Intrahepatic cholangiocarcinoma: review and update

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Abstract
Cholangiocarcinoma (CCA) is a heterogeneous group of malignancies that could develop at any level from the biliary tree. CCA is currently classified into intrahepatic (iCCA), perihilar and distal on the basis of its anatomical location. Of note, these three CCA subtypes have common features but also important inter-tumor and intra-tumor differences that can affect the pathogenesis and outcome. A unique feature of iCCA is that it recognizes as origin tissues, the hepatic parenchyma or large intrahepatic and extrahepatic bile ducts, which are furnished by two distinct stem cell niches, the canals of Hering and the peribiliary glands, respectively. The complexity of iCCA pathogenesis highlights the need of a multidisciplinary, translational and systemic approach to this malignancy. This review will focus on the advances of iCCA epidemiology, histo-morphology, risk factors, molecular pathogenesis, revealing the existence of multiple subsets of iCCA.

Keywords: Cholangiocarcinoma, classifications, inflammation, cells of origin, stem cells, molecular profiling

INTRODUCTION
Cholangiocarcinoma (CCA) is a heterogeneous group of malignancies emerging at any level from the biliary tree1-3. [Figure 1]. CCA is classified into intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) based on its anatomical location1-3. Of note, these three CCA subtypes have common features but also important inter-tumor and intra-tumor differences that can affect the pathogenesis and outcome1-9. The complexity...
of the pathogenesis and the pronounced heterogeneity affected in particularly iCCAs had impeded clinical goals in iCCA\textsuperscript{[10]}. This review will focus on the advances of iCCA epidemiology, classifications and histomorphology, risk factors, molecular pathogenesis and clinical presentation revealing the existence of multiple subtypes of iCCA.

**THE BURDEN OF iCCA**

The epidemiologic trend of CCA shows a constant and dramatic increase in incidence and mortality worldwide\cite{[1-3]}, clearly depicting CCA relevance among others types of cancer. A progressive increase in intrahepatic CCA incidence was reported, while the incidences of both perihilar CCA and distal CCA seem to be stable\cite{[1-3]}. The incidence of CCA in European countries ranges from 1 to more than 4 cases/100,000\cite{[1-3]} [Figure 1]. However, the difficulties with classification coding for CCA, and with the various terminology that is used, determined an underestimation of CCA burden. In a recent report, the four ICD-10 (International Classification of Diseases) sub codes were agreed on for CCA and used\cite{[11]}. This report showed that in England alone (not the whole of the UK), in 2013, 1965 new CCAs were diagnosed with an incidence rate of 3.65 per 100,000 population, while, 2161 deaths and a mortality rate of 4.01 per 100,000 population were registered. The number of deaths per 100,000 population for the CCA in the period from 2010 to 2013 in England tragically surpassed the ones for the hepatocellular carcinoma (HCC), with 7743 vs. 6899 deaths in 2013 for
CCA and HCC respectively. The trend in iCCA incidence is paralleled also by the fact that mortality for primary liver cancer has become more uniform across Europe over recent years with an evident decline of HCC mortality, but, in contrast, intrahepatic CCA mortality has substantially increased for the most part of Europe. Over recent years intrahepatic CCA accounted for over a fourth of all liver cancer deaths in men and 50% in women. Liver cancer mortality rates are expected to rise by 58% in the UK between 2014 and 2035, i.e., to 16 deaths per 100,000 people by 2035. Considering epidemiology trend in primary liver cancer, half of deaths for primary liver cancer will be determined by intrahepatic CCA. Furthermore, when the mortality rates for all malignancies are considered, the untargeted problem of CCA emerged clearly. Indeed, while a reduction of the mortality rate from 19 malignancies (comprising breast, lung, colon, etc.) was shown from 1990 to 2009 (US data), the mortality rate for malignancies of liver and bile ducts increased by more than 40% and 60% in females and males, respectively. Finally, it is noteworthy to mention that CCA is the most frequent cause of metastasis of unknown origin, and thus further highlights how we still do not know the real burden of CCA.

NEW INSIGHTS INTO iCCA CLASSIFICATIONS

A huge number of different classifications have been proposed for CCA. The most updated one, but still discussed, identify on the basis of the anatomical localization the iCCA, the pCCA and the dCCA. However, being a topographic classification it suffers several pitfalls, and, of course, it does not reflect different biological features. Firstly, it should be noted that, the diagnosis of CCA frequently occurs at an advanced stage, where, the differentiation between the intra-hepatic or extra-hepatic location results is very difficult, and sometimes impossible. Since, small bile ducts and ductules are also present in the perihilar liver parenchyma, then, pCCA as iCCA, may originate either from these smaller ducts and this cannot be discriminated based on gross morphology. Similarly, the iCCA may originate from larger or smaller portion of intrahepatic biliary tree. Third, recent studies demonstrated how, from a pathological and molecular point of view, differences between pCCA and the iCCA originated from larger bile ducts ceased to exist and, therefore, the distinction between these two forms of CCA is losing relevance. Taking into consideration the macroscopic pattern of growth, iCCA has been classified in mass-forming (MF), periductal infiltrating (PI), and intraductal growing (IG). As far as pCCA and dCCA are concerned, either a PI or IG pattern has been recognized. For pCCA a nodular + PI growth pattern predominates (> 80%). On the histological level, while, the vast majority of pCCA and dCCA are mucinous adenocarcinomas, iCCAs are highly heterogeneous tumors and several classifications have been proposed. The small bile duct type (mixed) iCCAs display an almost exclusively MF growth pattern, and are frequently associated with chronic liver diseases (viral hepatitis or cirrhosis), and are associated with cytokeratin (CK) 19-positive hepatocarcinoma (HCC). On the other hand, large bile duct type (mucinous) iCCAs may grossly appear as MF, PI or IG types; they are more frequently associated with PSC and can be preceded by pre-neoplastic lesions such as biliary intraepithelial neoplasm (BiIN) or intraductal papillary neoplasm (IPNB). Interestingly, the large bile duct type (mucinous) iCCAs share phenotypic traits with pCCA and pancreatic cancers.

In our opinion, this histological subtyping should be taken into serious consideration because it underlines different risk factors, molecular profile, and clinical management.

MULTIPLE RISK FACTORS REVEAL iCCA SUBTYPE-SPECIFIC PATHOGENESIS

Although CCA is a rare cancer (incidence < 6/100,000) in most countries, its incidence may reach an extremely high in some populations of Chile, Bolivia, South Korea and North Thailand. The different prevalence of risk factor in geographic areas may explain the variation in incidence rates of CCA. For example, in Thailand regions, the very high incidence of CCA is closely related to the incidence of liver flukes.
In order to review literature on risk factors associated with iCCA we have searched for case series of iCCA or case series with appropriate topographic classification of histologically verified iCCA. The risk factors of iCCA (diagnosed according the current recognized criteria, i.e. European RARECARE\(^{[33]}\)) could be classified on the basis of the tissue or the cell which is primarily targeted by diseases or conditions and therefore likely involved in the carcinogenic process as cell or tissue of origin. For instance, biliary diseases as cholangitis/PSC, secondary biliary cirrhosis, choleodocholithiasis, hepatolithiasis, cholecystitis, and liver flukes are pathologic conditions primarily affecting large intra-hepatic bile ducts [Table 1]\(^{[34-46]}\), and are risk factors for both iCCA and p/dCCA. Parenchymal liver diseases include chronic viral and non-viral liver diseases, recognize the interlobular bile ducts, bile ductules and the canals of Hering as the primary targets. Accordingly, these conditions are specific risk factors for iCCA [Table 1].

Other risk factors, like several toxic and environmental factors; amongst them nitrosamine-contaminated food, asbestos, dioxins, vinylchlorides, and thorotrast as was always the case in the past\(^{[47]}\), which hit multiple cellular targets, are considered risk factors associated to all CCA subtypes.

PSC, a disease affecting both intra-hepatic and extra-hepatic bile ducts, represents the strongest independent risk factor both for iCCA and for pCCA [Table 1]. Most of the studies evaluated the cumulative risk of CCA in PSC patients, but not the discrete risk of iCCA and/or pCCA to PSC\(^{[48-51]}\). The cumulative incidence of CCA in PSC patients ranges from 5% to 10%\(^{[52-55]}\). Clinical and pathological observations suggested that PSC is specifically associated with the development of bile duct (mucinous) type CCA\(^{[4,56]}\). Data on the role of inflammatory bowel diseases (IBD), associated with or preceding PSC, in affecting the risk of CCA are controversial. The coexistence and duration of IBD significantly increased the risk of CCA in PSC patients\(^{[51]}\). In IBD patients the RR estimated was 2.61 for iCCA vs 1.47 for pCCA\(^{[57]}\). Crohn’s disease (CD) seemed to have a lower risk of CCA than ulcerative colitis (UC)\(^{[57,58]}\). In contrast, in a study carried out in the USA, neither IBD nor its duration confers additional risk of CCA in PSC patients\(^{[59]}\).

In a study, Welzel et al\(^{[36]}\) described that duodenal ulcer disease was significantly more common among pCCA and iCCA cases than controls. Many studies have demonstrated associations between CCA and \(H.\ pylori\) but the correlation remains controversial and a direct cause-and-effect relationship has not been established\(^{[60-64]}\). In particular, in East-Asia, where iCCA represents a large proportion of primary liver cancers, a strong association exists between liver fluke infestation (Ophistorchis viverrini and Clonorchis sinensis) and the development of CCA [Table 1]\(^{[67,68]}\). Several epidemiological studies estimated the relationship between type II diabetes and CCA [Table 1]\(^{[66,69-72]}\). Notably, a possible explanation of this association is attributable to a recent demonstration that in a diabetes model and in human subjects affected by type II diabetes, PBGs underwent proliferation and expansion in relation to hyperglycemia\(^{[72]}\). It’s worthy to note that metformin reduced the risk of iCCA in diabetic patients by a significant margin up to 60%\(^{[70,74]}\). A recent meta-analysis confirmed that, in addition to type II diabetes, even obesity, alcohol use and smoking, have an association with iCCA\(^{[75]}\).

It is becoming increasingly evident that metabolic conditions predispose to the development of primary liver cancers\(^{[3,44,76]}\). Nonalcoholic fatty liver disease/non alcoholic steato-hepatitis (NAFLD/NASH) resulted in independent predictors of iCCA (not of pCCA development), even if with a less strong association compared with other risk factors (viral hepatitis, cirrhosis) [Table 1]\(^{[76]}\). Hemochromatosis resulted in an independent predictor of iCCA development, and it failed to predict pCCA [Table 1].

It has long been known that the presence of cirrhosis increases the risk of iCCA\(^{[58,57,40,44,77]}\). HBV- and HCV-related liver diseases have been identified as definitive risk factors for CCA, with a stronger association for iCCA than pCCA\(^{[77,78]}\). A meta-analysis by Palmer and Patel\(^{[75]}\) concerning 8 case control studies indicated that HCV was associated with an overall OR of 4.84 for iCCA. Where the prevalence of the HBV infection is higher, the association with iCCA and HBV is more significant (e.g. Asian countries)\(^{[79,80]}\). The range of the
The burden of HCV in the last decades has been associated with the specific increase of the iCCA as well as the HCC. Accordingly, clinical and pathological observations suggested that liver cirrhosis is specifically 

OR in the HbsAg positive subjects goes from 2.3 to 9.7 [Table 1](#). The presence of cirrhosis increases the risk of CCA [Table 1] even more by 2.5 fold (95% CI: 1.2-5.1; \( P = 0.02 \) in HBV, and 3.2 fold (95% CI: 1.23-8.148, \( P = 0.017 \) in HCV patients.

### Table 1. Summary of risk factors significantly associated to iCCA* as assessed by case control studies (odd ratios by multivariate analyses)

<table>
<thead>
<tr>
<th>Risk factors for iCCA</th>
<th>Odds ratios for increased risk</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bile duct diseases and conditions</strong></td>
<td></td>
</tr>
<tr>
<td>Cholecystitis [36]</td>
<td>8.5</td>
</tr>
<tr>
<td>Choledolithiasis [37,40,42,43,79]</td>
<td>10.23-13.5</td>
</tr>
<tr>
<td>Hepatolithiasis [38-40,42,43,79]</td>
<td>50.0-4.8; 6.7§</td>
</tr>
<tr>
<td>Choledochal cysts [37,44,45]</td>
<td>4.17-33.35</td>
</tr>
<tr>
<td>Choledocholithiasis [37,45]</td>
<td></td>
</tr>
<tr>
<td>Cholangitis/primary sclerosing cholangitis [38,44]</td>
<td>64.2-75.23</td>
</tr>
<tr>
<td>Biliary cirrhosis/PBC [37,44]</td>
<td>17.08-19.8</td>
</tr>
<tr>
<td>Cholecystectomy [36,39]</td>
<td>3.6-5.4</td>
</tr>
<tr>
<td><strong>Digestive diseases</strong></td>
<td></td>
</tr>
<tr>
<td>Inflammatory bowel diseases [38,55]</td>
<td>1.72-3.95</td>
</tr>
<tr>
<td>Crohn’s disease [36,44]</td>
<td>1.68-2.4</td>
</tr>
<tr>
<td>Ulcerative colitis [36]</td>
<td>3.3-4.5</td>
</tr>
<tr>
<td>Duodenal ulcer [36]</td>
<td>3.4</td>
</tr>
<tr>
<td>Chronic pancreatitis [36]</td>
<td>5.9</td>
</tr>
<tr>
<td><strong>Liver flukes</strong></td>
<td></td>
</tr>
<tr>
<td>Clonorchis sinensis infection [38,42]</td>
<td>8.6-13.6</td>
</tr>
<tr>
<td><strong>Endocrine disorders</strong></td>
<td></td>
</tr>
<tr>
<td>Thyrotoxicosis [36]</td>
<td>1.5</td>
</tr>
<tr>
<td>Diabetes mellitus type II [33,39,43,75,86]</td>
<td>1.8-3.2</td>
</tr>
<tr>
<td><strong>Metabolic conditions and general risks</strong></td>
<td></td>
</tr>
<tr>
<td>Obesity [36,44]</td>
<td>1.71-1.71</td>
</tr>
<tr>
<td>Alcohol intake &gt; 80 g/day [37,39,75]</td>
<td>1.52-5.21</td>
</tr>
<tr>
<td>Smoking [36,44]</td>
<td>1.3-2.1</td>
</tr>
<tr>
<td>Metabolic syndrome [449]</td>
<td>1.32-1.83</td>
</tr>
<tr>
<td>Dyslipoproteinemia [44]</td>
<td>1.63</td>
</tr>
<tr>
<td>Hyper tension [44]</td>
<td></td>
</tr>
<tr>
<td><strong>Chronic liver diseases</strong></td>
<td></td>
</tr>
<tr>
<td>Alcoholic liver disease [36,44]</td>
<td>3.1-5.69</td>
</tr>
<tr>
<td>Non specific cirrhosis [36,37,43,44,75]</td>
<td>18.24-28.79</td>
</tr>
<tr>
<td>Hemochromatosis [36]</td>
<td>2.6</td>
</tr>
<tr>
<td>Hepatic schitosomias [41]</td>
<td>11</td>
</tr>
<tr>
<td>Non alcoholic liver disease [36]</td>
<td>3</td>
</tr>
<tr>
<td>Unspecified viral hepatitis [44]</td>
<td>7.66</td>
</tr>
<tr>
<td>HCV infection [36,40,44,45,79]</td>
<td>2.41-9.71; 9.7§</td>
</tr>
<tr>
<td>HCV infection plus cirrhosis [40]</td>
<td>8.53</td>
</tr>
<tr>
<td>HBsAg positive [38,37,42,44,75,81]</td>
<td>2.3-9.7; *2.35-4.3</td>
</tr>
<tr>
<td>HBsAg positive plus cirrhosis [36,42,44]</td>
<td>13.18</td>
</tr>
<tr>
<td>HBsAg negative/HBeAb positive [45,81]</td>
<td>1.09-1.81*</td>
</tr>
<tr>
<td><strong>Occupational exposure</strong></td>
<td></td>
</tr>
<tr>
<td>Occupational exposure to asbestos [46]</td>
<td>4.81</td>
</tr>
</tbody>
</table>

*Histological verified cases; iCCA cases comprise 2 cases of cHCC-CCA; #according the 2001 U.S. NCEP-ATP III definition; °Risk of CCA only in Asia. The table was prepared summarizing findings by case control studies investigating risk factors associated to iCCA as assessed by multivariate analyses. The case-control studies were selected from the papers individuated by the following terms, that were searched on PubMed: ("cholangiocarcinoma"[MeSH Terms] OR "cholangiocarcinoma"[All Fields]) AND ("risk factors"[MeSH Terms] OR ("risk"[All Fields] AND "factors"[All Fields]) OR "risk factors"[All Fields] OR ("risk"[All Fields] AND "factor"[All Fields]) OR "risk factor"[All Fields])) NOT ("review"[Publication Type] OR "review literature as topic"[MeSH Terms] OR "review"[All Fields]) AND English[lang]. The criteria selections of the works comprise moreover the case definition of CCA: histological verified cases series of iCCA with appropriate topographic classification (Klatskin tumours classified as pCCA and excluded from the iCCAs).
associated with the development of small bile duct (mixed) type iCCA\cite{4}. Ductular reaction is a marker strongly associated with the evolution of chronic liver disease in cirrhosis. The origin of the small bile duct type iCCA may be associated with the chronic proliferative activation of hepatic stem cells and mature hepatocytes senescence in chronic liver diseases\cite{12,82}. Since cirrhosis, chronic hepatitis B and C, alcohol use, diabetes, and obesity are major risk factors for iCCA and HCC\cite{75}, a common pathogenesis of primary intrahepatic epithelial cancers has been suggested. The parallel worldwide reduction of mortality of HCC\cite{12}, which is highly correlated to viral infection and cirrhosis, and on the pandemic of metabolic disorders, suggests that metabolic risk factors are responsible for the rising clinical impact of iCCA. Interestingly we provided the pathologic basis of this epidemiology phenomenon since we demonstrated DM-induced proliferation of PBG cells\cite{72}.

**MOLECULAR PROFILING AND THE IDENTIFICATION OF MULTIPLE iCCA SUBSETS**

Although there exist enormous geographic and racial differences\cite{3,83}, generally, the prominent genetic alterations described in CCAs affect TP53 (DNA repair)\cite{84-86}, tyrosine kinase (KRAS, BRAF, SMAD4 and FGFR2)\cite{8,84-88}, protein tyrosine phosphatase (PTPN3)\cite{88}, deregulated WNT/CTNNB1\cite{88} and Notch pathways, epigenetic (IDH1 and IDH2)\cite{88,89,91,92}, and chromatin-remodeling factors (MLLs, ARID1A, PBRM1 and BAP1)\cite{84-86,88,91}.

Chronic bile duct inflammation characterizes CCA risk factors\cite{93-95}. Accordingly, it was demonstrated that the enzyme cyclooxygenase-2 (COX-2) is induced in CCA by both bile acids and oxysterols, the oxidation products of cholesterol that are increased in the bile during biliary inflammation\cite{96,97}. Inflammatory cytokines may upregulate the expression of inducible nitric oxide synthase (iNOS) in CCA. Notably, nitric oxide (NO) promotes DNA damage directly by inhibiting DNA repair mechanisms, thus promoting carcinogenesis\cite{98,99}. Moreover, iNOS activation stimulates further the expression of COX-2\cite{100}. Notably, the tumoral stroma seems to have a peculiar role in the amplification of the inflammation. While the tumor epithelium was defined by deregulation of the HER2 network and frequent over-expression of EGFR, the hepatocyte growth factor receptor (HGF/MET), pRPS6, and Ki67, the stroma was enriched in inflammatory cytokines\cite{101}.

In the chronic inflammation milieu of CCA emerging in hepatitis infection\cite{88}, recurrent genetic variants in the promoter of the human telomerase reverse transcriptase (TERT) were described\cite{88}. This could be correlated with the pivotal role of this “longevity” enzyme in controlling stem cells. These cells are extremely challenged in these conditions because the senescence of the mature hepatocytes determines the secondary stem proliferative activation (e.g. ductular reaction)\cite{12}.

A dissection of the molecular heterogeneity of iCCA, conducted by the evaluation of gene expression profile (transcriptome), clinic-pathological traits, and patient outcomes in iCCA cases, has allowed the identification of 2 main biological classes of iCCA. The first inflammation class (38% of IH-CCA), characterized by activation of inflammatory signaling pathways, overexpression of cytokines, and STAT3 activation and; the second proliferation class (62% of IH-CCA), characterized by activation of oncogenic signaling pathways (i.e. RAS, MAP-kinase and HGF/MET), DNA amplifications at 11q13.2, deletions at 14q22.1, mutations in KRAS and BRAF, and gene expression signatures previously associated with poor outcomes for patients with HCC\cite{7}.

Molecular studies of human iCCA associated with liver flukes demonstrated over-expression of genes involved in xenobiotic metabolism (UGT2B11, UGT1A10, CHST4, SULT1C1). Whereas non-OV-associated iCCA showed enhanced expression of genes related to growth factor signaling (TGFBI, PGF, IGFBP1, IGFBP3)\cite{52,102}. Possible mechanism associated with liver flukes carcinogenesis may emerge from the discovery of the draft genome of Clonorchis sinensis and transcriptomes of Clonorchis Sinensis and Ov\cite{103,104}. For instance, the evaluation of the putative signature of liver flukes associated CCA could help in screening and surveillance, with the perspective of an early diagnosis of infestation in subjects\cite{102}. A putative role of liver fluke infestation in modulating
epigenetic has been suggested by the demonstration of promoter hypermethylation in a handful of target genes in a large cohort of iCCA (n = 102) associated with liver fluke infection\(^{[105]}\).

CCA genetic susceptibility has been investigated in geographic areas where liver flukes are endemic. In these studies, specific haplotypes of COX2-coding gene (PTGS2) or IL8RB have been recently associated with a significant risk of CCA development\(^{[106]}\).

As far as CCA emerging in PSC, different molecular signatures of the high oncogenic risk were described in PSC patients. KRAS mutations were found in 30% of bile fluid of PSC patients without evidence of CCA\(^{[107]}\). Since KRAS mutations are frequently observed in CCA, and since the mutational profiling can be performed in cell-free DNA of bile supernatant, this early mutagenic event into the bile duct carcinogenesis could be evaluated for screening purposes in PSC patients\(^{[108]}\). The inflammatory microenvironment has also been associated with an aberrant DNA methylation profile in CCA emergence in PSC patients, which provides survival signals for the tumor\(^{[109]}\). Even, an inherited increase in the risk of CCA development in PSC patients was demonstrated by studies concerning the natural killer cell receptor G2D receptor, where specific genetic variants have been described in PSC patients\(^{[110]}\).

Heterogeneity of molecular profile of CCA provides a demonstration of how somatic mutagenesis and epigenome features are highly cell/lineage type-specific, and are largely driven by the pre-neoplastic tissue pathologic milieu (see inflammation). Indeed, at a molecular level, distinct patterns of genetic mutations, methylation, and expression profiling may differentiate iCCA from pCCA. iCCAs were significantly more frequently bcl-2+ and p53+\(^{[111]}\), whereas pCCAs were more often p53\(^{-}\)\(^{[111]}\). Miller et al.\(^{[112]}\) revealed 545 genes with altered expression in p/dCCA and 2354 in iCCA. Mutations in IDH1 and IDH2 were found only in iCCA (n = 9), but in none of the examined p/dCCA (n = 22) and gallbladder cancer (n = 75)\(^{[113]}\). Recent papers confirmed liver fluke negative iCCAs are enriched for IDH mutants\(^{[14,28]}\). A cross-platform comparison of iCCA with pancreatic cancer and HCC further emphasizes the presence of distinct tumor subsets, suggesting similarities of the IDH mutants CCAs with the HCCs rather than pancreatic cancers\(^{[28]}\). Conversely, mutations in KRAS by tumor site demonstrated predominance in pCCAs (53.3% of hilar vs. 6.7% of peripheral type)\(^{[7]}\). As far as epigenetic abnormalities are concerned, methylation of RASSF1A was more common in pCCA than in iCCA, while the opposite was demonstrated for methylation of GSTP gene\(^{[114]}\). Other reported alterations uniquely associated with iCCA, comprised fibroblast growth factor receptor (FGFR) pathways and ephrin type-A receptor 2 mutations\(^{[113]}\).

Finally, the histopathological distinction of cholangiolocellular differentiation of iCCA has been correlated with molecular features\(^{[115]}\). iCCA with cholangiolocellular differentiation resembling an inflammation-related subtype revealed less aggressive histopathological features compared to iCCA without cholangiolocellular differentiation resembling a proliferation subtype. Accordingly, the former showed more favorable clinical outcomes, including overall survival, than iCCA without cholangiolocellular differentiation\(^{[116]}\). The emerging therapeutic approaches based on the molecular targets in CCA have been recently reviewed by Rizvi and Gores\(^{[117]}\).

**VARIABLE CLINICAL PRESENTATIONS AND DIAGNOSTIC FEATURES**

Clinical presentation of CCA is largely influenced by anatomic location and pattern of growth, which ultimately belong from the cells of the origin. Accordingly, emerging concepts into CCA origins demonstrated that it comprises at least two separate entities which a distinct histology, progression and risk factors. These sub-types have been recently classified in large bile duct (mucinous) type CCAs and the small bile ducts or mixed-CCAs. According to different observations, pCCAs are more likely associated with pre-neoplastic lesions emerging in surface epithelium\(^{[6,3]}\) and PBGs\(^{[118]}\). On the other hand, iCCAs show inter-tumor heterogeneity leading to the classification into two main different histological subtypes\(^{[4,119]}\), with likely different cells of origin\(^{[4]}\): the CCAs of the small bile ducts or mixed-CCAs and the large bile duct
(mucinous) type iCCAs\(^{[22,119]}\). The last iCCA subtype displays IHC, gene expression and clinic-pathological profile that can be superimposed on pCCA\(^{[4,120-122]}\). Small bile ducts or mixed-CCAs usually showed a peripheral localization and a mass forming growing pattern. Differently, the large bile duct (mucinous) type usually showed a peri-ductal infiltrating and/or mass forming growth pattern\(^{[4]}\). Importantly, these separate entities displayed different prognosis (being worst the one of the mucin-producing iCCAs) and different associated diseases\(^{[4,10,82,123]}\). Indeed, parenchymal liver diseases, including chronic viral and non-viral liver diseases and liver cirrhosis, characterize the clinical-pathologic background for mixed-iCCAs\(^{[4,10,82,123]}\). In contrast, chronic biliary diseases or pathologies and conditions affecting the intrahepatic medium-large and extrahepatic bile ducts characterize the clinical-pathologic background for mucin-producing iCCAs and pCCAs\(^{[4,10,82,123]}\).

As far as the mixed type-mass-forming iCCA is concerned, the clinical presentation is similar to other intrahepatic liver malignancies, but different from that of pCCA\(^{[4,10,82,123]}\). iCCAs are usually asymptomatic in early stages (20%-25% of cases are incidental finding). Malaise, cachexia, abdominal pain, night sweats, fatigue and/or jaundice, associated or not with systemic manifestations, represent the clinical onset of symptomatic iCCA\(^{[4,10,82,123]}\). In contrast, a typically painless jaundice is the most frequent clinical onset in pCCA\(^{[4,10,82,123]}\). Regarding patients with PSC, CCA may present as the development of a rapid deterioration of clinical conditions or a dominant stricture during follow-up\(^{[3]}\). In general, the MF type represents the most frequent macroscopic presentation of iCCA (> 90%) appearing, at imaging, as a nodule\(^{[3,123]}\). In the context of cirrhotic liver, the first diagnostic challenge is the differential diagnosis of iCCA vs HCC. In the cirrhotic liver it was demonstrated that by contrast, enhanced MRI iCCAs showed constantly a lack of HCC hallmarks; however, by CT, this occurs only in large nodules (> 3 cm)\(^{[114-126]}\). Although, the HCC diagnosis belong from the demonstration of the typical contrast agent uptake, the identification of HCC with stem cell features (CK19+ – HCC), combined HCC-CCA, cholangiolocellular carcinoma and bile duct mixed type iCCA, by imaging procedures, still remains an unsolved challenge\(^{[3,4,10,123,127,128]}\). Biopsy is, therefore, necessary after excluding HCC in cirrhosis, or in the context of a nodule in non-cirrhotic liver\(^{[3,129]}\). From a histological point of view, differential diagnosis of iCCA vs. HCC or metastasis represents an unsolved problem\(^{[2,3,129,130]}\), also due to the lack of validation of specific markers.

Radiologically, iCCA may appear as a dominant stricture in the context of PSC or in patients without a documented specific hepatobiliary disease. This is a typical presentation of the pCCA. When a dominant stricture of the intrahepatic biliary tree is suspected, the MRI + MRCP represents the imaging procedure with the highest diagnostic accuracy for localizing and sizing the stricture\(^{[3]}\); the challenge being the definitive demonstration of malignancy\(^{[3]}\). In this respect, ERCP enables a number of procedures in order to obtain a microscopic confirmation, comprising, cytology, brushing, FISH-polisomy, biopsy, or further innovative techniques\(^{[3]}\). However, all these techniques show an unsatisfactory sensitivity\(^{[54,130-133]}\), and even, the FISH-polisomy in detecting CCA in PSC patients demonstrated a low sensitivity in a meta-analysis\(^{[133]}\).

In substance, diagnosis of CCA still requires a combination of clinical, radiologic and non-specific histologic/biochemical markers (see review by Banales et al.\(^{[3]}\)).

As already mentioned, no specific serum, urine, biliary or histological biomarkers are currently available for the diagnosis of CCA and a proposal by our group which has been recently refreshed by new confirmation, identifies biliary IGF1 as specific markers of CCA. However, the very promising role of biliary IGF1 has been confirmed only in CCA without PSC. Recently, Arbelaiz et al.\(^{[134]}\) evaluated the serum concentration of extracellular vesicles (EVs) and performed a careful analysis of the protein content in patients with CCA, PSC, and HCC. Proteomic signatures found in serum EV of CCA, PSC, and HCC patients show potential usefulness as diagnostic tools. As noted previously, the EV cargo in the two distinct EV populations (i.e., basolateral and apical) is evidently different as a large difference exists between the protein content of EVs released by normal cholangiocytes and cholangiocytes involved in chronic inflammation (i.e., PSC) or...
neoplastic transformation (i.e., CCA)\cite{135}. Further validation studies will be necessary to bring this important scientific advance into the clinical approach of CCA differential diagnosis.

**NEW ADVANCES INTO CCA THERAPY**

Surgery with complete resection, including liver transplantation in highly selected cases, is the only curative therapy for CCA. In patients with unresectable tumours, several types of loco regional therapy or chemotherapy (such as trans arterial chemoembolization, trans arterial radio embolization or radiofrequency ablation) can be considered. In substance, CCAs must be managed by dedicated centres with multidisciplinary expertise in which personalized diagnostic work-up and management can be performed, as clearly stated by a European Consensus (see review by Banales *et al.*\cite{3}).

Recently two important advances have been reached in therapy of iCCA. On one hand, the first clinical trial of adjuvant therapy has been concluded\cite{136}. In this clinical trial, 447 surgically resected patients were randomly assigned to capecitabine for 6 months or observation (> 80% of the patients were followed for at least 3 years). Interestingly, results showed a survival of 51 vs. 36 months in capecitabine arm vs. observation, and median time to cancer recurrence of 25 vs. 18 months, respectively. In 430 patients who received treatment per study protocol, capecitabine is associated with a 25% lower chance of death than observation\cite{136}. On the other, the first report of a molecular target therapy in chemotherapy-refractory CCA appeared. BGJ398 was a first-in-class FGFR kinase inhibitor with manageable toxicities showing meaningful clinical activity against chemotherapy-refractory CCA containing FGFR2 fusions. This promising antitumor activity supports continued development of BGJ398 in this highly selected patient population\cite{137}. Emerging therapeutic approaches based on the molecular targets are still in early phase of clinical study and have been recently reviewed by Rizvi and Gores\cite{117}.

**PERSPECTIVES**

A unique feature of CCA is that it recognizes as origin tissues, the hepatic parenchyma or large bile ducts, which are furnished by two distinct stem cell niches, the canals of Hering and the peribiliary glands (PBGs), respectively\cite{138}.

Stem cells have been identified as cells of origin of different cancer types, comprising primary liver cancers, both in experimental studies and in humans\cite{139-147}. Based on the grade of maturation of the cells of origin within the two lineages of the liver (hHpSC-derived and hBTSC-derived lineages), we have proposed that CCAs could be classified as:

- Primary liver parenchymal CCA: cholangio-lcarcinoma, small bile duct type (mixed) CCA. These tumors emerge within the liver parenchyma from canals of Hering, bile ductules and interlobular bile ducts and indeed originate from hHpSCs, immature NCAM+ cholangiocytes, or mature (NCAM-) interlobular cholangiocytes. A rigorous study, based on an integrative genomic analysis of HCC-CCAs, demonstrated that cholangio-lcarcinoma represents a distinct biliary-derived entity compared with the mixed/combined HCC-CCA, which, on the other side, comprised the stem-cell type, with an aggressive nature and poor outcome, and the classical type, with common cell lineage for both the HCC and the iCCA component\cite{148}.
- Primary biliary CCA: dCCA, pCCA, and large bile duct (mucinous) type iCCA. These tumors emerge from extra-hepatic biliary tree and larger intra-hepatic bile ducts and originate from PBGs or surface epithelium of corresponding bile ducts.

Thus, facing the origin of iCCA, a physio-pathology concept should be considered, instead of the cell of origin, the lineage of origin\cite{10,12,13,138}. An iCCA classification based on the cell-lineages-of origin is more coherent with current knowledge on the epidemiology and risk factors and may have important clinical implications for the definition of specific therapeutic targets. Moreover, it highlights a lineage dependency of the chronic
liver diseases and related molecular carcinogenesis. Being somatic mutagenesis and epigenome features highly cell/lineage type-specific, and largely driven by the pre-neoplastic tissue pathologic milieu (see inflammation), finally, the multiple lineages of origin plus the related diseases may explain the intratumoral heterogeneity observed at any level in iCCA, comprising molecular profiling, with clear implication into preventive strategies in patient with clinical or subclinical underlining hepatic or biliary diseases, therapy and in near future approaches of personalized medicine in iCCA patients.

DECLARATIONS

Authors’ contributions
Concept, design, definition of intellectual content, literature search, data acquisition and analysis, statistical analysis, manuscript preparation, editing and review: Cardinale V, Bragazzi MC, Carpino G
Data acquisition and analysis: Di Matteo S, Overi D, Nevi L
Manuscript editing and review: Gaudio E
Definition of intellectual content, literature search, manuscript preparation, editing and review: Alvaro D

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None.

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