE NORMAL LIVER

4.1 | Epidemiologic and histological definition of HCA

Hepatocellular adenoma (HCA) is a rare benign hepatocellular tumour usually developed in normal liver, with a broad predominance of the female gender. The main risk factors associated with HCA development are oral oestrogen-based contraception, obesity and anabolic androgen

use.[68-70] At the macroscopic level, it usually presents as a nodular lesion without a fibrous capsule. Microscopically, HCA is commonly defined as a monoclonal proliferation of well-differentiated hepatocytes and is characterized by the absence of portal triad and bile ducts.[71]

4.2 | Classification of HCAs

4.2.1 | Molecular classification

HCAs are heterogeneous lesions, with five main HCA subtypes (Figure 2) defined by the correlation between molecular alterations and histological, immunohistochemical and clinical features (Figure 2).[72,73]

Steatotic adenomas (hepatocyte nuclear factor 1 homeobox A *-HNF1-A* inactivated HCA, HHCA) represent about a third of HCAs and are characterized by biallelic inactivation (most of the times somatic alterations) of the *HNF1-α* gene. Sometimes, they can be found in the context of familial liver adenomatosis and maturity-onset diabetes of the young (MODY3) diabetes due to a germline inactivating mutation.[74]

Inflammatory adenomas (IHCA) are characterized by constitutive activation of the IL6/JAK/STAT pathway, mainly due to activating somatic alterations of interleukin 6 signal transducer (*IL6ST*), signal transducer and activator of transcription 3 *(STAT3)*, c-ros oncogene 1 *(ROS1)*, janus kinase 1 *(JAK1)*, guanine nucleotide binding protein alpha Stimulating activity polypeptide *(GNAS) or* fyn-related kinase (*FRK*).[75-77] They represent around 40% of HCAs and are often associated with alcohol intake, high exposure to oral contraceptive and obesity.[72] They may be responsible for systemic inflammation with fever, anaemia or secondary AA amyloidosis.[78]

Two other HCA subtypes are characterized by activating β-catenin somatic mutations. The mutation in exon 3 of the *CTNNB1* gene (b^{ex3}HCA) is observed in 10%-15% of HCAs, with an enrichment in male, and is associated with an increased risk of malignant transformation into HCC.[72,79] Mutations in exons 7 or 8 of the *CTNNB1* gene ($bex^{7,8}$HCA), associated with moderate activation of β-catenin, are present in about 10% of HCA and do not confer an increased risk of malignant transformation.[72]

The latest subtype described, Sonic Hedgehog HCA (shHCA), is associated with constitutive activation of the Sonic Hedgehog pathway (5% of HCAs) due to the overexpression of glioma-associated oncogene homolog 1 *(GLI1)* through an Inhibin betaE (*IHNBE)-GLI1* fusion. shHCAs present a significant risk of bleeding and are associated with oestrogen exposure and obesity.[72]

Finally, a minority of HCAs remain unclassified (UHCA, less than 10%).

4.2.2 | Mechanisms of malignant transformation of HCA

The high frequency of β-catenin pathway activation in HCCs developed on HCA and in borderline HCAs highlights the role of this pathway in the malignant transformation of HCAs. In addition, in borderline tumours, the presence of the same alteration of the *CTNNB1* gene in the adenoma and in parts of the tumour, suggests the possibility of the same clonal origin.[80] Pilati et al suggested a step-by-step carcinogenesis model in HCA, with $CTNNB1^{ex3}$ mutations playing a role at an early stage of the tumourigenesis, without being sufficient, alone, to induce HCC.[76] Accumulation of additional mutations is required to promote malignant transformation, mainly *TERT* promoter mutations, inducing telomerase reactivation in the tumour.[76,81] More data is needed to better understand the malignant transformation process of HCAs in other molecular subtypes.

4.2.3 | Pathologic correlation in clinical practice

The correlation between the genomic classification and the immunohistochemical profiles of HCA makes it possible to guide the diagnosis towards the different subtypes (Figure 2).

HHCAs are characterized by diffuse steatosis and the loss of expression of fatty acid binding protein 1 (FABP1) in the tumour at immunohistochemistry.[73] Inflammatory infiltrate and dystrophic vessels are useful findings for the diagnosis of IHCA and the diagnosis can be confirmed by C-reactive protein (CRP) or serum amyloid A (SAA) overexpression in the tumour at immunohistochemistry.[73] At histology, b^{ex3}HCAs are frequently cholestatic lesions and can be identified by the overexpression of glutamine synthase or the nuclear translocation of β-catenin at immunohistochemistry.[80] Finally, the overexpression of argininosuccinate synthetase 1 (ASS1) and prostaglandin D2 synthase (PTGDS) has been proposed as immunohistochemical markers to identify the shHCA subtype, even if their sensitivity and specificity remains to be better determined.[82]

Overall, the pathological examination with this HCA immunohistochemical panel is a useful tool to support the diagnosis of adenoma subtype in absence of genomic analysis, and to guide the clinical practice.

MRI can also play a role in distinguishing hepatocellular adenoma subtypes. Indeed, HHCA is characterized by a moderate enhancement on the arterial phase that does not persist on the portal and delayed one, and by a diffuse signal loss on out-of-phase T1-weighted sequences (sensitivity 87%, specificity 100%) due to the high content of intracellular fat.[83] IHCA is characterized by a strong and persistent enhancement in the arterial, portal and delayed phase. This feature, combined with the diffuse or peripheral (atoll sign) hyperintensity in T2-weighted images, allows the diagnosis of IHCA subtype with a sensitivity of 85%-88% and a specificity of 88%-100%.[84] Conversely to the two previous HCA subtypes, which usually appear hypointense in the hepatobiliary images, b^{ex3}HCA overexpress OATP1B3 and thus is able to concentrate gadolinium-based hepatobiliary MRI contrast agents appearing iso/hyperintense on the hepatobiliary phase.[85]

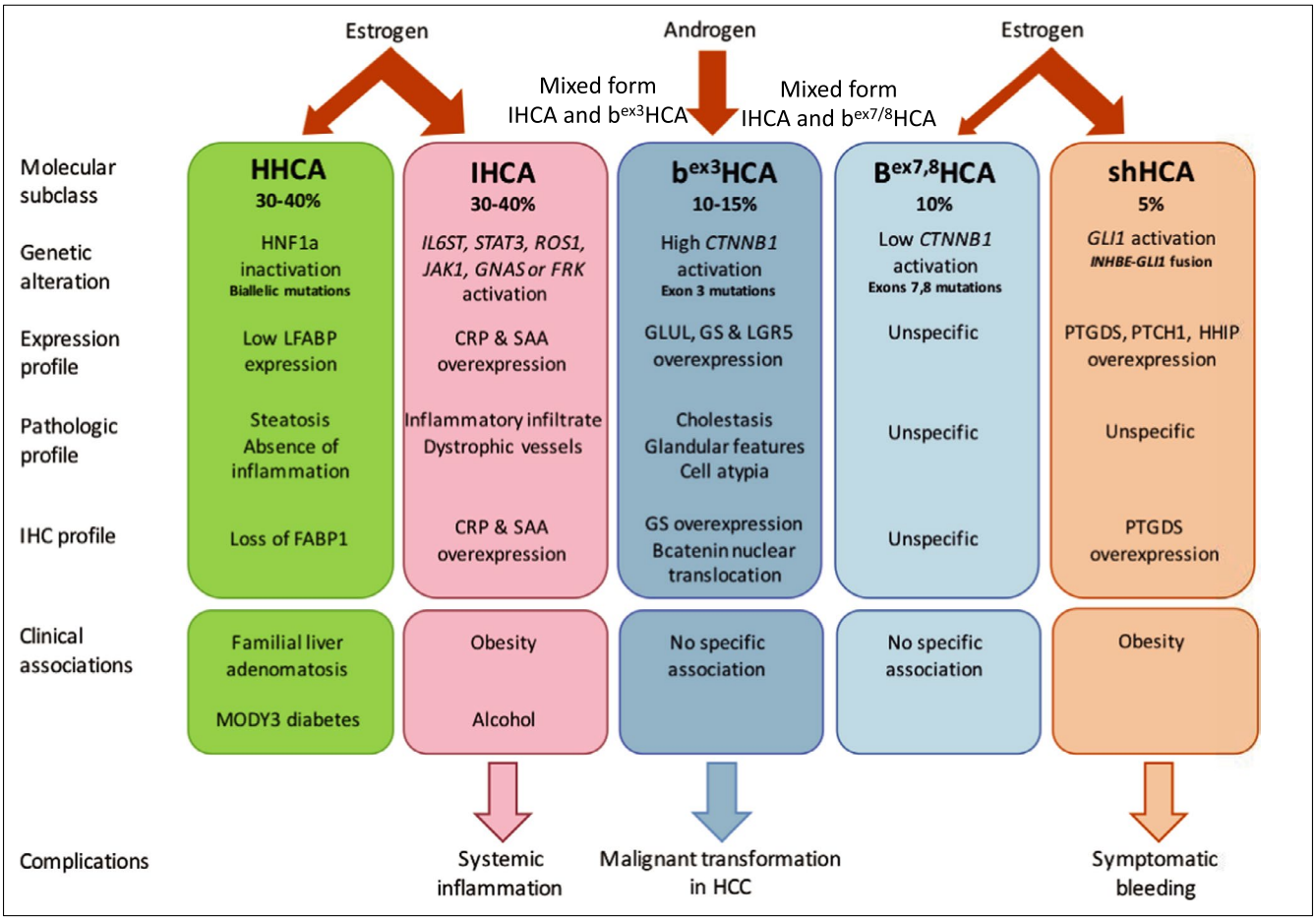


FIGURE 2 Molecular classification of hepatocellular adenomas. Risk factors, molecular alterations, pathological, immunohistochemical profiles and complication risks according to HCA's molecular subtype. HHCA, HNF1-A inactivated HCA; IHCA, inflammatory HCA; b^{ex3}HCA, CTNNB1 exon 3 mutated HCA; bex^{7,8}HCA, CTNNB1 exon 7 or 8 mutated HCA, shHCA, sonic hedgehog HCA; GS, Glutamine synthase

4.3 | From molecular characterization to personalized care

The two main complications of HCAs are symptomatic bleeding and malignant transformation into HCC. Identifying HCAs at risk for complications is, therefore, a major issue in the monitoring and therapeutic strategy.[86] The risk of symptomatic bleeding is mostly associated with the shHCA subtype, exophytic protrusion and size of the lesion.[72,87-89] The risk of malignant transformation concerns about 5% of patients in surgical series, with an association with male sex and mutations in exon 3 of the *CTNNB1* gene.[80,90-92]

The first step in the management of HCAs must be stopping oral contraception or androgens as well as weight loss.[93-95] Resection should be proposed for any HCA with *CTNNB1* exon 3 mutations or developed in male patients. For HCAs of more than 5 cm, if the lesion does not regress after the hormone withdrawal, surgical resection is the gold standard, but individualized management adjusted to the patient's risk of complications have been recently proposed.[87,96,97] In the other cases, such as small HCAs developed in women, the search for b^{ex3}HCA (immunohistochemical markers or mutation of exon 3 of *CTNNB1*) is useful to guide the resection depending on the risk of malignant transformation.[80,98,99]

5 | PRENEOPLASTIC LESIONS OF CHOLANGIOCARCINOMA

The incidence of CCA varies globally and the geographical variations in CCA seem to be related to different risks factors that cause chronic biliary inflammation and/or cholestasis. In East Asia, the most frequent risk factor for CCA is parasitic infection (*Opisthorchis viverrini* or *Clonorchis sinensis*) in contrast to primary sclerosing cholangitis in the West.[100] Chronic hepatitis B and C, cirrhosis, alcohol excess, smoke, obesity and diabetes have been also associated with CCA development.[100] Moreover, cases of CCA developed on Von Meyenburg complex have been reported in literature although its potential role as a CCA precursor lesion has not been clearly demonstrated.[101]

However, most of the CCA develop in patients without any chronic liver diseases. Based on the anatomical site of origin, CCA is classified as intrahepatic cholangiocarcinoma (bile ductules to

segmental bile ducts) (iCCA), perihilar CCA (right and/or left hepatic duct and/or their junction) (pCCA), and distal CCA (common bile duct) (dCCA).[102] CCA can generate from epithelial cells in the biliary tracts (i.e., cholangiocytes) and peribiliary glands. Moreover, some preclinical data had suggested that CCA could also arise from progenitor cells or even hepatocytes.[103] Hepatic stem or progenitor cell and cuboidal cholangiocytes have been linked to iCCA development, whereas columnar mucous cholangiocytes or peribiliary gland cells to pCCA and dCCA occurrence.[102]

In this part, we will focus on the two major premalignant lesions of CCA described in the literature: biliary intraepithelial neoplasia and intraductal papillary neoplasms of the bile duct (Figure 3). We will not discuss very rare lesions associated with a potential of transformation in CCA such as mucinous cystic neoplasm, von Meyenburg Complex and intraductal tubulo-papillary neoplasm.

5.1 | Histological definition

5.1.1 | Biliary intraepithelial neoplasia

Biliary intraepithelial neoplasia (BilIN) is the biliary counterpart of pancreatic intraepithelial neoplasia and includes lesions previously identified as atypical biliary epithelium, biliary dysplasia or carcinoma in situ.[104-106] BilIN is a flat, pseudo or micropapillary dysplastic lesion which can be found in intrahepatic or extrahepatic bile ducts.[105-107] These lesions have been mostly described in patients with PSC (19%-83%)[108-110] and hepatolithiasis.[111] In the majority of cases, BilIN is not macroscopically visible, although occasionally a thickened velvety or granular mucosa may be observed.[112] In 2007, a classification in three stages based on the degree of cellular and structural atypia has been proposed.[107] The major characteristics of each BilIN subtype are reported in Table 1.[105,107,113]

BilIN-1 has been observed in resection margins of biliary tree adenocarcinoma in about 48% of cases.[114] A higher prevalence (83%) of BilIN-2 and BilIN-3 has been observed in patients transplanted for PSC who had an associated CCA (most frequently tubular adenocarcinoma), confirming the role of a dysplasia-carcinoma sequence in CCA development.[112,115]

5.1.2 | Intraductal papillary neoplasms of the bile duct

Intraductal papillary neoplasm of the bile duct (IPNB) is a macroscopic epithelial papillary lesion which grows within the intrahepatic and/or extrahepatic bile ducts.[116] IPNBs were previously known as biliary papillomas, biliary papillomatosis or papillary adenomas.[116] Microscopically, IPNBs are composed of neoplastic epithelial cells covering fine fibrovascular stalks.[109] Based on the degree of cellular atypia, IPNBs have been classified as low-grade (LG) or high-grade (HG) IPNB.[116] LG-IPNB are usually less frequent (10%-20%) than HG-IPNB.[116] An invasive cholangiocarcinoma can be identified in approximately half of the cases of IPNB.[115,117]

According to the type of epithelial cells, IPNBs are further classified into four subgroups which may coexist, namely pancreato-biliary (PB-IPNB), intestinal (I-IPNB), gastric (GG-IPNB) and oncocytic (O-IPNB). The pancreas and hepatobiliary subtypes (PB-IPNB) are the most frequent in Western countries[112] and are frequently invasive and associated with the development of tubular adenocarcinoma, the same tumour that has been associated with as well as the G- and O-IPNBs.[112] Data concerning survival when IPNB become invasive are controversial, with some reports describing worse survival in patients with PB-IPNB compared to G- and I-IPNB.[118] The intestinal (I-IPNB) is the second most frequent subtype, and the most frequent subtype in Asia.[112] As opposed to the other subtypes, I-IPNB progresses to mucinous adenocarcinoma, which seems associated with better survival rates.[112]

5.2 | Mechanisms of malignant transformation into CCA

CCA is a highly heterogeneous tumour characterized by various somatic mutations, epigenetic modifications and alterations in DNA copy number, according to CCA subtypes and the underlying risk factors.[117,119] More specifically fibroblast growth factor receptor *(FGFR) 2* fusions, *TP53*, Kirsten rat sarcoma virus (*KRAS*), isocitrate dehydrogenase *(IDH) 1/2* and BRCA1-associated protein 1 *(BAP1)* mutations have been described as the most common alterations in iCCA in contrast to protein kinase CAMP-activated catalytic subunits alpha and beta *(PRKACA and PRKACB)* fusions and E74-like factor 3 *(ELF3)* mutations that usually occur in pCCA and dCCA.[102] Moreover, these mutational profiles differ in part from gallbladder cancer for which the most frequent mutations reported are *KRAS*, *TP53*, *CDKN2A/B*, Erb-B2 receptor tyrosine kinase *(ERBB) 2* and *ERBB3*.[120]

Several specific pathways involved in the early step of cholangiocarcinogenesis are highlighted below.

5.2.1 | Chronic inflammation and oxidative stress

Chronic biliary inflammation results in the sustained generation of reactive oxygen species (ROS) and nitric oxide (NO), which accumulate into cholangiocytes through the activation of several pro-inflammatory pathways.[121] Oxidative stress may favour CCA development and progression through DNA single or double-strand breaks, genetic instability, inhibition of DNA repairing enzymes and alteration of the balance between cell proliferation and apoptosis.[121,122]

Inducible nitric oxide synthase (NOS-2) and cyclooxygenase-2 (COX-2) are highly expressed both in reactive epithelium and in BilIN of any grade, suggesting their role in cholangiocarcinogenesis.[123] In addition, ROS accumulation favours the recruitment of TNF-producing kupffer cells, which promote biliary proliferation through c-Jun NH2-terminal kinase *(JNK)* signalling[124] and is associated with tumour progression and invasiveness.[125]

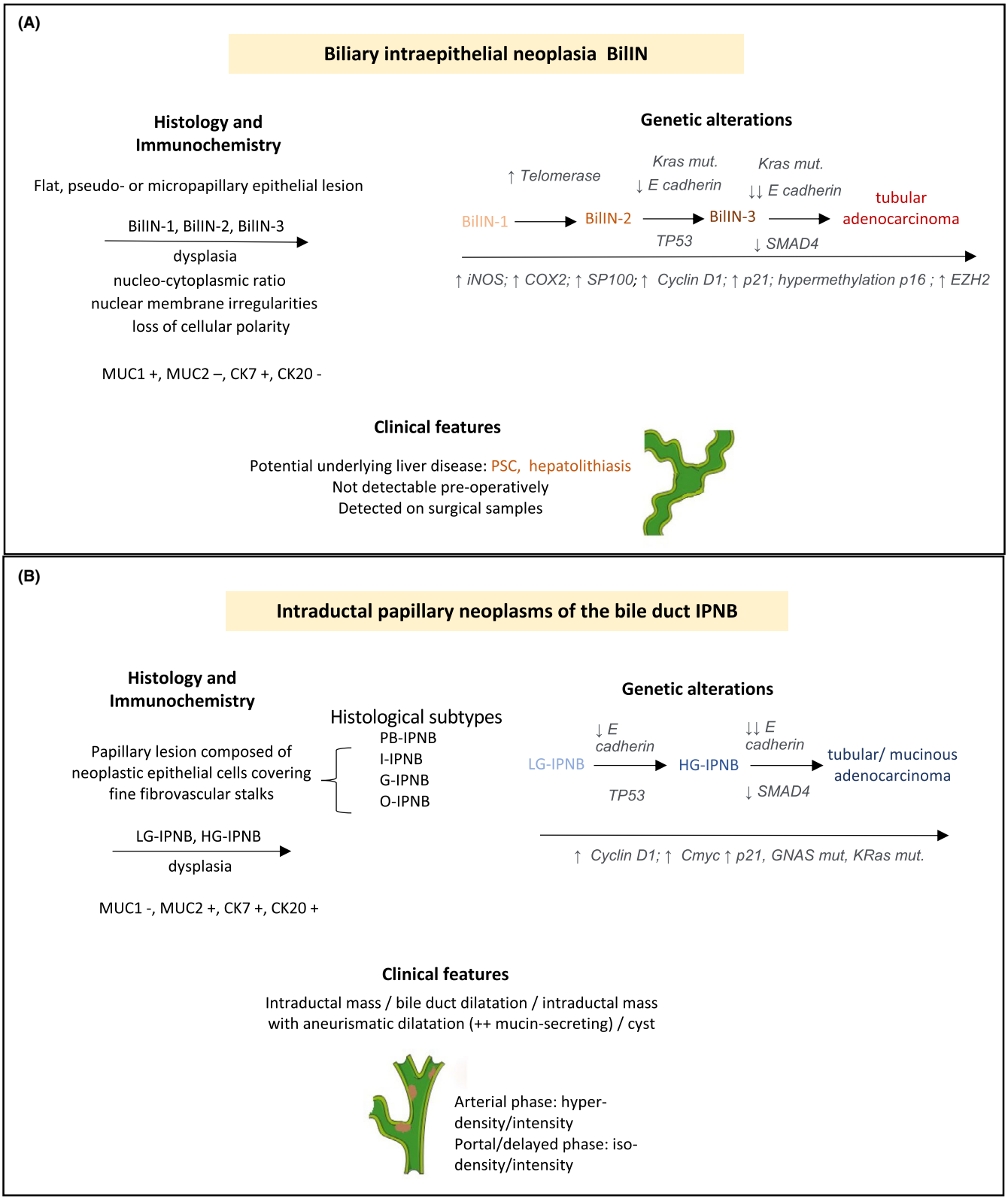


FIGURE 3 Preneoplastic lesion of cholangiocarcinoma. Representation of the main genetic alterations, clinical and histopathological features of (A) biliary intrahepitelial neoplasia (BilIN) and (B) intraductal papillary neoplasms of the bile duct (IPNB). CK: cytokeratin; COX2: cyclooxygenase 2; EXH2: enhancer of zeste homolog 2; G: gastric; GNAS: G protein alpha(s); HG: high grade; I: intestinal; iNOS: inducible nitric oxide synthase; KRAS: Kirsten rat sarcoma virus; LG: low-grade; MUC: mucin; O: oncocytic; PB: pancreato-biliary; SMAD 4: smad family member 4; SP100: speckled protein 100; TP 53: tumour protein 53

TABLE 1 Biliary intraepithelial neoplasia (BilIN) classification and histological features

	BilIN-1 (low-grade dysplasia)[96]	BilIN-2 (moderate grade dysplasia)[96]	BilIN-3 (high-grade dysplasia)[94,96,102]
Nucleus location	Conserved	Luminal surface	Huddled on the luminal surface
Nuclear features	Minor membrane irregularities, conserved shape	Moderate membrane irregularities, altered shape, hyperchromatic	Severe membrane irregularities, enlargement, hyperchromatic
Nucleo-cytoplasmic ratio	Slightly increased	Increased (++)	Increased (+++)
Cell size	Conserved	Altered (+)	Altered (++)
Cell shape	Conserved	Altered (+)	Altered (++)
Cellular polarity	Conserved	Lost (++)	Lost (+++)
Mitoses	Absent	Rare	Common
Basement membrane invasion	Absent	Absent	Absent

Note: Number of plus (+) represents the frequency of the feature.

During chronic inflammation, speckled protein 100 (S100P) expression progressively increases from reactive epithelium to high-grade BilIN and invasive CCA, mainly in perihilar CCA.[123,126] Loss of cell-adhesion properties can be considered late events leading to the development of an invasive CCA phenotype, both in BilIN and IPNB. E-cadherin expression is only slightly decreased in BilIN compared with normal biliary epithelium, whereas a strong downregulation is frequently observed in CCA arising both from BilIN and IPNB.[127] Further research is needed to support these findings and to confirm the role of the tumour microenvironment (TME) as a treatment target. Indeed, several lines of evidence are available about the involvement of TME in the proliferation, migration and invasion of CCA.[102,128-130]

5.2.2 | KRAS mutation

KRAS mutations are found in 20%-50% of CCA and are associated with poor prognosis.[131] *KRAS* mutations seem to occur early in cholangiocarcinogenesis both in IPNB (40%) and BiliIN (33%) whereas other mutations related to iCCA (*IDH1*, *FGFR2*) or eCCA (*ERBB3*) had never been identified in preneoplastic lesions.[123] They are more frequent in BilIN-3, BilIN associated with invasive carcinoma, and LG-IPNB, suggesting a role in malignant transformation.[118] In a recent study by Falcomatà et al in mice, an activating *Kras (G12D)* mutation was not able to induce neoplastic transformation of the extrahepatic bile duct but required the repression of tumour suppressor cyclin-dependent kinase inhibitor 1B *(P27kiP1)* or the *Pik3ca* mutation to induce the development of high-grade BilIN.[132]

Ras-Raf-MEK-ERK pathway can be activated also by alterations in FGFR pathway.[133] Fusion or rearrangements in *FGFR2* have been described in approximately 15%-30% of iCCA.[134] Several clinical trials in patients with iCCA using FGFR inhibitors have been published or are still ongoing: pemigatinib-INCB054828 [FIGHT-302; NCT03656536], infigratinib-BGJ398 [PROOF; NCT03773302], and futibatinib-TAS-120 [FOENIXCCA3; NCT04093362].[133] Moreover, *IDH1* inhibitor had been associated with a significant increase in progression-free survival in *IDH1* mutated iCCA (ivosidenib in IDH1-mutant, chemotherapy-refractory cholangiocarcinoma. However, it remains unknown whether the activation of the *FGFR2* signalling pathway leads to the onset and progression of IPNB, as for *IDH1* and *ERBB2-3*.[134]

5.2.3 | Dysregulation of cell cycle genes

Mutation of *TP53* has been described in iCCA, pCCA and dCCA,[135] and is one of the most common somatic mutations in iCCA (37%), especially in liver fluke and HBV-related CCA.[131] *TP53* mutations are rarely found in BilIN1 and BilIN-2, whereas they are recognized in approximately 75% of invasive CCA arising from BilIN.[109,127]

p21 overexpression accounts for the earlier mechanisms of cholangiocarcinogenesis in both BilIN and IPNB lineages.[116,118,127] Indeed, increases in p21 expression parallel the progression from BilINs or IPNBs to CCA.[108,118,127]

Cyclin D1 overexpression has been observed both in BilIN and IPNB with levels increasing in parallel to the degree of dysplasia, suggesting that cyclin D1 overexpression is involved in the acquisition of an invasive phenotype.[108,109,127] C-myc overexpression has been identified in up to 41% of resected CCA and in 50% of IPNB,[127] whereas its expression in BilIN does not exceed the 20%, suggesting a role in the IPNB lineage.[109] SMAD family member 4 (*Smad4)* mutations has been described in up to 16% of CCA subtypes and Smad4 expression gradually decreases from both BilIN and IPNB to invasive CCA suggesting that its loss is a late-occurring event.[108,118]

Finally, *GNAS* mutations have not been described across the BilIN lineage, whereas mutation in codon 201 have been described in IPNB, especially in I-IPNB, a reminiscence of the *GNAS* alteration observed in the intraductal papillary mucinous neoplasm of the pancreas.[109,118]

5.3 | Implications in clinical practice

5.3.1 | Identification of preneoplastic lesions

BilIN is an asymptomatic microscopic disease and is not detectable at imaging, but it is usually recognized in specimens of biliary tract

cancer.[106] Conversely, intermittent abdominal pain, acute cholangitis or jaundice are the most common clinical manifestations of IPNB, whereas less than one-third of patients have no symptoms.[109] US can detect up to 41% of IPNB as a hypo or hyperechoic mass, bile duct dilatation or both.[109,136] CEUS might facilitate the diagnosis of IPNB excluding sludge, stones and blood clots, but distinguishing invasive IPNB from non-invasive lesions can be challenging. As opposed to US, dynamic CT and MRI with their specific sequences (e.g. diffusion-weighted imaging, MR-cholangio-pancreatography) could be helpful to identify CCA in patients with IPNB.[137] In IPNB, the arterial enhancement is combined with an iso-dense/intense signal in the delayed phase rather than a strong centripetal contrast uptake as in CCA.[137] Moreover, a specific string-like filling defect ("thread sign") has been described on MRCP images for the diagnosis of mucin-secreting IPNB.[137] An additional unique feature of IPNB is represented by the appearance of an intraductal mass in an aneurysmatic dilatation. This feature is commonly observed in mucin-secreting IPNB where proximal dilatation caused by the lesion-related obstruction and distal dilatation related to mucin obstruction of the Vater papilla may be observed.[138,139] The appearance of IPNB as a mass associated with proximal duct dilatation, disproportionate dilatation without detectable mass or cystic appearance have also been described.[140]

Finally, percutaneous transhepatic cholangioscopy (PTCS) and peroral cholangioscopy (POCS) could be useful to assess the extent of the tumour but are burdened by the possible development of adverse events as well as by the difficulty of histological diagnosis, due to the possibility of mixed pathological findings in the same lesions.[109] ERCP is able to visualize mucin as filling defects but its sensitivity in diagnosing a malignancy is low (20%-55%).[119,136] Next-generation sequencing mutational analysis of bile cell-free DNA (cfDNA), has been tested in a cohort of 68 patients with biliary strictures, enabling the diagnosis and the early management of the more aggressive lesions.[141]

5.3.2 | Monitoring and treatment of preneoplastic lesions of cholangiocarcinoma

As BiliN could not be identified pre-operatively, no specific guidelines for clinical practice are available. In contrast, patients with IPNB, even in the absence of malignancy, should undergo surgery due to the risk of developing recurrent cholangitis and obstructive jaundice.[109] In order to establish the appropriate timing and type of surgical procedure, a precise preoperative assessment of tumour location and extension is mandatory.[109] In patients with IPNB without associated malignancy and with a limited extension of IPNB, partial hepatectomy should be considered.[109] Conversely, IPNB with an extensive superficial spread or with multifocal lesions has a high recurrence rate and should be treated with an extended hepatectomy and/or resection of the whole biliary tree.[109] Finally, data about liver transplantation are limited due to the difficulty in determining the presence of malignant transformation to CCA pre-operatively.

5.4 | Unmet needs and conclusion

Premalignant tumourigenesis is a complex phenomenon with an interplay between the underlying liver disease (cirrhosis and low-grade and high-grade dysplastic nodules) and environmental factors (hepatocellular adenoma developed in normal liver fostered by oestrogen exposure). Several unmet needs such as a better characterization of the percentages of low-grade and high-grade dysplastic nodules that will progress to HCC should be addressed. Moreover, more data on risk factors of malignant transformation of dysplastic nodules in cirrhosis, in addition to the mutations of the *TERT* promoter, must be obtained. In HCA, most of the HCC derives from HCA with *CTNNB1* exon 3 mutations, but a small subset of HCC develops in other molecular subtypes and the mechanisms of malignant transformation in these cases are still unknown.

Improvement in screening policies for early diagnosis of both HCC and CCA is urgently needed as the incidence of HCC among non-cirrhotic patients (e.g. NASH) is increasing and a known risk factor is only identified in a minority of CCA (approximately 20%). However, which patients should undergo surveillance and which test or combination of test should be used, are questions that have still not been answered.

Finally, liquid biopsies, aiming to detect biomarkers released from the tumours into the bloodstream (circulating tumour DNA or microRNA, extracellular vesicles, cytokines, circulating tumour cells) are minimally invasive technique tested in patients with HCC and CCA.[142,143] Further validation research is needed before we can assess the clinical relevance and feasibility of these new technologies to detect premalignant lesions and early HCC and CCA.

CONFLICT OF INTERESTS

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ORCID

Elvire Desjonqueres https://orcid.org/0000-0002-1444-8952
Claudia Campani https://orcid.org/0000-0003-3842-782X
Fabio Marra https://orcid.org/0000-0001-8629-0878
Jessica Zucman-Rossi https://orcid.org/0000-0002-5687-0334
Jean-Charles Nault https://orcid.org/0000-0002-4875-9353

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