Genomic Characterization of Cholangiocarcinoma in Primary Sclerosing Cholangitis Reveals Therapeutic Opportunities


BACKGROUND AND AIMS: Lifetime risk of biliary tract cancer (BTC) in primary sclerosing cholangitis (PSC) may exceed 20%, and BTC is currently the leading cause of death in patients with PSC. To open new avenues for management, we aimed to delineate clinically relevant genomic and pathological features of a large panel of PSC-associated BTC (PSC-BTC).

APPROACH AND RESULTS: We analyzed formalin-fixed, paraffin-embedded tumor tissue from 186 patients with PSC-BTC 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. Regardless 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, such as TP53 (35.5%), KRAS (28.0%), CDKN2A (14.5%), and SMAD4 (11.3%), as well as potentially druggable mutations (e.g., HER2/ERBB2). We found a high frequency of nontypical/nonductal histomorphological subtypes (55.2%) and of the usually rare BTC precursor lesion, intraductal papillary neoplasm (18.3%).

CONCLUSIONS: Genomic alterations in PSC-BTC include a significant number of putative actionable therapeutic targets. Notably, PSC-BTC shows 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. (Hepatology 2020;72:1253-1266).

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease, often associated with inflammatory bowel disease. In the absence of...
any effective medical treatment, progressive bile duct injury and cholestasis lead to end-stage liver disease in most 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. The reported cumulative risk of BTC development in PSC ranges from 6%-22% for CCA and 1%-4% for GBC. 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.

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In the United States and Europe, CCA is generally considered relatively rare (less than 6 per 100,000 population), with PSC as a predominant risk factor. CCA is more frequent in Southeast Asia (up to 113 per 100,000 person-years), primarily due to endemic fluke infections with Opisthorchis viverrini or Clonorchis sinensis. The main subtypes of CCA are represented by intrahepatic CCA, including perihilar CCA (pCCA or Klatskin tumors) and distal intrahepatic CCA (dCCA), and extrahepatic CCA (eCCA). 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 (i.e., not otherwise specified [NOS]) adenocarcinomas in about 90% of cases, whereas the histologic patterns of PSC-BTC have not yet been evaluated in comprehensive cohorts. Although there are data that 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 most cases. 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 the time of CCA diagnosis, and the local recurrence rate after surgery is above 60%. Liver transplantation following neoadjuvant radiotherapy with chemosensitization may provide improved survival for highly selected patients with early-stage, unresectable perihilar CCA.

The 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. The fraction of PSC patients in exome-sequencing studies of BTC has been low (less than 2% known cases with underlying PSC). Given the prospects for diagnostic and therapeutic improvements for patients with PSC, 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.

Materials 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 United States. After initial analysis, 38 samples were excluded based on the histomorphological criteria (mostly insufficient tumor material), tumor cellularity less than 10%, or low DNA content or quality (total dropout rate of 17%). The final panel submitted to further mutational profiling, consisting 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 (Tables 1 and 2 and Supporting Table S1). Two board-certified pathologists (B.G. and P.S.) validated the histopathological diagnosis of CCA and GBC. Staging of the tumors was performed according to the American Joint Committee on Cancer (AJCC) classification, 8th edition.
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 recruiting centers as well as the Regional Committees for Medical and Health Research Ethics of South East Norway (6.2008.1723) and the ethical board of the University Hospital Heidelberg, Germany (206/05).

**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 recruiting centers as well as the Regional Committees for Medical and Health Research Ethics of South East 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 Supporting Information. Massive parallel sequencing was performed using three panels: (1) a custom PSC-BTC panel that consisted of 284 primer pairs (amplicons) covering 165 exons of 40 genes frequently mutated in hepatopancreato-biliary cancers (2), a custom panel covering 27 hepatobiliary cancer–associated gene translocations, and (3) the commercial Oncomine BRCA (breast cancer antigen) panel covering all exons of BRCA1 (113 amplicons) and BRCA2 (152 amplicons) (Thermo Fisher Scientific, Waltham, MA) (see Supporting Table S2). All variants were inspected manually using the IGV browser, and the limit of detection 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 (Supporting Table S3). For immunohistochemical (IHC) staining and chromogen in situ hybridization (CISH), 3-µm sections of the TMA were used.

Technical details of the IHC and CISH analyses are provided in the Supporting Information and in Supporting Tables S3 and S5. For details on the molecular microsatellite instability (MSI) analysis, see the Supporting Information.

**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 interrelationship of mutations among 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 (Figs. 1 and 2 and Supporting Fig. 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 publicly available data of Wardell et al., J Hepatology 2018 were analyzed for the 12 genes with highest frequencies of alterations in our panel (TP53, KRAS, CDKN2A, SMAD4, PIK3CA [phosphoinositide-3-kinase, catalytic, alpha polypeptide], CDKN2B, ERBB2, ROBO1 [roundabout homolog 1], KDM6A [lysine demethylase 6A], FBXW7 [F-box and WD repeat domain containing 7], GNAS [guanine nucleotide binding protein (G protein), alpha
stimulating activity polypeptide 1], and TGFBR2) in the BTC subgroups iCCA, pCCA, dCCA, and GBC (Supporting Table S8).\(^{15}\)

We examined associations between genomic alterations and overall survival using the endpoint of BTC-related death. Only one patient died due to a PSC-unrelated cause and was therefore censored. The baseline time point used 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 the corresponding 95% confidence intervals 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

### Table 2. Clinical and Histopathological Data of the PSC-BTC Panel (n = 174)

<table>
<thead>
<tr>
<th>Category</th>
<th>Number (Percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with PSC*</td>
<td>174 (100.0)</td>
</tr>
<tr>
<td>Mean age at BTC diagnosis</td>
<td>48.1 years</td>
</tr>
<tr>
<td>Overall survival†</td>
<td></td>
</tr>
<tr>
<td>2-year survival (%)</td>
<td>47.0</td>
</tr>
<tr>
<td>5-year survival (%)</td>
<td>21.6</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>128 (73.6)</td>
</tr>
<tr>
<td>Female</td>
<td>46 (26.4)</td>
</tr>
<tr>
<td>Operation procedure‡</td>
<td></td>
</tr>
<tr>
<td>Biopsy</td>
<td>34 (19.5)</td>
</tr>
<tr>
<td>Resection</td>
<td>84 (48.3)</td>
</tr>
<tr>
<td>Liver transplantation</td>
<td>56 (32.2)</td>
</tr>
<tr>
<td>Subtype</td>
<td></td>
</tr>
<tr>
<td>iCCA</td>
<td>60 (34.5)</td>
</tr>
<tr>
<td>pCCA</td>
<td>64 (36.8)</td>
</tr>
<tr>
<td>dCCA</td>
<td>18 (10.3)</td>
</tr>
<tr>
<td>xCCA</td>
<td>4 (2.3)</td>
</tr>
<tr>
<td>GBC</td>
<td>28 (16.1)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>NOS(^{11})</td>
<td>74 (42.5)</td>
</tr>
<tr>
<td>Papillary</td>
<td>23 (13.2)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>41 (23.6)</td>
</tr>
<tr>
<td>Solid</td>
<td>19 (10.9)</td>
</tr>
<tr>
<td>Diffuse</td>
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<tr>
<td>Intestinal</td>
<td>3 (1.7)</td>
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<tr>
<td>Adenosquamous</td>
<td>3 (1.7)</td>
</tr>
<tr>
<td>NA</td>
<td>4 (2.3)</td>
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<tr>
<td>AJCC(^{4})</td>
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<td>9 (5.1)</td>
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<tr>
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<td>22 (12.5)</td>
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<tr>
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<td>45 (25.6)</td>
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<td>AJCC 4</td>
<td>38 (21.6)</td>
</tr>
<tr>
<td>NA</td>
<td>57 (32.4)</td>
</tr>
<tr>
<td>pT</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>18 (10.2)</td>
</tr>
<tr>
<td>T2</td>
<td>54 (30.7)</td>
</tr>
<tr>
<td>T3</td>
<td>32 (18.2)</td>
</tr>
<tr>
<td>T4</td>
<td>17 (9.7)</td>
</tr>
<tr>
<td>NA</td>
<td>53 (30.1)</td>
</tr>
<tr>
<td>pN</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>46 (26.1)</td>
</tr>
<tr>
<td>N1</td>
<td>67 (38.1)</td>
</tr>
<tr>
<td>NA</td>
<td>61 (34.7)</td>
</tr>
<tr>
<td>M</td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>93 (52.8)</td>
</tr>
<tr>
<td>M1</td>
<td>38 (21.6)</td>
</tr>
<tr>
<td>NA</td>
<td>43 (24.4)</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 afterward.  
\(^§\)Cases with NA for \(pN\) had no lymph nodes resected; therefore, AJCC status could not be assessed.  
\(^||\)NOS = typical ductal/glandular/tubular/acinar histologic phenotype of BTC.  
Abbreviations: G, grade of differentiation; L/V, invasion into lymphatic vessels/veins; M, distant metastases; NA, not available; pN, histopathologic lymph node evaluation; Pn, perineural invasion; pT, histopathologic tumor stage evaluation; R, resection margins.
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

FIG. 1. Mutational landscape of PSC-BTCs. (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, and CNAs with frequencies greater than 2% across the panel of PSC-BTC patients ($n = 186$). (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. Abbreviations: CNA, copy number alteration; TRUNC, truncation; QC, quality control.
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, TRUNCs, and CNAs of 146 patients with recurrent mutations are shown. Circos plots depicting interrelationships of mutations stratified by PSC-BTC-subtypes: iCCA (B), pCCA (C), dCCA (D), and GBC (E).
publicly available R-package circlize (see Supporting Information).\(^{16}\)

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 patients with PSC-BTC, including 174 (93.5%) invasive carcinomas and 12 (6.5%) high-grade noninvasive biliary neoplasms, were analyzed by panel sequencing (Table 1 and Fig. 1). The panel of patients with PSC-BTC showed a male preponderance (128 of 174; 73.6%; Table 2). The mean age at diagnosis of PSC was 41.6 years (range: 11.9-73.5 years), and at diagnosis of BTC was 48.1 years. Overall survival of patients with PSC- 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 of 186 patients (9 iCCAs, 24 pCCAs, 2 dCCAs, and 2 with unknown anatomical origin). Among the 37 patients with findings of CCA in explant liver, 30 of 37 (81.1%) were incidental findings, whereas 7 of 37 (18.9%) among the CCAs were diagnosed before the transplant.

Analysis of available clinicopathological data and histomorphological evaluation revealed a relatively high frequency of non-NOS histologic subtypes. In detail, only 74 of 174 (44.8%) of the invasive PSC-BTCs showed a typical NOS (ductal/glandular/tubular/acinar) histomorphology, whereas 96 of 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 of 60, 1.7%) iCCA. Moreover, 34 patients with PSC-BTCs (18.3%) showed intraductal or intracytic papillary neoplasms of the bile duct (PSC-IPNB). Of these 34 patients with PSC-IPNB, 30 were associated with invasive CCA, whereas 4 patients had IPNB without invasive CCA (Fig. 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 (Fig. 1A and Supporting Fig. S1). Tumor grading was performed for the invasive carcinomas and showed a predominance of moderate grade (131 of 174; 74.4%) (Table 2).

In 146 (78.5%) of the 186 BTC samples analyzed by massive parallel sequencing, a total of 247 nonsynonymous mutations and 89 copy number alterations (CNAs) in 30 of 42 targeted genes were identified (Fig. 1B and Supporting Table S6). Principal component analysis (PCA) of the mutational profiles revealed that patient samples did not cluster by clinical center or by AJCC staging (Supporting Fig. S2B). Among the 247 nonsynonymous mutations identified, 184 mutations were missense mutations, 56 were truncations, and 7 were in-frame insertions or deletions (Supporting Tables S6 and S7). Principal component analysis (PCA) of the mutational profiles revealed that patient samples did not cluster by clinical center or by AJCC staging (Supporting Fig. S2B). Among the 247 nonsynonymous mutations identified, 184 mutations were missense mutations, 56 were truncations, and 7 were in-frame insertions or deletions (Supporting Tables S6 and S7). 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, TGFBR2, GNAS, SMA1/3/4/7, SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4, BRAF, and ARID1A (AT-Rich Interaction Domain 1A) (Fig. 1B). Interrelationships among the genes harboring nonsynonymous mutations showed that co-occurrences of TP53 with KRAS alterations and KRAS with CDKN2A were most common (Fig. 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 ± SD) mutations per tumor. The mean number of nonsynonymous mutations per tumor was 2.06 ± 1.39 for GBC, 1.86 ± 1.10 for CCA, and 1.36 ± 0.50 for the noninvasive high-grade dysplastic precursor lesions (HGDs). Thus, CCA and GBC shared a comparable number of mutations per sample, whereas
the mean number of mutations in HGDs was significantly lower compared with the invasive BTC samples \((P = 0.01)\). CNAs were found primarily 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 \(BRCA1/2\) mutation and only one \(IDH1\) (p.R132C) mutation (in a single CCA with typical small-duct/cholangiolar histomorphology) were detected (Fig. 2A and Supporting Table S6).\(^{(18)}\)

**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.\(^{(17)}\) 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 Supporting 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 (Supporting Fig. S3). Using the guidelines for HER2 testing in gastric cancer, 8 of 95 (8.4%) cases showed HER2 amplification (see Supporting Table S3). Additionally, immunoreactivity was observed in 39 of 82 (47.6%) patients for epidermal growth factor receptor (EGFR), in 29 of 87 (33.3%) for c-Met, in 1 of 88 (1.1%) for c-Myc, and in 20 of 84 (23.8%) of the analyzed PSC-BTCs for programmed death ligand 1 (PD-L1). MSI analysis using mononucleotide MSI markers (BAT25, BAT26, and CAT25) and IHC for the DNA mismatch repair proteins (MSH2, MSH6, MLH1, and PMS2) revealed no MSI-high case in all analyzed PSC-BTCs (0 of 95). Detailed results of IHC analyses are found in Supporting 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; Fig. 2A and Supporting Figure S4). The interrelationship analysis by circos plots performed in all BTC subtypes separately showed a similar picture (Fig. 2B-E).

Clinical follow-up data were 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 (Supporting Fig. S5 and Table S4). Patient overall survival showed stratification by tumor AJCC stages \((P < 0.001;\) Fig. 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 nonsynonymous mutations in PSC-BTC subtype-specific analyses was not statistically significant (Fisher’s exact test). No statistically significant association between the number of detected molecular alterations in PSC-BTCs and patient overall survival was found (Supporting Fig. S6). Observed center-specific differences in patient overall survival were correlated with 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 Supporting Table S1 stratified by contributing centers.

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

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

**Discussion**

We present herein an integrated morphological and genomic analysis of a large and clinicopathologically
well-characterized PSC-BTC patient panel. Despite limitations posed by the FFPE basis, which restricted the assessment to targeted resequencing while allowing statistical power through sample size, our data 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 that is currently largely devoid of nonsurgical management options.

Previous studies have documented that genomic alterations in BTC differ according to etiology and anatomical location.\(^\text{[11-15]}\) With regard to etiology, our BTC panel is exceptionally homogenous, as all BTC samples in the cohort were derived from a monoetiological 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 among the anatomical subtypes have been driven primarily by mutations in certain genes, but also partly by variability in gene sets mutated across subtypes.\(^\text{[11,15]}\) A key finding of our study is that PSC-BTC exhibits a phenotype that is characteristic of extrahepatic, large-duct BTC, independent of the anatomical location of the tumor.\(^\text{[11-14]}\) When comparing the mutational frequencies of the most commonly mutated genes in our PSC-BTC panel with the data published on polyetiological, mostly non-PSC-associated BTCs, we observed that our PSC-BTC panel showed mutational frequencies that are well comparable to extrahepatic CCAs (including pCCA and dCCA).\(^\text{[15]}\) 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 tumors lacked the genomic alterations characteristic of iCCA (e.g., \(IDH1/2\) mutations and \(FGFR2\) translocations).\(^\text{[11,18]}\)

Among the 60 intrahepatic tumors in our panel, signature mutations of iCCA were absent, except for a single case with an \(IDH1\)-mutation that also included the only case in the patient panel with a small duct/cholangiolocellular histomorphology otherwise present in up to 40% of iCCAs.\(^\text{[18,20]}\) This mutation may have occurred sporadically in this patient. Noteworthy,

![Graph A](image1)

**FIG. 3.** Overall survival data of patients with PSC-BTC. (A) Overall survival data of 160 of the 186 analyzed patients with PSC-BTC stratified by AJCC staging. (B) Overall survival data of all analyzed patients with PSC-BTC stratified by histological phenotype. Abbreviations: AJCC, American Joint Committee on Cancer Classification; NA, not available.
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: (1) The small-duct type iCCA, now recognized as a distinct subtype of iCCA, was virtually not present in our monoetiological PSC-BTC cohort (1 of 60, 1.7%), and (2) all other PSC-iCCA showed the large-duct type (59 of 60, 98.3%), which is equivalent to an extrahepatic histomorphological phenotype, as seen in pCCA. In the non-PSC-BTC cohort, rarely seen non-NOS (i.e., non-ductal/glandular/tubular/acinar) histologic patterns were very frequent in our PSC-BTC cohort (i.e., 96 of 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 that presumptively result from common carcinogenic pathways. This is of relevance for molecular testing and for planning of clinical trials of targeted therapies.\(^{(18,20)}\)

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 intrahepatic and extrahepatic bile ducts.\(^{(20,21)}\) Differences between our PSC-BTC panel and available data from both sporadic-related and fluke-related BTC exist regarding the higher frequency of HER2 (ERBB2) alterations, identified in our panel predominantly in dCCAs (22.2%; 4 of 18) and GBC (14.3%; 4 of 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 (Supporting Table S8).\(^{(17)}\) We found KRAS mutations were identified in 2.7% (5 of 186) of our cases, suggesting a therapeutic potential of immune checkpoint inhibitors.\(^{(29)}\) Immunoreactivity for c-Met was observed in 33.3% (29 of 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 analyses in PSC-BTC have been limited to sequencing of KRAS and CDKN2A genes performed in considerably smaller PSC-BTC panels (n = 10-33 patients).\(^{(30-33)}\) We found KRAS mutations in 28% of PSC-BTC, a number that is in line with previous studies.\(^{(30,31)}\) Supported by our results, previous studies have also implicated CDKN2A inactivation in PSC-BTC carcinogenesis.\(^{(33)}\) 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 tissue by IHC.\(^{(30-32)}\) 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.\(^{(30-32)}\) 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.\(^{(34)}\) 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.\(^{(35)}\) These morphological findings may reflect the different environmental and molecular setting of PSC versus 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 are 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, in which prospectively collected fresh frozen tissue is extremely scarce, we focused our recruitment on establishing an adequately sized 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 of 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 the 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.

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Supporting Information

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