Genomic characterization of cholangiocarcinoma in primary sclerosing cholangitis reveals novel therapeutic opportunities

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**Abbreviations:**
AJCC American Joint Committee on Cancer classification
BilIN-3 Biliary intraepithelial neoplasm grade 3
BTC Biliary tract cancer
CCA Cholangiocarcinoma
CISH Chromogen-in-situ-hybridization
CNA Copy number alterations
dCCA Distal cholangiocarcinoma
eCCA Extrahepatic cholangiocarcinoma
FFPE Formalin fixed, paraffin embedded
GBC Gallbladder carcinoma
IBD Inflammatory bowel disease

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iCCA  Intrahepatic cholangiocarcinoma
IHC  Immunohistochemical
IPNB  Intraductal or intracystic papillary neoplasms of the bile duct
IPSCSG  International PSC Study Group
MSI  Molecular microsatellite instability
MSI-H  Molecular microsatellite instability-high
NOS  Not otherwise specified
pCCA  perihilar CCA or Klatskin tumors
PSC  Primary sclerosing cholangitis
PSC-BTC  Primary sclerosing cholangitis-associated biliary tract cancer

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ABSTRACT
Background & Aims
Lifetime risk of biliary tract cancer (BTC) in primary sclerosing cholangitis (PSC) exceeds 20% and BTC is currently the leading cause of death in PSC patients. To open new avenues for management, we aimed to delineate novel and clinically relevant genomic and pathological features of a large panel of PSC-associated BTC (PSC-BTC).

Approach & Results
We analysed formalin fixed, paraffin embedded tumor tissue from 186 PSC-BTC patients from 11 centers in eight countries with all anatomical locations included. We performed tumor DNA sequencing at 42 clinically relevant genetic loci to detect mutations, translocations and copy number variations, along with histomorphological and immunohistochemical characterization. Irrespective of the anatomical localization, PSC-BTC exhibited a uniform molecular and histological characteristic similar to extrahepatic cholangiocarcinoma. We detected a high frequency of genomic alterations typical of extrahepatic cholangiocarcinoma, e.g. \textit{TP53} (35.5\%), \textit{KRAS} (28.0\%), \textit{CDKN2A} (14.5\%), and \textit{SMAD4} (11.3\%), as well as potentially druggable mutations (e.g. \textit{HER2}/\textit{ERBB2}). We found a high frequency of non-typical/non-ductal histomorphological subtypes (55.2\%) and of the usually rare BTC precursor lesion, intraductal papillary neoplasia (18.3\%)

\textbf{Conclusion}

Genomic alterations in PSC-BTC include a significant number of putative actionable therapeutic targets. Notably, PSC-BTC show a distinct extrahepatic morpho-molecular phenotype, independent of the anatomical location of the tumor. These findings advance our understanding of PSC-associated cholangiocarcinogenesis and provide strong incentives for clinical trials to test genome-based personalized treatment strategies in PSC-BTC.
Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease, often associated with inflammatory bowel disease (IBD).\footnote{1} In absence of any effective medical treatment, progressive bile duct injury and cholestasis lead to end-stage liver disease in the majority of patients. In addition, patients with PSC experience a greatly increased risk of neoplasia arising from the biliary epithelium, including cholangiocarcinoma (CCA) and gallbladder carcinoma (GBC). Malignancy reduces overall patient survival significantly and currently serves as the most frequent cause of PSC-related death.\footnote{2} The reported cumulative risk of BTC development in PSC ranges from 6-22\% for CCA and 1-4\% for GBC.\footnote{1, 3, 4} Patients with PSC are young, and the high risk of an often incurable cancer poses an important unmet clinical need. The pathophysiological basis of the high risk of BTC in PSC is not clear, but chronic inflammation in the context of the biliary microenvironment is likely to play a key role.

In the US and Europe, CCA is generally considered relatively rare (less than 6 per 100,000 population) with PSC as a predominant risk factor.\footnote{5} CCA is more frequent in Southeast Asia (up to 113 per 100,000 person-years) mainly due to endemic fluke infections with Opistorchis viverrini or Clonorchis sinensis.\footnote{6} The main subtypes of CCA are represented by extrahepatic CCA (eCCA), including perihilar CCA (pCCA or Klatskin tumors) and distal extrahepatic CCA (dCCA), and intrahepatic CCA (iCCA). The spectrum of subtypes of CCA in PSC have not been precisely defined, but tumors are frequently located in the perihilar and distal extrahepatic regions. Histologically, CCA and GBC from other etiologies consist of ductal/glandular/tubular/acinar (=NOS; not otherwise specified) adenocarcinomas in ~90\% of cases, whilst the histologic patterns of PSC-BTC have not yet been evaluated in comprehensive cohorts. Although there are data that

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suggest there are effective measures for surveillance of cholangiocarcinoma in PSC, it is notoriously difficult to differentiate benign from malignant biliary strictures, leading to late diagnosis in the majority of cases.\(^{(7)}\) Surgery either by resection or liver transplantation represents the only curative intent treatment for PSC-CCA. Only one-third of the patients are candidates for radical surgery at time of CCA diagnosis and local recurrence rate after surgery is above 60%.\(^{(8)}\) Liver transplantation following neoadjuvant radiotherapy with chemosensitization may provide improved survival for highly selected patients with early stage, unresectable perihilar CCA.\(^{(9)}\) Benefit of current palliative systemic chemotherapy regimens is limited with median overall survival less than 12 months using first-line treatment with gemcitabine and cisplatin.\(^{(10)}\)

As an established branch of personalized medicine, profiling of somatic mutations in tumor DNA has identified clinically relevant genomic alterations in key pathways of prognostic and therapeutic relevance.\(^{(11)}\) In BTC derived from other etiologies than PSC, several molecular genetic alteration have been identified across multiple tumor suppressor genes and oncogenes, e.g.\(\text{KRAS, TP53, SMAD4, CDKN2A, ERBB1/2, FGFR and IDH1/2.}\)\(^{(11-14)}\) Previous efforts have revealed that genomic alterations in BTC differ according to the anatomical subtypes of BTC and causative etiology, guiding the transformation of findings from different anatomical and etiological subtypes into diagnostic- and treatment algorithms.\(^{(11, 15)}\) As such, molecular profiling of different BTC subtypes highlights different clusters of genomic alterations that converge into functional categories that may enable future precision oncology approaches.\(^{(14)}\)

The fraction of PSC patients in exome-sequencing studies of BTC has been low (less than 2% of cases with underlying PSC).\(^{(11, 14)}\) Given prospects for diagnostic and therapeutic improvements for PSC patients, we herein aimed to integrate findings from these studies with generic cancer gene panels to perform a focused assessment of clinically relevant mutations in PSC-associated BTC using targeted resequencing. We hypothesized that an enhanced understanding of the molecular carcinogenesis would delineate molecular driver lesions of relevance and potentially identify druggable targets.
MATERIAL AND METHODS

Patient samples and clinicopathological data
We used archived, formalin fixed, paraffin embedded (FFPE) specimens obtained from explanted livers, partial liver resections, cholecystectomies or biopsies performed between 1996 and 2016 for the purpose of diagnosis or treatment of PSC-BTC. In total, we collected 224 PSC-BTC samples from 11 centers in Europe and the U.S. After initial analysis, 38 samples were excluded based on the histomorphological criteria (mostly insufficient tumor material), tumor cellularity <10% or low DNA content or quality (total dropout rate 17%). The final panel submitted to further mutational profiling consisted of 186 PSC-BTC tissue specimens. Clinical follow-up data were available for 160 patients. Diagnosis of PSC was based on standard clinical, biochemical, cholangiographic and histological criteria. Detailed clinical data and histopathological information were available for 174 patients (Table 1, Table 2, and Supplementary Table S1). Two board-certified pathologists (BG and PS) validated the histopathological diagnosis of CCA and GBC. Staging of the tumors was performed according to the American Joint Committee on Cancer classification (AJCC, 8th Edition).

Ethical approval
Written informed consent was obtained from all study subjects at each center if possible. For long-time archived samples, where this was not possible, an exemption from informed consent was obtained by the local ethical committee to allow the use of the samples. Study protocols were approved by the ethics committees of all the recruiting centers, as well as the Regional committee for medical and health research ethics South Eastern Norway (6.2008.1723) and the ethical board of the University Hospital Heidelberg, Germany (206/05).

Panel sequencing
For DNA and RNA extraction and processing see Supplementary Methods. Massively parallel sequencing was performed using three panels: i) a custom PSC-BTC panel which consisted of 284 primer pairs (amplicons) covering 165 exons of 40 genes frequently mutated in hepato-pancreato-biliary cancers ii) a custom panel covering 27 hepatobiliary cancer associated gene translocations, and iii) the commercial Oncomine BRCA panel covering all exons of \textit{BRCA1} (113 amplicons) and \textit{BRCA2} (152 amplicons; Thermo Fisher Scientific) (see Supplementary Table S2). All variants were inspected manually using the IGV browser and the LOD was set at 5% to avoid false-positive results due to C>T transitions (deamination artifacts introduced by formalin fixation).

Immunohistochemistry, chromogen-in-situ-hybridization and molecular microsatellite instability analysis
Tissue microarrays (TMAs) were fabricated for all 95 cases of which sufficient tissue block material was available (Supplementary Table S3). For immunohistochemical (IHC) staining and chromogen-in-situ-hybridization (CISH), 3 \mu m sections of the TMA were used. Technical details of the IHC and CISH analyses are provided in the Supplementary Material and in Supplementary Table S3 and S5. For details on the molecular microsatellite instability (MSI) analysis see the Supplementary Material.

Statistical analysis
The extensive tissue panel with samples from tumors originating from all anatomical subsites, allowed for subgroup assessments, including analyses of frequencies of mutations and inter-relationsship of mutations between the anatomical subtypes. Graphical representations of mutational frequencies in the total BTC panel and in the anatomical BTC subtypes by oncoplot, circos plots and PCA were created by the publicly available R-packages ComplexHeatmap,
circlize and factoextra (Figure 1, 2 and Supplementary Figure S4). Due to limitations in visualizing multidimensional data, only pair-wise co-occurrences are represented in the circos plots. Frequencies shown by the oncoplots are on a sample per gene basis and do not take into account that some genes may contain more than one mutation in the same tumor sample.

The publically available data of Wardell et al., J Hepatologt 2018 was analysed for the twelve genes with highest frequencies of alterations in our panel (TP53, KRAS, CDKN2A, SMAD4, PIK3CA, CDKN2B, ERBB2, ROBO1, KDM6A, FBXW7, GNAS, TGFBR2) in the BTC subgroups iCCA, pCCA, dCCA and GBC (Supplementary Table S8). (15)

We examined associations between genomic alterations and overall survival using the endpoint of BTC-related death. Only 1 patient died due to a PSC-unrelated cause and was therefore censored. The baseline time point utilized in the survival analysis was BTC diagnosis. Statistical analysis and visualization was performed using the computing environment R (http://www.R-project.org/) and GraphPad Prism 6. Median survival and corresponding 95% confidence intervals (CI) were calculated by Kaplan–Meier survival analysis, and the survival distributions for each category were compared using the log-rank test. All reported *P*-values were two-sided, and *P* < 0.05 was considered statistically significant. Enrichment analysis was done using Fisher’s exact test. If more than two groups were compared, pairwise comparisons were done for all combinations using Fisher’s exact test and false-discovery rate correction of the *P*-values was performed according to Benjamini-Hochberg.

Graphical representations of mutational differences by oncoplot and circos plots were created by the publicly available R-package circlize (see Supplementary Methods). (16)

Putative actionable targets were identified using the TARGET (tumor alterations relevant for genomics-driven therapy) database version 3 by Broad Institute (http://archive.broadinstitute.org/cancer/cga/target). (17)
RESULTS

Clinical characteristics
Tumor samples from 186 PSC-BTC patients, including 174 (93.5%) invasive carcinomas and 12 (6.5%) high-grade non-invasive biliary neoplasms, were analysed by panel sequencing (Table 1, Figure 1). The PSC-BTC patient panel showed a male preponderance (128/174; 73.6%; Table 2). The mean age at diagnosis of PSC was 41.6 years (11.9-73.5 years), at diagnosis of BTC 48.1 years. Overall survival of PSC-BTC patients was poor with a 5-years survival rate of 21.6% (n=160; Table 2).

Histomorphology and panel sequencing revealed an extrahepatic phenotype of PSC-BTC
According to the anatomical location, the patient panel consisted of 60 iCCAs, 64 pCCAs, 18 dCCAs, 28 GBCs and 4 samples of unknown anatomical origin (Table 1). CCA was sampled from explant liver tissue obtained at liver transplantation in 37/186 patients (9 iCCAs, 24 pCCAs, 2 dCCAs, 2 with unknown anatomical origin). Among the 37 patients with findings of CCA in
explant liver 30/37 (81.1%) were incidental findings, while 7/37 (18.9%) among the CCAs were diagnosed pre-transplant.

Analysis of available clinicopathological data and histomorphological evaluation revealed a relatively high frequency of non-NOS (not otherwise specified) histologic subtypes. In detail, only 74/174 (44.8%) of the invasive PSC-BTCs showed a typical NOS (ductal/glandular/tubular/acinar) histomorphology, while 96/174 (55.2 %) showed a non-NOS histomorphology (i.e. papillary, mucinous, solid, diffuse, intestinal or adenosquamous) (Table 2). A cholangiolar/small-duct histology was observed in only one (1/60, 1.7%) iCCA. Moreover, 34 PSC-BTCs (18.3%) showed intraductal or intracystic papillary neoplasms (PSC-IPNB). Of these 34 PSC-IPNB, 30 were associated with invasive CCA, whereas 4 patients had IPNB without invasive CCA (Figure 1A). Two patients with high-grade precursor biliary intraepithelial neoplasm with grade 3 (BilIN-3) lesions were included; one BilIN-3 was associated with invasive BTC and one was not (Figure 1A and Supplementary Figure S1). Tumor grading was performed for the invasive carcinomas and showed a predominance of moderate grade (131/174; 74.4%) (Table 2).

In 146 (78.5%) out of the 186 BTC-samples analysed by massive parallel sequencing a total of 247 non-synonymous mutations and 89 copy number alterations (CNAs) in 30 of the 42 targeted genes were identified (Figure 1B and Supplementary Tables S5 and S6). Principal Component Analysis (PCA) of the mutational profiles revealed that patient samples did not cluster by clinical center or by AJCC staging (Supplementary Figure S2). Among the 247 non-synonymous mutations identified, 184 mutations were missense mutations, 56 truncations and 7 in-frame insertions or deletions (Supplementary Tables S5 and S6). Genomic alterations within TP53 (35.5%, including 34.4% missense mutations and truncations), KRAS (28.0%, including 24.7% missense mutations), CDKN2A (14.5 %, including 7% deletions), SMAD4 (11.4%), PIK3CA (9.1%), CDKN2B (8.6%), ERBB2 (8.1%), KDM5A/6A (7.0%), and ROBO1 (7.0%) were most common, with a second tier of less frequently mutated genes (2-5%) including FBXW7, TGFB2, GNAS, SMARCA4, BRAF, ARID1A (Figure 1B). Inter-relationships among the genes harboring non-synonymous mutations showed that co-occurrences of TP53 with KRAS alterations and KRAS with CDKN2A were most common (Figure 1C). Transitions of both C to T and G to A mutations (C>T|G>A) represented the predominant mutation in all subtypes. The number of mutations per tumor ranged from one to six with a mean of 1.84 ± 1.12 (mean ±standard deviation) mutations.
per tumor. The mean number of non-synonymous mutations per tumor was 2.06 ± 1.39 for GBC, 1.86 ± 1.10 for CCA and 1.36 ± 0.50 for the non-invasive high-grade dysplastic precursor lesions (HGD). Thus, CCA and GBC shared a comparable number of mutations per sample, while the mean number of HGD was significantly lower compared to invasive BTC samples (p=0.01).

CNAs were mainly found in CDKN2B (7.5%), CDKN2A (7.0%) and ERBB2 (4.3%).

In 40 tumors (21.5%), no mutation was detected by panel sequencing of the 42 targeted genes. In the entire patient panel including n=60 iCCAs, no FGFR-translocation or deleterious BRCA1/2-mutations and only one IDH1 (p.R132C) mutation (in a single CCA with typical small-duct/cholangiolar histomorphology) were detected (Figure 2A and Supplementary Table S6).

Putative actionable targets in PSC-BTC

Of the 30 genomically altered genes, 19 are considered actionable according to the TARGET database version 3 by the Broad Institute. A total of 116 (62.4%) samples had mutations within one or more potentially actionable genes, and 49 samples (26.3%) had two or more potentially actionable genes (see Supplementary Table S9 for full results).

For a subset of the PSC-BTC panel (n = 95), we were able to construct a TMA and perform additional analyses using IHC and CISH (Supplementary Figure S3). Employing the guidelines for HER2-testing in gastric cancer, 8/95 (8.4%) cases showed HER2-amplification (see Supplementary Table S3). Additionally, immunoreactivity was observed in 39/82 (47.6%) for EGFR, in 29/87 (33.3%) for c-Met, in 1/88 (1.1%) for c-Myc, and in 20/84 (23.8%) of the analysed PSC-BTCs for PD-L1. MSI analysis using mononucleotide MSI markers (BAT25, BAT26 and CAT25) and immunohistochemistry for the DNA mismatch repair proteins (MSH2, MSH6, MLH1, PMS2) revealed no MSI-High case in all analysed PSC-BTC (0/95). Detailed results of IHC-analyses are displayed in Supplementary Table S3.

Correlation of molecular alterations with clinicopathological data and follow-up

Comparison of the three anatomical subgroups of PSC-associated BTC, including CCA (iCCA, pCCA, and dCCA) and GBC, showed a homogenous mutation profile with no statistically significant differences in frequencies of detected genomic alterations (Fisher’s Exact test; Figure 2A and Supplementary Figure S4). The inter-relationship analysis by circos plots performed in all
BTC-subtypes separately showed a similar picture, with the exception of the co-occurrence of SMAD4 with PIK3CA alterations being more frequent in iCCA than in the other anatomical CCA subtypes and GBC (Figure 2B-E).

Clinical follow-up data was available for 160 of the 186 PSC-BTC patients. During follow-up, as many as 60% of the patients received liver transplantation, which was associated with significantly prolonged patient survival (Supplementary Figure S5 and Table S4). Patient overall survival showed stratification by tumor AJCC stages (p<0.001; Figure 3A). Correlation analyses between the mutational profiles did not show statistically significant associations with histological phenotype and AJCC staging (Fisher’s Exact test). In addition, comparison of the number of non-synonymous mutations in PSC-BTC subtype-specific analyses was not statistically significant (Fisher’s Exact test). No statistically significant association between number of detected molecular alterations in PSC-BTCs and patient overall survival was found (Supplementary Figure S6). Observed center-specific differences in patient overall survival were correlated to differences of tumor AJCC stages between the contributing centers. Patient subtypes and clinicopathological data are described in detail in Table 2 for the entire patient panel and in Supplementary Table S1 stratified by contributing centers.

The histomorphological phenotype was associated with overall survival in PSC-BTC patients. Solid growth pattern showed a significantly shortened overall survival compared to all other histological phenotypes, whereas, papillary histology showed a better overall survival compared to other histomorphological subtypes (Figure 3B).

The analysis of single mutations with overall patient survival revealed significant negative effects on overall survival in patients with tumors harboring mutations in KRAS (n = 46, P= 0.027; Figure 4A-D and Supplementary Figure S7). Patients with non-synonymous mutations in more than one of the analysed genes did not show statistically significant differences in overall survival (Supplementary Figure S6).
DISCUSSION

We present herein the first integrated morphological and genomic analysis of a large and clinically well-characterized PSC-BTC patient panel. Despite limitations posed by the FFPE basis, which restricted the assessment to targeted resequencing whilst allowing statistical power through sample size, our data for the first time define a common histomorphologic phenotype and the predominant molecular alterations in PSC-driven biliary carcinogenesis. Importantly, we identified a number of potential targets for individualized therapy in a patient group currently largely devoid of non-surgical management options.

Previous studies have documented that genomic alterations in BTC differ according to etiology and anatomical location.\(^{(11-15)}\) With regard to etiology our BTC-panel is exceptionally homogenous as all BTC samples in the cohort were derived from a monoetiologic background of PSC. The analysis of a large number of samples from a single BTC etiology allowed us to correlate the molecular findings with various clinicopathological data and anatomical location for common motifs of PSC-BTC. Genomic differences between the anatomical subtypes have mainly been driven by mutations in certain genes, but partly also by variability in gene sets mutated across subtypes.\(^{(11, 15)}\) A key finding of our study is that PSC-BTC exhibits a phenotype characteristic of extrahepatic, large duct BTC independent from the anatomical location of the tumor.\(^{(11-14)}\) When comparing the mutational frequencies of the most commonly mutated genes in our PSC-BTC panel with data published on polyetiologic, mostly non-PSC associated BTCs, we observed that our PSC-BTC panel showed mutational frequencies well comparable to extrahepatic CCAs (including pCCA and dCCA).\(^{(15)}\) Importantly, this observation includes a large number of tumors with an intrahepatic location (34.5% of the panel), which were still histologically and molecularly indistinguishable from PSC-CCAs of extrahepatic origin. Furthermore, these intrahepatic tumours lacked genomic alterations characteristic of iCCA (e.g. IDH1/2 mutations and FGFR2 translocations).\(^{(11, 18)}\)

Among the 60 intrahepatic tumors in our panel, signature mutations of iCCA were absent, except for a single case with an IDH1-mutation which also included the only case in the patient panel with a small duct/cholangiolocellular histomorphology otherwise present in up to 40% of
FGFR2 translocations, otherwise frequently observed in iCCA were completely absent from the entire PSC-BTC patient panel, including all intrahepatic tumors.(11, 14) Likewise, the histomorphological phenotype of our PSC-BTC cohort showed two main differences from non-PSC-BTC: i) the small-duct type iCCA, now recognized as a distinct subtype of iCCA, was virtually not present in our monoetiological PSC-BTC cohort (1/60, 1.7%), ii) all other PSC-iCCA showed the large-duct type (59/60, 98.3%), which is equivalent to an extrahepatic histomorphological phenotype, as seen in pCCA, and iii) in non-PSC-BTC patients rarely seen non-NOS (i.e. non-ductal/glandular/tubular/acinar) histologic patterns were very frequent in our PSC-BTC cohort, i.e. 96/174 (55.2 %) showed a non-NOS histomorphology (e.g. papillary, mucinous, solid, diffuse, intestinal or adenosquamous).

Taken together, PSC-BTC displays a predominantly large duct BTC genotype and phenotype presumptively resulting from common carcinogenic pathways. This is of relevance for molecular testing and for planning of clinical trials of targeted therapies.(18, 19)

The mutational profile of PSC-BTC is similar to that observed in liver-fluke related BTC. Corresponding to the genomic profile in our PSC-BTC panel fluke-positive CCAs are enriched in TP53, SMAD4, ERBB2 and GNAS alterations, while fluke negative CCAs frequently exhibit IDH1/2 and FGFR-related alterations.(12-14) One explanation may be that both PSC and liver fluke disease represent chronic inflammatory conditions which together with disruption of host bile homeostasis result in chronic damage of bile duct epithelia and oxidative stress predisposing to comparable oncogenic mutations. Another explanation may be the common cellular origin as both conditions primarily affect large intra- and extrahepatic bile ducts.(19, 20) Difference between our PSC-BTC panel and available data from both sporadic- and fluke related BTC exist regarding the higher frequency of HER2 (ERBB2) alterations in our panel identified predominantly in dCCAs (22.2%; 4/18) and GBC (14.3%; 4/28), but present in all anatomical subgroups.(11-15)

Unlike the situation in many other cancers, no targeted treatment options are yet approved for BTC. The scarcity of therapeutic options together with the lack of sensitive screening options leads to high mortality and poses an important unmet clinical need in patients with PSC. Thus, to identify genomic alterations amenable to drugs approved for other tumor types or currently tested in clinical trials is of major interest. We detected potentially actionable mutations in 116 (62.4%)
of the included patients according to the TARGET database (Supplementary Table 8). Examples include alterations in genes affecting P13K/AKT/MTOR- (e.g. PIK3CA, FBXW7), RAS/RAF/MEK/ERK- (KRAS, BRAF, NRAS) and tyrosine kinase receptor signalling (e.g. EGFR (HERB1), HER2 (HERB2), ERBB3, FGFR2). ERBB2 mutations were identified in 8.1% (15/186) of the PSC-BTCs in our panel and 8.4% (8/95) of the IHC/CISH accessible cases showed HER2-amplifications. Anti-Her-2 treatment is long term approved for breast cancer, stomach cancer and gastroesophageal junction cancer with Her-2 overexpression/amplification. Several anti-HER2 clinical trials and case reports show that HER2 is a promising target in BTC, but anti-HER2 agents are still not approved for routine administration in BTC. EGFR-alterations were identified in 2.7% (5/186) of our cases. The anti-EGFR antibody cetuximab is approved for treatment of metastatic colorectal cancer with wild type K- and N-RAS genes. Various EGFR antibodies (cetuximab, erlotinib and panitumumab) have been analysed in various combinations with gemcitabine in advanced BTC in Phase II and III clinical studies, but the clinical benefit of EGFR inhibitors in BTC is still unclear and biomarkers predicting potential response to EGFR inhibition needed.

Other targets with potential future treatment potential include CDKN2A/2B, which can be targeted by CDK4/6 inhibitors such as palbociclib that have been applied for the treatment of breast cancer and currently are tested in Phase III trials in pancreatic cancer (NCT03065062).

Although we failed to detect MSI-H tumors in our PSC-BTC panel, immunoreactivity for PD-L1 was observed in 23.8% (20/84) of our cases suggesting a therapeutic potential of immune checkpoint inhibitors. Immunoreactivity for c-Met was observed in 33.3% (29/87) cases, indicating a potential for Met targeted agents in PSC-BTC. Based on our data, further preclinical research and clinical studies are now warranted to explore the role of the molecular targets observed in PSC-BTC.

Prior genomic analysis in PSC-BTC have been limited to sequencing of KRAS and CDKN2A genes performed in considerably smaller PSC-BTC panels (n = 10-33 patients). We found KRAS mutations in 28% of PSC-BTC, a number that is in line with previous studies. Supported by our results, previous studies have also implicated CDKN2A inactivation in PSC-BTC carcinogenesis. Presence of TP53 mutations in PSC-BTC have previously only been investigated indirectly by estimating the accumulation of the TP53 encoded p53 protein in tumor

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tissue by IHC. Reported rates of p53 overexpression show large variability in previous studies (31-79%) which may be attributed to the detection mode of altered p53 expression as well as patient selection bias. The current TP53 mutation frequency of 35.5% in PSC-BTC is likely a robust estimate given the size and broad representation of the current patient panel.

Histomorphological evaluation of the PSC-BTC panel showed a high prevalence of rare histologic phenotypes. Most prominently, a papillary subtype was frequent in PSC-BTC of all anatomical subtypes. This is in line with the finding that IPNB lesions were also more frequent in comparison to previous reports on non-PSC-associated BTC. These morphological findings may reflect the different environmental- and molecular setting of PSC vs non-PSC-associated biliary carcinogenesis. Future mechanistic studies should attempt to elaborate on IPNB as a precursor lesion of PSC-BTC.

Obvious limitations of this study is the retrospective character of the tissue- and data collection as well as the limited genomic coverage intrinsic to the panel sequencing approach. To enable a statistically informative study in this rare group of BTC, where prospectively collected fresh frozen tissue is extremely scarce, we focused our recruitment on establishing an adequate size archived FFPE PSC-BTC material from explant livers, surgical resections or biopsies. The panel sequencing approach allowed for the use of FFPE material from these sources with an acceptable rate of excluded samples (17%; 38/224 samples) due to insufficient tumor material or low DNA content or quality. Selection bias, and putatively also a center bias, may have been introduced by indirectly enriching for resected and transplanted cases (i.e. an overrepresentation of lower stage cases). Despite this limitation, a distribution of patients across all four AJCC-stages was still achieved. Retrospective data also led to some degree of missing data and together with the high transplantation frequency, limits validity of survival analyses for certain subtypes, including the assessment of putatively prognostic mutations. Despite limitations, the study represents a major advance in being the largest molecular characterization of PSC-BTC to date, and the analysis of histopathological, clinical and follow-up data allowed for several significant conclusions to be drawn.

In conclusion, our study demonstrated a common morpho-molecular phenotype across all anatomical locations of PSC-BTC. We also detected several genetic abnormalities relevant for
future research into potential targeted therapies in this underserved patient group. Further characterization of PSC-BTC tissue panels and single cells using whole-exome and whole-genome sequencing in future studies is likely to expand on current observations.

**AUTHOR CONTRIBUTIONS**

Study concept and design: BG, TF, PS, THK; acquisition of biological material and clinicopathological data: BG, TF, KG; BG, ES, GMR, DNG, AM, AC, JV, JA, HM, TJE, SW, JCC, GM, GMH, CYP, AB, PM, KNL, CS, MPM, MF, AV, KMB; analysis and interpretation of data: BG, TF, SR, MK, ALV, VE, IB, AS, MMG, AF; drafting of the manuscript: BG, TF; critical revision of the manuscript for important intellectual content: BG, TF, SR, MF, AV, KNL, KMB, PS, THK; administrative, technical, or material support: IPSCSG, KMB, PS, THK; study supervision: PS, THK.

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Author names in bold designate shared co-first authorship.

**Figure legends**

**Fig. 1. Mutational landscape of PSC-associated biliary tract carcinomas.**
(A) Schematic overview of the study design. (B) Oncoplots of genes sorted by frequency of mutations in all BTC samples. Missense mutations, inframe mutations, truncations (TRUNC) and copy number alterations (CNA) with frequencies >2% across the PSC-BTC patient panel (n=186). (C) Circos plot representing co-occurrence of mutations. A band connecting genes represents co-occurring mutations in a given patient. The width of the band represents the frequency of this mutation pair within the data set.

Fig 2. Mutational landscape of different subtypes of PSC-associated BTC.

(A) Oncoplots of genes sorted by frequency of mutations in all BTC subtypes. Missense mutations, inframe mutations, truncations (TRUNC) and copy number alterations (CNA) of 146 patients with recurrent mutations are shown. Circos plots depicting inter-relationships of mutations stratified by PSC-BTC-subtypes: (B) iCCA, (C) pCCA, (D) dCCA, and (E) GBC.

Fig 3. Overall survival data of PSC-BTC patients.

(A) Overall survival data of 160 out of the 186 analysed PSC-BTC patients stratified by AJCC staging. (B) Overall survival data of all analysed PSC-BTC patients stratified by histological phenotype.

Fig 4. Overall survival data of PSC-BTC patients in correlation with specific molecular alterations.

(A) KRAS, (B) TP53, (C) CDKN2A/B, (D) SMAD4.
Table 1: Study samples and tumor subtype of the PSC-BTC panel (n=186)

<table>
<thead>
<tr>
<th>Tumor subtype</th>
<th>Invasive (n=174)</th>
<th>HGD (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iCCA</td>
<td>60 (32.3)</td>
<td>-</td>
</tr>
<tr>
<td>pCCA</td>
<td>64 (34.4)</td>
<td>6 (3.2)</td>
</tr>
<tr>
<td>dCCA</td>
<td>18 (9.7)</td>
<td>-</td>
</tr>
<tr>
<td>xCCA</td>
<td>4 (2.2)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>GBC</td>
<td>28 (15.1)</td>
<td>5 (2.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sampling procedure*</th>
<th>Biopsy</th>
<th>Resection</th>
<th>Liver explant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>36 (19.4)</td>
<td>96 (51.6)</td>
<td>42 (22.6)</td>
</tr>
</tbody>
</table>

iCCA = intrahepatic cholangiocarcinoma; pCCA = perihilar cholangiocarcinoma; dCCA = distal cholangiocarcinoma; xCCA = cholangiocarcinoma of unknown anatomical subtype; GBC = gallbladder carcinoma; HGD = high-grade dysplasia = non-invasive biliary neoplasia, i.e. IPNB (intraductal papillary neoplasm of the bile duct) or BilIN-3 (biliary intraepithelial neoplasm with grade 3). * Sampling procedure of the analysed samples according to assessment of the histological material and available clinicopathological data.
Table 2: Clinical- and histopathological data of the PSC-BTC panel (n=174)

<table>
<thead>
<tr>
<th>Number (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PSC patients</strong></td>
</tr>
<tr>
<td><strong>Mean age at BTC diagnosis</strong></td>
</tr>
<tr>
<td><strong>Overall survival</strong></td>
</tr>
<tr>
<td>2-year survival (%)</td>
</tr>
<tr>
<td>5-year survival (%)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td>male</td>
</tr>
<tr>
<td>female</td>
</tr>
<tr>
<td><strong>Operation procedure</strong></td>
</tr>
<tr>
<td>Biopsy</td>
</tr>
<tr>
<td>Resection</td>
</tr>
<tr>
<td>Liver transplantation</td>
</tr>
<tr>
<td><strong>Subtype</strong></td>
</tr>
<tr>
<td>iCCA</td>
</tr>
<tr>
<td>pCCA</td>
</tr>
<tr>
<td>dCCA</td>
</tr>
<tr>
<td>xCCA</td>
</tr>
<tr>
<td>GBC</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
</tr>
<tr>
<td>NOS</td>
</tr>
<tr>
<td>papillary</td>
</tr>
<tr>
<td>mucinous</td>
</tr>
<tr>
<td>solid</td>
</tr>
<tr>
<td>diffuse</td>
</tr>
<tr>
<td>intestinal</td>
</tr>
<tr>
<td>adenosquamous</td>
</tr>
<tr>
<td>NA</td>
</tr>
<tr>
<td><strong>AJCC</strong>****</td>
</tr>
<tr>
<td>AJCC 0</td>
</tr>
<tr>
<td>AJCC 1</td>
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<tr>
<td>AJCC 2</td>
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<tr>
<td><strong>pT</strong></td>
</tr>
<tr>
<td>T1</td>
</tr>
<tr>
<td>T2</td>
</tr>
<tr>
<td>T3</td>
</tr>
<tr>
<td>T4</td>
</tr>
<tr>
<td>NA</td>
</tr>
<tr>
<td><strong>pN</strong></td>
</tr>
<tr>
<td>N0</td>
</tr>
<tr>
<td>N1</td>
</tr>
<tr>
<td>NA</td>
</tr>
<tr>
<td><strong>M</strong></td>
</tr>
<tr>
<td>M0</td>
</tr>
<tr>
<td>M1</td>
</tr>
<tr>
<td>NA</td>
</tr>
<tr>
<td><strong>G</strong></td>
</tr>
<tr>
<td>G1</td>
</tr>
<tr>
<td>G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>NA</td>
</tr>
<tr>
<td><strong>R</strong></td>
</tr>
<tr>
<td>R0</td>
</tr>
<tr>
<td>R1</td>
</tr>
<tr>
<td>R2</td>
</tr>
<tr>
<td>NA</td>
</tr>
<tr>
<td><strong>L/V</strong></td>
</tr>
<tr>
<td>L/V0</td>
</tr>
<tr>
<td>L/V1</td>
</tr>
<tr>
<td>NA</td>
</tr>
<tr>
<td><strong>Pn</strong></td>
</tr>
<tr>
<td>Pn0</td>
</tr>
<tr>
<td>Pn1</td>
</tr>
<tr>
<td>NA</td>
</tr>
</tbody>
</table>
Detailed clinical data and histopathological information were available for 174 patients.

Overall survival was available for n = 160 patients.

Twelve patients with resection and 2 patients with biopsy at the time of diagnosis received liver transplantation afterwards.

Cases with pNx had no lymph nodes resected, therefore, AJCC (American Joint Committee on Cancer classification, 8th Edition) status could not be assessed.

NOS (not otherwise specified) = the typical ductal/glandular/tubular/acinar histologic phenotype of BTC.

Abbreviations pT, Histopathologic tumor stage evaluation; pN, Histopathologic lymph node evaluation; M, Distant metastases; G, Grade of differentiation; R, Resection margins; L/V, Invasion into lymphatic vessels/veins; Pn, Perineural invasion.
Clinicopathological data

- Invasive only (150 patients)
- Invasive & IPNB (24 patients)
- Dysplasia & Invasive BTC (7 patients)
- Dysplasia only (5 patients)
- IPNB (6 patients)
- BiIN grade 3 (1 patient)

Panel Sequencing

- High-grade Dysplasia (12 patients)
- PSC-BTC samples (186 patients)

QC

11 international PSC centers (224 patients)

Invasive BTC (174 patients)

BiIN grade 3 (1 patient)

Alterations

- TP53
- KRAS
- CDKN2A
- SMAD4
- PIK3CA
- CDKN2B
- ERBB2/3
- KDM5A/6A
- ROBO1
- FBXW7
- GNAS
- SMARCA4
- BRAF
- SMARCA4
- ARID1A
- EGFR
- FGFR2
- FGF23
- FGF10

Clinicopathological data

- Invasive only (150 patients)
- Invasive & IPNB (24 patients)
- Dysplasia & Invasive BTC (7 patients)
- Dysplasia only (5 patients)
- IPNB (6 patients)
- BiIN grade 3 (1 patient)
The image contains a series of bar charts and circular gene expression plots. The charts compare alterations in various cancer types (iCCA, pCCA, dCCA, GBC) for different genetic markers.

### iCCA
- TP53: 36.7%
- KRAS: 23.3%
- CDKN2A: 15.0%
- SMAD4: 11.7%
- PIK3CA: 3.3%
- CDKN2B: 5.0%
- ERBB2/3: 3.3%
- KDM5A/6A: 3.3%
- ROBO1: 1.7%
- FBXW7: 0.0%
- TGFBR2: 0.0%
- GNAS: 0.0%
- SMARCA4: 0.0%
- BRAF: 0.0%
- ARID1A: 0.0%
- EGFR: 0.0%
- FGFR2: 0.0%
- FGF23: 0.0%

### pCCA
- TP53: 39.1%
- KRAS: 40.6%
- CDKN2A: 12.5%
- SMAD4: 10.9%
- PIK3CA: 22.2%
- CDKN2B: 7.8%
- ERBB2/3: 6.2%
- KDM5A/6A: 5.6%
- ROBO1: 6.2%
- FBXW7: 5.6%
- TGFBR2: 5.6%
- GNAS: 5.6%
- SMARCA4: 5.6%
- BRAF: 5.6%
- ARID1A: 5.6%
- EGFR: 5.6%
- FGFR2: 5.6%
- FGF23: 5.6%

### dCCA
- TP53: 27.8%
- KRAS: 16.7%
- CDKN2A: 16.7%
- SMAD4: 16.7%
- PIK3CA: 14.3%
- CDKN2B: 11.1%
- ERBB2/3: 11.1%
- KDM5A/6A: 7.1%
- ROBO1: 7.1%
- FBXW7: 7.1%
- TGFBR2: 7.1%
- GNAS: 7.1%
- SMARCA4: 7.1%
- BRAF: 7.1%
- ARID1A: 7.1%
- EGFR: 7.1%
- FGFR2: 7.1%
- FGF23: 7.1%

### GBC
- TP53: 28.6%
- KRAS: 24.4%
- CDKN2A: 16.7%
- SMAD4: 16.7%
- PIK3CA: 14.3%
- CDKN2B: 11.1%
- ERBB2/3: 11.1%
- KDM5A/6A: 7.1%
- ROBO1: 7.1%
- FBXW7: 7.1%
- TGFBR2: 7.1%
- GNAS: 7.1%
- SMARCA4: 7.1%
- BRAF: 7.1%
- ARID1A: 7.1%
- EGFR: 7.1%
- FGFR2: 7.1%
- FGF23: 7.1%

The bar charts show the percentage of alterations for each marker in each cancer type. The circular plots illustrate the relationships between different markers.
A log-rank p<0.001
p-trend <0.001
NA was excluded

AJCC 1-2 (N=31)
AJCC 3 (N=44)
AJCC 4 (N=36)
NA (N=49)

B log-rank papillary-solid p=0.004
log-rank papillary-mucinous p=0.029
log-rank ductal-solid p=0.006

papillary (N=22)
ductal (N=67)
mucinous (N=37)
solid (N=18)
other (N=12)

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A. KRAS mut vs KRAS wt

B. TP53 mut vs TP53 wt

C. CDKN2A/B mut vs CDKN2A/B wt

D. SMAD4 mut vs SMAD4 wt

log-rank p = 0.027
log-rank p = 0.351
log-rank p = 0.696
log-rank p = 0.659
log-rank p = 0.696