



# Molecular profiling and prognostic analysis in Chinese cholangiocarcinoma: an observational, retrospective single-center study

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## Abstract

Cholangiocarcinoma (CCA) is a primary malignancy which is often diagnosed when it is advanced and inoperable due to the lack of effective biomarkers and poor sensitivity of clinical diagnosis. Molecular profiling may provide information for improved clinical management, particularly targeted therapy. The study aimed to improve the understanding of molecular characteristics and its association with prognosis in Chinese CCA. We enrolled 41 Chinese patients with CCA, including 6 intrahepatic CCA (iCCA), 14 perihilar CCA (pCCA), and 21 distal CCA (dCCA) cases, all patients underwent radical operations and tumor samples underwent next-generation sequencing (NGS) by Foundation One Dx, which analyzed 324 genes. The patients' genetic characteristics, clinical management, and prognosis were analyzed. The most mutated genes were TP53 (68%, 28/41), *CDKN2A* (37%, 15/41), and *SMAD4* (29%, 12/41). The genetic mutations in dCCA, pCCA, and iCCA were significantly different. For example, *NOTCH3* mutations were not found in dCCA. The gene mutations of *AXL* were specifically associated with lymph node metastasis in patients with CCA, whereas gene mutations of *SMAD4* were specifically associated with lymphovascular invasion. Furthermore, mutations in *APC*, *DAXX*, *FANCA*, *LTK*, *MAP2K4*, and *NOTCH1* were associated with a poor prognosis ( $P < 0.05$ ). This study provides an overview of genetic alterations in Chinese patients with CCA, which will provide novel potential biomarkers for the diagnosis of CCA and may guide targeted therapeutic strategies for Chinese patients with CCA.

**Keywords** Cholangiocarcinoma · Genomic mutation signature · Target therapy · Immune therapy · Progression-free survival · Biomarkers

## Introduction

Despite the fact that the epidemiology of cholangiocarcinoma (CCA) varies greatly across the globe, the overall incidence is increasing [1]. It is classified as intrahepatic (iCCA) or extrahepatic (eCCA) based on its anatomical origin, with the second-order bile ducts serving as the dividing line [2]. The second most common type of primary liver

cancer is intrahepatic cholangiocarcinoma (iCCA), accounting for 1% of all neoplasms [3, 4]. Furthermore, depending on whether it originates above or below the cystic duct, eCCA is classified as perihilar (pCCA) or distal (dCCA) [2]. The etiopathogenesis of these subtypes differs, with distinct risk factors [1, 2], proposed cells of origin [5, 6], and specific genomic aberrations [6, 7].

For patients in the early stages, surgical resection is currently the mainstay of curative-intent treatment; however, most patients will not benefit from radical surgery [3, 8, 9]. However, the insensitivity of CCA to radiotherapy and chemotherapy results in a poor prognosis [10, 11]. Biomarker-based targeted therapy and immunotherapy are effective treatments for some malignant tumors. There are, however, few effective biomarkers for CCA, and these need to be researched and developed to facilitate early detection and diagnosis.

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The heterogeneity of CCA hinders targeted treatment for CCA subtypes that differ in their response to treatment [12]. With the development of next-generation sequencing (NGS) technology, we can identify molecular differences between subtypes. Recent research has revealed that iCCA and eCCA have distinct molecular characteristics [13, 14]. NGS studies have revealed the genomic heterogeneity of CCA subtypes, which may impact future therapy trials [13]. Although extrahepatic CCA can be divided into pCCA and dCCA, their prognosis differs. Waseem et al. reported that the mean survival of pCCA was lower than that of dCCA, but comparable to that of iCCA [15]. However, few studies have isolated pCCA and dCCA and focused on their genomic characteristics.

We enrolled 41 Chinese patients with CCA to characterize their genomic profiles according to the different subtypes. We aimed to identify potential biomarkers for prognosis and provide evidence for targeted therapy and immunotherapy.

## Materials and methods

### Patient information and sample collection

From 2019 to 2020, 41 CCA patients were enrolled from Peking University People's Hospital according to the tumor locations. Informed consent was obtained from all patients and this study was approved by the Institutional Ethics Committee of Peking University People's Hospital (2021PHB279-001). The patients were given the necessary jaundice-reducing treatment based on the results of computed tomography or magnetic resonance imaging. Surgical resection was performed once the total bilirubin level was less than 5 times normal. The tumor tissue samples were fixed in formalin for 24 h before being embedded in paraffin. Tumor tissues that have been formalin-fixed, paraffin-embedded (FFPE), and contain at least 20% tumor cells are considered tumor tissue samples and can be used for further NGS detection. For those cases, relevant demographic and clinical data from the database were extracted, including age, gender, date of diagnosis, histology type, and evaluation of treatment responses based on clinical investigator reports. Pathologists confirmed each case's pathologic diagnosis and tumor content based on tumor tissue samples.

### DNA extraction and library preparation

Between May and November 2020, all tissue samples were sent to Foundation Medicine for analysis. NGS was performed in tumor tissue samples with at least 20% tumor cellularity and sequencing panels of 324 genes (FoundationOne® CDx) (<https://www.foundationmedicine.com/>

[genomic-testing/foundation-one-cdx](#)), respectively. Foundation Medicine provided the tests as part of an expanded access program at no cost to patients or institutions. The NGS report was sent to the ordering physician to discuss the treatment plan with the patient. Between May, 2020 and July, 2020, all the reports and baseline characteristics were collected at Peking University People's Hospital, and results were retrospectively analyzed.

### Sequence data processing

The qualified DNA libraries were sequenced on the Illumina NovaSeq6000 platform (Illumina, San Diego, CA) and produced 150 bp paired-end reads. Base calls from the Illumina NovaSeq6000 were made to FASTQ files. An automated data management system monitored the sequencers and started the analysis pipeline when the sequencing run finished. BWA MEM was used to align sequence reads to the human genome (hg19). To reduce alignment artifacts, ABRA was used to realign reads around indels, and the Genome Analysis Toolkit was used to recalibrate base quality scores. Duplicate reads were flagged for removal, and the resulting BAM files were used to discover variants. MuTect, Pindel, and Somatic Indel Detector union calls were then subjected to automated filtering to produce a comprehensive list of somatic mutation calls that included single nucleotide variants, short and long indels. Copy number changes were detected using an in-house developed algorithm, and structural variants were detected using Delly. Germline variants were eliminated by using patient-matched blood DNA. Each alteration identified by the pipeline was manually reviewed to ensure that no false positives were reported, complex events identified separately were merged and properly represented, and mutation annotations were Human Genome Variation Society (HGVS, <http://varnomen.hgvs.org>) compliant [16].

### The public cohort

All clinical and genomic data of 346 CCA patients were acquired from cBioPortal (<http://www.cbioportal.org>) [17] and the ICGC portal (<http://dcc.icgc.org/releases/current/Projects>). We selected the WES dataset for CCA patients only. The repositories used were BTCA-JP [18], BTCA-SG [19], TCGA-CHOL (TCGA, Pan Cancer Atlas) [20].

### Statistical analysis

The Kaplan-Meier curve analysis OS and PFS was compared using the log-rank test. The Cox proportional hazards regression was applied for univariable and multivariate analysis, and available confounding factors including sex,

age, subtype, and TMB level were adjusted. Student's t-test was used to compare continuous variables, and the  $\chi^2$  test or Fisher's exact test was used to compare categorical data. All reported P values were two-tailed, and  $P < 0.05$  was considered statistically significant. Statistical analyses were performed using R v. 4.0.3 (<https://www.r-project.org>).

## Results

### Clinical characteristics of Chinese CCA patients

A total of 41 patients with CCA, 34 (82.9%) males and 7 (17.1%) females, with a median age of 64 years (range 42–78 years) were enrolled in this study. Twenty-one of the 41 patients harbored hepatitis B virus and one harbored hepatitis C virus, 19 patients were negative. In all, 34.1% of the patients were lymph-node metastasis positive. There

were 14.6% iCCA, 34.1% pCCA, and 51.3% dCCA tumors. All patients were treated surgically and 39 (95.1%) patients had R0 resection. Thirty patients received postoperative adjuvant chemotherapy, 8 did not, and 3 patients' postoperative treatment was unknown. Intraoperative exploration showed 1 (2.4%) patient had vascular invasion, 39 (95.1%) had none, and 1 patient had no information. Histologically, 26 (63.4%) samples were well or moderately differentiated and 14 (34.2%) samples were poorly differentiated or undifferentiated. Histological information was not available for 1 (2.4%) patient. The patients' clinical and pathological information is shown in Table 1.

### Genomic alterations in Chinese CCA

A total of 545 clinically relevant genomic alterations in 199 genes were identified in 41 patients with CCA. The alterations included 351 (64.4%) substitution/indels, 97 (17.80%)

**Table 1** Clinicopathologic features of 41 Chinese CCA patients

Total	dCCA	pCCA	iCCA	P-value
<b>Age</b>				
Median (range)	65(56–74)	62(51–78)	59.5(42–65)	
< 65	7	6	5	0.093
≥ 65	14	8	1	
<b>TMB</b>				
Median (range)	1.26(0-11.35)	2.52(0-22.7)	1.26(1-5.04)	
<b>Gender</b>				
Male	17	12	5	0.934
Female	4	2	1	
<b>Hepatitis virus</b>				
HBV Positive	12	6	3	0.741
HCV Positive	1	0	0	
Negative	8	8	3	
<b>Metastatic lymph nodes</b>				
Yes	5	7	2	0.277
No	16	7	4	
<b>Vascular invasion</b>				
Yes	0	0	1	0.141
No	20	14	5	
Not available	1	0	0	
<b>Vascular cancer embolus</b>				
Yes	5	3	2	0.848
No	16	11	4	
<b>Resection margins, n (%)</b>				
R0	20	13	6	0.793
R1	1	1	0	
<b>Postoperative chemotherapy</b>				
Yes	13	11	6	0.261
No	5	3	0	
Not available	3	0	0	
<b>Histology</b>				
Well/moderately differentiated	13	10	3	0.486
Poorly/undifferentiated	8	3	3	
Not available	0	1	0	

truncations, 13 (2.39%) splice site mutations, 57 (10.46%) gene amplifications, and 27 (4.95%) gene deletion. The most mutated genes were *TP53* (68%, 28/41), *CDKN2A* (37%, 15/41), *SMAD4* (29%, 12/41), *ATM* (22%, 9/41), *MLL2* (22%, 9/41), *ERBB2* (22%, 9/41), *KRAS* (20%, 8/41), *IRS2* (17%, 7/41), and *ARID1A* (17%, 7/41) (Fig. 1A). The genetic mutations in dCCA, pCCA, and iCCA were significantly different. The most mutated genes in dCCA were *TP53* (76%, 16/21), *SMAD4* (33%, 7/21), and *ATM* (33%, 7/21). While in pCCA they were *TP53* (64%, 9/14), *CDKN2A* (57%, 8/14), and *SMAD4* (36%, 5/14). In iCCA they were *TP53* (50%, 3/6), *MLL2* (33%, 2/6), and *STK11* (33%, 2/6) (Fig. 1A). However, only the *NOTCH3* gene was significantly different between the three groups, as it did not mutate in the patients with dCCA (Fig. 1B).

We identified the tumor mutation burden (TMB) value of 41 patients with clinically relevant genomic alterations. The median TMB value was 1.26 mutations/million bases (Mb), ranging from 0 to 22.7 mutations/Mb. There was no significant difference in TMB among the three groups (Fig. 1C).

### Correlations between mutated genes and clinical characteristics

To explore potential biomarkers, we analyzed the association between mutations and clinical characteristics, such as visceral organ, nerve, and vascular invasion, lymph node metastasis, and lymphovascular invasion. Only lymph node metastasis and lymphovascular invasion were significantly

associated with gene mutations. Thirteen patients had lymph node metastases and 10 patients, lymphovascular invasion. There was a significant association between *AXL* mutation and lymph node metastasis ( $P=0.027$ ) (Fig. 2A), and *SMAD4* mutation and lymphovascular invasion ( $P=0.045$ ) (Fig. 2B).

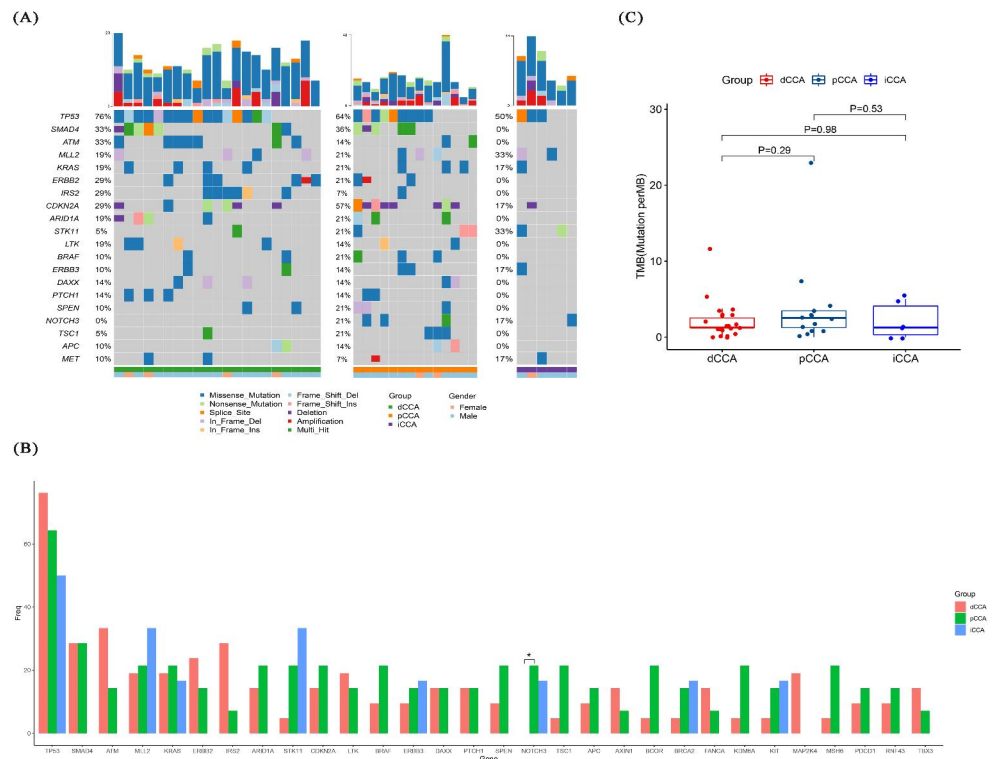
### Genetic mutations associated with progression-free survival (PFS)

Thirty-six patients who had radical surgery were followed up. The median progression-free survival (PFS) was not reached. Taking PFS as a continuous variable, it was associated with *APC*, *DAXX*, *FANCA*, *LTK*, *MAP2K4*, and *NOTCH1* mutations, and PFS. Survival curve analysis showed that patients with these mutations had a shorter PFS time (Fig. 3). To confirm this result, we performed multivariate Cox regression analysis of gender, age, TMB, and other clinical characteristics. The results showed that *APC*, *DAXX*, *FANCA*, *LTK*, and *MAP2K4* ( $P<0.05$ ) mutations were still significantly associated with a shorter PFS, while *NOTCH1* mutations were not (Fig. 4); the association between *NOTCH1* mutations and PFS was easily neutralized by other clinical characteristics.

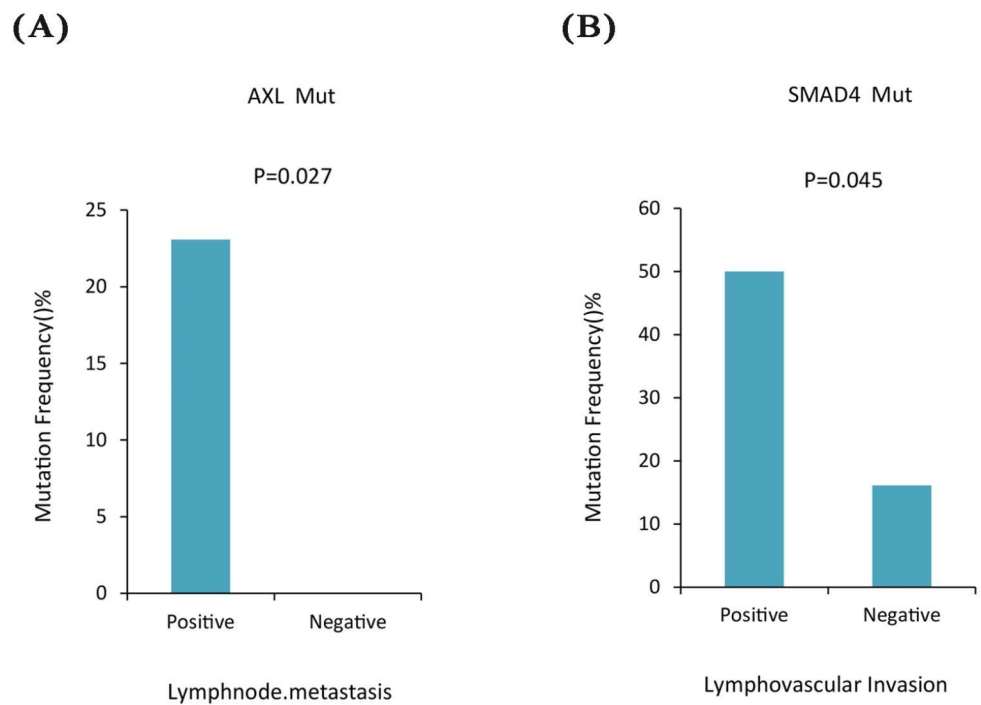
### Actionable target mutations of Chinese CCAs

Actionable alterations in various types of cancers were collected and summarized by the OncoKB team, and 17

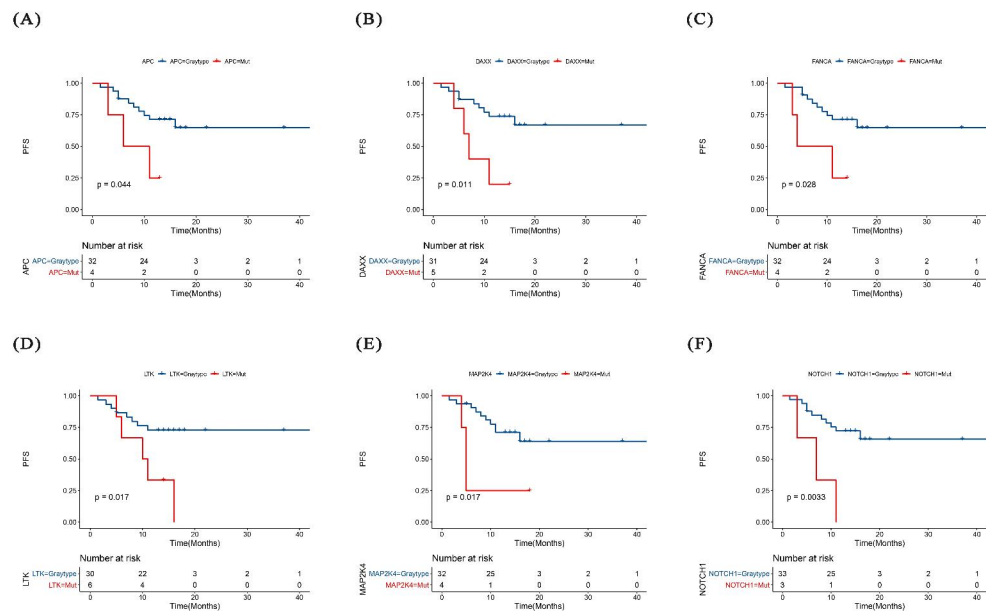
**Fig. 1** Mutation frequency and significantly mutated genes in Chinese CCA. **(A)** Mutational spectrum of patients with CCA. **(B)** Comparative analysis of high frequently mutated genes in pCCA (green), iCCA (blue), and dCCA (orange). The X-axis represents the most mutated genes and the Y-axis represents the mutation frequency of each gene in different CCA subtypes. The significant differences were marked with \* for  $P<0.05$ , \*\* for  $P<0.01$ , and \*\*\* for  $P<0.001$ , NA not available. **(C)** The tumor mutation burden (TMB) of non-synonymous mutations (substitutions and indels) between dCCAs (presented in red), pCCAs (presented in navy blue), and iCCAs (presented in blue)



**Fig. 2** The correlation between mutated genes and lymph node metastasis and lymphovascular invasion. **(A)** *AXL* mutation was associated with lymph node metastasis. **(B)** *SMAD4* mutation was associated with lymphovascular invasion



**Fig. 3** Correlation analysis between mutated genes and progression-free survival (PFS). Kaplan–Meier curves of the PFS in patients with (red)/without (blue) *APC* **(A)**, *DAXX* **(B)**, *FANCA* **(C)**, *LTK* **(D)**, *MAP2K4* **(E)**, and *NOTCH1* mutations

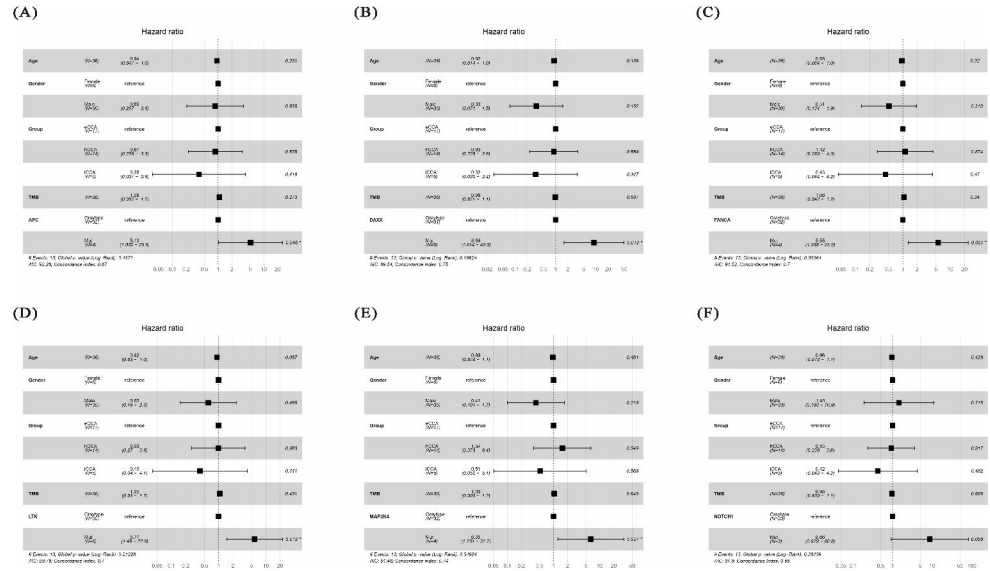


clinically relevant genes with 26 potential therapies for CCA were examined [21]. Potential target drugs for KRAS mutations included asadagrasib, cobimetinib, binimetinib, and trametinib, while debio1347, infgratinib, erdafitinib, and AZD4547 were potential target drugs for FGFR mutations. Trametinib and cobimetinib were potential target drugs for NF1 mutations. In this cohort, there were 7 actionable mutated genes in 41 (17.1%) patients with CCA. The most common drug target mutations were *KRAS* (17.1%), *BRAF* (12.2%), *NF1* (4.9%), and *FGFR3* (4.9%) (Table 2). Cobimetinib and trametinib are the potential target drugs

for both *KRAS* mutation and *NF1* mutations, and our data showed that 21.9% (9/41) of patients with CCA harboring *KRAS* mutations or *NF1* mutations might benefit from them.

Further, the actionable target mutations were different in the three groups. In patients with dCCA, the most common drug target mutations were *KRAS* (19.1%) and *BRAF* (9.5%). Each patient harbored *NF1*, *FGFR3*, and *CDK12* (Table 2). In patients with pCCA, 3 patients harbored *KRAS* (21.4%) and *BRAF* (21.4%), and only one patient had *NF1* (7.1%). In patients with iCCA, each patient harbored *KRAS*, *FGFR2*, *FGFR3*, and *MET*.

**Fig. 4** Multivariate cox regression analysis to confirm the correlation between PFS and genetic mutations. *APC*(A), *DAXX*(B), *FANCA*(C), *LTK*(D), *MAP2K4*(E), and *NOTCH1* mutations (F). Forest plot showed the risk of PFS in various sub-groups of patients such as gender, age, and tumor grade



**Table 2** Comparative analysis of druggable genes in pCCA, iCCA, and dCCA

Genes	Drugs	dCCA (n,%)	pCCA (n,%)	iCCA (n,%)	Total CCA
<i>KRAS</i>	Adagrasib, Cobimetinib, Binimetinib, Trametinib	4, 19.1%	3, 21.4%	1, 16.7%	7, 17.1%
<i>BRAF</i>	PLX8394	2, 9.5%	3, 21.4%	0, 0	5, 12.2%
<i>NF1</i>	Cobimetinib, Trametinib	1, 4.8%	1, 7.1%	0, 0	2, 4.9%
<i>FGFR3</i>	AZD4547, Erdafitinib, Debio1347, Infigratinib	1, 4.8%	0, 0	1, 16.7%	2, 4.9%
<i>FGFR2</i>	Infigratinib, Pemigatinib, Futibatinib	0, 0	0, 0	1, 16.7%	1, 2.4%
<i>CDK12</i>	Pembrolizumab, Nivolumab, Cemiplimab	1, 4.8%	0, 0	0, 0	1, 2.4%
<i>MET</i>	Crizotinib	0, 0	0, 0	1, 16.7%	1, 2.4%

**Table 3** Comparative analysis of hyperprogressive disease (HPD) associated genetic alterations in pCCA, iCCA, and dCCA

Genes	dCCA (n, %)	pCCA (n, %)	iCCA (n, %)	Total CCA
<i>CDKN2A</i> loss	3, 14.28%	5, 35.71%	1, 16.67%	9, 21.9%
<i>CDKN2B</i> loss	3, 14.28%	5, 35.71%	1, 16.67%	9, 21.9%
<i>CCND1</i> amplification	3, 14.28%	1, 7.14%	0, 0	4, 9.8%
<i>FGF3</i> amplification	3, 14.28%	1, 7.14%	0, 0	4, 9.8%
<i>FGF4</i> amplification	3, 14.28%	1, 7.14%	0, 0	4, 9.8%
<i>FGF19</i> amplification	3, 14.28%	1, 7.14%	0, 0	4, 9.8%
<i>EGFR</i> amplification	1, 4.76%	0, 0	1, 16.67%	2, 4.9%

**Hyperprogressive disease (HPD) associated genetic alterations**

In recent years, with the emergence of precision medicine therapies such as targeted therapy and immunotherapy, the treatment of biliary tract cancer (BTC) has entered a new era. The FDA approved durvalumab in combination with chemotherapy for the first-line treatment of locally advanced or metastatic BTC in 2022. Increasing clinical trial data supports the effectiveness of immunotherapy with chemotherapy in the treatment of CCA. However, some patients still did not benefit from the combination and were even at risk of hyperprogressive disease (HPD). Therefore, we analyzed gene variants associated with HPD (Table 3). There were 7 HPD-associated genetic alterations, *CDKN2A* loss

(9, 21.9%), *CDKN2B* loss (9, 21.9%), *CCND1* amplification (4, 9.8%), *FGF3* amplification (4, 9.8%), *FGF4* amplification (4, 9.8%), *FGF19* amplification (4, 9.8%), and *EGFR* amplification (2, 4.9%). The distribution of HPD-associated genetic alterations in the three groups differed. The pCCA group showed a higher rate in HPD-associated genetic alterations (36%, 5/14), followed by dCCA (24%, 5/21), and iCCA (17%, 1/6).

**Differences in genomic alterations between Chinese and western patients with CCA**

In 346 public cohort CCA samples in Western patients, the most common mutations were *TP53*, *KRAS*, *ARID1A*, *SMAD4*, and *APC*. In 41 Chinese patients with CCA, the

mutations of *TP53*, *SMAD4*, and *KRAS* also frequently occurred. Based on statistical analysis, the mutations of *TP53*, *ATM*, *IRS2*, and *LTK* were significantly higher in Chinese patients ( $P < 0.01$ ) (Fig. 5).

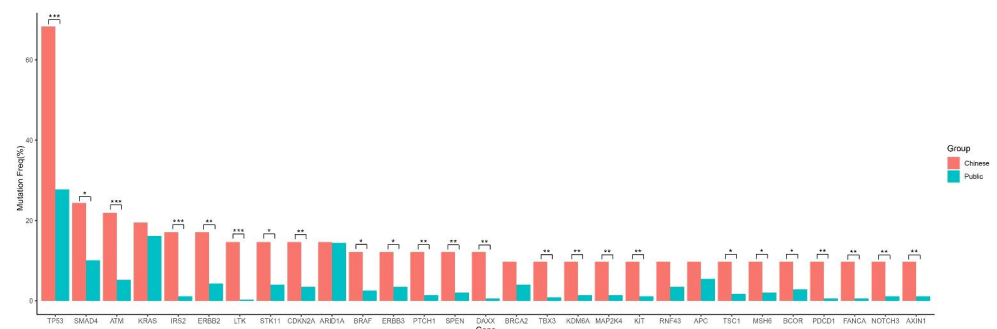
## Discussion

Cholangiocarcinoma (CCA) is a highly lethal and aggressive cancer of the biliary epithelium [22]. Although the CCA subtypes differ in their presentation and natural history, and necessitate different approaches to diagnosis and management [23, 24], there are few reports of the differences in their molecular characteristics. We enrolled 41 patients with CCA, including 6 iCCA, 14 pCCA, and 21 dCCA, and identified their mutational profiles. The greatest difference in genetic mutation occurred with *NOTCH3*, as none of the patients with dCCA showed *NOTCH3* mutations. NOTCH is a master regulator of cell fate in the mammalian liver, and reactivation of the NOTCH pathway has been reported to varying degrees in biliary malignancies. *NOTCH3* was found to be important in the development and progression of CCA [25]. O'Rourke et al. [26] discovered a network-wide imbalance of the NOTCH pathway in CCA, including a relationship between *NOTCH1* and *NOTCH3* and *NOTCH* ligands, and proposed  $\gamma$ -secretase modulation as a therapeutic option. However, because *NOTCH3* mutations were not found in dCCA patients, the pathogenesis of dCCA and iCCA/pCCA may differ, and these genes may be specific and potential tumor biomarkers in dCCA.

Many studies on potential biomarkers for CCA have been conducted, and revealed different molecular characteristics in patients from various regions [14, 23]. Another research focused on Chinese pCCA and iCCA founded that top 10 mutated genes were *TP53*, *KRAS*, *ARID1A*, *SMAD4*, *PBRM1*, *NF1*, *MACF1*, *GNAS*, *PIK3CA*, and *EPHA2*, while our pCCA and iCCA were *TP53*, *CDKN2A*, *MLL2*, *STK11*, *KRAS*, *NOTCH3*, *ERBB3*, *SMAD4*, *MET*, and *ERBB2*. The difference could be attributed to the relatively small number of our patients, particularly those with iCCA; on the other hand, it could be attributed to the fact that *MACF1* and *EPHA2* were not included in the FoundationOne® CDx

gene list. However, *CDKN2A* deletion one of the genes with significant copy number alteration, indicating that *CDKN2A* deletion was important for patients with pCCA and iCCA [27]. Our findings, when combined with gene mutation data of Japanese liver cancer displaying biliary phenotype (LCB, which means iCCA and combined hepatocellular cholangiocarcinoma) [28] and Thai iCCA [29] revealed that *KRAS* was important for Asian iCCA patients. Furthermore, the top mutated gene in Chinese and Thai iCCA patients was *TP53*. The gene mutations of the iCCA in our study were also significantly different from those of patients in Japan and Thailand. For example, the top mutated genes in iCCA were *TP53*, *MLL2*, *STK11*, *KRAS*, *IRS2*, *ERBB3*, *NOTCH3*, and *MET*. In Japan, they were primarily *TERT* promoter, *KRAS*, *IDH* family (*IDH1* or *IDH2*), *PBRM1*, *ARID2*, *BAP1*, *PCLCLO*, *ODZ1*, and *XIRP2*. The most common mutations in Thailand's iCCA were *TP53*, *ARID1A*, *KRAS*, *SMAD4*, *APC*, *KMT2C*, *GNAS*, *LRP1B*, *CSMD3*, and *ERBB2*. The differences could be attributed to regional differences (Japan, Thailand, and China), differences in sequencing technology (WGS vs. targeted panel sequencing), and large sample numbers. Our study and another Thailand's CCA genetic data [19] revealed that *TP53*, *ERBB2*, and *ARID1A* were the main driver genes. Additionally, the most frequently mutated genes in our CCA patients included *SMAD4*, *ATM*, *MLL2*, *KRAS*, *IRS2*, and *CDKN2A*, whereas in Thailand, the most commonly mutated genes contained *FGFR*, *BAP1*, and *IDH1/2*. Recently, Lowery et al. [24] reported the genomic profiling of patients with CCA, including 152 iCCA and 43 eCCA cases, from Caucasian (89.2%, 174/195), Asian (7.1%, 14/195), and African American (3.6%, 7/195) patients. *IDH1*, *TP53*, *ARID1A*, *BAP1*, *KRAS*, *PBKMI*, *SMAD4*, and *ATM* were the most common mutations. In addition, Tian et al. [14] reported that mutations of *TP53*, *KRAS*, and *SMAD4* were significantly higher in Chinese patients, whereas the mutations of *IDH1* and *BAP1* were significantly lower than in Western patients. We detected common high-frequency mutations (> 10%) of *TP53*, *KRAS*, *SMAD4*, *TERT*, and *ARID1A* in Chinese CCA patients; compared with Western patients, *TP53*, *ATM*, *IRS2*, and *LTK* mutations were significantly higher ( $P < 0.001$ ).

**Fig. 5** Comparative analysis of high frequency mutant genes in Chinese and Western patients with CCA. The X-axis represents the most mutated genes and the Y-axis represents the mutation frequency of each gene in different CCA subtypes. The significant differences were marked with \* for  $P < 0.05$ , \*\* for  $P < 0.01$ , and \*\*\* for  $P < 0.001$



There have been many studies of the association between genetic mutations and patients' clinical characteristics. Tian et al. [14] assessed gender-associated specific genomic characteristics in Chinese patients with CCA. They identified *CCND1*, *FBXW7*/*FGF3/4/19*, *PIK3C1*, *NF1* and *STK11* as mutations specific to male patients, while mutations in *ERBB2*, *AXIN2*, *CREBBP*, *ERBB3*, *MTOR* and *MYB* were specific to female patients. Feng et al. [30] identified that patients with *KRAS* mutations may have a higher risk of vascular invasion and poor prognosis. However, little is known about the genomic characteristics associated with lymph node metastasis and lymphovascular invasion in patients with CCA. The present study identified an association between *AXL* mutation and lymph node metastasis ( $P=0.027$ ), and *SMAD4* mutation and lymphovascular invasion ( $P=0.045$ ). *AXL* is a receptor tyrosine kinase involved in many pathological conditions, including carcinogenesis. It has been previously reported to be significantly associated with lymph node metastasis in CCA [31], and in most melanoma lymph node metastases [32]. Its expression was associated with differentiation grade, TNM stage, lymph node, and distant metastasis in solid malignancies [33]. The genetic mutations may affect the expression or function of *AXL*, which is then associated with lymph node metastasis in CCA. *SMAD4* is found to be mutated in many cancers. It acts as a tumor suppressor regulating the TGF- $\beta$  signaling pathway. The expression of *SMAD4* was associated with the histological grade, clinical stage, and metastasis of ICC ( $P<0.05$ ) [34]. A history of biliary surgery and mutations in *SMAD4* were independent protective factors for metastasis in patients with advanced CCA [35]. The association of *SMAD4* with lymphovascular invasion in CCA was first identified in the present study, and the specific molecular mechanisms need to be studied further.

We investigated the possible link between genetic alterations and prognosis and found worse outcomes for patients with certain genetic mutations although statistically, this was of borderline significance. Our results showed that *APC*, *DAXX*, *FANCA*, *LTK*, and *MAP2K4* mutations were significantly associated with a shorter PFS ( $P<0.05$ ). The adenomatous polyposis coli (*APC*) gene is the most frequently mutated in colon cancer and is responsible for familial adenomatous polyposis. Mutations in the *APC* gene play a critical role in the adenoma-carcinoma pathway of colorectal tumorigenesis [36, 37]. In addition, downregulated *APC* expression was associated with a poor prognosis in patients with esophageal squamous cell carcinoma [38]. Loss of heterozygosity was displayed close to the *APC* gene in iCCA [39]. It was first identified that the mutations of *APC* were associated with CCA prognosis. The death domain-associated protein encoded by the *DAXX* gene is involved in transcriptional regulation, cell apoptosis, carcinogenesis,

and anti-virus infection. *DAXX* is a transcriptional repressor that regulates p53 chromatin binding and DNA damage response [40]. It has been linked to a number of cancers, and its role in tumorigenesis and development is complex. By activating the ERK signaling pathway, it promotes ovarian cancer ascites, cell proliferation, and migration [41]. It also promotes prostate cancer tumorigenicity by suppressing autophagy [42], and inhibits cancer stemness and epithelial-mesenchymal transition in gastric cancer [43]. Its role in the development of CCA remains unknown. *FANCA* mutations exhibit a clinical impact in Fanconi anemia, which is characterized by congenital abnormalities, bone marrow failure, and cancer predisposition. It is rarely reported in CCA; however, there is one case report of a patient with stage IV CCA who developed fatal myelotoxicity after receiving palliative chemotherapy with cisplatin and gemcitabine [44]. Leukocyte tyrosine kinase (*LTK*) that has been found to be overexpressed in human leukemia. Its function is largely unknown, but it is very similar to anaplastic lymphoma kinase, which is frequently mutated in human cancer [45]. Mitogen-activated protein kinase 4 (*MAP2K4*) belongs to the family of mitogen-activated protein kinase (*MAPK*) activators. *MAPK* signaling is important for cell proliferation, differentiation, transcriptional regulation, and development. It promotes human prostate cancer metastasis [46], breast cancer pathogenesis [47], and functions as a tumor suppressor in lung adenocarcinoma [48]. However, the specific function and mechanism of *MAP2K4* in CCA have not been clarified.

Recently, several immune checkpoint inhibitor (ICI) therapies have been developed for treating advanced-stage cancers, although they currently benefit only a minority of patients. TMB, a novel predictive biomarker, can predict the clinical response to ICI and identify patients who may benefit from this therapy [49]. An increased TMB indicates increased levels of tumor antigen, which is beneficial for activating the body's immune response. Previous studies of TMB have found a strong link between increased TMB and improved response to ICI therapy. However, patients with CCA showed a relatively low TMB value; there were only two patients whose TMB was higher than 10. In addition, there was no significant difference between the TMB in the iCCA, pCCA, and dCCA groups. The importance of hyperprogressive disease (HPD) in ICI therapy cannot be overstated. However, few studies have explored HPD in CCA. In our study, we analyzed HPD-associated genetic mutations in Chinese CCA patients. Our results showed that there were 11 (27%) patients who harbored HPD-associated genetic mutations, and many patients had more than one. The pCCA group showed the highest ratio in HPD-associated genetic mutations. Therefore, immunotherapy in patients with CCA should be seriously considered.



## Conclusion

In conclusion, we had investigated the comprehensive genomic features of 41 Chinese patients with CCA. TP53 mutations were significantly higher in Chinese patients than in Western patients with CCA. This suggests that TP53 may be more suitable as a predictive and diagnostic biomarker in Chinese patients with CCA. Furthermore, gene mutations that were associated with clinical characteristics, such as lymph node metastasis and lymphovascular invasion, were detected. Overall, these data provide an insight into the diagnosis and prognosis of Chinese patients with CCA. The results may guide future therapeutic strategies, including targeted and immune therapies.

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**Data Availability** The datasets used in the study are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval** This study was approved by the Institutional Ethics Committee of Peking University People's Hospital (2021PHB279-001).

**Consent to participate** Informed consent was waived given the retrospective nature of the study.

**Consent to publish** Not applicable.

**Competing interests** The authors have no conflict of interests related to this publication.

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