Mutation spectrum associated with metastasis of advanced cholangiocarcinoma

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Abstract
Background: The mutations associated with metastasis in advanced-stage cholangiocarcinoma (CCA) have not been investigated.
Objective: To explore mutations in patients with advanced CCA and independent factors related to metastasis.
Methods: This retrospective study performed next-generation sequencing of tumor specimens from patients with advanced CCA treated between January 2017 and December 2019. Tumor mutational burden (TMB), microsatellite instability, and programmed cell death ligand (PD-L)1 positivity were determined. Factors independently associated with metastasis were explored via logistic regression.
Results: Ninety-one patients were included in this study. TP53 mutation frequencies were significantly higher in extrahepatic than intrahepatic CCA, while ARID1A mutations were significantly more frequent in intrahepatic CCA. Mutation frequencies in six selected genes did not differ according to patient age or sex. SMAD4 mutations were significantly less frequent in stage IV cancer; ARID1A and PBRM1 mutation frequencies were significantly higher in TMB >10 tumors. PBRM1 mutation frequencies were significantly higher in PD-L1-positive tumors, but lower in patients with metastasis. Multivariable analysis showed that a history of biliary surgery, SMAD4 mutations, and PBRM1 mutations were independently associated with CCA metastasis.
Conclusions: A history of biliary surgery and mutations in SMAD4 and PBRM1 are independent protective factors for metastasis in patients with advanced CCA.
Introduction

Biliary tract cancers are rare, accounting for less than 1% of all cancers and about 10% to 15% of all primary cancers arising in the liver. They mostly occur during or after the seventh decade of life, and are typically diagnosed at a late stage and characterized by poor outcomes. Cholangiocarcinoma (CCA) is an invasive carcinoma of the biliary tract, arising in the bile duct epithelium. It is the second most common form of primary liver cancer, accounting for about 10% to 15% of all hepatobiliary malignancies and 3% of all gastrointestinal tumors. Even after complete surgical resection, its recurrence rate remains high, and 5-year overall survival (OS) rates are poor at 20% to 35%. CCAs can be intrahepatic (ICC) or extrahepatic (ECC). They are slightly more common in men than women, except among people of Hispanic ethnicity. Common risk factors include advanced age, chronic inflammation, primary sclerosing cholangitis (PSC), and exposure to chemical agents such as Thorotrast and asbestos; obesity and overweight are considered possible risk factors.

Driver gene mutations in CCA include those in E74 like ETS transcription factor 3, AT-rich interactive domain-containing protein (ARID)1, and ARID2. CCAs also demonstrate the enrichment of genes involved in Wnt signaling, apoptosis, and oncogenic pathways, while genes such as N-sulfoglucosamine sulfohydrolase, eukaryotic translation initiation factor 5A, Bet1 golgi vesicular membrane trafficking protein like, glucosaminyl (N-acetyl) transferase 4, and phospholipase C gamma 2 were associated with the prognosis of CCA.

ICC and ECC display different mutation patterns, with isocitrate dehydrogenase 1 gene mutations occurring exclusively in ICC, and Erb-B2 receptor tyrosine kinase (ERBB)2 mutations in ECC. KRAS mutations are associated with progression-free survival (PFS) in ICC, while BRCA1 associated protein 1 gene mutations and changes in the fibroblast growth factor pathway are associated with the PFS of ECC. Of note, about 50% of CCAs carry mutations that have therapeutic implications.

The increasing popularity of next-generation DNA sequencing (NGS) provides an opportunity to tailor therapy to potential targets. NGS is a powerful technology that allows accurate, efficient, and large-scale genome sequencing. Tian et al. used NGS to reveal the mutational landscape of CCA in Chinese patients, identifying mutations specific to ICC, ECC, sex, age, tumor differentiation, and tumor mutational burden (TMB). Moreover, Feng et al. showed that microsatellite instability (MSI) was only found in CCA in older patients, and identified mutations specific to CCAs in young adults. Nevertheless, no study to date has examined the mutations found in patients with advanced-stage CCA or those associated with metastasis.

Therefore, this study aimed to explore the mutations in patients with advanced CCA and whether there are independent factors related to metastasis.
Patients and methods

Study design and patients

This was a retrospective study of patients with advanced CCA who were treated at the First Department of Biliary Surgery of the Eastern Hepatobiliary Surgery Hospital affiliated to the Second Military Medical University (Shanghai, China) between January 2017 and December 2019. This study was approved by the Ethics Committee of the Third Affiliated Hospital of the Naval Military Medical University (Shanghai, China; approval no: EHBHKY2020-01-008). All identifying patient details have been removed from this article. The need for informed consent was waived by the committee because of the retrospective nature of this study.

Inclusion criteria were: 1) patients with CCA confirmed by pathological examination;2,5 2) inoperable CCA because of the advanced stage or recurrence after previous surgery; and 3) genetic testing completed during diagnosis and treatment. Exclusion criteria were: 1) incomplete medical records; or 2) with another advanced tumor.

Patients were divided into ICC and ECC groups. Hilar CCA was grouped with ECC. Clinical staging was based on the 8th edition of the American Joint Committee on Cancer Guidelines for Biliary Tract Tumors.14 Stages 3 and 4 with vascular invasion or abdominal metastasis were defined as advanced stages.

Sample collection and targeted NGS

All tumor specimens were reviewed by two independent pathologists to confirm the pathologic diagnosis and select the appropriate areas for macrodissection, which was performed to evaluate tumor content and percentage of tumor cells. For each tumor sample, at least 15 unstained slides containing more than 20% tumor cells, a formalin-fixed paraffin-embedded (FFPE) block, or 0.5 cm³ of fresh-frozen tissue was required to extract DNA. At least 50 ng DNA was extracted from each 40 mm FFPE tumor sample using a DNA Extraction Kit (TianGen Biotech Co., Ltd. Beijing, China) in accordance with the manufacturer’s protocols. This panel encompassed all coding exons of 450 cancer-related genes and 64 selected introns of 39 genes that are frequently rearranged in solid tumors. The genes were captured and sequenced with a mean coverage of 900× for FFPE samples and 300× for matched blood samples using an Illumina NextSeq 500 Platform (Illumina, Inc., San Diego, CA, USA), and 50 to 250 ng of double-stranded DNA was sheared by ultrasound. Comprehensive genomic alteration analyses of the tumor and matched blood samples were performed using an assay panel that captured 450 cancer-related genes and selected introns of 38 genes frequently rearranged in cancer (Yuansu™, OrigiMed, Shanghai, China). NGS was then performed with a mean coverage of 900× for tumor tissues and 300× for paired blood cells using a NextSeq-500 platform (Illumina, Inc.).

Somatic alternations, including base substitutions, insertions and deletions (indels), copy number alterations, and gene fusions/rearrangements, were identified. Briefly, reads were aligned to the human genome with the reference sequence (hg19) using the Burrows–Wheeler Aligner,15 which was accompanied by removing duplicates from PCR using Picard tools.16 MuTect was used to identify single nucleotide variants (SNVs) and short indels after quality recalibration and realignment using the Genome Analysis Toolkit pipeline (Broad Institute, Cambridge, MA, USA). The Pindel program17 was used to calibrate short indels. Read depths were normalized in target regions by Exome Copy number Alterations/Variations annotATOR software.18
Customized algorithms were used to detect copy number changes and gene rearrangements. For copy number variations, genes with a threshold surpassing four copies were deemed to be amplified, and genes with zero copies to be homozygous deletions.

**Detection and determination of immunogenic markers**

TMB was estimated for each sample by counting its somatic mutations, including coding SNVs and indels per megabase. Driver mutations and germline alterations in the dbSNP database were not counted. MSI was determined by Shanghai Zhiben Medical Laboratory (Shanghai, China). The above indicators were all tested and reported by Shanghai Zhiben Medical Laboratory. PD-L1 expression was detected by immunohistochemical staining of FFPE tumor sections using an anti-PD-L1 antibody at 1:50 dilution overnight at 4°C (clone 22C3; OrigiMed, Shanghai, China). Slides were then stained with a mAb clone E1L3N secondary antibody (Xiamen Aid Biological Co., Ltd, Xiamen, China) at 37°C for 40 minutes. All slides were counterstained with hematoxylin. PD-L1 expression was interpreted as a combined positive score, which was defined as the number of PD-L1-positive cells divided by the total number of tumor cells, multiplied by 100. The threshold for PD-L1 positivity was set at >10% according to 2021 National Comprehensive Cancer Network (NCCN) guidelines. Biopsy samples containing ≥100 live tumor cells were required to evaluate PD-L1 expression. PD-L1 was considered positive when ≥1% of the tumor cells showed any intensity of complete or incomplete cell membrane staining.

**Statistical analysis**

SPSS software v.22.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Continuous variables are shown as means ± standard deviations or as medians (interquartile ranges) according to their distribution, as determined by the Kolmogorov–Smirnov test; comparisons between groups were performed with the Student’s t-test or Mann–Whitney U-test, as appropriate. Categorical variables were reported as frequencies with percentages and compared with the chi-square test or Fisher’s exact test, as appropriate. Univariable and multivariable logistic regressions were used to explore the factors independently associated with the occurrence of metastasis. Variables with P < 0.10 in the univariable analysis were included in the multivariable analysis (backward method). Common mutations of patients were compared according to subgroups of different clinical characteristics. These subgroups included age (30–39, 40–49, 50–59, 60–69, and 70–79 years), sex (male and female), clinical stage (II, III, and IV), TMB (high: >10; low: ≤10), PD-L1 expression (positive and negative), and metastasis status (metastasis and no metastasis). Two-tailed P-values <0.05 were considered statistically significant.

**Results**

**Patient characteristics**

Ninety-one patients with ICC or ECC were included (Table 1). Patient ages ranged from 30 to 78 years, and 29 patients (31.9%) were female. A history of biliary surgery was significantly more common in patients with ICC than with ECC (61.9% vs. 28.6%, P = 0.002), while stage 4 disease (63.3% vs. 38.1%, P = 0.02) and metastasis (85.7% vs. 64.3%, P = 0.02) were significantly more common in ECC than ICC.

**Mutations**

The six most commonly mutated genes in CCA were investigated for mutation
frequency between patients with ECC and those with ICC: TP53, ARID1A, KRAS, cyclin dependent kinase inhibitor (CDKN) 2A, SMAD family member (SMAD)4, and polybromo (PBRM)1. TP53 mutation frequencies were significantly higher in ECC than in ICC (49.0% vs. 38.1%, P = 0.04), while ARID1A mutation frequencies were significantly higher in ICC (28.6% vs. 8.2%, P = 0.01). There were no significant differences in the frequencies of mutated KRAS, CDKN2A, SMAD4, or PBRM1 between the two groups (Table 1).

Subgroup analyses (Figure 1) showed that the frequencies of mutations in the six genes were not significantly different among subgroups. However, the frequency of SMAD4 mutations was significantly lower in stage IV cancer than other stage cancers (P = 0.03), while ARID1A (P = 0.04) and PBRM1 (P = 0.02) mutation frequencies were significantly higher in tumors with a TMB >10. The PBRM1 mutation frequency was also significantly higher in PD-L1-positive than -negative tumors (P = 0.03), but significantly lower in patients with metastasis (P = 0.04).

**Multivariable analysis**

Univariable analyses showed that a history of biliary surgery (P < 0.001), carbohydrate

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**Table 1. Characteristics of patients with primary cholangiocarcinoma.**

| Characteristic                        | ICC (n = 42) | ECC (n = 49) | P-value |
|---------------------------------------|--------------|--------------|---------|
| Age (years), median (range)           | 57.88 (32.78) | 56.79 (30.74) | 0.529   |
| Sex (female), n (%)                   | 16 (38.1)    | 13 (26.5)    | 0.266   |
| Liver cirrhosis, n (%)                | 8 (19.1)     | 3 (6.1)      | 0.104   |
| HBV infected, n (%)                   | 12 (28.6)    | 7 (14.3)     | 0.123   |
| Bile duct stones, n (%)               | 2 (4.8)      | 4 (8.2)      | 0.683   |
| History of biliary surgery, n (%)     | 26 (61.9)    | 14 (28.6)    | 0.002   |
| Family tumor history, n (%)           | 6 (14.3)     | 10 (20.4)    | 0.583   |
| AJCC clinical stage, n (%)            |              |              |         |
| II                                    | 5 (11.9)     | 2 (4.1)      | 0.242   |
| III                                   | 21 (50.0)    | 16 (32.7)    | 0.134   |
| IV                                    | 16 (38.1)    | 31 (63.3)    | 0.021   |
| MSI, n (%)                            | 3 (7.1)      | 1 (2.0)      | 0.332   |
| TMB score, median (range)             | 3.9 (0.8,50) | 2.5 (0.59)   | 0.588   |
| PD-L1 positive, n (%)                 | 11 (26.2)    | 5 (10.2)     | 0.056   |
| CA199 (U/mL), median (range)          | 161 (8,1000) | 176 (1,1000) | 0.542   |
| Mutant genes with high mutation rate, n (%) |              |              |         |
| TP53                                  | 16 (38.1)    | 24 (49.0)    | 0.040   |
| ARID1A                                | 12 (28.6)    | 4 (8.2)      | 0.014   |
| KRAS                                  | 14 (33.3)    | 25 (51.0)    | 0.137   |
| CDKN2A                                | 7 (16.7)     | 8 (16.3)     | 1.000   |
| SMAD4                                 | 8 (19.1)     | 5 (10.2)     | 0.248   |
| PBRM1                                 | 6 (14.3)     | 3 (6.1)      | 0.293   |
| Tumor metastasis, n (%)               | 27 (64.3)    | 42 (85.7)    | 0.018   |

ICC: intrahepatic cholangiocarcinoma; ECC: extrahepatic cholangiocarcinoma; HBV: hepatitis B virus; AJCC: American Joint Committee on Cancer; MSI: microsatellite instability; TMB: tumor mutational burden; PD-L1: programmed cell death ligand 1; CA199: carbohydrate antigen 19-9; ARID1A: AT-rich interactive domain-containing protein 1A; CDKN2A: cyclin dependent kinase inhibitor 2A; PBRM1: polybromo 1.
antigen 19-9 levels (P = 0.05), SMAD4 mutation status (P = 0.08), and PBRM1 mutation status (P = 0.03) were significantly associated with CCA metastasis (Table 2). Multivariable analysis showed that a history of biliary surgery (odds ratio [OR] = 0.114, 95% confidence interval [CI]: 0.033–0.390, P = 0.001), SMAD4 mutations (OR = 0.202, 95% CI: 0.045–0.903, P = 0.04), and PBRM1 mutations (OR = 0.175, 95% CI: 0.033–0.922, P = 0.04) were independently associated with CCA metastasis (Table 2).

Discussion
To our knowledge, no study has previously examined the mutations found in patients with advanced-stage CCA or those associated with metastasis in CCA. We investigated this in the present study, and found that a history of biliary surgery, and mutations in SMAD4 and PBRM1 were independent protective factors for metastasis in patients with advanced CCA.

We also showed that mutations in TP53 were significantly more frequent in ECC than ICC, while mutations in ARID1A were significantly more frequent in ICC than ECC. Tian et al. previously reported significantly more frequent TP53 and ARID1A mutations in ECC; this discrepancy could be caused by the analyses of different study populations, but could also be a feature of advanced CCA because Tian et al. included CCAs of all stages in their study. Conversely, Lowery et al. showed that TP53 mutations were more frequent in ICC than in ECC, but they did not examine ARID1A mutations.

Our study included patients who had recurrence after the removal of a previous CCA tumor, as well as those with inoperable CCA. Our observation that a history of surgery for a previous cancer was an independent protective factor for the development of metastasis is in line with previous work, because surgery is part of the multidisciplinary management of CCA. Indeed, an R0 resection is a predictor of survival and recurrence.

The SMAD4 protein is part of the transforming growth factor-β pathway and is a tumor suppressor. SMAD4 mutations were detected in 24.2% of patients with CCA in the study by Tian et al. Yan et al. showed that the loss of SMAD4 expression was more frequent in metastatic ICC than in non-metastatic ICC, while the loss of SMAD4 expression has been
Table 2. Factors independently associated with metastasis (logistic regression).

| Characteristic                        | Metastasis (n = 69) | Non-metastasis (n = 22) | Univariable analysis | Multivariable analysis |
|---------------------------------------|---------------------|-------------------------|----------------------|------------------------|
|                                       | OR 95% CI P         | OR 95% CI P-value       |                      |                        |
| Age (years), median (range)           | 59 (30–78)          | 61 (35–72)              | 0.985 0.942, 1.031   | 0.525                  |
| Sex (female), n (%)                   | 20 (76.9)           | 6 (23.1)                | 1.088 0.372, 3.182   | 0.877                  |
| Liver cirrhosis, n (%)                | 10 (83.3)           | 2 (16.7)                | 1.695 0.342, 8.400   | 0.518                  |
| HBV infected, n (%)                   | 11 (15.9)           | 4 (18.2)                | 0.853 0.242, 3.011   | 0.805                  |
| Bile duct stones, n (%)               | 16 (23.2)           | 4 (18.2)                | 1.358 0.401, 4.598   | 0.622                  |
| History of biliary surgery, n (%)     | 20 (29)             | 17 (77.3)               | 0.120 0.039, 0.370   | <0.001                 |
| Family tumor history, n (%)           | 7 (10.1)            | 4 (18.2)                | 0.508 0.134, 1.932   | 0.320                  |
| TMB score, median (range)             | 2.65 (0–50)         | 4.3 (0.8–44.1)          | 0.966 0.916, 1.018   | 0.199                  |
| PD-L1 positive, n (%)                 | 11 (28.2)           | 6 (42.9)                | 0.524 0.147, 1.861   | 0.317                  |
| CA199 (U/mL), median (range)          | 211 (1–1000)        | 52.5 (8–1000)           | 1.003 1.003, 1.003   | 0.050                  |
| Tumor anatomy (ECC), n (%)            | 41 (59.4)           | 7 (31.8)                | 3.138 1.134, 8.682   | 0.028                  |
| Mutant genes with high mutation rate, n (%) |                   |                        |                      |                        |
| TP53                                  | 30 (43.5)           | 11 (50.0)               | 0.769 0.294, 2.013   | 0.593                  |
| ARID1A                                | 13 (18.8)           | 4 (18.2)                | 1.045 0.302, 3.610   | 0.945                  |
| KRAS                                  | 35 (50.7)           | 7 (31.8)                | 2.206 0.800, 6.079   | 0.126                  |
| CDKN2A                                | 13 (18.8)           | 5 (22.7)                | 0.789 0.246, 2.532   | 0.691                  |
| SMAD4                                 | 8 (11.6)            | 6 (27.3)                | 0.350 0.106, 1.153   | 0.084                  |
| PBRM1                                 | 4 (5.8)             | 5 (22.7)                | 0.209 0.051, 0.865   | 0.031                  |

OR: odds ratio; CI: confidence interval; HBV: hepatitis B virus; MSI: microsatellite instability; TMB: tumor mutational burden; PD-L1: programmed cell death ligand 1; CA199: carbohydrate antigen 19-9; ECC: extrahepatic; ARID1A: AT-rich interactive domain-containing protein 1A; CDKN2A: cyclin dependent kinase inhibitor 2A; SMAD4: SMAD family member 4; PBRM1: polybromo 1.
associated with the development of metastasis and resistance to chemotherapy. In the present study, mutations in SMAD4 were observed to be protective for the development of CCA metastasis. PBRM1 encodes the BAF180 protein, which is involved in various DNA repair mechanisms and centromere cohesion. Silencing of PBRM1 increases proliferation, migration, and colony formation, and PBRM1 can also act as an oncogene in some conditions, like in the absence of expression of hypoxia-inducible factor 1α. In the present study, PBRM1 mutations were found to be protective against metastasis. It is possible that specific mutations and the co-occurrence of different mutations drive oncogenic PBRM1, and that other PBRM1 mutations then protect the development of metastasis. The reasons for these discrepancies in the role of PBRM1 and SMAD4 in CCA are unknown, but they could reflect the study population, or the fact that only advanced CCA cases were included in our study. Additionally, the exact nature of the mutations was not assessed, the presence of activating mutations cannot be ruled out, and interactions with other genes and proteins were not examined. Future studies are needed to explore this.

In the present study, patient age was not associated with specific mutations in the six most commonly mutated genes in CCA. Feng et al. showed that young patients with CCA had more frequent mutations in ASXL1, lysine N-methyltransferase 2C, and ERBB3, but mutations in these genes were not common in the present study.

This study had some limitations, including its retrospective nature, small sample size, and single institution. The small sample size prevented the study of genes mutated at low frequencies. Therefore, a prospective, multi-center study with a larger sample size is needed to provide more in-depth evidence.

Mutations are thought to be a main cause of malignant tumors. Research into the tumor microenvironment and the function of various mutations aims to improve patient survival times and quality of life by enabling the targeted treatment of tumors.

Conclusions
The present study showed that a history of biliary surgery, as well as SMAD4 and PBRM1 mutations were independent protective factors for metastasis in patients with advanced CCA. These results could provide a genetic stratification for the risk of developing metastasis, and identify potential therapeutic targets for CCA.

Declaration of conflicting interest
The authors report no conflicts of interest.

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References
1. Valle JW, Borbath I, Khan SA, et al. Biliary cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 2016; 27: v28–v37. Epub 2016/09/25. doi: 10.1093/annonc/mdw324. PubMed PMID: 27664259.
2. NCCN clinical practice guidelines in oncology (NCCN guidelines). Hepatobiliary cancers. Version 5.2020. Fort Washington: National Comprehensive Cancer Network; 2020.
3. Razumilava N and Gores GJ. Cholangiocarcinoma. Lancet 2014; 383: 2168–2179. Epub 2014/03/04. doi: 10.1016/S0140-6736(13)61903-0.
4. Kanthan R, Senger JL, Ahmed S, et al. Gallbladder cancer in the 21st century. J Oncol 2015; 2015: 967472. Epub 2015/10/01. doi: 10.1155/2015/967472.

5. Rizzo A, Frega G, Ricci AD, et al. Anti-EGFR monoclonal antibodies in advanced biliary tract cancer: a systematic review and meta-analysis. In Vivo 2020; 34: 479–488; DOI: https://doi.org/10.21873/invivo.11798.

6. Nakamura H, Arai Y, Totoki Y, et al. Genomic spectra of biliary tract cancer. Nat Genet 2015; 47: 1003–1010. Epub 2015/08/11. doi: 10.1038/ng.3375.

7. Lin P, Zhong XZ, Wang XD, et al. Survival analysis of genome-wide profiles coupled with Connectivity Map database mining to identify potential therapeutic targets for cholangiocarcinoma. Oncol Rep 2018; 40: 3189–3198. Epub 2018/10/03. doi: 10.3892/or.2018.6710.

8. Mertens JC, Rizvi S and Gores GJ. Targeting cholangiocarcinoma. Biochim Biophys Acta Mol Basis Dis 2018; 1864: 1454–1460. Epub 2017/08/29. doi: 10.1016/j.bbadis.2017.08.027.

9. Churi CR, Shroff R, Wang Y, et al. Mutation profiling in cholangiocarcinoma: prognostic and therapeutic implications. PLoS One 2014; 9: e115383. Epub 2014/12/24. doi: 10.1371/journal.pone.0115383.

10. Lowery MA, Ptashkin R, Jordan E, et al. Comprehensive molecular profiling of intrahepatic and extrahepatic cholangiocarcinomas: potential targets for intervention. Clin Cancer Res 2018; 24: 4154–4161. Epub 2018/06/01. doi: 10.1158/1078-0432.CCR-18-0078.

11. Aitcheson G, Mahipal A and John BV. Targeting FGFR in intrahepatic cholangiocarcinoma: leading the way for precision medicine in biliary tract cancer. Expert Opin Investig Drugs 2021; 30: 463–477. doi: 10.1080/13543878.2021.1900821. Epub 2021 Apr 11.

12. Tian W, Hu W, Shi X, et al. Comprehensive genomic profile of cholangiocarcinomas in China. Oncol Lett 2020; 19: 3101–3110. Epub 2020/04/08. doi: 10.3892/ol.2020.11429.

13. Feng H, Tong H, Yan J, et al. Genomic features and clinical characteristics of adolescents and young adults with cholangiocarcinoma. Front Oncol 2019; 9: 1439. Epub 2020/02/06. doi: 10.3389/fonc.2019.01439.

14. Amin MB. AJCC Cancer Staging Manual, Eighth Edition. Chicago: American College of Surgeons; 2018.

15. Li H and Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009; 25: 1754–1760. Epub 2009/05/20. doi: 10.1093/bioinformatics/btp324.

16. Ebbert MT, Wadsworth ME, Staley LA, et al. Evaluating the necessity of PCR duplicate removal from next-generation sequencing data and a comparison of approaches. BMC Bioinformatics 2016; 17 Suppl 7: 239. Epub 2016/07/28. doi: 10.1186/s12859-016-1097-3.

17. Ye K, Schulz MH, Long Q, et al. Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. Bioinformatics 2009; 25: 2865–2871. Epub 2009/06/30. doi: 10.1093/bioinformatics/btp394.

18. Magi A, Tattini L, Cifola I, et al. EXCAVATOR: detecting copy number variants from whole-exome sequencing data. Genome Biol 2013; 14: R120. Epub 2013/11/01. doi: 10.1186/gb-2013-14-10-r120.

19. Nakagohri T, Asano T, Kinoshita H, et al. Aggressive surgical resection for hilar-invasive and peripheral intrahepatic cholangiocarcinoma. World J Surg 2003; 27: 289–293. Epub 2003/02/28. doi: 10.1007/s00268-002-6696-7.

20. Konstadoulakis MM, Roayaie S, Gomatos IP, et al. Fifteen-year, single-center experience with the surgical management of intrahepatic cholangiocarcinoma: operative results and long-term outcome. Surgery 2008; 143: 366–374. Epub 2008/02/23. doi: 10.1016/j.surg.2007.10.010.

21. Paik KY, Jung JC, Heo JS, et al. What prognostic factors are important for resected intrahepatic cholangiocarcinoma? J Gastroenterol Hepatol 2008; 23: 766–770. Epub 2007/09/18. doi: 10.1111/j.1440-1746.2007.05040.x.

22. Lang H, Sotiriopoulos GC, Sgourakis G, et al. Operations for intrahepatic
cholangiocarcinoma: single-institution experience of 158 patients. *J Am Coll Surg* 2009; 208: 218–228. Epub 2009/02/21. doi: 10.1016/j.jamcollsurg.2008.10.017.

23. Murakami Y, Uemura K, Sudo T, et al. Prognostic factors after surgical resection for intrahepatic, hilar, and distal cholangiocarcinoma. *Ann Surg Oncol* 2011; 18: 651–658. Epub 2010/10/15. doi: 10.1245/s10434-010-1325-4.

24. Ribero D, Pinna AD, Guglielmi A, et al. Surgical approach for long-term survival of patients with intrahepatic cholangiocarcinoma: a multi-institutional analysis of 434 patients. *Arch Surg* 2012; 147: 1107–1113. Epub 2012/08/23. doi: 10.1001/archsurg.2012.1962.

25. Zhao M, Mishra L and Deng CX. The role of TGF-beta/SMAD4 signaling in cancer. *Int J Biol Sci* 2018; 14: 111–123. Epub 2018/02/28. doi: 10.7150/ijbs.23230.

26. Yang G and Yang X. Smad4-mediated TGF-beta signaling in tumorigenesis. *Int J Biol Sci* 2010; 6: 1–8. Epub 2010/01/21. doi: 10.7150/ijbs.6.1.

27. Yan XQ, Zhang W, Zhang BX, et al. Inactivation of Smad4 is a prognostic factor in intrahepatic cholangiocarcinoma. *Chin Med J (Engl)* 2013; 126: 3039–3043. Epub 2013/08/29.

28. Xourafas D, Mizuno T and Cloyd JM. The impact of somatic SMAD4 mutations in colorectal liver metastases. *Chin Clin Oncol* 2019; 8: 52. Epub 2019/09/11. doi: 10.21037/cco.2019.08.04.

29. Kakarougkas A, Ismail A, Chambers AL, et al. Requirement for PBAF in transcriptional repression and repair at DNA breaks in actively transcribed regions of chromatin. *Mol Cell* 2014; 55: 723–732. Epub 2014/07/30. doi: 10.1016/j.molcel.2014.06.028.

30. Brownlee PM, Chambers AL, Cloney R, et al. BAF180 promotes cohesion and prevents genome instability and aneuploidy. *Cell Rep* 2014; 6: 973–981. Epub 2014/03/13. doi: 10.1016/j.celrep.2014.02.012.

31. Varela I, Tarpey P, Raine K, et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature* 2011; 469: 539–542. Epub 2011/01/21. doi: 10.1038/nature09639.

32. Murakami A, Wang L, Kalhorn S, et al. Context-dependent role for chromatin remodeling component PBRM1/BAF180 in clear cell renal cell carcinoma. *Oncogenesis* 2017; 6: e287. Epub 2017/01/17. doi: 10.1038/oncsis.2016.89.

33. Gordan JD, Bertout JA, Hu CJ, et al. HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. *Cancer Cell* 2007; 11: 335–347. Epub 2007/04/10. doi: 10.1016/j.ccr.2007.02.006.