Molecular Profiling

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Abstract

Molecular studies concerning cholangiocarcinoma (CCA) or gallbladder cancer are only at the beginning, and the epidemiologic, biologic, and pathological heterogeneity of these cancers constitutes a challenge for the future. Recent studies, in fact, highlighted how CCA is composed of different clinical–pathological subtypes with different cells of origin, pathogenesis, and risk factors. In this chapter, we discuss recent studies regarding the molecular profiling of CCA and gallbladder cancer, which aimed to clarify tumor etiopathogenesis, support diagnosis, and target treatments. Published studies have been critically analyzed taking into consideration the geographic and racial variability, and the pathologic features of the CCA.

1 Introduction

Cholangiocarcinoma (CCA) is a malignant tumor that arises in the biliary tree from the neoplastic proliferation of cholangiocytes, the epithelial cells lining bile ducts. According to current classifications, CCA is divided into intrahepatic (IH-CCA) and extrahepatic (EH-CCA), the latter comprising the perihilar and distal forms [1–3]. Neither gallbladder cancer nor ampullary cancer are considered part of the CCA classification. CCA is characterized by a desmoplastic nature, scarce cellularity, a pleiotropic marker expression, and frequent neuroendocrine differentiation [4–6]. A progressive increase in CCA worldwide incidence and mortality has been described [7, 8]. However, epidemiologic data are biased by a number of pitfalls including the absence of specific markers or specific radiologic features, the biologic and histologic heterogeneity, and, mainly, the lack of uniform classification [7, 8]. CCA still represents a challenge for clinicians at both the diagnostic and therapeutic levels [9].

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J. M. Herman et al. (eds.), *Biliary Tract and Gallbladder Cancer*, Medical Radiology. Radiation Oncology,
DOI: 10.1007/978-3-642-40558-7_7,

So far, basic science studies on CCA have been limited with scarce translation into the clinical setting, and this is particularly true for diagnostic and prognostic biomarkers [4, 10–13]. Recently, using a molecular approach, CCA has been demonstrated to represent the predominant cause of distant metastases when the primary malignancy is unknown, thus confirming a general belief among clinicians and oncologists [14]. This is a further demonstration of how basic science studies may impact general practice, and of the importance of promoting such studies.

Molecular profiling is the classification of pathological tissues for diagnostic or prognostic purposes based on multiple gene expression and is currently utilized to clarify tumor etiopathogenesis or to support diagnosis and targeted treatment [15, 16]. However, the use of these tests for clinical decisions presents many challenges since assay development and data analysis are strongly affected by a number of variables. Frequently, the performance of a certain assay is emphasized in basic studies, while the absolute sensitivity and specificity remain modest when tested in validation studies. With the exception of breast cancer, the real usefulness of molecular profiling is so far limited, especially in terms of cost-effectiveness [16]. Nevertheless, the potential of molecular technology deserves attention in the near future, and this is particularly relevant in the setting of cancer, where the etiopathogenesis is extremely complex. In CCA, molecular studies are only at the beginning, and this is further complicated by the epidemiologic, biologic, and pathological heterogeneity of this cancer. In addition, the availability of good quality CCA samples is mandatory for clinicopathological or basic science studies, but, unfortunately, the desmoplastic nature and the anatomical location make sampling very difficult in most cases.

2 Molecular Profiling and the Origin of Cholangiocarcinoma

Identification of key genetic and epigenetic signatures could aid the identification of biomarkers for diagnosis, screening, surveillance of CCA in categories at risk, and, finally, the development of potential therapeutic strategies [10–12]. In addition, these studies could provide insights into the mechanisms underlying neoplastic transformation of cholangiocytes. However, enormous geographic and racial differences exist with CCA [8]. As far as risk factors are concerned, for example, liver flukes represent the main risk factor in east countries, while hepatitis viruses and primary sclerosing cholangitis (PSC) represent main risk factors in western countries [8], but, in the majority of CCA cases, no risk factor is found [17]. This implies that molecular studies performed in a certain population are not always globally applicable.

Chronic inflammation is considered the background, which favors the emergence of the majority of primitive liver cancers, and this is even truer for CCA [4, 11, 17]. Indeed, all the putative risk factors so far identified for CCA share, as a common variable, the chronic inflammation of bile ducts [11]. However, only 40–50 % of CCA emerges in the setting of chronic liver disease or parasitic infestation; the remaining CCA cases emerge in the absence of an evident chronic liver disease [8, 11, 17]. To explain this variability, two models have been proposed for liver carcinogenesis [17]. According to the so-called clonal evolution model, sequential genetic and epigenetic changes in a cell in the setting of chronic inflammatory stimuli determine a multistep process of tumor development from precancerous lesions to metastatic carcinoma [17]. The alternative model contemplates the involvement of individual genetic and environmental factors [17].

Since all known CCA risk factors are associated with chronic bile duct inflammation, it is conceivable that molecular studies have focused on genetic/epigenetic abnormalities involving inflammation-related genes other than genes involved in the control of DNA repair, cell cycle, apoptosis, and proliferation [10–12, 17].

P53 is a pivotal cell cycle regulator at the G1/S regulation checkpoint, but it is also involved in controlling DNA repair and apoptosis [10, 11]. Nault and Zucman-Rossi observed that substitutions, insertions, or deletions associated with loss of heterozygosity (LOH) may occur in biliary tract cancers [10]. However, differences in *P53* mutations have not been reported when IH-, EH-CCAs (Table 1) and gallbladder cancer are compared [18]. Studies concerning *P53* in CCA highly reflect the complexity and heterogeneity of this cancer at molecular level and further sustain the relevance of the two models of carcinogenesis. Indeed, over 90 different types of *P53* mutations have been described in CCA [18]. As reported in Table 1, a total number of 330 CCA patients have been investigated by sequencing studies [18–31]. Studies from Europe, America, and Asia showed a 34 % (112/330 patients) overall percentage of *P53* mutations [18–31]. Overall, the most commonly reported type of mutation in CCA interests CpG sites. Mutation pattern showed G:C>A:T at CpG sites in 29.3 % of CCAs [18]. Interestingly, alkylating agents, such as N-nitroso compounds, tend to induce G:C–A:T transitions in *P53* via the formation of O-6-methylguanine. In northeast Thailand, the traditional habit of eating nitrosamine- and liver fluke-contaminated foods exposes the population to a synergistic effect of chemical carcinogens and liver fluke infection (Opisthorchis viverrini). Nitrosamines are assumed to act as genotoxicants, while liver flukes are assumed to play epigenetic role in CCA development in this exposed population. Consistently, Kamikawa et al. [19] found that mutational spectra are highly correlated with each carcinogen. A lower overall percentage

Table 1 *P53* mutations in human cholangiocarcinoma: sequencing studies

References	Country	No. of patients	CCA site	Overall number of patients with *P53* mutations (%)	Notes
Jonas et al. [23]	Germany	12	Perihilar	2 (16.6)	*P53* exons 5–8 evaluated
Sturm et al. [22]	USA	27	Perihilar	7 (26.31)	*P53* exons 5–8 evaluated
Petmitr et al. [21]	Thailand	20	IH-CCA	1 (5)	*P53* exons 5–8 evaluated
Kang et al. [24]	Korea	40	IH-CCA	14 (35.7)	*P53* exons 5–8 evaluated
Furubo et al. [25]	Japan	15	IH-CCA (peripheral) and perihilar	3[a] (20)	*P53* exons 5–8 evaluated
Kamikawa et al. [19]	Japan	22	IH-CCA	9 (41.6)	*P53* exons 5–8 evaluated; thorotrast exposed patients
Della Torre et al. [26]	Italy)	13	Not specified	2 (15.3)	*P53* exons 5–8 evaluated
Tullo et al. [27]	Europe	29	Perihilar	7 (24)	*P53* exons 5–8 evaluated
					3/7 cases carried germline heterozygous polymorphism in tumoral and non-tumoral DNA
Momoi et al. [28]	Japan	28	IH-CCA	2 (7.1)	*P53* exons 5–8 evaluated
Khan et al. [18]	UK	31	IH-CCA	24 (76)	Complete *P53* mutational signatures
					Three new frameshift mutations and two new intron mutations discovered
Liu et al. [29]	China	36	Not specified	22 (62)	*P53* exons 5–8 evaluated
Kiba et al. [20][c]	Thailand	26	IH-CCA	9 (35.7)	*P53* exons 5–8 evaluated
					2 patients with *KRAS* mutations, none carrying both *P53* and *KRAS* mutations
Kiba et al. [20][c]	Japan	12	IH-CCA	4 (33.3)	*P53* exons 5–8 evaluated 7 patients with *KRAS* mutations, none carrying both *P53* and *KRAS* mutations
Imai et al. [30][c]	Japan	7	IH-CCA[b]	2 (28.5)	*P53* exons 5–8 evaluated
Itoi et al. [31][c]	Japan	12	Not specified	4 (33.3)	6 patients with *KRAS* mutations, none carrying both *P53* and *KRAS* mutations
					KRAS and *P53* abnormalities not detected in non-neoplastic biliary tract tissues
					The same mutation patterns detected in bile and neoplastic tissue
		Total 330		Total 112 (34.0)	

Abbreviations: *IH-CCA* intrahepatic cholangiocarcinoma
[a] 1 case perihilar-type and 2 cases not defined
[b] Combined hepatocarcinoma-CCA; 1 patient with *KRAS* mutations, none carrying both *P53* and *KRAS* mutations
[c] Studies where also *KRAS* mutations were evaluated

of *P53* mutations were seen in CCA cases from European studies (14 %) with respect to Asian studies (23 %) [18]. Also, the pattern of mutations shows large geographic differences. For example, Kiba et al. [20] found that over 50 % of *P53* mutations in their Thai patients were G:C–A:T transitions at CpG sites, whereas a study on Korean patients found the same pattern in only 17 % of cases [24].

In the absence of definite environmental risk factors, *P53* mutations are more frequent in areas with high CCA incidence (United States of America high-incidence cluster area = 67 %) than in areas with low incidence (United States low-incidence cluster = 20 %) [22]. This could reflect the exposure to unidentified mutagen triggering *P53*, in high-incidence areas. Unfortunately, very little is still

known on environmental mutagens, and our current capability to disclose *P53* impairment is limited. In the western world, similar rates (on average, 51 %) of *P53* mutation have been found in CCA associated or not with PSC, indicating the lack of a PSC–CCA-specific molecular signature in *P53* gene. It has been previously suggested that *P53* alterations in CCA may be mediated by abnormal intracellular signaling cascades caused by cytotoxic biliary constituents [18]. In PSC, changes in bile composition are associated with bile duct inflammation and enhanced cholangiocyte proliferation, and this could favor, according to the clonal model of carcinogenesis, accumulation of mutations up to the threshold of neoplastic transformation. The alternative model of cholangiocarcinogenesis contemplates the involvement of individual genetic and environmental factors [17]. Several *P53* polymorphisms have been so far described. Their relevance is unclear, and only two of these variants are associated with abnormal amino acid sequence of the *P53* protein [18]. The lack of a specific *P53* molecular signature in sporadic CCA could be explained if a definite gene polymorphism predisposes to *P53* alterations in the presence of the pathological milieu (i.e., inflammation) determined by CCA risk factors. In comparison with the sporadic form, CCA associated with thorotrast exposure showed a different pattern of *P53* mutations [18, 19]. It is, however, important to note that the full-length *P53* cDNA has been insufficiently investigated. Indeed of the fourteen *P53* sequencing studies, thirteen have evaluated only *P53* exons 5–8, whereas the only study that evaluated the complete *P53* mutational signatures disclosed three new frameshift mutations and two new intron mutations and demonstrated the highest mutation rate in *P53* gene never reported (76 %).

In conclusion, the frequency and type of *P53* mutations occurring in CCA patients depends from environmental factors, including the nature and dose of exposure to environmental carcinogens, which vary in different populations [18].

Growth factors and growth factor receptors (e.g., the ErbB family, insulin-like growth factors (IGF), and hepatocyte growth factor (HGF/MET)) are pivotal growth signal regulators in cancers of different origin [10]. Among the pathways involved in the pathogenesis of IH-CCA, the family of ErbB receptors is perhaps the most relevant [10, 11]. ErbB-2 is an epidermal growth factor receptor (EGFR) homologue and is able to homodimerize or heterodimerize with other members of the EGFR superfamily, resulting in activation of the Raf/MAPK pathway [10, 11]. The most notable are the aberrant regulation of ErbB2 and the EGFR signaling [10, 11]. Constitutive overexpression of ErbB2 and/or ErbB1 in malignant cholangiocytes has been documented in more than 50 % of IH-CCA [32, 33]. In addition, experimental models of IH-CCA in rodents are associated with constitutive ErbB2 overexpression [11]. ErbB2 and ErbB1 interact with different relevant molecular signaling pathways associated with IH-CCA development and progression, including bile acids, IL (interleukin)-6/gp130, transmembrane mucins, HGF/MET, and vascular endothelial growth factor (VEGF) signaling [11, 32, 33]. Hydrophobic bile salts, such as deoxycholate, may play a carcinogenetic role through transactivation of EGFR and impairment of Mcl-1 functions, and this has been considered a mechanism favouring the intraductal pattern of growth characterizing a subset of CCAs [11]. The relevance of ErbB2- or ErbB1-related pathways in CCA has raised interest in exploring, for the treatment of CCA, agents selectively targeting these receptors. However, current experience with ErbB-targeted therapies produced only modest responses in patients with biliary tract cancers [10, 11]. Activation of EGFR triggers downstream Ras/Raf/Mek/Erk and PI3K/PTEN/Akt, two major cell survival pathways. Ras proteins (K-Ras, N-Ras, H-Ras, B-Raf), responsible for signal transduction downstream to growth factor receptors, have been largely investigated in CCA, and in this regard, *KRAS*-activating mutations represent one of the most frequent genetic alterations found in CCA (10–75 % of CCA cases) [34]. After binding and activation by GTP, Ras proteins recruit Raf that, in turn, activates, by phosphorylation, MAP kinases (MEK1/2 and ERK1/2) [10, 11]. Activation of MAP kinase pathways leads to enhanced proliferation and inhibition of apoptosis.

As reported in Table 2, a total number of 218 CCA patients have been investigated by sequencing studies aimed to identify *KRAS* mutations [20, 30, 31, 35–39, 40, 41]. Studies from 1992 to 2011 have evaluated CCA patient courts from Europe, America, and Asia, as shown in Table 2 [20, 30, 31, 35–39, 40, 41]. The total number of CCA patients with *KRAS* mutations resulted 88, the 40.4 % of all the CCAs. When classified by tumor site, 17 % of peripheral type CCAs were positive for *KRAS* mutations with the most frequent alteration in codon 12. Importantly, the incidence of mutations was higher in the hilar-type tumors (53 %) [34]. It is noteworthy that the frequency of *KRAS* mutations increases with tumor stage (stage I, 8 %; stage II, 15 %; stage III, 31 %; stage IV, 46 %) [39].

Another recently proposed mechanism linking chronic inflammation with CCA development is related to activation-induced cytidine deaminase (AID), a member of the DNA/RNA editing enzyme family, implicated in human cancerogenesis via its mutagenic activity [42]. AID was found to be increased in biopsies from patients with PSC or CCA, whereas only trace amounts of AID were detected in the normal liver [11, 42]. In in vitro studies, in human CCA cell lines, AID was induced by tumor necrosis factor-alpha that, in turn, was stimulated via IkappaB kinase-dependent nuclear factor-kappaB (NF-kappaB) pathway [11]. The aberrant expression of AID in biliary cells resulted in the

Table 2 *KRAS* mutations in human cholangiocarcinoma: sequencing studies

References	Country	No. of patients	CCA site	Overall number of patients with *KRAS* mutations (%)	Notes
Tada et al. [35]	Japan	18	IH-CCA (peripheral) and perihilar	9 (50)	The incidence of mutations higher in the perihilar CCA
Tannapfel et al. [37]	Germany	41	IH-CCA	22 (54)	All 22 cancers with *KRAS* mutations also exhibited methylated *P16*; in 2 cases, mutations were detected in non-neoplastic liver tissue surrounding the tumor (germline mutations)
Ahrendt et al. [38]	USA	12	Not specified	12 (33)	Patients with PSC-associated CCA
					Overall survival shorter in patients with *KRAS* mutation
Xu et al. [39]	China	13	Not specified	5 (38.2)	2 patients (5.9 %) harbored both *KRAS* and PIK3CA mutations
Isa et al. [41]	Japan	23	IH-CCA (peripheral) and perihilar	9 (39.1)	Patients with *KRAS* mutations worst survival rates; *KRAS* mutation rates higher in perihilar (6/8, 75.0 %) than in peripheral (3/5, 20.0 %) CCA
Rashid et al. [40]	China	33	Not specified	5 (15.2)	Mean survival of patients with *KRAS* mutations shorter (3.0 months) compared with patients without mutation (15.5 months)
Kiba et al. [20][b]	Thailand	26	IH-CCA	2 (7.6)	*P53* exons 5–8 also evaluated; 9 patients (35.7 %) with *P53* mutations
Kiba et al. [20][b]	Japan	12	IH-CCA	7 (58.4)	*P53* exons 5–8 also evaluated and the overall number of patients with *P53* mutations was 4 (33.3 %)
Ohashi et al. [36][b]	Japan	21	IH-CCA	10 (48)	*P53* exons 5–8 also evaluated; 2 patients (7.1 %) with *P53* mutations; *KRAS* mutations were prominent in the periductal growing CCA (4/6; 67 %) with respect to the mass-forming CCA (0/5)
Imai et al. [30][b]	Japan	7	IH-CCA[a]	1 (14.2)	*P53* exons 5–8 also evaluated; 2 patients (28.5 %) with *P53* mutations
Itoi et al. [31][b]	Japan	12	Not specified	6 (50)	*P53* exons 5–8 also evaluated; 4 patients (33.3 %) with *P53* mutations
					KRAS abnormalities were not detected in non-neoplastic tissues
					The same mutation patterns detected in bile and neoplastic tissues
		Total 218		88 (40.4)	

Abbreviations: *IH-CCA* intrahepatic cholangiocarcinoma, *PSC* primary sclerosing cholangitis
[a] Combined HCC-CCA
[b] Studies where also *P53* mutations were evaluated

generation of somatic mutations in tumor-related genes, including *P53*, *c-Myc*, and the promoter region of the *INK4A/P16* sequences [10, 11]. In contrast with hepatocellular carcinoma (HCC), mutations activating β-catenin are rarely found in CCA (0–8 % of CCA cases) [10]. Other genes such as *IDH1*, *SMAD4*, and *KEAP1* have been described to be frequently mutated in CCA tissue, but with large differences among studies. [10, 11, 43]. Aberrant epigenetic regulation, such as promoter hypermethylation, was demonstrated in numerous important cancer-associated genes in CCA [44, 45]. Promoter methylation of *P14*, a regulator of *P53*, has been found in CCA [10]. *P16* (*CDKN2*) is frequently silenced in CCA by genetic or epigenetic mechanisms [37].

The interleukin-6 (IL-6) is one of the most investigated genes in the pathogenesis of CCA, where it could be involved by different mechanisms [10, 11]. IL-6 is produced at high levels in CCA cells and elevated IL-6 serum concentrations have been reported in CCA patients [10, 11]. Constitutive activation of the IL-6/STAT3 pathway has been described in CCA cells, and this was associated with silencing of *SOCS3*. The methylation of *SOCS3* promoters

occurs in 61 % of IH-CCA together with down-regulation of gp130, a membrane protein that, when associated with SOCS3 protein product, inhibits the IL-6 pathway [44]. By autocrine and paracrine mechanisms, IL-6 activates via STAT3 the prosurvival *P38* mitogen-activated protein kinase [10, 11]. STAT3 is an activator of p44/42 and *P38* mitogen-activated protein kinase that has been frequently found, by immunohistochemistry, to be activated in IH-CCA [10, 11]. In addition, IL-6 up-regulated the expression of myeloid cell leukemia-1 (Mcl-1) through STAT3- and AKT-related signaling pathways [46, 47]. Mcl-1 increases cell resistance to TRAIL apoptotic signals [48]. Moreover, IL-6-related pathways can modulate epigenetic fate of the cells through DNA (cytosine-5)-methyltransferase 1 (DNMT1), and this has been demonstrated for IL-6-mediated up-regulation of EGFR and for down-regulation of *P53* expression, which occur by promoter hypo- or hypermethylation, respectively [10, 12]. Finally, IL-6 may act in CCA by autocrine and paracrine pathways since it is secreted by malignant cholangiocytes [11]. In light of these findings, IL-6 has been explored in the diagnostic setting and, in fact, serum levels of IL-6 have been correlated with tumor burden in CCA patients [13]. However, although these findings are encouraging, it should be considered that serum IL-6 is also elevated in many patients with HCC, benign biliary disease, and metastatic lesions, and therefore, the specificity of high IL-6 serum levels for CCA is still debated [13]. Recently, the induction of progranulin (PGRN) has been advanced as another mechanism by which IL-6 could enter CCA pathogenesis [49]. PGRN is involved in multiple steps of the tumor progression cascade, including cellular proliferation, anchorage independence, invasiveness, resistance to apoptosis, and promotion of resistance to certain cytotoxic drugs. In addition, PGRN may also act by promoting neoangiogenesis with a direct effect on endothelial cells as well as an indirect effect on VEGF synthesis. The expression and secretion of PGRN are up-regulated in human CCA, and this in part occurs viaIL-6-mediated activation of the Erk1/2/Rsk1/C/EBPb pathway [49]. Serum PGRN levels were higher in patients with CCA than in non-neoplastic controls, but it is unknown if this can discriminate CCA with respect to benign biliary pathologies, including PSC and benign strictures of the biliary tree [13]. IL-6 and other mediators of inflammation, including TNF-alpha, may enter CCA pathogenesis by inducing or synergizing a number of different growth factors [10, 11].

Cyclooxygenase 2 (COX-2), the rate-limiting enzyme in prostaglandin biosynthesis from arachidonic acid, activated by inflammatory cytokines and nitric oxide (NO), accelerates cell cycle via prostaglandin E2 (PGE2) and inhibits different apoptotic cascades. Indeed, increased COX-2 immunohistochemical expression has been documented in more than 70 % of CCA samples [50], and the COX-2 gene is frequently affected by epigenetic (methylation) perturbations in CCA. COX-2 is activated by oxysterols, oxygenated cholesterol derivatives formed in the bile of patients with inflammatory diseases of the biliary tree, and by hydrophobic bile acids [11]. Another COX-2-inducing molecule is the tyrosine kinase ErbB-2, which is overexpressed in CCA and involved in CCA origin and progression [11]. Current evidence supports a primary role played by NO, induced by proinflammatory cytokines (TNF-α, IL-6, etc.) [51]. These cytokines are able to activate inducible nitric oxide synthase (iNOS), which, at the immunohistochemical level, is overexpressed in more than 70 % CCA [11]. Increased iNOS activity results in generations of NO and reactive oxygen species, which are known to interact with cellular DNA and to inhibit DNA reparative mechanisms, thus triggering oncogenetic mutations. NO together with different cytokines can also inhibit cholangiocyte apoptosis by nitrosylation of caspase-9 and may also induce proliferation, thus favouring accumulation of somatic mutations [11]. Very recently, a relevant role in modulating CCA growth and proliferation has been attributed to estrogens, IGF1, leptin, opioid receptor modulators, endothelin, and serotonin [11]. As far as estrogens are concerned, recent studies suggest their synergistic action with growth factors (IGF1, VEGF) in sustaining the cholangiocyte proliferative machinery and in depressing apoptosis [52, 53]. Indeed, a cross talk between IGF1 and estrogens has been demonstrated to modulate CCA proliferation, whereas estrogens act at several points of the IGF1 signal transduction pathway [52]. In addition, it has been shown that the estrogen proliferative effect on CCA cells is also due to the stimulation of VEGF synthesis and secretion [52, 53]. In agreement with these data, IGF1 have been explored as CCA markers in a diagnostic setting. The IGF1 biliary concentration was shown to be capable of completely discriminating CCA from benign biliary pathologies and pancreatic cancer [54].

Recent technical improvement in molecular profiling platforms is adding new insights into the current knowledge of cholangiocarcinogenesis favoring the integration of the different proposed models. Unfortunately, few comparative genomic hybridization (CGH) studies on CCA have been performed during the past decade, and these studies are biased by the heterogeneous population investigated that included IH-CCA, EH-CCA, or even gallbladder cancers, making difficult any accurate interpretation. Evaluation of DNA copy number (CN) demonstrated CN gains in the region of several molecular targets: *ERBB2, MEK2, PDGFB, MTOR, VEGFR-3, PDGFA, RAF1, VEGFA,* and *EGFR* [55]. Technological advances also allow the differential characterization of genomic and genetic features of CCA epithelial and stromal compartments [56]. The tumor epithelium was defined by deregulation of the HER2 network and frequent

overexpression of EGFR, the HGF/MET receptor, pRPS6, and Ki67, whereas stroma was enriched in inflammatory cytokines [56]. Recently, the comparative evaluation of gene expression profile (transcriptome), clinicopathological traits, and patient outcomes in IH-CCA cases has allowed the identification of 2 main biologic classes of IH-CCA: (1) the inflammation class (38 % of IH-CCA), characterized by activation of inflammatory signaling pathways, overexpression of cytokines, and STAT3 activation and (2) the 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 [57]. As previously discussed, an optimal approach to CCA molecular profiling should be the comparative investigation of subtypes such as CCA emerging in a definite category at risk, including PSC or liver fluke infestation. Unfortunately, very few studies followed this type of approach. PSC is a major risk factor for IH- and EH-CCAs, and these patients experienced a cumulative risk of 11.2 %, 10 years after diagnosis [7]. Unfortunately, predictive factors or standardized screening or surveillance strategies are lacking. Different molecular signatures of the high oncogenic risk have been described in PSC patients. *KRAS* mutations have been found in 30 % of bile fluid of PSC patients without evidence of CCA [58]. Since *KRAS* mutations are frequently observed in CCA, this could be an early event of bile duct carcinogenesis in PCS patients. Notably, mutational profiling can be performed in cell-free DNA of bile supernatant [59]. The inflammatory microenvironment has also been associated with an aberrant DNA methylation profile in PSC-derived CCA, which provides survival signals for the tumor [60]. Genetic susceptibility of PSC patients for CCA development has been demonstrated by studies concerning the natural killer cell receptor G2D receptor [61], where specific genetic variants have been described in PSC patients.

The association between liver flukes and CCA has been evaluated by the International Agency for Research on Cancer (IARC) since 1994. Opisthorchis viverrini (OV) infestation, endemic in Southeast Asia, is now considered a definitive carcinogen. The molecular mechanism of OV-associated CCA has been also studied in experimental models. Up-regulation of 23 transcripts and down-regulation of 1 transcript related to CCA induced in OV-infected hamsters has been identified. The up-regulated genes include signal transduction protein kinase A regulatory subunit Ia (PRKAR1a), myristoylated alanine-rich protein kinase C substrate, transcriptional factor LIM-4-only domain, oxysterol-binding protein involved in lipid metabolism, splicing regulatory protein 9, ubiquitin-conjugating enzyme involved in protein degradation, β-tubulin, β-actin, and collagen type VI. Interestingly, PRKAR1a expression tended to increase during the progression from hyperplasia to precancerous lesions and to CCA [62]. In humans, molecular studies of IH-CCA associated with liver flukes demonstrated overexpression of genes involved in xenobiotic metabolism (*UGT2B11*, *UGT1A10*, *CHST4*, *SULT1C1*), whereas, in contrast, non-OV-associated IH-CCA showed enhanced expression of genes related to growth factor signaling (*TGFBI*, *PGF*, *IGFBP1*, *IGFBP3*). Thus, the evaluation of the putative signature of OV-associated IH-CCA in OV-infected patients could help in screening and surveillance, with the perspective of an early diagnosis [63]. The draft genome of *Clonorchis sinensis* and transcriptomes of *Clonorchis sinensis* and OV have been recently elucidated [64, 65]. Recently, a study in a large IH-CCA cohort (N = 102) associated with liver fluke infection demonstrated promoter hypermethylation in a handful of target genes, when CCA specimens were compared with adjacent non-tumoral tissues [66]. These results could help in identifying molecules linked with the development of liver fluke-induced CCA. CCA genetic susceptibility has been investigated in geographic areas endemic for liver flukes. In these studies, specific haplotypes of COX-2-coding gene (PTGS2) or IL8RB have been recently associated with a significant risk of CCA development [67].

3 Molecular Profiling and the Diagnosis of Cholangiocarcinoma

Immunohistochemical markers specific to CCA are lacking, and the definite diagnosis in bioptic or surgical samples is still based on a panel of markers aimed at excluding HCC or metastatic cancer. Therefore, for many years, studies have been focused on the search for CCA-specific markers. Different proposals appear in recent literature, but none of these reached clinical routine application. Recently, high-throughput techniques based on DNA microarray technology [68] have been tested in human CCA samples. The first study using DNA microarray technology (Affymetrix U133A) in a series of surgically resected biliary cancers, biliary cancer cell lines, and biliary epithelial scrapings was carried out in 2003 by Hansel et al. [69]. They reported 282 genes overexpressed threefold or greater in biliary malignancies or cancer cell lines, including proliferation and cell cycle-related genes (e.g., cyclins D2 and E2, cdc2/p34, and geminin genes), transcription factors (e.g., homeobox B7 and islet-1), growth factors and growth factor receptors (e.g., hepatocyte growth factor, amphiregulin, and insulin-like growth factor 1 receptor), two important downstream mediators of the mitogenic Akt/mTOR signaling pathway (ribosomal protein S6 kinase and eukaryotic translation initiation factor 4E), enzymes modulating sensitivity to chemotherapeutic agents (e.g., cystathionine beta synthase,

dCMP deaminase, and CTP synthase), and cytosolic phospholipase A2 [69]. After this first report, other studies aimed to investigate the utility of transcriptomic in CCA diagnosis have been performed. A genome-wide cDNA microarray containing 27,648 cDNAs carried out in IH-CCA specimens and non-cancerous biliary tissues, showed 52 genes up-regulated and 421 genes down-regulated. The overexpressed genes are related to a variety of functions, such as signal transduction (*GNAZ*, *MDK*), transcription (*FOXM1*, *HOXB7*, *DRIL1*), DNA synthesis (*TOP2A*, *TOP2B*, *NAV2*, *BUB1B*, *CKS2*), antiapoptosis (*BIRC5*, *S100P*), angiogenesis (*ECGF1*), cytoskeleton (*FSCN1*, *PRC1*, *ANLN*, *KIF2C*), and cytokinesis or adhesion (*CDH3*, *CIT*, *ECT2*). On the contrary, the down-regulated genes are mainly involved in growth suppression (*EGR1* and *EGR2*, AXIN1, *AXUD1*, *DLC1*, *DOC1*). From the 52 up-regulated genes, P-cadherin and survivin were selected for further investigation, and the enhanced expression of their protein products in CCA tissues was demonstrated by immunohistochemical staining [70]. Recently, oligonucleotide arrays (Affymetrix U133A) were used to establish a specific gene expression profile of IH-CCA in comparison with adjacent non-malignant liver tissue. Most of the strongly overexpressed genes are related to cell cycle regulation and DNA replication (15 genes, including *ribonucleosidediphosphate reductase M2, calgizzarin, calcyclin, BUB1B*) intracellular signaling (15 genes, including *CD24* and *MARCKS*), genes encoding transcription factors (6 genes, such as *SOX9*), or genes involved in nuclear organization and nucleic metabolism (13 genes, such as *thymidylate synthetase*). Other up-regulated genes include those coding for extracellular matrix and cell adhesion molecules (37 genes, for example *OPN, ADAM9, thymosin beta-10, integrin alpha-6*), cytoskeleton structure proteins (16 genes, such as *tropomyosin 2, cytokeratin 7* and *19*), or enzymes involved in protein biosynthesis (4 genes). The gene encoding for OPN was identified as the highest and most consistently overexpressed gene (33.5-fold change) in all analyzed CCA samples. Most of the genes encoding proteins involved in cellular apoptosis (7 genes including growth arrest-specific protein 2, *CIDE-B*) were found to be down-regulated in IH-CCA [71]. The genes overexpressed in IH-CCA, have been confirmed at protein level by immunohistochemical analysis, and included osteopontin, *P38* δ/MAPK-13, cadherin, and survivin. In conclusion, oligonucleotide microarray analysis shows a specific gene expression profile of IH-CCA, which could discriminate this cancer with respect to other malignancies or non-malignant lesions. These data, however, need further validation in independent cohorts of samples.

The differential diagnosis between IH-CCA and some subtypes of HCC is frequently challenging because of the existence of many overlapping features. Indeed, detailed studies on immunohistochemical profile have revealed that a whole range of phenotypical traits of hepatocytes, cholangiocytes, and progenitor cells can be shared by IH-CCA, combined HCC-CCA, fibrolamellar HCC, and HCC with stem cell features. This is consistent with a common origin of these cancers from the hepatic stem cell compartment within canals of Hering [72]. A substantial number of HCCs, ranging from 28 to 50 % of human HCCs, express markers of progenitor cells or cholangiocytes including CK7, CK19, and OV6, which suggest an origin from bipotential stem/progenitor cells located within canals of Hering [73]. Some of these markers in HCC, especially CK19, have been associated with a worse prognosis and higher rates of recurrence after surgical treatment [73]. The emergence of HCC and IH-CCA in the same pathological context of chronic liver diseases does not help in differential diagnosis, and radiologic features may overlap. Differential diagnosis between HCC and IH-CCA deserves important clinical implications since, for example, IH-CCA is excluded from liver transplantation programs. Recently, mutations of *BRAF* and *KRAS* were evaluated in 25 HCC and in 69 CCA by direct DNA sequencing analyses after microdissection. Using this molecular profiling approach, *RAS* or *BRAF* mutations have been detected in approximately 62 % of CCA, but not in HCC [74]. The diagnostic utility of evaluation of active intermediates of the MAPK pathway was assessed by microarray gene expression. The study identified a *P38* MAP kinase, *P38* δ (also known as MAPK13 or SAPK4) as a protein that is up-regulated in CCA relative to HCC and to normal biliary tract tissues. Consistently, *P38* δ immunohistochemical staining distinguished CCA from HCC with a sensitivity of 92.6 % and a specificity of 90.7 %. *P38* δ is important for motility and invasion of CCA cells, suggesting an important role in CCA metastasis. Therefore, *P38* δ could represent a novel diagnostic marker for CCA and may also serve as a new target for molecular-based targeted therapy [75]. Evaluation of markers of apoptosis and cell proliferation, such as bcl-2, c-myc, Fas, Lewis(y), and *P53* in human CCA and HCC, showed that Lewis(y) antigen was expressed in some CCA, whereas it was not found in HCC [76] The diagnostic workup of EH-CCA usually starts with the evidence of biliary tract obstruction [2, 9]. The definitive diagnosis is obtained during endoscopic retrograde cholangiopancreatography (ERCP) with cytology on bile samples, brushing, or endoscopic biopsies. Unfortunately, endoscopic biopsies can be obtained almost exclusively in the case of CCA with an intraductal pattern of growth and located at the distal part of the bile duct [2, 9]. Furthermore, these samples are often of poor quality given the scarce cellularity of this tumor. For the same reasons, cytology on bile samples or brushing has a low diagnostic yield, which is markedly increased by fluorescent in situ hybridization (FISH) analysis of

chromosomal aberrations (mainly polysomy) [2, 9]. Even recent guidelines indicate FISH analysis of chromosomal aberrations in cells collected by bile sampling or brushing as the procedure to be performed during the diagnostic workup of EH-CCA [2, 9]. Another unresolved issue is the differential diagnosis of biliary strictures, especially in the setting of PSC. Recently, microarray analysis has been applied to endoscopic biliary brushing from patients with benign and malignant biliary disease. Despite the variable quantity and poor quality of analyzed RNA, a differential gene expression profile by microarray analysis was demonstrated in patients with CCA with respect to benign pathologies. Specifically, comparing malignant versus benign biliary strictures by quantitative polymerase chain reaction (qPCR) and microarray analysis of endoscopic biliary brushings, 45 up-regulated genes have been identified in malignant strictures including various *HOX* genes, collagens, *PVT1*, *MUC4*, *MUC5AC*, and *LEF1*. Immunohistochemistry of surgically resected tissues showed elevated CD9, Serpina, and PNMA2 protein expression in CCA [77]. Notably, mutational profiling of cell-free DNA in residual supernatant fluid improves sensitivity of microscopic examination of biliary cytobrush specimens and demonstrated *KRAS* mutations as distinctive feature of CCA with respect to benign biliary strictures. Molecular analyses of biliary brushings using microarray and qPCR have the potential to provide valuable information on the biology of biliary diseases [78]. As a clinical translation of studies exploring CCA pathogenesis, the IGF1 biliary concentration was shown to be capable of completely discriminating CCA from benign biliary pathologies and pancreatic cancer [54].

4 Molecular Profiling and the Prognosis of Cholangiocarcinoma

CCA prognostic factors represent the basis for recently proposed staging systems, but not without certain criticisms and controversies. In general, the histologic grade, the size and number of the primary tumor, the tumor growth type, the depth of tumor invasion, local and distant metastatic disease, tumor-associated vascularization, vascular encasement, and lobar atrophy have been considered factors affecting survival. Biomarkers and molecular markers of local invasiveness and early metastatic behavior would help to assess prognosis as well as the eligibility of CCA patients to potential curative treatments, but, to this regard, still little is known. Indeed, no molecular marker entered the staging systems so far proposed for IH- or EH-CCA. [2, 6, 9, 11]. Several molecular markers have been investigated in relationship to CCA prognosis, and some of these have been found of potential clinical utility, including P-cadherin, p27, Skp2, *P16*, matrix metalloproteinases, and vitamin D receptor [79]. The frequency of *KRAS* mutations progressively increases with increasing tumor stage (stage I, 8 %; stage II, 15 %; stage III, 31 %; stage IV, 46 %) [39]. Molecular profiling could open new perspectives for identifying valid and reproducible predictors of survival based on protein or gene profiles. Gene expression profiling demonstrated the periostin gene as markedly overexpressed in CCA, and, by multivariate analysis, high levels of periostin were found to represent an independent negative prognostic factor, also predictive of chemoresistance [80]. Moreover, recent studies of gene expression profiling in node-positive with respect to node-negative CCA cases have shown a significantly higher expression of the genes coding for: BRCA1-associated protein 1, cyclin I, collagen type IV alpha-1 chain, collagen type IV alpha-2 chain, DR3, TL1A, heparin-binding EGF-like growth factor, urocortin receptor, bradykinin receptor B1, calpain 1, nitric oxide synthase 2, RAB10, and scavenger receptor class B member 1. In contrast, the following gene products were found down-regulated: caspase-7, BCL2/adenovirus E1B 19kD-interacting protein 1, cadherin-8, phosphodiesterase 4D, c-Abl, MEK Kinase-4 [81]. The same authors were able to select several expressed genes capable of predicting, in 100 % of the cases, the perineural invasion: *MMP-14, HSD3B, Wip1, COL2A1, CNP, Integrin 4, ING1, Wnt-10b, IL15RA, Fbn-1*, *Spectrin, ARF1* [81]. Recently, gene expression cluster analysis performed in large series of IH-CCA demonstrated how CCA could be separated into two distinct subclasses with large different survival (5-year survival rate after resection: 72 % in cluster 1 vs. 30 % in cluster 2). Major networks controlled by key molecules, such as tumor necrosis factor, transforming growth factor, and mitogen-activated protein kinase-1/2, were found to be deregulated in the poor prognosis cluster. Thirty-six genes were strongly associated with poor survival, and these genes were found to be enriched in key networks controlled by *VEGF/ERRB*, *CTNNB1/MYC*, and *TNF*. At a protein level, three of the survival genes (*ITGA2, TMPRSS4*, *CEACAM6*) as well as pRPS6, a marker of mTOR, and Ki67 staining showed significant over expression in CCA with poor prognosis. Moreover, all patients with mutated *KRAS/BRAF* have been retrieved in poor prognosis cluster [57]. These new insights received confirmation by another independent study, which showed two main biologic classes of IH-CCA. The so-called proliferation class (62 % of IH-CCA), characterized by activation of oncogenic signaling pathways (including RAS, mitogen-activated protein kinase, and MET), DNA amplifications at 11q13.2, deletions at 14q22.1, and mutations in *KRAS* and *BRAF*, showed reduced survival with respect to the so-called inflammation class (38 % of IH-CCA), which is characterized by activation of inflammatory signaling pathways, overexpression

of cytokines, and STAT3 activation [82]. In this study, an association of various genes with the histopathological grading has been demonstrated. Indeed, a trend toward higher expression of specific cell surface proteins (EMP1, EVA1, proteoglycan2) and intermediate filaments (cytokeratin 6, 7, 13, 15, 17) in well-differentiated tumors (G1–G2) was observed, whereas samples of high-grade (G3) IH-CCA showed an elevated expression of genes involved in G-protein signaling and nuclear transcription [71].

Stem cell markers have been extensively investigated as prognostic markers in CCA. The expression of SALL4, for example, correlates with tumor growth and resistance to 5-fluorouracile, while its suppression results in differentiation and delayed tumor growth [83]. The expression of neural cell adhesion molecule 1 (NCAM1), a known hepatic stem/progenitor cell marker, has been found to be predictive of poor overall survival in patients with IH-CCA [84]. In immunohistochemical investigated specimens, strong expression of CD133, a cancer stem cell marker, was strictly associated with lymph node involvement and positive surgical margins in resected CCA [72]. Recently, S100A4, a member of the S100 family of small calcium-binding proteins, expressed by macrophages and epithelial cells in mesenchymal transition, was proposed as a biomarker of increased metastasization and reduced survival after resection in CCA [5].

MicroRNA (miRNA) profile analyses have identified various microRNAs associated with either the progression or prognosis of CCA. MicroRNAs can thus serve as potential prognostic biomarkers. Recently, a transcriptomic profile has revealed hepatic stem-like gene signatures and interplay of miR-200c and epithelial–mesenchymal transition in IH-CCA. Integrative analyses of the IH-CCA-specific mRNA and microRNA expression profiles revealed that a common signaling pathway linking miR-200c signaling with epithelial–mesenchymal transition (EMT) was preferentially activated in IH-CCA with stem cell trait and poor prognosis [84].

5 Molecular Profiling and Classification of Cholangiocarcinoma

The distinction between IH- and EH-CCA, which has been reported for many years in different classifications, has become increasingly important since these two CCA forms showed enormous differences in epidemiologic features (i.e., incidence and risk factors), biologic and pathological characteristics, and clinical course [7, 8]. Recent studies comparing clinicopathological features with molecular profiling are bringing new insights into CCAs classification, further supporting the concept that IH- and EH-CCAs are two different tumors. Indeed, in vitro studies on cell cultures prepared from IH-CCA or EH-CCA have shown that they express different cellular proteins, cellular shape, doubling time, chromosome karyotype, and chemosensitivity [85]. Consistently, researchers from France have demonstrated that perihilar EH-CCA expresses with respect to IH-CCA higher levels of MUC5AC (60 vs. 22 %), Akt2 (64 vs. 36 %), K8 (98 vs. 82 %), annexin (56 vs. 44 %), and less VEGF (22 vs. 78 %) [86]. At a molecular level, distinct patterns of genetic mutations, methylation, and expression profiling may differentiate IH-CCA from EH-CCA. IH-CCAs, for example, were significantly more frequently bcl-2+ and *P16*+, whereas EH-CCAs were more often *P53*+ [87]. Miller et al. [88] investigated gene expression and copy number in biliary cancers and correlated their changes with the anatomical site of origin, histopathology, and outcomes. They revealed 545 genes with altered expression in EH-CCA and 2,354 in IH-CCA. Mutations in *IDH1* and *IDH2* were found only in IH-CCA ($n = 9$), but in none of the examined EH-CCA ($n = 22$) and gallbladder cancer ($n = 75$) [43]. *KRAS*-activating mutations appear to be less frequent in EH-CCA (9–33 %) than in IH-CCA (21–54 %). As far as epigenetic abnormalities are concerned, methylation of *RASSF1A* was more common in EH- than in IH-CCAs, while the opposite was demonstrated for methylation of GSTP gene [89].

More recently, new updated classifications of CCAs are emerging in which the IH-CCA is comprised of a pure mucin-secreting form similar to EH-CCA and a peripheral non-mucin-secreting form [4, 72, 90, 91]. These new classifications are based on cells of origin. Their rationale derives from recent scientific advances in the heterogeneity of cholangiocytes lining bile ducts of different diameters and in the nature and distribution of stem cell niches along the biliary tree [4, 72]. As far as cholangiocyte heterogeneity is concerned, small bile ducts are lined by cuboidal non-mucin-secreting cells, while large intrahepatic and extrahepatic bile ducts are lined by cylindric mucin-secreting cells. Molecular profiling of small and large mouse bile ducts have been analyzed by Alpini's group [92]. Isolated total RNAs were hybridized with microarrays, which detect 4850 cDNA expressions. Of these, 230 cDNAs were differentially expressed between small and large cholangiocytes, with aquaporin 8, IL-2 receptor beta chain, and caspase-9 being strongly expressed by large cholangiocytes [92]. In general, this study demonstrated how genes controlling proliferative activities were strongly expressed in cholangiocytes lining small ducts, while genes controlling transport processes were strongly expressed in large cholangiocytes lining large ducts. These findings are consistent with the role of small cholangiocytes as precursor cells linked with liver regeneration. As far as stem cell niches are concerned, two types have been so far identified in the biliary tree. The first type is located in the canals of

Hering and bile ductules and is composed of bipotential progenitor cells, named human hepatic stem/progenitor cells (hHpSCs) [93, 94]. The second type is located in the peribiliary glands (PBGs) and is composed of multipotent stem cells of endodermal origin, named human biliary tree stem/progenitor cells (hBTSCs) [95, 96]. Based on these concepts, the clinicopathological heterogeneity of CCAs could reflect the different lineage of origin. Nakanuma et al. [90] stressed the concept of CCA heterogeneity and proposed a small duct type (peripheral type) and a large bile duct type (or perihilar type) IH-CCA [90], with the first type originating from canals of Hering/hHpSCs and the second from peribiliary glands/hBTSCs in large ducts. The small duct type IH-CCA is mainly described as a tubular adenocarcinoma, while the large bile duct type involves the IH large bile ducts and is composed of mucin-producing elements [90]. Aishima et al. [97] investigated 87 cases of IH-CCA smaller than 5 cm in diameter and described a perihilar type showing IH large bile duct involvement within the tumor and a peripheral type containing a preserved architecture of the portal triad. They demonstrated that the frequency of perineural invasion, lymph node metastasis, vascular invasion, intrahepatic metastasis, and recurrence of IH-CCA from large ducts were significantly higher than that of IH-CCA from small ducts. In addition, the survival of patients with IH-CCA from large ducts was worse than that of patients with IH-CCA from small ducts [97]. Recently, Roskams et al. [91] carried out a study investigating the CCA histologic diversity in relation to the heterogeneity of cholangiocytes lining the biliary tree: perihilar mucin-producing cells versus peripheral cuboidal ductular cells or hHpSCs. They investigated the clinicopathological and molecular features of 79 resected CCAs and their relationship with hHpSCs and compared the spectrum of CCAs with respect to K19-positive or K19-negative HCCs. They described a subtype IH-CCA with mixed features (mixed CCAs) showing a peripheral location, a larger tumor size, less microvascular invasion, and less lymph node involvement when compared to pure mucin-producing CCAs which, in contrast, showed a hilar location, a smaller tumor size, more microvascular invasion, and more lymph node involvement. S100P expression was seen only in mucin CCAs, while neural cell adhesion molecule (NCAM) expression was only present in mixed CCAs [91]. Phenotype profiling showed high homology between mixed CCAs and K19-positive HCCs, suggesting that these two primitive liver cancers could arise from the same cell type, i.e., hHpSCs. In keeping, indeed, in 2006 Lee et al. [98], analyzing the transcriptional characteristics of HCCs by integrating gene expression or rat fetal hepatoblasts, adult hepatocytes, and HCCs from human and mouse models, showed that a gene expression profile that distinguishes HCC subtypes with poor prognosis includes well-known markers of progenitor cells (i.e., KRT7, KRT19, and VIM). This probably reflects the derivation of these HCCs from hepatic progenitor cells. Notably, at multivariate analyses where all relevant pathological and molecular variables were included, only the hepatoblast subtype was independently associated with both recurrence and worse overall survival [98].

These recent results are opening a completely new scenario and break many paradigms in the field of primitive liver cancers. Indeed, the large bile duct mucin-producing IH-CCA has similarities with EH-CCA. In contrast, the small bile duct type (peripheral) or mixed type IH-CCA has features in common with ductular type cholangiolocellular carcinoma and with CK19+ HCC [99], further reflecting the different cells of origin [4, 72]. The clinical implications of these recent advances in terms of diagnostic tools, targeted therapy and indications for surgery or transplantation need accurate evaluations in the near future. In substance, the existence of two different stem cell compartments and the associated cell lineages may result in multiple cells of origin of CCA and could represent the basis of the clinicopathological, epidemiologic, and molecular heterogeneity of CCA.

6 Molecular Profiling of Gallbladder Cancer

Mutations and epigenetic alterations of K-ras, *P53*, and *P16* have been frequently considered to be involved in the development of gallbladder cancer (GBC) and precancerous lesions [31, 100–109]. As reported in Table 3, a total number of 327 patients affected by GBC have been investigated by sequencing studies to evaluate *KRAS* mutations [104–109], with a 25 % (80 patients) overall rates of mutations. A high heterogeneity of the mutation rates among studies is clearly evident. The observed differences may recognize several causes including methods, the quality of DNA, the diversity of the ethnic background, and the different etiologies of the GBC under investigation [102]. Adenoma and dysplasia are considered to represent precancerous lesions, the latter being frequently associated with carcinoma. The mutation rates of *KRAS* in GBC, dysplasia, and adenoma have been reported, in different studies, to be 0–73 %, 0–59 %, and 0 %, respectively [102]. Controversy exists on whether *KRAS* mutations may participate in early step of cancerogenesis or, alternatively, drives adenoma formation. To this regard, two recent studies achieved opposite results. Indeed, Kim et al. [102] demonstrated that *KRAS* gene was mutated in 20 % of the GBC, but never in dysplasia or adenoma [102]. In sharp contrast, Pai et al., in 29 GBC, 16 adenomas, and 5 cases of high-grade dysplasia, analyzed for activating missense

Table 3 Sequencing studies detailing *P53* and/or *KRAS* mutations in human gallbladder cancer

References	Country	No. of patients	Overall number of patients with *P53* mutations (%)	Overall number of patients with *KRAS* mutations (%)	Notes
Yokoyama et al. [100]	Japan	22	13 (58)	ND	*P53* exons 5–8 evaluated
Yokoyama et al. [100]	Chile	20	12 (60)	ND	*P53* exons 5–8 evaluated
Nigam et al. [101]	North India	22	2 (9.1)	ND	*P53* exons 5–8 evaluated
Itoi et al. [31]	Japan	7	3 (42.9)	4 (57)	*P53* exons 5–8 evaluated; none patient with both *P53* and *KRAS* mutations
					KRAS and *P53* abnormalities not detected in non-neoplastic tissues
Kim et al. [102]	South Korea	15	3 (20.0)	5 (35.7)	*P53* exons 5–8 evaluated; none patient with both *P53* and *KRAS* mutations
					P53 and *KRAS* mutations were not found in five dysplasias around cancers and three adenomas; 30.7 % of GBC patients carried also *P16* mutations
Nagahashi et al. [103]	Japan	22	11 (50)	0 (0)	*P53* exons 5–8 evaluated; None patient with both *P53* and *KRAS* mutations
					Dysplastic epithelia obtained from gallstone patients demonstrated less frequent *P53* mutations (11 %)
Nagahashi et al. [103]	Hungary	18	6 (33.3)	1 (5.5)	*P53* exons 5–8 evaluated; none patient caring both *P53* and *KRAS* mutations
					Dysplastic epithelia obtained from gallstone patients demonstrated less frequently *P53* mutations (11 %)
Imai et al. [104]	Japan	23	ND	9 (39)	No mutations detected in normal, hyperplastic, dysplastic epithelium, adenomyomatous hyperplasia, cholesterol polyps, and cystitis glandularis proliferans
Ajiki et al. [105]	Japan	51	ND	30 (59)	Mutations in *KRAS* detected also in 8/11 gallbladder dysplasias in gallstone patients but not in normal gallbladder epithelium
Hanada et al. [106]	Japan	39	ND	15 (38)	In GBC associated with anomalous junction of the pancreaticobiliary duct (AJPBD) the prevalence of *KRAS* mutations were 100 % (stage II–IV carcinomas), whereas in the GBC without AJPBD were 38 %
Rashid et al. [40]	China	75	ND	2 (2.7)	Mean survival of GBC with *KRAS* mutation shorter (3.0 months) in comparison with GBC without mutation (15.5 months)
Parwani et al. [108]	USA	27	ND	8 (30)	
Saetta et al. [107]	Greece	21	ND	4 (19)	*BRAF* mutations observed in 7/21 (33 %) GBC; *KRAS* and *BRAF* mutations never in the same specimen
Pai et al. [109]	USA	29	ND	2 (7)	
			Total 50/126 (39.6)	Total 80/327 (25)	

Abbreviations: *ND* not determined, *GBC* gallbladder cancer

mutations in *KRAS* codons 12 and 13 and *BRAF* V600E mutations, demonstrated that *KRAS* mutations were infrequently found in GBC (2/29, 7 %) or high-grade dysplastic lesions (0/5, 0 %) but, in more than 30 % (5/16, 31 %) adenomas where, *KRAS* codon 12 mutations have been detected [107]. Based on these controversial findings, the role played by *KRAS* mutations in the stepwise malignant transformation of dysplasia to carcinoma or as mutational event in adenoma formation is still indefinite. However, it is possible that controversial findings depend on the background favoring GBC emergence. To this regard, *KRAS* mutations have been reported more frequently in GBC

arising in patients with anomalous union of the pancreatobiliary duct (AUPBD) (50 %) than without AUPBD (6 %) [106]. In the study by Kim et al. [102], the high frequency of *KRAS* mutation in GBC was found in patients without gallstones, but this is not the case in patients investigated by Pai et al. [109]. The polymorphisms of *KRAS* gene were investigated in different studies. For example, Pramanik et al. analyzed 60 GBC (13 men and 47 women) with histologically proven diagnosis and 90 controls (14 men and 76 women) in eastern India. They found a novel polymorphism in codon 25 of the *KRAS* gene associated with GBC. This novel polymorphism was found at codon 25 (CAG>CAT; Gln25His) in exon 1 of the *KRAS* gene in both germline and tissue DNA and appeared significantly associated with GBC also in multivariable logistic regression analysis after adjustment for age and sex. Silico analysis validated the *KRAS* p.Q25H polymorphism as a disease-causing variant [109].

As far as *P53* is concerned (Table 3), using sequencing methodology, several rates of *P53* mutations (0, 30, 37.5, and 50 %) have been described in GBC but not in gallbladder adenoma [102]. As reported in Table 3, a total number of 126 patients affected by GBC have been investigated by sequencing studies to evaluate *P53* mutations [31, 100–103] with a 39.6 % overall mutation rate (50 patients). It is, however, important to note that the full-length *P53* cDNA has been insufficiently investigated. Indeed, all the studies have evaluated only *P53* exons 5–8. *P53* mutations have been found mostly in the advanced stages of GBC, and therefore, *P53* has been considered to be involved only in the late events of GBC carcinogenesis favoring an aggressive behavior. Reports concerning *P16* point mutation in GBC showed alteration rate of 40 and 80 %. Similar to *P53*, the *P16* mutations or down-regulation occurred only at the advanced stage of GBC [102]. Point mutations of serine or threonine phosphorylation sites in exon 3 of β-catenin have been detected at higher rates in GBC than in bile duct carcinomas [110]. Finally, substitution and deletion of the *CTTNB1* gene causing Wnt/β-catenin activation and associated with chromosomal stability has been described in the majority of GBC (from 58 to 62 %), while substitution and insertion in the *KEAP1* gene have been described only in 30 % of GBC cases. Mutations of *PIK3CA* have been also described in GBC [111]. A mass spectrometry-based platform evaluating common cancer-associated mutations across a panel of 77 formalin-fixed paraffin-embedded biliary tree cancer specimens (32 GBC, 45 CCA) demonstrated how activating mutations in *PIK3CA* occur only in GBC (4/32, 12.5 %) [111]. This was confirmed in a recent study by sequencing analysis where even higher rates of *PIK3CA* mutations (32.4 %) were found in GBC [39]. Finally, LOHs of multiple chromosomes have been described not only in GB cancers, but also in the dysplastic lesions of gallbladder mucosa.

Given the silent clinical presentation, early diagnosis of GBC is very difficult. In light of the discussed findings, the screening and surveillance of patients affected by serious risk factors such as AUBPD could be performed by searching for *KRAS* p.Q25H polymorphism, but this needs further evaluations in different geographic areas. Biomarkers helping diagnosis have been recently investigated by evaluating gene and protein expression profile (proteomic) of GBC, compared with benign pathologies or normal tissues. A largely different profile of proteins expression marks GBC, since 46 differentially expressed proteins have been individuated by two-dimensional gel electrophoresis and by mass spectrometry. The increased level of PEBP1 protein in GBC with respect to normal mucosa has been confirmed by immunohistochemical analysis [112]. The connective tissue growth factor (CTGF) transcripts were significantly overexpressed in microdissected GBC when compared to non-neoplastic gallbladder epithelium by real-time qPCR [113]. Using a similar proteomic analysis, it has been shown that annexin A3 expression is significantly higher in GBC cancer than in chronic cholecystitis (74.0 vs. 21.1 %) [112].

Molecular profiling of GBC has been also investigated in relation to prognostic factors. Different studies suggest that gene expression or proteomic profiles can be predictive of progression and invasiveness of GBC. For example, gene expression profile evaluated by cDNA array technology showed a significantly higher expression in node-positive with respect to node-negative GBC cases of the following genes: arginine vasopressin receptor 2, sulfotransferase family, cytosolic 2B member 1, CD152 antigen. In contrast, phosphodiesterase 4C and CD1A antigen were markedly down-regulated [81]. By a proteomic evaluation, overexpression of annexin A3 gene resulted correlated significantly with lymphonode positivity or distant metastasis (40.9 vs. 100 %) or a shorter survival time after operation (50.0 vs. 93.8 %) [112]. Connective tissue growth factor (CTGF) gene overexpression has been observed in microdissected primary GBC, but not in metastatic GBC, compared with non-neoplastic gallbladder epithelium. High CTGF antigen labeling by immunohistochemistry has been significantly associated with better survival on univariate analysis [113]. The expression of MK-1, a tumor-associated antigen encoded by the *GA733-2* gene, was demonstrated in 79 % of GBCs but with large changes in relation to histologic grade. MK-1 expression, in fact, occurred in approximately 90 % of well-differentiated tubular adenocarcinomas but only in approximately 10 % of poorly differentiated adenocarcinomas. In addition, multivariate analysis showed that MK-1 expression is an independent prognostic marker, significantly correlated with increased

overall survival [114]. Therefore, MK-1 could be a useful prognostic marker for GBC. Recently, CD44 and CD133 emerged as cell surface markers for CSCs in GBC [115].

7 Conclusions

The biliary tract and gallbladder cancers are still a challenge for scientists and clinicians. These tumors usually progress insidiously, are difficult to diagnose, and have a bad prognosis. Unfortunately, treatment options are discouraging. In fact, radical surgery, the only effective treatment, is applicable in a minority of patients due to the late clinical presentation and diagnosis. Thus, to improve survival, the early detection of biliary tract and gallbladder cancers seems to be essential. Molecular biomarkers or gene polymorphisms allowing screening and surveillance of population at risk represent a necessity for the near future. Furthermore, molecular profiling analyses providing a detailed tissue evaluation for diagnosis, prognosis, and staging other than guiding therapeutic decisions are absolutely demanding. As discussed in this article, several studies have evaluated gene mutations in CCA and GBC and their impact as diagnostic or prognostic tool. Unfortunately, conclusive data are limited by the small number of samples analyzed, the CCA heterogeneity, and, mainly, the requirement of validation studies in independent cohorts of samples.

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