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New genomic landscapes and therapeutic targets for biliary tract cancers

Michele Simbolo[1], Matteo Fassan[1], Andrea Mafficini[1], Rita T. Lawlor[2], Andrea Ruzzenente[3], Aldo Scarpa[2]

[1]ARC-Net Research Centre, University and Hospital Trust of Verona, Piazzale L. Scuro 2, 37134 Verona, Italy, [2]ARC-Net Research Centre and Department of Pathology and Diagnostics, University and Hospital Trust of Verona, Piazzale L. Scuro 2, 37134 Verona, Italy, [3]Department of Surgery and Oncology, University of Verona, Piazzale L. Scuro 2, 37134 Verona, Italy

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1. ABSTRACT

Biliary tract cancers (BTCs) are a heterogeneous group of neoplasms characterized by a dismal prognosis. At variance with most solid tumors, no effective molecular targeted agent has been currently approved for BTCs treatment and their molecular landscape has only been recently investigated. Comprehensive mutational profiling studies identified *IDH1/2* and *BAP1* as characteristic of intrahepatic cholangiocarcinomas, while extrahepatic cholangiocarcinomas and gallbladder carcinomas were characterized by frequent *KRAS* and *TP53* alterations. Moreover, targeted next-generation sequencing has uncovered alterations in several key cellular pathways. BTC-specific alterations include disorders of major regulators of cell cycle and chromatin remodeling processes, as well as deregulation of the mTOR-, TGF-beta/Smad- and receptor tyrosine kinases signaling. The next step will be the correlation of these findings with clinical trials to identify predictive biomarkers for the development of personalized therapies. This will permit early access for BTC patients to innovative drugs.

2. INTRODUCTION

Biliary tract cancers (BTCs) are a heterogeneous group of cancers, representing only about 3% of all gastrointestinal malignancies (1). They may arise from the gallbladder (i.e. gallbladder carcinomas; GBC) or the biliary tree. These latter can be separated according to their anatomical origin into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC) (2, 3).

The mortality rate for these neoplasms is increasing worldwide (4). Surgery represents the only curative option at early stages of the disease, which is, however, affected by a high rate of tumor recurrence (5).

Remarkably, early diagnosis has been demonstrated to increase the survival rate of only 5% (6), and by the clinical point of view, a definite diagnosis of cholangiocarcinoma can be significantly delayed also in advanced stages because of the wide range of alternative diagnoses, including benign biliary tree strictures (i.e. choledocholithiasis and iatrogenic bile duct injuries), primary sclerosing cholangitis and other primary and metastatic carcinomas (7).

At variance with most solid tumors, no effective molecular targeted agent has been approved for BTCs, and consequently BTC patients have limited access to innovative clinical trials. It has to be underlined that the most important issue hampering the introduction of new therapeutic options for BTCs' patients is represented by the limited information available on their molecular background (Table 1 and Figure 1).

Table 1. Most frequently mutated genes in cholangiocarcinomas by subtype (numbers represent percentage of mutated cases)

Gene	ICC	ECC	GBC	Ref
ARID1A	19	NA	0	65
	NA	NA	0	39
	11.4.	12.3.	11.5.	22
	20	5	NA	75
BAP1	25	NA	0	65
	NA	NA	0	39
	14.3.	0	3.8.	22
	9.1.	10	NA	75
BRAF	0	NA	0	65
	NA	NA	5.9.	39
	4.3.	0	0	22
	5.3.	0	NA	75
CDKN2A	3	NA	0	65
	NA	NA	5.9.	39
	1.4.	0	3.8.	22
	5.3.	10	NA	75
EGFR	0	NA	12.5.	65
	NA	NA	3.9.	39
	0	1.7.	3.8.	22
	1.8.	5	NA	75
ERBB2	0	NA	0	65
	NA	NA	9.8.	39
	0	0	3.8.	22
	1.8.	20	NA	75
ERBB3	0	NA	0	65
	NA	NA	11.8.	39
	NA	NA	NA	22
	0	0	NA	75
FBXW7	0	NA	12.5.	65
	NA	NA	5.9.	39
	1.4.	3.5.	0	22
	5.5.	15	NA	75
IDH1/2	19	NA	0	65
	NA	NA	0	39
	20	0	0	22
	23.6.	0	NA	75
KRAS	3	NA	0	65

(Cond...)

Table 1. *(Contiuned...)*

Gene	ICC	ECC	GBC	Ref
	NA	NA	7.8.	39
	15.7.	47.4.	19.2.	22
	23.6.	40	NA	75
NRAS	3	NA	0	65
	NA	NA	0	39
	9.3.	1.7.	0	22
	0	5	NA	75
PBRM1	17	NA	25	65
	NA	NA	0	39
	14.3.	3.5.	7.7.	22
	10.9.	5	NA	75
PIK3CA	6.2.	NA	12.5.	65
	NA	NA	5.9.	39
	5.7.	8.7.	7.7.	22
	5.3.	10	NA	75
PTEN	6.2.	NA	0	65
	NA	NA	0	39
	1.4.	3.5.	3.8.	22
	5.3.	5	NA	75
SMAD4	0	NA	12.5.	65
	NA	NA	3.9.	39
	1.4.	10.5.	7.7.	22
	3.6.	20	NA	75
SMARCB1	0	NA	0	65
	NA	NA	3.9.	39
	0	0	7.7.	22
	0	0	NA	75
TP53	6.2.	NA	63	65
	NA	NA	47.1.	39
	8.6.	17.5.	46.2.	22
	29.1.	45	NA	75

This minireview summarizes the current evidence on the most frequently deregulated pathways in BTCs, focusing on their potential as novel therapeutic targets.

3. ROLE OF CHRONIC INFLAMMATION AND RELATED PATHWAYS

Major BTC risk factors are primary sclerosing cholangitis (PSC), long-standing intraductal gallstone

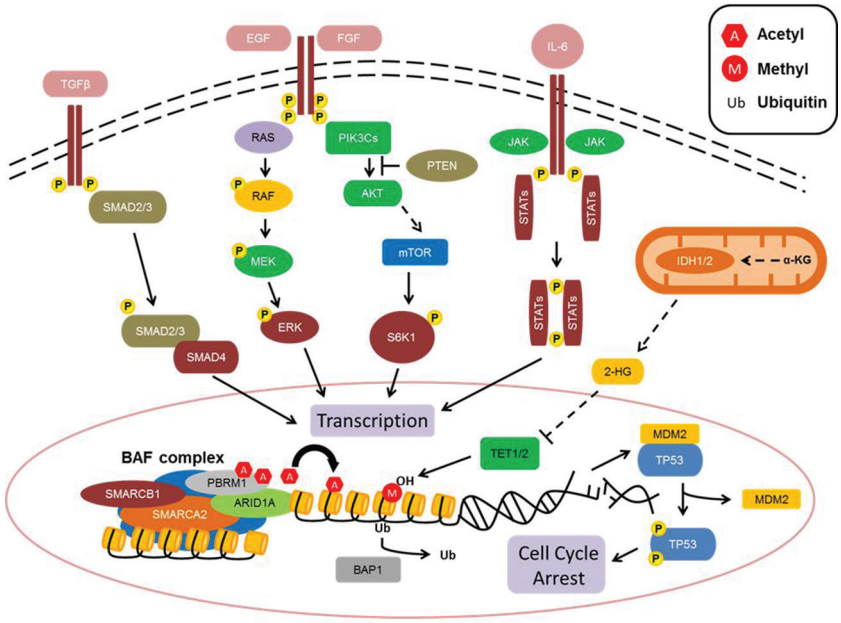

Figure 1. Pathways most frequently altered in biliary tree tumors: inflammation related cytokines (IL-6), chromatin remodeling (*PBRM1*, and *BAP1*), mTOR and TGF-beta/Smad signaling, and receptor tyrosine kinases (EGF and FGF). *IDH1/2* and *BAP1* are characteristic of intrahepatic cholangiocarcinomas, while *KRAS* and *TP53* genes alterations are more frequent in extrahepatic cholangiocarcinomas and gallbladder carcinomas.

disease, and infestation by the liver flukes *Clonorchis sinensis* and *Opisthorchis viverrini* (8). These data demonstrate an important role for chronic inflammation on biliary tree carcinogenesis.

Similar to what observed in the hepatitis-associated multistep liver carcinogenesis (9), several studies pinpointed a key role of inflammation-related cytokines on the induction of the malignant transformation of cholangiocytes. Among the others, interleukin 6 (IL-6) and its associated pathways have been demonstrated to be an important proinflammatory signaling in the biliary tree chronic inflammatory setting (10). Increased levels of IL-6 have been demonstrated in serum from cholangiocarcinoma (CCA) patients (10). Moreover, IL-6 levels significantly correlated with tumor burden and were significantly decreased two weeks after therapeutic surgery. Of note, IL-6 levels were able to discriminate between CCA and benign biliary disease with a sensitivity and specificity of 71% and 90%, respectively (11).

IL-6 has an autocrine role on CCA cell survival. Through the activation of the p38 and p44/p42 MAPK signaling (12), IL-6 promotes telomerase activity (13), up-regulation of Mcl-1 (14), and induces STAT3 activation (15). Notably, STAT3 expression is significantly up-regulated in dedifferentiated CCAs, and is associated with shorter survival of CCA patients (16).

Another important player in the promotion of malignant transformation of cholangiocytes is COX-2. Cholestasis and the presence of oxysterols, oxidized forms of cholesterol, can significantly induce the overexpression of COX-2, which subsequently promotes cell growth (17). Moreover, inflammatory cytokines significantly up-regulate the inducible nitric oxide synthase (iNOS); iNOS directly binds to COX-2 and enhances COX-2 activity (18). It has been demonstrated a significant overexpression of both iNOS and COX-2 in reactive biliary epithelia and biliary intraepithelial neoplastic lesions (19).

A recent whole-exome sequencing study investigated the impact of *O. viverrini* infection on CCA mutational profile (20). Of interest, *BAP1 (*10.5.% versus 2.8.%) and *IDH1*/*IDH2 (*9.3.% versus 2.8.%) mutation rates were significantly higher in non–*O. viverrini* CCAs. On the other hand, *TP53 (*9.3.% versus 39.8.%), *SMAD4 (*5.8.% versus 19.4.%), *MLL3 (*3.5.% versus 13.0.%) and *GNAS* (0% versus 5.6.%) were more frequently involved in *O. viverrini*-related tumors (20). Overall, these data strongly suggest distinct mutational profiles based on the different etiologies.

4. CELL SURVIVAL PATHWAYS ACTIVATION

mTOR signaling is one of the most involved pathway in the maintenance of cell survival and inhibition of apoptosis in solid tumors. Aberrant expressions of activated forms of mTOR (phospho-mTOR) and of its downstream effectors (ph-AKT1, ph-p70S6K and/or ph-4EBP1) is a common finding in BTCs (21, 22).

Moreover, the overexpression of ph-mTOR has been associated to well-to-moderately differentiated tumors, and to tumors without metastatic spread (21). Elevated activated mTOR levels have been also associated to a significantly shorter overall survival for BTCs' patients (23).

Another important mTOR-related gene is the phosphatase and tensin homologue (*PTEN*) which is a key tumor suppressor frequently altered in BTCs. Low PTEN expression and decreased PTEN/ph-AKT1 and PTEN/ph-mTOR expression ratios are poor prognostic factors in ECCs (24).

The role of *PIK3CA* alterations in BTCs is still under study. In a Chinese series, 32% of CCAs showed a mutation in the *PIK3CA* gene (25); another study found only one mutated case out of 11 (26).

Our group recently further supported mTOR pathway relevance in BTCs by demonstrating a significant up-regulation of ph-mTOR and of its downstream effectors in 51% of a large series of BTCs (22). This altered activation could be partly explained by the concomitant presence of a mutation in one of the genes involved in the mTOR pathway (i.e., *AKT*, *FBXW7*, *PIK3CA*, *PIK3C2A*, *PIK3C2G*, *PTEN*).

5. TYROSINE KINASES RECEPTORS

The ErbB family of class I receptor tyrosine kinases comprises four distinct receptors: ErbB1 (EGFR), ErbB2 (HER2), ErbB3 and ErbB4 (27, 28).

Aberrant EGFR and/or HER2 expression and deregulation of their downstream signaling are frequently observed in ICCs. Different independent studies demonstrated a significant up-regulation of EGFR in ICC (29-32). Among these, Ito *et al.* (30) further observed that 39.5.% and 10.5.% of their ICCs showed also an overexpression of ErbB3 and ErbB4, respectively.

HER2 immunohistochemical overexpression has been demonstrated in variable percentages of noncancerous biliary proliferative disorders associated to CCA, including choledocholithiasis and PSC (33-35). Overall, these findings support an early involvement of HER2 deregulation in biliary carcinogenesis. Interestingly, Su *et al.* (36) demonstrated a higher level of HER2 in the bile collected from CCA patients than in the bile collected from patients with biliary tract infection, biliary stone disease, or normal controls.

Somatic mutations in the tyrosine kinase domain of *EGFR* have been described in a subgroup of patients with either cholangiocarcinoma or gallbladder carcinoma (37, 38). However, the clinical impact of these mutations should be further investigated. More recently, a whole-exome sequencing study identified recurrent mutations in the ErbB signaling (including *EGFR*, *ERBB2*, *ERBB3*, *ERBB4* and their downstream genes) in GBCs (39).

It is well known that c-Met plays an important role in carcinogenesis in many cancer types, including hepatic tumors (34, 40). Terada *et al.* reported a significant c-Met overexpression in 81% of proliferative lesions associated to choledocolithiasis (41). Of note, c-Met was also significantly overexpressed in 58% of ICCs and correlated with tumor differentiation (41).

Also defects in the TGF-beta signaling play a significant role in many malignancies, including BTCs. Transforming growth factor signaling pathway disturbance in biliary cancers results from intragenic mutations in either the TGF-beta type I and II receptor genes, or the TGF-beta gene itself (42, 43). Moreover, homozygous or heterozygous deletions of the TGF-beta type I receptor gene has also been reported in biliary carcinomas (42, 43). Of note, deregulation of the TGF-beta signaling is associated to an increased production of peritumoral fibrosis (44), which is a hallmark of extrahepatic BTCs. In fact most EECs are often accompanied by abundant fibrous stroma (5).

Members of the *FGFR* family (i.e. *FGFR1-4*) have been associated with mutations, amplifications and translocation events with oncogenic potential (45). As observed in other solid tumors, the presence of *FGFR* gene fusions have been recently demonstrated also in CCAs (46, 47); several ICC cases characterized by *FGFR2-BICC1* fusions and a single case with *FGFR2-AHCYL1*.

6. INTRACELLULAR SIGNALING

Several studies associated *KRAS* gene alterations to GBCs. However, the reported frequency of gene mutations (in codons 12, 13, and 61) varies from 0% to 80% (22, 48). Of interest, in Western countries, where GBC is most related to chronic cholecystitis and cholelithiasis, *KRAS* mutations are rarely encountered (0-10%). In contrast, in Japanese series, where GBCs may often be associated with a congenital abnormality, the frequency of *KRAS* mutations is markedly higher.

Activation of the *KRAS* oncogenes has been described also for other BTCs (49). The variable incidence of *KRAS* mutations reflects the anatomical location and the type of duct on which CCAs develop. In fact, *KRAS* mutations are more frequently observed in ECCs and ICCs of the periductal-spreading type, whereas are almost absent in the ICCs mass-forming type (50, 51). These different rates may also reflect different etiologies; the prevalence ranges from 58% in sporadic CCAs to 8% in CCAs related to *O. viverrini* in Thailand (52).

BRAF mutations have been observed mainly in ICCs. In our series all three *BRAF* mutations observed

clustered in ICC tumor subtype and were mutually exclusive with mutations in *KRAS* and/or *NRAS* (22). Similarly, Goeppert *et al.* observed *BRAF* mutations only in ICCs and this specific alteration affected 3% of the series. Tannapfel *et al.* characterized a large series of CCAs for *BRAF* mutations, and found activating *BRAF* missense mutations (V599E and also V599D) in 15/69 (22%) cases (53). Saetta *et al.* observed a high incidence of *BRAF* exon 15 mutations in GBCs (33%); of note, *BRAF* and *KRAS* mutations were mutually exclusive (54).

SMAD4 tumor suppressor gene is an important downstream component of TGF-beta signaling (55). Mutations determining loss of expression of *SMAD4* gene have been described in biliary malignancies and in particular in ECCs (56). Of interest, the liver-specific targeted simultaneous disruption of *SMAD4* and *PTEN* tumor suppressors generated a mouse model that develops biliary malignancies (57).

7. CELL CYCLE CHECKPOINT

The p16/cyclin D1/Rb pathway is one of the most important cell-cycle regulator in many different types of cancers (58). Evidence for a role in biliary tract carcinogenesis derives from the demonstration of frequent cyclin D1 overexpression and inactivation of *CDKN2A* (p16) by a variety of mechanisms, including mutation, deletion, and abnormal hypermethylation (59). Ishikawa *et al.* reported a significant reduction of p16 expression due to promoter methylation in intraductal papillary neoplasms of livers with hepatolithiasis; intraductal papillary neoplasms are identified as precursor lesions of ICC (60). Methylation of the *CDKN2A* promoter and homozygous deletion of the gene has also been frequently observed in ICCs (61, 62).

Several studies pinpointed a central role for *TP53* gene alterations in BTCs (63). Also *MDM2* gene amplification has been demonstrated as an important *TP53* master deregulator in CCAs (64). In our mono-institutional series of 153 CCAs, the presence of *TP53* mutations was the only significant independent prognostic factor (22). It has to be noted that although *TP53* mutations are frequent in ICCs, their prevalence is significantly higher in ECCs and GBCs. In fact, somatic mutations of *TP53* occur in almost half of GBCs (65).

8. METABOLIC RELATED FACTORS

Somatic mutations in the cytoplasmic and peroxisomal isocitrate dehydrogenase gene *IDH1* and its mitochondrial counterpart *IDH2* have been identified through large genomic screenings of human tumors (66). These mutations cause a single amino acid change at a conserved arginine residue within the isocitrate binding site of *IDH1* (R132) or *IDH2* (R172, R140) resulting in decreased enzymatic activity for oxidative decarboxylation of isocitrate to alfa-ketoglutarate (67).

A seminal study on the mutational profiling of 287 gastrointestinal cancers identified *IDH1* mutations in a significant subset of patients affected by ICC (68). In the same study it was shown that the prevalenceof *IDH1* and *IDH2* gene mutations was greater than the combined prevalence of activating mutations in *AKT1*, *KRAS*, *NRAS*, and *BRAF* in ICC, making it the most prevalent drug targetable gene alteration in this cancer type (68). This finding was further supported by deep sequencing studies, which identified *IDH1* and *IDH2* gene mutations in almost 20% of ICCs (22, 65, 68).

The presence of *IDH1/2* mutations in a significant proportion of ICCs may offer novel insights on how to develop targeted approaches against this treatment-refractory disease. The physiological IDH1/2 function is the catalyzation of the oxidative carboxylation of isocitrate to alpha-ketoglutarate. IDH1/2 mutations confer neomorphic activity through the reduction of alpha-ketoglutarate to the metabolite R(–)-2-hydroxyglutarate (2HG), resulting in the accumulation of 2HG in tumor tissues (67, 69). A recent study suggests that circulating 2HG may serve as a surrogate biomarker of *IDH1* or *IDH2* mutation status in ICC, and its levels are related to tumor burden (70).

9. CHROMATIN REMODELING GENES

Genome-wide sequencing has identified frequent mutations in chromatin remodeling genes across different tumor types. These discoveries have profound implications in understanding the mechanistic basis of epigenetic changes that have recently been demonstrated as widespread in most tumors (71).

The seminal report of Jiao *et al.* highlighted the key role of mutations in chromatin-remodeling genes in ICC with frequent inactivating mutations in three different genes (*ARID1A*, *BAP1*, *PBRM1*) affecting almost half of the tumors sequenced. These studies point to chromatin remodeling as a crucial area of investigation for innovative therapeutic strategies. Moreover, comparison of somatic mutation data for cholangiocarcinoma and gallbladder carcinoma suggests that, while both tumor types arise from biliary epithelium, they are genetically distinct (65).

Mutations in *PBRM1* (which encodes BAF180) were identified in 41% of renal cell carcinomas, making *PBRM1* the second most frequently mutated gene in these cancers after *VHL* (72). BAF180 contains six tandem bromodomains that bind acetylated histones, two bromo-adjacent homology (BAH) domains that mediate protein–protein interactions and a high-mobility group (HMG) domain that binds nucleosomal DNA. Given the function of these domains, BAF180 may recruit

PBAF complexes to specific loci (73). BAF180 binds preferentially to different acetylated lysine configurations of histone tails, indicating that it may contribute to the recognition of the histone code (74). The frequency of mutations of this gene in CCAs was attested at between 5 and 20% depending on histopathological subtype (Table 1), further underlying its importance in human cancers (22, 39, 65, 75).

The *ARID1A* subunit of SWI/SNF complexes was also recently found to be specifically mutated in primary human cancers. The *ARID1A* subunit contains an ARID domain that binds DNA in a sequence-nonspecific manner (76). However, the role of this domain during the engagement and repositioning of a nucleosome is still not fully understood as *PBRM1*, *ARID1A* mutation frequencies significantly differ among CCA subtypes. Most ICC and ECC studies reported a mutation frequency of 5-20% (22, 65, 75). In GBCs, *ARID1A* seems to be infrequently altered (22, 39).

Protein ubiquitination was initially seen as a mechanism to label proteins for degradation; however, this idea has evolved as we have come to understand that ubiquitination and deubiquitinating enzymes (DUBs) regulate various cellular processes, including DNA repair, gene transcription, cell membrane trafficking, cell cycle progression, stress response, cell communication, differentiation and apoptosis, and they also have a role in cancer (77). *BAP1* was discovered in a yeast two-hybrid screen owing to its interaction with the RING finger domain of the tumor suppressor *BRCA1*. *BRCA1* forms a heterodimer through its RING domain with *BRCA1*-associated RING domain 1 (*BARD1*), and this *BRCA1–BARD1* tumor suppressor complex has E3 ubiquitin ligase activity that regulates the DNA damage response (DDR) (78). *BAP1* binds and deubiquitylates *BARD1*, thus modulating the E3 ligase activity of *BRCA1-BARD1 (*79). Mutations in the *BAP1* gene seem to be specific of ICC tumors. Over 20% of ICC cases showed mutation in *BAP1* in exome sequencing studies (20, 39, 65). Differently studies performed on ECCs and GBCs showed no or low mutation frequency in *BAP1* gene (20, 22, 39, 75).

10. CONCLUSIONS

BTCs are characterized by an unpredictable clinical behaviour. A significantly poorer prognosis is usually observed for ECCs and GBCs in comparison to mass-forming ICCs; however, significant clinical differences are observed also among tumors characterized by similar clinico-pathological features. As such, no biomarker prognostically stratify BTCs' patients, so far. However, the remarkable progress in molecular biology has led to a better understanding of the complex molecular landscape of BTCs'. Of note, particular mutational profiles have been identified according to tumor location through targeted next generation sequencing. Mutations in *IDH1/2* and *BAP1* have been recognized as characteristic of ICCs, while ECCs and GBCs are characterized by frequent *KRAS* and *TP53* alterations. Alterations of the *TP53* gene have also been associated with a worse patient prognosis.

Several other key cellular pathways, including disorders of major regulators of cell cycle and chromatin remodeling processes, as well as deregulation of the mTOR-, TGF-beta/Smad- and receptor tyrosine kinases signaling, have recently been shown to be altered in these tumors. This emerging knowledge is of utmost importance for cancer research and may be the breakthrough to novel innovative, targeted therapies.

The next step will be the correlation of these findings with clinical trials to identify predictive biomarkers for the development of personalized tailored therapies. This will also permit early access for BTCs patients to innovative drugs.

11. ACKNOWLEDEMENTS

Current address of Matteo Fassan is Department of Medicine (DIMED), University of Padua, via Gabelli 61, 35121 Padua, Italy. M. Simbolo and M. Fassan equally contributed to this article. All authors participated in writing and approved the final, submitted manuscript. No conflict of interest to be declared. This work was partly supported by theItalian Cancer Genome Project grants from the Italian Ministry of University and Research (FIRB - RBAP10AHJB) and Fondazione Italiana Malattie Pancreas – Ministry of Health (J33G13000210001), AIRC grant n. 12182, and FP7 EU project CAM-PaC no: 602783.

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Abbreviations: ICC, intrahepatic cholangiocarcinoma, ECC, extrahepatic cholangiocarcinoma, GBC, gallbladder carcinoma

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Send correspondence to: Matteo Fassan, ARC-Net Research Centre; University of Verona, Policlincio G.B. Rossi, 37134, Verona- Italy, Tel: 390498211312, Fax:390498272277, E-mail: matteo.fassan@gmail.com