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The frequency of *KRAS* and *BRAF* mutations in intrahepatic cholangiocarcinomas and their correlation with clinical outcome

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Abstract

The incidence of intrahepatic cholangiocarcinoma (ICC) is increasing worldwide. The prognosis of ICC is poor and a better understanding of ICC tumor biology is needed to more accurately predict clinical outcome and to suggest potential targets for more effective therapies. v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and *BRAF* are frequently mutated oncogenes that promote carcinogenesis in a variety of tumor types. In this study, we analyze a large set of ICC tumors (N = 54) for mutations in these genes and compare the clinical outcomes of wild type versus *KRAS* and *BRAF* mutant cases. Out of 54 cases, 7.4% were mutant for *KRAS*, 7.4% were mutant for *BRAF* and these were mutually exclusive. These mutant cases were associated with a higher tumor stage at time of resection and a greater likelihood of lymph node involvement. These cases were also associated with a worse long-term overall survival. Therefore, testing for *KRAS* and *BRAF* mutations could be a valuable adjunct in improving both prognosis and outcome stratification among patients with ICC.

Introduction

Cholangiocarcinoma is the second most common type of liver cancer after hepatocellular carcinoma [1]. In the United States, there are 5000 cases annually, constituting nearly 3% of all gastrointestinal cancers [2]. Approximately two-thirds of cases arise from the extrahepatic biliary tree while the remainder arise within the liver. Although intrahepatic (ICC) and extrahepatic cholangiocarcinomas (ECC) arise from similar epithelium, each tumor type has distinct risk factors, clinical presentations, genetic changes and management.

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Alarming, the incidence of ICC is rising while the incidence of ECC has remained stable [3]. The factors driving the increased incidence of ICC are not well-understood, but increases in hepatitis C infection and nonalcoholic fatty liver disease may be partially responsible [4]. Other risk factors for ICC include hepatitis B infection, primary sclerosing cholangitis, advanced age, hepatolithiasis, chemical exposure and liver fluke infection [2].

ICC has a dismal prognosis and surgical resection is the only therapy with the possibility of long-term cure. Unfortunately, most patients present with advanced lesions and are not suitable candidates for resection [1]. While chemotherapy provides improved quality of life and a modest increase in survival, it is not curative [5]. At these later stages, the median survival for patients with unresectable ICC ranges from five and eight months [6]. Even with surgical resection, five-year overall survival is still poor and ranges from only 14% to 40% [1]. Certainly, a better understanding of cholangiocarcinoma tumor biology is needed to advance therapeutic strategies to improve survival.

The molecular alterations that drive tumorigenesis in cholangiocarcinomas are beginning to be identified. Single-gene studies have identified a variety of genetic derangements, most of which involve well-known tumor genes including *KRAS*, *BRAF*, *TP53* and *epidermal growth factor receptor (EGFR)* [7–12]. More recently, several studies have used comprehensive genomic and proteomic analyses to identify additional molecular determinants as well as to suggest multi-gene signatures to stratify cholangiocarcinomas into different functional and prognostic groups. In many of these studies, mutations in *KRAS* and *BRAF* are reported, however, the frequency of these mutations varies considerably between studies. With respect to *KRAS*, the reported frequency ranges from as low as 8% to as high as 53% [9,13]. Similarly, the frequency of *BRAF* mutations has been reported over a wide incidence ranging from 0% to 22% [7,8].

The large variance across studies is likely the result of small samples sizes. Furthermore, some studies report aggregated data on cases consisting of both ICC and ECC, which have very different frequencies of *KRAS* mutation as suggested by two recent reports [14,15]. Similarly, a subset of liver neoplasms also have both neoplastic ductal and hepatocellular epithelial elements. These tumors almost certainly have different underlying pathogenesis, possibly arising from hepatic stem cells rather than more differentiated cholangiocytes [16]. To more accurately define the mutational frequency, a more carefully defined study with a large and carefully defined set of intrahepatic tumors is needed.

An accurate report of *KRAS* and *BRAF* mutations has important clinical implications since epidermal growth factor receptor (EGF-R) inhibitor therapy is largely ineffective in the presence of *KRAS* and *BRAF* mutations [17–19]. On the other hand if *KRAS* or *BRAF* mutations are frequent, these should be specifically targeted.

Moreover, the prognostic and predictive implications of *KRAS* and *BRAF* mutations are not defined. There are no studies that report clinical follow-up data in conjunction with *KRAS* and *BRAF* mutational status. One study examined a mixed population of sixty-nine ICC and ECC cases and mutations in *KRAS* and *BRAF* were not associated with differences in prognosis [8]. Given the limited data on *KRAS* and *BRAF* in ICC, additional studies linked to clinical outcome are clearly indicated. As such, in the present study we sought to examine a carefully defined set of patients with ICC tumors to define the frequency of *KRAS* and *BRAF* mutations and compare the mutational status with patient demographics, tumor pathological characteristics and clinical outcome.

Materials and Methods

Patients and tissue samples

The use of human tissues in this study was approved by the Johns Hopkins Institution Review Board. All human liver samples are from patients undergoing surgical resection at the Johns Hopkins Hospital, Baltimore, MD. Fifty-four cases of ICC derived from patients treated with curative intent surgical resection at The Johns Hopkins Hospital from 1990 through 2011 were identified. From these surgical cases, formalin-fixed paraffin-embedded tumor tissue was retrieved. Data on patient and treatment-related variables were also obtained. Specifically, information on tumor number, size, as well as margin and nodal status were ascertained. Tumor staging was assigned according to the AJCC cancer staging manual, 7th edition [20]. Date of last follow-up, vital status and date of recurrence were collected on all patients.

Pathology Review

The pathology of all cases was reviewed by an experienced hepatobiliary pathologist to verify the tumor type and evaluate other pathologic parameters including degree of differentiation, vascular invasion, perineural invasion, biliary invasion and satellite lesions. The pathology was independently reviewed by two additional pathologists. All tumors were composed of glandular differentiated cells. No tumors contained areas with hepatocellular differentiation (biphenotypic tumors).

DNA Extraction and Pyrosequencing

Paraffin embedded blocks with tumor tissue were selected and tumor DNA was macro-dissected and isolated using the Pinpoint DNA Isolation System (Zymo Research, Irvine, CA). Extracted DNA was then purified using the QIAamp DNA Mini Kit (Qiagen Valencia, CA), according to the manufacturer's "Blood and Body Fluid Spin" protocol. Pyrosequencing for detecting codon 12 and 13 mutations of the *KRAS* gene was performed as described previously [20]. Pyrosequencing for detecting codon 600 mutations of the *BRAF* genes was conducted using forward primer 5' - GAAGACCTCACAGTAAAAATAG-3' and biotinylated reverse primer 5' - ATAGCCTCAATTCTTACCATCC-3' for PCR and primer 5' - GACCTCACAGTAAAAATAGGTGATTTTG-3' for sequencing. PCR conditions were: 95°C for 15 min, 42 cycles of 95°C for 20 sec, 53°C for 30 sec and 72°C for 20 sec, and 72°C for 5 min. The nucleotide dispensation order for pyrosequencing was: 5' - GTAGCTAGCTATCAGCATCGACTCTCGATGAGTG-3'. The limit of mutant allele detection by pyrosequencing was 5%, based on sequencing colon margin of resection negative control samples.

Statistical analysis

Summary statistics were obtained using established methods and presented as percentages of median values. Categorical and continuous variables were compared using χ^2 exact and Kruskal-Wallis tests, respectively. Median overall and recurrence free survival times were estimated using the Kaplan-Meier method and differences were compared using the log-rank test, where $p < 0.05$ was considered statistically significant. All statistical tests were two-tailed. Statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC).

Results

Frequency of *KRAS* and *BRAF* mutations

A total of fifty-four cases of ICC were evaluated. The demographic, disease and treatment characteristics are summarized in Table 1. Median patient age was 64 years and the cohort had a predominance of white females. The tumor from each case was sequenced for *KRAS* (codon 12 and 13) and *BRAF* (codon 600) mutations by pyrosequencing, with a mutant allele detection limit of 5% [20]. Representative pyrograms showing mutant *KRAS* and *BRAF* sequences are shown in Figure 1. We found that the tumor from four cases (7.4%) had mutations in *KRAS* and an additional, but distinct four cases (7.4%) had mutations in *BRAF*. All *KRAS* mutations were G12D substitutions and all *BRAF* mutations were V600E substitutions. When cases with either a *KRAS* or *BRAF* mutation were grouped together, we found that mutant cases were more likely to have a higher TNM stage at the time of surgical resection. Two of four (50%) *KRAS* mutant cases and two of four (50%) *BRAF* mutant cases presented as T4 lesions compared with only seven of 46 (16%) wild type (WT) lesions ($p=0.03$). Similarly, two of four (50%) *KRAS* mutant cases and two of four (50%) *BRAF* mutant cases presented with lymph node involvement at the time of surgical resection compared with only three of forty-six (7%) without either mutation ($p=0.01$). Evaluation of other pathologic parameters including tumor size, tumor multifocality, tumor differentiation, vascular invasion, perineural invasion, biliary invasion and satellite lesions showed no correlation with mutational status (all $p>0.05$).

Clinical Outcome

Overall survival (OS) and recurrence free survival (RFS) were analyzed for each group and are summarized in Table 2. Median overall survival was 13.5 months for *KRAS* mutant cases and 24.4 months for *BRAF* mutant cases, compared to 37.3 months for wild type cases. Combining *KRAS* mutant cases with the *BRAF* mutant cases resulted in a median OS of 23.2 months ($p = 0.05$, compared with the wild type group). The Kaplan-Meier survival curves for these two groups are shown in Figure 2.

Discussion

In this study, we examined the frequency of *KRAS* and *BRAF* mutations in a set of surgically resected intrahepatic cholangiocarcinomas. We identified *KRAS* mutations in 7.4% of cases and *BRAF* mutations in an additional 7.4% of cases. When correlated with pathological parameters and survival data, we found that either mutation is associated with higher TNM stage at presentation and decreased overall survival. These findings suggest that *BRAF* and *KRAS* mutational analysis could serve as a useful adjunctive tool for evaluating patients with ICC. Furthermore, *BRAF* and *KRAS* mutational analysis is commonplace in the workup of other tumor types and the infrastructure needed for this analysis is already widely available.

The identification of several *BRAF* mutated tumors in our data, in agreement with several previous studies, also suggests a potential application for targeted therapy in these cases. Vemurafenib, for example, has demonstrated dramatic antitumor activity in malignant melanomas with *BRAF* codon V600 mutations [22]. Perhaps, this agent could also be of use in the context of appropriately selected biliary malignancies. On the other hand, the 15% of patients with either *KRAS* or *BRAF* mutations would likely not be good candidates for chemotherapy trials involving EGF-R inhibitors. Given the low frequency of these mutations, this information would only benefit a subset of patients. However, any improvement in survival would be a significant step forward considering the current poor prognosis of this disease.

It is important to note that almost all studies investigating gene mutations in cholangiocarcinomas, including the current study, have used tissue obtained at the time of surgical resection. Since only a subset of patients presenting with ICC have disease limited enough to allow for surgery, these cases only represent a subset of total ICC, one enriched for less aggressive disease. Therefore, estimations about the frequency of gene mutations may underestimate the true incidence of *KRAS* and *BRAF* mutation if these genes are indeed associated with more aggressive and advanced disease. Further work is needed to determine if the characteristics of resectable tumors are representative of the larger set of all cholangiocarcinomas regardless of the amenability to resection.

Lastly, while our data suggest that mutations in *KRAS* and *BRAF* portend a poor prognosis, this is only applicable to a minority of cases, a mere 15% in our study. In a report by Andersen et al., the authors examined global gene expression in cholangiocarcinomas and reported that by integrating the *KRAS* mutational status with a 238-gene molecular signature, grouped all patients with mutated *KRAS/BRAF* into a poor prognostic group [14]. However, a *KRAS* mutation was not an independent prognostic factor in this circumstance. Interestingly, these data are similar to the outcomes presented in the current study. Collectively, these data suggest that stratification of patients into prognostic groups is feasible based on certain genetic profiles. Other means of evaluating ICC to identify additional potential gene candidates for better patient stratification into prognostic categories is still needed to address the majority of cases. Recent studies employing whole genome expression profiling have revealed fascinating insights into the tumor biology of cholangiocarcinomas and suggest that individual cases can be placed into prognostically significant categories based on an array of gene expression changes [13–15,23].

References

1. Poultides GA, Zhu AX, Choti MA, Pawlik TM. Intrahepatic Cholangiocarcinoma. *Surgical Clinics of North America*. 2010; 90:817–37. [PubMed: 20637950]
2. Lazaridis KN, Gores GJ. Cholangiocarcinoma. *Gastroenterology*. 2005; 128:1655–67. [PubMed: 15887157]
3. Patel T. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer*. 2002; 2:10. [PubMed: 11991810]
4. Yamamoto S, Kubo S, Hai S, Uenishi T, Yamamoto T, Shuto T, et al. Hepatitis C virus infection as a likely etiology of intrahepatic cholangiocarcinoma. *Cancer Sci*. 2004; 95:592–5. [PubMed: 15245596]
5. Glimelius B, Hoffman K, Sjöden PO, Jacobsson G, Sellström H, Enander LK, et al. Chemotherapy improves survival and quality of life in advanced pancreatic and biliary cancer. *Ann Oncol*. 1996; 7:593–600. [PubMed: 8879373]
6. Chou FF, Sheen-Chen SM, Chen YS, Chen MC, Chen CL. Surgical treatment of cholangiocarcinoma. *Hepatogastroenterology*. 1997; 44:760–5. [PubMed: 9222685]
7. Goldenberg D, Rosenbaum E, Argani P, Wistuba II, Sidransky D, Thuluvath PJ, et al. The V599E BRAF mutation is uncommon in biliary tract cancers. *Modern Pathology*. 2004; 17:1386–91. [PubMed: 15181454]
8. Tannapfel A, Sommerer F, Benicke M, Katalinic A, Uhlmann D, Witzigmann H, et al. Mutations of the BRAF gene in cholangiocarcinoma but not in hepatocellular carcinoma. *Gut*. 2003; 52:706–12. [PubMed: 12692057]
9. Tannapfel A, Benicke M, Katalinic A, Uhlmann D, Köckerling F, Hauss J, et al. Frequency of p16INK4A alterations and k-ras mutations in intrahepatic cholangiocarcinoma of the liver. *Gut*. 2000; 47:721–7. [PubMed: 11034592]
10. Ahrendt SA, Rashid A, Chow JT, Eisenberger CF, Pitt HA, Sidransky D. p53 overexpression and K-ras gene mutations in primary sclerosing cholangitis-associated biliary tract cancer. *Journal of Hepato-biliary-pancreatic Surgery*. 2000; 7:426–31. [PubMed: 11180865]

11. Furubo S, Harada K, Shimonishi T, Katayanagi K, Tsui W, Nakanuma Y. Protein expression and genetic alterations of p53 and ras in intrahepatic cholangiocarcinoma. *Histopathology*. 1999; 35:230–40. [PubMed: 10469215]
12. Gwak G-Y, Yoon J-H, Shin CM, Ahn YJ, Chung JK, Kim YA, et al. Detection of response-predicting mutations in the kinase domain of the epidermal growth factor receptor gene in cholangiocarcinomas. *J Cancer Res Clin Oncol*. 2005; 131:649–52. [PubMed: 16032426]
13. Sia D, Hoshida Y, Villanueva A, Roayaie S, Ferrer J, Tabak B, et al. Integrative Molecular Analysis of Intrahepatic Cholangiocarcinoma Reveals 2 Classes That Have Different Outcomes. *Gastroenterology*. 2013; 144:829–40. [PubMed: 23295441]
14. Andersen JB, Spee B, Blechacz BR, Avital I, Komuta M, Barbour A, et al. Genomic and genetic characterization of cholangiocarcinoma identifies therapeutic targets for tyrosine kinase inhibitors. *Gastroenterology*. 2012; 142:1021–1031.e15. [PubMed: 22178589]
15. Borger DR, Tanabe KK, Fan KC, Lopez HU, Fantin VR, Straley KS, et al. Frequent mutation of isocitrate dehydrogenase (IDH)1 and IDH2 in cholangiocarcinoma identified through broad-based tumor genotyping. *Oncologist*. 2012; 17:72–9. [PubMed: 22180306]
16. Zingh S, Chakraborty S, Bonthu N, Radio S, Hussain SM, Sasson A. Combined hepatocellular cholangiocarcinoma: a case report and review of literature. *Dig Dis Sci*. 2013; 58:2114–23. [PubMed: 23397471]
17. Massarelli E, Varela-Garcia M, Tang X, Xavier AC, Ozburn NC, Liu DD, et al. KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. *Clin Cancer Res*. 2007; 13:2890–6. [PubMed: 17504988]
18. Lièvre A, Bachet J-B, Le Corre D, Boige V, Landi B, Emile J-F, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res*. 2006; 66:3992–5. [PubMed: 16618717]
19. Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M, et al. KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med*. 2005; 2:e17. [PubMed: 15696205]
20. Edge, S.; Byrd, D.; Compton, A.; Fritz, F. *AJCC Cancer Staging Manual*. 7. New York, NY: Springer; 2010.
21. Tsiatis AC, Norris-Kirby A, Rich RG, Hafez MJ, Gocke CD, Eshleman JR, et al. Comparison of Sanger sequencing, pyrosequencing, and melting curve analysis for the detection of KRAS mutations: diagnostic and clinical implications. *J Mol Diagn*. 2010; 12:425–32. [PubMed: 20431034]
22. Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med*. 2012; 366:707–14. [PubMed: 22356324]
23. Voss JS, Holtegaard LM, Kerr SE, Fritcher EGB, Roberts LR, Gores GJ, et al. Molecular profiling of cholangiocarcinoma shows potential for targeted therapy treatment decisions. *Hum Pathol*. 2013; 44:1216–22. [PubMed: 23391413]

Figure 1.
Representative pyrograms of *KRAS* (A) and *BRAF* (B) mutations detected in ICC by pyrosequencing.

Figure 2. Kaplan-Meier plot demonstrating overall survival for patients with either *KRAS* or *BRAF* mutated tumors compared to wild type ($p = 0.05$).

Table 1

Demographic, disease, and treatment characteristics of patients

	No Mutation (n=46)	KRAS (n=4)	BRAF (n=4)	P-value
Age, median (range)	64 (37–81)	67.5 (55–78)	59 (45–78)	0.12
Male gender	14 (30.4)	1 (25.0)	2 (50.0)	0.82
Race				
White	39 (84.8)	3 (75.0)	4 (100)	0.75
Other	7 (15.2)	1 (25.0)	0	
Body mass index, median	26.0 (16.0–44.1)	24.0 (20.9–28.1)	36.2 (26.6–45.9)	
HBV infection	3(6.5)	0	0	1.00
Cirrhosis	4(8.7)	0	0	1.00
CA 19–9, median (range)	31 (11–56)	81 (–)	503 (1–1005)	0.08
Size, median (range)	5.5(2.5–19.0)	5.2 (3.7–5.7)	4.6(3.5–5.6)	0.22
Bilobar involvement	11(23.9)	1(25.0)	0	0.81
Nodes				
N0	43 (93.5)	2(50.0)	2(50.0)	0.01 [*]
N1	3 (6.5)	2 (50.0)	2(50.0)	
AJCC stage				
0	1 (2.2)	0	0	0.03 [*]
1	28(60.9)	0	2(50.0)	
2	10(21.7)	1(25.0)	0	
3	0	1(25.0)	0	
4	7(15.2)	2(50.0)	2(50.0)	
Multiple tumors	9(19.6)	2(50.0)	1(25.0)	0.38
Liver resection				
< Hemihepatectomy	11(23.9)	0	3(75.0)	0.18
Hemihepatectomy	24(52.2)	3(75.0)	1(25.0)	
Extended Hepatectomy	11(23.9)	1(25.0)	0	
Margins				
R0	38 (82.6)	2 (50.0)	3(75.0)	0.20
R1	8(17.4)	2(50.0)	1(25.0)	
Tumor Differentiation				
Well	8(17.3)	2(50.0)	0	0.32
Moderate	20(43.5)	2(50.0)	2(50.0)	
Poor	18(39.1)	0	2(50.0)	
Vascular Invasion	11(23.9)	2(50.0)	1 (25.0)	0.52
Perineural invasion	7(15.2)	2(50.0)	0	0.24
Biliary invasion	6(13.0)	2(50.0)	0	0.19
Satellite lesions	7(15.2)	2(50.0)	1(25.0)	0.16

	No Mutation (n=46)	<i>KRAS</i> (n=4)	<i>BRAF</i> (n=4)	P-value
Intrahepatic metastases	2 (4.4)	0	0	1.00
Adjuvant therapy	20 (43.5)	3 (75.0)	1 (25.0)	0.39

*
p< 0.05

Table 2Description of patients with a mutation of *KRAS* or *BRAF* (n=4 each)

	Age	Sex	Race	Tumor Size	Nodal status	AJCC Stage	Overall Survival (m)	Median OS (m)	P-value OS (WT* vs. <i>KRAS</i> + <i>BRAF</i>)
<i>KRAS</i>	55	F	Asian	5.4	N0	4a	13.5		
	66	M	White	5	N0	2	6.8	13.5	
	78	F	White	3.7	N1	3c	8.4		
	69	F	White	5.7	N1	4a	13.0		0.05
<i>BRAF</i>	45	F	White	4.5	N1	4a	85.4		
	78	M	White	3.5	N0	1	25.6	24.4	
	63	M	White	4.7	N0	1	23.2		
	55	F	White	5.6	N1	4a	2.7		

* OS for WT = 37.3 months