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Review

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Combined hepatocellular-cholangiocarcinoma: morpho-molecular updates and considerations

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Abstract

Combined Hepatocellular-Cholangiocarcinoma is a heterogenous primary malignant epithelial tumor of the liver with variable morphological and immunophenotypical features. Although the biology of this tumor has been described in the literature, changes in classification and its heterogeneity imply difficulties in collecting reliable homogenous groups to compare. The article aims to review available data on morphology and immunohistochemistry for practicing pathologists integrated with original data from our referral Center.

Keywords: Combined hepatocellular-cholangiocarcinoma, WHO classification, immunohistochemistry, molecular analyses

INTRODUCTION

Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a heterogenous primary liver carcinoma (around 2%-5%)[1] in which both hepatocellular and cholangiocellular differentiation is present. Diagnosis relies on morphological aspects with routine haematoxylin-eosin (H&E) which can be confirmed, if needed,

by immunohistochemistry (IHC). Classification has changed and evolved through the years due to the heterogenous presentation of this tumor type. In the nineteen-forties, Allen and Lisa[2] proposed to differentiate between tumors with features of both hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA), and distinct morphological lesions of HCC and iCCA in the same liver in which three different entities could be described in the liver: (1) Distinct concomitant HCC and iCCA lesions; (2) Contiguous HCC and iCCA lesions; and (3) a single lesion with cellular characteristics of both hepatocytes and cholangiocytes[2]. Only five years later, Edmondson and Steiner reported 4 cases of morphologically distinct cHCC-CCA tumors among 100 cases in an autopsy series[3].

With the advent of IHC, classification was integrated to include this additional descriptor, and with the turn of the century, the WHO suggested novel assays, pCEA and HepPar1 markers, to better describe and classify hepatocellular differentiation within mixed HCC-CCA, which they first defined as tumor characterized by both HCC and iCCA *intimately admixed*[4].The 4th edition, published in 2010[5], further refined the definition of this tumor type, subdividing cHCC-CCA into classical cHCC-CCA and cHCC-CCA with stem cell features; three subtypes, typical, intermediate-cell, and cholangiolocellular, were also identified.

The 5th and latest edition of the WHO (2019[1]) simplified subtypes and returned to descriptors used in the 3rd edition (2000[4]), which were adopted, from the conclusions of the International Consensus Group reported by Brunt *et al.*[6]. It defines cHCC-CCA as consisting of its two components, HCC and CCA, which must be very close or *intermingled*, without a clear transition and without limits in their proportion and, therefore, with no cut-off for the percentage of the two components. Furthermore, IHC is a support for the diagnosis and is not sufficient alone without an adequate morphological description. The terminology used in the previous version, with stem cell features[5], was no longer recommended since subclassification was applied with difficulty in routine practice due to disagreement between pathologists and "stem cell" features may be found in all cHCC-CCA and because immunohistochemical markers used for the confirming stem cell phenotype, such asCD56, EpCAM, CD133, and CK19[7], are not specific for stem cells. Current classification thus recognizes two broad subgroups of cHCC-CCA; a neoplastic mass made of two clear components of HCC and CCA and a mass made of tumoral cells with intermediate phenotype, morphologically and phenotypically, a mix of hepatocytes and cholangiocytes. The evolution of nomenclature is compared in Figure 1.

The correct diagnosis implies extensive sampling of the liver mass (at least 1 sample for each cm of the maximum diameter) since cHCC-CCAs do not have specific macroscopic aspects and one of the two morphological components may be lost with incorrect tissue probing. Morphological patterns are very variable and include the different histological aspects described in HCC and iCCA. The iCCA component is generally characterized by a malignant glandular proliferation and is subdivided between "small duct type" and "large duct type"[1]; the first is more peripheral, the latter usually arises close to the hilum, is mucus-secreting and involves the perihilar extrahepatic tissue. The HCC component also tends to be very heterogeneous, with malignant differentiated proliferation of cells arranged in trabeculae, pseudoglands, and acinar structures[1], characterized by bile duct secretion, fat accumulation in the cytoplasm or cytoplasmic inclusions, probably due to accumulation of proteins[8]. Correct differentiation between iCCA and HCC is difficult as treatment guidelines are not well established, due to the heterogeneity of these tumors, their rare presentation, and limited data from which to draw conclusions. Biopsy differential diagnosis can be very challenging and may lead to an incorrect diagnosis of cancer of unknown primitive (CUP) due to poorly undifferentiated tissue, incomplete clinical information, inaccurate choice of correct immunohistochemical markers, and a non-specific grading system for cHCC-CCA[1], in particular for

2000 WHO CLASSIFICATION 3° EDITION	2010 WHO CLASSIFICATION 4° EDITION		2019 WHO CLASSIFICATION 5° EDITION	
Combined hepatocellular and cholangiocarcinoma	cHCC-CCA, classical type	cHCC-CCA, stem cell features - Typical cell subtype - Intermediate cell subtype - Cholangiolocellular subtype	cHCC-CCA, classical type	Intermediate cell carcinoma
- A rare tumour containing unequivocal elements of both hepatocellular and cholangiocarcinoma, intimately admixed («different from collision tumour») - Recommended to look for bile and mucus secretion in the two components	- Contains areas of typical HCC and typical iCCA. - Confirmation is provided by IHC - In many cases there are foci of intermediate morphology at the interface between the 2 components	-**Typical cell subtype**: nests of mature appearing hepatocytes with peripheral clusters of small cells with high nucleus:cytoplasm ratio. -**Intermediate-cell subtype**: tumour cells with features intermediate between hepatocytes and cholangiocytes. They are small, oval-shaped, arranged in trabeculae, solid nests or strands. -**Cholangiolocellular subtype**: small atypical cells growing in a tubular, cord-like, anastomosing pattern («antler-like»), embedded in a fibrous stroma, recapitulating the canals of Hering or cholangioles -recommendation to apply IHC (markers for stem cells are considered: CD117, CD56, EpCAM).	-The diagnosis is based on H&E morphology and can be confirmed by IHC. -The hepatocytic and cholangiocytic tumour areas may show all the architectural and cytological differentiation patterns described for HCC and iCCA; -The 2 components are either close to each other or intermingled. -No minimal cut off amounts of each component. -«stem cell like population» can be found in different proportions and *can be* confirmed by IHC.	-It's a primary liver cancer with monotonous morphological features intermediate between hepatocytes and cholangiocytes. -The cells are usually small with scant cytoplasm, arranged in cords, stands, trabeculae and occasional gland like structures
	Introduction of different subtypes describing the heterogeneity of cHCC-CCA, with a large group called «stem cell features» including other 3 subtypes. IHC is recommended to identify the «stem cell population»		**Being a rare tumor, the WHO 2010 is difficult to apply in routine practice, so the latest WHO cuts down the subtypes to 2 groups with the intermediate cell carcinoma replacing the «stem cell features». The diagnosis relies of H&E and can be confirmed with IHC**	

Figure 1. WHO classification: change over time in classification (2010 and 2019)(modified from 1,4,5).

intermediate cell category tumors.

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To date, specific biomarkers for biliary differentiation are not able to distinguish normal and non-normal tissue; only specific markers for hepatocellular and cholangiocellular differentiation can be considered reliable [Figure 2].

If bile production is not visible on the embedded slide, hepatocyte-specific antigen/Hep-par1 (clone OCH1E5)[9] can be useful. This marker is also expressed in normal hepatocytes and associated with the urea cycle enzyme carbamoyl phosphatase synthetase 1, CPS1[10]. Unfortunately, it works well only when the tumor is well or at least moderately differentiated, but it has very poor sensitivity in poorly differentiated tumors. It also is not completely specific for hepatocellular neoplasms because occasionally gastric, esophageal, and lung adenocarcinomas show strong positive reactions and are thus called *hepatoid* adenocarcinomas[11]. When the tumors are less differentiated, Arginase 1 is very useful; this enzyme of the urea cycle[12], mainly expressed in the periportal hepatocytes, was first described as a powerful tool for diagnosis of origin in 2010[13]. Expressed both in normal and neoplastic hepatocytes, both in the nucleus and the cytoplasm. Detection sensitivity and specificity are superior compared to HepPar1, also in the poorly differentiated HCC[1] but can also be positive in hepatoid adenocarcinomas, similarly to HepPar1[14]. Glypican 3 is an oncofetal gene that encodes a protein of the prototypical cell-surface heparan sulfate proteoglycans and can be useful, especially in moderately and poorly differentiated HCC. It is not specific to HCC since it stains other different and very important tumors such as yolk sac tumors and squamous cell carcinoma of the lung[15]. For this reason, a strong correlation with morphology is mandatory for its interpretation. Nevertheless, testing for Arginase1 and Glypican 3 seems to increase sensitivity to approximately 100% in poorly differentiated HCC[16].

Markers for hepatocellular differentiation	Markers for biliary differentiation
HepPar1/Hepatocyte specific antigen	CK7
Arginase 1	CK19
Glypican 3	CA19.9
CD10	
pCEA	
Alpha-fetoprotein	
In situ hybridization of albumin	
Bile salt export pump (BSEP)	
Multidrug resistant protein 3 (MDR3)	

Figure 2. Immunohistochemical markers of hepatocellular and biliary differentiation.

Alpha-fetoprotein (AFP) is a sugar-containing protein produced by the yolk sac and liver in the fetus. It is one of the most widely used serum biomarkers for the early detection and follow-up of HCCs[17]. AFP is expressed in only about one-third of cases of HCC[18] but can be useful in poorly differentiated HCCs[19].

Other commonly used markers are polyclonal antibodies against carcinoembryonic antigen (p-CEA) and CD10, with low sensitivity and specificity (less than 50%) in poorly differentiated HCC[20]. Moreover, CD10 is not lineage-specific; it is also expressed in numerous normal tissues, and also in epithelioid hemangioendothelioma[21], which can be tricky to diagnose on liver biopsy.

Albumin was considered as a potential tool for the identification of HCC almost 40 years ago[22], but only recently, with branched chain *in situ* hybridization assay[23], has been shown to be applicable in routine diagnostics. Albumin is produced by hepatocytes, so it should be specific for hepatocellular origin, but like anti-hepatocyte specific antigen/HepPar1, it is also expressed in acinar cell carcinoma[24] and together with Arginase 1, can also be expressed in hepatoid carcinomas[25].

Other markers for hepatocellular origin are ATP-binding cassette (ABC) transporters which are the members of efflux pumps called bile salt export pump (BSEP) and the multidrug-resistance protein 3 (MDR3) which metabolize and remove cytotoxic agents. Both have high sensitivity and are specific for HCC, but not useful in poorly differentiated HCC[26]. In a recent study[26], their expression was investigated in hepatoid carcinomas, where they were found to be overexpressed in this subset of tumors, suggesting their value in this differential diagnosis.

To date, there are no specific markers for cholangiocytes, and the most commonly used remain CK7 and CK19 which, though, can also be expressed in HCC and other tumors. Poor prognosis remains correlated to CK 19 expression[27]; up to 10% of HCC can co-express CK7 and CK19 and approximately 18% can express at least CK7 or CK19[28]. Thus, diagnosis always relies on the combination between morphology and immunophenotype.

MOC31 is an antibody that targets EpCAM, a surface glycoprotein. Initially, it was considered as a good marker for differentiating CCA from HCC[11,29], but it is specific for diagnosis since it stains carcinomas of different origins[30]. In addition, it can be expressed in poorly differentiated HCC[31]. Another marker that is used for biliary differentiation is CA19.9, which was found to be positive in around 60% of CCA, but not

present in HCC[32].

MOLECULAR STUDIES

Molecular characteristics of cHCC-CCA are described only in a few reports in literature, mainly due to the rarity of these tumors. Changes in classification definitions have also complicated the collection of reliable series since the family of cHCC-CCA is very heterogeneous in terms of molecular alterations.

The larger series come from Eastern countries where the main etiology is still HBV infection, which is not frequent in western countries. In fact, one of the most recent and extensive studies by Xue *et al.* is a pan-Asia multi-center study including 8 hospitals with 133 cases[33]. Of these, 75% were HBV positive, 5.3% HCV positive, 18.8% double negative and only one double positive. The authors used whole-exome and whole-genome RNA with single-cell nucleus sequencing to investigate the different molecular aspects. It is noteworthy that 22.9% of cases had the same hotspot mutation C228T in the TERT promoter and FGFR fusions were found in 6.5% of cases. Authors subdivided the cases between cHCC-CCA, characterized by clearly defined areas of HCC and CCA components in the same tumor, and mixed HCC-CCA, in which the two components were deeply intermingled without clear boundaries. They interestingly found that the first subgroup is more like CCA in terms of different gene pathways, while the second group is closer to HCC[33]. Another important result in this series was identifying a monoclonal cell of origin both for combined and mixed type[2], also supported by other studies (Fujii *et al.* 2000[34] and Moeini *et al.*[35]). Moeni *et al* studied a series of 18 cHCC-CCA including 6 cholangiolocarcinoma, 8 stem cell features, and 4 classical combined, according to WHO 2010. Cholangiolocarcinoma was characterized by chromosomal stability and activation of TGF pathway which was not found in the other categories. They also confirmed that the classical combined tumor shared features of both HCC and CCA and that the stem cell category had different molecular signatures, mainly regarding activation of proliferation[35], and characteristically expressed SALL4 as progenitor phenotype in 75% of cases.

Another important study by Joseph *et al.* from the University of California studied a series of 30 cases classified according to WHO 2010[5] which included 20 classical types cHCC-CCA and 10 CCA, with variable etiology; in particular, 60% of the cHCC-CCA were concomitant with HCV-related cirrhosis[36]. They applied broad capture-based sequencing of the genomic DNA collected, separating the HCC and CCA components. Eighty percent of cHCC-CCA showed TERT promoter mutations and 70% also had p53 alterations. iCCA in cirrhosis showed different alterations, mostly IDH1/2, FGFR2, CDKN2A, and those involving chromatin regulators, demonstrating that cHCC-CCA were more similar to HCC than CCA[36].

FOCUS ON OUR CASES

We retrospectively reviewed our internal histopathological series from 2012 to September 2022 and found 21 cases of cHCC-CCA in the 1263 intrahepatic resected liver tumors (1,7%) available. Tumor tissue was reclassified according to WHO 2019[1]; 6 were reclassified as cholangiolocarcinoma, considered iCCA, and 15 as cHCC-CCA, representing 1,2% of the intrahepatic liver tumors resected. The median age was 71 years and only 2 were female subjects (13,3%). Eleven were classified as classical type (73,3%) [Figure 3 A-F], 2 were intermediate cell phenotype (13,3%, Figure 3 G and H) and 2 showed both subtypes, including mature components of HCC and CCA but also a minor group of cells with intermediate phenotype (13,3%) [Figure 4A-C] confirmed by IHC [Figure 4, 1-4].

Regarding liver condition, four patients had HBV hepatitis (26%) and were associated with hemochromatosis in one case and non-alcoholic steatohepatitis (NASH) in another. Six patients had background liver fibrosis grade S3 (according to Ishak,[37]) or more; 5 cases showed no or mild fibrosis. In 4

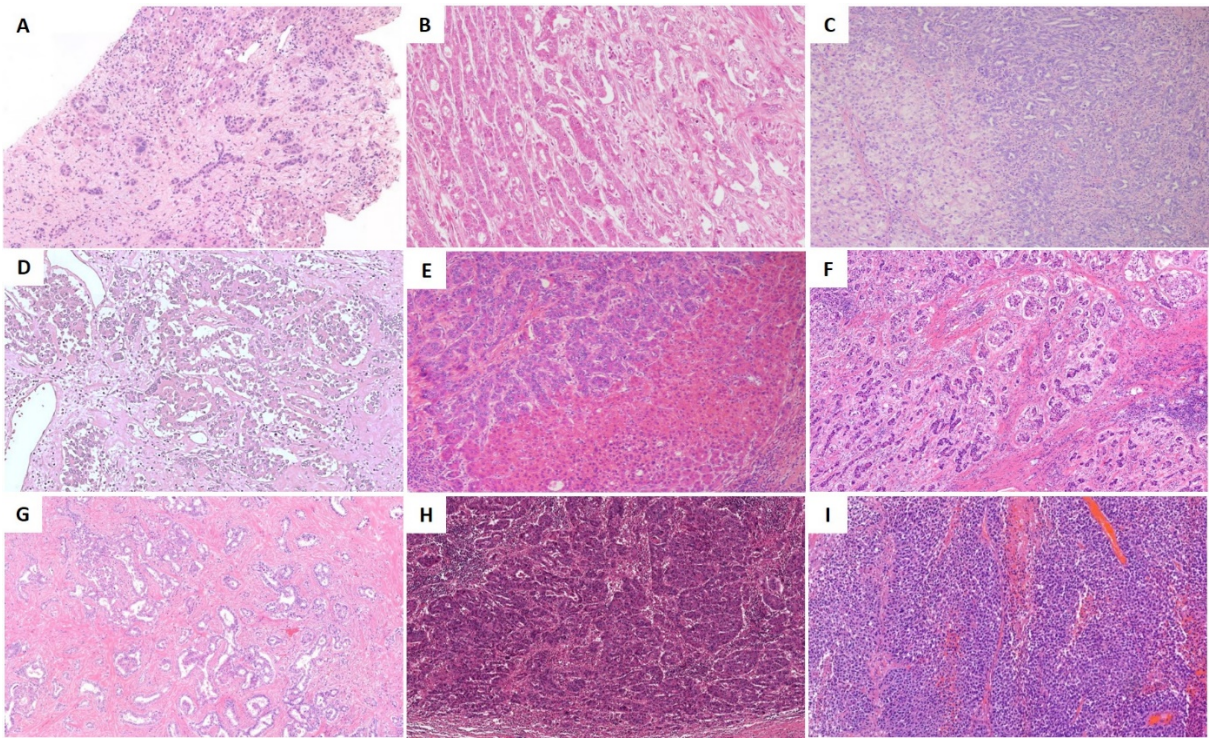

Figure 3. Morphological aspects of cHCC-CCA in our series. H&E, classical HCC-CCA can show different aspects, depending on how the cells intermingle with each other. There can be a sharp transition (10x, A) or a deep intermingle (20x, B,C,D,E,F,G); intermediate cell carcinoma is composed of small cells with glandular or solid organization and hyperchromatic nuclei (20x, H,I).

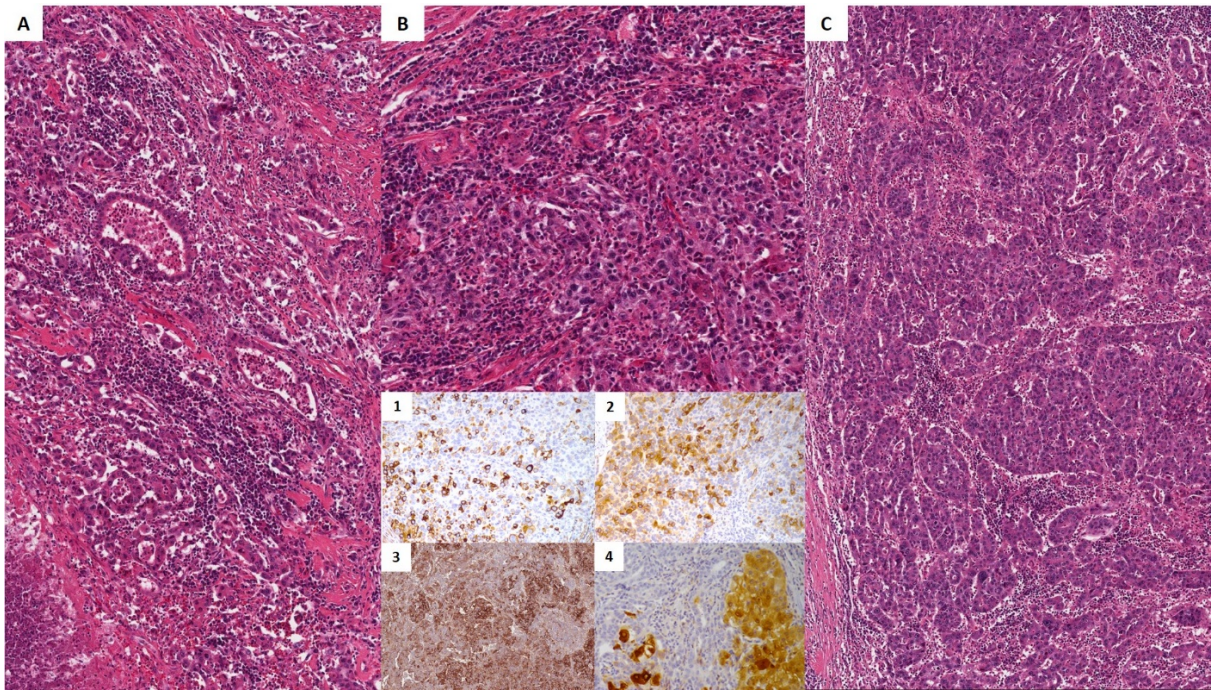

Figure 4. A case of classical HCC-CCA. This case shows an adenocarcinoma component (20x, A), a solid hepatocellular carcinoma part (20x, C), and an area of transition (20x, B) made of smaller cells which are partially positive for CK7 (40x, 1), glypican 3 (40x, 2), alpha-fetoprotein (40x, 3) and arginase 1 (40x, 4).

cases, fibrosis could not be evaluated histologically because collected tissue was from tumor biopsies or resections of the nodule [Figure 5].

Another interesting feature to report is that in 3 cases of classical HCC-CCA, the tumor had aspects of ductal plate malformation (DPM). One case [Figure 6A], in particular, had areas resembling DPM [Figure 6B] but also recalled biliary adenofibroma [Figure 6C]. It was peculiar because the hepatocellular mature component was growing inside biliary neoplastic structures organized in glands [Figure 6D, blue arrow], as also demonstrated by IHC for arginase1 which stains the neoplastic hepatocytes [Figure 6E, green arrow]. Therefore, intermediate cell components can be found together with a classical HCC-CCA, supporting the hypothesis of transdifferentiation between the two mature components [Figure 7]. In addition, one case of these cases was found to have a mutation on TERT promoter and amplification of ERBB2.

HCC with partial biliary differentiation following transarterial chemoembolization (TACE) [Figure 8], as described in the literature[38], was found in two cases. They have the typical presentation of and HCC, but in the proximity of the embolized foreign material, a neoplastic glandular component [Figure 8A and B] showing biliary differentiation (CK7 positive, Figure 8C) can be seen.

In cases in which lymphadenectomy was performed (in patients with biopsy diagnosis of adenocarcinoma, $n = 5$), two poorly differentiated cHHCC-CCA were metastatic; of these, one also had a sarcomatoid component.

FINAL CONSIDERATIONS

cHCC-CCA is a very heterogeneous malignant primary liver tumor that can be difficult to diagnose, especially on biopsies, and whose classification has changed over the years, complicating correct reporting and case presentations.

Clinical management is still not "standardized" and is left to personalized approaches in which the role of the pathologist is fundamental in order to aid in correct sampling procedures and accurate diagnosis of all the components, according to WHO 2019[1]. According to the literature, the main prognostic factors for cHCC-CCA are size (> 5 cm), multiple satellite nodules, lymph node metastases, vascular invasion, levels of tumoral markers (CA19-9 and CEA), and resection margins[39].

cHCC-CCA are fascinating tumors because, in the same mass, many different histological aspects can be found, from a classical HCC to a classical iCCA with different grades of differentiation and intermediate cells. These aspects recapitulate molecular characteristics[40]. The "stem cell-like" components, no longer recommended in reporting, can be nevertheless recognized by pathologists as smaller cells with scant cytoplasm and hyperchromatic nucleus, organized in ductular structures (different presentation from ductular reactions) and generally associated with a desmoplastic stroma. IHC can help with several markers [Figure 2], but morphology drives the diagnosis as no immunohistochemical staining is entirely specific. Reviewing our cases, we found that HBV infection was present in 26% of cases with cHCC-CCA, and approximately 5% in patients with iCCA. This interesting result could be the consequence of HBV integration in the DNA of the hepatocytes as previously investigated in the literature[41], but its biological meaning needs further investigation and the collection of well characterized cases of cHCC-CCA can help to better understand its biology.

Classical cHCC-CCA	Intermediate cell carcinoma	Classical cHCC-CCA with intermediate cell component
1 HBV, DPM (S3)	NAFLD (S1)	1 ASH (S6)
1 HBV, HFE mut (S5)	HBV (wedge resection)	1 NASH (S4)
1 HBV, ASH (S3)		
1 HCV (S4)		
1 NASH (S2) (1 with DPM)		
1 ASH (S not eval)		
1 NAFLD (S0)		
2 steatosis <5% (S0) (1 with DPM)		
2 tumoral biopsies		

Figure 5. A detailed description of background liver function in our cases. S: staging of fibrosis according to Ishak[44], HFE mut-homeostatic iron regulator mutation; NASH- non-alcoholic steatohepatitis; ASH-alcoholic steatohepatitis.

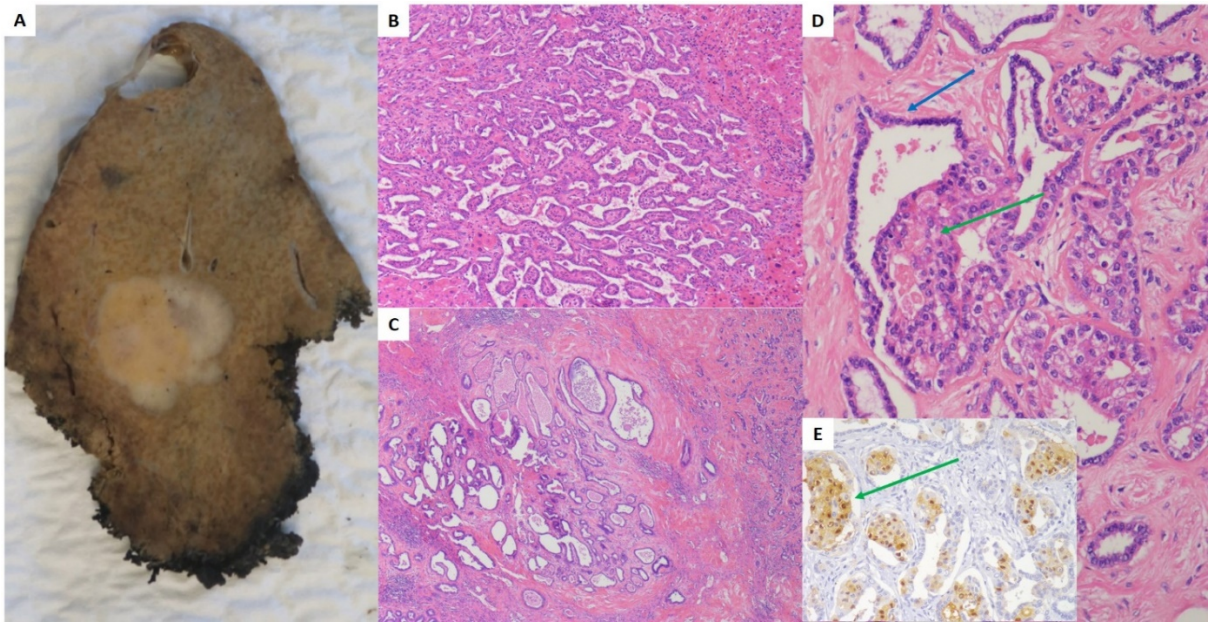

Figure 6. Case of cHCC-CCA with features of biliary adenofibroma. (A) Gross features of the 2.5 cm nodule with yellow-greyish color, in an HBV-positive patient with S3 fibrosis. (B) H&E, 10x, areas resembling ductal plate malformation (DPM). (C) H&E, 10x, areas resembling biliary adenofibroma. (D) H&E 40x, Peculiar neoplastic glandular component (blu arrow) with intraglandular growth of HCC (green arrow), confirmed by IHC with arginase-1. (E) 20x (green arrow).

The incidence of lymph node metastases in cHCC-CCA is around 20%, similar to that of intrahepatic CCA[40]; in our cases, it reached 33%, considering that only 5/12 patients performed lymphadenectomy.

Molecular analyses have a fundamental role as a descriptor of individualized, targetable alterations, but also in terms of confirmation of diagnosis. In fact, in our reported case above, both a mutation in the promoter of TERT, typically found in HCC[36], and ERBB2 amplification, described in cHCC-CCA but more frequent in biliary tract cancer found in extrahepatic biliary adenocarcinomas[40], were present.

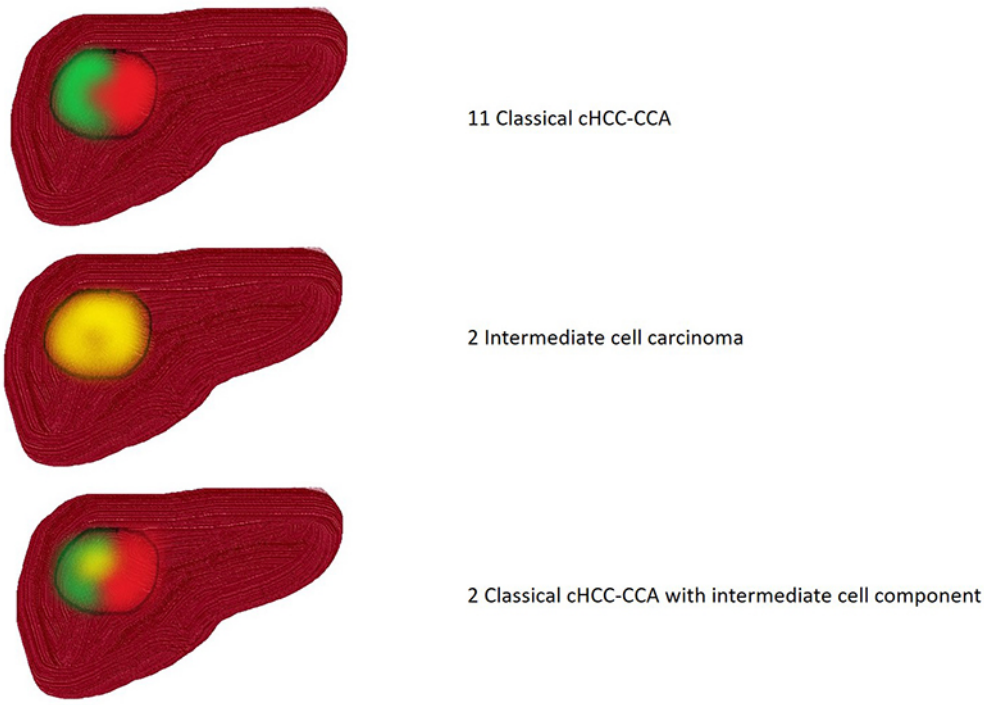

Figure 7. Morphological aspects observed in our cases.

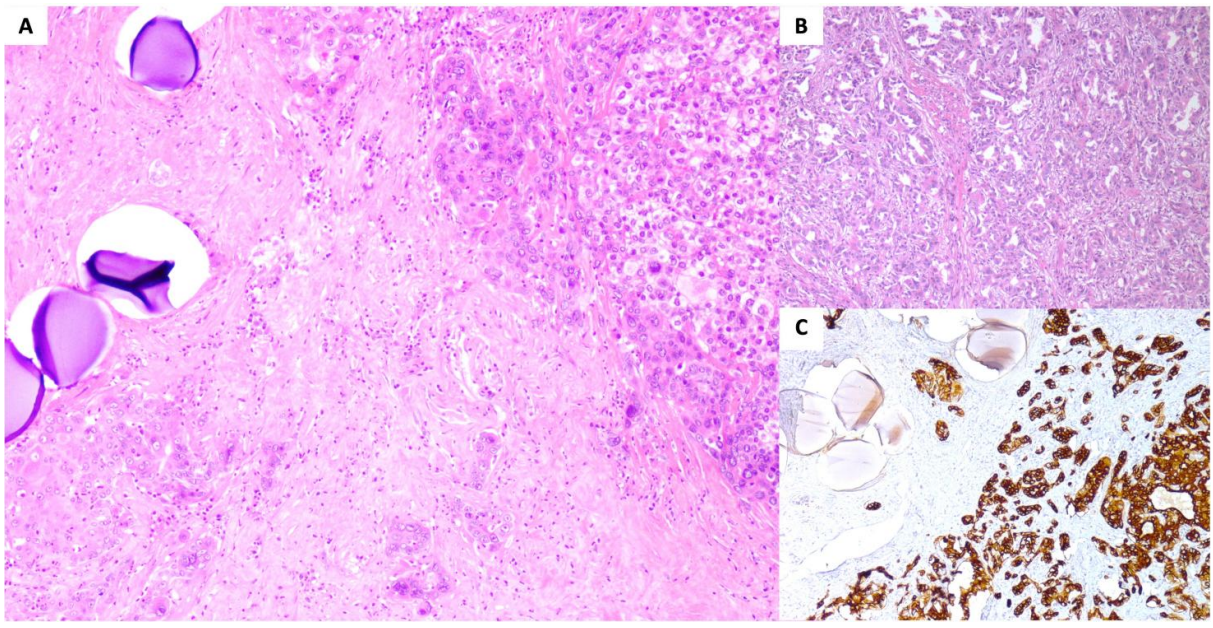

Figure 8. A case of biliary differentiation post-TACE. (A) H&E 20x, TACE foreign material, on the left, with close neoplastic glandular component and hepatocellular carcinoma on the right. (B) H&E, 20x, Another view of the adenocarcinoma component. (C) IHC for CK7 demonstrating clear biliary differentiation.

The molecular profiles of cHCC-CCA seem to recapitulate the two different histological populations, yet the current WHO classification does not contemplate this, mostly due to the limited data available for this rare tumor type. The lack of data[42] also hinders official recommendations on lymphadenectomy, which has

been shown to improve survival in patients with iCCA[43]. Correct differential diagnosis (HCC *vs.* CCA *vs.* cHCC-CCA) is also fundamental in terms of locoregional treatments for disease control, such as microwave ablation or transarterial chemoembolization[44] in HCC, or liver transplant[45], still debated but available treatment option in patients with HCC[46].

Correct sampling methods for unclear cases, precise morphological description, adequate molecular analysis, and up-to-date knowledge of the current scenario of hepatic mixed tumor types must be all proper to the pathologist in order to best describe the tumor and aid in offering the most adequate therapeutical option for these patients.

DECLARATIONS

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Authors' contributions

Made substantial contributions to study conception and design, data analysis and interpretation: Ahmed N, Falcinelli F, Rimini M, Burgio V, Casadei-Gardini A, Aldrighetti L, Ratti F, Pedica F

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Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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