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ORIGINAL RESEARCH

Molecular alterations and clinical prognostic factors for cholangiocarcinoma in Thai population

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Abstract: This study explores genomic alterations in cholangiocarcinoma (CCC) tissues in Thai patients. We identified and reviewed the records of patients who had been diagnosed with CCC and for whom sufficient tumor samples for DNA and RNA extraction were available in our database. The specimens were explored for *EGFR*, *KRAS*, *BRAF*, and *PIK3CA* mutations and *ROS1* translocation in 81 samples. Immunohistochemistry staining for HER2, ALK, and Ki-67 expression was tested in 74 samples. Prevalence of *EGFR*, *KRAS*, and *PIK3CA* mutations in this study was 21%, 12%, and 16%, respectively. No *BRAF V600* mutation or *ROS1* translocation was found. Patients with *T790M* mutation had a significantly longer overall survival (18.84 months) than those with the other types of *EGFR* mutations (4.08 months; hazard ratio [HR]: 0.26, *P*=0.038) and also had a significantly lower median Ki-67 (22.5% vs 80%, *P*=0.025). Furthermore, patients with *PIK3CA* mutations had a significantly longer median progression-free survival (15.87 vs 7.01 months; HR: 0.46, *P*=0.043). Strongly positive HER2 expression was found in only 1 patient, whereas ALK expression was not found. The presence of *EGFR* and/or *PIK3CA* mutations implies that targeted drugs may provide a feasible CCC treatment in the future.

Keywords: cholangiocarcinoma, targeted therapy, gene alterations

Introduction

Biliary tract cancer (BTC) consists of both cholangiocarcinoma (CCC) and gallbladder cancer. Approximately 90% of BTCs are adenocarcinoma arising from the epithelial lining of the gallbladder and intrahepatic and extrahepatic bile duct. CCC is classified, according to its anatomical location in biliary tract, into 3 subtypes: intrahepatic, perihilar, and distal extrahepatic.

BTC shows differences in etiology, prevalence, and molecular alterations between Caucasian and Asian populations. BTC is relatively rare in Europe and the USA. Age-adjusted rates of CCC are reportedly lowest in non-Hispanic white people and black people (both 2.1 per 100,000) and highest in Hispanic and Asian populations (2.8–3.3 per 100,000).[1,2] The highest rates are found in Eastern Asia especially the northeast of Thailand (85 per 100,000),[3] whereas rates are low in South, Central, and Western Asia, as well as in Northern and Eastern Europe.[4] The National Cancer Institute in Thailand estimated that liver and bile duct cancers were the most common cancers in men, with an estimated 8,000 new cases per year; the highest incidence was found in Khon Kaen, north-eastern region of Thailand.[5]

The etiology of CCC in Asian countries is liver fluke infestation especially *Opisthorchis viverrini* and *Clonorchis sinensis*. It induced chronic inflammation leading to oxidative DNA damage of the biliary epithelium and malignant transformation.

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C. sinensis infestation is common in rural area of Korea and China, whereas *O. viverrini* infestation is highly prevalent in the northeast of Thailand.[6]

Thailand has the highest incidence of intrahepatic CCC in the world, perhaps related to a tradition of eating raw fish, which may be contaminated with *O. viverrini*.[7]

Although surgery is the only curative treatment for CCC, the resection rate is quite low and variable, as most patients present with advanced disease. Median survival after CCC resection is 10–40 months. Patients with locally advanced or metastatic BTC have poor prognoses, with 5-year survival rates of 5%–10%.[8] The response rate (RR) of first-line systemic chemotherapy (gemcitabine- or 5-fluorouracil [5-FU]-based regimens) is 10%–40%.[9] Patients who received single-agent gemcitabine had median overall survival (mOS) of 6.5–11.5 months,[10,11] whereas patients treated with 5-FU/leucovorin (LV) had mOS of 6–6.5 months.[12–14] For patients who received combined 5-FU and cisplatin, mOS was 9.5–10 months.[15–17] The other gemcitabine combination regimen was studied in a Phase II trial that showed an RR of 9%–36% and mOS of 11–15.4 months. In addition, a Phase III randomized study (ABC-02) showed longer overall survival (OS) from gemcitabine/cisplatin over gemcitabine alone and led to this combined regimen becoming a standard first-line treatment.[18] However, survival of patients with advanced disease remains poor with the current treatments.[19]

In the era of individualized medicine and targeted therapy, the molecular pathogenesis of CCC is worthy of study. Established mutations and amplification of known oncogenes had been shown in previous studies, including various molecular differences between Caucasian and Asian populations; for example, 8%–22% of Caucasians with CCC showed *BRAF* mutations, whereas no *BRAF* mutations were seen in Asian patients, who had higher rates of *KRAS* and *PIK3CA* mutations and *ROS1* gene rearrangements; however, *EGFR* mutation rates were similar in Asian and Caucasian patients (14%–17%).[20–38]

In our previous pilot study by Detarkom,[39] we found that some clinical prognostic factors affected survival, including staging, Eastern Cooperative Oncology Group (ECOG) performance status, surgical resection, and carbohydrate antigen 19-9 (CA19-9) pretreatment level. The study showed a trend of better OS in a patient with strong ALK expression, but due to the shortage of tissue sample, fluorescent in situ hybridization (FISH) for ALK could not be performed. Therefore, in this study, we explored clinical factors more extensively to predict prognosis and further studied genomic alteration in CCC in Thai patients with an aim to develop new treatment for this lethal disease.

Materials and methods

Study cohort, data, and clinical characteristics

This study used a computerized search of the tumor registry database of Ramathibodi Hospital for patients treated from November 2007 to December 2013. The data were accessed using the International Classification of Disease-10 (ICD-10) and the database from the tumor bank of the Pathology Department. We selected patients who had been diagnosed with BTC, and from whom adequate tumor tissue for extracting DNA was available. We fully reviewed their medical records, with particular regard to the natural history of their disease including clinical and tumor characteristics (age, sex, smoking status, staging, tumor type, presenting symptoms, CA19-9 and carcinoma embryonic antigen [CEA] levels at diagnosis, viral hepatitis B surface antigen [HBsAg], and anti-hepatitis C virus [HCV] status, patient's birthplace, resection procedure, lymph node status, and medical treatment).

This study was approved by the ethics committee of Ramathibodi Hospital, Mahidol University, Bangkok, Thailand (EC approval number 11-56-03). As data collection and further analyses were performed without disclosure of the identity and private information of patients, informed consents for the review of medical records and the use of archived tissue samples were not required by the ethics committee of Ramathibodi Hospital.

We categorized tumor into 2 groups (intrahepatic and hilar/extrahepatic/gallbladder) by Bismuth classification that defines intrahepatic as a tumor that is located in intrahepatic bile duct, hilar type is located from common hepatic duct to position of cystic duct, extrahepatic is located at common bile duct to ampulla of Vater, and gallbladder type is tumor located at gallbladder and cystic duct.

Age at diagnosis was divided into 4 ranges: ≤50, 51–60, 61–70, or ≥71 years. Staging of disease was performed by using American Joint Committee on Cancer (AJCC) TNM staging system (seventh edition, 2010) according to the diagnosis.

Performance status was evaluated by ECOG scale and criteria. ECOG criteria was defined as follows: 0= fully active, able to carry on all pre-disease performance without restriction; 1= restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, eg, light house work, office work; 2= ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours; 3= capable of only limited self-care, confined to bed or chair more than 50% of waking hours; 4= completely disabled, cannot carry on any self-care. Totally confined to bed or

chair; 5= death. We grouped patients into 2 groups as ECOG 0–1 and ECOG 2–4 for analyzing the data.

Presenting symptoms were categorized into 2 categories: asymptomatic or symptomatic with any symptoms (gastrointestinal-related symptoms and other symptoms that were not related to gastrointestine, eg, weight loss, back pain, and dyspnea).

Surgery was categorized into curative, diagnostic, or palliative procedure. Two categories of systemic chemotherapy were gemcitabine-based regimen (gemcitabine single agent, gemcitabine with cisplatin, and gemcitabine with carboplatin) or non-gemcitabine-based regimen (capecitabine, or 5-FU with LV). Lymph node status was obtained by pathological report from surgical specimen. HCV infection was evaluated by enzyme immunoassay to detect HCV antibodies. Hepatitis B virus (HBV) infection was evaluated by enzyme-linked immunosorbent assay (ELISA) to detect HBsAg. Blood for CA19-9& CEA was collected at the time of first visit. Regarding hometown, patients from northern and north-eastern part of Thailand were analyzed compared with those who came from central, western, and southern part of Thailand, because north and north-eastern part of Thailand has highest incident rate of CCC. The result of treatment was assessed at the time after complete treatment with surgery, radiation therapy, or chemotherapy by clinical or radiological examination with Response Evaluation Criteria In Solid Tumors (RECIST) criteria.

Molecular alterations study: formalin-fixed, paraffin-embedded (FFPE) tissue blocks from our archives, with tumor cellularity ≥50%, were routinely prepared by a pathologist at the Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University. This study was approved by the research ethics committee of Faculty of Medicine Ramathibodi Hospital, Mahidol University (approval ID 11-56-03).

We randomly selected 81 FFPE tissue blocks from the years 2010–2013 and prepared them for ALK, HER2, and Ki-67 immunohistochemistry (IHC) staining. Molecular testing for mutations in *KRAS*, *BRAF*, *EGFR*, and *PIK3CA* from DNA extractions and RNA extractions was performed for FISH (only for ALK+ samples), and *ROS1* translocation tests.

Molecular testing for *PIK3CA*, *BRAF*, *KRAS*, and *EGFR* mutations

Paraffin-embedded tissue was dissolved in xylene and followed by 2 washes with 100% ethanol to remove residual xylene. Tissue was digested at 56°C for 1 hour, then at 90°C 1 hour, with 180 µL of ATL buffer and 20 µL of proteinase K. After digestion, 200 µL of alkaline lysis (AL) buffer was added. The solution was transferred into a spin column and washed with the wash buffers provided in the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA was eluted in 30 µL of ATE buffer and was ready for use in amplification reactions or for storage at −20°C.

PIK3CA, *BRAF*, *KRAS*, and *EGFR* mutation testing by amplification-refractory mutation system-based quantitative polymerase chain reaction (ARMS-qPCR)

DNA samples were subjected to *PIK3CA*, *BRAF*, *KRAS*, and *EGFR* mutant analysis using AmoyDx *PIK3CA* 5 Mutations, *BRAF V600* Mutations, *KRAS* 7 Mutations, and *EGFR* 29 Mutations Detection Kits (Amoy Diagnostics, Xiamen, China). These kits employ ARMS-real-time (RT) PCR technology to detect 5 common mutations in the *PIK3CA* gene, *V600* mutation in the *BRAF* gene, 7 mutations in *KRAS* codons 12 and 13, and 29 mutations in *EGFR* gene (Table S1). The experiments and analyses were performed according to the manufacturer's instructions, using Bio-Rad CFX96 RT-PCR (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Sanger sequencing for *EGFR* mutations

We assembled 10 ng DNA with 10 µL AmpliTaq Gold® PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 10 µM forward and reverse *EGFR*-specific primers (Table S2). PCRs were amplified, and the PCR product was then purified. Sequencing reactions were performed using chain-terminating dideoxynucleotides (BigDye® 1.0; Thermo Fisher Scientific) and loaded into an automated ABI 310 sequencer. Eventually, the data were analyzed by Sequencer 3.1.1 Software (Thermo Fisher Scientific).

Droplet digital PCR (ddPCR) for *EGFR* mutations

ddPCR reagents were ordered from Bio-Rad Laboratories Inc. Combined nucleic DNA and primer/probe mixes for *EGFR* T790M were custom made by Thermo Fisher Scientific. PCRs were performed from a DNA template, 1× ddPCR Mastermix (Bio-Rad Laboratories Inc.), TaqMan probe, and 20× custom primers made specifically for each assay. Each ddPCR mix was loaded into the wells of a droplet generator cartridge (Bio-Rad Laboratories Inc.). The target DNA and background DNA were randomly distributed in droplets, which were transferred to a 96-well PCR plate. The plate was sealed and subjected to the PCR protocol. The 96-well PCR

plate was loaded into the QX-100 droplet reader (Bio-Rad Laboratories Inc.). Data were read and analyzed by QuantaSoft analysis software (Bio-Rad Laboratories Inc.).

ROS1 translocation testing by RT-PCR

Paraffin was removed from FFPE tissue sections by treatment with xylene. Samples were incubated at 56°C for 15 min; then, at 80°C for15 min with lysis buffer, which contained proteinase K, treat lysate with DNase was then mixed with buffer red blood cell and ethanol. The solution was applied to an RNeasy MinElute spin column. RNA was eluted into 14 µL of RNase-free water and was ready for use or for storage at −80°C.

We evaluated FFPE tumor samples for *ROS1* fusion using the AmoyDx*ROS1* Gene Fusions Detection Kit (Amoy Diagnostics). About 50 ng/mL of RNA OD260/OD280 value (1.9–2.0 of RNA) sample was used for reverse transcription and RT-PCR of 4 reactions of *ROS1* Fusion Gene Detection Kit according to the manufacturer's instruction. This kit detected 14 *ROS1* gene fusions with various spliced genes and exons. We analyzed reaction sample reference gene Ct value ≤20. If sample 6-carboxyfluorescein (FAM) Ct value was <30, the sample was considered positive for one of the variants detected by reaction mixture.

IHC staining

All IHC staining was performed on 4 µm-thick FFPE tissue sections. The slides were deparaffinized, and antigen retrieval was performed. We identified ALK, HER2, and Ki-67 expression by IHC using D5F3, HER2/neu, and Ki-67 antibodies, respectively, on the sections. For ALK, FFPE tissues were sectioned at 4 µm thickness and stained with anti-ALK rabbit monoclonal antibody (clone D5F3; 1:20 dilution; Ventana Medical Systems, Tucson, AZ, USA), using the Optiview DAB IHC Detection Kit and Optiview Amplification Kit with the Ventana Benchmark XT Stainer (Ventana Medical Systems) according to the manufacturer's protocol. Immunoreactivity was scored as follows: 0, no staining; 1+, faint cytoplasmic staining; 2+, moderate cytoplasmic staining; 3+, strong granular cytoplasmic staining in ≥10% of tumor cells. Immunoreactivity was evaluated as positive or negative according to the manufacturer's protocol. If the specimen had positive IHC staining for ALK, we then performed FISH to confirm *ALK* rearrangement.

Tissue sections were IHC stained for HER2 on BenchMark XT IHC/ISH staining module (Ventana Medical Systems), using the technical protocol XT UltraView DAB V3 by incubation with anti-HER2/neu (4B5) rabbit monoclonal primary antibody. Antigen detection was carried out using UltraView Universal DAB IHC Detection Kit (Ventana Medical Systems). IHC staining was assessed and scored by a pathologist. HER2 expression scores of 0 and 1+ were considered to be HER2 negative, and 2+ and 3+ as HER2 positive. A standard criterion for HER2 scoring was utilized.[40]

Tissue sections were IHC stained for Ki-67 on a BenchMark XT IHC/ISH staining module (Ventana Medical Systems) using the technical protocol XT Ultra View DAB V3 by incubation with Confirm anti-Ki-67 (30-9) rabbit monoclonal primary antibody. Antigen detection was carried out using UltraView Universal DAB IHC Detection Kit. IHC staining was assessed and scored by a pathologist. The labeling index of the Ki-67 in each tumor was estimated as a percentage of positive cells out of 100–1,000 counted tumor cells.

Statistical analysis

Statistical analyses were performed using STATA software v.13 (StataCorp LP, College Station, TX, USA). OS and progression-free survival (PFS) were calculated and censored on January 31, 2015. OS and PFS curves were drawn using the Kaplan–Meier method. The log-rank test was used to compare survival rates by each variable. Univariate analysis of OS and PFS used the Cox proportional hazards regression model. Significantly, prognostic factors were included in subsequent multivariate analyses. $P \leq 0.05$ was considered significant. The correlation between molecular alteration and survival was compared by log-rank test. Mean Ki-67 in each molecular alteration group was compared by the Mann–Whitney *U* test.

Results

EGFR mutation

We found that 17 out of 81 samples (21%) were positive for *EGFR* mutations, comprising 9 with *T790M*, 4 with *S768I*, 2 with *L861Q*, 1 with *G719X*, and 1 with insertion (Table 1). The *EGFR*$^+$ group showed a nonsignificant trend of longer

Table 1 Molecular alteration in CCC

Gene	Positive samples	%
EGFR mutation	17 in 81	21
T790M	9 in 17	53
S768I	4 in 17	24
L861Q	2 in 17	12
G719X	1 in 17	6
Exon 20 insertion	1 in 17	6
KRAS mutation	10 in 81	12
BRAF mutation	0 in 81	0
PIK3CA mutation	13 in 81	16
ROS1 translocation	0 in 81	0
ALK expression	0 in 74	0
HER2 expression	3 in 74	4
Ki-67 expression	74 in 74	Range 4%–85%

Abbreviation: CCC, cholangiocarcinoma.

mOS than the *EGFR*$^{-}$ group (7.92 vs 11.52 months, *P*=0.927) (Table 2 and Figure 1A). Median PFS (mPFS) was comparable between the *EGFR*$^{Mut+}$ and *EGFR*$^{Mut-}$ groups (6.45 vs 7.60 months, *P*=0.724; Table 2 and Figure 2A). Patients with *T790M*$^{Mut+}$ mutations group showed a nonsignificant trend of longer mOS (18.84 vs 11.52 months, *P*=0.199) and mPFS (10.15 vs 7.60 months, *P*=0.371; Table 2 and Figures 1B and 2B) than the *EGFR*$^{Mut-}$ group. However, patients with *T790M* mutations had a significant longer mOS (18.84 months) than the *non-T790M* mutation group (4.08 months, *P*=0.038; Table 2 and Figure 1C); the *T790M* group also had a trend of longer mPFS (10.15 vs 3.63 month, 0.063; Table 2 and Figure 2C).

We further validated the positive results of *EGFR*$^{Mut+}$ mutation results by the Sanger direct sequencing method which is a gold standard method for mutation analysis, and by ddPCR, which is a highly sensitive method for detecting low percentages of gene mutations (0.1%–5% of gene frequency) compared with ARMS-qPCR (1%–5% of frequency).[41] We found 100% concordance of *EGFR T790M* mutation by ddPCR, compared with qRT-PCR, but only 5 of 9 (55.55%) *T790M* mutations were detected by direct sequencing (Table 3 and Figure 3). We did not confirm exon 20 insertion and *L861Q* in ddPCR, because we did not have enough DNA. However, we confirmed *L861Q* with Sanger direct sequencing, but unfortunately we did not find it.

PIK3CA mutation

In 81 samples, 13 (16%) were positive for *PIK3CA* mutations (*PIK3CA*$^{Mut+}$), which comprised 6 with *E545K*, 3 with *E542K*, 3 with *H1047R*, and 1 with double mutations of *E542K* and *E545K* (Table 1). The *PIK3CA*$^{Mut+}$ had a trend of longer mOS than the *PIK3CA*$^{Mut-}$ group (18.84 vs 7.56 months, *P*=0.072) and significantly longer mPFS than the *PIK3CA*$^{Mut-}$ group (15.87 vs 7.01 months, *P*=0.043; Table 2 and Figures 1D and 2D).

KRAS mutation, *BRAF* mutation, and *ROS1* translocation

We found that 10 out of the 81 samples (12%) were positive for *KRAS* mutation (*KRAS*$^{Mut+}$), comprising 2 with *G12Asp*, 1 with *G12Ala*, 3 with *12V*, 1 with *G12S*, 1 with *G13Asp*, and 1 with double mutations of *G12Asp* and *G12* (Table 1). The *KRAS*$^{Mut+}$ and *KRAS*$^{Mut-}$ groups did not significantly differ in mOS or mPFS at $P<0.05$ (Table 2 and Figures 1E and 2E). We detected no *BRAF* mutation or *ROS1* translocation in the 81 samples (Table 1).

Clinical characteristics and prognostic factors based on significant gene alterations

The clinical characteristics and clinical data categorized based on each molecular alterations (*EGFR*, *EGFR T790M*, *PIK3CA*, and *KRAS*) of CCC patients were similar as listed in Table 4, except staging that was significantly different based on *KRAS* mutation and CEA baseline level at cut point of 15 μg/L was significantly different based on *PIK3CA* mutation.

IHC

Not all 81 patients were tested for ALK expression due to the exhaustion of tumor tissue. We tested 74 samples for ALK expression, all of which showed negative ALK expression (Table 1). We found strongly positive staining (IHC 2+ and 3+) for HER2 expression in 3 out of 74 (4%) samples.

Table 2 OS and PFS of CCC by molecular analysis (N=81)

Molecular analysis	Patients, n (%)	mPFS (months)	HR (95% CI)	*P*-value	mOS (months)	HR (95% CI)	*P*-value
EGFR mutation (N=81)							
Negative (reference)	64 (79)	7.60	1.11 (0.62–2.00)	0.724	11.52	0.97 (0.51–1.82)	0.927
Positive	17 (21)	6.45			7.92		
EGFR mutation (*T790M*) (N=73)							
Negative (reference)	64 (88)	7.60	0.68 (0.29–1.58)	0.371	11.52	0.55 (0.22–1.37)	0.199
T790M	9 (12)	10.15			18.84		
EGFR mutation (*T790M*) (N=17)							
Non-T790M (reference)	8 (47)	3.63	0.34 (0.11–1.06)	0.063	4.08	0.26 (0.08–0.93)	0.038
T790M	9 (53)	10.15			18.84		
PIK3CA mutation (N=81)							
Negative (reference)	68 (84)	7.01	0.46 (0.22–0.98)	0.043	7.56	0.50 (0.24–1.06)	0.072
Positive	13 (16)	15.87			18.84		
KRAS mutation (N=81)							
Negative (reference)	71 (88)	7.60	1.18 (0.58–2.38)	0.643	9.24	1.07 (0.52–2.26)	0.847
Positive	10 (12)	7.01			9.36		

Abbreviations: CCC, cholangiocarcinoma; HR, hazard ratio; mOS, median OS; mPFS, median PFS; OS, overall survival; PFS, progression-free survival.

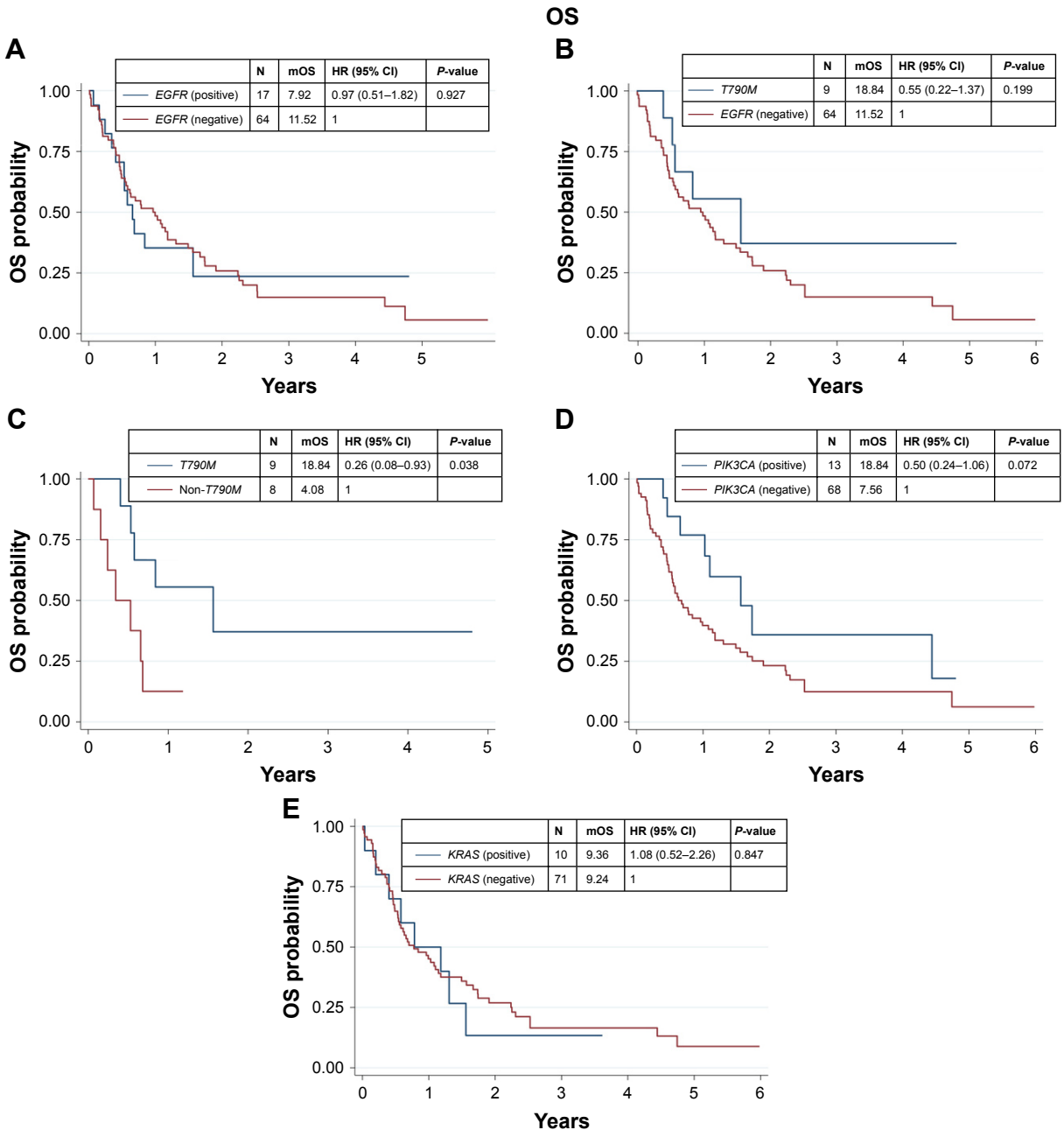

Figure 1 Kaplan–Meier curve for OS of CCC according to gene alteration.
Notes: (**A**) OS by *EGFR* mutation. (**B**) OS by *EGFR T790M* mutation and negative *EGFR* mutation. (**C**) OS by *EGFR T790M* mutation and other positive *EGFR* mutation. (**D**) OS by *PIK3CA* mutation. (**E**) OS by *KRAS* mutation.
Abbreviations: CCC, cholangiocarcinoma; HR, hazard ratio; mOS, median OS; OS, overall survival.

Another 9 samples had IHC 1+ staining for HER2 (Table 1). Median Ki-67 was 42.5% (interquartile range: 4%–85%). The $EGFR^{Mut+}$ and $EGFR^{Mut-}$ groups did not significantly differ in median Ki-67 (65% vs 40%, P=0.121). However, *T790M* samples had a significantly lower median Ki-67 than did samples without *T790M* mutations (22.5% vs 80%, P=0.025). The $PIK3CA^{Mut+}$ group had lower median Ki-67 but not significantly so (25% and 47.5%, P=0.273). The $KRAS^{Mut+}$ and $KRAS^{Mut-}$ groups did not significantly differ in median Ki-67 (22.5% vs 45%, P=0.260; Table 5).

Discussion

CCC is a lethal malignancy that usually presents at an advanced stage. Due to the difficulty of early diagnosis and limited effective treatment, CCC has a poor prognosis. Few new treatments seem to be in development or in trials,

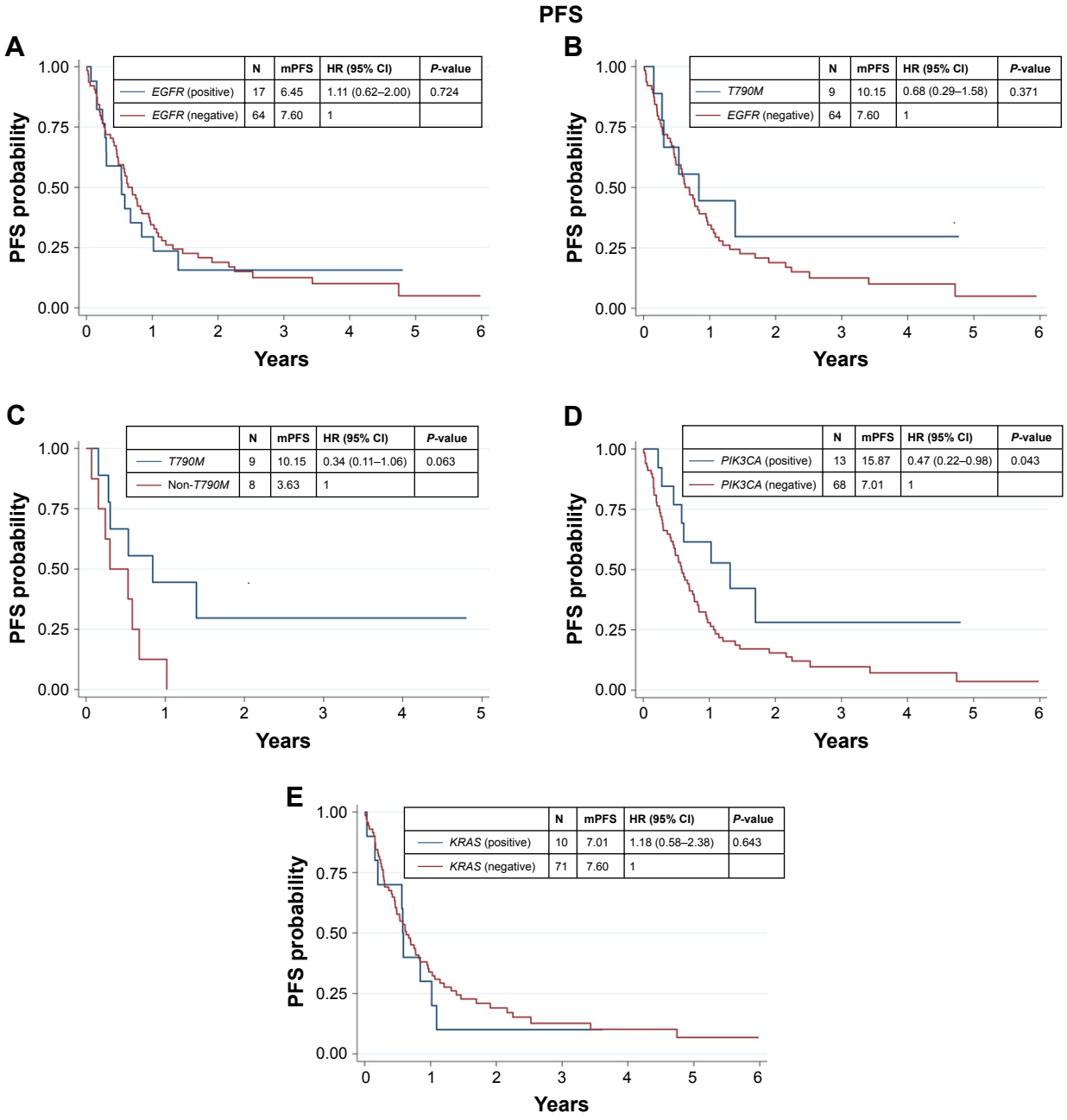

Figure 2 Kaplan–Meier curve for PFS of CCC according to gene alteration.
Notes: (**A**) PFS by *EGFR* mutation. (**B**) PFS by *EGFR T790M* mutation and negative *EGFR* mutation. (**C**) PFS by *EGFR T790M* mutation and other positive *EGFR* mutation. (**D**) PFS by *PIK3CA* mutation. (**E**) PFS by *KRAS* mutation.
Abbreviations: CCC, cholangiocarcinoma; HR, hazard ratio; mPFS, median PFS; PFS, progressive-free survival.

because of the lack of clinical data and oncogenic data. However, as most relevant studies show differences in clinical characteristics and genetic alterations in Western and Asian populations, we have therefore investigated the clinical data and genomic alterations in Thai patients.

In our study, we found 21% of Thai patients with CCC had $EGFR^{Mut+}$ tumor tissues, which was higher than that seen in other populations investigated in previous studies.[32,42–44] Types of *EGFR* mutation included 1 case in exon 18 (*G719X*), 13 cases in exon 20 (9 of *T790M*, 4 of *S768I*), and 3 cases in exon 21 (2 of *L861Q* and 1 of insertion). Consistent with a Taiwan study,[43] no exon 19 mutation was identified, but contrast to Italian study,[42] which found an exon 19 substitution (*K757R*) in 1 out of 40 samples. Interestingly, we found a high rate of *T790M* mutation (50% of *EGFR* mutation), and this group had significant longer OS and a trend of longer

Table 3 Detection of EGFR mutation by qRT-PCR, Sanger sequencing, and ddPCR

EGFR mutation	qRT-PCR	Sanger sequencing	ddPCR
EGFR	17	7	14
T790M	9	5	9
S768I	4	2	4
L861Q	2	ND	NA
G719X	1	ND	1
Exon 20 insertion	1	NA	NA

Abbreviations: ddPCR, droplet digital polymerase chain reaction; NA, not available; ND, not determined; qRT-PCR, quantitative real-time polymerase chain reaction.

PFS than did the *non-T790M* group. However, the study by Chang et al[43] reported *EGFR* mutation to be the strongest independent predictor of shorter OS. In addition to longer survival, patients with the *T790M* mutation in our study also had a significant lower median Ki-67 compared with the *non-T790M* group – interestingly, as we know that Ki-67 indicates cell proliferation.

To our knowledge, no study has previously reported this correlation between *T790M* mutation and clinicopathological factors or prognosis in patients with CCC.

Approximately 12% of patients had *KRAS* mutations, most of which were located in codon 12 and only 1 in codon 13. One of the previous studies reported 16% of *KRAS* mutation in codon 12 in Thai population.[45] Other populations have shown a reported 13%–50% prevalence of *KRAS* mutations in BTCs.[22,36,37,43,46] *BRAF* mutation was not detected in this study, which was similar to the report by Xu et al[36] in a Chinese population, whereas Tannapfel et al[22] demonstrated 22% (15/69) had positive *BRAF* mutations but found no significant correlation with the other clinicopathological factors and patient survival. We could not find the *ROS1* translocation in our Thai CCC cohort, although a 2011 study found 8.7% of *ROS1* translocations in an Asian population.[35]

PIK3CA mutation was rare in a previous study; Riener et al[47] reportedly found it in only 1 out of 11 (9%) intrahepatic CCC samples and 1 out of 23 (4%) in gallbladder carcinoma samples.

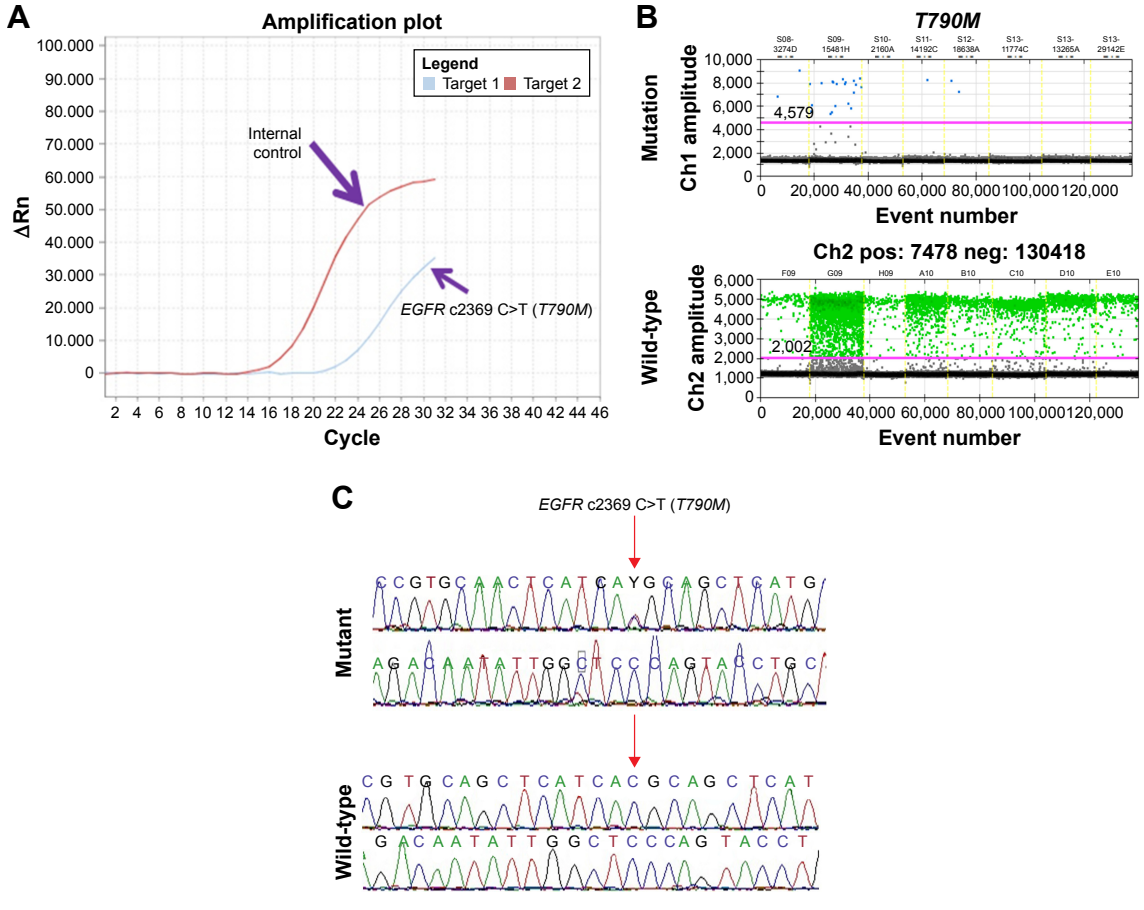

Figure 3 Analysis of *EGFR T790M* mutation using RT-PCR, ddPCR, and Sanger sequencing.
Notes: (**A**) Amplification curve of sample with *EGFR* c2369 C>T (*T790M*) mutation (blue line) and internal control (red line). (**B**) ddPCR amplitude scatter plot, single-well data for wild-type DNA; mutation assay (FAM, channel 1), wild-type assay (Hex, channel 2). (**C**) Chromatograms *EGFR* c2369 C>T (*T790M*).
Abbreviations: Ch1, channel 1; Ch2, channel 2; ddPCR, droplet digital polymerase chain reaction; FAM, 6-carboxyfluorescein; Hex, hexachloro-6-carboxyfluorescein; neg, negative; pos, positive; RT-PCR, real-time polymerase chain reaction.

Table 4 Demographic and clinical characteristics of patients categorized by mutations

Characteristic	*EGFR* mutation		*P*-value	*EGFR T790M*		*P*-value	*PIK3CA* mutation		*P*-value	*KRAS* mutation		*P*-value
	Positive	Negative		*T790M*	*Non-T790M*		Positive	Negative		Positive	Negative	
Age, years (N=81)												
≤50	3 (3.70)	8 (9.88)	0.733	2 (11.77)	1 (5.88)	0.430	3 (3.70)	8 (9.88)	0.710	2 (2.47)	9 (11.11)	0.572
51–60	6 (7.41)	24 (29.63)		4 (23.53)	2 (11.77)		4 (4.92)	26 (32.10)		2 (2.47)	28 (34.57)	
61–70	7 (8.64)	22 (27.16)		2 (11.77)	5 (29.41)		4 (4.92)	25 (30.86)		4 (4.92)	25 (30.86)	
≥71	1 (1.23)	10 (12.35)		1 (5.88)	0 (0)		2 (2.47)	9 (11.11)		2 (2.47)	9 (11.11)	
ECOG performance status (N=81)												
0–1	16 (19.75)	58 (71.61)	1.000	9 (52.94)	7 (41.18)	0.471	13 (16.05)	61 (75.31)	0.591	8 (9.88)	66 (81.48)	0.206
2–4	1 (1.23)	6 (7.41)		0 (0)	1 (5.88)		0 (0)	7 (8.64)		2 (2.47)	5 (6.17)	
Sex (N=81)												
Male	6 (7.41)	39 (48.15)	0.059	2 (11.77)	4 (23.53)	0.335	8 (9.88)	37 (45.68)	0.636	5 (6.17)	31 (38.27)	0.745
Female	11 (13.58)	25 (30.86)		7 (41.18)	4 (23.53)		5 (6.17)	31 (38.27)		5 (6.17)	40 (49.38)	
Type (N=81)												
Intrahepatic	15 (18.52)	62 (96.88)	0.192	7 (41.18)	8 (47.06)	0.471	12 (14.81)	65 (80.25)	0.511	9 (11.11)	68 (83.95)	0.416
Extrahepatic + GB	2 (2.47)	2 (3.13)		2 (11.77)	0 (0)		1 (1.24)	3 (3.70)		1 (1.24)	3 (3.70)	
Lymph node status (N=51)												
Positive	7 (13.73)	24 (47.06)	0.454	5 (55.56)	2 (22.22)	1.000	6 (11.76)	25 (49.02)	0.382	5 (9.8)	26 (50.98)	0.724
Negative	2 (3.92)	18 (35.29)		2 (22.22)	0 (0)		6 (11.76)	14 (27.45)		4 (7.84)	16 (31.37)	
Staging (N=81)												
I	4 (4.94)	14 (17.28)	1.000	2 (11.77)	2 (11.77)	1.000	5 (6.17)	13 (16.05)	0.121	2 (2.47)	16 (19.75)	0.030
II	1 (1.24)	6 (7.41)		1 (5.88)	0 (0)		1 (1.24)	6 (7.41)		0 (0)	7 (8.64)	
III	0 (0)	2 (2.47)		0 (0)	0 (0)		1 (1.24)	1 (1.24)		2 (2.47)	0 (0)	
IV	12 (14.82)	42 (51.85)		6 (35.29)	6 (35.29)		6 (7.41)	48 (59.26)		6 (7.41)	48 (59.26)	
Smoking status (N=81)												
Current	3 (3.70)	4 (4.94)	0.157	0 (0)	3 (17.65)	0.082	1 (1.24)	6 (7.41)	1.000	1 (1.24)	6 (7.41)	1.000
No smoking/ ex-smoker	14 (17.28)	60 (74.07)		9 (54.24)	5 (29.41)		12 (14.82)	62 (76.54)		9 (11.11)	65 (80.25)	
Group of symptoms (N=81)												
Asymptomatic	2 (2.47)	15 (18.52)	0.503	0 (0)	2 (11.77)	0.206	2 (2.47)	15 (18.52)	0.726	1 (1.24)	16 (19.75)	0.680
Symptomatic	15 (18.52)	49 (79.01)		9 (54.24)	6 (35.29)		11 (13.58)	53 (65.43)		9 (11.11)	55 (67.90)	
Surgery (N=81)												
Yes	11 (13.58)	44 (54.32)	0.751	8 (47.06)	3 (17.65)	0.050	12 (14.82)	43 (53.09)	0.052	9 (11.11)	46 (56.79)	0.156
No	6 (7.41)	20 (24.69)		1 (5.88)	5 (29.41)		1 (1.24)	25 (30.86)		1 (1.24)	25 (30.86)	
Aim of surgery (N=55)												
Curative	9 (16.36)	40 (72.73)	0.413	6 (54.55)	3 (27.27)	1.000	11 (20)	38 (69.09)	1.000	9 (16.36)	40 (72.73)	0.645
Diagnosis	2 (3.64)	3 (5.45)		2 (18.18)	0 (0)		1 (1.81)	4 (7.27)		0 (0)	5 (9.09)	
Palliative	0 (0)	1 (1.82)		0 (0)	0 (0)		0 (0)	1 (1.81)		0 (0)	1 (1.81)	
Receive first-line systemic chemotherapy (N=81)												
No	11 (13.58)	40 (49.38)	0.867	6 (35.29)	5 (29.41)	1.000	8 (9.88)	43 (53.09)	1.000	7 (8.64)	44 (54.32)	0.737
Yes	6 (7.41)	24 (29.63)		3 (17.65)	3 (17.65)		5 (6.17)	25 (30.86)		3 (3.70)	27 (33.33)	
Regimen of systemic chemotherapy (N=31)												
Gemcitabine based	6 (19.35)	22 (70.97)	1.000	3 (50)	3 (50)	N/A	5 (16.13)	23 (74.19)	1.000	2 (6.45)	26 (83.87)	0.271
Non-gemcitabine based	0 (0)	3 (9.68)		0 (0)	0 (0)		0 (0)	3 (9.68)		1 (3.23)	2 (6.45)	
Site of metastases (N=54)												
≤1	9 (16.67)	35 (64.81)	0.674	5 (41.67)	4 (33.33)	1.000	5 (9.26)	39 (72.22)	1.000	6 (11.11)	38 (70.37)	0.580
≥2	3 (5.56)	7 (12.96)		1 (8.33)	2 (16.67)		1 (1.85)	9 (16.67)		0 (0)	10 (1.85)	
HBsAg (N=56)												
Positive	3 (5.36)	9 (16.07)	1.000	1 (7.69)	2 (15.39)	0.559	1 (1.79)	11 (19.64)	0.671	2 (3.57)	10 (17.86)	0.635
Negative	10 (17.86)	34 (60.71)		6 (46.15)	4 (30.77)		9 (16.07)	35 (62.50)		5 (8.93)	39 (69.64)	
Anti-HCV (N=53)												
Positive	0 (0)	0 (0)	N/A	0 (0)	0 (0)	N/A	0 (0)	0 (0)	N/A	0 (0)	0 (0)	N/A
Negative	12 (22.64)	41 (77.36)		6 (50)	6 (50)		10 (1.89)	43 (81.13)		6 (11.32)	47 (88.68)	
Birthplace (N=81)												
N + NE	8 (9.88)	28 (34.57)	0.807	3 (17.65)	5 (29.41)	0.347	5 (6.17)	31 (38.27)	0.636	7 (8.64)	29 (35.80)	0.100
C + S + W	9 (11.11)	36 (44.44)		6 (35.29)	3 (17.65)		8 (9.88)	37 (45.68)		3 (3.70)	42 (51.85)	

(*Continued*)

Table 4 (*Continued*)

Characteristic	*EGFR* mutation		*P*-value	*EGFR T790M*		*P*-value	*PIK3CA* mutation		*P*-value	*KRAS* mutation		*P*-value
	Positive	Negative		*T790M*	*Non-T790M*		Positive	Negative		Positive	Negative	
CEA (N=68)												
<15	8 (11.77)	41 (60.29)	0.727	4 (33.33)	4 (33.33)	0.576	13 (19.12)	36 (52.94)	0.014	7 (10.29)	42 (61.76)	1.000
≥15	4 (5.88)	15 (22.06)		3 (25.00)	1 (8.34)		0 (0)	19 (27.94)		2 (2.94)	17 (25.00)	
CA19-9 (N=75)												
<180	6 (8.00)	36 (48.00)	0.163	3 (20.00)	3 (20.00)	1.000	10 (13.33)	32 (42.67)	0.095	8 (10.67)	34 (45.33)	0.170
≥180	9 (12.00)	24 (32.00)		5 (33.33)	4 (26.67)		3 (4.00)	30 (40.00)		2 (2.67)	31 (41.33)	

Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; ECOG, Eastern Cooperative Oncology Group; GB, gallbladder; HBsAg, hepatitis B surface antigen; N, northern; N/A, no data; NE, north-eastern; C, central; S, southern; W, western.

Another study from China showed that 32.4% of patients with CCC had *PIK3CA* mutations.[36] However, our study is the first to report the prevalence of *PIK3CA* mutation in Thai patients with CCC. Interestingly, the $PIK3CA^{Mut+}$ group had significantly longer mPFS than did the $PIK3CA^{Mut-}$ group. However, no previous study had analyzed patient survival in this group, although Xu et al[36] found that $PIK3CA^{Mut-}$ CCC was detected at a more advanced stage and in more aggressive forms.

We identified HER2 expression in only 3 out of 74 samples (4%), whereas Nakazawa et al[26] identified HER2 overexpression in 15.7% of gallbladder carcinoma samples and 5.1% of extrahepatic bile duct carcinoma samples. We found strongly positive (3+) HER2 in 1 sample, from a patient who was diagnosed with early-stage disease and underwent curative surgery; 4 months later, he developed metastatic disease at lung, liver, adrenal gland, and lymph nodes. He received palliative chemotherapy, but unfortunately the disease was very aggressive, and his OS was 6 months after his diagnosis. This may imply that high HER2 expression is associated with more aggressive tumors.

IHC for Ki-67 showed a median Ki-67 of 42.5%. A previous study showed that high Ki-67 expression was correlated with advanced stage disease and could be used as a prognostic biomarker for CCC.[48] We also found low median Ki-67 (22.5%) in the *T790M* mutation group, which correlated with longer survival.

We have validated our study's results by both Sanger direct sequencing technique and ddPCR technique; we found 100% concordance between ARMS-RT-PCR and ddPCR. This result was also similar to that of Zhang et al[41] in a Chinese population. However, we found only 55.6% concordance between Sanger direct sequencing technique and RT-PCR technique. Previous studies found that RT-PCR was significantly more sensitive in detecting mutation than Sanger DNA sequencing. Each technique has different limitations in detecting solid tumor mutations; for example, Sanger direct sequencing technique can detect 15%–20% mutant alleles, whereas ddPCR can detect low abundance mutations, present in only 0.02% of alleles.[49,50] The ddPCR is a new technology to detect invasive genotyping of cfDNA in plasma, which can thus help detect acquired resistance in lung cancer.[51–53]

Our study had some limitations. First, because of the retrospective nature of the study, some data had been missing. Second, archival tissue specimens may suffer DNA damage, which can affect genomic alteration testing.

Table 5 Median Ki-67 in each group of molecular analysis and tumor marker

Characteristics	Median Ki-67	*P*-value
EGFR mutation: negative vs positive	40% vs 65%	0.121
EGFR mutation: *non-T790M* vs *T790M*	80% vs 22.5%	0.025
KRAS mutation: negative vs positive	45% vs 22.5%	0.260
PIK3CA mutation: negative vs positive	47.5% vs 25%	0.273

Conclusion

Cancer genetics may guide direct therapeutic decision in CCC, including the use of targeted drugs. *EGFR* mutations and *PIK3CA* mutations may imply and guide targeted therapy for CCC in the future, but due to the small sample size of this study, thus, we need to further explore in the larger population. Our study also suggested ARMS-RT-PCR or ddPCR for detecting low percentage of gene mutations.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 List of mutation and cosmic ID identities of *EGFR*, *PIK3CA*, *KRAS*, and *BRAF* for ARMS-qPCR Amoy Kit

Mutation	Exon	Base change	Cosmic ID
G719A	18	2156G>C	6239
G719S	18	2155G>A	6252
G719C	18	2155G>T	6253
E746_A750del (1)	19	2235_2249del15	6223
E746_A750del (2)	19	2236_2249del15	6225
L747_P753>S	19	2240_2257del18	12370
E746_T751>I	19	2235_2252>AAT (complex)	13551
E746_T751del	19	2236_2253del18	12728
E746_T751>A	19	2237_2251del15	12678
E746_S752>A	19	2237_2254del18	12367
E746_S752>V	19	2237_2255>T (complex)	12384
E746_S752>D	19	2238_2255del18	6220
L747_A750>P	19	2238_2248>GC (complex)	12422
L747_T751>Q	19	2238_2252>GCA (complex)	12419
L747_E749del	19	2239_2247del9	6218
L747_T751del	19	2239_2253del15	6254
L747_S752del	19	2239_2256del18	6255
L747_A750>P	19	2239_2248TTAAGAGAAG>C	12382
L747_P753>Q	19	2239_2258>CA (complex)	12387
747_T751>S	19	2240_2251del12	6210
L747_T751del	19	2240_2254del15	12369
L747_T751>P	19	2239_2251>C (complex)	12383
T790M	20	2369C>T	6240
S768I	20	2303G>T	6241
H773_V774insH	20	2319_2320insCAC	12377
D770_N771insG	20	2310_2311insGGT	12378
V769_D770insASV	20	2307_2308insGCCAGCGTG	12376
L858R	21	2573T>G	6224
L861Q	21	2582T>A	6213
PIK3CA mutations			
H1047R	20	CAT>CGT	775
H1047L	20	CAT>CTT	776
E542K	9	GAA>AAA	760
E545K	9	GAG>AAG	763
E542D	9	GAG>GAT	765
KRAS mutations			
Gly12Asp (G12D)	2	GGT>GAT	521
Gly12Ala (G12A)	2	GGT>GCT	522
Gly12Val (G12V)	2	GGT>GTT	520
Gly12Ser (G12R)	2	GGT>AGT	517
Gly12Arg (G12R)	2	GGT>CGT	518
Gly12Cys (G12C)	2	GGT>TGT	516
Gly13Asp	2	GGC>GAC	532
BRAF mutations			
V600E1	15	1799T>A	476
V600K	15	1798_1799GT>AA (complex)	473
V600E2	15	1799_1800TG>AA (complex)	475
V600R	15	1798_1799GT>AG (complex)	474
V600D1	15	1799_1800TG>AC (complex)	478
V600D2	15	1799_1800TG>AT (complex)	477

Abbreviation: ARMS-qPCR, amplification-refractory mutation system-based quantitative polymerase chain reaction.

Table S2 List of primers for direct sequencing *EGFR* (Ref seq: NG_007726.3)

Target	Primer name	Primer sequence (5′→3′)	Length (bp)	Tm	%GC	Product Size (bp)
EGFR, exon 18	EGFR-Ex18_719FP	TTGTGGAGCCTCTTACACCCAG	22	72.6	54.5	185
	EGFR-Ex18_719RP	GCTCCCCACCAGACCATGAGAG	22	76.5	63.6	
EGFR, exon 20	EGFR-Ex20_768+790FP	ATTCATGCGTCTTCACCTGGAA	22	72.9	45.5	252
	EGFR-Ex20_768+790RP	GAGCAGGTACTGGGAGCCAATA	22	71.5	54.5	
EGFR, exon 21	EGFR-Ex21_858+861FP	TTTCAGGGCATGAACTACTTGG	22	70.2	45.5	171
	EGFR-Ex21_858+861RP	CCTCCTTACTTTGCCTCCTTCTG	23	71.5	52.2	

Abbreviations: G, guanine; C, cytosine.