REVIEW

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Biphenotypic (hepatobiliary) primary liver carcinomas: the work in progress

Hepatic Oncology

Elizabeth M Brunt¹, Valerie Paradis², Christine Sempoux³ & Neil D Theise^{*,4}

Practice points

- Diversity is increasingly recognized in primary liver carcinomas (PLCs), changing the dichotomy of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) as distinctly separate tumors in all situations.
- PLCs with mixed hepatobiliary differentiation have been described in the literature for over 100 years, first by histopathology, then with the addition of immunohistochemistry to identify markers of biliary and/or progenitor cell or stem cell differentiation. The presence of such markers is commonly associated with a worse prognosis.
- PLCs may have mixed 'hepatobiliary' features (i.e., biphenotypic [hepatobiliary] PLC) at many levels: at the gross tissue level, at the microscopic (cellular) morphologic level or by immunophenotypes. The latter group includes the 'pure' HCC with biliary findings by immunophenotype, pure CC with hepatocytic findings by immunophenotype, cholangiolocellular carcinomas and PLC without typical morphology but with mixed hepatobiliary immunophenotype.
- Classification of biphenotypic (hepatobiliary) PLC must always begin with the light microscopic morphologic assessments; immunophenotyping is reserved as a secondary means of identification.
- Molecular studies to date of microdissected biphenotypic (hepatobiliary) PLCs have shown surprisingly more homogeneity than the histologic and/or immunohistochemical phenotypes, and are closer to CC than to HCC.
- Many clinical, pathologic and molecular questions remain unanswered regarding these complex tumors; the first task to promote studies that can answer these questions is to establish consistent consensus terminology and method(s) for analysis.

Recent WHO classification for combined hepatocellular–cholangiocarcinoma and recognized stem cell subtypes has increased attention to such tumors; however, the resulting burst of reporting and research indicates that this classification, while provocative, is incomplete for description of the full array of primary liver carcinomas with biphenotypic (hepatobiliary) differentiation. We review the history of such lesions and consider the wider array of such tumors previously described. Mixed hepatobiliary phenotypes and immunophenotypes are found in individual tumors at the tissue level – with architectural and cytologic features

¹Department of Pathology & Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA

²Department of Pathology, Beaujon Hospital, 92118 Clichy, France

³Institute of Pathology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

⁴Departments of Pathology & Medicine, Mount Sinai Beth Israel Medical Center, Icahn School of Medicine at Mount Sinai, New York, NY 10002, USA

*Author for correspondence: Tel.: +1 212 844 1747; Fax: +1 212 420 4373; ntheise@chpnet.org

KEYWORDS

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- cholangiolocellular carcinoma • combined hepatocellular–cholangiocarcinoma
- hepatocarcinogenesis
- hepatocellular carcinoma
- liver progenitor cell
- liver stem cell • mixed hepatobiliary carcinoma
- primary liver carcinoma
- stem cell tumor • stemness

supportive of both differentiation states – and at the cellular level, with individual cells that display cytology of one cell type, but immunophenotypically showing mixed expression. Pathobiologic and clinical questions to be answered by future research are suggested.

Traditional teaching has it that there are two interconnected, but separate epithelial compartments in the liver: hepatocytes and cholangiocytes. Traditional teaching follows then that primary liver carcinomas (PLCs) reflect this dichotomy: hepatocellular carcinoma (HCC), and cholangiocarcinoma (CC), each with variants. We now know that hepatobiliary lineages are more flexible as displayed most prominently by ductular reactions that are present in liver injury of all forms in varying degrees. Just as these reactive lesions display a range of epithelial phenotypes between the hepatocytic and cholangiocytic differentiation, likewise, increasingly, similar diversity is being recognized in PLCs.

The other relatively uncommon kinds of primary liver cancers (e.g., mucinous cystic neoplasms, biliary intraductal papillary neoplasms) and special types of HCC (e.g., fibrolamellar carcinoma) have established diagnostic criteria that are readily applied. However, increasingly commonly, new forms of PLC are being seen worldwide: tumors of mixed hepatobiliary phenotypes and immunoexpression that are presenting with a wide range of clinical, imaging and histopathologic complexities and, thus confusion. These include not only the most well-described ‘combined hepatocellular–cholangiocarcinoma’ (cHCC-CC), but also several variants – including those with ‘stem cell features.’

In this review, we discuss and show examples of PLC with such mixed hepatobiliary features and explore many of the clinicopathologic questions they raise. Some of these tumors may indeed be new forms of PLC, related to the changing incidences of important, premalignant conditions; they may be a reflection of newly recognized PLC variants either because of detailed hepatobiliary immunophenotyping not previously available to pathologists or the newly sensitive imaging techniques in practice today. Though there are few absolute answers for the questions that recognition of these tumors raise, we take this opportunity to put the discussion first into historical context, then to detail currently published diagnostic categories, to summarize existing molecular data concerning the lesions and finally to point to diagnostic approaches needed to further refine our understanding of these tumors.

A final note concerns terminology: there is currently no accepted consensus terminology for these tumors. Indeed, expert liver pathologists in the field are not in full agreement. Some favor the broad term ‘biphenotypic PLC’; others argue that this may be imprecise, since there may be many different forms of biphenotypia outside the theme of this paper (e.g., HCC squamous, adenosquamous, sarcomatoid HCC). On the other hand ‘mixed hepatobiliary carcinomas’ is preferred by some. The argument against this term is the implication of specific differentiation when in fact, some of these tumors may appear to be monomorphic by light microscopy, and only the application of immunohistochemistry (IHC) highlights the true biphenotypic expression of the cells within the tumor. For this review, we chose to observe the inelegant, albeit unsimplified convention of this paper’s title: biphenotypic (hepatobiliary) PLC, abbreviated to b(HB)-PLC.

An established nomenclature – that is scientifically valid and clinically meaningful – still needs time to undergo community wide discussion, consensus building, understanding of pathophysiology and acceptance over the coming years.

Historical overview

During the last three decades, many publications, partly reviewed in [Table 1](#), have reported and analyzed liver tumors that are clearly to be considered b(HB)-PLC. The presence of tumors displaying features of both hepatocellular and biliary origin was described for the first time by HG Wells more than 100 years ago [1] although Allen and Lisa’s description of five cases is often cited as the first [2]. In their series, the tumors were noted as separate or contiguous tumors with differentiation typical for HCC and CC. Further description showed two separate tumors arising distantly in the same liver, two contiguous tumors intermingling at their borders and one single mass showing both hepatocellular and biliary features. In the milestone paper of Edmondson and Steiner published in 1954 [3], 4% of their entire series of PLC showed both hepatocellular and biliary differentiation. Their recommendation for terminology was ‘hepatobiliary cancers,’ but also recommended that they

Table 1. Literature review on biphenotypic (hepatobiliary) primary liver carcinomas.

Study (year), (n, if given)	Proposed nomenclature	New concept proposed	Ref.
Wells (1903), (n = 1)	Primary carcinoma of the liver	First description	[1]
Allen and Lisa (1949), (n = 5)	Combined liver cell and bile duct carcinoma	Three types: <ul style="list-style-type: none"> Two distinct tumors in different parts of liver Two separate tumors adjacent and intermingling One tumor with intimately associated cellular components due to origin from same focus 	[2]
Steiner <i>et al.</i> (1959), (n = 11)	Cholangiolocarcinoma	First description	[4]
Goodman <i>et al.</i> (1985), (n = 24)	Combined HCC-CC	Three types: <ul style="list-style-type: none"> Collision: separate HCC and CC in same liver Transitional: single tumor with features of both HCC and CC Fibrolamellar: primarily FL-HCC but with mucin-producing foci 	[5]
Maeda <i>et al.</i> (1995), (n = 36)	Combined HCC-CC	First survival rates after resection: combined HCC-CC has worse outcomes	[6]
Wu <i>et al.</i> (1996), (n = 290 HCC); Wu <i>et al.</i> (1999), (n = 64 from the previous series)	HCC with dual (hepatocellular/biliary) phenotype	Marked difference in survival: shorter if biliary features; expression of K14 means either derived from progenitor cells or regressed to the bipotential progenitor cell phenotype during carcinogenesis	[7,8]
Robrechts <i>et al.</i> (1998), (n=1)	Intermediate cell tumor; dense stroma	Possibly derived from progenitor cell; by light and electron microscopy, expression of hepatocyte and biliary keratins	[9]
Tickoo <i>et al.</i> (2002), (n = 27)	Combined HCC-CC	All had 'transitional' areas; utilized albumin ISH to prove HC differentiation; 96% showed both ISH and biliary keratins = 'biphenotypic' differentiation	[10]
Theise <i>et al.</i> (2003), (n = 4)	Combined HCC-CC	Tumors contained undifferentiated cells with morphological and immunohistochemical features of hepatic progenitor cells	[11]
Yano <i>et al.</i> (2003), (n = 26)	Combined HCC-CC	More similar to HCC, than to CC clinically except more advanced disease stage; survival worse than HCC and CC	[12]
Kim <i>et al.</i> (2004), (n = 54)	Primary hepatic carcinoma of intermediate (hepatocyte-cholangiocyte) phenotype	Morphologically intermediate can arise from progenitor cells	[13,14]
Kim <i>et al.</i> (2011)	HCC only: impact of stemness features	K19 correlated with clinicopathologic features of tumor aggressiveness, more invasive characteristics, compared with K19-negative HCCs through the upregulation of EMT-associated genes	
Cazals-Hatem <i>et al.</i> (2004), (n = 9)	Combined HCC-CC	Closer in mutations to CC than to HCC	[15]
Durnez <i>et al.</i> (2006), (n = 109)	HCC only: impact of progenitor cell component	>5% K19 positivity = higher recurrence rate and worse prognosis	[16]
Aishima <i>et al.</i> (2006), (n = 40)	Combined HCC-CC	Four subtypes based on the amount of CC component: outcomes relies on ≥60% CC component and/or sarcomatous component	[17,18]
Aishima <i>et al.</i> (2007), (n = 35)	Combined HCC-CC	HCC smaller than 3 cm in diameter can have biliary differentiation and prognosis is worse	
Komuta <i>et al.</i> (2008), (n = 30)	Cholangiolocellular carcinoma	Investigated relationship to HPCs, HCC and CC: CLC and K19 HCC have high homology: both are likely progenitor cell derived association with HCC and sometimes with CC	[19]
WHO (2010)	Combined HCC-CC	Unequivocal components of both HCC and CC in same tumor; does not include two separate tumors either in same liver or simply adjacent to each other; four types: classic or stem cell features (three subtypes: typical, intermediate and cholangiocellular)	[20]

?: Questioned if tumor was of progenitor cell origin; CC: Cholangiocarcinoma; CLC: Cholangiolocellular carcinoma; HCC: Hepatocellular carcinoma; HCC-CC: Hepatocellular-cholangiocarcinoma; HPC: Hepatic progenitor cell; IHC: Immunohistochemistry; ISH: *In situ* hybridization; PLC: Primary liver carcinoma.

Table 1. Literature review on biphenotypic (hepatobiliary) primary liver carcinomas (cont.).

Study (year), (n, if given)	Proposed nomenclature	New concept proposed	Ref.
Roncalli <i>et al.</i> (2010)	Three types: <ul style="list-style-type: none"> • HCC with stem/progenitor cell immunophenotype • Mixed hepatobiliary, classical type • Mixed hepatobiliary with stem/progenitor phenotype and immunophenotype 	All are subtypes of 'progressed HCC' <ul style="list-style-type: none"> • >5% of tumor cells express stem/progenitor IHC markers: K19, EpCAM, CD133, etc.; not recognizable by H/E ? origin from progenitor/stem cell or dedifferentiation from mature hepatocytes; increased likelihood recurrence, resistance to treatment, mets • Uncommon; combined HCC-ChC; can be detected by HE and confirmed with IHC:HC component by HepPar, pCEA, CD10; ChC by K7, K19; mucin for mature biliary component; may have intermediate components at interface: K7, 19, CD56, c-kit, EpCAM (derivation from stem/progenitor; px is poorer than usual progressed HCC) • Rare; majority of tumor is stem/progenitor or intermediate; other names: hepatic stem cell; PLC of intermediate phenotype; cholangiolocellular [14,22–25]; Laennec society proposed name: mixed hepatobiliary carcinoma with stem/progenitor cell features <p>Px uncertain due to small numbers Large-sized CLC had high recurrence Intermediate ca survival was intermed b/w HCC and CCA</p>	[21]
Woo <i>et al.</i> (2010)	HCC, CC and combined HCC-CC	By gene expression studies, distinct tumors but some HCC close to CC	[22]
Ikeda <i>et al.</i> (2013), (n = 36)	Combined HCC-CC	Added DLK-1 to IHC panel; separated cases into groups A and B according to < or >5% stem cells cases with >5% stem cells had worse prognosis; DLK-1 was usually pos in those cases	[26]
Akiba <i>et al.</i> (2013), (n = 54)	Combined HCC-CC	To confirm the classification of four subtypes of WHO, no effect of this classification	[27]

?: Questioned if tumor was of progenitor cell origin; CC: Cholangiocarcinoma; CLC: Cholangiolocellular carcinoma; HCC: Hepatocellular carcinoma; HCC-CC: Hepatocellular–cholangiocarcinoma; HPC: Hepatic progenitor cell; IHC: Immunohistochemistry; ISH: *In situ* hybridization; PLC: Primary liver carcinoma.

be contained within the group of HCC as they were mostly observed in men on a cirrhotic background, as with classical HCC. This combined type of cancer was considered by the authors as 'a problem difficult to solve at the present', a relevant opinion today, 60 years later.

The first part of the 20th century was the era of histopathology alone and extensive descriptions of the histological and cytological characteristics of b(HB)-PLC were supported by numerous photomicrographic figures. Since that time, a diagnosis of b(HB)-PLC requires the unequivocal histological presence of both hepatocellular and cholangiocellular elements within the same tumor, as stated by the 2000 WHO classification of the digestive tumors [28].

With the developments of IHC new concepts emerged. In 1985, Goodman [5] reported the experience of the Armed Forces Institute of Pathology with 24 cases and classified them in three categories, only slightly modified from those of Allen and Lisa. The first was termed the 'collision type,' corresponding to the

coincidental occurrence of both HCC and CC, distinctly separate, in the same liver. The second was the 'transitional type,' with intermediate differentiation and areas of transition between HCC and CC. The third was the 'fibrolamellar type,' resembling fibrolamellar HCC, but containing pseudoglands producing mucin. α -fetoprotein, a marker of hepatocytic differentiation, and staining for 'keratins' (polyclonal antikeratin antibodies unspecified in the study, but probably against those typically expressed in cholangiocytes alone, such as K7 and K19), markers of cholangiocytic differentiation, were both found to be expressed in these mixed tumors collectively termed 'combined hepatocellular cholangiocarcinomas' according to the authors.

IHC was subsequently used more and more in order not only to help in diagnosing PLCs of all kinds and to distinguish between them and poorly differentiated metastatic carcinomas, but also to assess their origin(s), and to study and subtype HCC and, to a lesser extent,

CC [6–7,29–37] to yield prognostic information. It has since been shown that approximately 25–30% of HCC diagnosed by histology show an expression of biliary markers, such as K7 and/or K19 and this has been correlated to a worse prognosis [7,13,16–18,38]. Furthermore, in a study from 2002, Tickoo *et al.* investigated 27 mixed tumors with IHC for biliary markers and with *in situ* hybridization for albumin mRNA, a specific marker for hepatocyte differentiation [10]. A positive albumin signal was found in 96% of PLCs and the authors concluded in favor of a biphenotypic differentiation.

Because of these results and the developing evidence for the existence of human hepatobiliary stem cells during the same era [39–42], the idea of a stem/progenitor cell origin for b(HB)-PLC gained increasing traction. The first direct evidence of this possibility was in a collection of four cases of cHCC-CC ‘with stem cell features.’ [11]. In all four cases, there were populations of small cells, with high nuclear:cytoplasmic ratio, dense nuclear chromatin, arrayed around nests of hepatocytic and/or cholangiocytic cells. In all of these cases cells of intermediate morphology lay between these stem cell-like components and the more differentiated components, suggesting a visible maturation lineage.

Furthermore, different authors using different immunomarkers such as K19, K14 (cluster of differentiation) CD117/c-kit or EpCAM (epithelial cell adhesion molecule) identified progenitor cell expression in b(HB)-PLC and/or in otherwise typical HCC [8,13–14,16,23–24,36]. It was postulated that HCC in which a subpopulation

is found expressing K19 arise from progenitor cells [16,25] or result from dedifferentiation or transdifferentiation of tumoral hepatocytes yielding expression of ‘stemness’ features. This characteristic has always been associated with a worse prognosis [13,22].

Studies in the past decade undertaken to search for a relationship between b(HB)-PLC and classical CC or HCC have shown contradictory results, probably related to the differing terminology as well as diagnostic criteria used by the different investigators [12,15,22,43–44]. Moreover, the spectrum of b(HB)-PLC was expanded with reports of new histological features, associated with progenitor cell IHC markers. An example is the most recently proposed tumor. In 2001, Shiota *et al.*, [45] reported a series of cholangiolocellular carcinoma (CLC), a particular type of PLC that had been described initially by Steiner in 1959 [4], but only by routine histochemical evaluation. CLC are usually [19], but not always [46] associated with HCC in continuity or elsewhere within the liver. These may or may not also contain overt CC. The characteristic histologic feature is anastomosing regular ductules without lumina resembling canals of Hering in a dense, sclerotic stroma in which the epithelial component resembles the benign counterpart, that is, the ductular reaction [33]. In fact, this appearance itself led Shiota *et al.* [45] to consider a possible stem cell origin for the tumor. Detailed studies by Komuta *et al.* in a study of 30 cases of CLC by morphology (both light and electron microscopy), IHC and molecular biology strongly support a stem/progenitor cell origin for CLC [19].

Table 2. Descriptive versus WHO classification of primary (mixed hepatobiliary) liver carcinomas.

Descriptive classification	2010 WHO classification
Stem cell/biliary marker positive (particularly K19+) ‘pure’ HCC	HCC
HCC, small cell subtype	HCC
HCC with stem cell features and IHC markers	Unclassified
Hepatocyte marker positive pure CC	Unclassified
cHCC-CC	cHCC-CC
cHCC-CC with ‘typical’ stem cell features	cHCC-CC, typical stem cell subtype
cHCC-CC with ‘intermediate’ features	cHCC-CC, intermediate stem cell subtype
Cholangiolocellular carcinomas with HCC component	Unclassified
Cholangiolocellular carcinomas with cHCC-CC	cHCC-CC, cholangiolocellular subtype
Primary liver carcinoma with mixed hepatobiliary IHC features (non-HCC, non-CC)	Unclassified

CC: Cholangiocarcinoma; cHCC-CC: Combined hepatocellular–cholangiocarcinoma; IHC: Immunohistochemistry.

As a consequence of such IHC studies, criteria to accurately diagnose b(HB)-PLC became confused. Are morphologically pure HCC or CC that display immunophenotypes of either stem, progenitor or mature epithelial cells of the ‘other’ cell type actually a different tumor class? Or are they merely a subclass of the pure tumors with different prognostic markers (similar to the prognostic markers in clinical breast oncology)? Do overt histologic ‘stem cell features’ have clinical significance that warrants a separate diagnostic category? Furthermore, if stem cell features are only apparent by immunostains, should these be considered ‘stem cell tumors’? These and other important questions that arise from them are undergoing investigation, but currently there are no consensus answers.

The clinical impact of these reports led to a revision of the classification of such tumors by the WHO in the 2010 publication [20]. In this publication, for the first time, there is an entirely separate chapter to explore b(HB)-PLC (therein referred to as ‘combined hepatocellular–cholangiocarcinoma’). This redefinition is preliminary, inherently reflecting our early, incomplete understanding and describes two different forms: the classical cHCC-CC containing histologically typical areas of HCC together with those of CC within the same tumor; and the cHCC-CC with stem cell features. In the WHO 2010 chapter, this latter subtype is further divided into three categories: typical (nests of mature hepatocyte-like tumor cells surrounded by small cells exhibiting IHC markers of progenitor cells), intermediate (small homogeneous tumor cells comprising most of the tumor that are intermediate between hepatocytes and cholangiocytes and showing IHC markers of both) and CLC [20]. In all of these groups, the histologic assessment is diagnostic, with IHC playing only a supportive, confirmatory role in tumor classification.

The revised WHO classification was utilized recently by Akiba *et al.* [27] in a study of 54 cases; the authors underlined the complexity of the histological features in b(HB)-PLC named and classified according to the 2010 WHO guidelines (i.e., cHCC-CC with or without stem cell features) and the difficulties in its application, as only 1 of the 54 cases fit criteria of ‘typical.’ The difficulty was also emphasized by the study of Ikeda *et al.* [26] who recommended evaluating the amount of stem cell features (as a percentage) in order to predict the prognosis.

Sasaki *et al.* [47] also highlighted the variability of stem cell features in any given b(HB)-PLC, but also pointed out that different ‘stem cell feature’ subtypes were often present in the same tumor and, moreover, could be identified in many classical HCC with careful attention. Significant clinicopathologic associations were found with the presence of stem cell features [47].

b(HB)-PLC are frequent in cases of preexisting liver disease, especially HCV and advanced fibrosis [33,48]. These tumors have also been reported in persistent or recurrent tumors after transarterial chemoembolization. This outcome of treatment in some cases may potentially impact the prognosis of patients after liver transplantation [49,50] and raises again the question of transdifferentiation versus stem/progenitor cell origin. However, in clinical practice, these tumors are being noted with increasing frequency in nondiseased liver as well [EM BRUNT, UNPUBLISHED DATA].

Importantly, new, cutting edge work by Holczbauer *et al.* [51] confirms that human HCC cell lines showing hepatocytic features can change genetic programming to become cancer stem cells with bipotent hepatobiliary differentiative potential. As Zucmann-Rossi and Nault pointed out in an accompanying editorial [52], this work confirms that PLC may variously derive from malignant transformation of adult hepatocytes, liver stem/progenitor cells and fetal hepatoblasts, all of which can give rise to the mature-appearing cells of these tumors as well as to cancer stem cells within them that sustain tumor self-renewal and resistance to treatment. Raggi *et al.* [53] have further established that DNA methylation is a key epigenetic regulatory mechanism determining the pool of PLC cancer stem cells.

Histologic & immunophenotypic diversity of b(HB)-PLC

Leaving aside variations of straightforward ‘pure’ (i.e., typical) HCC and pure CC, which are not reviewed, we summarize the histologic and immunophenotypic features of the various forms of b(HB)-PLC reported to date (Table 2). Two ‘broad’ groups of tumors show a mixture of biphenotypic (mixed hepatobiliary) phenotypes expressed at either the tissue or cellular levels.

- Tumors with biphenotypic/mixed hepatobiliary features at the tissue level show co-mingling of malignancies of the two different

cytologic and architectural features of HCC and CC, sometimes with cells of intermediate cytology/immunophenotype between them. These are commonly recognizable by light microscopy.

- Tumors with biphenotypic/mixed hepatobiliary features at the cellular level are not often recognized as such by light microscopy. These are largely monomorphic, and may be of varying differentiation with or without a stromal component. However, with use of IHC, these tumors display mixed immunophenotypes either focally or diffusely within the tumor. These may be ‘traditional’ HCC or CC with immunostaining for markers of the other cell type or monomorphic tumors that cannot be classified histologically as either type, but in which immunophenotypic evidence is demonstrated for combined hepatobiliary features at the cellular level.

Figure 1 is a schema to organize our knowledge of PLC. The areas denoted within each segment are not intended to reflect prevalence or incidence of each type within the clinical spectrum of PLC, although obviously straightforward HCC and CC are the most common. The schematic is meant as a device for organizing our knowledge of these tumors and as a stepping stone toward development of a robust, clinically relevant nomenclature.

Immunomarkers that are commonly used or that have been used in research efforts will also be discussed in each section. A variety of hepatocyte markers, cholangiocyte markers and stem cell markers are recognized, some of which are common in many clinical laboratories, others of which are uncommon outside of research settings. These are collectively highlighted in **Table 3**. It is gratefully acknowledged that that this table was generated through

Figure 1. Schematic diagram representing our present interpretation of primary liver carcinoma. The areas assigned to each segment area are not reflective of prevalence or incidence of each type.

Table 3. Immunohistochemical markers for evaluating differentiation in biphenotypic (hepatobiliary) primary liver carcinoma.

	Hepatocyte differentiation	Biliary differentiation	Stem cell differentiation with stem cell morphology	Stem cell differentiation without stem cell morphology
Common markers (use as panel or use in sequence until a positive result is obtained)	HepPar1, Arginase-1 , canalicular staining with CD10 , canalicular staining with pCEA, AFP	Keratin 7, Keratin 19 , cytoplasmic staining for CEA (pCEA or mCEA), EMA (Muc-1)	Keratin 19, NCAM (CD56), EpCAM, EMA (Muc-1), c-kit (CD117)	No available common markers
Experimental markers (supplemental to 'common markers')	Nuclear staining for HNF4a	Nuclear staining for Sox-9	Nestin, Keratin 14, CD133 , nuclear staining for Sox-9	Nuclear staining for Oct-4 Nuclear staining for Nanog Nuclear staining for Sall-4

Bold text highlights the antibodies to be used.
For each differentiation state within a b(HB)-PLC – hepatocellular, biliary and stem cell – several markers are available; however, availability or cost may limit the utility of such large panels. On the other hand, lack of consistency between studies and/or centers can result in data that cannot be easily compared between different studies. To limit these difficulties, the panels in the upper row are recommended to be used either: as a panel with all antibodies applied to every case; or in a sequence, top to bottom, stopping with the first positive antibody. The markers in the bottom row are recognized as having utility for studying b(HB)-PLC, but are considered supplemental to the primary panels listed first. It is emphasized that morphology is primary in the assessment of subpopulations and immunophenotyping is secondary. In particular, stem cell components are assessed by routine light (H&E) microscopy before confirming by immunohistochemistry.

group discussion and with approval from the members of the 2013 meeting of the Laennec Hepatopathology Society held in Singapore (Aileen Wee, MD, USA host).

• **Pure HCC with biliary (stem cell) immunophenotype**
Hepatocyte-like tumor with stem cell immunophenotype

Keratin 19 positive HCC is defined as a tumor with purely typical features of hepatocellular differentiation and which expresses keratin 19 in ≥5% of its cells; these cells are usually or mostly hepatocyte-like in morphology (Figure 2A & B). Such tumors (with the arbitrarily selected 5% cut off) have been shown to have a poorer prognosis with diminished disease free survival, increased likelihood of metastasis, greater chemoresistance and diminished life expectancy [16,54]. Some have argued that this indicates that it should be treated as a separate, distinct subclass of HCC [36]; however, one can also argue that it should merely be considered a prognostic indicator. The latter position is supported by the apparent emergence of keratin 19 expression or even overt cHCC-CC in recurrent HCC that previously had undergone ablative therapy [55]. Thus, rather than a distinct type of b(HB)-PLC, K19-positive HCC may perhaps better be considered in the spectrum of HCC itself. Future prospective clinical studies may be of value in further addressing these questions, keeping in mind that of the biliary markers K19 may also correlate with other marker expression of biliary and/or stemness antigens such as CD56, EpCAM and CD133.

Isolated tumor cells with stem cell morphology confirmed by stem cell immunophenotype

Among the predominant hepatocyte-like cells in a pure HCC, are small, round to cuboidal, cells phenotypically similar to those of the canal of Hering in normal livers. Such stem cell-like tumor cells may be recognized by immunostaining for K19, CD56, EpCAM, CD133 and/or CD117/c-kit (at least). They are scattered individually or in clusters within the tumor (Figure 2C & D) or they may distribute along stromal boundaries either at the edges of the HCC or, more frequently along fibrous bands, within the tumor (Figure 2E & F) [11,55–56]. While the marker immunostaining is usually sharply defined and limited to these smaller cells, morphology may reveal a pattern of cellular changes suggestive of a maturation lineage: from small, marker-positive, stem cell morphology to overt, marker-negative hepatocyte morphology. The frequent juxtaposition of the small cells against stroma is tempting to consider as a malignant variant of an hepatobiliary stem cell niche, complete with cell:matrix localization that would potentiate interactions. The clinical implications of these stem cell-like findings, however, remain uncertain.

• **Pure CC with hepatocytic immunophenotype**

The presence of some form of hepatocytic markers in CC has not been well studied. Hepatocyte in paraffin-1 (HepPar1) and arginase-1 have both been reported to be expressed in a minority of CC that are otherwise devoid of hepatocyte

morphology [57,58]. There are no reports to suggest that such hepatocytic expression indicates CC with better prognosis; clearly these are otherwise typical CC in terms of their biology. It is also uncertain whether this should be taken as evidence of stem cell origin of some CC or redifferentiation reflecting the flexible gene expression of non-neoplastic hepatobiliary lineages. It is worth noting, after all, that one may sometimes observe hepatocytes within non-neoplastic bile ducts.

• cHCC-CC

By initial H&E evaluation, these tumors will show two intermixed cytologic/architectural patterns (**Figures 3–5**): areas suggestive of CC, for example, gland formation or strands of small cells with little cytoplasm or angulated ‘glands’ with poorly formed lumina, all of which usually occur in dense acellular stroma, and of HCC, that is, variably pleomorphic hepatocyte-like cells often with growth showing distortions of normal liver parenchyma, such as thickened trabeculae or massively dilated canaliculi (‘pseudoglands’ or ‘pseudoacini’). The HCC component may have any of the common findings in pure HCC, such as steatosis, ballooning with Mallory-Denk bodies and intercellular or intracellular bile accumulation.

Where the CC and HCC components meet the change may be abrupt or there may be transitional cytologic, immunophenotypic or architectural features. For example, there may be pseudoacinar formations lined not by hepatocytes, but by cuboidal, cholangiocyte-like cells, there may be what appear to be malignant variations of ductular reactions in which immunophenotyping demonstrates a lineage-like range of cytology and immunophenotypes ranging between cholangiocyte-like and hepatocyte-like. These transitional areas may include not only the epithelial portions of the tumor, but also the amount and nature of the stroma; dense hyaline stroma readily characterizes many types of cholangio/cholangiolo/stem/progenitor cell carcinomas. These areas of dense hyalinized stroma may also include central areas of nearly ‘empty’ stroma in which epithelial components are absent and only ghost-like spaces reminiscent of the structures remain.

By convention, fibrolamellar carcinoma is not considered such a tumor even though phenotypic markers that could be utilized to argue in favor of b(HB)-PLC and a unique ‘lamellar’ fibrous

Figure 2. ‘Pure’ hepatocellular carcinoma with biliary (stem cell) immunophenotype.

(A & B) Hepatocyte-like tumor cells with stem cell immunophenotype: the features are those of hepatocellular differentiation with keratin 19 expressed in $\geq 5\%$ of the cells (in this case, 80%; hematoxylin and eosin [H&E] and K19, 15-times). (C & D) Hepatocyte-like tumor cells containing isolated tumor cells with stem cell morphology confirmed by stem cell immunophenotype, in this case, K19 (expressed in 5%; H&E and K19, 10-times). (E & F) Hepatocyte-like tumor cells comprise the majority of the tumor, but tumor cells with stem cell morphology can be seen at (E) the stromal interface or (F) within the hepatocellular carcinoma (H&E, 10-times and 15-times, respectively).

Figure 3. Biphentotypic (hepatobiliary) primary liver carcinoma. Immunohistochemistry highlights the mixed hepatobiliary nature of the tumor. (A) By hematoxylin and eosin stain, the tumor has an appearance of a monomorphic tumor both architecturally with cords and nests of tumor cells in a dense stroma, and cytologically with very little tumor cell pleomorphism (hematoxylin and eosin, 15-times). (B) Immunohistochemical detection of HepPar-1 in scattered tumor cells highlights foci of tumor cells with hepatocyte differentiation (10-times). (C) Immunohistochemical detection of K19, a biliary or stem cell marker, is noted diffusely and uniformly strongly throughout the tumor (10-times).

stroma are present [59]. It is excluded from consideration in this group of tumors because neither the clinical presentation (typically in younger

individuals and without association with chronic liver diseases) nor its clinical outcomes are similar to b(HB)-PLC being discussed herein. Likewise, the still rarer variant of scirrhous HCC, despite similar mixed immunophenotypes, is not included in this category [58].

• cHCC-CC with stem cell features

This is a heterogeneous group of tumors that have been included in the current WHO tumor classification; however, that classification is imperfect in many respects, though it has served to raise interest in and stimulate research about these lesions and others described in this paper. Those with the most overtly stem cell-like features were the first ones described [11], and were thus designated ‘typical’ in the WHO classification, though subsequent series make it clear that these are uncommon at best [26–27,47]. What makes them so evocative is the presence of small stem cell-like cells that are contiguous with regions of HCC and with regions of CC, but also surrounding islands of well-differentiated hepatocytes without significant cytologic atypia, simulating a ductular reaction surrounding a cirrhotic nodule, as though these presumably neoplastic cells are capable of giving rise to ‘normal’ hepatocytes. The striking appearance of these areas even without any immunohistochemical staining is what drew attention to the lesions in the first place [11].

Additionally, there are cHCC-CC in which the majority of tumor cells show ‘intermediate’ morphology or what may appear to be ‘small hepatocytes’ [14]. The pattern may include trabeculae or elongated, ill-defined gland-like structures in dense stroma, but true gland formation with well-defined lumens and mucin production are absent. These cells stain for a combination of hepatocyte and cholangiocyte makers, and often for CD117/c-kit (Figure 6).

Though the current WHO classification includes this group of historically well-recognized tumors as a stem cell subtype of cHCC-CC, its relative frequency compared with those other types and its longstanding recognition as a separate category of tumor perhaps still merits it being separated as a distinct subtype. When the cholangiolocellular component predominates in the tumor, rather than as appearing as a small subtype within a larger, different tumor mass, this designation is warranted. Many of these, though not all, will have either a clear HCC component or a clear CC component or both [19,46]. These recent data indicate, therefore,

Figure 4. Biphenotypic (hepatobiliary) primary liver carcinoma. (A) By routine hematoxylin and eosin staining, it is apparent there are morphologic features of both hepatocellular carcinoma and cholangiocarcinoma in this tumor. The former is appreciated by trabeculae, and the latter by glands (hematoxylin and eosin 15-times). (B) Immunohistochemical reactivity of the glandular components is strong with K19; this stain also highlights the negative reactivity of much of the tumor (15-times). (C) Immunohistochemical detection of pCEA is in the canalicular components of the hepatocellular carcinoma, and lining of well-formed glandular components. No cytoplasmic reactivity is appreciated, as would have been expected in a cholangiocarcinoma (15-times). (D) Immunohistochemical detection of CD10 is similar to pCEA and is restricted to the canalicular components of the hepatocellular portions of the carcinoma (15-times).

Figure 5. Combined hepatocellular–cholangiocarcinoma. (A) By hematoxylin and eosin (H&E), there is an hepatocellular carcinoma (HCC) area showing hepatocyte-like tumor cells associated with tumor cells with stem cell morphology (H&E, 100-times). (B) A cholangiocarcinoma (CC) area shows cuboidal cells arranged in gland formation associated with an acellular fibrous stroma (H&E, 100-times). (C) This photomicrograph shows a transitional area with closely approximated, small homogeneous tumor cells intermediate between hepatocytes and cholangiocytes (H&E, 100-times). (D) Immunohistochemical detection of glypican 3 is reactive in HCC area (200-times). (E) Immunohistochemical detection of K7 is reactive in CC area (200-times). (F) On macroscopic examination, this primary liver carcinoma appears relatively monomorphic, with features that are similar to CC than to HCC. These gross features include the white-tan color, firmness of the tumor and lack of obvious necrosis or bile.

that not all of these tumors, regardless of the recent WHO classification, are in fact cHCC-CC. It is felt that the dominant, cholangiolocellular compartment recapitulates features of the normative canal of Hering/ductule and perhaps the name should be limited to those tumors in which this compartment is well differentiated

with minimal pleomorphism; cases in which higher grade, pleomorphic variants mimic this pattern are perhaps not the same entity. This bland tumor can be a ‘mimic’ of ductular reaction [33] in cirrhotic septum on a small biopsy, but without the expected inflammation and vascular spaces of shunting present in the latter.

- **PLC without typical morphology but with mixed hepatobiliary immunophenotype**

These apparently novel PLCs which fit neither into the general categories of HCC, CC or cHCC-CC have most dramatically made the case that a tripartite division of hepatobiliary malignancy is insufficient. These are tumors whose histomorphology differs from these three well-established PLCs, whose immunophenotypes often show considerable diversity both with heterogeneous staining for different hepatobiliary markers, but also including, occasionally, nonhepatobiliary antigens, for example, those associated with squamous or endocrine or sarcomatous differentiation. Behavior of these tumors is not certain given that they are not in fact a uniform class, though the significant biliary features (either as overt CC, IHC expression of biliary/stemness markers or in

behaviors such as perineural and lymphatic invasion) suggest that their behavior will be likely more akin to CC than to HCC.

An example of such a tumor is shown in **Figure 7**. This figure should not be taken to be a new and specific tumor type; it is just one example of a quite heterogeneous group of previously poorly recognized or described b(HB)-PLC. Indeed, whether these represent a 'new' kind of PLC or have been present, but only now being recognized, due new emphasis on stem/progenitor cell interplay in hepatic neoplasia and more extensive immunostaining available globally, remains to be determined.

Molecular aspects of b(HB)-PLC

In recent years, molecular approaches dedicated to human liver neoplasms have provided in depth

Figure 6. Cholangiolocellular carcinoma. (A) This is a needle biopsy that illustrates the bland nature of the proliferative tumor cells in cholangiolocellular carcinoma. The tumor has the low power appearance of ductular reaction in cirrhotic septa, but the typical vascular proliferation and inflammation of septa are notably absent (hematoxylin and eosin four-times). (B) Immunohistochemical detection of HepPar1 shows occasional positivity in this small area of the tumor (20-times). (C) Immunohistochemical detection of K7 highlights the diffuse positivity of this marker of mature biliary cells and also shows the anastomosing nature of the tumor tubules (10-times). (D) Immunohistochemical detection of CD56 is also diffusely positive; this marker can be positive in a ductular reaction in certain types of reactive cases, as well as in certain cholangiolocellular carcinoma (10-times).

Figure 7. Primary liver carcinoma without typical morphology but with mixed hepatobiliary immunophenotype. (A & B) Hematoxylin and eosin photomicrographs illustrate small and large glandular-like formations with tumor cells of little pleomorphism, high N:C, vacuolated nuclei embedded within varying degrees of stroma (20-times). (C & D) Immunohistochemical detection of HepPar and CA19-9, respectively, show scattered reactivity, highlighting 'biphenotypia' (20-times). (E & F) Immunohistochemical detection of K19 and EpCam, respectively highlight 'stem-ness' (20-times).

insights into biological behavior and pathogenesis, in both benign and malignant hepatocellular tumors. Whether morphological heterogeneity, a critical hallmark of b(HB)-PLC as emphasized above, reflects molecular heterogeneity has not been specifically addressed. Molecular studies focusing on such complex tumors would ideally include the preliminary step of tumor microdissection, in order to obtain gene expression profiles according to the different, distinct morphologic patterns. Nevertheless, based on the few studies of a relative low number of b(HB)-PLC, the following relevant information is known to date.

- **b(HB)-PLC: a clonal tumor**

HCC and CC areas from a series of 11 b(HB)-PLC were subjected to laser microdissection for clonal analysis by studying allelic status of a number of selected chromosomes' arms [60]. Unexpectedly, loss of heterozygosity (LOH) at multiple chromosomal loci were identified in all tumors with three different LOH patterns, including biclonal neoplasms in three cases, consistent with the previously so-called collision tumors, and single clonal neoplasms in eight cases, including four with homogeneous genetic changes and four with genetic divergence. The common allelic losses shared by both tumor components were strongly suggestive of a single clonal derivation. Although performed on a limited number of cases, these clonal data are critically relevant, highlighting the relative discordance between morphologic heterogeneity and molecular homogeneity.

- **Molecular overlap of b(HB)-PLC with cholangiocarcinoma**

There are several studies of chromosomal changes and gene expression patterns in b(HB)-PLC. To note, none have included a preliminary tumor microdissection step. Thus, a series of 15 b(HB)-PLC (specifically typical cHCC-CC) was screened for LOH using 400 microsatellite markers, p53 and β -catenin mutations and compared with three collision tumors, nine CC and a set of 137 HCC [15]. A high level of chromosome instability was found in both CC and cHCC-CC with recurrent specific LOH identified at 3p and 14q, more frequently observed in both CC and cHCC-CC compared with HCC. According to these results, the authors suggested that cHCC-CC is genetically closer to CC than HCC. This was further confirmed in a recent study, aiming to investigate the molecular pathways associated

with pathogenesis of CLC. To address this issue, a genome wide transcriptional analysis was performed in a set of 20 CLC [43]. The comparative analysis with CC and HCC demonstrated that CLC clustered with CC by hierarchical analysis while HCC and normal liver samples clustered together and CLC displayed biliary differentiation gene signature in parallel with downregulation of the hepatocyte differentiation program. These data are consistent with previous immunophenotypic analysis performed in a set of 30 CLC. As noted above, in this series, all cases had HCC-like areas, while 63% had CC-like areas. Strong positivity of mature cholangiocytic markers (K7 and K19) was present in all cases in both cholangiolocellular areas and areas of overt CC, when present, while hepatocellular markers (HepPar1 and canalicular staining with anti-CD10 and pCEA antibodies) were positive in HCC-like areas [19].

- **Molecular pathways involved in b(HB)-PLC**

Notably, TGF- β -signaling pathway has shown to be specifically deregulated in CLC, with a significant number of upregulated fibrosis-associated genes, including genes encoding profibrogenic cytokines, extracellular matrix components and remodeling enzymes [43]. Such molecular features might be expected in CLC which is usually rich in stroma. Whether a similar extracellular matrix signature could be a common feature of other b(HB)-PLC subtypes certainly merits investigation. Interestingly, increase in TGF- β -signaling pathway expression, including TGF- β , TGF β R1 and Smad4, has also been reported in a subset of HCC characterized by the presence of prominent fibrous stroma, namely 'scirrhous HCC' [61]. In addition, scirrhous HCC displayed a CC-like gene expression trait that could be at least partly attributed to the presence of the tumoral fibrous stroma [61].

- **Nomenclature & research methodologies**

The 2010 WHO designation of cHCC-CC with and without stem cell features, and the three subtypes of 'cHCC-CC with stem cell features,' has several organizational benefits: defining and cataloging some of the diversity of b(HB)-PLC, recognizing that the CLC is likely a progenitor cell carcinoma with poor clinical behavior and 'grouping' of otherwise disparate primary tumors within the liver by immunophenotype and poor clinical outcomes. However, the attempted brevity reflected by these subtypes does not, in fact, encompass all that one encounters either in

practice or in experimental settings. The previously referenced study by Akiba *et al.* [27] of b(HB)-PLC has shown the challenges of utilizing this WHO classification. While the careful delineation of the subgroups by IHC analysis documented the rarity of the typical subtype compared with intermediate and cholangiolocellular subtypes, the categorization led to no significant differences in clinical outcomes between the groups or subgroups. Furthermore, the authors noted that while confirmation of progenitor cell phenotype could be made by their study, the spectra of histology was still not entirely met by the WHO criteria and more work would be beneficial to further understand these tumors. The study by Sasaki *et al.* [47] further emphasizes these problems by showing that many b(HB)-PLC display more than one subtype of stem cell features.

Thus, it is clear that for further, efficient development of a rational, clinically useful classification scheme for b(HB)-PLC, a uniform approach to study would be of benefit. At a recent meeting of attending members (see Acknowledgements) of the Laennec Hepatopathology Society 2013 Meeting held in Singapore consensus regarding nomenclature was judged premature, but a proposed consensus regarding efficient research standards was achieved. These recommendations were:

- Multiple areas of each tumor deserves evaluation in order to be appropriately classified, based on:
 - Radiologic evaluation and subsequent careful radiologic-gross pathologic correlation at the bench, or
 - Sampling of grossly different areas of tumor at the time of gross description or
 - Laser capture microdissection from slides.
- Identification of subpopulations within a tumor begins with morphologic assessment for hepatocyte-like (large cells, arrayed in pseudoglands, thickened trabeculae or round, tubular structures, surrounded by CD34+ endothelium); cholangiocyte-like (forming glands, with or without mucin production) and stem-like (small cells round, oval or cuboidal cells with high nuclear:cytoplasmic ratio, hyperchromatic nuclei).
- Immunophenotypic evaluation follows with standardization of immunohistochemical

markers for hepatocellular, cholangiocellular and stem/progenitor cell differentiation (Table 3). To promote uniformity of approach between research laboratories and to foster data sets that can be easily compared across geographic and institutional boundaries these antibodies are listed, and ranked in a proposed sequence of use. Thus, the markers within a group may all be utilized at the same time or utilized sequentially until one is positive. If all are completed and none is positive, the recommendation is to move to the next set. One deviation from the above ‘morphology before immunophenotype’ proposal is the cautious, provisional recognition that any morphology with positive nuclear Oct 3/4, Sox 2, Nanog or Sall4 would also represent a stem cell immunophenotype. The members also discussed and agreed that any other antibodies one chose to use in any of the above three categories are optional, including, for example, K14 and c-kit (CD117).

Conclusion

The identification and analysis of b(HB)-PLC raise many questions crucial for understanding minimum diagnostic criteria (clinically, radiologically and pathologically), prognostic assessments, development of appropriate treatments and monitoring the possibly changing epidemiology of PLC worldwide. These include (but are not limited to) the following questions:

- These tumors may be seen with and without chronic liver disease: are they becoming more common? Or were they simply ‘underappreciated’ or ‘underevaluated’ in prior studies?
- If they are more common is this due to changing epidemiology of underlying chronic diseases (e.g., the denouement of HCV disease and, subsequently, increased prevalence and incidence of malignancy in NAFLD)? Could the increasingly common setting of post-treatment, but unremitted liver disease be creating a new tumor environment in which b(HB)-PLC are more likely to emerge (e.g., patients with unsuccessful antiviral regimens)? Is the increasing prevalence of mixed diseases (e.g., viral hepatitis and NAFLD, mixed viral infections) related to b(HB)-PLC development?
- How much (if at all) do the clinical features and natural histories of these different kinds of

b(HB)-PLC differ from their single phenotype, 'classic' counterparts? Are stem cell features a curiosity only relevant for understanding hepatocarcinogenesis or do they have true prognostic and/or treatment implications?

- Given the heterogeneity within b(HB)-PLC what is sufficient tumor sampling? Is single needle core sampling of tumors with suspiciously varied radiographic appearance sufficient? Or do separate biopsy specimens need to be obtained from areas with different radiologic appearances?
- What are the minimal criteria for including immunostains for subclassification? Further, what will be the role for the more sophisticated molecular studies that are becoming increasingly available?
- Does treatment of pure HCC sometimes lead to emergence of b(HB)-PLC or are recurrences of b(HB)-PLC post-treatment of a classical HCC evidence that it originally was, in fact, an undiagnosed b(HB)-PLC, prior to treatment? Or are both pathways for post-treatment tumor appearance possible?
- What is the post-treatment history of different types of b(HB)-PLC?

The literature cited and observations described in this review as well as the attempts to answer questions such as those above will vastly extend our knowledge of the biology of hepatocarcinogenesis. In terms of clinical practice, however, the growing acceptance of b(HB)-PLC poses major challenges to all professionals involved in the care of patients with liver cancer: imaging and interventional radiology colleagues, clinical oncologists, liver surgeons and transplant surgeons as the 'natural history' of this tumor phenotype is still being actively studied. As demonstrated, these tumors are certainly challenging for pathologists in terms of histological diagnosis and nomenclature.

Future perspective

We are continuing and also at the beginning of a fascinating journey for all those clinicians

and scientists interested in cancers that arise in the liver. It is exciting to recognize that all of our eventual progress will come from extensive interdisciplinary and multi-institutional research efforts. In the coming years we can look forward to greater understanding as to the histogenesis and pathobiology that lead to these complex forms of hepatobiliary malignancy. The first achievement will be a consensus terminology to describe these tumors to facilitate all subsequent work. As tumors are better classified according to consistent clinical and histological features, the most important molecular (genetic and epigenetic) features of the tumors will emerge. Such information, through interdisciplinary collaborations with molecular biologists, diagnostic and interventional radiologists, hepatologists and pathologists will lead to improved, more sensitive and specific screening protocols for early detection. Our ultimate expectation is that targeted therapies specific to individual, histologically characterized and (epi-) genetically defined subcomponents within a patient's tumor may result in improved, tailored therapies. Furthermore, these protocols may also be tailored to the specific background chronic liver diseases in which most of these tumors arise.

The authors acknowledge that we are simply four academic liver pathologists of a larger community. We invite all our liver colleagues, in pathology and other diverse, but related clinical and scientific fields, to join us for what is proving to be an exciting intellectual adventure. Inevitably, together, we will produce significant advances in patient care and further insights into hepatobiliary carcinogenesis.

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