Targeted deep sequencing contributes to guiding personalized targeted therapy for advanced biliary tract cancer patients with non-radical resection: A real-world study

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Abstract. Targeted therapy based on specific genetic alterations has been proven to be an effective treatment for various types of cancer. In the present study, we aimed to explore the efficacy of personalized targeted therapy guided by targeted deep sequencing for patients with advanced biliary tract cancer (BTC) after non-radical resection. Targeted deep sequencing was performed on 49 patients with BTC, to whom biologic agents were recommended. Among 32 patients with stage IV and R2 resection (a non-radical resection), 21 patients underwent conventional chemotherapy (mGEMOX), while the remaining 11 patients received a personalized targeted agent. The genomic landscape of the 49 patients with BTC was determined and the results showed that genetic alterations were enriched in the ERBB family and cell cycle pathway. After a median follow-up of 12 months, the 11 BTC patients with personalized targeted therapy showed a median progression-free survival (PFS) of 4.5 months (2.5-20.5 months), a median overall survival (OS) of 12.9 months (4.7-24.8 months) and a disease control rate (DCR) of 63.6%. In the other 21 BTC patients, who were undergoing conventional chemotherapy, the BTC patients had a median PFS of 1.5 months (0.5-11.6 months), a median OS of 4.1 months (1.3-18.4 months), and a DCR of 33.3%. In addition, 36.4% of the patients in the personalized targeted therapy group experienced grade >2 treatment-related toxicity vs. 19.0% of patients in the conventional chemotherapy group. This real-world study suggests that targeted deep sequencing contributes to the guidance of personalized targeted therapy based on individual actionable mutations, which may benefit advanced BTC patients undergoing non-radical resection.

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Abbreviations: BTC, biliary tract cancer; NGS, next generation sequencing; PFS, progression-free survival; OS, overall survival; DCR, disease control rate; GBC, gallbladder carcinoma; TKIs, tyrosine kinase inhibitors; NSCLC, non-small cell lung cancer; PR, partial response; SD, stable disease; PD, progressive disease

Key words: personalized targeted therapy, biliary tract cancer, non-radical resection, targeted deep sequencing, prognosis

Introduction

Biliary tract cancer (BTC) is a devastating disease of the digestive system with poor prognosis, and mainly includes bile duct carcinoma and gallbladder carcinoma (GBC). The 5-year survival rate is less than 10% in patients with advanced or metastatic BTCs according to the National Cancer Database of the American College of Surgeons and the Surveillance, Epidemiology, and End Results (SEER) Program (1). The potentially best treatment for patients with BTCs is radical resection, but many patients are not suitable for curative surgery. For patients with advanced BTC undergoing non-radical resection, the prognosis is very poor. Therefore, the exploration of a new strategy of more precise and effective treatment is critical to improve the prognosis of patients with advanced BTCs undergoing non-radical resection.

Precision medicine means matching the right patients with the right drugs. The use of genetic mutation testing is often required prior to issuing a matching prescription. The availability of appropriate molecular profiling is the key to precision medicine in routine clinical settings (2). A few studies have reported genetic mutation profiling in biliary tract tumors (3-7). In intrahepatic bile duct carcinoma, genes from the mitogen-activated protein kinase (MAPK) signaling pathway (8) are frequently mutated, and gene fusions from the fibroblast growth factor receptor (FGFR) family are also

common (9). The erythroblastic leukemia viral (v-erb-b) oncogene homolog (ERBB) family including epidermal growth factor receptor (EGFR, also known as ERBB1), ERBB2 (also known as HER2), ERBB3 and ERBB4 has a central role in the tumorigenesis and development (10). The ERBB family receptors are able to activate several downstream pathways, including the RAS-ERK and PI3K-AKT pathways (11). In the Chinese population, alterations in the ERBB family and its downstream signaling pathway account for up to 36.8% of the alterations detected in gallbladder cancer, and further multivariate analyses have revealed that cases with ERBB pathway mutations have worse outcomes (12). Studies in BTC cell lines have confirmed that the ERBB pathway may be a suitable candidate for disruption as part of treatment for BTC patients (13). Many mutated genes in biliary tract tumors, including *ERBB2*, *PIK3CA*, *FGFR* and *IDH1*, are targets for biological drugs. There are several studies indicating the potential of comprehensive genomic profiling for improving outcomes in advanced BTC patients (14,15). However, the clinical efficacy of personalized targeted therapy based on the genetic alterations found in BTC patients undergoing non-radical resection has not yet been reported. We aim to investigate the efficacy and safety of personalized targeted therapy with excellent potential for actual use based on specific genetic alterations for patients with advanced BTC after non-radical resection in this real-world study.

Materials and methods

Patients and data collection. The present study was approved by the Ethics Committee of the Shanghai Eastern Hepatobiliary Surgery Hospital, Navy Military Medical University, Shanghai, China (no. EHBHKY2015-02-010). Forty-nine patients with BTC, which was confirmed by surgical pathology, were enrolled for targeted deep sequencing in the Department of Biliary I, Shanghai Eastern Hepatobiliary Navy Hospital from August 2014 to June 2016 (Table I). Exclusion criteria were stage I disease (AJCC 7th edition) (http://aboutcancer.com/AJCC_stage.htm) at diagnosis, no tumor tissue sample available for targeted deep sequencing, disagreement on conducting target deep sequencing, known or current evidence of HIV, pregnant or lactating females. Thirty-two patients had stage IV disease and R2 resection (Table II). To investigate the impact of targeted medicine on patients with advanced BTC, eligibility criteria included R2 resection of biliary cancer at TNM stage IV, at least one measurable lesion at baseline and the detection of targeted deep sequencing. These BTC patients began drug treatment one month after the operation. For these patients, a regimen of mGEMOX (16) (gemcitabine 900 mg/m^2 and oxaliplatin 80 mg/m^2 i.v. infusion on days 1 and 8 every 3 weeks until disease progression or intolerable toxicity) was initially proposed by the local multidisciplinary team as the standard first-line chemotherapy (chemotherapy group). In patients found to have at least one targetable variant and who declined chemotherapy, a biological agent was recommended as an alternative treatment. The specific agent was selected according to the potentially targetable altered gene (targeted therapy group). The information of targetable altered genes corresponding to the biological agent is shown (Table SI). When a patient had some targetable altered genes, we recommended the drugs for patients according to different levels of evidence and gave them all the information they required. The highest level of evidence is that targeted drugs addressing specific gene mutations have been approved by the US Food and Drug Administration (FDA) or China Food and Drug Administration (CFDA) in this tumor. The second kind of evidence is that targeted drugs addressing specific gene mutations have been approved by the FDA or CFDA in other tumors. The third kind of evidence is that also drugs specific to this gene mutation are currently being assessed in clinical trials. The weakest evidence is some preclinical data about the relationship of the drug-gene mutation associations. In this real-world clinical study, the patients made decisions themselves according to the availability of specific drugs and what they were able to afford. The recorded data also included clinicopathological features, operative morbidity, drug administration, number of treatment cycles, and therapy-related toxicity (Tables III and IV).

Study assessment. Progression-free survival (PFS), overall survival (OS) and disease control rate (DCR) were used to assess the efficacy of treatments. The PFS of patients was measured as the duration from the beginning of chemotherapy or precision therapy to the time of disease progression [according to RECIST 1.1 (17)] or death. OS was defined as the period from the start of chemotherapy or targeted therapy to death. The PFS and OS were calculated for all enrolled patients (Table SII). Disease control was defined at the first response assessment as indicated by imaging. Response and disease progression were evaluated using the international criteria proposed by the revised Response Evaluation Criteria in Solid Tumors (RECIST 1.1) Committee (17). The grade of treatment-related toxicities was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0) (18), in order to assess the safety of treatments.

Targeted deep sequencing and variant calling. According to our previous study (19), this panel of targeted deep sequencing consisted of 4,557 exons of 365 tumor-associated genes, and 45 introns from 25 genes where frequent gene fusions could be captured in cancer (Table SIII). Targeted deep sequencing was carried out on hybridization-captured, adaptor ligation–based libraries using DNA extracted from 4 formalin-fixed paraffin-embedded (FFPE) sections cut at 10 μm from 49 BTC patients. All of the sequencing assays were performed at the 3D Med Clinical Laboratory Co., Ltd. (Shanghai) and successfully passed the Proficiency Testing (PT) on NGS solid tumor (NGSST) developed by the College of American Pathologists (CAP) (https://www.cap.org/). DNA was isolated from FFPE slides containing at least 20% tumor cells and the library was prepared using IDT Xgen hybridization buffer (Integrated DNA Technologies, Inc.) for capture and sequenced on an Illumina Nextseq 500 (Biostar Technology Ltd.).

Short reads were mapped by BWA 0.7.12-r1039 with default settings (20). Somatic single-nucleotide variants (SNVs) were identified using Mutect 1.1.4 with default settings, and Indels (smaller than 50 bp) were identified by Pindel v0.2.5b8 and VarScan v2.4.0 with default settings (21-23). For somatic copy number alterations (CNAs), coverage in the tumor was

normalized to that in the matched control (blood DNA). Our in-house program combined with modified algorithm was applied to call somatic CNAs (24). Actionable genomic alterations were defined as being linked to commercially available targeted therapies or to targeted therapies currently in ongoing clinical trials. To further annotate the genetic variants found in each patient, an in-house manually reviewed clinical database (3D Medicines Inc.) was used to develop plans for targeted therapy.

Statistical methods. The difference in age between the two groups was tested by using the Student's t test. Fisher's exact test was used to assess the significance of differences between groups, such as sex, cancer type, TNM stage, and operative complication. The heatmap was plotted by R package GenVisR 1.0.2 (25), which was used to demonstrate somatic mutations spectrum across BTC cohorts. Statistical analyses were performed using R packages. The log-rank test and Kaplan-Meier analyses were performed for PFS and OS. $P<0.05$ was considered statistically significant. Kaplan-Meier analyses were performed using SPSS statistical software (version 19.0; SPSS Inc.).

Results

Clinical characteristics of the patients with biliary tract cancer (BTC). In the present study, we enrolled 49 BTC patients with a median age of 59 years (range, 26-72) at diagnosis (Table I) from August 2014 to June 2016 for targeted deep sequencing. These patients consisted of 32 males and 17 females, and were composed of 21 patients with gallbladder carcinoma and 28 with bile duct cancer (21 intrahepatic, 5 perihilar, and 2 distal cholangiocarcinomas). Most patients (n=42, 85.7%) had cTNM stage IV disease (AJCC 7th edition) at diagnosis. Out of the 49 patients, 8 patients with BTCs received radical resection (R0), while the others (n=41, 83.7%) received R1/R2 resection or biopsy. Follow-up was completed for all 49 patients.

Genetic alterations are enriched in the ERBB family and cell cycle pathway in patients with BTC. The genomic landscape of the 49 patients established that *TP53* (n=31, 63.3%) variants were most prevalent, followed by variants in *KRAS* (n=12, 24.5%), *ARID1A* (n=6, 12.2%), *PIK3CA* (n=6, 12.2%), *SMAD4* (n=6, 12.2%), *CDKN2A* (n=5, 10.2%), and *ERBB4* (n=5, 10.2%) (Fig. 1). Further analysis of copy number alterations (CNAs) showed low levels of recurrent amplified genes, such as *PIK3CA*, *SMAD4*, *FGFR3*, *SRC*, *PIK3R2*, *CDK4*, *ERBB2*, and *CDK6*. Among these genes, *PIK3CA*, *ERBB2*, *CDK4*, and *CDK6* may be suitable drug targets for these BTC patients. In 21 patients with gallbladder cancer (GBC), 8 had mutations in the ERBB pathway. Further analysis of all of the alterations demonstrated that these altered genes were highly enriched in the ERBB family or the cell cycle pathway (Fig. 2A and B).

Personalized targeted therapy in advanced BTC patients with R2 resection. Because tumor staging and resection margins can significantly impact the prognosis of patients with BTCs (26,27), only 32 patients who met the eligibility criteria of having stage IV disease and R2 resection were enrolled in the treatment study (Table II). The patient characteristics, age, sex, cancer type, pTNM stage, and operative morbidity, were similar between the targeted therapy and conventional chemotherapy groups (Table II). In the targeted therapy group, 11 patients received corresponding targeted therapy as an alternative to chemotherapy (Tables III and IV). The mutated genes, recommended drugs, drug administration (drug usage, dosage, and cycle), operative morbidity, prognosis, and treatment-related toxicities of the two groups are described in Tables III and IV. The targeted drugs lapatinib, everolimus, dasatinib, imatinib, pazopanib, and regorafenib were administered to 11 BTC patients in the targeted therapy group, while mGEMOX was given to 21 patients in the chemotherapy group. The administration of these targeted agents and chemotherapeutic regimens was performed by the local multidisciplinary team in this study.

According to the revised RECIST guideline (version 1.1), the targeted therapy group had partial response (PR) in 3 patients, stable disease (SD) in 4 patients, and progressive disease (PD) in the remaining 4 patients, while the chemotherapy group had 2 patients with PR, 5 with SD, and 14 with PD (Tables III and IV). The targeted therapy group had a 63.6% disease control rate (DCR), while the chemotherapy group had a 33.3% DCR. Moreover, the targeted therapy group had a median PFS of

Table I. Characteristics of the 49 patients with biliary tract cancer that received surgery.

Characteristics	Data values
Age, median (range) in years	59 (26-72)
Sex, n	
Male	32
Female	17
Cancer type, n	
Gallbladder carcinoma	21
Cholangiocarcinoma	28
AJCC stage, n	
I	0
II	2
III	5
IV	42
Type of surgical operation, n	
R0	8
R1	8
R2	32
Biopsy	1
Treatment, n	
Targeted therapy	14
Chemotherapy	35
Follow-up, n	
PFS	49
OS	49
NGS detection, n	49

AJCC, American Joint Committee on Cancer; PFS, progression-free survival; OS, overall survival; NGS, next generation sequencing.

Table II. Characteristics of the 32 patients with stage IV biliary tract cancer after R2 resection.

Characteristics	Targeted therapy (n=11)	Conventional chemotherapy (n=21)			
		All patients (n=21)	P-value	With recommendation (n=13)	P-value
Age, median (range) in years	60 (26-65)	58 (35-66)	0.645	61 (35-66)	0.897
Sex					
Male	7	13	1.000	9	1.000
Female	4	8		4	
Cancer type					
Gallbladder carcinoma	6	10	1.000	5	0.682
Cholangiocarcinoma	5	11		8	
pTNM stage (AJCC 7th edition)					
IVA	3	4	0.668	2	0.630
IVB	8	17		11	
Operative complication (Clavien-Dindo)					
Grade II	10	20	1.000	13	0.458
Grade IIIa	1	1		0	

AJCC, American Joint Committee on Cancer.

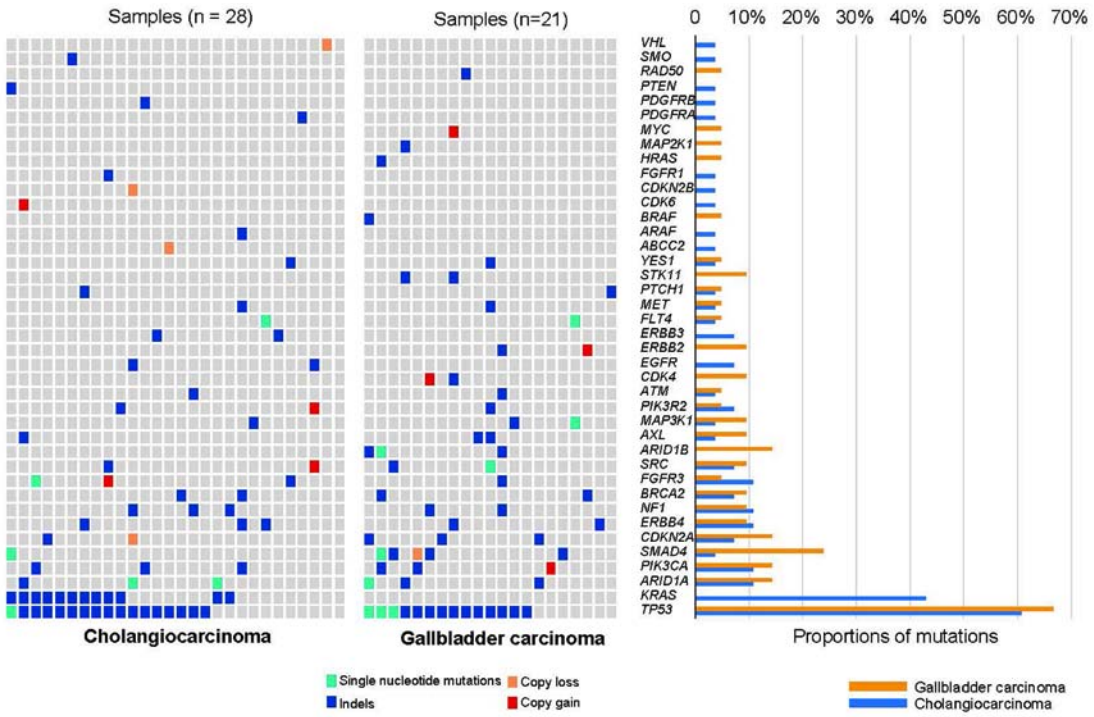

Figure 1. Mutational landscape of biliary tract cancers (BTCs). Mutational spectrum of the BTC patients as determined by targeted deep sequencing (left and middle panels). Overall, 28 cholangiocarcinomas and 21 gallbladder cancers were included. The genetic variants landscape showed that *TP53*, *KRAS*, *ARID1A*, and *PIK3CA* were frequently mutated. Mutation subtypes (single nucleotide variant, indel, copy gain and loss) are denoted by color. The right panel shows the frequency of recurrent mutated genes. The histogram with different colors shows the frequency of corresponding genes in cholangiocarcinoma or gallbladder carcinoma, respectively. The colors indicating the frequency of corresponding genes in cholangiocarcinoma and gallbladder carcinoma are reversed in the right panel. *TP53*, tumor protein P53; *KRAS*, KRAS proto-oncogene, GTPase; *ARID1A*, AT-rich interaction domain 1A; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α.

4.5 months (2.5-20.5 months), and a median OS of 12.9 months (4.7-24.8 months) (Fig. 3A and B), while the chemotherapy group had a median PFS of 1.5 months (0.5-11.6 months) and a median OS of 4.1 months (1.3-18.4 months) (Fig. 3C and D). Subgroup analysis of 13 patients from the chemotherapy group who received recommendations of targeted therapy, showed

Table III. Targeted drugs, the corresponding altered genes, prognosis and toxicity for the 11 patients with BTC receiving targeted therapy.

Patient_ID	Sex	Age (years)	Cancer type	Mutated genes			Recommended drugs	Drug usage	Dosage	Cycle	Operative morbidity	Prognosis[a] (PD\SD\ PR\CR)	Treatment-related toxicities[b]
BTC-007	M	59	Gallbladder carcinoma	*TP53*	*MEK1*	*PIK3CA*	Trametinib, everolimus	Everolimus	10 mg/day (2 months); 5 mg/day (4 months)	6	II	SD	Grade 2: Pruritis/ itching
BTC-010	M	58	Cholangiocarcinomas	*ERBB3*			Trastuzumab, lapatinib	Lapatinib	1250 mg/day	3	II	PR	Grade 2: Gastrointestinal reaction (vomiting/ nausea/diarrhea)
BTC-011	F	59	Cholangiocarcinomas	*ERBB4*	*PTCH1*	*KRAS*	Lapatinib, vismodegib	Lapatinib	1250 mg/day	3	II	PR	Grade 2: Pain
BTC-012	M	60	Cholangiocarcinomas	*ERBB3*			Trastuzumab, lapatinib	Lapatinib	1250 mg/day	2	II	PD	Grade 2: Gastrointestinal reaction (constipation/ vomiting/nausea)
BTC-018	M	60	Gallbladder carcinoma	*MET*	*PIK3R2*	*SRC*	Crizotinib, dasatinib, cabozantinib	Dasatinib+ rapamycin	Dasatinib 140 mg/day; Rapamycin 40 mg/day	1	II	SD	Grade 2: Pain
BTC-020	F	71	Gallbladder carcinoma	*SRC*	*TP53*	*SMAD4*	Dasatinib	Dasatinib	140 mg/day	1	II	PD	Grade 2: Central nervous system
BTC-026	M	65	Cholangiocarcinomas	*KRAS*	*PIK3CA*	*TP53*	Everolimus, Trametinib	Everolimus	10 mg/day	1	II	SD	Grade 3: Hepatic damage
BTC-034	M	62	Cholangiocarcinomas	*PDGFRA*			Imatinib, Bevacizumab	Imatinib	800 mg/day	6	II	PR	Grade 3: Hepatic damage
BTC-040	M	63	Cholangiocarcinomas	*FGFR3*			Pazopanib	Pazopanib	800 mg/day	1	II	PD	Grade 3: Platelets
BTC-043	F	56	Gallbladder carcinoma	*ERBB2*	*FGFR3*	*NF1*	Trastuzumab, Lapatinib	Lapatinib	1250 mg/day	4	II	PD	Grade 2: Hepatic damage

Table III. Continued.

Patient_ID	Sex	Age (years)	Cancer type	Mutated genes	Recommended drugs	Drug usage	Dosage	Cycle	Operative morbidity	Prognosis[a] (PD\SD\ PR\CR)	Treatment-related toxicities[b]
BTC-048	F	26	Gallbladder carcinoma	*RET* *CTNNB1*	Regorafenib	Regorafenib	160 mg/day	5	IIIa	SD	Grade 3: Fatigue; Grade 2: Nausea, vomiting, diarrhea, stomatitis

[a]Prognosis: PR, partial response; SD, stable disease; PD, progressive disease. [b]Treatment-related toxicities: The grade of the side effect was evaluated according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (version 3.0). BTC, biliary tract carcinoma; M, male; F, female. *TP53*, tumor protein P53; *MEK1*, MAPK/ERK kinase 1, also known as mitogen-activated protein kinase kinase 1 (*MAP2K1*); *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; *ERBB3*, Erb-B3 receptor tyrosine kinase; *ERBB4*, Erb-B4 receptor tyrosine kinase; *PTCH1*, protein patched homolog 1; *KRAS*, KRAS proto-oncogene, GTPase; *MET*, MET proto-oncogene, receptor tyrosine kinase; *PIK3R2*, phosphoinositide-3-kinase regulatory subunit 2; *SRC*, SRC proto-oncogene, non-receptor tyrosine Kinase; *SMAD4*, SMAD family member 4; *PDGFRA*, platelet derived growth factor receptor α, receptor tyrosine kinase; *FGFR3*, fibroblast growth factor receptor 3, receptor tyrosine kinase; *NF1*, neurofibromin 1; *RET*, Ret proto-oncogene; *CTNNB1*, catenin β1.

that they had a median PFS of 1.5 months (2.5-20.5 months), a median OS of 2.8 months (4.7-24.8 months) and a DCR of 30.8% (Fig. S1 and Table IV).

Safety of personalized targeted therapy. In the targeted therapy group, there were 4 patients (BTC-026, BTC-034, BTC-040, and BTC-048) who experienced Grade 3 treatment-related toxicity, including hepatic damage, thrombocytopenia, and fatigue (Table III). The chemotherapy group had 3 patients (BTC-009, BTC-039, and BTC-041) with Grade 3 treatment-related toxicities and 1 patient (BTC-044) with Grade 4 renal impairment (Table IV). In addition, 36.4% patients in targeted therapy group experienced Grade >2 treatment-related toxicity, while 19.0% patients in the conventional chemotherapy group did.

Three BTC cases of personalized targeted therapy. In this cohort, 11 patients received the corresponding targeted therapies based on their genetic variants. Positive clinical responses were observed in 3 patients and are described herein. A 26-year-old female patient (BTC-048) was diagnosed with Stage IVb Grade 2 gallbladder adenocarcinoma with metastases to the peritoneum in December 2014 (Table III and Fig. S2A). Based on her genetic alterations (Fig. 4A), a targeted drug, regorafenib, targeting RET (160 mg/day) was recommended and was administered on January 25, 2015. On April 21, 2015, the sum of the diameters of all of the measurable lesions was decreased by 29.2% (SD) according to RECIST 1.1 (Fig. S2B). The patient's performance status was found to have improved a month later, with a reported increase in body weight and decrease in abdominal pain. The patient continued to experience stable disease based on CT imaging (Fig. S2C). However, the patient ceased regorafenib treatment on July 18, 2017 due to Grade 3 fatigue and Grade 2 nausea, vomiting, diarrhea, and stomatitis. On July 30, the CT images showed PD and the sum of the diameters had increased to the pre-treatment RECIST measurements (Fig. S2D). For this reason, a PFS period of 6 months was recorded for this patient from initiation of treatment in January to disease progression in July (Fig. 4B).

There were also 2 patients with BTC, who received personalized precise therapy with satisfying results. One patient (BTC-010) with stage IVa intrahepatic cholangiocarcinoma who underwent R2 resection, harbored an ERBB3 p.R1127H mutation and the ERBB inhibitor lapatinib was administered (Table III). The PFS and OS of this patient were 20.5 and 24.8 months, respectively. The other intrahepatic cholangiocarcinoma patient (BTC-034), also with stage IVa disease, carried a PDGFRA p.T1066I mutation and was treated with imatinib as recommended (Table III). The PFS and OS were 7.5 and 15.8 months respectively.

Discussion

In the present study, the landscape of genomic variants in 49 Chinese biliary tract cancer (BTC) patients was obtained. Several studies on whole-exome sequencing and whole genome sequencing of BTCs have recently been published (3,12,28-30). *TP53* and *KRAS* were reported as the mostly frequently mutated genes in previous studies (9,31), and the majority of the variants are single nucleotide variants. These findings

Table IV. Targeted drugs, the corresponding altered genes, prognosis and toxicity for the 21 patients with BTC receiving standard chemotherapy.

Patient_ID	Sex	Age (years)	Cancer type	Mutated genes	Recommended drugs	Drug usage	Dosage	Cycle	Operative morbidity	Prognosis[a] (PD\SD\ PR\CR)	Treatment-related toxicities[b]
BTC-001	M	64	Gallbladder carcinoma	*STK11* *TP53* *MYC* *FANCA* *MDM2*	Everolimus, temsirolimus	mGEMOX	Gemcitabine 900 mg/m^2 and oxaliplatin 80 mg/m^2 i.v. infusion on days 1 and 8 every 3 weeks until disease progression or intolerable toxicity	2	II	PD	-
BTC-002	F	46	Cholangiocarcinomas	*KRAS* *TP53*	None			1	II	PD	-
BTC-003	F	66	Cholangiocarcinomas	*KRAS* *TP53* *SF3B1* *CDK6*	Palbociclib			1	II	PD	-
BTC-006	F	51	Gallbladder carcinoma	*PIK3CA* *TP53* *AXIN1* *SMAD4* *CCNE1*	Everolimus, temsirolimus			6	II	PR	-
BTC-009	M	58	Cholangiocarcinomas	*ERBB4* *FLT4* *MLL3*	Lapatinib, axitinib, pazopanib, sunitinib			1	II	PD	Grade 3: Gastroin testinal reaction (vomiting/ constipation/ diarrhea)
BTC-016	F	63	Gallbladder carcinoma	*PIK3CA* *FLT3*	Rapamycin, everolimus, sorafinib, sunitinib			1	II	PD	-
BTC-017	M	35	Cholangiocarcinomas	*PIK3CA* *PDGFRB*	Rapamycin, everolimus			2	II	PD	-
BTC-019	M	46	Cholangiocarcinomas	*ERBB4*	Lapatinib			1	II	PD	-
BTC-021	M	66	Cholangiocarcinomas	*KRAS* *APC*	Cetuximab (invalid), panitumumab (invalid), Gefitinib			1	II	PD	-

Table IV. Continued.

Patient_ID	Sex	Age (years)	Cancer type	Mutated genes				Recommended drugs	Drug usage	Dosage	Cycle	Operative morbidity	Prognosis[a] (PD\SD\ PR\CR)	Treatment-related toxicities[b]
								(invalid), Erlotinib (invalid), everolimus (invalid)						
BTC-022	F	61	Cholangiocarcinomas	*KRAS*	*SMAD4*	*PTEN*	*TP53*	Trametinib combined with everolimus, cetuximab (invalid), panitumumab (invalid), gefitinib (invalid), erlotinib (invalid), everolimus (invalid)			12	II	PR	-
BTC-023	M	58	Gallbladder carcinoma	*CDKN2A*	*BRAF*	*ARID1B*	*ARID1A*	Palbociclib, sorafinib, trametinib			1	II	PD	-
BTC-025	M	51	Cholangiocarcinomas	*EGFR*	*PIK3R2*	*SRC*		Cetuximab, panitumumab, gefitinib, erlotinib, nimotuzumab, everolimus			4	II	SD	-
BTC-031	F	65	Gallbladder carcinoma	*RB1*	*TP53*			None			2	II	PD	-
BTC-036	M	65	Gallbladder carcinoma	*RAD50*				Cisplatin			1	IIIa	PD	-
BTC-039	F	52	Gallbladder carcinoma	*SMAD4*				Cisplatin			4	II	SD	Grade 3: Thrombocy-topenia

Table IV. Continued.

Patient_ID	Sex	Age (years)	Cancer type	Mutated genes				Recommended drugs	Drug usage	Dosage	Cycle	Operative morbidity	Prognosis[a] (PD\SD\ PR\CR)	Treatment-related toxicities[b]
BTC-041	F	55	Gallbladder carcinoma	*ATRX*				None			3	II	SD	Grade 3: Gastrointestinal reaction (vomiting/ nausea)
BTC-042	M	53	Cholangiocarcinomas	*TAF1*				None			2	II	PD	-
BTC-044	M	65	Cholangiocarcinomas	*ATM*	*NF1*	*TP53*	*PTPN11*	Daunorubicin, olaparib, rapamycin			4	II	SD	Grade 4: Impairment of renal function
BTC-045	M	46	Cholangiocarcinomas	*IDH2*	*MEN1*			None			6	II	PD	Grade 2: Gastrointestinal reaction (vomiting/ nausea)
BTC-047	M	55	Gallbladder carcinoma	*FANCA*	*KEAP1*	*BRCA1*	*MAP3K1*	None			4	II	SD	Grade 2: Thrombocytopenia
BTC-049	M	64	Cholangiocarcinomas	*VHL*				Sorafinib, sunitinib, bevacizumab			3	II	PD	-

[a]Prognosis: PR, partial response; SD, stable disease; PD, progressive disease. [b]Treatment-related toxicities: The grade of side effect was evaluated according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (version 3.0). BTC, biliary tract carcinoma; M, male; F, female. *STK11*, STK11 serine/threonine-protein kinase; *TP53*, tumor protein P53; *MYC*, MYC proto-oncogene, bHLH transcription factor; *FANCA*, fanconi anemia complementation group A; *MDM2*, MDM2 proto-oncogene; *KRAS*, KRAS proto-oncogene, GTPase; *SF3B1*, Splicing Factor 3b Subunit 1; *CDK6*, cyclin dependent kinase 6; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; *AXIN1*, Axin 1; *SMAD4*, SMAD family member 4; *CCNE1*, cyclin E1; *ERBB4*, Erb-B4 receptor tyrosine kinase; *FLT4*, Fms related tyrosine kinase 4; *MLL3*, myeloid/lymphoid or mixed-lineage leukemia protein 3; *FLT3*, Fms related tyrosine kinase 3; *PDGFRB*, platelet derived growth factor receptor β, receptor tyrosine kinase; *APC*, APC regulator of WNT signaling pathway; *PTEN*, phosphatase and tensin homolog; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *ARID1B*, AT-rich interaction domain 1B; *ARID1A*, AT-rich interaction domain 1A; *EGFR*, epidermal growth factor receptor; *SRC*, SRC proto-oncogene, non-receptor tyrosine kinase; *RB1*, RB transcriptional corepressor 1; *RAD50*, RAD50 double strand break repair protein; *ATRX*, ATRX chromatin remodeler; *TAF1*, TATA-box binding protein associated factor 1; *ATM*, ATM serine/threonine kinase; *NF1*, neurofibromin 1; *PTPN11*, protein tyrosine phosphatase non-receptor type 11; *IDH2*, isocitrate dehydrogenase (NADP(+)) 2; *MEN1*, Menin 1; *KEAP1*, Kelch like ECH associated protein 1; *BRCA1*, BRCA1 DNA repair associated; *MAP3K1*, mitogen-activated protein kinase kinase kinase 1; *VHL*, von Hippel-Lindau tumor suppressor.

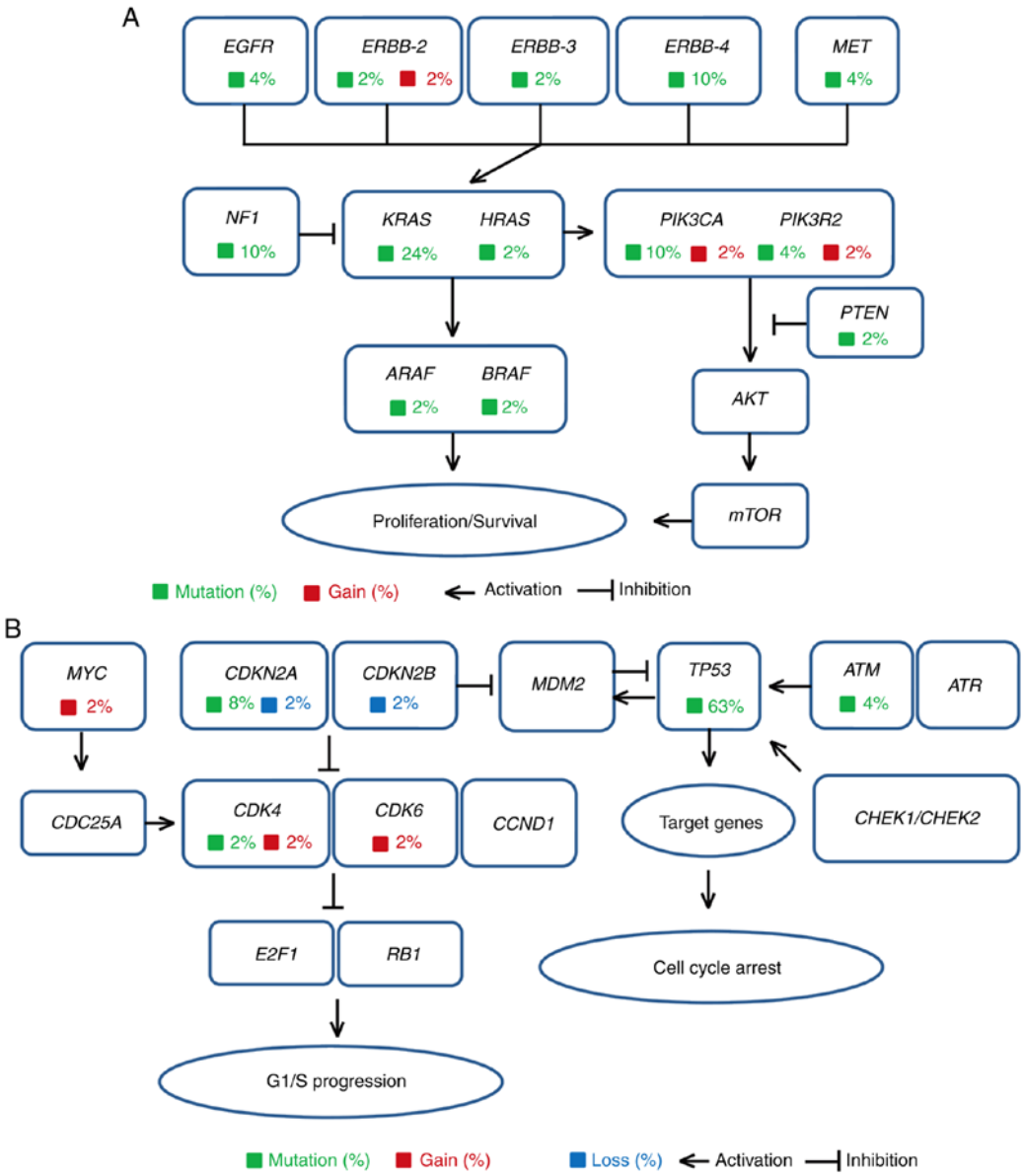

Figure 2. Cellular signaling pathways associated with the mutated genes of the biliary tract cancer (BTC) cases. The mutated genes in 49 patients with BTCs were found to be mainly distributed in the (A) ERBB family signaling pathway and (B) cell cycle signaling pathway. Genes responsible for somatic cell variants and the proportion of mutated genes in 49 patients are indicated in the signal transduction pathway. Different types of variants are marked with different colors, such as mutation (green), gain (red), and loss (blue). *EGFR*, epidermal growth factor receptor; *ERBB2*, Erb-B2 receptor tyrosine kinase; *MET*, MET proto-oncogene, receptor tyrosine kinase; *NF1*, neurofibromin 1; *KRAS*, KRAS proto-oncogene, GTPase; *HRAS*, HRas proto-oncogene, GTPase; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; *PIK3R2*, phosphoinositide-3-kinase regulatory subunit 2; *ARAF*, A-Raf proto-oncogene, serine/threonine kinase; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *PTEN*, phosphatase and tensin homolog; *AKT*, protein kinase B; *mTOR*, mechanistic target of rapamycin kinase; *MYC*, MYC proto-oncogene, bHLH transcription factor; *CDKN2A*, cyclin dependent kinase inhibitor 2A; *CDKN2B*, cyclin dependent kinase inhibitor 2B; *MDM2*, MDM2 proto-oncogene; *TP53*, tumor protein P53; *ATM*, ATM serine/threonine kinase; *ATR*, ATR serine/threonine kinase; *CDC25A*, cell division cycle 25A; *CDK4*, cyclin dependent kinase 4; *CDK6*, cyclin dependent kinase 6; *CCND1*, cyclin D1; *CHEK1*, checkpoint kinase 1; *CHEK2*, checkpoint kinase 2; *E2F1*, E2F transcription factor 1; *RB1*, RB transcriptional corepressor 1.

are consistent with our results. However, we found a higher frequency of *CDKN2A* loss in comparison to Western cohorts (14). High *BRCA* and *IDH* mutations were reported in cholangiocarcinoma of Western populations (3-5,14), while no such mutations were found in our study. These aforementioned studies only described the genomic variant landscape and the relationship between prognosis and genomic variants. The use of this genomic profiling information to guide clinical treatment has not been available to use (14,15). Our study focused on advanced BTC patients with non-radical resection, and we assessed the clinical efficacy and safety of personalized targeted therapy guided by targeted deep sequencing in these patients.

In recent years, biomarker-driven clinical trials have been carried out in a wide variety of cancers. Targeted deep sequencing that can achieve high sequencing depth is crucial to accurately identify genomic variants in formalin-fixed paraffin-embedded samples with low tumor cell content and high heterogeneity (32-34), and has also been recognized as a practical method for clinical genetic alteration detection in many types of cancers (35-37). Nevertheless, no studies have been reported on the application of genomic profiling

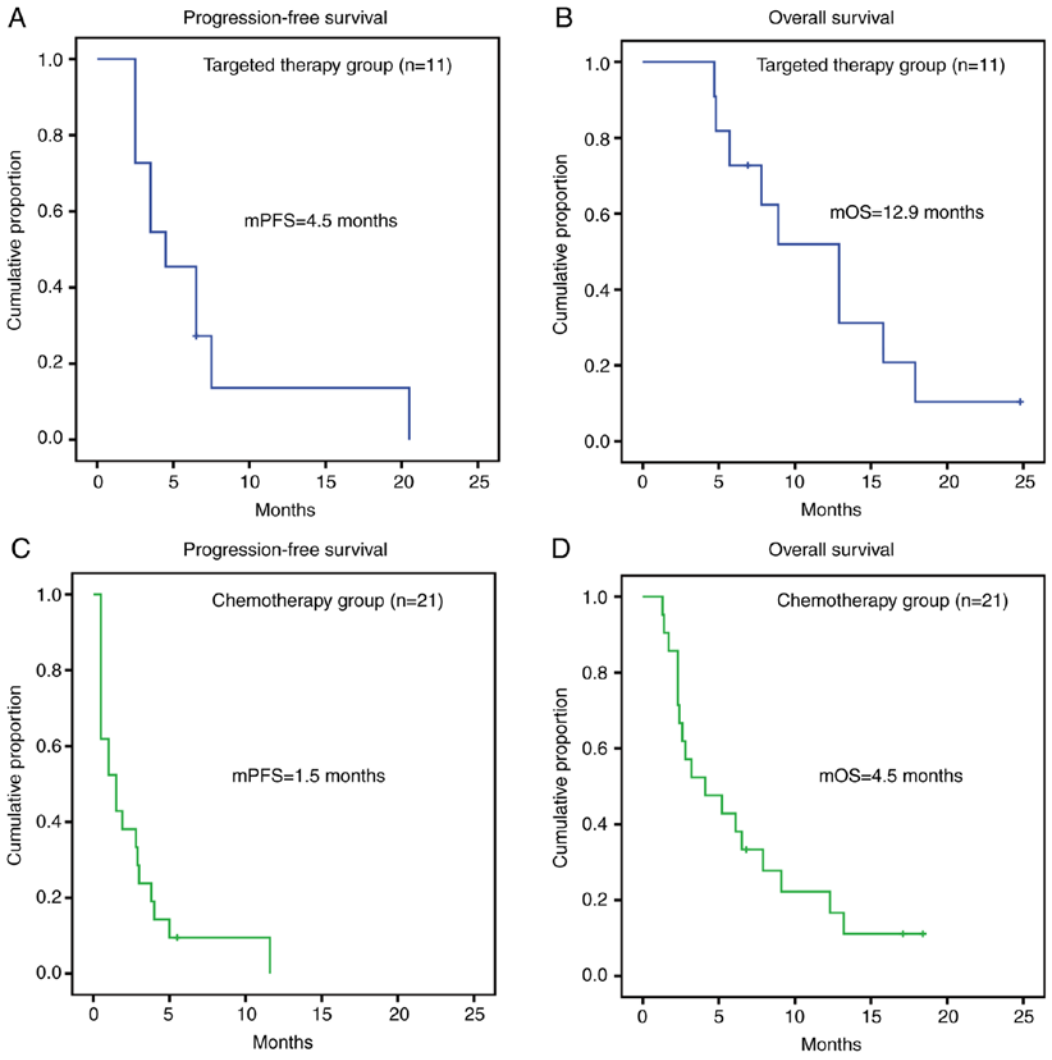

Figure 3. Prognostic analysis of personalized targeted therapy and conventional chemotherapy in patients with biliary tract cancers (BTCs). The genetic variants were detected by targeted deep sequencing in 32 BTC patients with stage IV disease and R2 resection, including 11 patients who received targeted therapy and 21 patients who received conventional chemotherapy. Kaplan-Meier curves of the (A) progression-free survival (PFS) and (B) overall survival (OS) in the targeted therapy group were constructed. Kaplan-Meier curves of the (C) PFS and (D) OS of the chemotherapy group are displayed.

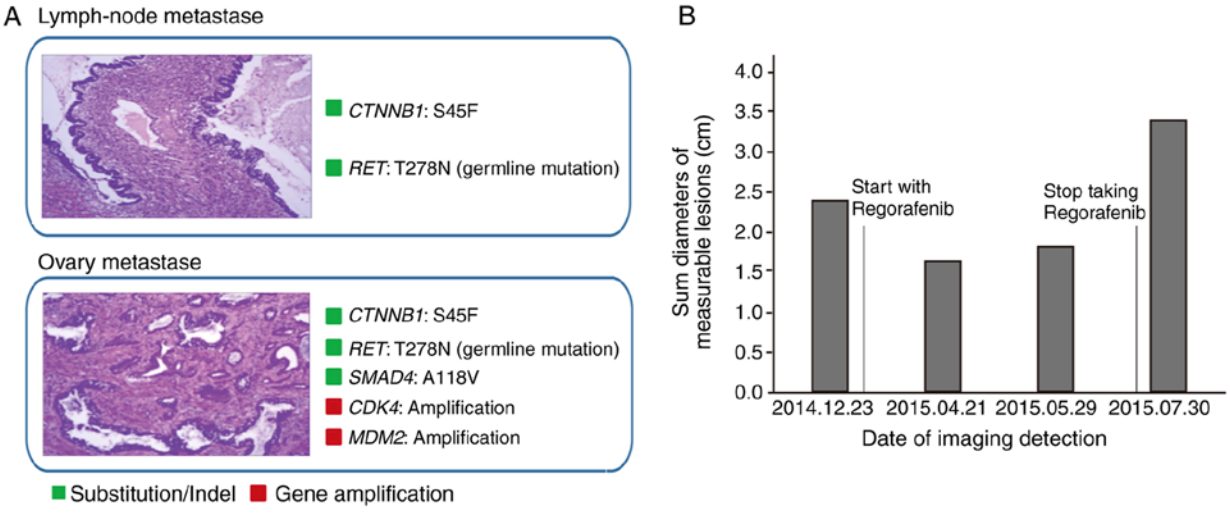

Figure 4. A case of precise therapy for gallbladder carcinoma. (A) Hematoxylin and eosin staining results and genetic variants of metastatic lesions on lymph node and subsequently on the ovary as identified by targeted deep sequencing. Different types of variants are marked with different colors, such as substitution/Indel (green) and gene amplification (red). (B) Sum of diameters of the measurable lesions derived from the four imaging examinations before and after medication. Histogram shows the sum of the diameters of measurable lesions at different dates. *CTNNB1*, catenin β1; *RET*, Ret proto-oncogene; *SMAD4*, SMAD family member 4; *CDK4*, cyclin dependent kinase 4; *MDM2*, MDM2 proto-oncogene.

information to guide the precision treatment for a group of advanced BTC patients with non-radical resection. Our study was designed to use targeted deep sequencing for the detection of genetic mutations to guide clinical decision-making in advanced BTC patients with non-radical resection. The personalized targeted therapy group had a median PFS of 4.5 months, a median OS of 12.9 months and a 63.6% DCR, while the chemotherapy group had a median PFS of 1.5 months, a median OS of 4.1 months, and a 33.3% DCR. These results may provide preliminary evidence to support the development of a novel treatment strategy of personalized targeted therapy for advanced BTC patients with non-radical resection.

Gemcitabine plus cisplatin (GC) is the standard treatment for advanced BTC for this decade, demonstrating a median OS of gemcitabine regimen of 8.1 months and GC of 11.7 months, respectively (38). The OS of GC reported is longer than that explored in our study. However, there are some differences between their research and ours. Regarding group selection, we focused on the patients with R2 resection, while they choose patients who did not receive surgery. The two sets of patients are not comparable. The staging system is also different. The 32 patients we used to analyze prognosis were all stage IV patients (with metastatic tumors) in our study, while part of their group was made up of patients with locally advanced cancer but no metastatic tumors. The prognosis of these patients by stage is quite different. Our study more closely reflects real-world clinical practice for advanced BTC with non-radical resection, in which the standardization of drug use and other factors are not as strict as in clinical drug trials.

Treatment-related toxicity is a crucial factor that influences clinical drug use and effects (39,40). In this study, all of the patients in the targeted therapy group experienced Grade 2 or 3 treatment-related toxicities, while five patients in conventional chemotherapy group did. When Grade 2 and 3 toxicity occurred, the drugs could be continuously used by adjusting the drug dosage and drug properties. In both groups, some patients with Grade 2 or 3 toxicities continued taking medicine by reducing the dosage or making other changes. Only one patient in the chemotherapy group with Grade 4 renal impairment stopped taking the drugs. Most patients were on medication regimens for only a short time and the patients in the chemotherapy group did not experience any treatment-related toxicity because of the rapid progression of the disease, which does not mean that these chemotherapeutic drugs had low toxicity. Overall, both targeted therapy and chemotherapy were found to pose some risk of toxicity for BTC patients in real-world clinical practice. The key is finding a way to reduce treatment-related toxicity through drug adjustment or other means so that BTC patients can continue and complete their medication regimens.

In our cohort, 8 of 21 (38.1%) GBC patients had mutations in the ERBB pathway. It has also been reported that approximately 36.8% of GBC patients have aberrant ERBB signaling, and multivariate analyses revealed that patients with ERBB pathway mutations had worse outcomes (12). However, there are no clinical studies that have explored if interrogating ERBB signaling can improve the prognoses of such GBC patients. In this study, 3 advanced GBC patients who received therapy specifically targeting alterations in the ERBB pathway achieved marginally longer PFS and OS (Fig. S3), in comparison to the 5 GBC patients who underwent conventional chemotherapy. Despite the small sample size of GBC patients treated with targeted therapy, the preliminary results have shed light on precision therapy for GBC patients with mutations in the ERBB signaling pathway.

Furthermore, we observed that most BTC patients who progressed rapidly such that those in the chemotherapy group experienced only 1-2 cycles of chemotherapy (Table IV). Some BTC patients experienced treatment-related toxicity and had to stop taking chemotherapy drugs. Overall, BTC patients with PR and SD underwent more cycles than those with PD did. Here, BTC-045 was an outlier. BTC-045 had PD and underwent six cycles of chemotherapy. To some extent, chemotherapy cycles are related with disease progression in this real-world clinical study.

The present research has several limitations. Some recommended targeted drugs are difficult to obtain because some of them have been approved by the United States Food and Drug Administration (FDA) but not by the China Food and Drug Administration (CFDA). In consideration of high medical costs, many patients chose the cheaper option, even if another option was favored that may have been more effective. Furthermore, genetic testing did not show which mutations were related to the resistance of chemotherapy in the present study. We do not have access to any other information on BTC patients with targetable altered genes resistant to chemotherapy. In addition, toxicity caused a dose reduction in the targeted drugs, optimization of the medication plan, or finally discontinuation in the medication in this study, which affected the evaluation of the drug effect. Although this real-world clinical study included the greatest number of patients with R2 resection undergoing personalized precision therapy of any such study, total sample size and the proportion of BTC patients taking the medicine were relatively small. Large umbrella trials of personalized precision therapy are needed to confirm our findings. Despite various limitations, this study reflects real-world clinical practice as it relates to personalized targeted therapy guided by targeted deep sequencing in patients with advanced BTC undergoing non-radical resection in China.

In conclusion, the results of this clinical study suggest that targeted deep sequencing offers a promising method of detecting actionable genetic alterations in BTC cases for precision therapy. This study provides preliminary evidence that personalized targeted therapy based on actionable genetic alterations may benefit patients with advanced BTC undergoing non-radical resection. Large umbrella trials covering personalized precision therapy are needed to confirm the clinical efficacy and safety of this therapeutic strategy for patients with advanced BTC undergoing non-radical resection.

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Availability of data and materials

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

FF designed the targeted therapy study, contributed to data interpretation and wrote the manuscript. QC designed the targeted therapy study of gallbladder carcinoma patients and wrote the manuscript; DZ designed the targeted deep sequencing study, contributed to data interpretation and composed the manuscript. BL performed the targeted therapy study of gallbladder carcinoma patients and interpreted the data; HQ analyzed the data from targeted deep sequencing and contributed to data interpretation. CX collected tumor tissue from cholangiocarcinoma patients and contributed to data interpretation; MH analyzed the clinical information and interpreted the data. YY collected the tumor tissue from gallbladder carcinoma patients and collected the clinical information; ZL performed the extraction of genomic DNA from tumor tissue samples and contributed to data interpretation. JYL performed the quality control analyses on targeted deep sequencing and interpreted the data; ZQ performed the targeted therapy study of cholangiocarcinoma patients and interpreted the data. LX discussed the hypothesis, interpreted data, and composed the manuscript; CL performed the construction of sequencing library, analyzed the clinical information of patients, and contributed to data interpretation. FL performed the analyses on the SNV/Indel and copy number variations of tumor tissue samples, contributed to data interpretation, and composed the manuscript; BY led the project, conceived the study, and composed the manuscript; XJ conceived the study and participated in its design and coordination. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This study protocol was approved by the Institutional Review Board of Shanghai Eastern Hepatobiliary Surgery Hospital, Navy Military Medical University (no. EHBHKY2015-02-010). Written informed consent was obtained from all of the BTC patients.

Patient consent for publication

Not applicable.

Competing interests

All of the authors affiliated with 3D Medicines Inc. are current or former employees. The other authors have no potential or actual conflicts of interest to disclose.

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