Research Article

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Mixed hepatocellular cholangiocarcinoma tumors: Cholangiolocellular carcinoma is a distinct molecular entity

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Background & Aims: Mixed hepatocellular cholangiocarcinoma (HCC-CCA) is a rare and poorly understood type of primary liver cancer. We aimed to perform a comprehensive molecular characterization of this malignancy.

Methods: Gene expression profiling, DNA copy number detection, and exome sequencing using formalin-fixed samples from 18 patients with mixed HCC-CCA were performed, encompassing the whole histological spectrum of the disease. Comparative genomic analysis was carried out, using independent datasets of HCC (n = 164) and intrahepatic cholangiocarcinoma (iCCA) (n = 149).

Results: Integrative genomic analysis of HCC-CCAs revealed that cholangiolocellular carcinoma (CLC) represents a distinct biliary-derived entity compared with the stem-cell and classical types. CLC tumors were neural cell adhesion molecule (NCAM) positive (6/6 *vs.* 1/12, p <0.001), chromosomally stable (mean chromosomal aberrations 5.7 *vs.* 14.1, p = 0.008), showed significant upregulation of transforming growth factor (TGF)-β signaling and enrichment of inflammation-related and immune response signatures (p <0.001). Stem-cell tumors were characterized by spalt-like transcription factor 4 (SALL4) positivity (6/8 *vs.* 0/10, p <0.001), enrichment of progenitor-like signatures, activation of specific oncogenic pathways (i.e., MYC and insulin-like growth factor [IGF]), and signatures related to poor clinical outcome. In the classical type, there was a significant correlation in the copy number variation of the iCCA and HCC components, suggesting a clonal origin. Exome sequencing revealed an average of 63 non-synonymous mutations per tumor (2 mean driver mutations per tumor). Among those, *TP53* was the most frequently mutated gene (6/21, 29%) in HCC-CCAs.

Conclusions: Mixed HCC-CCA represents a heterogeneous group of tumors, with the stem-cell type characterized by features of poor prognosis, and the classical type with common lineage for HCC and iCCA components. CLC stands alone as a distinct biliary-derived entity associated with chromosomal stability and active TGF-β signaling.

Lay summary: Molecular analysis of mixed hepatocellular cholangiocarcinoma (HCC-CCA) showed that cholangiolocellular carcinoma (CLC) is distinct and biliary in origin. It has none of the traits of hepatocellular carcinoma (HCC). However, within mixed HCC-CCA, stem-cell type tumors shared an aggressive nature and poor outcome, whereas the classic type showed a common cell lineage for both the HCC and the intrahepatic CCA component. The pathological classification of mixed HCC-CCA should be redefined because of the new molecular data provided.

Keywords: Liver cancer; Molecular subclass; Progenitor-like origin; TGF-β signaling.

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Introduction

Liver cancer is the second leading cause of cancer-related deaths, with more than 850,000 new cases annually worldwide [1]. Mixed hepatocellular cholangiocarcinoma (HCC-CCA) is a rare type of primary liver cancer accounting for less than 1% of all primary liver malignancies [2,3]. Diagnosis is based on histological examination and requires the intimate mix of both hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA) elements [2]. Due to its low incidence and the lack of an established pathological diagnosis, the features and clinical behavior of these tumors remain ill-defined. The patient age, sex specific incidence and geographical distribution are similar to those for HCC [2,4,5], and the median overall survival rates of HCC-CCA are similar to iCCA [3,6–8]. To date, clinical practice guidelines

do not include a specific treatment recommendation for HCC-CCA, and surgical resection remains the standard of practice, when feasible.

Histologically, mixed HCC-CCA is a heterogeneous group of primary liver tumors. According to the 2010 World Health Organization (WHO) classification [2], they are divided into two main categories: the 'classical' type and the 'stem-cell features' type. The classical type is characterized by areas of typical HCC and iCCA with an intermediate transition which holds mixed features of both. The stem-cell features type is further subdivided into typical, intermediate and cholangiolocellular carcinoma (CLC). Subtypes with stem-cell features are composed of tumor cells with intermediate histological features between hepatocytes and cholangiocytes. In addition, recent studies have suggested the presence of distinct properties for each subtype of HCC-CCA with stem-cell features, given their association with different clinicopathological factors [9,10].

Unlike HCC or iCCA, there is no genome-wide characterization of mixed HCC-CCA tumors. Indeed, it is unclear whether histologic subtypes correlate at the molecular level. Gene expression profiling on a small series of HCC-CCA samples has suggested that HCC-CCA might share common characteristics with poorly differentiated HCC and iCCA with stem-cell traits [11–15]. Furthermore, WNT/beta-catenin and TGF-β signaling were reported to be significantly activated in mixed HCC-CCA when compared to progenitor-like HCC [13]. Mutational analysis has suggested common recurrent driver mutations in HCC and HCC-CCA in comparison to iCCA, such as larger frequency of telomerase reverse transcriptase (*TERT*) promoter mutations and a lower frequency of *KRAS* and isocitrate dehydrogenase (*IDH1/2*) mutations [14]. However, genome-wide allelotyping analyses of classical HCC-CCA has suggested a closer genomic proximately to iCCA than to HCC [16].

In this study, a comprehensive molecular characterization of mixed HCC-CCA including histological characterization, whole-genome expression profiling, single-nucleotide polymorphism array, and whole-exome sequencing, is carried out. Integrated analysis to evaluate the genomic overlap with a large independent set of HCC and iCCA samples was also performed. Overall, integrative genomic analysis indicates that CLC is a distinct entity with a biliary molecular profile, low chromosomal instability, and enrichment of TGF-β and immune-related signaling. The other mixed tumors can be distinguished into two main subclasses: the stem-cell subclass, characterized by the presence of the early progenitor marker (spalt-like transcription factor 4 [SALL4]) and signatures of a more aggressive phenotype; and the classical subclass, with components of both HCC and iCCA from a clonal origin. Thus, a molecular classification that encompasses two groups within the mixed HCC-CCA tumors (stem-cell and classical) is proposed. In addition, the data suggest that CLC stands alone as an independent biliary-derived entity, not sharing any molecular traits of HCC.

Materials and methods

Human samples and nucleic acid extraction

Once local Institutional Review Board (IRB) approval was granted, analysis was carried out on 4728 consecutive patients who underwent surgery for primary liver cancer between 1994 and 2013 at the Icahn School of Medicine at Mount Sinai (HCC [4307, 91%], iCCA [360, 7.7%], and mixed HCC-CCA [61, 1.3%]). Among the 61 mixed HCC-CCA cases, 43 were excluded due to: i) pre-treatment with locoregional therapies (such as transarterial chemoembolization, TACE), ii) lack of sample availability, or iii) low tumor cell viability. The diagnosis of mixed HCC-CCA was confirmed by two expert hepatopathologists (MIF and ST). A final set of 18 patients with fixed paraffin-embedded (FFPE) samples and clinical data were selected and categorized according to the latest WHO classification [2], as classical (n = 4) or with stem-cell features (n = 14). The stem-cell features subgroup included typical (n = 2), intermediate (n = 6), and CLC (n = 6). For molecular profiling in the classical mixed tumors, nucleic acids were extracted separately from the HCC-like and CCA-like components (four patients, eight tumor samples in total). Table 1 summarizes the main clinicopathological features of the 18 patients included in the study. For integrative genomic analysis, molecular data previously reported by our group on HCC (n = 164) and iCCA (n = 149) was used [17,18]. For the identification of driver mutations in stem-cell features subtype, fresh-frozen optimal cutting temperature compound (FF-OCT) embedded tumor tissues and corresponding normal tissue (n = 6 pairs including three overlapping cases with above) were provided by the Mount Sinai Institutional Biorepository after IRB committee approval. For a detailed description of nucleic acid extraction, see the Supplementary materials and methods section.

For further details regarding immunohistochemistry, whole-genome gene expression profiling, genome-wide analysis of DNA copy number alteration, whole-exome sequencing, and statistical analyses, see the Supplementary materials and methods section.

Results

CLC, stem-cell and classical types are distinct entities

To understand if the different histological subtypes of mixed HCC-CCA represent distinct subgroups of the disease, we performed gene expression-based unsupervised clustering. The unsupervised clustering analysis (Fig. 1; Fig. S1) revealed three distinct clustered groups: 1) CLC tumors in cluster C, ($p = 0.0013$); 2) stem-cell feature tumors in cluster D ($p = 0.0062$); 3) classical tumors, depending on its components (HCC component in clusters A and B, $p = 0.024$). In addition, iCCA-like components of the classical subtype co-clustered with either stem-cell feature tumors or CLC, suggesting the presence of common molecular traits (Fig. 1A). CLC had a different molecular profile to other stem-cell feature tumors, which was confirmed by integrative genomic analysis with an independent set of HCC (n = 164) and iCCA (n = 149) samples (Fig. 2). CLC tumors significantly co-clustered together, suggesting a high genomic similarity among them, in comparison to other primary liver tumors ($p < 0.001$). Significant genomic proximity was also observed for stem-cell HCC-CCA ($p < 0.001$). Moreover, CLC tumors co-clustered with iCCA from the proliferation class, whereas the stem-cell mixed tumors co-clustered with HCC with progenitor-like traits (Fig. 2, $p < 0.001$). Analysis of cell lineage with specific marker genes, further corroborated the observation that CLC may represent a separate entity. This was indicated by the expression of a biliary phenotype, with significant upregulation of biliary-specific genes (e.g., *KRT7*, *KRT19*, *ITGB4*) and downregulation of hepatocyte-related genes (e.g., *ADH1A*, *ALB*, *APOB*, *HNF1A*) [19] (Fig. 1). These findings were in concordance with the immunostaining profile (Fig. 1B and Fig. 3), which defined CLC tumors as negative for the hepatocyte marker HepPar1 (0/6 in CLC *vs.* 10/12 in others, $p = 0.015$, Table S1), but positive for biliary markers (CK7, CK19), and more specifically, the progenitor-like marker neural cell adhesion molecule (NCAM) (6/6 in CLC *vs.* 1/12 in others, $p < 0.0001$, Table S1).

The stem-cell molecular subclass was characterized by the expression of both hepatocyte and biliary markers (Fig. 1B, Fig. 3), and the early progenitor cell marker SALL4 (6/8 *vs.* 0/10 in rest of mixed tumors, $p = 0.0004$, Table S1). Conversely, the