Recent Topics Concerning Combined Hepatocellular-Cholangiocarcinoma

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Summary: Combined hepatocellular-cholangiocarcinoma (CHC) is a relatively rare tumor with an incidence range of 1.0-4.7%. CHC is defined as a tumor containing unequivocal, intimately mixed components of both hepatocellular carcinoma and intrahepatic cholangiocarcinoma. The recent development of biochemical methodologies and cancer stem cell theory have paved the way for a clearer understanding of the histogenesis of CHC. The latest edited WHO classification published in 2010 adopted the concept of stem cell/hepatic progenitor cells in the pathological classification of CHC. Although this classification includes novel and unique concepts of histogenesis and facilitates the recognition of CHC, there are several problems with it in practice. To reduce confusion, an international group of hepatic pathologists, radiologists, surgeons, and clinicians formulated a nomenclature for CHC and issued a consensus article in 2018. In this review article, we discuss the problems with the latest WHO classification and introduce recent topics concerning CHC from pathologic and genetic points of view.

Key words combined hepatocellular-cholangiocarcinoma, WHO classification, immunohistochemical stain, consensus article, genetic findings

INTRODUCTION

Most cases of primary liver carcinoma (PLC) are hepatocellular carcinoma (HCC), followed by intrahepatic cholangiocarcinoma (CCA) and combined hepatocellular-cholangiocarcinoma (CHC). CHC is a relatively rare tumor with an incidence range of 1.0-4.7% [1-6]. CHC is defined as a tumor containing unequivocal, intimately mixed components of both HCC and CCA [7]. Although criteria for the pathological diagnosis of CHC have been proposed [8-10], the histogenesis of CHC has remained elusive. The recent development of biochemical methodologies has contributed to the clarification of the histogenesis of various malignancies, including not only hematopoietic tumors but also solid tumors, and cancer stem cell theory has paved the way for understanding the histogenesis of various malignancies. This theory explains the characteristics of cancer, such as heterogeneity, drug resistance and recurrence after long time periods. The origin of CHC could be hepatic progenitor cells (HPC), which can be differentiated into both hepatocytes and cholangiocytes [11-14]. The markers of HPC are reported to be CD13, CD44, CD56, CD133, OV6, c-kit (aka: CD117), keratin (K) 19 and epithelial cell adhesion molecule (EpCAM, aka:CD326) [15-24]. The latest edited WHO classification published in 2010 adopted the concept of stem cell/HPC in the pathological classification of CHC.

Abbreviations: c, combined; CCA, intrahepatic cholangiocarcinoma; CHC, combined hepatocellular-cholangiocarcinoma; EMA, epithelial membrane antigen; EpCAM, epithelial cell adhesion molecule; HCC, hepatocellular carcinoma; HPC, hepatic progenitor cell; IHC, immunohistochemical; K, keratin; PLC, primary liver carcinoma.
Histological subtypes are described in detail in Fig. 1. This classification is useful, but there are several problems in practice [25,26]. In this review, we discuss recent topics concerning CHC from pathologic and genetic aspects.

PATHOLOGICAL DIAGNOSIS OF CHC

The latest WHO classification for CHC includes a novel and unique concept regarding the histogenesis of CHC. This classification facilitates the recognition of rare tumors such as PLC, CHC. However, since this classification does not mention precise rules for pathological diagnosis, pathologists and hepatopathologists have been confused. The following practical problems have been reported. First, CHCs are generally composed of various components, including other types of CHC, HCC and CCA, within the same nodule. Sasaki et al. reported that all 62 cases of CHC contained CHC, HCC and CCA components in various amounts and combinations [26]. However, there is no definitive description of how tumor amount should be reflected in the pathological diagnosis and handling of minor components. Secondly, immunohistochemical (IHC) markers showing stem cell/HPC characteristics are not specific although IHC findings are described in detail. As we mentioned before, several markers are recognized as stem cell/HPC markers, and some of them are used as biliary differentiation markers, such as CD56, K19 and EpCAM. However, this classification does not clarify the necessity of IHC for the pathological diagnosis of CHC.

To reduce confusion, an international group of hepatic pathologists, radiologists, surgeons, and clinicians have formulated a nomenclature for CHC and issued a consensus article [27]. The goals of this project were as follows: i) to create a uniform histological approach for diagnostic and research purposes and ii) to facilitate scientific studies. In this consensus report, diagnostic categories of CHC among PLCs and diagnostic terminologies of CHC have been clearly rearranged. Several important findings are described. First, the association among CHC, HCC, and CCA is clearly defined. Second, classification of PLC must always begin with a morphological assessment by light microscopy. Third, IHC is a supportive tool, but is not essential for the diagnosis of CHC.

PLCs show a wide histologic spectrum from HCCs, which show hepatocytic differentiation, through CHC to CCAs, which show cholangiocytic differentiation. CHC locates in the middle between the HCC and CHC morphological spectrum. CHC shows varying degrees of hepatocytic and cholangiocytic cytologies and architectures, either admixed or as separate areas within the same tumor. The following are excluded from CHC: 1; distinct HCC and CCA, 2; collision tumors of HCC and CCA arising separately in the same liver, 3: any form of hepatoblastoma or variant, 4: HCC morphology with immunophenotype of cholangiocytic markers or other stem cell markers, 5: CCA with immunophenotypes of hepatocytic markers or other stem cell markers, or CCA with in situ hybridization markers for hepatocytic differentiation, 6: sclerosing/scirrhouc HCC. In the latest WHO clas-

**Fig. 1.** The latest WHO classification of combined hepatocellular-cholangiocarcinoma (CHC). CHC was divided into two subtypes. One is classical type. The other is subtypes with stem-cell features. Subtypes with stem-cell features were subsequently divided into 3 histological subtypes; i.e. typical subtype, intermediate-cell subtype, and cholangiolocellular subtypes. Classical CHC is composed of hepatocellular carcinoma and intrahepatic cholangiocarcinoma components. HCC: hepatocellular carcinoma, CCA: intrahepatic cholangiocarcinoma.
sification, intermediate cell carcinoma and cholangiocl
cellular carcinoma are categorized within CHC. Whether these tumors should be categorized within
CHC, or as unique and dependent entities, has yet to
be fully determined. Intermediate cell carcinoma is
histologically composed of tumor cells smaller than
normal hepatocytes, but larger than the below-de-
scribed stem/progenitor cell phenotypes, and have
features intermediate between hepatocytes and chol-
angiocytes. The tumor has a trabeculae, cord, solid-
nested, or strand character. Each tumor cell may be
cuboid to oval-shaped, with a pale or pink cytoplasm
(Fig. 2a). Cholangiolocellular carcinoma histologi-
cally consists of thin, malignant ductular-like struc-
tures that may appear to radiate from or surround a
portal tract in a tubular, cord-like, anastomosing pat-
tern (aka: antler-like pattern) within a dense, hyalin-
ized stroma (Fig. 2b). In the latest WHO classifica-
tion, stem/progenitor cells components are recognized
as formal diagnostic subtypes. However, these com-
ponents are no longer distinct entities in this consen-
sus report. In brief, stem/progenitor cell components
consist of small cells with scant cytoplasm, a high nu-
clear/cytoplasmic ratio and hyperchromatic nuclei.
These cells are most often found at the interface be-
tween a nest of carcinoma and the adjoining tumoral
fibrous or desmoplastic stroma (Fig. 2c). If these com-
ponents are observed, the presence of these compo-
nents could be documented. CHC shows a wide histo-
logical diversity in the same nodule. If plural
components are seen, a pathologist should assess the
combinations, such as combined (c) HCC-CCA,
chCC-CLC, cCCA-CLA, cHCC-CCA-CLC, cHCC-
CCA-intermediate cell carcinoma, and so forth. Fur-
thermore, some reports recommend describing the
percentage of each component present.

The classification of PLC, such as HCC morphol-
ogy with immunophenotype of cholangiocytic mark-
ers or CCA with immunophenotypes of hepatocytic markers, has been unclear. However, those tumors showing discrepancies between morphologic and immu
nophenotypic findings should be diagnosed prior to
the morphologic findings. Therefore, these tumors
should be diagnosed as follows: classical HCC with
cholangiocyte IHC or classical CCA with hepatocytic
IHC. Since HCCs with K19 expression, which is a
representative cholangiocyct marker, show worse
prognosis compared to HCC without K19 expression,
this category should be preserved [28,29].

SPECIAL STAIN AND
IMMUNOHISTOCHEMICAL STAIN FOR CHC

Special stains, such as mucicarmine and alcian blue, are useful for confirming mucin production. Mu-
cin production leads to the diagnosis of the CCA or
CCA component of CHC. IHC may not be essential for the diagnosis of PLC, including CHC. However,
IHC may be useful in some cases. In the diagnosis of
CHC, markers with hepatocytic and cholangiocytic
differentiations, as well as stem cell differentiation,
are used. As hepatocytic differentiation markers, Hep-
Par-1, arginase-1 and alfa fetoprotein are used. As
cholangiocytic differentiation markers, K7, K19, and
epithelial membrane antigen (EMA) are used. As stem
cell differentiation markers, K19, CD56, EpCAM, e-
kit and CD133 are known. However, as we described
before, some markers overlap with cholangiocytic and
stem cell differentiation markers.

We previously reported that EpCAM and CD133
were stained only in CHC subtypes with stem cell fea-
tures [25]. Furthermore, we reported that EpCAM-
positive cells, which were isolated from a CHC cell
line, showed hepatic stem cell-like features and high
tumorigenicity, and developed tumors with CHC fea-
tures in vivo [30].

Fig. 2. Representative microphotographs of combined hepatocellular-cholangiocarcinoma.
Figure 2a, 2b, and 2c showed intermediate cell carcinoma, cholangiolocellular carcinoma and
stem/progenitor cells component of CHC, respectively.
Intermediate cell carcinoma is composed of tumor cells with features intermediate between hepatocytes and cholangiocytes. The WHO classification indicates that intermediate cell carcinoma shows simultaneous expression of both hepatocyte and cholangiocyte markers. However, we previously reported that intermediate cell carcinoma showed a high positive rate of biliary markers, but the expression of HepPar-1 was low. To confirm the property of hepatocytic differentiations of intermediate cell carcinoma, we conducted IHC of arginase-1, K8 and K18 using 32 surgically resected intermediate cell carcinomas [31]. Out of these, arginase-1 and K8 were useful markers. We reported that arginase-1 showed hepatocytic differentiation and K8 showed cholangiocytic differentiation and that these markers were useful in the pathological diagnosis of intermediate cell carcinoma combined with other markers, such as K7 and K19.

We previously conducted cluster analysis of 53 CHC cases with only IHC findings (Figure 3)[32]. The HCC component and the CCA component of 10-CHC, classical type were divided. Therefore, 63 samples were analyzed with 12 IHC markers. Twelve markers were divided into three categories. Marker 1 is cholangiocytic markers and is composed of K7, K18, K19, EMA. Marker 2 is stem cell markers/mesenchymal markers and is composed of K8, EpCAM, CD56, CD133, c-kit and vimentin. Marker 3 is hepatocytic markers and is composed of HepPar-1 and arginase-1. Samples were clustered into the HCC component of classical CHC and the others. The CCA component of classical CHC, intermediate cell carcinoma and cholangiolocellular carcinoma could not be clearly divided. However, these tumors tended to be divided into two clusters. One is the CCA parts of classical CHC and intermediate cell carcinoma. The other is intermediate cell carcinoma and cholangiolocellular carcinoma. Therefore, these tumors, especially intermediate cell carcinoma, could not be distinguished even when using more than 10 markers.

IHC is not mandatory to make a diagnosis of CHC, however, it is sometimes useful in daily practice. When pathologists observe PLC with hepatocytic and/or cholangiocytic differentiation, it may be useful to archive IHCs of two hepatocytic markers, such as HepPar-1 and arginase-1, and two cholangiocytic markers, such as K19 and EMA, as well as mucin stains. As mentioned in Fig. 3, HepPar-1 is not a sensitive marker, however, its specificity is relatively high. We reported that arginase-1 is useful for recognition of hepatocytic differentiation in intermediate cell carcinoma. As Fig.3 shows, K19 and EMA are widely stained with intermediate cell carcinoma, CLC and CCA components of CHC, but not stained with HCC components of CHC. Furthermore, the staining pattern of EMA is reported to be useful in the diagnosis of CLC [33].

**MOLECULAR ANALYSIS OF CHC**

There are far fewer articles on the molecular analysis of CHC than on that of HCC or CCA. The following articles are representative.

Cazals-Hatem et al. reported that TP53 and β-catenin mutation were identified in 26% and 0% cases of CHC, respectively. Loss of heterozygosity (LOH) at 3p and 14q was frequently observed in CHC (60% and 53%, respectively) and CCA (55% and 66%, respectively) compared with HCC (11% and 13%, respectively). They concluded that CHC was genetically closer to CCA than HCC [34].

Homayounfar et al. examined the patterns of chromosomal aberrations in 7 CHCs as well as 49 HCCs and 22 CCA by comparative genomic hybridization. CHC showed HCC-like chromosomal changes (+8q, +1q and -8p) and a tendency for CCA-like higher chromosomal instability, suggesting that primary genetic events may be shared with HCC, but further genetic tumor progression may be associated with CCA [35].

Coulouarn et al. reported that CHC exhibited stem/progenitor features, a down regulation of the hepatocyte differentiation program and a commitment to the biliary lineage. They also reported that TGFβ and Wnt/β-catenin were two major signaling pathways activated in CHC despite a lack of Wnt/β-catenin mutation in any case [36].

Fujimoto et al. comprehensively analyzed 30 liver cancers, including CHC and CCA, by whole genome sequencing and RNA-seq and compared their genomic landscapes with those of 60 HCCs. They identified recurrent mutation in TERT promoter (53%), followed by chromatin regulators, such as ARID2 (27%) and PBRM1 (20%), PCLO (20%), and XIRP2 (13%) in CHC. The rate of TERT promoter mutation, which is the most frequent mutation site in HCC, was similar to that of HCC. No mutations of IDH1/2 and KRAS were detected in CHC cases, however, these mutations were detected in approximately 10% of CCA cases. They also reported that HCC and hepatitis-positive CHC and CCA were tightly clustered together, whereas the hepatitis-negative CHC and CCA were more spread out, suggesting that the chronic inflammation involved with hepatitis strongly influenced the somatic substi-
Sixty-three samples were analyzed with 12 IHC markers. Twelve markers were divided into three categories. Marker 1 is cholangiocytic markers and is composed of Keratin (K) 7, K18, K19, epithelial membrane antigen (EMA). Marker 2 is stem cell markers/mesenchymal markers and is composed of K8, epithelial cell adhesion molecule (EpCAM), CD56, CD133, c-kit and vimentin. Marker 3 is hepatocytic markers and is composed of HepPar-1 and arginase-1. Samples were clustered into HCC components of classical CHC and the others. The CCA component of classical CHC, intermediate cell carcinoma and cholangiolocellular carcinoma could not be clearly divided. However, these tumors tended to separate into two clusters. One cluster is CCA from classical CHC and intermediate cell carcinoma. The other cluster is intermediate cell carcinoma and cholangiolocellular carcinoma. Indicator shows immunohistochemical (IHC) score:

- IHC score 0, no staining;
- IHC score 1, 1 to 5% positive cells;
- IHC score 2, 6 to 25% positive cells;
- IHC score 3, 26 to 50% positive cells;
- IHC score 4, > 50% positive cells.
Moeine et al. examined a comprehensive molecular characterization of 18 CHCs by using gene expression profiling, DNA copy number detection, and exome sequencing. To the best of our knowledge, this is the first genetic article on a strict pathological assessment in accordance with the latest WHO classification. Out of 18 cases, 6, 8 and 4 cases were diagnosed as cholangiocellular carcinoma, stem-cell tumors, including intermediate cell carcinoma and typical subtype, and classical type, respectively. CHCs of the classical type were separately analyzed for HCC and CCA components. Cholangiocellular carcinoma showed CD56 expression in IHC, chromosomal stability, significant upregulation of TGF-β signal, enrichment of inflammation related and immune response signature, and mutations of IDH1 and TP53. Stem cell tumors demonstrated SALL4 positivity in IHC, chromosomal instability (gains: 1q, 8q, losses: 4q, 8p, 9q, 16q, 16p), enrichment of progenitor-like signature, activation of specific oncogenic pathways, such as MYC and insulin-like growth factor, and mutations of FGFR2-BICCI, TP53 and BRAF. CHC, classical type showed a significant correlation in the copy number variations of CCA and HCC components, indicating a clone origin. They concluded that cholangiocellular carcinoma represented a distinct biliary-derived entity compared with other subtypes of CHCs [38].

Lin et al. conducted whole genome sequence analysis, whole exome sequencing, and RNA-seq using 10 CHCs as well as 10 CCAs. TP53, RYR2, FBN2, CTNNB1 and ARID1A were identified as exhibiting mutations in CHCs. They also identified specific mutations in TP53 (chr17:7577534C>T), which were strongly associated with worse prognosis, using the HCC database from The Cancer Genome Atlas. This type of mutation was detected in 2 CHCs in their series [39].

Very recently, Jeon et al. analyzed the genetic characteristics of 4 CHCs using the Oncomine Comprehensive Panel. To date, this is the first genetic study on CHC using targeted sequencing. Out of 4 cases, 2 cases showed bicolonal signatures in their HCC and CCA components, while 2 cases shared monoclonal signatures in two components. These genetic characteristics corresponded almost completely with the histological ones. Out of several muted genes, TP53 and PTEN mutation were detected in both HCC and CCA components.

Collectively, molecular or genetic characteristics of CHC lack consistency. Several reasons for this inconsistency have been considered. First, there may be confusion regarding the pathological assessment for CHC. As molecular studies are based on definitive pathological diagnosis, it is important for pathologists to have a common recognition of morphological observations. Second, sampling for genetic analysis may be another problem. As CHC usually contains plural components in a nodule, sampling of the proper portion may sometimes be difficult. Contamination with other components could affect the result. Therefore, Jeon et al. recommend separate sequencing of each component of CHC [40].

**FUTURE DIRECTION**

A recently published consensus report states that the classification of PLC must always begin with a morphological assessment by light microscopy [27]. However, even when conducting a detailed morphological observation in addition to conducting IHGs, it may be difficult to distinguish PLCs lacking stereotypical morphologies, such as poorly differentiated HCC, CCA showing hepatoid/HCC-like features, cholangiocellular carcinoma and intermediate cell carcinoma. Intermediate cell carcinoma may be misdiagnosed as CCA when present in a desmoplastic stroma [27]. A previous report stated that the concordance rate of pathological diagnosis for CHC was not high even among hepatopathologists [41]. To date, there may not be classifications to clearly distinguish between these tumors. Practically, pathological diagnosis of these tumors depends on the evaluation of each pathologist. It may be important for pathologists to recognize the presence of PLCs, which are diagnosed as ambiguous, as well as to recognize typical morphologies of PLCs.

CHC is a relatively rare primary tumor and curative treatment is still limited in surgical resection. Treatment for unresectable and/or recurrent cases has not been standardized yet. Nowadays, molecular targeted therapies are widely performed in the case of various neoplasms. In some malignancies, such as primary lung carcinoma and brain tumor, molecular classifications dominate conventional morphological diagnosis and molecular testing is essential. To pave the way for molecular targeted therapies in CHC, pathologists should try to reach a consensus as the pathological diagnosis of CHC is still controversial. These efforts will facilitate scientific studies and contribute to establishing molecular classifications.
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