The significance of genetics for cholangiocarcinoma development

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Abstract: Cholangiocarcinoma (CCA) is a rare malignancy of the liver, arising from bile ducts. The incidence is increasing worldwide, but the prognosis has remained dismal and virtually unchanged in the past 30 years. Although several risk factors have been associated with the development of this cancer, none of them are normally identified in most patients. Diagnosis in advanced stages of the disease and limited therapeutic options contribute to poor survival rates. The recent analysis of genetic and epigenetic alterations occurring in CCA has shed new light in the understanding of the molecular mechanisms leading to the malignant transformation of biliary cells. Further studies in this direction may foster new diagnostic, prognostic and therapeutic approaches. This review provides a global overview of recent advances in CCA and describes the most important genetic mutations and epigenetic alterations so far reported in CCA.

Key Words: Genetics; cholangiocarcinoma (CCA)

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Introduction

Cholangiocarcinoma (CCA) is a rare malignant cancer arising from cholangiocytes, the epithelial cells lining the bile ducts (1). Anatomically, CCA is classically divided in intrahepatic or extrahepatic. The intrahepatic form arises within the liver parenchyma, and the extrahepatic variant may be further subdivided in perihilar (also called Klatskin tumor) or distal tumor, with the landmark at the insertion of the cystic duct. The extent of the perihilar CCA may be described according to the Bismuth-Corlette classification (1,2).

Symptoms of CCA are often nonspecific and appear late in the course of the disease; therefore, extrahepatic cancer may show signs and symptoms related to cholestasis, such as jaundice without pain, pale stools, dark urine and pruritus, whereas intrahepatic CCA is often an incidental hepatic lesion (3,4).

To date, no specific CCA markers have been found. However, CA 19-9 (i.e. carbohydrate antigen 19-9) and CEA; (i.e. carcinoembryonic antigen) usually support the diagnosis in association with clinical, radiologic, and endoscopic findings (5-7). Despite not being specific for CCA, classical cholestatic serum parameters are often increased.

The therapeutic options for this cancer are very limited. CCA is characterized by high chemoresistance and is usually late diagnosed, providing few possibilities for surgery. These features result in low survival rates: about 50% of patients who did not receive surgery die in 3-4 months after diagnosis due to liver failure or infectious complications associated with the progressive biliary obstruction (8). On the other hand, survival rates after five-years liver resection were 20-32% for intrahepatic, 30-42% for perihilar, and 18-54% for distal CCA (9).

The election of the adequate surgery procedure is often

complex and depends on the tumor stage and localization. In general, the liver resection size for intrahepatic and perihilar CCA is driven by the histological pattern and usually needs partial hepatectomy in order to achieve negative resection margins, which correlates to a better survival. On the other hand, pancreatoduodenectomy (also called Whipple resection) is indicated for distal CCA (10-12). So far, the beneficial effects of orthotopic liver transplantation (OLT) have not been completely established in terms of survival improvement compared to partial hepatectomy. However, cases of liver transplantation in selected conditions have shown promising results with regards to survival improvement (11).

CCA is usually not affected by common chemotherapies. Several studies using monotherapy or drugs combination have been performed but currently none of the antineoplastic regimens show a sufficient efficacy in CCA (13).

When surgery is not indicated, the treatment is palliative and mainly aims to reduce the biliary obstructions and infection, as well as the relative symptoms (8).

Epidemiology of CCA

Although CCA is overall a rare neoplasm accounting for 3% of all gastrointestinal tumors worldwide (14), it is the second most common primary hepatic neoplasm, after hepatocellular carcinoma (15). Several studies have reported that while the incidence and mortality rates for intrahepatic CCA are increasing worldwide, a slight decrease or stabilization for extrahepatic CCA might be occurring. In particular, the age-adjusted annual incidence of intrahepatic CCA appears to have a progressive increase in USA, from 0.13 per 100,000 persons in 1973 to 0.67 in 1997 (16) and to 0.85 during the period from 1995 to 1999 (17). In contrast, the age-adjusted incidence of extrahepatic CCA decreased from 1.08 per 100,000 in 1979 to 0.82 in 1998. Moreover, comparable trends have been shown also in the United Kingdom (18,19) and Germany (20), whereas in Italy increasing incidences for both intra and extrahepatic CCA have been reported (21). By contrast, the incidence trends in Denmark and France seem to be declining (22,23). However, over the past few years, some authors have started to investigate a number of biases that might have influenced the results of former studies on the epidemiology of CCA (24,25). The lack of a uniform classification of the heterogeneous group of CCA, the unification of biliary malignancies and other hepatocellular neoplasms (such as hepatocellular carcinoma and gallbladder cancer) in most cancer registries, and the frequent misclassification due to diagnosis in advanced stage and histological heterogeneity, are critical issues affecting not only epidemiologic studies but also the understanding of the pathophysiology of the disease (8,15).

Despite these possible controversies, a slight male preponderance and possibly differences between races are generally acknowledged (17,26). Moreover, a clear relative difference between the incidence in Eastern and Western countries is well established (17). The highest incidence rates are observed in Eastern and South-Eastern Asia, with a peak registered in Thailand [33.4 per 100,000 in men, and 12.3 per 100,000 in women, with important differences within the country itself (27,28)]. In these regions, a plain association between the infection with Opisthorchis *viverrini* and the development of CCA has been demonstrated (28).

Several risk factors have been extensively studied and associated with the development of CCA, such as, primary sclerosing cholangitis (PSC), liver fluke infection, hepatolithiasis or biliary malformations (4), however, the majority of patients do not develop any of these features. In addition, other risk factors such as genetic polymorphisms and life style might also contribute (26,29,30), although further studies are eagerly awaited.

Genetic alterations in cancer

Carcinogenesis is considered a multistage process that causes the malignant transformation of cells (31). Most of the gene mutations are somatic and occur as sporadic events; conversely hereditary cancer, which results from mutations inherited from parents, is less common (32,33). Up to 90% of somatic mutations are dominant, whereas only 10% of the tumors need both alleles mutation to induce tumorigenesis (33). Mutations can target the genome by changing a single nucleotides [i.e. the so called "point mutations or single nucleotide polymorphism (SNP)]", or by altering more nucleotides, thus resulting in deletions, insertions, translocations or amplifications (34). Although mutations may occur as sporadic or inherited events, the targeted genes may be classified in: (I) oncogenes; (II) tumor suppressor; or (III) stability genes (35,36).

Mutations in oncogenes, which in physiological conditions participate in several intracellular pathways, result in their aberrant activation and therefore in loss of cell proliferation control (37).

Oncogenes-related products consist of a wide class of proteins such as transcription factors, growth factors and their

receptors, signal transducers, and apoptosis regulators (35,37). Transcription factors modulate the expression of genes involved in signaling pathways via downregulation or upregulation of their transcription. For example, mutations of Fos/Jun/AP1 are detected in lymphoid cancers as Hodgkin lymphoma (38).

ERBB receptors and c-MET are both members of the growth factor receptors; the binding of specific ligands initiates intracellular cascades via tyrosine kinase autophosphorylation resulting in cell proliferation, decreased apoptosis, enhanced cancer cell motility, and regulating cell differentiation (39-42). Overexpression of ERBB receptors in several tumors is the rational to treat these cancers with drugs that inhibit tyrosine kinase activity (40,43). Among the signal transducers, K-ras mutations are widely detected in a variety of tumors such as colon cancer, pancreatic cancer, and melanoma (44). Finally, oncogenes can modify the antiapoptotic activity of some molecule as Bcl-2; aberrant activation might be thus correlated to excessive proliferation as, for example, in diffuse large B-cell lymphoma (45).

Tumor suppressor genes (TSGs) are typically recessive genes; both alleles need to be mutated in order to induce tumorigenesis, according to the so-called "two hit hypothesis" (46). Many human cancers, such as retinoblastoma and familial adenomatous polyposis (FAP), have been associated with inactivation of TSGs (47). In this regard, p53 is a fundamental regulator of the cell cycle that in case of DNA damage blocks the cell cycle and leads to cellular apoptosis (46,48).

Moreover, there is a class of cancer genes called "stability genes" composed by the mismatch repair (MMR), the nucleotide-excision repair (NER) and the base-excision repair (BER) genes. The role of these genes is to correct mismatches of bases generated during normal DNA replication or induced by mutagens. Alterations of MMR genes can induce mistakes during the DNA replication; slipped strand mispairing mutations lead to different length in DNA regions and since that condition facilitates gene mutation is called microsatellite instability (49,50).

The predisposition to develop HPCC is due to mutations in members of MMR genes as MLH1, MSH2, MSH6, and PMS2 (51).

Epigenetic alterations in cancer

The research of the last decade has highlighted that human cancers also harbor a number of other heritable abnormalities in gene expression that are not caused by mutation in any region of the genome, termed epigenetic changes (52). The most studied epigenetic changes that occur in cancer comprise DNA methylation and histone modification and, broadly, include non-coding RNAs.

Methylation of the bases that constitute the genome plays a key role in a variety of physiologic processes such as embryologic development (53), genomic imprinting (54), inactivation of the X-chromosome in females (55), and preventing DNA instability caused by transposable DNA sequences (56).

DNA methylation takes place in mammals when a methyl group is added to the cytosine that directly precedes a guanine in the genome (also called CpG site, for Cytosine-phosphate-Guanine). CpG sites are not randomly distributed throughout the genome. Indeed, stretches of CpGs, termed CpG islands, can be found in many genes at the 5' end, which corresponds to their promoter region (57). These regions are typically not methylated in normal conditions, but become hypermetylated in TSGs genes in a broad variety of tumors (52,58,59). As a result of promoter hypermethylation, the gene transcription is silenced or downregulated, and thus epigenetic changes can influence the carcinogenetic process in a similar fashion to genetic mutations. The list of TSGs found to be hypermethylated in cancer is wide and constantly growing; well-known examples are VHL in renal carcinoma (60), $p16^{INK4a}$ in many cancers (61), and hMLH1 in colorectal carcinoma (61). Although hypermethylation of CpG islands appears to be a major event in many cancers, hypomethylation of CpG sites is also described for many tumors (62).

An alternative epigenetic change that occurs in cancer is histone modification (63). Histones are alkaline proteins that serve as scaffolds around which DNA winds in structures called nucleosomes (64). Post-transcriptional modifications, such as acetylation, methylation and phosphorylation are common events that regulate the biology of histones. In this context, the acetylation by histone acetyltransferases (HATs) of lysine residues and the deacetylation by histone deacetylases (HDACs) are the most prominent modifications influencing histone function, and the balance between the two processes regulates, at least in part, the gene expression. Indeed, the removal of the acetyl group by HDAC leads to chromatin condensation and inhibition of transcription of the involved gene, whereas the action of HATs favors gene transcription, possibly via a more favorable DNA conformation for the binding of RNA polymerases and transcription factors (65,66).

Non-coding RNAs (ncRNAs) are a group of

Table 1 Genes most frequently altered in CCA

Gene	Mutation	Cellular effect	Reference(s)
RAS/BRAF	Hyperactivation	Activation of Ras/Raf/Mek/Erk pathway	(80,81)
EGFR/ERBB2	Hyperactivation	Activation of MAPK, PI3K/Akt, mTOR and STAT	(82,83)
c-MET	Hyperactivation	Activation of MAPK, PI3K/Akt, mTOR and STAT	(84)
p53	Suppression	Loss of cell cycle control and apoptosis	(85)
SMAD4	Suppression	Suppression of TGF-beta downstream targets	(86)
APC	Suppression	Accumulation of β-catenin	(87)

endogenously transcribed RNA molecules that are not translated into proteins. The large family of ncRNAs comprises different members generally divided in two major subgroups: small ncRNAs and long ncRNAs (67). In this manuscript, we will only highlight microRNAs [for a comprehensive review on ncRNA see Esteller *et al*., Knowling *et al*. (68,69)].

MicroRNAs are small RNA sequences (19 to 25 nucleotides) that are involved in many biological processes such as embryonic development, proliferation, differentiation, and cell death (70). MicroRNAs are encoded in the genome, transcribed into precursor transcripts, and undergo a series of tightly regulated processes leading to their incorporation in the RNA-inducing silencing complex (RISC). RISC then directs the modulation of mRNAs translation by the binding of the microRNA to the 3' untranslated region of the target mRNA through a partial or complete sequence homology; as a result, the translation of the mRNA may be downregulated or blocked, respectively (71). MicroRNAs have been linked to many aspects of cancer, from initiation and progression of tumors to response to therapy, and development of new treatment (72).

Genetic alterations in CCA

The specific mechanisms that occur during biliary carcinogenesis are still unclear. However, chronic inflammation, partial bile flow obstruction (i.e. cholestasis), and bile duct injury are recognized to be major features for malignant transformation (1,13).

Chronic inflammation induces the secretion of pro-inflammatory cytokines from both cholangiocytes and inflammatory cells (73). Interleukin (IL)-6 and other mediators such as endotoxins and tumor necrosis factor (TNF)-α are important cytokines produced during inflammation (74). IL-6 can activate different pathways leading to mitogenic responses and cell survival (75). IL-6 is also able to induce nitric oxide synthase (iNOS) expression, which in turn increases nitric oxide (NO) production resulting in DNA damage (76,77). In addition, such inflammatory scenario can also lead to cyclic oxygenase (COX)-2 activation, the enzyme involved in prostaglandin secretion. Bile acids and other bile components have been associated to COX-2 overexpression, resulting in cell growth, anti-apoptosis and angiogenesis (78,79).

To date, many genes have been related to cholangiocarcinogenesis (*Table 1*) (88). However, the specific mechanisms responsible for tumorigenesis in CCA are still under investigation. Among the growth factor receptor family, c-MET mutation was reported to frequently occur in bile duct cancer, event that correlates with high grade of invasiveness and a poor prognosis (84,89,90). On the other hand, gain-of-function mutations in ERBB2 and EGFR genes are frequently observed in several heterogeneous tumors such as breast, lung, and colon cancers (91). In this regard, EGFR overexpression correlates with malignancy in human cholangiocytes since such mutation has been detected in both gallbladder and bile duct tumors but not in physiological conditions (82,92). Similarly to EGFR, ERBB2 overexpression has also reported in CCA (83,93). The simultaneous expression of ERBB2 and COX-2 may indicate a prostaglandins secretion induced by ERBB2, which is known to be strongly mitogenic (94). Moreover, the correlation between ERBB2 mutations and tumor progression is suggested by the fact that rat cells transfected with the ERBB2/neu oncogene show features similar to human CCA (95). In terms of prognosis, EGFR mutation correlates with poor survival and cancer progression, whereas ERBB2 is suggested to be overexpressed in early tumor stages (96,97). The significant role of EGFR and its mutations for CCA development suggested the employment of Tyrosine Kinase inhibitors (TKi) as a promising therapeutic strategy, similar to what is currently under use in advanced carcinomas (43,76). However, TKi therapy showed only modest benefits in certain CCA patients (98).

Ras and *Raf* are oncogenes and members of the MAPK

pathway. *Ras* mutations have been associated with both intrahepatic and extrahepatic CCA. Indeed, frequent (i.e. G/A transitions in codon 12) and less frequent (i.e. GGT/ GAT and CCA/CAC transitions in the 12th and 61st codons, respectively) *Ras* point mutations have been reported (80,99,100). On the other hand, mutations of the *Raf* isoform Braf, contributes with Ras to CCA development. Indeed, no Braf expression was found in human HCC. The most frequent mutation is localized in exons 15 and leads to a T/A change (81).

Beside oncogenes, TSGs are also involved in CCA development and progression. p53, for instance, is involved in protection against aberrant proliferation, including cell cycle arrest and apoptosis (101). p53 inactivation is one of the most common mutations in human cancers and the most frequent among the class of TSGs (102). In CCA, p53 mutations are well-known and many studies have been performed to determine the specific incidence and the type of mutations (85,103) Thus, the aberrant p53 expression was detected by both immunohistochemistry and sequencing studies, as reviewed by Khan *et al*. (104). p53 mutations occur mainly in exons 5, 6, 7, and 8 as transitions (G:C/A:T) or less commonly as transversion (G-T) (105).

SMAD4 is another TSG that mediates the transforming growth factor (TGF)-β signals (106). The SMAD4/TGF-β signal transduction pathway also negatively regulates epithelial cell growth (107). Loss of SMAD4 activity is a frequent hallmark of gastrointestinal tumors, and has been most frequently observed in CCA arising the distal common bile duct, close to the pancreas, which is, noteworthy, the organ where that mutation occurs more often (86,108,109).

Adenomatous Polyposis Coli (APC) is an additional TSG that regulates different intracellular pathways (110). The typical mechanism of inactivation is characterized by a mutation in one allele followed by loss of heterozygosity (LOH) with complete gene inactivation. The mutation of APC was originally observed in colorectal cancer, but it is currently associated with many other human cancers (111). In CCA cells, APC mutation occurs quite frequently and may be responsible for the early stages of carcinogenesis (87).

Among the allelic losses, lack of 8p22 was found in intrahepatic CCA and may correlate to tumor progression (112).

Epigenetic alterations in CCA

The role of epigenetic alterations in the pathophysiology of CCA is attacking increasing interest (113-115). Although the current knowledge is sparse, the recent technological advances and the attractive possibility to develop novel diagnostic, prognostic and therapeutic options warrant future research. Here, we will provide an overview of the principal and most relevant epigenetic alterations found in CCA.

DNA hypermethylation

DNA methylation is perhaps the most studied epigenetic change occurring in CCA. The main targets of epigenetic silencing through DNA hypermetilation are TSGs (including those implicated in the regulation of cell cycle and induction of apoptosis), stability genes, and genes involved in inflammatory processes and cell adhesion (*Table 2*).

Among the group of genes involved in the regulation of cell cycle, hypermethylation of *p16^{INK4a}* is probably the best characterized. *p16^{INK4a}*, also called cyclin-dependent kinase inhibitor 2A (CDKN2A), binds to cyclin-dependent kinase 4 and inhibits its ability to interact with cyclin D2, thereby preventing the cell to enter in the cell cycle S phase (132). The *p16^{INK4a}* promoter hypermethylation leads to cell proliferation and oncogenesis. Rates of hypermethylation in the *p16^{INK4a}* promoter range from 17% to 83% in different studies (116-121). Interestingly, not only *p16^{INK4a}* hypermethylation seems to be a common event in PSC-related CCA (122) but it has also been associated with a poor clinical outcome (117). Moreover, *p16^{INK4a}* hypermethylation is thought to be an early event in the progression of CCA: indeed, downregulation of *p16^{INK4a}* expression was found from intraductal papillary neoplasm of liver and CCA arising from hepatolithiasis (123,124).

Closely related to p16^{INK4a} is p14^{ARF}, the β transcript of the same gene located on chromosome region 9p21. In normal cells, p14^{ARF} blocks the progression from G1 to G2 phase of the cell cycle and inhibits growth of abnormal cells by indirectly p53 activation (133,134). In different studies, the reported methylation frequencies in CCA range from 24% to 40.2%, with the highest value registered in liver fluke-related CCA (105,118,119,125). Interestingly, methylation of *p14^{ARF}*, *DAPK*, and/or *ASC* (see below), together with *p53* mutations, were recently reported to correlate with poorly differentiated tumors and poor prognosis (105).

On the same chromosome region 9p21, adjacent to *p16^{INK4a}*, is located the *p15^{INK4b}* sequence, which is thought to be an effector of TGF-β-mediated cell cycle arrest (135). Hypermetilation of *p15^{INK4b}* promoter was reported in 50% of 72 cases of CCA (119). Similarly, 36% methylation of the *p73* promoter was also shown in CCA. p73 is a member of the p53 family that is also able to induce cell cycle arrest

Table 2 Most frequently methylated genes in CCA

Target gene	Function	Methylation frequency (%)	Reference(s)
$p16^{INK4a}$	Cell cycle control	17-83	(116-124)
$p14^{ARF}$	Cell cycle control	24-40.2	(105,118,119,125)
$p15^{INK4b}$	Cell cycle control	50	(119)
p73	Cell cycle control	36	(119)
RASSF1A	Cell cycle control	27-69 (83 in extrahepatic CCA)	(119,121,126)
RUNX3	Apoptosis	56.8	(121)
DAPK	Apoptosis	3-32	(117,119-121)
SEMA3B	Apoptosis	100	(127)
TMS1/ASC	Apoptosis	36.1	(128)
hMLH1	DNA mismatch repair	8-46 (0 in intraductal papillary neoplasm)	(119-121,129,130)
MGMT	DNA repair	0-46	(116,117,119,120)
SOCS-3	Cytokine regulation	88	(131)
E-cadherin	Cell adhesion	21.5-43	(117,119-121)

and apoptosis (136).

RASSF1A, a gene involved in cell cycle regulation, is epigenetically inactivated in CCA. This TSG has been shown to block the cell cycle progression by inhibiting the accumulation of cyclin D1 (137) and the progression of cellular mitosis (138). Hypermethylation of *RASSF1A* promoter occurs in up to 69% of the patients (126) and, of note, a higher prevalence has been reported in extrahepatic CCA compared with intrahepatic CCA (83% *vs.* 47%, respectively) (120).

A second subclass of TSGs comprises those involved in promoting apoptosis, the programmed cell death. Hypermethylation in the promoter region of a number of these genes has been found in different studies. Runt-related transcription factor 3 (RUNX3) is a TSG involved in cell growth regulation and TGF-β-induced apoptosis (139). Hypermethylation of *RUNX3* promoter was described in up to 56.8% of biliary tract cancers (121). In the same study, the methylation of *RUNX3* promoter was more frequent in elderly patients, and was associated with a lower survival rate compared to patients with an unmethylated gene. The methylation of *RUNX3* promoter gradually increases from normal samples to biliary intraepithelial neoplasia and eventually CCA (140). In addition, an assay for the analysis of *RUNX3*, *CCND2*, *CDH13*, *GRIN2B*, and *TWIST1* promoter methylation showed increased values in extrahepatic CCAs compared to control tissues (141).

A second member of this subclass of TSGs is the death-associated protein kinase (DAPK). DAPK is a pro-apoptotic mediator of interferon-γ-induced programmed cell death. Hypermethylation of *DAPK* promoter ranges from 3% to 32% in biliary cancers (117,119-121). Furthermore, it is likely that *DAPK* methylation correlates with poor prognosis and less survival (105,119,121,142). Additional pro-apoptotic genes found hypermethylated in CCA are semaphorin 3B (*SEMA3B*) and Target of Methylation-mediated Silencing/Apoptosis Speck like protein containing a CARD (*TMS1/ASC*). *SEMA3B* was found to be hypermethylated in 100% of 15 CCA tissue samples (127), while *TMS1/ASC* showed a 36.1% methylation (128).

Enzymes that participate in DNA repair compose the class of stability genes. Loss-of-function mutations of these genes lead to accumulation of mutations and genomic instability (143). The genes involved in DNA mismatch repair are important for cell protection to possible errors occurring during DNA replication. Defects in DNA mismatch repair machinery have been linked to microsatellite instability (144,145) and demonstrated in a variety of tumors (146,147). Human mutL homologue 1 (*hMLH1*) is a DNA mismatch repair gene located at 3p21.3 locus. The methylation frequencies of the *hMLH1* promoter vary in different studies between 0% in intraductal papillary neoplasms of the biliary tract (129) and 46% in a cohort of 37 patients with biliary tract cancers including gallbladder tumors (120). Interestingly, a high prevalence (62.5%) of microsatellite instability was reported in Thorotrast-induced intrahepatic CCA, suggesting the hypermethylation of the *hMLH1* promoter may be in part the cause of this phenomenon (148). Moreover, the same epigenetic process has been reported in 44.6% of cases of

liver fluke-related CCA with a significant association with poorly differentiated subtype (130).

An alternative enzyme involved in DNA repair is the O6-methylguanine-DNA methyltransferase (MGMT). The frequency of *MGMT* promoter methylation seems to vary between different CCA reports, from 33-49% (119,120) to 0% depending on the selected group of patients with CCA (116,117). However, interestingly, the lack of MGMT immunohistochemical staining correlates with poor prognosis of extrahepatic CCA (149).

As mentioned above, chronic biliary inflammation predisposes to the development of CCA (110,150). In this context, IL-6, which is found upregulated in the course of inflammation, is a pivotal growth and survival cytokine in CCA (75) by promoting the expression of the potent anti-apoptotic protein myeloid cell leukemia 1 (MLC1) via phosphorylation of STAT-3 (151). Under physiological conditions, IL-6 induces the expression of the suppressor of cytokine signal 3 (SOCS-3), which in turn inhibits IL-6 signal in a classic feedback loop (152). Interestingly, experimental hypermethylation of *SOCS-3* promoter that occurs in a subset of CCAs is responsible for sustained IL-6/STAT-3 signaling and enhanced MLC1 expression (131). These data suggest the use of demethylating agents as a therapeutic approach to revert this process.

Cell adhesion proteins may also be affected by epigenetic silencing in CCA through their gene promoter hypermethylation. Thus, alterations in the expression and function of cadherins, important cell adhesion proteins; are thought to be involved in the epithelial to mesenchymal transition (EMT) (153) and therefore to contribute to tumor progression and metastasis (154,155). The hypermethylation rates of *E(epithelial)-cadherin* promoter in CCA ranges between 21.5% and 43% (117,119-121). In this regard, a correlation between promoter methylation and reduced protein expression, measured by immunohistochemistry, was reported (117).

Histone modification

To date, limited evidences about the role of histone modifications in CCA exists. However, the intriguing possibility to open novel therapeutic approaches guarantees future research efforts in the upcoming years (156). So far, experimental incubation of different human CCA cell lines with HDAC inhibitors (i.e. MS-275, trichostatin A, NVP-LAQ824, and NVPLBH589) resulted in cell growth arrest and reduced survival in a dose-dependent manner (157-159). Moreover, the combination of conventional cytostatic drugs, such as gemcitabine or doxorubicin, or new agents such as sorafenib or bortezomib, and MS-275 resulted in additive or synergic growth inhibitory effect (157), via induction of apoptosis and cell cycle arrest (157,159). Importantly, initial evidences for the potential therapeutic role of HDAC inhibitors *in vivo* were reported. Thus, administration of the histone deacetylase inhibitor NVPLBH589 to nude mice with subcutaneously generated CCA tumors significantly reduced the tumor mass and also potentiated the efficacy of gemcitabine (159). Moreover, HDAC1 overexpression correlates with malignant behavior and poor intrahepatic CCA prognosis (160).

MicroRNAs

An evident role for microRNAs in CCA biology has been emerging in the last years (*Table 3*). Previous reports have focused on the study of microRNA expression in different CCA cell lines and shed light, at least in part, on the mechanisms governing their biology and function. A number of microRNAs (e.g., miR-141, miR-200b, miR-21, miR-29b) have been described to be either up or downregulated in CCA cell lines (161,163), and their predicted targets were found to be associated with cell growth and apoptosis.

The first microRNA profile comparing human intrahepatic CCA and normal cholangiocyte cell lines was based on cloning methodology and identified eight microRNAs specifically downregulated in cancer cell lines (i.e. miR-22, miR-125a, miR-127, miR-199a, miR-199*, miR-214, miR-376a, and miR-424) (170). In addition, a complex interplay between promoter hypermethylation, inflammation signals and microRNAs expression has been described. Thus, overexpression of IL-6 in human CCA cell lines was shown to increase the levels of microRNA let-7a, which in turn contributes to the survival effect of IL-6 by increasing the phosphorylation of STAT-3 (164). Furthermore, this cytokine increases the expression of the DNA methyltransferase enzyme-1 (DNMT-1) that epigenetically inhibits the transcription of miR-370, resulting in MAP3K8-dependent cell growth (165). Moreover, IL-6 can directly modulate the expression of both miR-148a and miR-152, which in turn regulate the expression of DNMT-1 and TSG (166).

An interesting interplay between epigenetic regulation of microRNAs and Hepatitis C core proteins has also been recently reported (167). The authors showed that

Table 3 MicroRNAs involved in CCA development and progression

MicroRNA	Target gene	Function	Change in CCA	Reference(s)
miR-141	CLOCK	Circadian rhythm	Increased	(161)
miR-200b	PTPN12	Tumor suppressor	Increased	(161)
miR-21	PTEN	Tumor suppressor	Increased	(161,162)
miR-29b	Mcl-1	Anti-apoptotic gene	Decreased	(163)
Let-7a	NF2	Negative regulator of inflammation	Increased	(164)
miR-370	MAP3K8	Oncogene	Decreased	(165)
miR-148a	DNMT-1	Methyltransferase	Decreased	(166)
miR-152	DNMT-1	Methyltransferase	Decreased	(166)
miR-124	SMYD3	Cell migration and invasion	Decreased	(167)
miR-26a	GSK-3b	Serine/threonine kinase	Increased	(168)
miR-214	Twist	Oncogene	Decreased	(169)

downregulation of miR-124—characteristic of HCV-related intrahepatic CCA—is induced *in vitro* by the HCV core proteins through epigenetic silencing via DNMT-1 upregulation.

SMYD3 was identified as a potential target gene of miR-124 and found to be involved in miR-124 mediated migration and invasion of CCA cells.

The role of microRNAs has been also investigated in human tissue samples. Thus, a genome wide microRNA expression pattern was performed using laser micro dissection techniques comparing 27 intrahepatic CCAs, 10 normal cholangiocyte cell samples, and normal liver tissues. The results showed 38 microRNAs differentially expressed between normal and tumoral samples (171).

miR-21 and miR-26a were found highly overexpressed in CCA. While miR-21 expression was detected with a sensitivity of 95% and 100% of specificity (162) miR-26a was found in 90.5% of the CCA samples and only in 33.3% of controls (168). In addition, miR-26a was shown to promote CCA growth both *in vitro* and *in vivo* by direct targeting the levels of glycogen synthase kinase 3β (GSK-3β), which normally regulates the degradation of β-catenin. The subsequent accumulation of β-catenin stimulated the transcription of different genes involved in tumor growth, such as c-Myc, cyclinD1, and peroxisome proliferator-activated receptor δ (168).

On the other hand, microRNAs were indicated to play an important role in the regulation of the metastasis of intrehepatic CCA. Indeed, miR-214 expression was found downregulated in intrahepatic CCAs from patients who developed metastasis compared to non-metastatic CCA tumors (169). The authors showed an indirect correlation between miR-214 levels and Twist, an important inhibitor of E-cadherin transcription, suggesting a potential role of miR-214 regulating the epithelial-mesenchymal transition of the tumor.

Conclusions

CCA is a deadly disease with an incidence increasing worldwide. Although the knowledge on the pathogenesis and the clinical features of the disease has significantly been improved, CCA still represents a major challenge for clinicians. Diagnosis is mostly performed when the disease is already at an advanced stage, thus making the medical and surgical therapy largely ineffective. The successes achieved in the management of different cancers have been commonly based on the identifications of categories of patients at risk and on the consequent set up of surveillance protocols. In this regard, in colon cancer, the identification of familial genetically-based predispositions have led to the determination of specific endoscopic surveillance for offsprings affected patients, increasing the rates of early diagnosis and survival. Identification of patients with high risk for CCA development is the next challenge for the translational research in the upcoming years. In particular, the identification of how genetic and epigenetic modifications may play a major role in CCA development, progression, and metastasis may open a new era for the management of CCA, and may represent a potential strategy for the treatment of this devastating malignancy.

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