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Defining the biology of intrahepatic cholangiocarcinoma: molecular pathways and early detection of precursor lesions

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Abstract. – Intrahepatic cholangiocarcinoma (ICC) is a rare malignant tumor of the biliary tract with growing incidence and dismal prognosis. It is responsible for 10-20% of primary liver cancer worldwide, but early diagnosis is still a challenge and few treatment options are available.

Aim of this review is to summarize the current knowledge about biological features and arising molecular patterns of this disease. The identification of emerging biomarkers and early detection of precursor lesions shall play a key role in the perspective of future tailored targeted therapies.

Key Words:
Biliary tract cancer, Cholangiocarcinogenesis, Diagnosis, Biomarker, Endoscopic detection, targeted biological therapy.

Abbreviations

Intrahepatic cholangiocarcinoma (ICC), cholangiocarcinoma (CCA), extrahepatic cholangiocarcinoma (EH-CCA), peripheral cholangiocarcinoma (pCC), distal chlangiocarcinoma (dCC), Biliary Intraepithelial Neoplasia (BilIN), Intraductal papillary neoplasm of the bile duct (IPBN), Von Meyerburg Complex (VMC) and bile duct adenoma (BDA.

Introduction

Intrahepatic cholangiocarcinoma (ICC) is a malignant tumor involved in the heterogeneous group of cancers of the biliary tract. It arises from the intrahepatic biliary tract and is responsible for 10-20% of primary liver cancer worldwide[1]. The aim of this review is to give an updated overview of the known complex biology of this tumor and its peculiar molecular pathways. The importance of investigating the genomic nature of this tumor is considered the key approach to reach targeted therapies. Precancerous lesions in the biliary tract are one of the most attractive conditions for both pathologists and endoscopists involved in this field. Despite its rarity, the growing incidence and the dismal prognosis of ICC are highlighting the importance to reach an earlier diagnosis aiming for a curative management.

Cholangiocarcinoma (CCA) comprises a heterogeneous group of cancers with pathologic features of biliary tract differentiation and it is presumed to arise from both intra- and extrahepatic biliary tract[1]. The classification of cholangiocarcinomas into intra- and extrahepatic cancers is based on differences in anatomy, etiology, pathogenesis, molecular signature, and treatment. The second-order bile ducts are the anatomic margin for the distinction between these two subsets [2]. Intrahepatic cholangiocarcinoma is best classified anatomically as perihilar or peripheral[3]. It is the second most important primary liver malignancy, which adds up to 10-20% of primary liver cancer diagnosed globally.

The incidence of this tumor varies worldwide, with the highest rates in Northeast Thailand (> 80 per 100,000 population) and much lower rates in the Western world, with rates of 0.3-2.1 per 100,000. Despite its significant geographical and ethnic variations (Hispanics females and American Indian/Alaskan Native and Asian Pacific groups seem the most affected ethnic groups), recent studies have reported a global increasing incidence over the last few decades[4-6] and simulta-

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neous higher mortality. Similar trends are observed in both sexes with an overall slight male predominance (M: F 1.2-1.5:1).

Cholangiocarcinogenesis is a complex multistep process. It may occur in normal liver or with underlying liver disease, and in these cases it appears as a mixed type hepatocellular-cholangiocarcinoma instead of traditional adenocarcinoma. Unfortunately, many studies do not differentiate between ICC, pCC or dCC, but most recent surveys are supporting the different underlying biology of these tumors. Several risk factors of chronic inflammatory damage and increased cellular turnover have been established, such as hepatobiliary flukes (*Opistorchis viverrini* and *Clonorchis sinensis,* classified as group 1 carcinogens in WHO classification 2009), primary sclerosing cholangitis (PSC), biliary tract cysts, hepatolithiasis and toxins[7]. Cirrhosis, chronic hepatitis B and C, obesity, diabetes mellitus and alcohol liver disease are known risk factors for HCC and they are emerging now as warning conditions for ICC, as well[8]. There is growing interest in suspected association with inflammatory bowel disease and smoking, and even for defining the role of genetics polymorphisms. These, indeed, could be the key roles in understanding the single predisposition in developing these tumors. Variations in genes coding for some enzyme systems with several functions in our cells, such as 5,10- Methylenetetrahydrofolate reductase or glutathione-S transferases, may be associated with increased ICC risks[9].

Despite this awareness, ICC early diagnosis is still a major clinical challenge since patients with early stage disease are often asymptomatic. Weight loss, abdominal discomfort, biliary tract obstruction with jaundice and hepatomegaly or palpable abdominal mass are usually observed at more advanced stages. A recent metanalysis from 57 studies and 4756 patients showed that vascular and perineural invasion were present in 38% and 29% of the patients, respectively; biliary invasion was present in 29% of the cases. Among patients who underwent a lymphadenectomy, approximately one-third had lymph node metastases (34%; range, 9%-100%). These results clearly highlight that we are still discovering the tumor when detectable dissemination is almost certain[10].

The only potentially curative treatment option for patients who have a resectable disease is surgery. Unfortunately, even after curative-intent surgery, the clinical outcomes of patients undergoing liver resection are disappointing, with a 5-year survival rate of 20% to 35%. Furthermore, the role of adjuvant therapies, including systemic chemotherapy and radiotherapy, remain poorly defined and have been reported to have only a modest therapeutic effect.

Traditional classification of ICC included well, moderately and poorly differentiated adenocarcinomas. Most recent evidence affirmed a new pathological concept to classify ICC into conventional ICC, bile ductular ICC, intraductal neoplasms and rare variants (combined hepatocellular CCA, undifferentiated type, squamous/adenosquamous type)[11]. This attention to a more defined pathological assessment reflects the current interest in the emerging biology of this tumor.

Defining its molecular pathways is the first step to know its specific features to assess an earlier diagnosis and also a tailored targeted therapy. Furthermore, there are different subgroups of this disease with different behavior, according to the high incidence of recurrence associated with certain tumor-specific factors.

Future research should, therefore, target the identification of novel agents with more activity toward ICC aiming to increase the goal of prolonging survival among this challenging group of patients.

The era of individualized medicine and targeted therapies needs a better understanding of tumour biology and molecular pathogenesis. The importance of knowledge of the genetic landscape of tumor cells could even highlight the complex interaction between the disease and the tumor microenvirement.

Molecular Pathology of Intrahepatic Cholangiocarcinoma

Carcinogenesis involves specific cell genome derangements and several molecular pathways contribute to the selective growth advantage of cancer cells. The wide genetic landscape of ICC is referred to signals governing cells differentiation, proliferation, cell survival and maintenance of genome integrity. A discrete number of chromosomal aberrations, mutations, deregulated signalling pathways and epigenetic changes have been described in the most recent literature (Figure 1).

Going to the Origin

The first section deals with the cell of origin and processes governing cell fate and differentiation. The origin of ICC, indeed, has been long debated and interesting theories emerged.

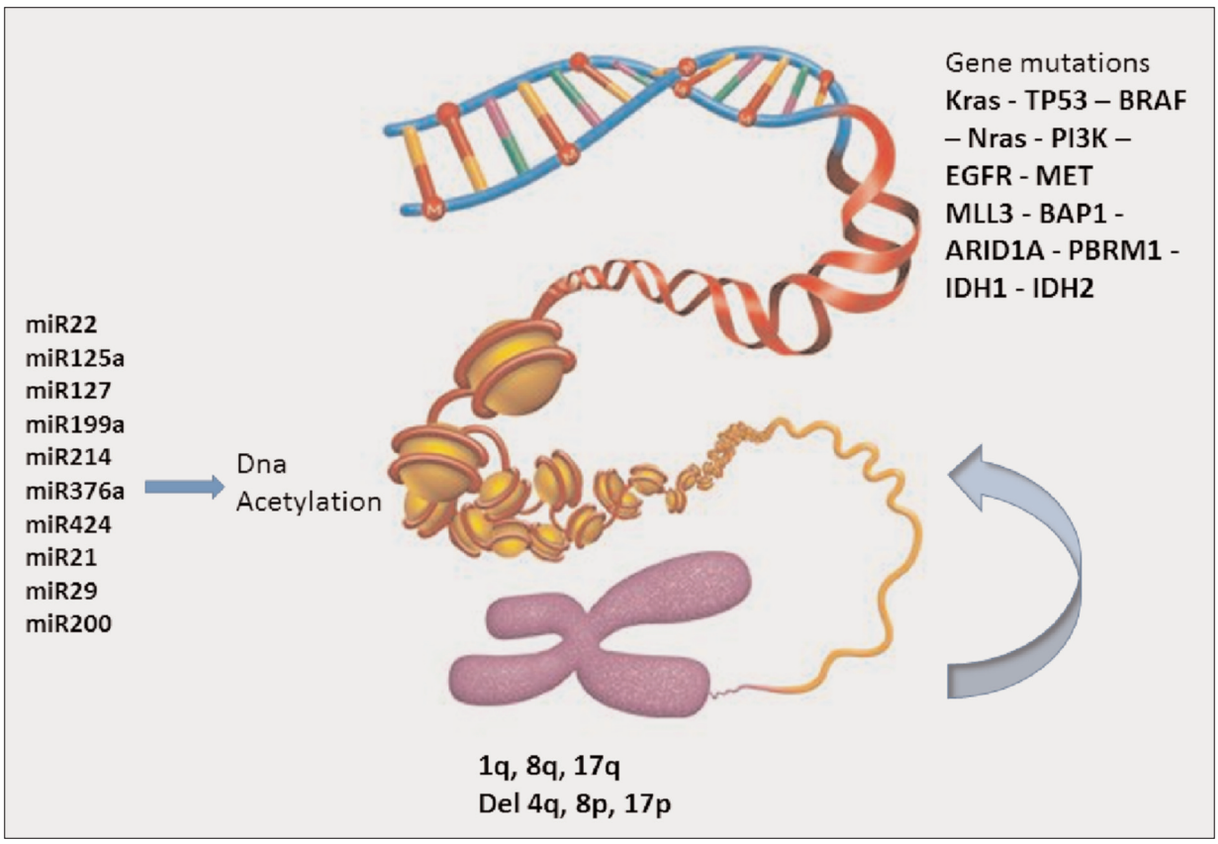

Figure 1. Multistep biology of intrahepatic cholangiocarcinoma.

In the past, it was presumed that ICC was a simple malignant transformation of cholangiocytes. Then, the complex heterogeneity of colangiocarcinomas was linked to different sites of the biliary tree and different stem cell niches described in the liver and biliary tree, considered hepatic bipotential progenitor cells. Cardinale et al[12] also defined the "cancer stem cell" as the cellular subset within the tumor that uniquely sustains malignant growth, while the "cell of origin" is the normal cell that acquires the first cancer-initiating mutations. Discovering heterogenous stem cells in the canals of Hering and peribiliary glands gave the chance to hypotize a physiopathological classification of CCAs based on the cell-lineage-of-origin. Following the process of maturation from the two different stem cell niches, it was hypotized that some ICCs originate from cells within the lineage starting in the canals of Hering, while other ICCs and the EH-CCAs could originate from endodermal origin stem cells of peribiliary glands lineage within the medium-large IH and EH bile ducts. The former could be classified on the basis of the grade of maturation of the cell-of-origin, in: (1) combined HCCCCA (hHpSCs); (2) CCL or ductular type CCA (immature cholangiocytes); and (3) CCA of the small bile ducts or mixed-CCA (mature cholangiocytes of the interlobular bile ducts). The latter could be classified in mucin-producing CCA of large bile ducts recognizing as cells-of-origin the ones within the BTSC-derived lineages (i.e., BTSCs in PBGs, cholangiocytes of large IH and EH bile ducts and goblet cells)[12].

More recent researches surprisingly showed that intrahepatic cholangiocarcinoma can arise from trans- differentiations of differentiated mature hepatocytes[12-15]. In this reprogramming subset the Notch pathway stands the key mechanism as a driver of biliary transformation and these studies challenged the theory that cholangiocarcinoma cells are derived from cholangiocytes, peribiliary glandular cells or hepatic progenitor cells. The plasticity of the liver cells at the differentiated state opened wider hypothesis about the overlap in hepatocellular carcinoma and cholangiocarcinoma signatures. Constitutive Notch2 activation emerged as a marker of poorly differenti-

ated hepatocellular carcinoma with features of biliary epithelium (SOX9 positivity). In another work[14] the overexpression of both Notch1 and AKT leaded to lethal ICC formation, through the transformation of hepatocytes into cholangiocyte precursors.

The exact definition of the origin of this disease is still a challenging and unsolved matter; however, it is suggested that mature hepatocytes, hepatoblasts and undifferentiated bipotential stem cells can produce a wide range of tumors, ranging from cholangiocarcinomas to hepatocellular carcinoma.

Chromosomal Aberrations

The chromosomal imbalance is one of the first features emerging in the wide genomic landscape of ICC. Recent advancements in genome definition[16,17] allowed a specific detection of defined chromosomal regions, candidate as main targets for future therapies. To date, innovative whole-genome technologies combined with integrative multidimensional analytical systems have become the most specific way of studying these alterations.

In the past, comparative genomic hybridization was reviewed in five studies focused on copy number variations in 98 typical UCC and revealed frequent copy number losses on chromosomal arm 1p, 4q, 8p, 9p, 17p and 18q, while copy number gains were found on chromosome 1q, 5p, 7p, 8q, 17q and 20q, in at least 3 studies with greater than 20% overall change[18].

Then, few investigations[19,20] analyzed whole-genome chromosomal copy-number alterations (CNAs) in ICC[19-21]. Among them, Sia et al[19] used single nucleotide polymorphism arrays in 149 patients and identified frequent CNAs, reporting a poor outcome associated with 14q22.1 deletion. It was performed, indeed, an analysis of a series of 149 archived formalin-fixed tumor tissues and reported the presence of two distinct transcriptional classes: the so-called “inflammation class” characterized by activation of inflammatory signaling pathways and the so-called “proliferation class” characterized by activation of oncogenic signaling pathways (including EGFR and MET), the latter being associated with a worse outcome.

More recently, a study described specific chromosomal patterns in 53 patients with intrahepatic cholangiocarcinoma who underwent surgical resection with curative intent. They performed DNA hybridization on a genome-wide SNP array and implemented a tree-based algorithm to classify genomic regions into four meaningful CNA states: copy loss, copy neutral, copy gain and copy neutral loss of heterozygosity. This allowed the identification of exclusively deleted and amplified recurrent genomic areas. Deleted genomic areas were 1p, 3p and 14 q and main amplified were 1q, 7p, 7q and 8q. Some of the recurrent exclusive chromosomal copy number alterations are supposed to harbor candidate target genes. Above all, the use of SNP array advanced technology allows the identification of high-level amplifications and focal deletions in order to explore novel oncogenes and oncosuppressors. Moreover, latest resources as molecular inversion probe single nucleotide polymorphism (MIP SNP) assay could facilitate the perspective of a faster routine copy number and marker gene identification for gene targeted therapy[16,17].

Interestingly, the study of these alterations suggest that ICC and HCC may be closely related at the molecular level as they share common chromosomal gains (chrom. 1q, 8q, 17q)and losses(4q, 8p, 17p). A close genomic similarity between ICC and a subset of HCCs with Hepatic Progenitor cells (HPCs) characteristics was shown in several recent studies[22].

Single Somatic Gene Mutations and Epigenetic Changes

The definition of the detailed molecular portrait of ICC is dominant as well. This is achieved by new opportunities of studying driver mutations using either targeted or whole- genome/exome sequencing technologies.

Collectively, genes affected by recurrent somatic mutations in ICC can be functionally grouped into those involved in genomic stability, cell cycle control, Wnt signaling, cytokine signaling, TGF-beta signaling, MAPK signaling, AKT/PI3K signaling and epigenetic regulation.

Activating mutations of KRAS are the most frequent emerged in ICC (22% cases described, ranging 5-57%). KRAS is an oncogene capable of inducing this tumor in genetically engineered mouse models and this leads to a more aggressive phenotype. It is linked to the activation of ERB-B family receptors activation, as KRAS is activated downstream of EGFR. Interestingly, the incidence of activating KRAS mutations is increasing moving from the liver towards the pancreas and also with increasing tumor stage. It is supposed to be an early event in the growth of malignancy.

Loss of function of the cell cycle regulator TP53 occurs in 15%, ranging 1-37% of cases and

have been proven oncogenic in experimental models frequently inactivated and associated with loss of heterozygosity. More than 90 different mutations are described, most of which associated with chemical carcinogen environmental exposure (e.g. thorotrast).

BRAF (7%), NRAS, PI3K, EGFR (2%) and MET mutations are rare events involving < 5% cases[22].

Next generation sequencing has enabled comprehensive mutation profiling of ICC. This approach was first used to describe genetic mutations in liver-fluke related ICC in endemic areas. Eight tumors were characterized by a total of 206 somatic mutations affecting 187 genes. The predominant somatic substitution was C: G > T: A and frequent somatic mutations emerged in key genes as TP53, KRAS and SMAD4. Furthermore, this analysis revealed alterations in previously unrecognized genes as MLL3, ROBO2, RNF43 and PEG3 and GNAS. These genes are involved in histone modification, genome stability and G protein signaling[23].

Subsequent studies showed that liver-fluke associated ICC encompasses a higher mutational burden[24], but most importantly, confirmed the emerging role of epigenetic complex architecture: indeed, up to 47% cases of ICC in some series have been linked to alterations in almost one chromatin-modifying gene. This revealed a significantly reduced 3-year survival rate of 33% compared to 81% for patients with the wild-type Isocitrate dehydrogenase gene (IDH)[25].

Concurrently, several recent studies[20] highlighted how next-generation DNA sequencing in everyday clinical practice has enabled oncologists to personalize therapy decisions. In this perspective, FGFR2 and NTRK1 tyr kinase receptors have been already described and FGFR inhibitors proposed as the MAPK signalling pathway inhibitors[20]. More specifically, PTPN3 mutations and other PTP family genes supposed to be a marker of ICC in Chinese population, as shown in a study involving exome-sequencing 7 tumor and even surrounding non-tumoral tissue[26].

Recent discoveries about chromatin -modifying genes emphasize how coding genoma determines the phenotype of the cell and these mechanisms play a key role in carcinogenesis. It is noteworthy, indeed, that epigenetics refers to the complex architecture overheading the first level DNA sequence expression and include histone modification, DNA methylation and non-coding RNAs[27].

In this field, a key group of genes that were found to be highly mutated in ICC through next-generation sequencing studies are chromatin modifying genes as MLL3, BAP1, ARID1A, PBRM1 and IDH. Latest studies focused on hotspots mutations of genes encoding Isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2), identified in the 14% of 340 ICCs[1]. These co-occurred with TP53 mutations and DNA hyper methylation. The aberrant DNA methylation occurs in CpG rich areas in the promoter regions of tumor suppressors. The resetting of the cell transcriptome program is associated with atypical gene silencing, affecting genes involved in tumor progression, DNA repair and metastasis[28,29]. Special AT-rich sequence-binding protein 1 (SATB1) are overexpressed in several kinds of malignant tumors and have been correlated with a more aggressive tumor phenotype and worse prognosis. Thus, it emerged as a significant marker even in ICC tissues and was associated with a metastatic phenotype, though the regulatory activity on ICC cell proliferation and invasion through the activation of MYC.

To resume, next-generation sequencing studies have highlighted the importance of chromatin remodeling factors conditioning the altered sensitivity to drugs targeting as histone deacetylase inhibitors or demethylating agents. This will affect the future clinical decisions and, as future perspective, these enzymes products could be detected in the serum and used as a biomarker of early diagnosis.

Decoding Biological Significance of Non-coding miRNAs

MicroRNAs are small non-coding RNAs, 20-22 nucleotides in length, that function as critical rheostats of the genome, regulating key properties such as cell survival, autophagy, stemness and response to therapy. A promising biological significance has been linked to these fragments with aberrant expression in cholangiocarcinoma cell lines[30-32]. Kawahigashi et al[33] first provided a profile of different expressed miRNAs in ICCA derived-cells: a 27 miRs signature was identified, including downregulation of eight miRs (miR22, miR125a, miR127, miR199a, miR199a, miR214, miR376a and miR424) specific for normal bile duct epithelium.

Recently, the role of miRNAs in the establishment and progression of ICC has become evident, and some of these nucleotides have been identified as tumor suppressor genes or oncogenes in ICC.

Wang et al[34] showed that the expression level of miR21 could distinguish ICC from the normal bile duct. It was also found that MiRNA21 is responsible for PTEN-dependent activation of PI3K signaling and this is indeed linked to cell growth and resistance to chemotherapy. PTPN14 and PTEN are the main targets of this miRNA. High expression levels of miR-21 were closely related to adverse clinical features, reduced survival, and poor prognosis in ICC patients. More recently, it was confirmed that MiR21 is an effective noninvasive screening and prognostic tool in patients with ICC[34].

Another interesting analogue is miR29, which emerged as a potential tumor suppressor in multiple human neoplasms as part of the TGF- 1/miR-29a/HDAC4 pathway. Several studies have shown that miR-29a was significantly downregulated in gastric, lung, and hepatocellular cancer. As expected, a recent study demonstrated that miR-29a was significantly reduced in cholangiocarcinoma cells and tissues and when overexpressed, instead, inhibited cholangiocarcinoma cell growth and metastasis by targeting HDAC4[34]. In addition, miR25 seems to have an apoptotic effect whereas miR26a could mediate intracellular accumulation of beta-catenin[35]. Other dysregulated miRNAs include miRlet7a and miR42[36-38].

Above all these new molecules, MiRNA 200-family expression profile and epithelial to mesenchymal transition represent another interesting area of investigation[39]. Mi200c was studied to assess its role in determining hepatic-stem like phenotype and, as well, miRNA 294 is suspected for playing an important role in epithelial-mesenchymal transition in ICC lines in Chinese population[40]. MiRNA 203 functions as a tumor suppressor and recently described as decreased in CCA tissues. The expression of miR-203 was dramatically decreased in CCA tissues, with significant association with tumor progression and predicted poor prognosis in the ICC patients. These findings indicate that miR-203 can serve as a novel prognostic marker and potential treatment target in ICC[40,41].

New protein profiles linked to these MiRNAs are progressively discovered and, for example, Novel (nua) kinase family (NUAK)1 functions as oncogenes in various cancers and are a putative target of miR-145 regulation. When miR-145 significantly decreases in ICC tissue and cell lines, it corresponds to an increase in NUAK1 expression. On the other side, the overexpression of miR-145 in ICC cell lines inhibits proliferation, growth, and invasion by suppressing NUAK1 expression, through a decrease in Akt signaling and matrix metalloproteinase protein expression[42].

Key Role Signaling Pathways

The ongoing definition of the complex nature of this disease can be reviewed as main activated significative pathways, considering even the determining biology of the nearly stromal tissue. This is the basis to determine a future molecular classification of this tumor. Defining these alterations in the microenvironment as well, is similarly crucial, considering that cholangiocarcinoma is a highly desmoplastic tumor and the surrounding tissue is a key component of the tumor development and progression.

Sia et al[19] interestingly showed the importance of the two distinct activated aspects, according to inflammation molecules and proliferation pathways. In the analysis of 149 ICCs, they identified two molecular subgroups, inflammation and proliferation, with distinct genomic profiles and clinical outcome. 40% consisted of inflammation subclass with enrichment of inflammation and cytokine pathway signatures, over-expression of IL-6, IL10 and IL17 with constitutive activation of STAT3. The proliferation subclass (60%) was characterized by enrichment of oncogenic pathways including RAS/MAPK and MET, high-level DNA amplifications at 11q13, deletions at 14q22.1 and poor clinical outcome.

ROBO2 and Wnt signaling pathway, including Hedgehog and Notch signals, could be the element to understand the cross-talk between epithelial tumoral cells and cancer-associated stromal tissue, with significant implications in chemoresistance. Profibrotic signals and tumorigenesis are supposed to be a novel target in treatment strategy[43,44] and periostin is in a product of cancer associated fibroblasts within the tumor stroma[45].

The Hippo signaling pathway is another novel-tumor suppressor pathway involved in development and stem cell biology. Deletion of MSt1/2 was recently shown to cause both HCC and CCA, with the activation of the oncogene YAP1 and resistance to FAS-induced apoptosis. If acutely damaged Mst1/2, it relates to activation of mTOR complex 1 and its downstream phosphorylation increases. YAP1 overexpression also seems to mediate activation of Notch- WNT signaling, according to a cross-talk between the two pathways[46-51].

Main Features of Precursor Lesions: Early Detection and Diagnostic Challenges

A wide range of premalignancy has been described according to the heterogeneous features of this tumor. In most recent literature, ICC is traditionally divided into perihilar and peripheral types[3,52]. The perihilar type, involving the large bile ducts, is characterized by mucin production, according to a large tubular component or papillary proliferation of tall columnar epithelium. The peripheral type, involving smaller ducts and segmental branches, is composed of a proliferation of relatively small, tubular, closely packed cord-like structures or ductular pattern lined by small cuboidal epithelium[3]. Perihilar type, conventional ductal carcinoma, bile duct type and mucin-producing type are considered part of the same group, while the peripheral, bile ductular, cholangiolar and cholangiolocellular types are distinguished by other similarities[3,11]. Some lesions are presently considered part of the suspected premalignancy pattern group, which comprises Biliary Intraepithelial Neoplasia (BilIN), Intraductal papillary neoplasm of the bile duct (IPBN), Von Meyerburg Complex (VMC) and bile duct adenoma (BDA)[53]. As previously reported, ICCs is not a unique disease and distinctions are needed in the discrimination of early lesions. Notably, perhilar large duct type ICCs may develop from biliary intraepithelial neoplasia (BilIN) and intraductal papillary neoplasm of the bile duct (IPNB)[54,55]. Otherwise, ICCs may evolve even without define premalignant lesions, as no intraepithelial or intraductal preneoplastic, dysplastic or neoplastic lesions have been demonstrated in the small bile ducts or ductules in some tumors.

BilIN epithelial lesion is a precursor to both intrahepatic and extrahepatic cholangiocarcinoma. It is characterized by epithelial cells with nuclear pseudostratification and atypia, often with micropapillary projections into the bile duct lumen. Frequently described as the biliary counterpart to pancreatic intraepithelial neoplasia, it is subdivided into Bil-1, -2, -3 as cells progressively lose differentiation and polarity going to the upper part of the epithelium and acquire invasive cytological characteristics, without the invasion of basement membrane. Macroscopic and radiologic identification of these lesions is not possible. BilIN shares several molecular alterations with cholangiocarcinoma, and the study of BilIN alongside cholangiocarcinoma has helped to elucidate several of the key molecular mechanisms in cholangiocarcinogenesis. Many of these molecular changes accumulate in conjunction with increasing grade of BilIN. KRAS has been shown to occur early in this lesion and it is present in approximately 33% of BilIN lesions. Also, p21, p53, cyclin D1 and EZH2 have all been described and, importantly, their expression has been shown to increase while the expression of Dcp4 and p16INK4A decreases in tandem with increasing grade of BilIN. Moreover, the study of the expression of autophagy-related proteins is another interesting point about molecular features during the progression to BilIN-3[53].

IPNB is usually described as the biliary analogue of the intraductal papillary mucinous neoplasm (IPMN) of the pancreas. As IPMN, it is characterized by fibrovascular cores lined with noninvasive papillary or villous epithelium and filling a dilated bile duct lumen. Grossly, IPNB shows dilated, fusiform to cystic bile ducts with soft papillary lesions ranging from white to tan to red. Histology shows that the aforementioned fibrovascular cores are lined by any of four cell types between pancreatobiliary, intestinal, gastric and oncocytic types, parallel to those defined in IPMN. Anyway, similarities between IPNB and IPMN are not all as expected. Mucin production, for example, is typical of only 1/3 of IPNB, while in IPMN it is almost certain; the presence of gastric epithelium, as well, is rarely observed in IPNB but usually present in IPMN. However, the grading of IPNB follows the same evolution of IPMN. Low, intermediate and high grade are assigned based on cytological and architectural characteristics, where low- and intermediate-grade tumors comprise one diagnostic entity, high-grade tumors another, and IPNB with associated invasive carcinoma a final separate entity. These lesions are still poorly characterized, but it has been assessed that cyclin D1 and p21 expression increase occurs in IPNB just as it does in BilIN. Similar to pancreatic neoplasms, loss of SMAD4/DPC4 has been shown in IPNB and BilIN with increased loss associated with higher grade[56].

Von Meyenburg complex (VMC) is suggested to be a possible premalignant lesion of ICC due to the occasional association of VMC with ICCs and reports of VMC-like cystic ICCs. VMCs, also known as bile duct or biliary hamartomas or biliary microhamartomas, are small (< 5mm), well-circumscribed, encapsulated nodules consisting of irregularly shaped, often dilated bile ducts lined by a low cuboidal epithelium, embed-

ded with a dense collagenous stroma. Multiple case reports showed VMCs with histologic evidence of transformation to cholangiocarcinoma, reporting benign, hyperplastic and dysplastic epithelium with adjacent progression to invasive carcinoma. This phenomenon was linked to molecular evidence of mutations shared between cholangiocarcinoma and VMCs. Specific molecular genetic changes have not been definitively established, but in one study of two patients with multiple VMCs and cholangiocarcinoma, loss of heterozygosity (LOH) was examined at 20 key genetic loci in the cholangiocarcinoma tumor, in VMCs distant from the tumor, and in intermediate lesions where VMCs showed transformation to cholangiocarcinoma. LOH was seen in VMCs at some of the same key loci as seen in cholangiocarcinoma, affecting oncogenes p16, p53, APC and PTEN which have been shown to play a role in the development of cholangiocarcinoma.

Progression of bile duct adenoma (BDA) to ICC has also been reported in the case of BDA or Peribiliary Gland Hamartoma, consisting of small (< 2 cm) lesions located beneath the liver capsule. They are rare, firm, well-circumscribed and not encapsulated. Histologically, they are comprised of uniform tubular or curvilinear ductules within a fibrous stroma. The ductules are lined by cuboidal cells with bland, round to oval nuclei and without mitotic activity, and sometimes show mucinous metaplasia, 1-antitrypsin droplets and neuroendocrine differentiation. Malignant transformation of BDA has not been unequivocally demonstrated. Identification of oncogenic mutations in BDA supports a benign neoplasm rather than reactive process and suggests that BDA may be an early lesion in the pathogenesis of ICC. Few cases of biliary adenofibroma have been reported to date. It is a benign tumor, with complex tubulocystic nonmucin secreting biliary epithelial and an abundant fibroblastic stromal components and the malignant transformation of the epithelial component has been reported. Preinvasive neoplastic lesions of ICCs are still not definitely pinpointed and evidences in this field arose from surgical histological examination, according to late diagnosis and attempts to curative resections[53].

The diagnosis of intrahepatic cholangiocarcinoma is challenging because of the paucicellular features of the malignancy and requires a multidisciplinary approach that includes clinical evaluation and laboratory, endoscopic, and radiologic studies[2]. Noninvasive early detection is even more challenging and endoscopic visualization is still not reached in the routinary management of this disease. Several devices are presently available to diagnose the progression of precursor lesions in several gastrointestinal cancers and endoscopic visualization of the main biliary duct and ramifications is proposed, as well. Considering resources used in the diagnosis of biliary tract disease, endoscopic retrograde coledocopancreatography (ERCP) is the first line approach and cholangioscopy, endoscopic and intraductal ultrasound and confocal laser endomicroscopy are additional methods which can be applied for the diagnosis of CCA[57]. Since the 1970s, peroral cholangioscopy was supposed to give a good visualization of the biliary tract, comprising intrahepatic components. Single operator cholangioscopy with Spyglass system has been recently proposed as an innovative method of direct visualization combined with histological sampling[58,59]. Another magnifying technique added to traditional endoscopic view has been proposed with the use of Narrow-bind imaging (NBI) to allow high-contrast observation of mucosal structures and vascular patterns[60,61]. A recent study showed its power in detecting dysplasia progression in primitive sclerosing cholangitis (PSC). In a group of 30 patients, four had a final diagnosis of CCA (2 extrahepatic, 2 intrahepatic). NBI detected only the 2 extrahepatic CCAs and allowed determination of tumor margins. The bile duct mucosa by NBI visual appearance in patients with PSC was variable and no correlation with CCA development could be determined. There was a 48% increase in suspicious lesions biopsied with NBI compared with white-light imaging, although NBI-directed biopsies did not improve dysplasia detection rate. In conclusion, NBI allowed visualization of tumor margins in CCA as compared with traditional fluoroscopy-based ERCP, but an improvement in dysplasia detection in patients with PSC could not be demonstrated despite an increase in the biopsy rate[61]. To date, most common conditions requiring this approach are indeterminate biliary strictures and difficult common bile duct stones, while we are still far from a considerable endoscopic approach to intrahepatic biliary cholangiocarcinoma. Nevertheless, promising devices are always introduced and most recent studies are focusing on microscopic instruments more dedicated to histological pattern, as confocal laser endomicroscopy. It allows the acquisition of histological architecture and gives more information

such as blood flow evaluation. New systems consist of probe-based confocal laser endomicroscopy, which is a mini-probe inserted in the operative channel of a traditional endoscope. Tissue pattern is visualized by parallel scans, instead of the orthogonal perspective of traditional histology. Imaging generation is achieved by the use of a fluorescent contrast agent and consists of virtual optical biopsies acquisition. The neoplastic pattern is easily suggested in case of irregular, winding vascularization, with fluorescein spreading and irregular and dark cellular pattern. In other gastrointestinal tracts, it works as a guide to reach targeted biopsies and this chance is expected even in the biliary tract. Effectively, it is a fluorescence-based technique and this could be a common point with advances in molecular biology. Combining endomicroscopic study with molecular probes highlights new perspectives even in the diagnosis of ICCs, especially in the perihilar types. Barrett esophagus detection, for example, is an established application of this device and some results are emerging even in the biliary setting. A recent study reported 14 patients with suspected indeterminate biliary stenosis studied with confocal mini-probes, leading to more accuracy than histology and cytological brushing[62]. An additional study consisting of 102 cases, supported a better diagnostic yield with the use of ERCP combined with confocal mini-probe instead of traditional ERCP with simple cytological sampling[63].

In the wide filed of endoscopic ultrasound, high-frequency (15-20 MHz) mini probes should be finally mentioned as further devices proposed for the pancreaticobiliary duct system, even if there are still no studies reporting its evidence in intrahepatic cholangiocarcinoma. Probably, the role of these mini-probes as previous techniques, such as Optical coherence tomography (OCT), proposed in this specific setting should be consigned to the history[64-67].

Conclusions

The heterogeneous and difficult nature of the intrahepatic cholangiocarcinoma is becoming a leading condition to define, in accordance with its growing incidence and poor prognosis[68, 69]. Many studies have assessed the key role molecules involved in the carcinogenesis and detectable at main levels of cell's biology, from single gene mutations to proteins aberration. There are many candidates for targeted therapies as MET, EGFR and ERBB2, FGFR2, JAK/STAT, RAS/RAF/MAPK, PI3K/AKT/mTOR pathways and even IDH mutations. At the same time, an earlier stage diagnosis probably is the next challenge in this field, considering the promising studies about the detection or visualization of precursor lesions and specific biomarkers in the blood. In this perspective, more efforts are needed to perform other studies and achieve substantial clinical results.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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