

High throughput molecular profiling reveals differential mutation patterns in intrahepatic cholangiocarcinomas arising in chronic advanced liver diseases

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Intrahepatic cholangiocarcinomas occur mostly in the normal liver but they also arise in chronic advanced liver diseases. However, genetic differences between two groups have yet to be examined. High throughput mass spectrometry-based platform was used to interrogate mutations in intrahepatic cholangiocarcinomas and to compare the mutation profiles between 43 intrahepatic cholangiocarcinomas with normal liver and 38 with chronic advanced liver diseases. Forty seven mutations in 11 genes were identified in 38 of 81 cases (46.9%). The most commonly mutated gene was *KRAS* (11/81, 13.6%), followed by *MLH1* (7/81, 8.6%), *NRAS* (7/81, 8.6%), *GNAS* (6/81, 7.4%), and *EGFR* (6/81, 7.4%). *BRAF*, *APC*, *PIK3CA*, *CDKN2A*, *PTEN*, and *TP53* mutations were found with less than 5%. Overall mutation rate of intrahepatic cholangiocarcinomas with chronic advanced liver disease (15/38, 39.5%, 95% confidence interval: 23.9–55.0) was lower than that of intrahepatic cholangiocarcinomas with normal liver (23/43, 53.5%, 95% confidence interval: 38.5–68.3). Intrahepatic cholangiocarcinomas with chronic advanced liver disease showed higher *EGFR* mutation rate (5/38, 13.2% vs 1/43, 2.3%) and lower mutation rates of *KRAS* (3/38, 7.9% vs 8/43, 18.6%), *MLH1* (2/38, 5.3% vs 5/43, 11.6%), and *GNAS* (1/38, 2.6% vs 5/43, 11.6%), compared with those in intrahepatic cholangiocarcinomas with normal liver. Mutations in *PIK3CA*, *PTEN*, *CDKN2A*, and *TP53* were harbored only in intrahepatic cholangiocarcinomas with normal liver. *KRAS* ($P=0.0075$) or *GNAS* mutations ($P=0.0256$) were associated with poor overall survival in all patients with intrahepatic cholangiocarcinoma. Differential mutation patterns of intrahepatic cholangiocarcinomas with chronic advanced liver disease suggest different cholangiocarcinogenesis depending upon the predisposing factors, and support that different strategy for targeted therapy should be applied in intrahepatic cholangiocarcinoma subtypes.

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Cholangiocarcinoma is very aggressive tumor and is associated with poor prognosis because surgical resection of the tumor is often impossible in far

advanced cases at initial diagnosis. Furthermore, chemotherapy for the advanced cholangiocarcinomas has failed to improve survival. Consequently, 5-year survival rate of cholangiocarcinoma patients is less than 50% and there is a high intrahepatic recurrence even after surgical resection.¹ Therefore, in patients with advanced cholangiocarcinomas and in those with therapeutically resected tumors with poor prognostic factors such as lymph node metastasis, macroscopic vascular invasion, and positive surgical margins, and a background of liver cirrhosis,^{2,3} the potential utility of

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targeted therapy has become a focus of interest. However, somatic mutations in cholangiocarcinoma which can be targets for chemotherapy have been still very rarely studied.^{4,5}

Cholangiocarcinomas are largely divided into the intrahepatic, perihilar, or distal cholangiocarcinomas according to the anatomical location.⁶ Their clinical features and biological behaviors are also different, which suggest that cholangiocarcinomas are heterogeneous in their genotypes and must be studied separately by anatomic location. Of these, intrahepatic cholangiocarcinomas are the second most commonly occurring primary liver malignancy worldwide after hepatocellular carcinoma.⁷ Furthermore, their incidence has been variably increasing not only in Western countries but also in Asia where biliary diseases are prevalent.⁷

Intrahepatic cholangiocarcinomas usually arise in the normal liver but some are associated with chronic biliary diseases, such as primary sclerosing cholangitis, and hepatolithiasis.⁸ A minority of intrahepatic cholangiocarcinomas occurs in the setting of non-biliary chronic liver diseases, associated with HCV- and HBV-related hepatitis, leading to cirrhosis.^{9–11} Recently intrahepatic cholangiocarcinomas arising in biliary and non-biliary diseases are described to differ from those arising in normal liver in terms of gross and histopathological features including the tumor size, growth pattern, and vascularity.^{12,13} Therefore, the occurrence of intrahepatic cholangiocarcinoma arising in these different settings suggests differences in the underlying genetic profiles.

In the present study, we carried out a high-throughput mutation analysis based on mass spectrometry¹⁴ using formalin-fixed paraffin-embedded tissue samples of intrahepatic cholangiocarcinomas in order to interrogate common mutations occurring in these tumors, revealing different somatic mutation profiles of intrahepatic cholangiocarcinomas arising in non-biliary chronic advanced liver diseases compared with those without liver diseases.

Materials and methods

Patients and Samples

Pathology records from the Department of Pathology of ASAN Medical Center from January 1998 to December 2008 were reviewed to retrieve cases of surgically resected intrahepatic cholangiocarcinoma arising in patients with non-biliary chronic advanced liver diseases. The search yielded 38 cases of intrahepatic cholangiocarcinoma arising in either pre-cirrhotic or cirrhotic chronic advanced liver diseases. Among the 38 cases of intrahepatic cholangiocarcinoma with chronic advanced liver diseases, 29 cases were associated with HBV infection, three with alcohol intake, two with HCV infection, and one with autoimmune hepatitis, and the remaining three were of unknown origin. For a comparison group, we selected randomly 43 cases of 143 cases of intrahepatic cholangiocarcinoma that developed in patients with normal livers from January 2003 to December 2008. There were no differences in two groups in terms of age, sex ratio, and other clinical parameters. The patients' demographics are summarized in Table 1.

The ASAN Medical Center Institutional Review Board granted its approval for use of the formalin fixed paraffin embedded samples. As the samples were all anonymized by coding independent numbers from any clinical data, the Review Board waived the need for patient consent to use the samples. Histopathological findings as well as pathology reports were reviewed by two pathologists (SMH and HP) in order to select the appropriate formalin fixed paraffin embedded tissue blocks and to mark those areas on the slides with the highest percentage of cancer involvement for DNA extraction.

DNA Extraction

The two pathologists microscopically reviewed all H&E slides to confirm the tumor type, as well as to ensure that the sample was representative of the

Table 1 Clinicopathological features of patients with intrahepatic cholangiocarcinoma as a function of background liver disease

Parameter	Intrahepatic cholangiocarcinomas with normal liver (n = 43)	Intrahepatic cholangiocarcinomas with chronic advanced liver disease (n = 38)	P value
Age, median (range)	59 yrs (from 48 to 78)	58 yrs (from 33 to 71)	0.070
Sex			1.000
Male:female	30:13	27: 11	
Differentiation			0.159
Well:moderate:poor	2:38: 3	5: 27: 6	
Gross type			0.918
Intraductal	0	1	
Periductal infiltrative	4	3	
Mass-forming	38	34	
Mixed	1	0	
Tumor size (mean ± s.d.)	5.8 cm ± 3.0	5.3 cm ± 2.7	0.425

tumor and that normal surrounding tissues were not included. For each formalin fixed paraffin embedded block, 10–20 sections of 6 μm thickness were used for the extraction of genomic DNA, depending on the tumor size on the matched slides. After deparaffinization with xylene and ethanol, DNA was purified using the QIAamp DNA formalin fixed paraffin embedded tissue kit (#56404; Qiagen, Hilden, Germany), then followed by quantification using the Quant-iTTMPicoGreen ds DNA assay kit (Invitrogen) and normalized to concentrations of 5 ng/ μl .

Genotyping Using OncoMaP_v4.4-Core Panels

Mutations in 41 critical genes related to tumor development were profiled using OncoMap version 4.4-Core (OncoMap_v4.4C) under the SequenomMassARRAY technology platform (Sequenom, San Diego, CA). OncoMap_v4.4C comprises 32 pool iPLEX panels that allow the interrogation of 471 unique mutation sites in 41 oncogenes and tumor suppressor genes (Table 2), together referred to as actionable targets. This OncoMap panel is an upgraded version of OncoMap_v1, developed and published by MacConaill *et al.*¹⁴ Multiplex amplification for 32 different pools was performed with 10 ng of genomic DNA per each PCR reaction. An additional 50–80 ng of DNA was used in homogeneous mass extension (hME) validation of the mutation candidates identified in the iPLEX reaction. After multiplex PCR, the samples were treated with shrimp alkaline phosphatase (SAP) from the iPLEX-Prokit (cat.# 10142-2, Sequenom, San Diego, CA) to inactivate residual deoxynucleotides, and then subjected to single-base extension (SBE) by the addition of 2 μl of the iPLEX-Gold chemistry mixture. After SBE, around 10 nl of the desalted product was spotted onto a 384-format SpectroCHIP II, then followed by mass determination with MALDI-ToF mass spectrometer. Genotypes were called using the cluster analysis algorithm developed by the Center for Cancer Genome Discovery of the Dana-Farber Cancer Institute and then further reviewed manually by two independent researchers

Table 2 Number of mutations in genes tested in this study using OncoMap_v4.4C

Gene	No.	Gene	No.	Gene	No.	Gene	No.
<i>ABL1</i>	16	<i>ERBB2</i>	8	<i>IDH2</i>	2	<i>NRAS</i>	22
<i>AKT1</i>	1	<i>FGFR1</i>	2	<i>JAK2</i>	1	<i>PDGFRA</i>	20
<i>AKT2</i>	2	<i>FGFR2</i>	6	<i>JAK3</i>	3	<i>PIK3CA</i>	23
<i>APC</i>	13	<i>FGFR3</i>	8	<i>KIT</i>	27	<i>PIK3R1</i>	14
<i>BRAF</i>	50	<i>FLT3</i>	9	<i>KRAS</i>	24	<i>PTEN</i>	15
<i>CDK4</i>	1	<i>GNA11</i>	2	<i>MAP2K1</i>	7	<i>RB1</i>	11
<i>CDKN2A</i>	11	<i>GNAQ</i>	3	<i>MET</i>	6	<i>RET</i>	14
<i>CSF1R</i>	7	<i>GNAS</i>	3	<i>MLH1</i>	1	<i>SRC</i>	1
<i>CTNNA1</i>	33	<i>HRAS</i>	16	<i>MYC</i>	6	<i>STK11</i>	12
<i>EGFR</i>	51	<i>IDH1</i>	3	<i>NPM1</i>	3	<i>TP53</i>	7
<i>VHL</i>	7						

to undo any uncertain calls resulting from clustering artifacts. Sample quality was considered adequate for analysis if more than 80% of the attempted genotypes resulted in identifiable products. A total of 46 candidate mutations from mutation calling were validated by hME genotyping of whole samples according to the previously described method.¹⁵ In brief, PCR amplification and SAP treatment was performed in 8 different pools with up to 6-plex reaction, and hME reaction was conducted using mixture of dNTP and ddNTP depending on the type of DNA change for each mutation candidates, and followed by desalting, spotting, and MALDI-ToF analysis. Information regarding the oligonucleotides used in the hME reactions is presented in the Supplementary Table 1. The genotypes obtained from the iPLEX and hME assays were compared; only concordant calls were regarded as validated mutations.

Direct DNA Sequencing Analysis

The exon 19 deletion of the *EGFR* gene was additionally validated by direct Sanger sequencing as follows: Exon 19 was PCR-amplified using a 2720 thermal cycler (Applied Biosystems, USA). The 20 μl reaction volume contained a 250 μM mixture of dATP, dGTP, dTTP, and dCTP, 0.5 U of AmpliTaq-Gold DNA polymerase, and 0.2 μM of each primer pair (*EGFR*_exon19_f: 5'-CATGTGGCACCATCTCAA-3', *EGFR*_exon19_R: 5'-GACATGAGAAAAGGTGGGCC-3'). The amplification protocol consisted of initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 10 min. Amplified PCR product with 221 bp size in wild type was sequenced using the forward primer and the Big Dye terminator v3.0 cycle sequencing reagents (Applied Biosystems). PCR amplification consisted of 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min. Each DNA sequence was read on an ABI-Prism 3100 automatic sequencer (Applied Biosystems).

Statistical Analysis

Overall survival was determined using Kaplan-Meier method, and survival curves were compared using the log-rank test. Survival was calculated from the date of surgery until the last follow up visit of the patients. Association test was performed using Fisher's exact test for categorical data and Wilcoxon rank-sum test for continuous data. The Odds Ratio was calculated in mutation occurrence between two genes to reveal the tendency of mutually exclusivity or co-occurrence. All tests were two-sided and *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed using Stata/IC statistical software (version 12, StataCorp Ltd., College Station, TX).

Results

Mutation Profile in Intrahepatic Cholangiocarcinomas

Overall, 47 mutations in 11 oncogenes or tumor suppressors were identified in 38 of the total of 81 cases (46.9%). Concordance rate between iPLEX and hME genotyping of 46 mutation candidates for total 81 samples was 98.44% (3,668 among 3726). Among these 38 cases, a single mutation was determined in 30 (78.9%), two mutations in seven (18.4%), and three mutations in one (2.6%). The most commonly mutated gene was *KRAS* oncogene (11/81, 13.6%), followed by *MLH1* (7/81, 8.6%), *NRAS* (7/81, 8.6%), *GNAS* (6/81, 7.4%), and *EGFR* (6/81, 7.4%). Other, less frequently mutated genes were *BRAF* (3/81, 3.7%), *APC* (2/81, 2.5%), *PIK3CA* (2/81, 2.5%), *CDKN2A* (1/81, 1.2%), *PTEN* (1/81, 1.2%), and *TP53* (1/81, 1.2%). Mutation occurrences in most genes have strong tendency toward mutual exclu-

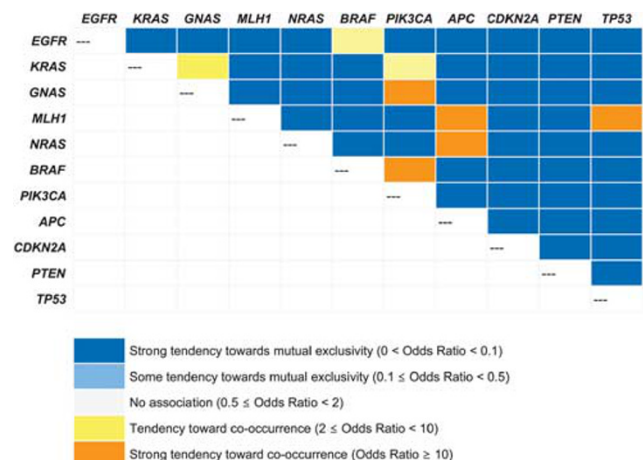


Figure 1 Overall tendency toward mutual exclusivity or co-occurrence in mutation occurrence between genes.

sivity ($0 < \text{Odds Ratio} < 0.1$, Figure 1). Some genes (for example *MLH1* vs *APC*, *MLH1* vs *TP53*, *NRAS* vs *APC* etc), however, have tendency toward co-occurrence ($\text{Odds Ratio} \geq 10$). Overall tendency between genes toward mutual exclusivity or co-occurrence was shown in Figure 1.

Comparison of the Mutation Rates for Intrahepatic Cholangiocarcinomas in Normal Versus Chronic Advanced Liver Disease

There were different mutation profiles in frequency and type of genes between intrahepatic cholangiocarcinomas from patients with chronic advanced liver disease and those from patients with normal livers (Figure 2). Overall mutation rate of intrahepatic cholangiocarcinomas with chronic advanced liver disease (15/38, 39.5%, 95% confidence interval: 23.9–55.0) was lower than that of intrahepatic cholangiocarcinoma with normal liver (23/43, 53.5%, 95% confidence interval: 38.5–68.3). However, intrahepatic cholangiocarcinoma with chronic advanced liver disease showed higher *EGFR* mutation rate (5/38, 13.2%) than that of intrahepatic cholangiocarcinoma with normal liver (1/43, 2.3%). Interestingly, *EGFR* mutations for current target therapy of the tyrosine kinase inhibitor, such as gefitinib, were present only in intrahepatic cholangiocarcinoma with chronic advanced liver disease (*EGFR_E746_A750del* in 2 patients and *EGFR_L747_P753Q* in 2 patients). Mutation rates of *KRAS* (3/38, 7.9% vs 8/43, 18.6%), *MLH1* (2/38, 5.3% vs 5/43, 11.6%), and *GNAS* (1/38, 2.6% vs 5/43, 11.6%) were lower in intrahepatic cholangiocarcinoma with chronic advanced liver disease than in intrahepatic cholangiocarcinoma with normal liver. The frequencies of *NRAS*, *BRAF*, and *APC* mutations were similar between the two groups. Mutations in *PIK3CA*, *PTEN*, *CDKN2A*, and *TP53*

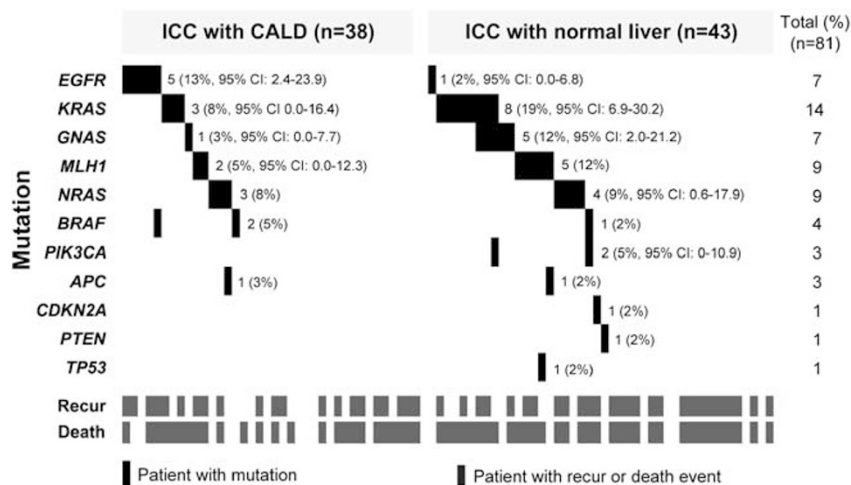


Figure 2 Mutation profiles and clinical features in intrahepatic cholangiocarcinomas (ICC) arising in chronic advanced liver disease (CALD) and intrahepatic cholangiocarcinomas arising in normal liver. Mutation frequency and 95% confidence interval was present in each gene between two groups.

were harbored only in intrahepatic cholangiocarcinomas with normal liver. Mutations of each gene including specific mutation site were demonstrated in Table 3.

Significantly shorter overall survival rates were observed in patients with *KRAS* mutations (18.2%, 95% confidence interval: 2.9–44.2) than those without *KRAS* mutation (36.6%, 95% confidence interval: 25.4–47.9, $P=0.0075$) (Figure 3). Similarly, overall five-year survival rates in patients with *GNAS* mutations (16.7%, 95% confidence interval: 0.8–51.7) were shorter than in those without *GNAS* mutations (35.5%, 95% confidence interval: 24.8–46.4, $P=0.0256$). When the patients were classified into two groups as intrahepatic cholangiocarcinoma with chronic advanced liver disease and intrahepatic cholangiocarcinoma with normal liver, *KRAS* mutation was still significantly associated with poor prognosis in intrahepatic cholangiocarcinomas with

chronic advanced liver disease ($P=0.037$, Figure 3). Survival impact for other somatic mutations except for *KRAS* and *GNAS* was not found.

Identification of *EGFR* Mutations Using iPLEX/hME Validation Compared with Sanger Sequencing

For *EGFR* mutations, Sanger sequencing was also conducted in four cases of intrahepatic cholangiocarcinoma with chronic advanced liver disease in which an exon 19 deletion mutation was detected. The four cases were of interest because they might be potential therapeutic targets of the tyrosine kinase inhibitor gefitinib. The exon 19 deletion mutation was identified both in the iPLEX/hME validation and by Sanger sequencing in one of the four cases (Figure 4), while the mutation was not revealed by Sanger sequencing in the other three cases. These differences may be explained by the higher sensitivity of iPLEX/hME validation method for mutation detection. The estimated allele frequency of the mutated *EGFR* by the OncoMap method was less than 10% which is below the detection sensitivity of the Sanger method.¹⁶

Discussion

Intrahepatic cholangiocarcinomas with specific etiologies have only rarely been subjected to high-throughput genome profiling.^{17,18} Instead, most investigations have focused on the genetic variations in several specific genes, typically *KRAS*, *EGFR*, and *BRAF* using fresh tissues.^{5,19} The present study, however, examined numerous mutations of oncogenes or tumor suppressor genes in formalin fixed paraffin embedded intrahepatic cholangiocarcinoma tissues using OncoMap system. In addition, this study is the first to provide mutational data for intrahepatic cholangiocarcinomas in the setting of non-biliary chronic advanced liver diseases such as HBV- or HCV-associated cirrhosis. It is not a hard thing to anticipate differences in the genetic profiles between intrahepatic cholangiocarcinomas arising in non-biliary chronic advanced liver disease and intrahepatic cholangiocarcinomas with normal liver, because of histopathological differences in the tumor as well as the background liver.¹³ Although many studies on genetic profiling of cholangiocarcinomas have been done in heterogeneous groups involving even gallbladder cancers, our cases were rather homogeneous, consisting of mass forming peripheral intrahepatic cholangiocarcinomas in about 90% and central intrahepatic cholangiocarcinomas with involvement of perihilar bile ducts in about 10%, none of which from either group have undergone prior chemotherapy.

Interestingly, there was a difference in the type and frequency of mutated genes between the two groups: *EGFR* mutations were detected mostly in

Table 3 Comparison of mutated genes with frequencies of mutations between intrahepatic cholangiocarcinomas arising in normal liver and those arising in chronic advanced liver diseases

Genes	Intrahepatic cholangiocarcinomas with normal liver (n = 43)	Intrahepatic cholangiocarcinomas with chronic advanced liver disease (n = 38)
	No. of cases with mutations (%)	No. of cases with mutations (%)
<i>KRAS</i>	8 (19)	3 (8)
<i>KRAS_G12D</i>	2	2
<i>KRAS_G12V</i>	2	0
<i>KRAS_Q61H</i>	2	0
<i>KRAS_A146T</i>	1	0
<i>KRAS_G12A</i>	1	0
<i>KRAS_G13D</i>	0	1
<i>MLH1</i>	5 (12)	2 (5)
<i>MLH1_V384D</i>	5	2
<i>GNAS</i>	5 (12)	1 (3)
<i>GNAS_R201H</i>	4	0
<i>GNAS_R201C</i>	1	1
<i>NRAS</i>	4 (9)	3 (8)
<i>NRAS_Q61R</i>	4	1
<i>NRAS_G12D</i>	0	1
<i>NRAS_Q61K</i>	0	1
<i>PIK3CA</i>	2 (5)	0
<i>PIK3CA_E542K</i>	1	0
<i>PIK3CA_E545K</i>	1	0
<i>EGFR</i>	1 (2)	5 (13)
<i>EGFR_E746_A750del</i>	0	2
<i>EGFR_L747_P753Q</i>	0	2
<i>EGFR_E709K</i>	0	1
<i>EGFR_V786M</i>	1	0
<i>BRAF</i>	1 (2)	2 (5)
<i>BRAF_N581S</i>	1	1
<i>BRAF_G469A</i>	0	1
<i>APC</i>	1 (2)	1 (3)
<i>APC_R1450*</i>	1	1
<i>CDKN2A</i>	1 (2)	0
<i>CDKN2A_W110*</i>	1	0
<i>PTEN</i>	1 (2)	0
<i>PTEN_R233*</i>	1	0
<i>TP53</i>	1 (2)	0
<i>TP53_R273C</i>	1	0

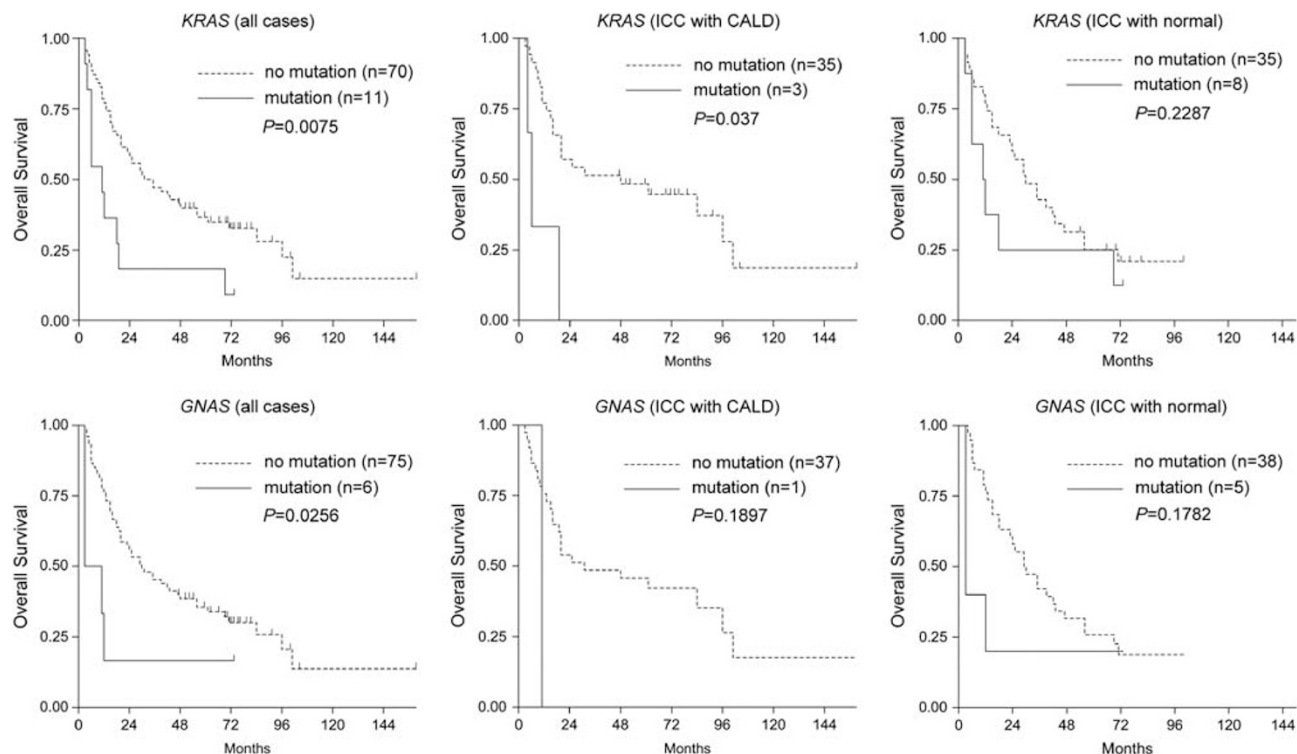


Figure 3 Prognostic significance of *KRAS* and *GNAS* mutations in intrahepatic cholangiocarcinomas (ICC) arising in chronic advanced liver disease (CALD) and intrahepatic cholangiocarcinomas arising in normal liver.

intrahepatic cholangiocarcinomas from patients with chronic advanced liver diseases, while *PIK3CA*, *CDKN2A*, *PTEN*, and *TP53* mutations occurred only in intrahepatic cholangiocarcinomas from patients with normal livers.

EGFR mutations have been rarely reported in resected cholangiocarcinomas or cholangiocarcinoma cell lines^{4,5,18,20,21} and do not occur in cholangiocarcinomas that are related to *Opisthorchis viverrini* infection.¹⁷ The frequency of *EGFR* mutations, especially in the kinase domain, in intrahepatic cholangiocarcinomas arising in chronic advanced liver disease was much higher than that described in a previous report using the same OncoMap platform.¹⁹ Another study of Asian patients with cholangiocarcinoma also found a few different types of mutations in the tyrosine kinase domains of *EGFR*,⁴ in addition to the same mutation detected in the present study. The difference in these studies may be explained by different underlying liver diseases and ethnicity of patients bearing tumors with *EGFR* mutations, as is well known in pulmonary adenocarcinoma.²¹ Taken together, it may be suggested that *EGFR* mutations play an important role in intrahepatic cholangiocarcinomas arising in the setting of non-biliary chronic advanced liver diseases.

Most of the *EGFR* exon 19 mutations examined in this study are identical to previously described deletions identified in non-small cell lung cancers.²² The deletion of exon 19 can result in an

enhanced sensitivity to the effect of EGF and also to gefitinib, secondary to an increased binding affinity to both adenosine triphosphate and anilinoquinazolines.²³ Thus, intrahepatic cholangiocarcinomas with these mutations are likely candidates for targeted therapy with gefitinib.

For patients with unresectable or metastatic cholangiocarcinoma, a combination regimen consisting of gemcitabine and cisplatin is the first-line treatment, with 5-FU monotherapy as the second line.²⁴ Additional radiation therapy may also be deemed necessary. Although none of the trials with targeted agents, including anti-*EGFR*-based approaches, have succeeded, modifications based on the genomic data of the various tumor targets may eventually lead to therapeutic improvements.

In this study, some of mutations in *EGFR* are rare events, affecting less than 10% of tumor cells. These patients even with *EGFR* mutation are unlikely to respond to anti-*EGFR* therapy. However, it was reported that the presence of the drug-resistance mutation of *EGFR*, *T790M*, at such a low frequency did not preclude significant responses to therapy with tyrosine kinase inhibitors among patients with *EGFR* mutant tumors, but it was associated with a striking shorter progression-free survival in patients with a detectable *T790M* allele.²⁵ Therefore, the detection of *EGFR* mutation, either sensitive or insensitive mutation to gefitinib and erlotinib, in only a small number of cells may be important and

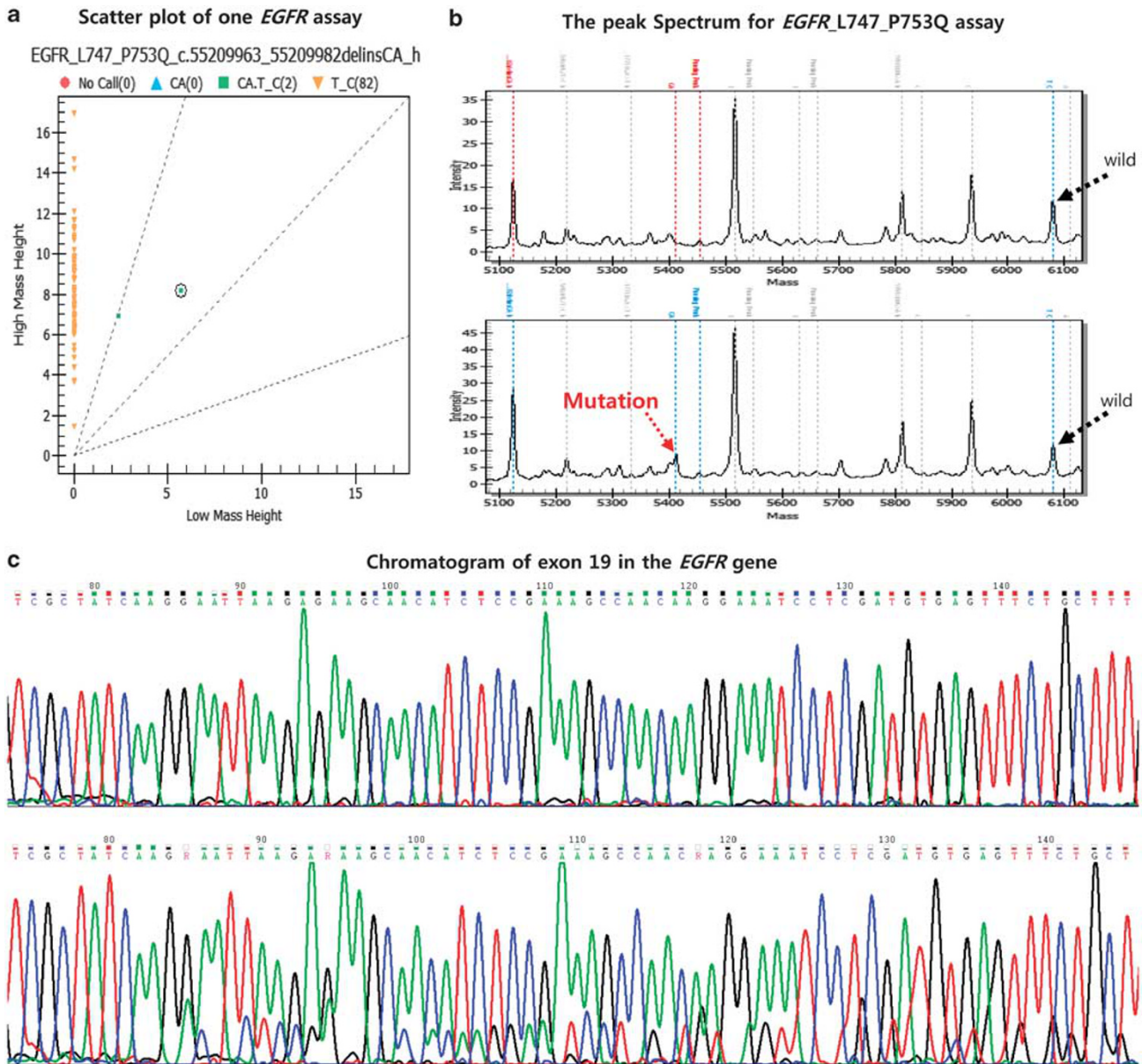


Figure 4 Identification of exon 19 deletion in the *EGFR* using iPLEX/hME validation and direct sequencing. (a) Scatter plot from a hME assay for validation of the exon 19 deletion in the *EGFR*. Orange symbols located along the y-axis indicate the wild-type gene. The two green spots indicate genomic DNA with *EGFR* mutation. (b) Representative peak spectrums from genomic DNA with no mutation (upper) and with *EGFR* mutation (the green spot circled in A) show a peak derived from mutation allele in addition to wild allele peaks. (c) Chromatogram of exon 19 deletion in *EGFR* detected by hME. A double peak in the exon 19 amplicon only that is absent in normal allele (upper) is observed in a mutation allele (lower).

need to further study for their clinical significance in cholangiocarcinoma. The low allele frequency of the mutated *EGFR*, as identified in this study, may be attributed to the result of a subclonal event during cholangiocarcinogenesis. To develop a targeted therapy for advanced intrahepatic cholangiocarcinoma with the low allele frequency, subsequent cell-based or xenograft studies will be necessary to confirm whether intrahepatic cholangiocarcinoma with *EGFR* mutations is addicted to the oncogene or not.

KRAS mutations occurring mutually exclusively with *EGFR* mutations are well-known markers to determine the therapeutic strategy for patients with colon cancer or non-small cell lung cancer.²⁶ In this work, the overall frequency of *KRAS* mutations was similar to, but each frequency in our two subgroups of intrahepatic cholangiocarcinomas was different from the previously reported *KRAS* mutations in intrahepatic cholangiocarcinomas¹⁸ that may have occurred only in the setting of normal liver. In another previous study using the same platform as

ours, the frequencies of *KRAS* mutations were analyzed regardless of underlying hepatobiliary diseases, and were higher in peripheral cholangiocarcinoma unlikely in our mass forming central intrahepatic cholangiocarcinomas in which *KRAS* mutations were not detected.¹⁹ Large-scale studies of cholangiocarcinomas, assessing both the tumor site and predisposing factors including various types of liver diseases are needed to elucidate the role of *KRAS* mutations in intrahepatic cholangiocarcinoma.

The high frequency of *GNAS* mutations in intrahepatic cholangiocarcinomas from patients with non-biliary chronic advanced liver diseases is a new and interesting finding to come out of this study. *GNAS* located on chromosome 20q13 encodes the G protein alpha subunit (*Gsz*). *GNAS* mutations, including somatic mutations and copy-number amplification, result in various diseases. In girls with McCune-Albright syndrome that includes precocious puberty, a relationship between the nature of the *GNAS* mutation and disease severity has been reported.²⁷ Somatic mutation of *GNAS* in codon 21, resulting in the replacement of arginine with cysteine or histidine, causes constitutive activation of *Gsz*, followed by the activation of adenylate cyclase. The subsequent increase in cAMP levels induces the production of protein kinase A, which is essential for the hypoxia-mediated epithelial-mesenchymal transition, as well as the migration and invasion of these tumorigenic cells in lung cancer.²⁸ Therefore, intrahepatic cholangiocarcinoma patient with this mutation may be investigated in a clinical trial for targeted therapy with protein kinase A inhibitors developed for that purpose.

Mutations in *PIK3CA* kinase domains are also candidates for targeted therapy. *PIK3CA* mutations have been identified either only in gallbladder¹⁹ or rarely in intrahepatic cholangiocarcinomas.¹⁸ Because the two cases of intrahepatic cholangiocarcinoma with *PIK3CA* mutations occurred in normal liver, those cases can benefit much more from a targeted therapy than intrahepatic cholangiocarcinoma patients with chronic advanced liver disease. With the availability of tools to detect target genes such as OncoMap platform, the liver biopsy samples are needed to be processed for molecular profiling to determine the optimal drugs for personalized cancer treatment.

Mutations in the tumor suppressor gene, such as homozygous deletion at the *p16* region and loss of heterozygosity of *CDKN2A* have been reported in intrahepatic cholangiocarcinomas and extrahepatic cholangiocarcinomas, while *p16* gene missense mutations have not been detected in intrahepatic cholangiocarcinoma.²⁹ In the present study, we identified a *CDKN2A_W110** mutation in one of the 43 intrahepatic cholangiocarcinomas arising without biliary diseases such as primary sclerosing cholangitis or hepatolithiasis in which promoter mutations and methylation of the *p16* gene leading

to a loss of transcriptional activity or the decreased expression of *CDKN2A* have been described.^{30,31}

Two of the *BRAF* mutations detected in the present study, G469A (c.1406G>C) and N581S (c.1742A>G), have not been previously reported in intrahepatic cholangiocarcinomas and they are distinct from the *BRAF* mutation V600E (c.1799T>A) commonly found in malignant tumors. The relevance of these mutations in intrahepatic cholangiocarcinoma remains to be determined.

OncoMap is a genotyping system for mutation profiling of well-known oncogenes and tumor suppressor genes in which common mutations occurring in hot spots and with high frequencies are detected in various tumors, using pre-designed probes for the corresponding mutation sites. Therefore, mutations that are unique for a specific cancer or that occur at low frequencies in common tumors are probably not detected by the OncoMap system. Despite these limitations, we were able to identify frequent actionable mutations in intrahepatic cholangiocarcinoma, one of the most notorious forms of cancer. Drug-targetable mutations, such as in *EGFR*, *GNAS*, *BRAF*, and *PIK3CA*, account for roughly 20% of both intrahepatic cholangiocarcinomas occurring in normal liver and those arising in chronic advanced liver diseases.

In summary, our results highlight the need for a modified OncoMap system that will be more effective in evaluating intrahepatic cholangiocarcinomas, focusing on *KRAS*, *EGFR*, *MLH1*, *GNAS*, *NRAS*, *BRAF*, and *PIK3C*, and new optimized mutation profiling platforms that can detect additional novel drug-targetable cancer genes. Then those improved high throughput molecular profiling with formalin fixed paraffin embedded liver samples will allow patient-tailored therapies, taking into account predisposing factors and underlying liver status, for postoperative or inoperable intrahepatic cholangiocarcinoma patients.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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