Critical Reviews in Oncology / Hematology 140 (2019) 8–16

Contents lists available at ScienceDirect

Critical Reviews in Oncology / Hematology

journal homepage: www.elsevier.com/locate/critrevonc

Crossing borders: A systematic review with quantitative analysis of genetic mutations of carcinomas of the biliary tract

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ARTICLE INFO

Keywords:
Biliary tract
Cholangiocarcinoma
Cancer biology
Genomics

ABSTRACT

Biliary tract carcinoma (BTC) comprises gallbladder and intra-/extrahepatic cholangiocarcinoma (GBC, ICC, EHC), which are currently classified by anatomical origin. Better understanding of the mutational profile of BTCs might refine classification and improve treatment.

We performed a systematic review of studies reporting on mutational profiling of BTC. We included articles reporting on whole-exome/whole-genome-sequencing (WES/WGS) and targeted sequencing (TS) of BTC, published between 2000-2017. Pooled mutation proportions were calculated, stratified by anatomical region and sequencing technique. A total of 25 studies with 1806 patients were included. Overall, *TP53* was the most commonly mutated gene in BTC. GBC was associated with mutations in *PFKFB3*, *PLXN2* and *PGAP1*. Mutations in *IDH1*, *IDH2* and *FGFR* fusions almost exclusively occurred in ICC patients. Mutations in *APC*, *GNAS* and *TGFBR2* occurred exclusively in EHC patients.

In conclusion, subtypes of BTCs exhibit minor differences in mutational profile, which is likely influenced by the cell of origin.

1. Introduction

Biliary tract carcinoma (BTC) is the collective name for a group of heterogeneous tumours arising from the epithelial cells in the biliary tract. These tumours are traditionally classified according to their anatomical origin: intrahepatic (ICC), extrahepatic (EHC) cholangiocarcinoma and gallbladder carcinoma (GBC). Additionally, EHC can be further subdivided into periampullary and perihilar cholangiocarcinoma (Khan et al., 2005; Banales et al., 2016a; Blechacz, 2017). The global incidence of BTC ranges from 0.6-0.8 per 100 000 people per year in Western countries to 40–90 per 100 000 people per year in Asian countries (Khan et al., 2005; Petrick et al., 2017). These high incidences in Asian countries are likely related to the high prevalence of liver fluke infection and other risk factors such as hepatolithiasis, hepatitis, and primary sclerosing cholangitis (PSC) (Chan-On et al., 2013a; Kongpetch et al., 2015; Jusakul et al., 2015; Boberg et al., 2000; Maemura et al., 2014; Carpino et al., 2015). Median survival is 9–12 months for the majority (60–80%) of patients with unresectable BTC and 5-year survival rates after resection range between 16–54% (Valle et al., 2010; Bridgewater et al., 2014; Aloia et al., 2015; Aljiffry et al., 2009; Esnaola et al., 2016; Sternby Eilard et al., 2017).

The biological behaviour and genomic characteristics of BTC show similarities to other upper gastrointestinal tract tumours (Nakanuma, 2010; Gandou et al., 2013). However, the oncogenesis of BTCs is very heterogeneous, which is partly related to the anatomical origin. Therefore, adequate understanding of the embryologic development of

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https://doi.org/10.1016/j.critrevonc.2019.05.011
Received 22 January 2019; Accepted 21 May 2019
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the biliary tract is indispensable. At week 4 of gestation, a ventral-medial outgrowth forms from the foregut endoderm, which eventually becomes the hepatic diverticulum (Fan et al., 2012). The biliary tract is derived from different parts of this hepatic diverticulum. The cranial parts of the hepatic diverticulum develop into the liver and intrahepatic bile ducts, whilst the caudal part forms the gallbladder and cystic duct. The pancreatic bud is also derived from the hepatic diverticulum, which differentiates into the pancreatic head. The original hepatic diverticulum elongates and forms the common bile duct (Gandou et al., 2013; Raggi et al., 2015; Holczbauer et al., 2013). Thus, the liver, bile ducts and pancreatic ducts are embryologically related, which explains genetic commodities encountered in tumours derived from either one of these structures.

Histologically, ICC shows two different subtypes: the mixed or cholangiolar subtype (originating from the small bile ducts) and the mucinous or ductular subtype (originating from the larger bile ducts) (Komuta et al., 2012a; Liau et al., 2014; Farshidfar et al., 2017a; Andersen and Thorgeirsson, 2013; Sia et al., 2013). The large intrahepatic ducts are lined by cylindrical mucin-producing cholangiocytes, similar to the extrahepatic bile ducts. The smaller intrahepatic ducts (canals of Herring) are lined by mucin-negative cuboidal cells. The small bile ducts contain hepatic progenitor cells (HPCs), which can differentiate into both hepatocytes and the mucin-negative cholangiocytes (Carpino et al., 2015; Cardinale et al., 2015a; Roskams, 2006. Taking into consideration these differences in the cell of origin, it follows that the mutational landscape of ICC shows a spectrum. On one end, tumours derived from the HPCs in the small hepatic ducts resemble the cell of origin in hepatocellular carcinoma (HCC). On the other end are tumours derived from the mucin-containing cholangiocytes in the large hepatic ducts, which show overlap with extrahepatic cholangiocarcinoma and pancreatic cancer (Roskams, 2006; Lanzoni et al., 2016).

Extrahepatic cholangiocarcinoma is thought to arise from adult cholangiocytes or pluripotent stem cells and progenitor cells that originate in the peribiliary glands located at branching points of the biliary tree, such as the hilum and periampullary region (Banales et al., 2016a; Cardinale et al., 2012, 2015b).

Recent studies have shown that mutational profiles differ between intra- and extrahepatic cholangiocarcinoma and gallbladder carcinoma, which reflects the above mentioned differences in aetiology (Andersen, 2015; Jusakul et al., 2017a; Farshidfar et al., 2017b). *IDH1* and *IDH2* mutations as well as *FGFR* fusion events are more frequently observed in ICC of a cholangiolar or mixed subtype than in those of a mucinous or ductular subtype (Komuta et al., 2012a; Liau et al., 2014; Goyal et al., 2019). Mutations in *RAS* are found more often in EHC (Komuta et al., 2012a; Liau et al., 2014). Except for more frequent *TP53* mutations in periampullary cholangiocarcinoma, there are no known major mutational differences between the two different anatomical varieties of EHC. TP53 and KRAS are most commonly mutated in GBC, as well as mutations *ErBB* pathway genes (*EGFR*, *HER2*, *ERBB3*, *ERBB4*) (Nakamura et al., 2015; Deshpande et al., 2012; Li et al., 2014; Jusakul et al., 2017b).

Chronic inflammation is the most important risk factor for BTC. This can be induced by inflammatory disease (PSC and gall stones), infectious agents (liver fluke infections and hepatitis), and occasionally chemical factors (organic solvents) (Maemura et al., 2014; Carpino et al., 2015). There is evidence that the different agents induce mutations in specific genes. Liver-fluke associated ICC shows more frequent mutations in *KRAS, SMAD4, CDKN2A* and *MLH1*, whereas fluke-negative ICC showed more frequent mutations in *BAP1, ARID1A, IDH1* and *IDH2*. *TP53* and *TERT* was more commonly mutated in patients with hepatitis (Jusakul et al., 2015; Chan-On et al., 2013b; Ong et al., 2012).

For many cancer types, a better understanding of the molecular background has provided new opportunities in diagnosis and treatment selection (i.e. molecular diagnostics, liquid biopsy and the discovery of targets for therapy). In BTC, molecular diagnostics may be valuable as well (Boberg et al., 2000; Timmer et al., 2016; Roos et al., 2019). Currently, differentiation of cholangiocarcinoma from benign disease -such as PSC and IgG4-associated cholangitis- is based on pathology and imaging, which is far from perfrect (Roos et al., 2019; Rassam et al., 2018).

A systematic overview of the mutational landscape of BTC is lacking and the available data is still very scattered. Primary articles are often subtype specific, hindering the comparison of the differences between subtypes. The aim of this study was to combine sequencing data from the current literature and provide an accurate overview of mutations in biliary tract cancers.

2. Methods

2.1. Search strategy and data selection

A systematic literature search was performed in PubMed and EMBASE to identify articles reporting on NGS results in BTC patients in English, published between January 2000 and December 2017. Pubmed was searched using the following terms: biliary tract neoplasms, bile duct tumours, bile duct cancers, cholangiocarcinoma and mutations, genetic associations, genome/exome sequencing. For all terms used, see supplementary file 1. Abstracts were screened for eligibility if they included the following tumour types and criteria: cholangiocarcinoma, gallbladder carcinoma, intraductal papillary neoplasm of the bile duct, intrahepatic-, perihilar or distal cholangiocarcinoma, human or in-vivo studies, sequencing type: next generation or sanger sequencing. Abstracts were excluded if they assessed pancreatic carcinoma, periampullary carcinoma, expression profiling only, immunohistochemistry only, proteomics only or methylation only.

Articles published before 2000 were excluded, as the number of interrogated genes and cohort sizes were very limited and the human genome project was only completed in 2003. Studies using targeted sequencing (TS) or whole exome sequencing (WES) or whole genome sequencing (WGS) in more than five patients were selected for full review after title and abstract screening. Studies with overlapping patient cohorts were included after removal of duplicates. Screening and judging of eligibility were performed by two independent investigators (ER and ECS) using the Covidence systematic review software (Veritas Health Innovation, Melbourne, Australia). Conflicts were resolved through mediation by a third investigator. In addition to genome sequencing, type of sequenced tissue, sequencing method and risk factors for BTC were also extracted. Corresponding authors were asked to provide additional data if necessary. Risk of bias tools are currently only available for genome wide association studies, therefore the risk of bias could not be assessed formally (Sohani et al., 2016). However, information on pathologic assessment, the fixation method of tissue, sequencing platform, panel design, patient characteristics i.e. disease stage, country of origin and associated risk factors were included to enable the reader to assess applicability of studies and their risk of bias.

2.2. Summary measures and quantitative analysis

The samples included in the quantitative analysis were stratified according to their localization in the biliary tree (i.e. ICC vs EHC vs GBC) and the sequencing technique (TS vs WES/WGS).

Mutations in an intronic region and non-silent mutations within the exonic region and the promotor and enhancer region were included in the analysis. In WES/WGS studies, non-somatic mutations were removed. If a patient had more than one mutation in a gene, it was counted as one mutation event. Genetic alterations were grouped according to their annotated pathways and cellular function. Other findings, including epigenetic changes like methylation and copy number aberrations were not systematically explored as this lies beyond the scope of this article. When available, morphological subtype of ICC (mixed or cholangiolar subtype versus mucinous or ductular), infection with liver flukes or hepatitis B or C and geographical background was

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recorded.

A quantitative analysis was performed using crude pooled proportions. For studies on TS, crude mutation proportions were calculated per gene by dividing the total number of patients with a mutated gene by the total number of patients in whom the gene was sequenced. For studies on WES/WGS, crude mutation proportions were calculated per gene by dividing the total number of patients with a mutated gene by the total number of patients in all WES/WGS studies combined, under the assumption that all relevant genetic regions were included in these studies. To determine subtype-specific gene mutations, WES, WGS and TS data were pooled together. Mutations were considered unique if their pooled prevalence was higher than 5% for one subtype and lower than 1% for other subtypes. For genes to be considered unique or overlapping, they had to be assessed in all three anatomical subtypes.

2.3. Additional statistics

All confidence intervals were 95% and derived from the normal distribution. These were calculated according to the Clopper-Pearson test for binomial confidence intervals. All data were analysed using STATA version 14.1 (StataCorp LP, College Station, TX, US) and visualised using Prism version 6.0 h (GraphPad Software, Inc, La Jolla, CA, US).

3. Results

3.1. Study characteristics

The search strategy returned 1358 unique articles. After reaching consensus, 183 articles were retrieved in full-text form and assessed for eligibility. After consensus meetings, eligibility assessment yielded 28 articles that reported data about mutational profiles of cholangiocarcinoma and/or gallbladder carcinoma found with next generation sequencing (NGS). Of these, 25 were included in the final analysis. Three articles were excluded as patient-level data could not be retrieved (Fig. 1, Sup Table 1) (Goyal et al., 2019; Ahn et al., 2016; Zhu and Hezel, 2011).

A total of 1806 patients were included. Of 25 studies, eight (32%) studies reported WES data or WGS data, three (12%) studies reported both WES and TS data, and 14 (56%) studies reported TS data only. The total study size ranged from 6 to 489 patients. 556 samples were sequenced with WES or WGS: 398 (72%) patients with ICC, 90 (16%) patients with EHC and 68 (12%) patients with GBC. 1250 samples were sequenced with TS: 652 (52%) patients with ICC, 340 (27%) patients with EHC and 258 (21%) patients with GBC. Five studies included samples that were analysed with both WGS/WES and TS. These samples were de-duplicated for the quantitative analysis (supplementary table 4).

In 11/25 (44%) of studies, it was mentioned that at least one slide of the tumour tissue used for DNA-extraction was assessed by a pathologist. In 4/25 (16%) of studies, pathological reassessment was implied, but not explicitly stated. Fresh frozen samples were used in nine studies (36%), paraffin embedded (FFPE) in 14 studies (56%), one (4%) study used both and one (4%) did not report on the fixation method.

3.2. Disease stage, risk factors, morphological subtypes and geographical location

13/25 (52%) studies reported on patients who had underwent a curative resection (resection samples), 3/25 (12%) studies reported on advanced disease (i.e. locally advanced or metastasized), 8/25 (32%) studies reported on both resectable and metastasized disease, issue from and in one (4%) study the disease stage was not stated.

In 12/25 (48%) studies risk factors were reported; two of these specifically reported on fluke positive cholangiocarcinoma (Table 1). Increased *TERT* promotor region mutations were more frequent in hepatitis-positive tumours. Especially TP53 was more frequently mutated in liver fluke-positive cases. 7/25 (28%) studies used samples from different geographical localisations. *ROBO1* and *ROBO2* mutations were more common in Asian cohorts, while mutations in the epigenetic regulator *MLL3* was more common in Caucasian cohorts. Data on risk factors, morphological subtype and geographical origin per sample could not be included in the analysis because of a lack of consistent and reliable reporting among the included studies.

3.3. Quantitative analysis

The results are presented in Table 2. *TP53* was the most frequently mutated gene among all anatomical subtypes of BTC. Although ICC and EHC show overlap in their profile, certain genes appeared to be almost exclusively mutated in ICC.

3.3.1. Intrahepatic cholangiocarcinoma

A total of 398 samples and 3299 genes were interrogated in the combined WES/WGS dataset. The top five mutated genes with highest pooled prevalence were *TP53* (26%, 95%CI 21.4–30.2), *ARID1A* (15%, 95%CI 11.7–18.9), *KRAS* (14%, 95% CI 10.8–17.9), *BAP1* (12%, 95%CI 8.8–15.4) and *PBRM1* (9%, 95%CI 6.0–11.7) (Table 2, supplementary Table 2, supplementary Fig. 1A). Among TS studies, the number of interrogated samples ranged from 28 to 652 and a total of 538 unique genes were interrogated. The top five mutated genes with highest pooled prevalence detected with TS were *TP53* (28%, 95%CI 23.9–33.2), *ARID1A* (25%, 95%CI 20.6–29.7), *MCL1* (18%, 95%CI 10.5–28.1), *IDH1* (15%, 95%CI 11.6–18.1) and *KRAS* (14%, 95%CI 11.6–18.1) (Table 2, supplementary table 3, supplementary Fig. 1B).

Many of the genes affected play a central part in cell growth, cell differentiation and apoptosis (*TP53, KRAS, EPHA2*, and *MCL1*). Genes controlling transcription at the ultrastructural level, either by methylation (*IDH1, MML3*), chromatin remodeling (*ARID1A, BAP1,* and *PBRM1*) are commonly mutated as well. The influence of these genes on transcription is more difficult to quantify. Mutations in genes involved in cellular structure and epidermal-to-mesenchymal (EMT) were also common (*MUC16, SYNE1*).

A few mutations are found almost exclusively in ICC patients. These were alterations in epigenetic regulators *IDH* (13%, 82/616 samples interrogated) and *IDH2* (3%, 21/613 samples interrogated). *FGFR* fusion events were also nearly exclusive to ICC patients (4%, 18/457 samples interrogated). *TERT* mutations, which are common in hepatocellular carcinoma, were reported in 4/398 samples in the promotor region.

There are a few differences between the WES/WGS and TS group. *EPHA2* and *CSMD3*, which are in the top 10 mutated genes in the WES/WGS group, but were never interrogated in any panel used in a TS study.

3.3.2. Extrahepatic cholangiocarcinoma

A total of 90 samples and 385 genes were interrogated in the combined WES/WGS dataset. The top five mutated genes wit highest pooled prevalence were *TP53* (37%, 95%CI 27.8–47.5), *SMAD4* (17%, 95%CI 9.6–26), *KRAS* (16%, 95%CI 8.8–24.7), *SYNE1*(13%, 95%CI 7.1–22.1 and *ARID1A* (13%, 95%CI 7–22) (Table 2, supplementary Table 2, supplementary Fig. 2A). Among TS studies, the number of interrogated samples ranged from 8 to 340 and a total of 563 unique genes were interrogated. The top five mutated genes wit highest pooled prevalence found with TS were *TP53* (19%, 95%CI 15.1–23.7), *MUC16* (18%, 95%CI 10.0–28.9), *SACS* (15%, 95%CI 7.9–25.7), *KRAS* (14%, 95%CI 9.9–18.1) and *FSIP2* (14%, 95%CI 6.8–24.1) (Table 2, supplementary table 3, supplementary Fig. 2B).

As in ICC, most of the genes affected play a central role in cell growth, cell differentiation and apoptosis (*TP53, KRAS, APC,* and *CDKN2A*). Genes controlling transcription at the ultrastructural level, either by methylation (*MML2, MML3*) or chromatin remodeling

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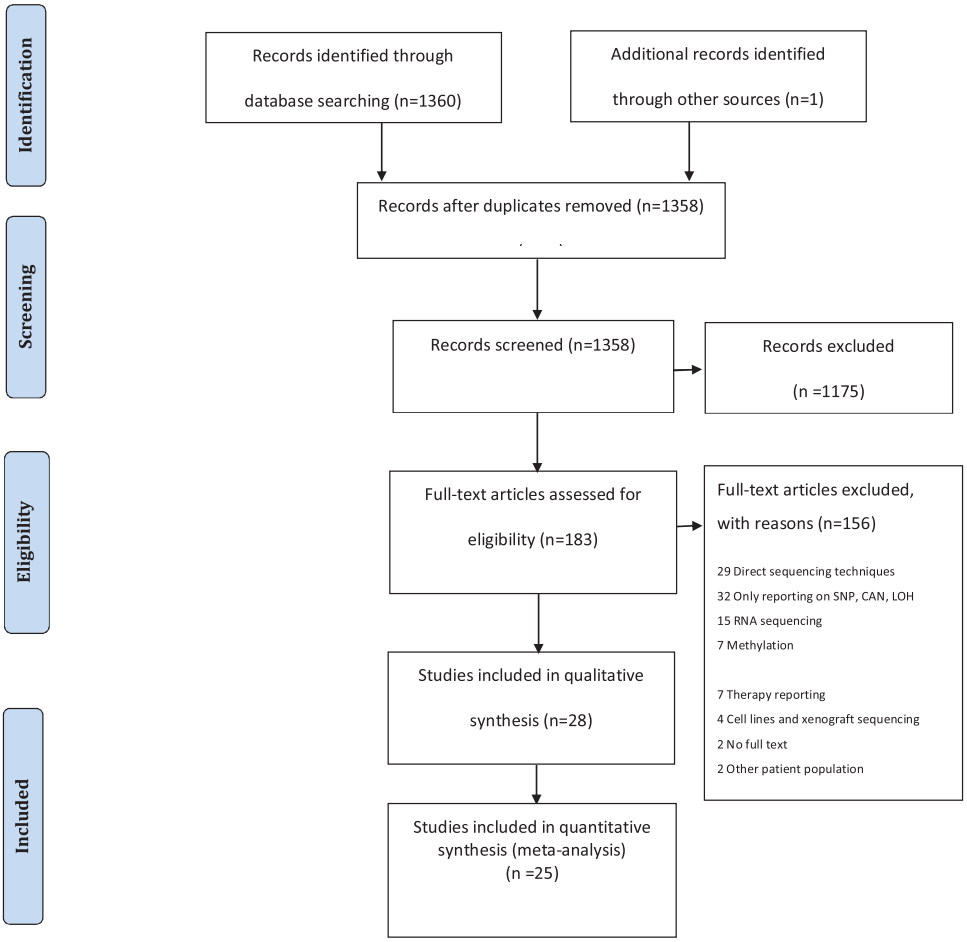

Fig. 1. Study flow-chart.

(*ARID1A, PBRM1*) were affected as well. Mutations in genes involved in cellular structure and/or EMT were common (*MUC16, SYNE1, ELF3, PCLO, SACS*). *FSIP2* and *LRP1B* mutations were also frequently reported. Although these are thought to be driver genes, their mode of action is still unknown.

3.3.3. Gallbladder carcinoma

A total of 68 samples and 10,631 genes were interrogated in the combined WES/WGS dataset. In the series of Nakamura et al., GBC cases were frequently hyper-mutated, which causes the high number of mutated genes. The mutated genes wit highest pooled prevalence found were *TP53* (29%, 95%CI 19.0–41.7), *PFKFB3* (15%, 95%CI 7.2–25.4), *PGAP1* (15%, 95%CI 7.2–25.4), *PLXNA2* (15%, 95%CI 7.2–25.4) and *SYNE1* (15%, 95%CI 7.2–25.4) (Table 2, supplementary Table 2, supplementary Fig. 3A). Among TS studies, the number of interrogated samples ranged from 15 to 258 and a total of 402 unique genes were interrogated. The mutated genes wit highest pooled prevalence found with TS were *TP53* (31%, 95%CI 24.3–39.0), *CSDM1* (14%, 95%CI 5.7–26.3), *CDKN2A/B* (13%, 95%CI 1.7–40.5), *MCL1* (13%, 95% CI 1.7–40.5) and *EYS* (12%, 95%CI 4.4–23.9) (Table 2, supplementary table 3, supplementary Fig. 3B).

TP53, ERBB2, MCL1 and *CDKN2A/B* play a central role in cell growth, cell differentiation and apoptosis. Frequently mutations occurred in genes involved in cellular structure and/or EMT (*PLXNA2, SYNE1,* and *FAT4*). *PFKFB4, PGAP1* and *PFKM* play an important role in metabolism and inhibiting formation of free radicals. Mutations in *CSDM1* were also frequently reported. Although this is thought to be a driver gene, its mode of action (like *FSIP2* and *LRP1B* mutations in EHC) is unknown as well.

3.3.4. Sub analysis of overlapping mutations

All WES, WGS and TS data were pooled to determine the most frequently mutated genes in all three anatomical subtypes and investigate the percentage of exclusively mutated genes per subtype. Top five mutations in the overall BTC group were *TP53* (26%, 95%CI 24–29), *ARID1A* (16%, 95%CI14-18), *SYNE1* (13%, 95%CI 9–17), *KRAS* (13%, 95%CI 12–15) and *MLL3* (10%, 95%CI 8–13). *MUC16* (8%, 95%CI 6–10) and anti-proliferative gene *ELF3* (8%, 95%CI 5–11). Frequency of these mutations varied per anatomical subtype, for confidence intervals per subtype see Fig. 2 and supplementary Table 4. ICC and GBC had the least overlap in mutation profiles. EHC and GBC both showed frequent mutations in *CDKN2A*, *MLL2*, *ERBB2, LAMA1* and *PIK3CA*. ICC and EHC both showed mutations in *SMAD4, SACS* and *BAP1* (supplementary table 4). *MCL1* and *PBRM1* where mutated in ICC and GBC. Mutations in *IDH1* (95/877) and *FGFR2* translocations with several different fusion partners were specific for ICC and almost exclusively were found in that subset. Mutations in *APC* (27/303), *GNAS* (8/90) and *TGFBR2* (18/175*)* occurred only in EHC. GBC had mutations in *PFKFB3* (10/68), *PLXN2* (10/68) and *PGAP1* (10/68).

Pooled data of EHC, ICC and GBC. The most frequently mutated genes are displayed on the Y-axis. Mutation proportion displayed on the X-axis. Each bar denotes the point-estimate and 95% confidence interval.

4. Discussion

BTC are heterogeneous tumors and, as a result, subtype-specific gene mutations generally affect less than 30% of the subtype population. As expected, this quantitative analysis showed that ICC, EHC and

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Table 1

Study Characteristics: +: positive. -: negative. WES: whole exome sequencing. WGS: whole genome sequencing. TS: targeted sequencing. Abbreviations for countries were used in the origin of sample column. FFPE: formalin fixed paraffin embedded. FF: fresh frozen. Unk.: unknown. Fluke: liver fluke. HBV: hepatitis B virus infection. HCV: hepatitis C virus infection. Res: resectable disease. Adv: advanced disease. AUS: Austria. BE: Belgium. CH: China. FR: France. IN: India. IT: Italy. JP: Japan. KOR: South-Korea. RO: Romania. SG: Singapore. TH: Thailand. TW: Taiwan. UK: United Kingdom. US: United States.

Study	Year	N	Pathological reassessment	WES/ WGS/ TS	Platform	Sample origin	Tissue type	Risk factors	Disease stage	BTC origin	Study overlap
Andersen	2012	69	No	TS	qPCR	US, AU, BE	FFPE	Unkn.	res.	15 PHC, 54 ICC	No
Borad	2014	6	No	WGS/ WES	Illumina	US	FF	Fluke (-)	adv.	ICC	No
Borger	2012	87	No	TS	SNaPshot genotyping assay	US	FF, FFPE	Unkn.	res., adv.	40 ICC, 22 EHC, 25 GBC	No
Chan-On	2013	219	No	WES/TS	Illumina	86 SG, 45 RO, 108 TH	FFPE	Fluke (+), fluke (-)	res., adv.	ICC, EHC	Ong et al., Jusakul et al.
Churi	2014	75	Yes	WES/TS	Illumina	US	FFPE	Unkn.	res.	55 ICC, 20 EHC	No
Chang	2014	137	No	TS	PCR	TW	FFPE	Unkn.	res.	57 ICC, 45 EHC 35 GBC	No
Deshphande	2011	77	Yes	TS	Mass spectrometric genotyping	US	FFPE	Unkn.	res., adv.	24 ICC, 3 PHC, 33 GBC, 15 distal	No
Farshidfar	2017	38	Yes	WES	Illumina	89% US	FF	38 Fluke (-), 37 HBV/ HCV (-)	unkn.	38 ICC	No
Fujimoto	2015	30	Yes	WGS/TS	Illumina, Sanger sequencing	JP	FF	Fluke (unkn.), 7 HBV (+), 9 HCV (+), 14 HBV/HCV (-)	res.	22 ICC, 7 mixed ICC/HCC, 1 cholangiocellular carcinoma	No
Javle	2014	72	Yes	TS	Sequenom MassARRAY, Illumina NGS	US	FFPE	Unkn.	res.	GBC	No
Jiao	2013	41	No	WES	Illumina	US, RO	FF	No	res.	32 ICC	No
Jusakul	2017	489	No	WES/ WGS/TS	Illumina	SP, RO, TH, IT, FR, UK, CH, TW, JP, KOR	FF	Fluke (+), fluke (-)	res.	310 ICC	Yes Ong et al., Chan-On et al., Nakamura et al.
Kumari	2014	49	Yes	TS	Sequenom Massarray and Sanger sequencing if low confidence interval of massarray	IN	FFPE	Unkn.	res., adv.	49 GBC	No
Kim	2015	10	No	WES	Agilent Technologies and Illumina	KOR	FF	4 HBV (+)	res.	10 ICC	No
Li	2014	57	Uncertain	WES/TS	Illumina	CH	FF	Unkn.	res.	57 GBC	No
Nakamura	2015	216	No	WES/TS	Illumina	JP	FF	12 HBV (+), HTLV-1 2	res.	137 ICC, 74 EHC, 28 GBC	Jusakul et al.
Ong	2012	54	Yes	WES/TS	Illumina	TH	Unkn.	Fluke (+)	adv.	54 ICC	Chan-on et al., Jusakul et al.
Putra	2015	16	Yes	TS	IonTorrent	UK	FFPE	Unkn.	adv.	8 ICC, 8 EHC	No
Ross	2014	28	Uncertain	TS	Illumina	US	FFPE	Unkn.	res., adv.	28 ICC	No
Ruzzenente	2016	91	Uncertain	TS	IonTorrent	IT	FFPE	Unkn.	res.	35 ICC, 38 EH-PHC, 18 IH-PHC	No
Sia	2013	153	Yes	TS	Sanger sequencing	IT, US, SP	FFPE	HBV/HCV (+), HBV/ HCV (-)	res., adv.	153 ICC	No
Sia	2015	116	Yes	1 WGS/ 8 WES/ 107 TS	Sanger sequencing, Illumina	IT, US, SP	FFPE	HBV/HCV (+), HBV/ HCV (-)	res., adv.	116 ICC	Sia et al. 2013
Simbolo	2014	153	Uncertain	TS	IonTorrent	IT	FFPE	HBV/HCV (+), HBV/ HCV (-)	res.	70 ICC, 57 EHC, 26 GBC	No
Voss	2012	94	Yes	TS	Sequenom MassArray	US	FFPE	Unkn.	res.	67 ICC, 27 EHC	No
Zou	2014	103	No	WES	Illumina	CH	FF	HBV/HCV (+), HBV/ HCV (-)	res.	103 ICC	No

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Table 2
Pooled results: Most frequently mutated genes per locus, stratified by WES/WGS or TS and anatomic location. Abbreviations: *: both called in top 10 TS and WES/WGS, ICC = intrahepatic cholangiocarcinoma, EHC: extrahepatic cholangiocarcinoma, GBC: gallbladder carcinoma, WES: whole exome sequencing, WGS: whole genome sequencing, TS: targeted sequencing.

Gene affected	Total number	Percentage of samples mutated (95%CI)	Function/ Pathway	Gene affected	Total Number	Percentage of samples mutated (95%CI)	Function/ Pathway
ICC				**ICC**			
WES/WGS				**TS**			
TP53*	398	25.63 (21.41-30.21)	DNA damage response	TP53*	312	27.88 (22.98-33.22)	DNA damage response
ARID1A*	398	15.08 (11.71-18.97)	DNA binding regulatory protein	ARID1A*	369	24.93 (20.60-29.67)	DNA binding regulatory protein
KRAS*	398	14.07 (10.81-17.88)	Signal transduction	MCL1	83	18.07 (10.48-28.05)	Cell fate determination, protein binding
BAP1*	398	11.81 (8.81-15.39)	Deubiquitinase	IDH1*	479	14.61 (11.57-18.10)	Epigenetic regulator
PBRM1*	398	8.54 (5.99-11.73)	Chromatin remodelling, cell cycle	KRAS*	652	13.69 (11.39-16.86)	Signal transduction
SMAD4	398	7.54 (5.14-10.59)	TGFβ signal transduction	MLL3	216	12.04 (8.02-17.14)	Epigenetic regulator
IDH1*	398	6.28 (4.11-9.13)	catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate	BAP1*	369	11.65 (8.56-15.37)	Ubiquitin hydrolase subfamily
MUC16	398	6.03 (3.90-8.84)	Membrane bound member of the mucin family	SYNE1	97	11.34 (5.80-19.39)	Protein involved in subcellular spatial organization
EPHA2	398	5.78 (3.70-8.55)	Tyrosine kinase receptor	PBRM1*	222	10.36 (6.68-15.14)	Chromatin remodelling, cell cycle
CSMD3	398	5.53 (3.50-8.25)	Plasma membrane protein with unknown function	ACAN	97	10.31(5.06-18.14)	extracellular matrix proteoglycan
EHC				**EHC**			
WES/WGS				**TS**			
TP53*	90	36.67 (26.75-47.49)	DNA damage response	TP53	340	19.12 (15.08-23.71)	DNA damage response
SMAD4	90	16.67 (9.64-26.00)	SMAD protein complex	MUC16	72	18.06 (9.98-28.89)	Membrane bound member of the mucin family
KRAS*	90	15.56 (8.77-24.72)	Signal transduction	SACS	72	15.28 (7.88-25.69)	Nucleus, protein folding and binding
ARID1A	90	13.33 (7.08-22.13)	Gene activity regulation nBAF complex	FSIP2	72	13.89 (6.87-24.06)	Sperm fibrous-sheath associated protein
SYNE1*	90	13.33 (7.08-22.13)	Protein involved in subcellular spatial organization	PCLO	72	13.89 (6.87-24.06)	Cytoskeleton
APC	90	12.22 (6.26-20.82)	Tumour suppressor	KRAS	293	13.65 (9.94-18.12)	Signal transduction
FSIP2	90	11.11 (5.46-19.49)	sperm fibrous-sheath associated protein	CDKN2A	214	13.55 (9.27-18.88)	Cyclin dependent kinase
MLL2	90	10.00 (4.68-18.14)	Epigenetic regulator	ELF3	72	12.50 (5.88-22.41)	RNA transcription factor activity
MLL3	90	10.00 (4.68-18.14)	Epigenetic regulator	SYNE1	72	12.50 (5.88-22.41)	Protein involved in subcellular spatial organization
MUC16*	90	10.00 (4.68-18.14)	Membrane bound member of the mucin family	LRP1B	92	11.96 (6.12-20.39)	Member of the LDL receptor family
GBC				**GBC**			
WES/WGS				**TS**			
TP53*	68	29.41 (18.98-41.71)	DNA damage response	TP53*	166	31.33 (24.36-38.97)	DNA damage response
PFKFB3	68	14.71 (7.28-25.39)	Cell metabolism	CSDM1	51	13.73 (5.70-26.26)	Plasma membrane protein with unknown function
PGAP1	68	14.71 (7.28-25.39)	Nuclease activity	CDKN2A/B	15	13.33 (1.66-40.46)	Cyclin dependent kinase
PLXNA2	68	14.71 (7.28-25.39)	Protein binding, plasma membrane	MCL1	15	13.33 (1.66-40.46)	Cell fate
SYNE1	68	14.71 (7.28-25.39)	Cytoskeletal protein	EYS	51	11.76 (4.44-23.87)	Protein coding for multiple epidermal growth factor (EGF)-like and LamG domains
PFKFB4	68	13.24 (6.23-23.64)	Bifunctional kinase/phosphatase	ERBB2	92	8.70 (3.83-16.42)	Tyrosine kinase growth factor recetor
PFKM	68	13.24 (6.23-23.64)	Phosphofructokinase isozyme	KRAS	233	8.15 (4.98-12.44)	Signal transduction
PFN3	68	13.24 (6.23-23.64)	Single exon genes, cytoskeleton	ARID2	51	7.84 (2.18-18.88)	Gene activity regulation nBAF complex
PGK1	68	13.24 (6.23-23.64)	Cell migration	CSDM3	51	7.84 (2.18-18.88)	Plasma membrane protein with unknown function

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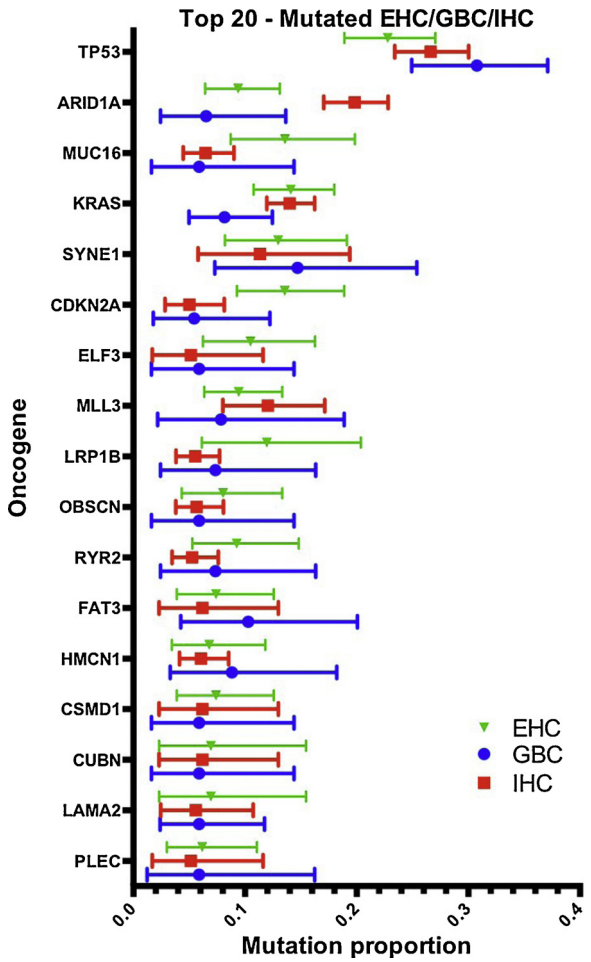

Fig. 2. Top 20 overlapping gene mutations per anatomical origin.

GBC have some overlap in mutational profiles (*KRAS, TP53, ARID1A, SYNE1, MLL3, MUC16* and *ELF3*). Notably, EHC and ICC appear most closely related, whereas ICC and GBC share the fewest gene mutations.

Although they only appear in a minority of patients, there are a few subtype specific mutations. *FGFR2* fusion genes and *IHD1/IDH2* mutations are found nearly exclusively in a ICC patients; this possibly reflects the differences in cell of origin (small bile duct vs. large bile ducts) and morphological subtype (mixed/cholangiolar vs. ductular/mucinous) within ICC.

More evidence for these findings is found in literature. Patients with small-duct type cancer often have a history of chronic liver disease, whereas large-duct type is associated with the presence of precursor lesions (biliary intraepithelial neoplasia (BilIN)). The small-duct type is associated with a longer 5-year overall survival (Akita et al., 2017). The mutational profile also differs with aberrations in *IDH1/IDH2, BAP1*, and *FGFR* being more common in small-duct cancers, and mutations in *SMAD4* and *KRAS* occurring more frequently in the large duct type (Komuta et al., 2012a; Liau et al., 2014; Akita et al., 2017; Hayashi et al., 2016). As large-duct ICC shares many clinicopathological and molecular features with EHC, it has been suggested that these cancers are biologically similar, and the current separation according to the anatomical location is suboptimal (Akita et al., 2019). Unfortunately, as most ICC samples lacked annotation on histological growth pattern, we are unable to make the distinction in this review. The overlap between EHC and a subset of ICC might be due to their common cell of origin in the larger bile ducts (Lanzoni et al., 2016; Bragazzi et al., 2018; Banales et al., 2016b). This could explain why they share mutations such as *ELF3,* also a driver in periampullary cancer, and *MUC16* (Gingras et al., 2016; Yachida et al., 2016). *MUC16* mutations were previously described in mass-forming ICC, a growth pattern seen in mucinous and ductular ICC. It stands to reason that these mass-forming ICC and EHC both derive from dedifferentiated mature cells that line the large bile duct, explaining this shared mutation (Higashi et al., 2012; Komuta et al., 2012b).

We found mutations that are associated with an intestinal subtype (i.e. *APC, GNAS* and *TGFB2*) to be exclusive to EHC. These mutations all involve the *Wnt*-pathway and they are rarely reported in cholangiocarcinoma (Cong et al., 2001; Kim et al., 2016). Possibly, part of the samples exhibiting these mutations were of ampullary origin with an intestinal subtype and incorrectly regarded as perihilar cholangiocarcinoma; correctly assessing the site of origin in periampullary cancers is notoriously difficult (Bledsoe et al., 2015). Unfortunately, it is impossible to explore this supposition. The mutational profile of GBC looks most distinct from the mutational profiles of ICC, with a higher frequency of *TP53* mutations and mutations in the *ERBB* pathway. These mutations are associated with chronic inflammation, such as caused by bile stones and infectious diseases (Wistuba et al., 2002; Wistuba and Gazdar, 2004). Genes involved in cellular structure and/or EMT (*PFKFB3, PGAP1* and *PLXN2*) as well as metabolism (*PFKFB4*, *PGAP1* and *PFKM*) were also frequently observed. The cell of origin in GBC is unknown. As of yet, it is poorly understood what the explanation of this difference is.

This review has several limitations that need to be addressed. First, various suggested methods for meta-analysis of genome wide association studies have been published (Gwinn et al., 2014; Boffetta et al., 2012; Ioannidis et al., 2008; Little et al., 2006). However, for meta-analyses reporting on the mutational landscape of cancer, there is no guideline or precedent. The current study was a first attempt at providing a reliable estimation of mutational proportions for BTC. Second, although a random-effects analysis of mutational profiles would be valuable, such analysis is currently not informative given the limited amount of data. For such an analysis, only nonzero mutational proportions can be included. For instance, if a small study reports zero mutations in TP53, this gene cannot be included in a random-effects model. Therefore, our analysis was based on pooling crude proportions. Third, a validated risk of bias tool is lacking and risk of bias could therefore not be established reliably. Finally, the mutational profiles found with WES/WGS and TS did not always overlap. This could have several causes including coverage differences between techniques (e.g. standardised panels) or the usage of work-up for FFPE or fresh frozen tissue (Stiller et al., 2016; Mittempergher et al., 2011). As a result, bias could have been introduced. For instance, estimation of mutation proportions for TS may have been over- or underestimated depending on the attention a certain disease receives. Data on clinical parameters, such as pathology assessment and risk factors were frequently missing. Furthermore, the exact anatomical location and tumour morphology was often not reported. Unfortunately, data are rarely published in open source datasets such as The Cancer Genome Atlas (TCGA), hindering a comprehensive overview and integration of genomics data (Chen et al., 2014; Creighton, 2018; Wood et al., 2018). It was therefore not possible to provide a more in-depth overview that correlates these factors to findings on a genomic level. This might be due to a very different perspective on BTC by researchers in the basic science field and clinicians in the hospital. For example, the term cholangiocarcinoma is interchangeably used for ICC, perihilar, periampullary cancer and sometimes even GBC without further specification on the location of the tumor, whereas this is important information for clinicians. Genomic coordinates or HGVS (human genome variation society) annotation were frequently not available, therefore these data are not included in the meta-analysis, although this would have provided more insight. For this reason, we provided an overview of mutated genes without the genomic coordinates, and stratified for anatomical subtype, providing a first step for more insight into the different subtypes of BTC.

In conclusion, BTC share commodities in their mutation profile but also exhibit some more exclusive genetic alterations that are associated

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with anatomical subtype. This might reflect the common embryological ancestry of the biliary tract. Possibly genetic background of BTC could be seen as a gliding scale, with genetic characteristics more related to the cell of origin then to anatomical localization alone. In case of perihilar EHC and ICC, the cell of origin (large or small bile duct origin) appears most relevant and may be of use in distinguishing different clinical and biological subgroups. With this study we hope to contribute to the ongoing debate about classification of BTC (Cardinale et al., 2013). Over the past years, publications of individual studies on BTC have greatly increased the understanding on the mutational landscape of this disease. To achieve a consensus molecular classification, collaboration and the sharing of data is essential to establish larger cohorts that are less prone to bias. Open source publication and adequate annotation would tremendously improve research accuracy in the field of BTC.

Conflicts of interest and source of funding

No funding was received for this systematic review. The authors declare no conflicts of interest. **(i) Guarantor of the article**: prof. M.J. van de Vijver. **(ii) Specific author contributions:** ER, ECS, SK, JV and MJV performed the research, ER, ECS and SK collected and analysed the data, ER, ECS, SK, JV, TVG, FD, MH, EG and MJV designed the research study and wrote the paper, ER, ECS, SK, LLM, MGB, EG, MHB, GK, HJK, RBT, HW, TW, FD, TMG, JV and MJV contributed to the design of the study and paper. **(iii) A statement indicating that all authors approved of the final version of the manuscript:** All authors approved of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.critrevonc.2019.05.011.

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