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Sequencing of intraductal biopsies is feasible and potentially impacts clinical management of patients with indeterminate biliary stricture and cholangiocarcinoma

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Abstract

Background: Definite diagnosis and therapeutic management of cholangiocarcinoma (CCA) remains a challenge. The aim of the current study was to investigate feasibility and potential impact on clinical management of targeted sequencing of intraductal biopsies.

Methods: Intraductal biopsies with suspicious findings from 16 patients with CCA in later clinical course were analyzed with targeted sequencing including tumor and control benign tissue ($n = 55$ samples). A CCA-specific sequencing panel containing 41 genes was designed and a dual strand targeted enrichment was applied.

Results: Sequencing was successfully performed for all samples. In total, 79 mutations were identified and a mean of 1.7 mutations per tumor sample (range 0–4) as well as 2.3 per biopsy (0–6) were detected and potentially therapeutically relevant genes were identified in 6/16 cases. In 14/18 (78%) biopsies with dysplasia or inconclusive findings at least one mutation was detected. The majority of mutations were found in both surgical specimen and biopsy (68%), while 28% were only present in biopsies in contrast to 4% being only present in the surgical tumor specimen.

Conclusion: Targeted sequencing from intraductal biopsies is feasible and potentially improves the diagnostic yield. A profound genetic heterogeneity in biliary dysplasia needs to be considered in clinical management and warrants further investigation.

Translational impact: The current study is the first to demonstrate the feasibility of sequencing of intraductal biopsies which holds the potential to impact diagnostic and therapeutical management of patients with biliary dysplasia and neoplasia.

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Introduction

Cholangiocarcinoma (CCA) is a gastrointestinal neoplasia derived from the biliary epithelium or peribiliary glands[1]. The majority of CCA emerge from the

extrahepatic bile ducts (eCCA) in the perihilar (50%) and distal (40%) region of the biliary tree whereas intrahepatic CCA (iCCA) occurs in <10%[2]. The current standard treatment in advanced stages consists of a systemic platinum-based chemotherapy and gemcitabine[3]. Many approaches of personalized therapy regimens have been evaluated, but so far, none could demonstrate a benefit in survival[4]. One reason might be a profound intertumoral heterogeneity requiring precise characterization of mutated targetable genes of every tumor[4].

Besides therapeutic management, definite diagnosis of CCA remains challenging, especially in patients with only small lesions in the extrahepatic biliary tract. These patients frequently present with indeterminate biliary stricture and obtaining a representative histopathological specimen is necessary to confirm diagnosis and avoid potentially unnecessary surgery. However, percutaneous puncture is often hindered due to the longitudinal growth of perihilar CCA and distal CCA, which also impedes reliable detection of the lesions in cross sectional imaging modalities. Therefore, the standard approach to obtain material for cytology or histopathology in non-operative cases or prior to surgery is by endoscopic retrograde cholangiography (ERC). Note that, due to the small size of specimens as well as the disconnected tissue structure and desmoplastic growth pattern of CCA, sensitivity of intraductal tissue brushings and intraductal biopsies remains below 50%[5]. Many approaches such as fluorescence in-situ hybridization or immunohistochemical techniques have been evaluated, but these are not widely established in diagnostic routine and sensitivity remains limited[6–9]. Small size and poor tissue quality might be reasons, that to date, data on technical feasibility of next-generation sequencing of intraductal biopsies are lacking.

In the current study, suspicious biopsies of patients with indeterminate biliary stricture, who were diagnosed with CCA in further clinical course, were investigated with targeted sequencing. Therefore, we designed a customized panel of genes frequently mutated in CCA and used an enrichment technique specifically adapted to low input and low quality of DNA extracted from formalin-fixed, paraffin embedded (FFPE) tissue. The aim of the study was to investigate the feasibility of next-generation sequencing (NGS) from intraductal biopsies as a potential theranostic tool for both therapeutic management and diagnosis of CCA. Moreover, we aimed to further characterize the genetic landscape of biliary dysplasia.

Methods

Patients

Intraductal biopsies and matching surgical specimen collected from patients with a diagnosis of dysplasia or CCA between 2005 and 2015 at Frankfurt University Hospital were included in this study ($n = 16$). Samples with low or high-grade dysplasia as well as samples in which dysplasia could not be excluded were regarded as suspicious findings. If multiple biopsies were available, all were included. In total, 21 biopsies with either inconclusive findings ($n = 3$), biliary dysplasia ($n = 15$), or adenocarcinoma ($n = 3$) were included as well as corresponding surgical tumor specimens ($n = 16$), and benign control tissue ($n = 16$). If multiple biopsies were included, they were either obtained within the same intervention (P42, P43) or in two interventions within 3 months (P86b1-3 and P86b4). In two patients, tumor adjacent adenoma was microdissected from the FFPE sample of the surgical specimen and investigated as well (43d, 141d). Classification of iCCA, pCCA, and dCCA was based on pathological and clinical reports (if pathological reports were inconclusive) of the surgical specimens. All extrahepatic CCA involving the perihilar region were classified as pCCA, whereas all other extrahepatic CCA were classified as dCCA. The majority of CCA specimen was extrahepatic (distal: $n = 9$, perihilar: $n = 3$), whereas four cases were intrahepatic. Three cases with suspicion for carcinoma of the ampulla of Vater were included, since biliary etiology could not be excluded in these cases (P1, P139, P141). CCA were staged according to the 7th edition of the classification of the Union for International Cancer Control (UICC). Biomaterial and clinical data were obtained from the biobank and the tumor documentation of the UCT Frankfurt (University Cancer Center, Frankfurt, Germany). Informed consent was obtained from all patients. The study protocol was approved by the local ethics committee of the University of Frankfurt (Approval No. SGI-02-2015).

Intraductal biopsies were obtained during ERC with standard duodenoscopes under fluoroscopic guidance (Olympus V-Scopes, TJF 160VF, TJF-Q180 V; Olympus Europe, Hamburg, Germany). A large capacity forceps (Radial Jaw 4, M00513320, 2.8 mm diameter working channel, Boston Scientific, Marlborough, MA) as well as forceps with lower diameter (EndoJaw FB-231K, 1.9 mm, Olympus; EndoBite BIO1-C4-18-260, 1.8 mm, Medwork, Hochstadt/Aisch, Germany) were used.

Sample preparation

Hematoxylin and eosin (H&E) stained slides were reviewed by an expert gastrointestinal pathologist and representative FFPE blocks of biopsy, tumor and non-tumor tissue were selected. Non-infiltrated tissue of liver or small intestine served as non-tumor tissue. H&E stained slides were examined by an expert gastrointestinal pathologist to determine the content of neoplastic or dysplastic cells. Biopsy samples were reviewed and classified according to grade of biliary intraepithelial neoplasia (BilIN[10,11]). Before DNA purification, size of the biopsies was measured on the FFPE block and all biopsies

with a diameter ≤ 1 mm ($n = 6$) were assessed as 0.5 mm. To enhance tumor content of surgical tumor specimens, laser capture microdissection (PALM MicroBeam IV Laser Capture System, ZEISS, Oberkochen, Germany) or macrodissection was applied as appropriate. Due to the small amount of tissue, intraductal biopsies were not dissected. DNA of surgical specimens was extracted with GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) or Maxwell 16 System DNA Purification Kit (Promega, Fitchburg, WI) according to manufacturer's recommendations. DNA from intraductal biopsies was extracted with the truXTRAC FFPE DNA Kit according to manufacturer's recommendations (Covaris, Woburn, MA). Purified DNA was quantified with Quantus Fluorometer (Promega, Madison, WI). Representative H&E stained sections of BilIN 1–3 are shown in Supplementary Figure S1.

Custom panel design

A CCA-specific sequencing panel was created based on recurrently mutated genes in large scale or targeted sequencing projects on iCCA (all genes reported to be mutated in ≥two independent publications[12–20]) as well as genes already described to be mutated in ≥3% of biliary tract cancer in the COSMIC database (data extracted May 2016)[21]. The following genes were completely covered: *ARID1A, ARID2, AXIN1, BAP1, BRAF, BRCA1, CDH1, CDKN2A, CTNNB1, ELF3, EPHA2, ERBB2, ERBB3, FAM47A, FBXW7, FGFR2, FGFR3, GNAS, IDH1, IDH2, KRAS, MDM2, MET, MUC4, NRAS, PBRM1, PIK3CA, PTEN, RNF43, ROBO2, SMAD4, SMARCA4, STK11, TERT,* and *TP53*. For the following large genes, only functional relevant domains and known mutagenic positions were selected: *ADAMTSL3, APC, ATM, FAT1, MUC16*, and *NF1*. The potential tumor suppressor gene *CSMD1* was not included in the panel due to large size and missing data on cancer relevant domains, although described to be mutated in CCA. Based on the mutation frequency of the Cosmic database entry for biliary tract cancer (data extracted 05/2016), an estimated mutation frequency of 2.1 mutations per tumor sample was calculated for our cohort of 16 patients (Supplementary Table S1). Amplicon size of the designed panel was 175 base pairs (bp) and the final panel had a size of 1588 amplicons with a total length of 223,083 bp. Chromosomal positions of the panel design are provided in Supplementary Table S2.

Sequencing and variant calling

Detailed information on enrichment and variant calling pipeline is provided in the supplementary information. Briefly, an enrichment method targeting both DNA strands was used and library preparation was performed with TruSeq Custom Amplicon Low Input Panel (Illumina, San Diego, CA) according to the manufacturer's instructions. For sequencing, an Illumina NextSeq500 (150 bp paired-end runs) with two High Output cartridges v2 (300 cycles) was used. Variants were first called separately for each probe set and only variants present in both probe sets with a cumulative depth of 1000× or average depth of 500× per probe set were called. Only non-synonymous variants in exons or splice sites of the targeted genes with a variant allele frequency of ≥5% (mean of both probe sets) were further analyzed. All mutations with suspicion for sequencing or alignment error in manual review were removed. Sanger or pyrosequencing were used for validation, as appropriate. Primer sequences are listed in Supplementary Table S3.

Statistics

Data were collected and analyzed by BiAS (version 11.01, BiAS for Windows; Epsilon-Verlag, Frankfurt, Germany). Descriptive statistics, such as calculation of mean value, range, and standard deviation (SD) were determined using BiAS as well. Box-plot diagrams for supplemental Figure S2 included quartiles and were generated based on mean and SD. Survival was calculated as time from date of surgery to death or date of last contact. Uniformity was defined as % of reads with >0.2× mean coverage.

Results

Patients

Mean age at surgery was 68 years (range 48–82, SD 9.6) and 11/16 (69%) patients were male. Mean time between date of obtaining the intraductal biopsy and surgery was 30 days (range 8–84, SD 21.9). P86 had a biliary papillomatosis and P140 a biliary cystadenoma as precancerous lesions. All other patients had no underlying diseases in the biliary tract or the liver. 13/16 patients had already died at the time of study closure and mean survival was 9.6 mo (range 0–28 mo, SD 7.6). One patient is still alive (P51), while for two patients (P36, P139) no follow up was available. 7/16 tumors had stage III or IV. Data on CA19-9 level prior to surgery were available only for 8/16 patients and were elevated in four of these eight patients. Clinicopathological characteristics are shown in Table 1.

Sequencing performance

One aim of the study was to evaluate technical feasibility of NGS from intraductal FFPE biopsies in our cohort of 16 patients. Mean diameter of included intraductal biopsies was 2.2 mm (range 0.5–8 mm). Estimated mean content of neoplastic or dysplastic cells was 45% (range 20–80%) for surgical resected tumor samples and 57% (10–90%) for intraductal biopsies. Sufficient DNA for library preparation (>10 ng) could be purified in all cases. Mean amplicon coverage of all samples was 6603× (range

Table 1 Clinical characteristics of all patients

ID	Age at surgery	Sex	Location	UICC	TNM	N	L	V	PN	R	G	M	Surgery	CA 19-9 (KU/L)
P1	82	M	AAC[a]	4	4	1	1	0	1	0	1	0	n.r.	N/A
P6	66	F	dCCA	2b	3	1	1	0	0	0	3	0	cur.	3159
P8	77	M	pCCA	2	2b	0	1	1	1	0	3	0	cur.	111
P36	53	M	dCCA	4								1	n.r.	N/A
P38	61	M	pCCA	4b								1	n.r.	N/A
P42	68	F	dCCA	3	4	1							n.r.	750
P43	73	F	dCCA	2b	3	1	1	0	1	0	2	0	cur.	N/A
P51	76	M	pCCA	1	1	0	0	0	0	0	2	0	cur.	N/A
P59	77	M	iCCA	4b								1	n.r.	N/A
P67	64	M	iCCA	4b								1	n.r.	1000
P73	77	M	dCCA	1a	1	0	1	1	1	0	2	0	cur.	< 37
P86	75	F	iCCA	4b	3	0	0	0	1	x	3	1	cur.	N/A
P87	60	M	iCCA	4a	2a	1	1	0	1	0	2	0	cur.	<37
P139	48	M	AAC[a]	2	2	0	0	0	0	0	2		cur.	N/A
P140	61	M	dCCA	1b	2a						3		cur.	<37
P141	68	F	AAC[a]	1a	1	0				0	1		cur.	<37

dCCA distal cholangiocarcinoma (CCA), *pCCA* perihilar CCA, *iCCA* intrahepatic CCA, *UICC* staging was performed according to the 7th edition of the classification of the Union for International Cancer Control (UICC), *n.r.* non resectable, *cur.* resection in curative intention
[a]These samples were classified as ampullary adenocarcinoma (AAC), but distal CCA could not be excluded. N/A: not available

3269–11,670×, SD 1772) and a mean uniformity of 92.5% (range 79.3–94.6, SD 2.7) was yielded. Mean amplicon coverage and uniformity of biopsies (6523×, 91.5%, SD 1752), tumor (6988×, 93.1%, SD 1543), and control samples (6231×, 92.7%, SD 2015) were comparable. Notably, mean coverage of very small biopsies (<1 mm, $n = 6$: 6205×, range 5328–8923, SD 1364) was only slightly lower compared to the rest of biopsies (6650×, range 4140–11,670, SD 1913) and mean uniformity was equal (91.5% vs 91.7%). Representative SNVs ($n = 5$) and Indels ($n = 7$) were successfully validated with Sanger or pyrosequencing (validation rate 100%). An overview of sequencing performance of biopsies is provided in Table 2. A comparison of sequencing performance of biopsies and surgical specimen is given in Supplementary Figure S2 and full sequencing data is shown in Supplementary Table S4 as well.

Molecular analysis

In total, 79 mutations in 44 positions of 20 genes in all dysplastic and neoplastic samples ($n = 39$) were identified. A mean of 1.7 mutations per surgical specimen (range 0–4) and 2.3 (range 0–6) per biopsy specimen was detected. The majority of the detected mutations were missense (49/79, 62%) and frameshift variants (17/79, 22%). Mean allele frequency was higher in surgical tumor specimens (27%, range 4.66–53.8%) compared to biopsy samples (17.7%, range 4.8–79.9%). C > T/G > A (52% of SNVs) and T > C/A > G (19.3%) transitions were found more frequently than C > A/G > T (18%) and T > G/A > C (12%) transversions. Interestingly, no C > G/G > C and T > A/A > T transversions were observed. An overview of mutational characteristics is provided in Fig. 1.

Some genes were found to be recurrently mutated. Mutations of *ARID1A*, *ARID2, BAP1, EPHA2, ERBB3, GNAS, MET, NF1, PBRM1, PIK3CA,*and *RNF43* were found in two different patients, while *SMAD4* was found to be mutated in four and *TP53* in five patients, respectively. One position was mutated in two cases (17:7578263 of *TP53* in P36 and P86).

In two patients, no mutations were found in both biopsy and tumor specimen (P6, P8). All identified mutations are provided in Supplementary Table S5.

Mutational load of biopsies in relation to degree of dysplasia and sample size

Another aim of this study was to analyze mutational profiles of biliary dysplasia and intraductal samples with BilIN 1 ($n = 5$), BilIN 2 ($n = 7$), and BilIN 3 ($n = 6$) and tumor adjacent dysplasia ($n = 2$). Notably, in three cases it was not possible to conclusively differentiate between a BilIN and reactive changes by means of histopathology due to inflammation. The mean mutational load was 2.7 for BilIN 1 (range 2–3), 2 for BilIN 2 (0–5), and 2.8 for

Table 2 Sequencing performance of intraductal biopsies

Sample	Diameter (mm)	Dysplastic cell content (%)	Coverage	Uniformity
1B	2	50	6907.5	92.5%
6B	0.5	n.e.	8923.1	93.5%
8B	2	10	6043.3	91.2%
36B	2	30	5454.8	94.0%
38B	4	65	11,669.9	93.9%
42B1	3	10	5914.6	94.1%
42B2	0.5	n.e.	5396.1	90.0%
43B1	3	70	6092.7	92.5%
43B2	0.5	90	6081.7	93.1%
51B	0.5	40	5932.3	89.8%
59B	0.5	60	5570.2	89.7%
67B	0.5	40	5328.3	92.8%
73B	2	80	6209	93.5%
86B1	2	50	10,190.5	79.3%
86B2	2	55	7306	86.6%
86B3	4	60	5935	93.0%
86B4	8	80	5601.4	94.3%
87B	2	50	6735.7	93.6%
139B	2	80	5170.9	91.8%
140B	2	85	6388	92.9%
141B	3	80	4140.9	88.7%

Diameter of intraductal biopsies as well as estimated tumor content (based on hematoxylin–eosin stained sections, HE) and corresponding detected mutations and sequencing data. All biopsies with a diameter <1 mm were assessed as 0.5 mm
n.e. not evaluable

BilIN 3 (2–5). Overall, at least one mutation was detected in 14/18 (78%) biopsies with dysplasia or inconclusive findings. Of note, in one sample with inconclusive histological findings three mutations were found as well. In very small biopsies (≤1 mm), a mean of 1.2 (2–3) mutations was found compared to 2.7 mutations (0–6) in the larger biopsies (mean diameter 2.9 mm). Data on dysplasia grade including amount of detected mutations is provided in Table 2. A representative case with indeterminate biliary stricture including endoscopic and histopathological evaluation is shown in Fig. 2.

Furthermore, we wanted to determine a potential correlation of the most frequently mutated genes (*TP53*, *SMAD4*) with the degree of dysplasia. Of note, apart from one specimen with BilIN 2 (P86b1), all samples with *TP53* mutations were either BilIN3 or CCA. Mutations in *SMAD4* were found in CCA samples as well as BilIN 1 (43b1, 1b, 1t) and BilIN 2 (42b2). Collectively, in 7/15 cases (47%) with dysplastic biopsies either *SMAD4* or *TP53* were found to be mutated.

Genetic heterogeneity

We further aimed to investigate a potential presence of subclonality within biliary dysplasia and neoplasia. The majority of variants was detected in both biopsy and corresponding tumor tissue (54/79, 68%). Notably, 22 mutations (28%) were found only in the biopsy samples while three mutations (4%) were found in the tumor sample but not in the biopsy. Two of these mutations were found in a patient with a very small biopsy (<0.5 mm, P67) with a comparably low (40%) content of dysplastic cells. In cases with multiple biopsies obtained, different mutations were identified such as in P86 where three mutations were found in one biopsy (b1) compared to five mutations in the other biopsies (b2, b3, and b4). Moreover, in another patient (P43b1), three mutations were found in the biopsy but none in the tumor sample. There was no difference of detected mutations in the two samples of tumor adjacent adenoma and the corresponding tumor sample. Figure 3 gives an overview of mutated genes of all samples of this study.

Potential driver mutations and therapeutical relevance

Of the 44 identified mutated positions, 32 were classified as likely pathogenic mutations since they had a SIFT score of ≤0.05, or were nonsense or frameshift mutations[22]. The other twelve mutations were assessed as variants of uncertain significance. Interestingly, all three mutations detected in the biopsy of Patient 1, which was inconclusive in histopathological examination, were likely pathogenic mutations (Fig. 2). The 22 mutations detected only in the biopsy but not in the tumor included both likely pathogenic mutations ($n = 14$) and variants of uncertain significance ($n = 8$).

To investigate a potential theranostic value of targeted sequencing of intraductal biopsies, genes known as potentially relevant in targeted cancer therapy were assessed as well. In 6/16 patients (37.5%), mutations in therapeutically relevant genes were identified and likely pathogenic mutations were found in *EPHA2*, *ERBB3*, *PIK3CA*, and *PTEN*. Mutations of therapeutically relevant genes are shown in Table 3.

Discussion

In the present study we analyzed FFPE specimens of patients with biliary dysplasia in the intraductal biopsy and CCA in further clinical course by applying a targeted, CCA-specific dual strand NGS-based analysis. Thereby, 1.8 non-synonymous mutations per tumor and 2.3 non-synonymous mutations per biopsy were identified. This is consistent with an estimated amount of 2.1 mutations per

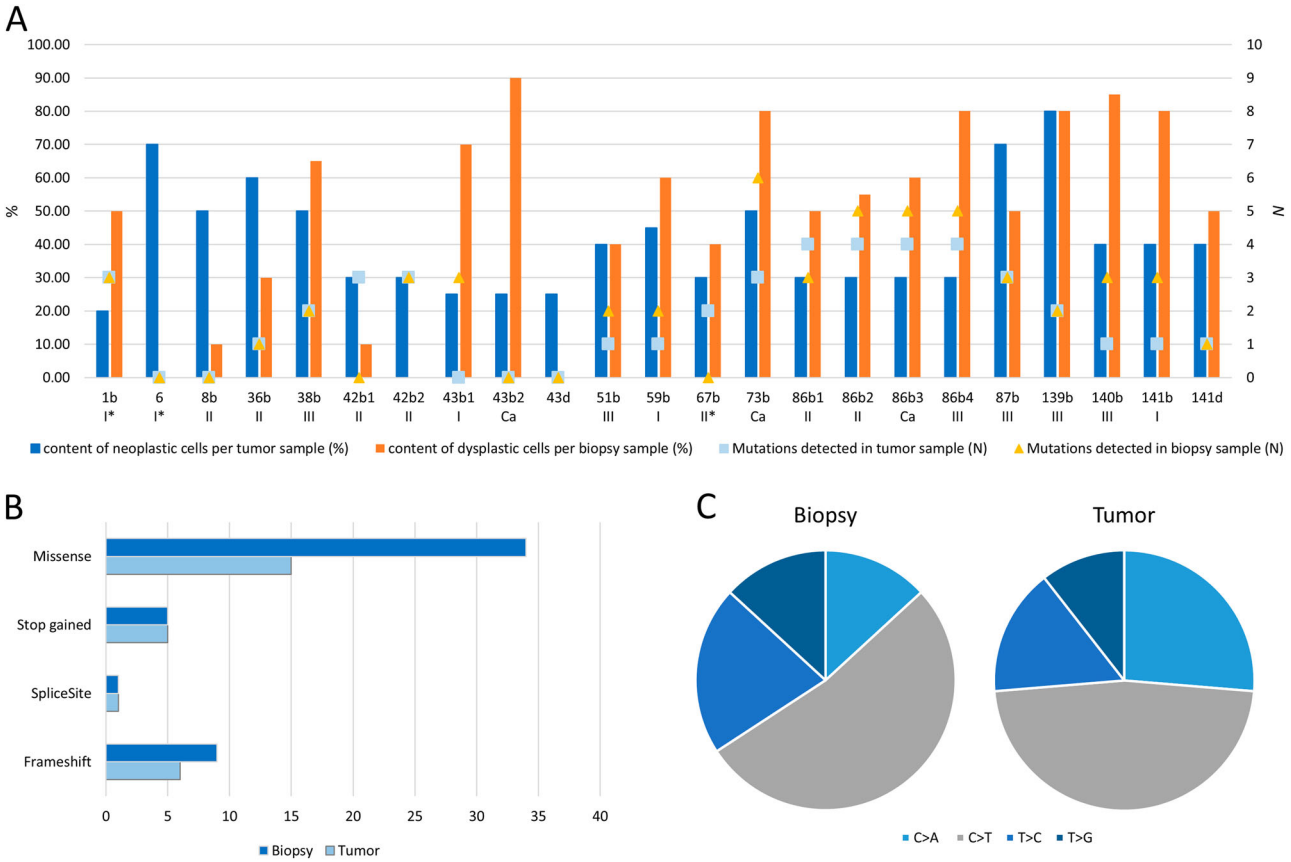

Fig. 1 Mutational characteristics. Characteristics of mutations detected in biopsies and resected cholangiocarcinoma. **a** Estimated content of tumor or dysplastic cells based on stained sections of the respective tumor or biopsy samples and corresponding amount of detected mutations in biopsy and surgical specimens. Values below sample IDs indicate degree of biliary intraepithelial neoplasia (BilIN). Asterisk marks samples in which a BilIN could not be conclusively differentiated from only reactive epithelial alterations. 43d and 141d were dissected from the surgical specimen and histopathologically diagnosed as adenoma. Samples 1, 139, 141: these samples were classified as ampullary adenocarcinoma, but distal CCA could not be excluded. **b** Categories of detected biopsies in mutations and tumor samples. **c** Mutational spectral of point mutations

tumor sample, which was calculated based on the mutation frequency in biliary tract cancer of the Cosmic database (Supplementary Table S1)[21]. Library preparation as well as sequencing was successful in all samples and no difference in sequencing performance in relation to biopsy size was observed. Notably, a lower number of variants was observed in very small biopsies (<0.5 mm) compared to the larger ones potentially indicating that sensitivity of NGS from these biopsies might be decreased. Even so, these data demonstrate that the dual strand approach can be regarded as a suitable method to perform targeted sequencing of very small FFPE biopsies. Notably, NGS from low FFPE tissue amounts such as bronchial biopsies was reported, but these are the first data of NGS from the considerably smaller intraductal biopsies[23].

Besides technical feasibility, our results revealed a substantial genetic heterogeneity of biliary dysplasia and neoplasia in the investigated gene set, as 28% of mutations were only found in biopsy samples but not in the corresponding surgical specimen. Furthermore, a slightly higher mutation rate was found in biopsy samples compared to tumor samples while allele frequency was lower. These data show that biliary dysplasia contain a marked subclonality and indicate that CCA might derive from a minority subclone of dysplasia. This is exemplified in intraductal biopsy 86b3 which was diagnosed as dysplasia as well as invasive CCA: this sample shares mutations with the resected tumor as well as with other biopsies of the patient with only biliary dysplasia. These findings are in line with recent reports on mutational landscape of Barrett's esophagus and esophageal adenocarcinoma as well as oral precancerous lesions where a profound subclonal heterogeneity was shown[24,25]. On the other hand, a study on pre-invasive stages of esophageal carcinogenesis reported that the majority of recurrently mutated genes were found in both metaplastic never-dysplastic Barrett's esophagus and esophageal adenocarcinoma, but *TP53* and

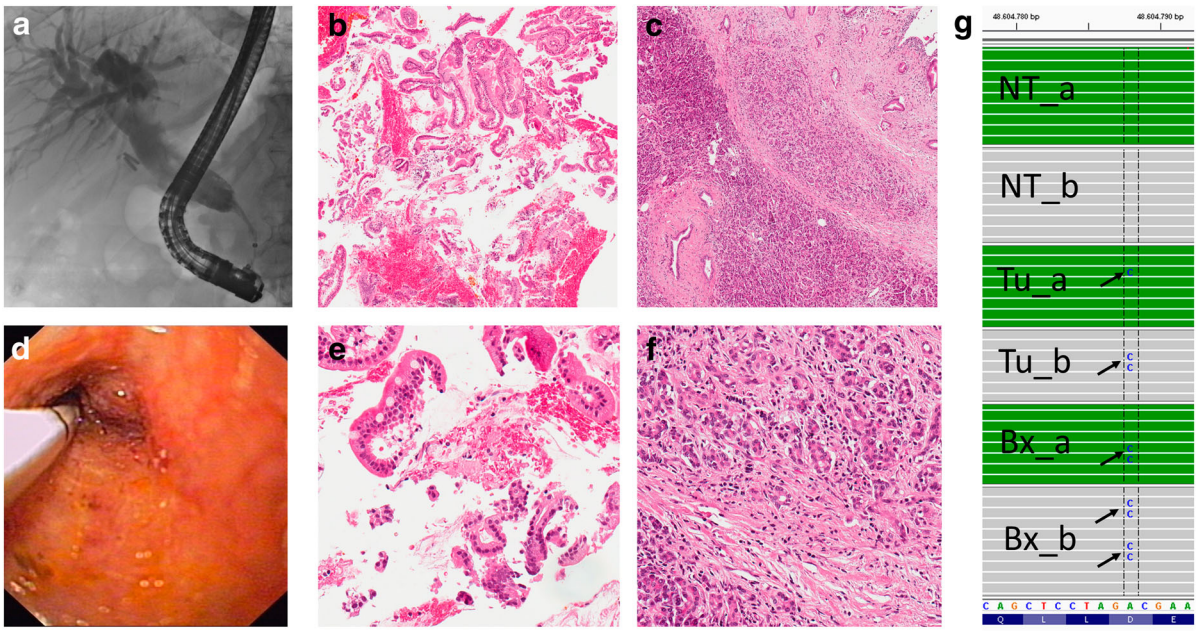

Fig. 2 From endoscopy to targeted sequencing of intraductal biopsies. Representative results of endoscopical, histopathological and molecular analysis of Patient 1. In fluoroscopy (**a**), a high-grade stenosis of the distal common bile duct with concomitant intra- and extrahepatic cholestasis was diagnosed. In cholangioscopy (**b**), an ulcerative lesion highly suspicious for malignancy was identified to cause the biliary stricture. Histopathological analysis of hematoxylin–eosin stained sections of the biopsy (**c** 50×, **d** 200×) was inconclusive: a definite differentiation of reactive and dysplastic alterations of the biliary epithelium was not possible due to artificial degradation of the obtained tissue. Due to clinical suspicion for malignancy, surgery was performed and histopathological analysis established diagnosis of adenocarcinoma (**e** 50×, **f** 200×). After targeted sequencing (**g**), the same three mutations were observed in both the intraductal biopsy and the tumor sample such as shown for the mutation in *SMAD4* (Chr.18:48604788, A > C), which was observed in both sequencing libraries of the tumor (Tu-a, Tu-b) as well as the biopsy sample (Bx-a, Bx-b). There was no mutation observed in any library of the non-tumor control sample (NT-a, NT-b)

SMAD4 were confined to high-grade dysplasia and carcinoma indicating that these mutations represent major tumor drivers in this entity[26]. This corresponds to our findings, where *TP53* and *SMAD4* were found being mutated in 47% of dysplastic biopsies. *TP53* was mutated only in BilIN 3 and CCA in contrast to *SMAD4*, which was found to be mutated in BilIN 1 and 2 as well as CCA, indicating the need of further characterization of *SMAD4* mutations in non-CCA associated biliary dysplasia. In particular, while a recent study of our group revealed a small amount of intratumoral heterogeneity in iCCA, these are the first data on genetic heterogeneity of biliary dysplasia[27]. Further studies are warranted including more samples and more patients to determine presence of different mutated cancer pathways in pre-invasive and invasive disease. These observations have to be kept in mind when including patients to targeted therapy trials based on mutations from a single biopsy.

Added to the revealed genetic heterogeneity, our results indicate that NGS might be a potentially promising tool to enhance the diagnostic yield of intraductal biopsies in patients with indeterminate biliary stricture. For example, likely pathogenic mutations were observed in a tissue sample with inconclusive histopathological findings (P1) and the majority of BilINs was found to harbor mutations within the CCA-specific gene panel. Moreover, the correlation of TP53 with BilIN 3 and CCA indicates that sequencing of TP53 in intraductal biopsies helps to strengthen a suspicion for malignancy in inconclusive cases. Although TP53 is the most frequently mutated gene in CCA, mutation rate is still below 40% (Supplementary Table S1) demonstrating that sequencing of a larger gene panel such as used in the current study might increase the diagnostic value. These data point to a potential impact of NGS on future clinical management of patients with indeterminate biliary stricture by strengthening or weakening suspicion for a malignancy: The additional information might be essential for patients with CCA, since in these cases surgery should be performed as soon as possible to improve chances for a curative resection. On the other hand it might help to avoid surgery in non-malignant etiologies of biliary stricture which was reported in up to 17%[28,29]. Final diagnosis after surgery in cases with a benign CCA-mimicking etiology were mostly inflammatory strictures which are unlikely to harbor CCA associated mutations. However, prospective validation studies with larger cohorts including dysplastic lesions without development of invasive disease are

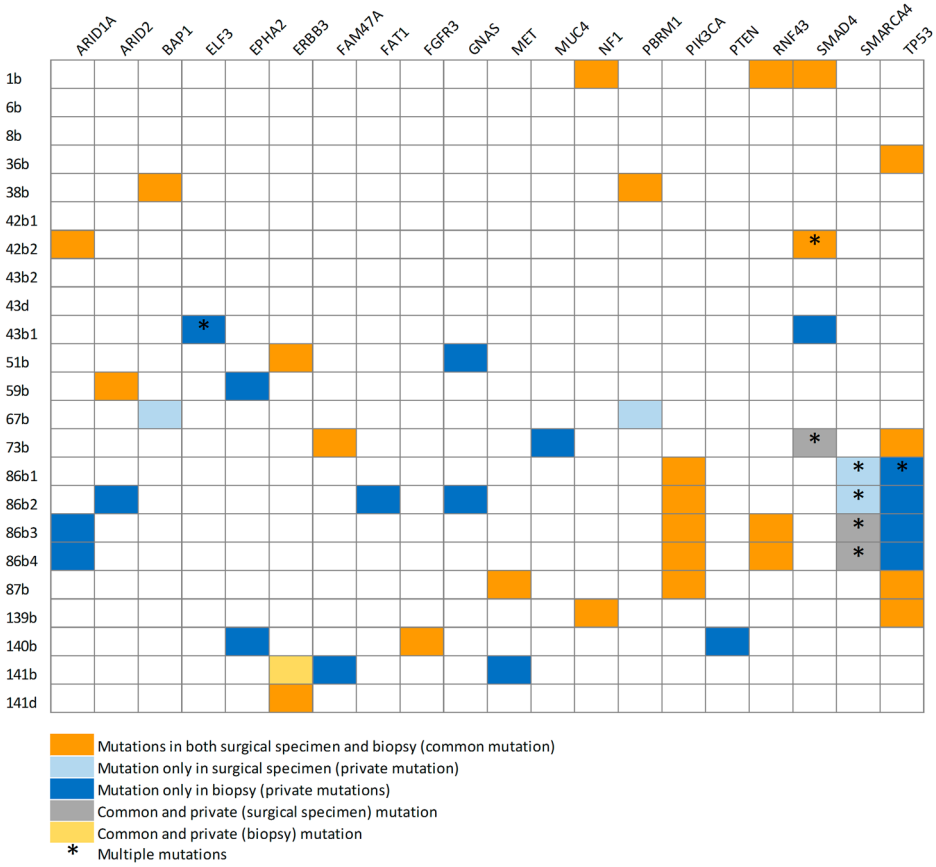

Fig. 3 Overview of mutated genes. Overview of mutated genes in the study cohort of all biopsy samples and corresponding surgical specimens. b: biopsy; 43d and 141d: These samples were dissected from the surgical specimen and histopathologically diagnosed as adenoma

Table 3 Potential therapeutically relevant mutations

ID	Sample	Gene	Base exchange	Category	Amino acid exchange	Clinical relevance
P59	b	EPHA2	1:16461663 C > T	Missense	Val484Met	VUS
P140	b	EPHA2	1:16475274 T > C	Missense	Lys141Arg	Likely pathogenic
P141	b	ERBB3	12:56481856 C > T	Missense	Pro262Ser	VUS
P51	b, t	ERBB3	12:56482607 C > T	Missense	Thr355Ile	Likely pathogenic
P141	b, d, t	ERBB3	12:56491666 T > G	Missense	Phe853Cys	Likely pathogenic
P140	b	PTEN	10:89692901 G > T	Stop gained	Gly129Ter	Likely pathogenic
P141	b	MET	7:116339760 G > A	Missense	Asp208Asn	VUS
P140	b, t	FGFR3	4:1801248 C > T	Missense	Thr126Ile	VUS
P87	b, t	MET	7:116339201 G > T	Missense	Arg21Ser	VUS
P87	b, t	PIK3CA	3:178916944 A > G	Missense	Lys111Glu	Likely pathogenic
P86	b1–b4, t	PIK3CA	3:178919231 T > C	Missense	Leu239Pro	Likely pathogenic

List of mutations in potentially therapeutically relevant genes identified in this study. Variants with a SIFT score of ≤0.05 as well as nonsense and frameshift mutations were categorized as likely pathogenic
b biopsy, *t* tumor, *d* tumor adjacent dysplasia, *VUS* variant of uncertain significance

needed to adequately assess the diagnostic yield of NGS. It has to be considered that NGS is still a very costly method, and in the present study a highly adapted approach with a dual strand enrichment technique and a customized CCA-specific panel was used. Even so, implementation of cancer gene panels in routine diagnostics has already begun, areas of clinical application are increasing and costs are decreasing.

Apart from a potential diagnostic value, our data indicate that NGS of intraductal biopsies might serve as a potential screening tool for personalized therapy regimens. Mutations were identified in potentially therapeutically relevant genes such as *EPHA2*, *FGFR3*, *ERBB3*, *MET*, *PIK3CA*, and *PTEN*. This highlights that NGS of intraductal biopsies might help to assign patients to targeted therapy trials according to the mutated cancer pathway. For example, trials including patients with CCA and FGFR alterations are currently ongoing (NCT01752920, NCT02150967). Moreover, the mutational profile can be assessed in further clinical course in case of disease progression or recurrent disease to detect potential additional driver mutations or mutations in genes associated with resistance to personalized therapy. In addition, obtaining intraductal biopsies is a safe method rarely causing complications and since most patients need recurrent ERC for stent placement, it reduces additional effort and risk (e.g., in comparison to transcutaneous biopsy) for the patient[30]. However, due to the genetic heterogeneity, results from a single biopsy should always be interpreted with caution. Moreover, as it is recommended for histopathological analysis to obtain multiple biopsies, NGS should also be performed based on DNA isolated from multiple biopsies to reduce the bias of genetic heterogeneity[30].

Some limitations must be considered to interpret data of our study. First of all, all dysplasias analyzed were most likely directly tumor adjacent and NGS data on BilIN without appearance of CCA such as in a chronological study are still lacking. Moreover, no patient with primary sclerosing cholangitis was included—a well-known risk factor for CCA with frequent occurrence of BilIN[31]. Secondly, we included tumors from different areas of the biliary tract (iCCA, pCCA, dCCA, ampullary carcinoma) as well as a varying number of dysplastic samples per patient resulting in a heterogeneous study cohort. Thirdly, genetic heterogeneity is most likely far more complex than revealed by our panel-sequencing approach and highly comprehensive data on eCCA are still due.

In conclusion, the present study demonstrates that NGS of intraductal biopsies is technically feasible and potentially improves the diagnostic yield in indeterminate biliary stricture and CCA. Moreover, a substantial genomic heterogeneity in biliary dysplasia and neoplasia was revealed, which needs to be considered in therapeutic management of patients with CCA and targeted therapy trials.

Study Highlights

What is current knowledge

- Sensitivity of intraductal biopsies is limited to date
- Prognosis of biliary cancer is poor and no personalized therapy has been established so far

What is new here

- Targeted sequencing of very small intraductal biopsies is feasible
- Sequencing results potentially improve diagnostic yield
- Sequencing can identify potential driver mutations

Translational impact

- These results demonstrate the potential of sequencing intraductal biopsies to impact diagnostic and therapeutic management of patients with dysplastic and neoplastic biliary lesions

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Conflict of interest

Guarantor of the article: Dirk Walter, MD.
Specific author contributions: planning and/or conducting the study: D.W., J.P.-O., K.B., J.G.A., S.Z.; collecting and/or interpreting data: D.W., J.P.-O., K.B., R.W., W.O.B., M.L.H., S.H., M.S., C.D.; drafting the manuscript: D.W., J.P.-O., K.B., S.Z., W.O.B., M.L.H., M.S. All authors approved the final manuscript.
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