Mutation spectrum associated with metastasis of advanced cholangiocarcinoma

Hao Song[1] [ID]**, Yao Huang[2] and Xiaoqing Jiang[3]**

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.

[3]Department of Biliary Surgery, Eastern Hepatobiliary Surgery Hospital Affiliated to Shanghai Second Military Medical University, Shanghai, China

[1]Department of Organ Transplantation, Eastern Hepatobiliary Surgery Hospital Affiliated to Shanghai Second Military Medical University, Shanghai, China
[2]Department of Transplantation, Xinhua Hospital Affiliated to Shanghai JiaoTong University School of Medicine, Shanghai, China

Corresponding author:
Xiaoqing Jiang, Department of Biliary Surgery, Eastern Hepatobiliary Surgery Hospital Affiliated to Shanghai Second Military Medical University, No. 700 Moyu North Road, Jiading District, Shanghai 201805, China.
Email: jxq1225@sina.com

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.[1] They mostly occur during or after the seventh decade of life,[1] and are typically diagnosed at a late stage and characterized by poor outcomes.[2–4] Cholangiocarcinoma (CCA) is an invasive carcinoma of the biliary tract, arising in the bile duct epithelium.[2–4] 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%.[5] CCAs can be intrahepatic (ICC) or extrahepatic (ECC).[1–3] They are slightly more common in men than women, except among people of Hispanic ethnicity.[3] 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.[2–4]

Driver gene mutations in CCA include those in E74 like ETS transcription factor 3, AT-rich interactive domain-containing protein (ARID)1, and *ARID2*.[6] CCAs also demonstrate the enrichment of genes involved in Wnt signaling, apoptosis, and oncogenic pathways,[7] 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.[8]

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.[9] 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.[9] Of note, about 50% of CCAs carry mutations that have therapeutic implications.[10]

The increasing popularity of next-generation DNA sequencing (NGS) provides an opportunity to tailor therapy to potential targets.[11] NGS is a powerful technology that allows accurate, efficient, and large-scale genome sequencing. Tian et al.[12] 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.[13] 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 $0.5\,cm^3$ 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 $900\times$ for FFPE samples and $300\times$ 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[TM], OrigiMed, Shanghai, China). NGS was then performed with a mean coverage of $900\times$ for tumor tissues and $300\times$ 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 program[17] 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 counter stained 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

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-LI: 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.

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
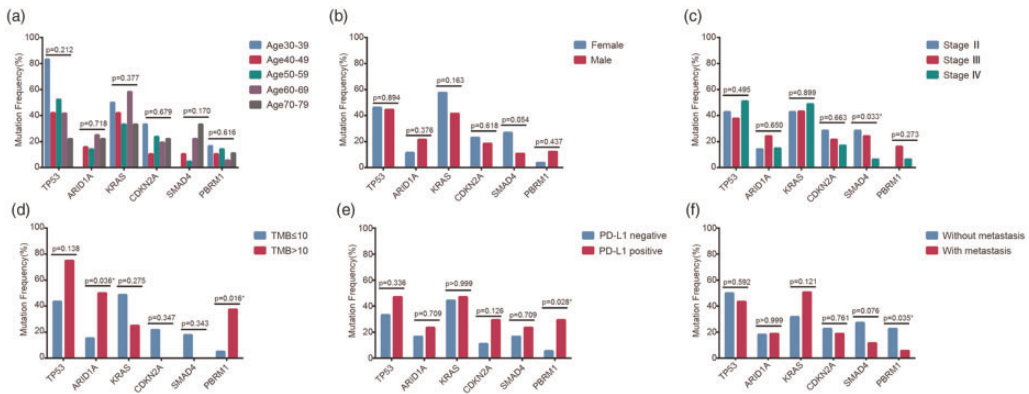
Figure 1. Genetic mutations with respect to patient clinical characteristics. (a) Age. (b) Sex (male and female). (c) Clinical stages (II, III, and IV). (d) Tumor mutational burden (TMB) levels (high: >10; low: ≤10). (e) PD-L1 expression (positive, negative). (f) Metastasis status (metastasis, no metastasis).

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.[12] 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.[10] 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.[1,2] Indeed, an R0 resection is a predictor of survival and recurrence.[19–24]

The SMAD4 protein is part of the transforming growth factor-β pathway and is a tumor suppressor.[25,26] *SMAD4* mutations were detected in 24.2% of patients with CCA in the study by Tian et al.[12] Yan et al.[27] 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	0.114	0.033, 0.390	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-LI 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, 1.006	0.050	1.003	1, 1.006	0.057
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	0.202	0.045, 0.903	0.036
PBRM1	4 (5.8)	5 (22.7)	0.209	0.051, 0.865	0.031	0.175	0.033, 0.922	0.040

OR: odds ratio; CI: confidence interval; HBV: hepatitis B virus; MSI: microsatellite instability; TMB: tumor mutational burden; PD-LI: programmed cell death ligand I; 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.[28] 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.[29,30] Silencing of *PBRM1* increases proliferation, migration, and colony formation,[31] and *PBRM1* can also act as an oncogene in some conditions, like in the absence of expression of hypoxia-inducible factor 1α.[32,33] 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.[13] 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.

ORCID iD

Hao Song 🆔 https://orcid.org/0000-0001-7180-3343

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