**KRAS Mutation as a Potential Prognostic Biomarker of Biliary Tract Cancers**

Masaaki Yokoyama¹, Hiroaki Ohnishi², Kouki Ohtsuka², Satsuki Matsushima², Yasuo Ohkura³, Junji Furuse⁴, Takashi Watanabe², Toshiyuki Mori⁴ and Masanori Sugiyama¹

¹Department of Surgery, Kyorin University School of Medicine, Tokyo, Japan. ²Department of Laboratory Medicine, Kyorin University School of Medicine, Tokyo, Japan. ³Department of Pathology, Kyorin University School of Medicine, Tokyo, Japan. ⁴Department of Medical Oncology, Kyorin University School of Medicine, Tokyo, Japan.

**ABSTRACT**

**BACKGROUND:** The aim of this study was to identify the unique molecular characteristics of biliary tract cancer (BTC) for the development of novel molecular-targeted therapies.

**MATERIALS AND METHODS:** We performed mutational analysis of KRAS, BRAF, PIK3CA, and FBXW7 and immunohistochemical analysis of EGFR and TP53 in 63 Japanese patients with BTC and retrospectively evaluated the association between the molecular characteristics and clinicopathological features of BTC.

**RESULTS:** KRAS mutations were identified in 9 (14%) of the 63 BTC patients; no mutations were detected within the analyzed regions of BRAF, PIK3CA, and FBXW7. EGFR overexpression was observed in 5 (8%) of the 63 tumors, while TP53 overexpression was observed in 48% (30/63) of the patients. Overall survival of patients with KRAS mutation was significantly shorter than that of patients with the wild-type KRAS gene (P = 0.005). By multivariate analysis incorporating molecular and clinicopathological features, KRAS mutations and lymph node metastasis were identified to be independently associated with shorter overall survival (KRAS, P = 0.004; lymph node metastasis, P = 0.015).

**CONCLUSIONS:** Our data suggest that KRAS mutation is a poor prognosis predictive biomarker for the survival in BTC patients.

**KEYWORDS:** biliary tract cancer, KRAS, molecular targeted therapy, biomarker

---

**Introduction**

Biliary tract cancer (BTC) is characterized by significant geographic variation; it is rarely detected in Europe and North America but has a high incidence rate in some areas of Latin America and Asia.² BTC comprises aggressive tumors and has extremely poor prognosis; the five-year survival rates are 30%–50% for resectable tumors and less than 5% for unresectable cases.² Although surgical resection currently remains the only potentially curative treatment, most patients are already at the advanced unresectable stage of the disease at the time of diagnosis. Systemic chemotherapy based on the combination of 5-fluorouracil with cisplatin and gemcitabine can improve the quality of life; however, the impact on survival is minimal.³ Therefore, new therapeutic modalities, including molecular targeting therapy, need to be explored for advanced BTC patients to improve treatment outcomes.

To date, several studies have assessed the incidence of molecular abnormalities in BTC, but the results are inconsistent.⁴–⁶ For example, the incidence of mutations of KRAS, BRAF, and PIK3CA genes in BTC patients varies between 0% and 60%, 0% and 22%, and 0% and 12.5%, respectively. In recent data of TCGA (the cancer genome atlas and c-bioportal), the frequencies of mutation of KRAS, BRAF, PIK3CA, and FBXW7 in cholangiocarcinoma are reported to be 5.7%, 2.9%, 5.7%, and 2.9%, respectively. Overexpression of EGFR and TP53 has been detected in ~10% and 6%–35%, respectively.⁷ In addition, different carcinogenic mechanisms involving molecular abnormalities have been reported among each subdivision.⁸–⁹ However, previous studies analyzing aberrations of oncogenes or tumor suppressor genes in BTC have been conducted on relatively small patient populations, probably because of low BTC prevalence in Western countries. Therefore, unlike other major solid tumors, the molecular mechanisms underlying BTC development remain poorly understood, and their clinical significance remains elusive.

In the present study, we examined the mutation and expression of several molecules that have been reportedly associated with the development of BTCs and analyzed their correlation with patients' clinical features. The main purpose of this study was to identify the molecular characteristics of BTC that could help developing novel molecular-targeted therapies for BTC.
Materials and Methods

Subjects. A total of 63 BTC patients who had undergone tumor resection and had been histologically diagnosed for adenocarcinoma of the bile duct or gall bladder at the Kyorin University Hospital between January 2005 and December 2011 were enrolled in the present study. According to the anatomical location of original tumors, BTC is subdivided into gall bladder adenocarcinoma (GBC), intrahepatic cholangiocarcinoma (IHCC), ampulla of Vater adenocarcinoma (AC), and extrahepatic cholangiocarcinoma (EHCC). Among the BTC patients enrolled in this study, 23 (37%) patients had GBCs, 7 (11%) patients had IHCCs, 29 (46%) patients had EHCCs, and 4 (6%) patients had ACs. Patients’ clinicopathological characteristics including gender, age, lymph node metastasis, tumor differentiation, location, pT/TEM pathological classification according to the Union for International Cancer Control10 and long-term outcome by 2014 were retrieved from medical records. The patient population included 38 (60%) males and 25 (40%) females, with a median age of 71 years (Table 1). Although BTC treatment was heterogeneous, no patient had been administered molecular-targeted drugs. This retrospective study was approved by the Ethics Committee of the Kyorin University School of Medicine. Our research complied with the principles of the Declaration of Helsinki.

Mutational analysis of KRAS, BRAF, PIK3CA, and FBXW7. Paraffin-embedded tissues were sectioned to 10-μm thicknesses and mounted as three separate slides per tissue. The resulting slides were treated three times with xylene and washed with ethanol. To minimize contamination by normal tissue, regional samples were dissected under a binocular microscope and used for DNA extraction with the DNeasy Blood & Tissue Kit (QIAGEN). We obtained polymerase chain reaction products by using Veriti® Thermal Cycler (Applied Biosystems), and they were then purified by QIAamp DNA FFPE Tissue Kit (QIAGEN). Regions of the KRAS, BRAF, PIK3CA, and FBXW7 genes were amplified using gene-specific primers and subjected to direct DNA sequencing as previously described.11-13 KRAS point mutations were screened for codons 12 and 13 within exon 2, two hot spots that cumulatively include >95% of this gene’s mutations.12 BRAF was screened for V600E mutation within exon 15, where >95% of point mutations occur.14,15 PIK3CA mutations were screened within exons 9 and 20 where >80% of point mutations occur.11,16,17 FBXW7 mutations were screened within exons 8, 11, and 12, since most of the FBXW7 mutations (eg, R465C, R465H, R505C, and Y519C) occur within these regions.18

Immunohistochemistry of EGFR and TP53. The overexpression of EGFR and TP53 was examined by immunohistochemistry. Based on the HercepTest™ (Dako) criteria, EGFR signals were defined in terms of staining intensity of the cancer cell membrane: 0, between 0% and 10% stained cells; 1+, faint and/or partial staining in >10% cells; 2+, moderate staining in >10% cells and strong and complete staining in 10%–30% cells; 3+, strong and complete staining in >30% cells.19 For the cases showing mixed intensities, predominant signal was selected as the final score. The samples with the final score of 2+ or 3+ were considered positive for EGFR overexpression (Fig. 1A-1). TP53 staining was classified according to previous reports20 as follows: (grade 1) absent; (grade 2) present in a minority of the cells (below 10%); (grade 3) present in approximately 10%–75% of the cells; or (grade 4) present in virtually all the cells (Fig. 1B-1). Only the tumor cells with dense nuclear staining were graded as positive. Tumors with TP53 expression in the majority of the cells (grades 3 and 4) were classified as exhibiting TP53 overexpression.

The EGFR and TP53 staining intensities were evaluated by a surgeon (MY) and a pathologist (YO) independently.

Statistical analysis. Associations between molecular factors and clinicopathological parameters were assessed by χ^2 test or Fisher’s exact test. Overall survival (OS) after surgery was calculated using the Kaplan–Meier method; the log-rank test was performed to compare survival curves. To identify independent predictive biomarkers for survival, multivariate analyses were performed using the Cox regression model (a log-rank test) for OS. Two-tailed P values of <0.05 were considered significant. All analyses were performed using the SPSS software (SPSS for Macintosh Version 21; IBM Corporation).

Results

KRAS, BRAF, PIK3CA, and FBXW7 mutations in BTC. KRAS mutations were identified in 9 (14%) of the 63 BTC patients: 2 of 23 GBC cases (9%) and 7 of 29 IHCCs (24%). The G12V mutation was detected in 3 BTC patients, G12D in 5 BTC patients, and G12S in 1 BTC patient (Table 2). OS of KRAS mutation-bearing patients was significantly worse than that of the patients with the wild-type
Figure 1. Representative immunohistochemical analyses and HE results of EGFR and TP53: (A-1) 2+ immunoreactivity for EGFR, (A-2) HE, (B-1) positive immunoreactivity for TP53, and (B-2) HE.

Table 2. Molecular features of patients with BTC.

| PATIENTS (n = 63) | N (%) |
|------------------|-------|
| EGFR (IHC)       |       |
| Score 0          | 58 (92) |
| Score 1+         | 0 |
| Score 2+         | 5 (8) |
| Score 3+         | 0 |
| TP53 (IHC)       |       |
| (-)              | 33 (52) |
| (+)              | 30 (48) |
| KRAS mutant      |       |
| G12V             | 3 (5) |
| G12D             | 5 (8) |
| G12S             | 1 (1) |
| BRAF mutant      | 0 |
| PIK3CA mutant    | 0 |
| FBXW7 mutant     | 0 |

Abbreviations: BTC, biliary tract cancer; IHC, immunohistochemistry.

KRAS gene (Fig. 2A; P = 0.005 by log-rank test). In addition, patients with lymph node metastasis showed higher rate of KRAS mutation than those without metastasis, although the difference was statistically only marginal (P = 0.066).

No mutations were identified within the analyzed regions of the BRAF (exon 15), PIK3CA (exons 9 and 20), and FBXW7 genes (exons 8, 11, and 12) in BTC patients.

EGFR overexpression. EGFR expression was absent in 58 of the 63 tumors (92%). The remaining 5 (8%) samples were scored as 2+ and considered positive for EGFR overexpression; no samples showed scores of 1+ or 3+ (Table 2). There was no statistically significant difference in clinicopathological features between the patients with and without EGFR overexpression (Table 3a and Fig. 2B).

TP53 overexpression. TP53 overexpression was found in 30 of the 63 patients (48%; Table 2). However, there was no statistically significant difference in clinicopathological features between the patients positive and negative for TP53 overexpression (Table 3b). Similarly, no association between TP53 overexpression and OS was detected (Fig. 2C).

Multivariate analysis for OS. Multivariate analysis of the molecular and clinicopathological parameters of BTC
Yokoyama et al

Japanese Clinical Medicine 2016:7

(listed in Tables 1 and 2) identified *KRAS* mutation (*P* = 0.004) and lymph node metastasis (*P* = 0.015) as independent factors for shorter OS (Table 4).

**Discussion**

To the best of our knowledge, this is the first study to reveal the prognostic significance of molecular abnormalities in BTC. In the present study, *KRAS* mutations were detected in 14% of BTC patients, which is within the 0%–60% range reported by other studies including TCGA. Most strikingly, the treatment outcome of *KRAS* mutation-positive patients was significantly worse than that of *KRAS* wild-type patients. To date, no studies have demonstrated the prognostic impact of *KRAS* genetic alterations in BTC, although *KRAS* has been reported as one of the most frequently mutated genes in this type of cancer. Our present data suggest that mutations in the *KRAS* gene can be used as a prognostic biomarker for BTC patients. In addition, *KRAS* may be associated with the efficacy of molecular-based therapeutic approaches to treat BTC. Currently, no approved *KRAS*-targeting molecular drugs have been established. However, *KRAS* mutational status is crucial for the application of anti-EGFR antibody drugs, because *KRAS* mutations usually confer resistance to these drugs in colon or lung cancers. In BTC, relationship between *KRAS* status and efficacy of anti-EGFR antibody drugs remains uncertain. Future studies are required to investigate the efficacy of anti-EGFR antibody-based therapy for BTC patients with *KRAS* mutations.

*BRaf* belongs to the mitogen-activated protein kinase signaling pathways, which mediate cellular response to

---

**Figure 2.** OS according to molecular features by Kaplan–Meier analysis. (A) OS of BTC patients classified according to the presence of *KRAS* mutations. (B) OS of BTC patients classified according to the presence of EGFR overexpression. (C) OS of BTC patients classified according to the presence of *TP53* overexpression.
Table 3. Relationship between molecular and clinicopathological features in biliary tract cancer.

|                          | IHC (−) | IHC (+) (%) | P    |
|-------------------------|---------|-------------|------|
| **A) EGFR OVEREXPRESSION** |         |             |      |
| Age                     |         |             |      |
| 65<                     | 15      | 0           | 0.326|
| 65≥                     | 43      | 5 (10)      |      |
| Gender                  |         |             |      |
| Male                    | 35      | 3 (8)       | 1.000|
| Female                  | 23      | 2 (8)       |      |
| GBC                     | 21      | 2 (9)       |      |
| IHCC                    | 6       | 1 (14)      | 0.851|
| EHCC                    | 27      | 2 (7)       |      |
| AC                      | 4       | 0           |      |
| Stage                   |         |             |      |
| 0                       | 1       | 1 (50)      | 0.180|
| I                       | 8       | 1 (11)      |      |
| II                      | 34      | 3 (8)       |      |
| III                     | 11      | 0           |      |
| IV                      | 4       | 0           |      |
| Lymphnode metastasis    |         |             |      |
| (−)                     | 30      | 4 (12)      | 0.363|
| (+)                     | 28      | 1 (3)       |      |
| **B) TP53 OVEREXPRESSION** |         |             |      |
| Age                     |         |             |      |
| 65<                     | 5       | 10 (67)     | 0.254|
| 65≥                     | 24      | 23 (49)     |      |
| Gender                  |         |             |      |
| Male                    | 19      | 18 (49)     | 0.443|
| Female                  | 10      | 15 (60)     |      |
| GBC                     | 10      | 13 (57)     |      |
| IHCC                    | 2       | 4 (67)      | 0.548|
| EHCC                    | 16      | 13 (45)     |      |
| AC                      | 1       | 3 (75)      |      |
| Stage                   |         |             |      |
| 0                       | 2       | 0           | 0.208|
| I                       | 3       | 6 (67)      |      |
| II                      | 19      | 18 (49)     |      |
| III                     | 5       | 6 (55)      |      |
| IV                      | 0       | 3 (100)     |      |
| Lymphnode metastasis    |         |             |      |
| (−)                     | 17      | 17 (50)     | 0.617|
| (+)                     | 12      | 16 (57)     |      |
| **C) KRAS MUTATION**    |         |             |      |

Table 3. (Continued)

| WILD TYPE | MUTANT (%) | P     |
|-----------|------------|-------|
| **C) KRAS MUTATION** |         |       |
| Age       |           |       |
| 65<       | 11        | 2 (15)| 1.000|
| 65≥       | 39        | 7 (15)|      |

**Abbreviations:** IHC, immunohistochemistry; GBC, gallbladder adenocarcinoma; IHCC, intrahepatic cholangiocarcinoma; EHCC, extrahepatic cholangiocarcinoma; AC, ampulla of Vater adenocarcinoma.

growth factors, including EGF. **BRAF** mutations have been reported as a relatively common event (22%) in IHCC but not in EHCC, in the Anglo-Saxon population; on the other hand, they were not observed in Chinese patients with IHCC. Our present findings are consistent with the latter report, suggesting ethnic variations in the frequency of **BRAF** mutations in BTC. These results suggest that BRAF inhibitors that are effective only in the patients with **BRAF** mutation, such as vemurafenib, may not be effective in Asian patients with BTC.

**PIK3CA** encodes the catalytic subunit of PI3K that activates a downstream Akt kinase upon stimulation with various growth factors. The response rate to PI3K/Akt/mTOR pathway inhibitors has been found to be significantly higher in patients with **PIK3CA** mutations than in those with the wild-type gene. In addition, **PIK3CA** mutations in colorectal cancer have been associated with clinical resistance to anti-EGFR monoclonal antibodies. Our present study did not identify mutations in **PIK3CA** in Japanese patients with BTC, suggesting that the efficacy of PIK3CA inhibitors may be minimal in this ethnic group. However, anti-EGFR
antibody-based therapy may be effective in the Japanese BTC population, provided that PIK3CA in BTC plays a similar role as in colorectal cancer.

Consistent with the previous report, EGFR overexpression was not detected in most BTC tumors (92%).\(^{30}\) Unlike tyrosine kinase inhibitors, cetuximab is effective in colorectal cancer patients with EGFR-negative tumors.\(^ {31} \) If such is the case in BTC, cetuximab therapy may be applicable for BTC even if most cases are negative for EGFR expression. Future studies treating BTC patients with low EGFR expression by cetuximab will be necessary to address this issue.

TP53 is one of the major tumor suppressor genes involved in cell cycle, DNA repair, and apoptosis. In contrast to the previous reports, we found that the incidence of TP53 overexpression was similar between GBC and other BTC categories. In addition, no clinicopathological features were associated with TP53 overexpression. Our results suggest that TP53 overexpression may have little clinical relevance in Japanese patients with BTC.

FBXW7 is a member of the F-box family of proteins, which function as substrate recognition components of the multisubunit ubiquitin ligase SCF.\(^ {16} \) FBXW7-inactivating mutations have been detected in diverse human cancers with an overall frequency of approximately 6%;\(^ {26} \) the highest mutation incidence has been reported for cholangiocarcinomas in Sweden (35%).\(^ {16} \) However, we did not detect FBXW7 mutations in Japanese BTC patients. The reason for this discrepancy remains unknown; however, differences in ethnicities and the prevalence of histological subtypes could be involved.

In our study, IHCC represented only 11% of all BTCs, which is consistent with previous reports of BTC histological classification in Japan.\(^ {22} \) In contrast, IHCC is the dominant BTC category in Western countries.\(^ {33} \) IHCC may harbor FBXW7 mutations more frequently than other BTC subtypes. Of note, recent TCGA data show that the mutation rate of FBXW7 in cholangiocarcinoma is relatively low (2.9%), which is consistent with our result. Future studies recruiting a larger number of patients with diverse ethnic background are necessary to elucidate this issue.

This study had some limitations. It was performed retrospectively in a relatively small, heterogeneously treated population; insufficient sample size may contribute to the lack of statistical significance in some analyses. The discrepancy observed between the results of univariate and multivariate analyses may have been caused by these factors. Our findings, therefore, should be validated in subsequent prospective studies on a large number of patients before application in clinical practice.

Conclusions

The present study identified KRAS mutations as a poor prognostic marker for BTC patients. These findings warrant future prospective studies for elucidating the efficacy of molecular-targeting drugs and its association with predictive biomarkers in BTC.

Acknowledgment

The authors acknowledge the previous publication of “Molecular analysis of BTCs identified KRAS mutation as a potential prognostic biomarker”\(^ {34} \), a summary in Japanese of the work presented fully here.

Author Contributions

Conceived and designed the experiments: MY, HO, KO, TW, JF, and MS. Analyzed the data: MY, HO, and KO. Helped with the experiment: SM. Supported pathological diagnosis: MY, YO. Write the first draft of the manuscript: HO. Contributed to the writing of the manuscript: MY, HO, and TM. All authors reviewed and approved of the final manuscript.

REFERENCES

1. Randi G, Franceschi S, Lo Vecchia C. Gallbladder cancer worldwide: geographica l distribution and risk factors. J Natl Cancer Inst 2006;19:1591–1602.
2. Miyakawa S, Ishihara S, Horiguchi A, Takada T, Miyazaki M, Nakagawa K. Biliary tract cancer treatment: 5,584 results from the Biliary Tract Cancer Statistics Registry from 1998 to 2004in Japan. J Hepatobiliary Pancreat Surg 2009; 16:1–7.
3. Romiti A, D’Antonio C, Zullo A, et al. Chemotherapy for the biliary tract can cers: moving toward improved survival time. J Gastrointest Cancer 2012;43(3): 396–404.
4. Pignochino Y, Sarotto I, Peraldo-Neia C, et al. Targeting EGFR/HER2 pathways enhances the antiproliferative effect of gemcitabine in biliary tract and gall bladder carcinoma. BMC Cancer 2010;10:16.
5. Rashid A, Ueki T, Gao YT, et al. K-ras mutation, p53 overexpression, and mic rosatellite instability in biliary tract cancers: a population-based study in China. Clin Cancer Res 2002;8(10):3156–3163.
6. Kim YT, Kim J, Jung YH, et al. Genetic alterations in gallbladder adenoma, dysplasia and carcinoma. Cancer Lett 2001;169(1):59–68.
7. Watanabe H, Date K, Itoi T, Matsuyasahi H, Yokoyama N, Yamanaka M. Hist ological and genetic changes in malignant transformation of gallbladder adenoma. Ann Oncol 1999;10(suppl 4):136–139.
8. Hezel AF, Deshpande V, Zha AX. Genetics of biliary tract cancers and emerg ing targeted therapies. J Clin Oncol 2010;28(21):3531–3540.
9. Geynisman DM, Catenacci DV. Toward personalized treatment of advanced biliary tract cancers. Diso Med. 2012;14(74):41–57.
10. Sobin LH, Gospodorawics MK, Wirtelекind C. TNM Classification of Malignan t Tumours. 7th ed, Wiley, New Jersey; 2009.
11. Saridaki Z, Tzardl M, Padapaki C, et al. Impact of KRAS, BRAF, PIK3CA mutations, PTEN, AREG, EREG expression and skin rash in ≥2 line cetux imab-based therapy of colorectal cancer patients. PLoS One 2011;6(3):e15980.
12. Inno A, Dn Salvatore M, Cenci T, et al. Is there a role for IGFR and c-MET pathways in resistance to cetuximab in metastatic colorectal cancer? Clin Colorectal Cancer 2011;10(4):325–332.
13. Ohishi H, Ohtsuka K, Oside A, Matsuhashina S, Goya T, Watanabe T. A simple and sensitive method for detecting major mutations within the tyrosine kinase domain of the epidermal growth factor receptor gene in non-small-cell lung car cinoma. Diagn Mol Pathol 2006;15(2):101–108.
14. De Roock W, Claes B, Bernasconi D, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consorti um analysis. Lancet Oncol 2010;11(8):753–762.
15. Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and pan itumumab in colorectal cancer. J Clin Oncol 2010;28(7):1254–1261.
16. Sartore-Bianchi A, Martini M, Molinari F, et al. PIK3CA mutations in colorec tal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. Cancer Res 2009;69(5):1851–1857.
17. Soulakos J, Philips J, Wang R, Marwha S, Silver M, Tzardl M. Prognostic and predictive value of common mutations for treatment response and survival in patients with metastatic colorectal cancer. Br J Cancer 2009;101(3):465–472.
18. Akhondi S, Sun D, von der Lehr N, et al. FBXW7/6CDD4 is a general tumor suppressor in human cancer. Cancer Res 2007;67(19):9006–9012.
19. Ellis IO, Bartlett J, Dowsett M, Humphreys S, Jasani B, Miller K. Best Practice No 176: updated recommendations for HER2 testing in the UK. J Clin Pathol 2004;57(3):233–237.
20. Kandziora-Eckersberger D, Ludwig C, Rudas M, et al. TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast can cer patients. Clin Cancer Res 2000;6(1):50–56.

38 I JAPANESE CLINICAL MEDICINE 2016;7
21. Harder J, Waiz O, Otto F, et al. EGFR and HER2 expression in advanced biliary tract cancers. *World J Gastroenterol*. 2009;15(36):4511–4517.

22. Deshpande V, Nduaguba A, Zimmerman SM, et al. Mutational profiling reveals PIK3CA mutations in gallbladder carcinoma. *BMC Cancer*. 2011;11:60.

23. Qiu LX, Mao C, Zhang J, et al. Predictive and prognostic value of KRAS mutations in metastatic colorectal cancer patients treated with cetuximab: a meta-analysis of 22 studies. *Eur J Cancer*. 2010;46(15):2781–2787.

24. Linardou H, Dahabreh IJ, Kanaloupiti D, Siannis F. Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. *Lancet Oncol*. 2008;9(10):962–972.

25. Tannapfel A, Sommerer F, Benicke M, et al. Mutations of the BRAF gene in cholangiocarcinoma but not in hepatocellular carcinoma. *Gut*. 2003;52(5):706–712.

26. Xu RF, Sun JP, Zhang SR, et al. KRAS and PIK3CA but not BRAF genes are frequently mutated in Chinese cholangiocarcinoma patients. *Biomed Pharmacother*. 2011;65(1):22–26.

27. Janku F, Wheler JJ, Westin SN, et al. PI3K/AKT/mTOR inhibitors in patients with breast and gynecologic malignancies harboring PIK3CA mutations. *J Clin Oncol*. 2012;30(8):777–782.

28. Janku F, Tsimberidou AM, Garrido-Laguna I, et al. PI3K mutations in patients with advanced cancers treated with PI3K/AKT/mTOR axis inhibitors. *Mol Cancer Ther*. 2011;10(3):558–565.

29. Mao C, Yang ZY, Hu XF, Chen Q, Tang JL. PIK3CA exon 20 mutations as a potential biomarker for resistance to anti-EGFR monoclonal antibodies in KRAS wild-type metastatic colorectal cancer: a systematic review and meta-analysis. *Ann Oncol*. 2012;23(6):1518–1525.

30. Nakazawa K, Dobashi Y, Suzuki S, Fujii H, Takeda Y, Ooi A. Amplification and overexpression of c-erbB-2, epidermal growth factor receptor, and c-met in biliary tract cancers. *J Pathol*. 2005;206(3):356–365.

31. Chung KY, Shia J, Kemeny NE, et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol*. 2005;23(9):1803–1810.

32. Ikai I, Ariti S, Okazaki M, et al. Report of the 17th Nationwide Follow-up Survey of Primary Liver Cancer in Japan. *Hepatol Res*. 2007;37(9):676–691.

33. McGlynn KA, Tarone RE, El-Serag HB. A comparison of trends in the incidence of hepatocellular carcinoma and intrahepatic cholangiocarcinoma in the United States. *Cancer Epidemiol Biomarkers Prev*. 2006;15(6):1198–1203.

34. Yokoyama M, Ohnishi H, Ohsuka K, et al. Molecular analysis of biliary tract cancers identified KRAS mutation as a potential prognostic biomarker. Tan to sui (Igakutosyo-syuppan, Tokyo, Japan). 2015;36(2):93–98.