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Ultrasensitive Detection of *KRAS2* Mutations in Bile and Serum from Patients with Biliary Tract Carcinoma Using LigAmp Technology

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Patients with biliary tract carcinoma have a poor prognosis. Early detection efforts are urgently needed to ameliorate the dismal prognosis for these patients. Mutations of the *KRAS2* gene are one of the most common genetic aberrations in this cancer. In this study, we used LigAmp, an ultrasensitive technology for detecting point mutations, to analyze *KRAS2* mutations in patients with a variety of neoplastic and non-neoplastic pancreatobiliary diseases. DNA was isolated from 64 samples, including 44 bile samples and 20 serum samples. Oligonucleotides specific for *KRAS2* G35A (GAT, G12D), G35T (GTT, G12V), and G34A (AGT, G12S) mutations were used. *KRAS2* mutations were detected in 14 of 16 (87.5%) neoplastic bile samples and in 9 of 28 (32.1%) non-neoplastic bile samples. However, the mutation levels were significantly lower in the non-neoplastic bile (median = 0.4%) compared with those in the neoplastic bile (median = 5.1%). *KRAS2* mutations were also detected in 9 of 11 (81.8%) serum samples from patients with biliary tract carcinoma, which was further confirmed by cloning BstN1-refractory PCR products and DNA sequencing. However, *KRAS2* mutations were not present in the sera from eight patients with benign pancreatobiliary diseases. These data demonstrate that *KRAS2* mutations are detectable in both bile and serum using LigAmp. This technology has the potential for early biliary tract carcinoma detection and possibly for residual disease monitoring post-therapy. ***(J Mol Diagn 2009, 11:583–589; DOI: 10.2353/jmoldx.2009.090061)***

Biliary tract carcinoma arises within the bile duct system, and is anatomically divided into carcinoma of the gallbladder, as well as intrahepatic and extrahepatic cholangiocarcinoma. It carries a poor prognosis, with an overall 5 year-survival of less than 5%.[1] In the United States, intrahepatic cholangiocarcinoma is the second most common primary hepatic cancer, and cholangiocarcinoma as a whole accounts for >4500 cancer-related deaths each year. The dismal outcome of biliary tract carcinoma is largely because the majority of cancers are diagnosed at an unresectable advanced stage. Patients with early biliary tract carcinoma have a survival rate as high as 40% following a curative surgical resection,[2,3] whereas those diagnosed late have a survival rate of only 5%. Early detection therefore could have a significant impact on overall survival. Although recent progress has been made in various imaging modalities such as endoscopic retrograde cholangiopancreatography, endoscopic ultrasonography, and magnetic resonance imaging, diagnosis of biliary tract carcinoma at an early stage is still difficult in many cases.[4,5] In addition, it is technically challenging to perform endoscopic biopsy because of the presence of biliary strictures associated with small lesions. Although biliary tract carcinoma is derived from the bile duct epithelium, endobiliary brush cytology typically yields a low sensitivity of detection for malignancy.[6] Furthermore, reactive biliary epithelial atypia associated with inflammatory biliary stricture mimics cancerous epithelium, which makes it problematic to diagnose biliary tract carcinoma cytologically and histologically. On the other hand, since this type of tumor tends to grow less coherently and the neoplastic cells are easily shed into bile, molecular analysis of tumor DNA in bile could be an important ancillary testing for definitive diagnosis of early biliary tract carcinoma.

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Table 1. Oligonucleotides and Probes

KRAS2 upstream	GAT	5′-ACTGTAAAACGACGGCCAGTGT-*TCCCCTCAAACTGGCAGATGCACG-C*-**TTGTGGTAGTTGGAGCCGA*-3′**
	GTT	5′-ACTGTAAAACGACGGCCAGTGT-*TCCCCTCAAACTGGCAGATGCACG-C*-**TTGTGGTAGTTGGAGCCGT*-3′**
	AGT	5′-ACTGTAAAACGACGGCCAGTGT-*TCCCCTCAAACTGGCAGATGCACG-C*-**TTGTGGTAGTTGGAGTTA*-3′**
	GGT (Wild-type)	5′-ACTGTAAAACGACGGCCAGTGT-*CGTATTACCGCGGCTGCTGGCACC*-**TTGTGGTAGTTGGAGCTGG*-3′**
KRAS2 common (downstream)	12b	5′-PO^4-**TGGCGTAGGCAAGAGTGCC**-TGGTCATAGCTGTTTCCTGCA-3′
M13 primers	Forward	5′-CTGTAAAACGACGGCCAGTG-3′
	Reverse	5′-TGCAGGAAACAGCTATGACCA-3′
TaqMan probes	*lacZ*	FAM-5′-TCCCCTCAAACTGGCAGATGCACG-3′-BHQ-1
	16S rDNA	ROX-5′-CGTATTACCGCGGCTGCTGGCAC-3′-BHQ-2

Underlined, M13 primer binding regions; Italics, probe binding regions (*lacZ* or *16S rDNA*); Bold, target-specific regions; Asterisk, terminal bases with perfect homology to either the wild-type or mutant sequences; FAM, 6-carboxyflurorescein; ROX, 6-carboxy-X-rhodamine; BHQ, black hole quenche; Boxed base, an additional mis-pair in the upstream mutant oligonucleotides was introduced at the third base from the 3' end to improve the specificity of the assay.

Patients with biliary tract carcinoma who harbor locally advanced or distant metastatic disease are not candidates for surgery, and receive either systemic chemotherapy or local irradiation. However, there are few avenues available to monitor the effectiveness of therapy, or to assess for minimal residual disease in the face of therapeutic response. To ensure that these patients receive effective treatment and avoid unnecessary toxicities, it is mandatory to have a sensitive and specific test to monitor the therapeutic response *in vivo* using a relatively non-invasive assay.

Activation of oncogenes and inactivation of tumor suppressor genes have been identified as important contributors in the development of biliary tract carcinoma.[7–9] The incidence of *KRAS2* mutations that have been reported in biliary tract carcinoma varies widely ranging from 0% to 100%, depending on what techniques have been used. *KRAS2* mutations most commonly occur at codon 12, including GGT to AGT, GAT, and GTT.[9,10] Detection of *KRAS2* mutations in bile has been explored as a potential diagnostic test by several groups. However, in these studies, although *KRAS* mutations were detected in the primary tumors of more than 80% cases, a much lower frequency of *KRAS2* mutations was observed in bile from the same population,[6,11,12] which probably resulted from the presence of excessive wild-type DNA in the bile. An identical technological pitfall exists for detecting mutant DNA in serum. A sensitive and specific assay could improve the detection of mutant *KRAS2* to a great extent.

Recently, we developed an ultra-sensitive technology, LigAmp, for detection of single base mutations in the presence of large amounts of wild-type DNA.[13] We have demonstrated that LigAmp is able to detect one mutant in the presence of ~10,000 wild-type molecules or cells. In the present study, we used LigAmp to analyze *KRAS2* mutations in the bile and serum from patients with a variety of non-neoplastic diseases and those with biliary tract carcinoma.

Materials and Methods

We obtained appropriate institutional approval for all experiments involving human subjects.

Sample Collection

Bile was collected during endoscopic retrograde cholangiopancreatography from 16 patients with biliary tract carcinoma (as confirmed by subsequent histology on surgical resection, diagnostic biopsy, or imaging), and from 28 patients with benign pancreatobiliary conditions (as confirmed by serial imaging or clinical follow up). The 16 biliary tract carcinomas included 12 extrahepatic cholangiocarcinomas, 3 intrahepatic cholangiocarcinomas and 1 carcinoma of the gallbladder. Bile samples were kept at −70°C until further use. Blood (10 ml) from patients with biliary tract carcinoma (n = 11, including 9 extrahepatic cholangiocarcinomas, 1 intrahepatic cholangiocarcinoma and 1 carcinoma of the gallbladder) and those with benign pancreatobiliary diseases (n = 9) was drawn into Becton-Dickinson SSAT tubes (Franklin Lakes, NJ) and placed immediately on ice. Then, these samples were centrifuged at 1500 × g for 30 minutes at 4°C. Serum was removed and stored at −20°C for further use.

DNA Extraction

Genomic DNA from a *KRAS2* wild-type cell line (HeLa), *KRAS2* mutant cell lines LS513 (GAT), and SW480 (GTT) was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA). QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) was used to extract DNA from serum (200 μl) and bile (200 μl). DNA was eluted in a final volume of 30 μl.

LigAmp Oligonucleotides and Probes

Ligation oligonucleotides for wild-type and mutant *KRAS2* and modified M13 forward and reverse primers (Table 1) were purchased from Invitrogen, Corp. (Carlsbad, CA). The downstream common oligonucleotides were phosphorylated at the 5′ end. The *LacZ* and *16S rDNA* Taqman probes containing different fluorophores and quenchers (Table 1) were purchased from Integrated DNA Technology (Coralville, IA).

LigAmp Analysis of KRAS2 Mutations

LigAmp analysis of *KRAS2* mutations has been previously described in detail.[13,14] First, a region of *KRAS2*

including *KRAS2* codon 12 (hot spot) was PCR amplified. For ligation, 1 pmol upstream mutant oligonucleotide, 1 fmol wild-type oligonucleotide, and I pmol downstream oligonucleotide were incubated with approximately 200 pg PCR amplified *KRAS2* DNA and 4 U *Pfu* DNA ligase in 1× *Pfu* Ligase buffer (Stratagene, La Jolla, CA). The ligation condition included denaturation at 95°C for 3 minutes, followed by 90 two-step cycles of 95°C for 30 seconds, alternating with 65°C for 4 minutes. For quantification of both wild-type and mutant *KRAS2* DNA in the serum samples, mutant *KRAS2* DNA was serially diluted into wild-type DNA (from 200 pg to 0.2 pg into 200 pg Hela DNA), the wild-type DNA was also serially diluted (2000 pg, 200 pg, and 20 pg). LigAmp analyses of mutant DNA mixtures and serial diluted wild-type DNA were used as standard quantitative controls.

Q-PCR amplification of ligated products was performed in a SmartCycler (Cepheid, Sunnyvale, CA). Each reaction (25 μl) contained 5 pmol forward and 5 pmol reverse M13 primers, 2 μl of the unpurified ligation reaction, 12.5 μl platinum Quantitative PCR SuperMix-UDG (Invitrogen), and 2.5 pmol *lacZ* and 16S rDNA probes. To simultaneously quantify mutant and wild-type *KRAS2*, we included both *lacZ* (for mutated *KRAS2*) and *16S rDNA* (for wild-type *KRAS2*) probes in the reaction. The PCR reaction included pre-incubation at 50°C for 2 minutes and 95°C for 2 minutes, followed by 50 two-step cycles of 95°C for 10 seconds alternating with 64°C for 20 seconds. For each experiment, a cycle threshold (Ct) was manually set to the middle of the linear range (log scale) of the growth curves.

BstN1 Restrict Digestion-PCR, TA Cloning, and DNA Sequencing

To eliminate wild-type and enrich mutant *KRAS2*, *KRAS2* DNA was first amplified using a forward mutant primer to produce a BstN1 site in only wild-type *KRAS2* PCR amplified products. The forward and reverse primers are 5′-AATATAAACTTGTGGTAGTTGGACCT-3′ and 5′-TCAAAGACAAGGCGATATGCT-3′ respectively, where an underlined base in the forward primer is the mutant base required to create a BstN1 site in wild-type *KRAS2* DNA. A 30-cycle PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) was performed, yielding a 1031 bp PCR product. A second BstN1 restriction site for both wild-type and mutant *KRAS2* PCR products was located 136 bases upstream of the reverse primer. Three fragments (136, 888, and 18 bp) for wild-type *KRAS2* and two (136 and 906 bp) for mutated *KRAS2* were produced after BstN1 enzyme digestion.

Following purification using a Qiagen PCR purification Kit, the PCR product (5 μl) was digested with BstN1 (20 units, New England Biolabs, Beverly, MA) at 65°C for 2 hours. The digestion reaction was analyzed by 2% agarose gel. The restriction digest product (1 μl) was then re-amplified for 35 cycles using the same forward primer and a second reverse primer (R: 5′-CCCTGACATACTCCCAAGGA-3′) located upstream of the second restriction site, and produced a 304 bp PCR product. Wild-type DNA from HeLa cells was used as a negative control. The BstN1-refractory PCR products were saved for TA cloning.

TA Cloning and Sequencing

Purified BstN1 refractory PCR products were cloned into pCRII-TOPO cloning vectors using the TOPO TA Cloning Kit, Version N (Invitrogen). PCR was performed on 20 white colonies using M13 forward and reverse primers. These PCR products were then subjected to BstN1 digestion. The BstN1-refractory PCR products were sequenced using a M13 forward primer, the BigDye Terminator 3.1 Cycle Sequencing Kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Results

LigAmp Analyses of KRAS2 Mutations in Bile Samples Obtained from Patients with Biliary Tract Carcinoma Versus Those with Benign Pancreatobiliary Diseases

We first analyzed *KRAS2* mutations in bile from 16 patients with biliary tract carcinoma, including 12 extrahepatic cholangiocarcinomas, three intrahepatic cholangiocarcinomas, and one carcinoma of the gallbladder. We had no obvious difficulty amplifying DNA isolated from bile, although bile is known to contain PCR inhibitors.[15] The amounts of mutant *KRAS2* DNA relative to wild-type *KRAS2* are shown in Figure 1A. We detected *KRAS2* mutations in 14 of 16 samples (87.5%), and multiple *KRAS2* mutations were present in 9 of these 14 positive samples. GAT mutation was the predominant variant in all these samples. The amounts of *KRAS2* mutations varied over a wide range, from less than 1% to over 90%. However, majority of the positive samples contained more than 2% of mutant DNA. Preliminary analysis comparing level of mutant KRAS2 with the size and stage of the tumor shows no obvious correlation, although a larger sample size would need to be analyzed for proper analysis. Initial analysis of the cases with multiple mutations versus those with single mutations revealed no obvious correlation to histological grade, size, or stage.

KRAS2 mutations were also evaluated in bile from patients with benign pancreatobiliary diseases ($n = 28$). *KRAS2* mutations were detected in nine cases (31.4%). However, the levels of the mutations in these positive samples were significantly lower than those from the cancer group, ranging from 0.01% to 3.6% (Figure 1B). Of these positive samples, only two cases had a mutation level of more than 2%. These two cases were obtained from one patient with serous cystadenoma and another with primary sclerosing cholangitis (PSC), respectively. Of note, PSC is the most common precursor lesion for cholangiocarcinoma in the United States,[16–19] so the presence of mutant *KRAS2* in this condition is somewhat expected.

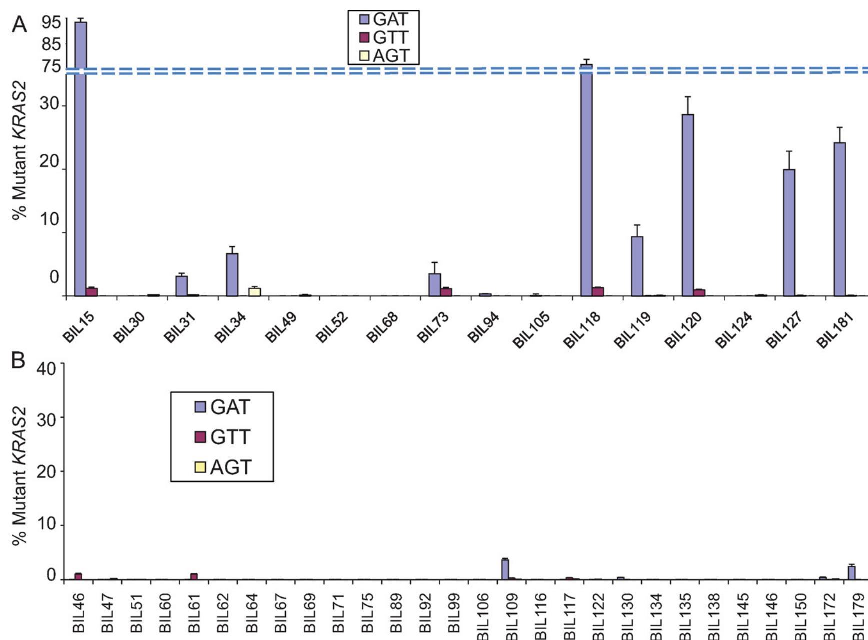

Figure 1. LigAmp quantification of *KRAS2* mutations in bile from patients with and without biliary tract carcinoma. **A:** Percentage of mutant *KRAS2* relative to wild-type *KRAS2* detected in bile from 16 patients with biliary tract carcinoma. LigAmp analysis of GAT (G12D), GTT (G12V), and AGT (G12S) *KRAS2* mutations shown as blue, red, and yellow bars, respectively. The mean data were obtained from four individual experiments. Percent mutant *KRAS2* = mutant *KRAS2*/(mutant *KRAS2*+wild-type *KRAS2*). The data are presented as mean ± SEM. **B:** Percentage of mutant *KRAS2* relative to wild-type *KRAS2* detected in serum from 28 patients with benign pancreatobiliary diseases. The data are presented as mean ± SEM from four independent experiments.

We compared *KRAS2* mutation levels between the benign and malignant groups. At a threshold of 2% mutational levels, 57% of the samples from the patients with biliary tract carcinomas were positive for *KRAS2* mutations, while only 7% of the benign samples are positive ($\chi^2 = 7.333$, $P < 0.01$). The *KRAS2* mutation levels in the cancer group was significantly higher than that in the benign group (16.61 ± 7.11% versus 0.24 ± 0.15%, $P < 0.01$). The median value was 5.1% in the cancer group versus 0.4% in the benign group.

LigAmp Analyses of KRAS2 Mutation in Serum from Patients with Biliary Tract Carcinoma Versus Those with Non-Neoplastic Diseases

We next investigated circulating *KRAS2* DNA status in serum from 11 patients with biliary tract carcinoma. LigAmp analysis revealed detectable mutant *KRAS2* DNA in 9 of 11 cases (81.8%) (Figure 2A). In three of these positive cases, multiple *KRAS2* mutations were detected. Additionally, as we observed in the bile samples, GAT *KRAS2* mutation was predominant in most cases. The relative amount of *KRAS2* mutations detected in the patients' serum ranged from 0.1% to 24% with a median value of 1.5%.

To rule out whether the detection of low levels of *KRAS2* mutations in the serum is caused by a nonspecific reaction, we further confirmed its existence by T-A cloning BstN1-refractory PCR products and DNA sequencing in two cases, designated as Bil100 and Bil166. Bil166 contained approximately 0.1% mutant *KRAS2*, which was the lowest level of mutant DNA detected among the *KRAS2* positive samples. Sequencing of cloned BstN1-refractory PCR products confirmed GAT *KRAS2* mutation in both Bil100 (data not shown) and Bil166 (Figure 2B).

Serum samples from nine patients with benign pancreatobiliary disorders were also analyzed as negative controls. Eight of nine samples were amplified by PCR. LigAmp analyses of these eight samples demonstrated wild-type *KRAS2* except one sample, which showed a minimal level of GAT mutation (0.03%). This experiment was repeated, however, in the second experiment, no GAT or other *KRAS2* mutations were detected.

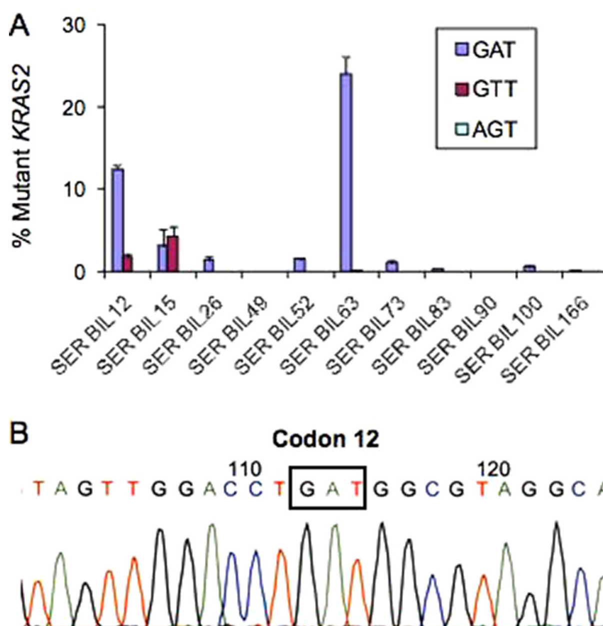

Figure 2. Detection of *KRAS2* mutations in serum from 11 patients with biliary tract carcinoma. **A:** The relative amount of mutant to wild-type *KRAS2* DNA detected by LigAmp in serum ($n = 11$). LigAmp analysis of GAT (G12D), GTT (G12V), and AGT (G12S) *KRAS2* mutations shown as blue, red and yellow bars, respectively. The mean data were obtained from four individual experiments. The date is presented as mean ± SEM. **B:** A representative *KRAS2* DNA sequence of a cloned BstN1 refractory PCR product from serum of Bile166. A GGT to GAT mutation at codon 12 is shown in the box.

Table 2. Correlation of *KRAS2* Mutation Status between Bile and Serum of Four Patients with Biliary Tract Carcinoma

	Location	Bile	Serum
Bil 15	Extrahepatic	GAT (93.4%) GTT (1.2%)	GAT (3.2%) GTT (4.3%)
Bil 49	Extrahepatic	AGT (<0.1%)	Wild type
Bil 52	Gallbladder	Wild type	GAT (1.2%)
Bil 73	Intrahepatic	GAT (3.5%) GTT (1.2%)	GAT (1.0%)

Correlation of KRAS2 *Mutation Status between Bile and Serum of Same Patients with Biliary Tract Carcinoma*

Four patients with cholangiocarcinoma had matched bile and serum samples available (Table 2). For case Bil15, GTT and GAT *KRAS2* were detected in bile, and both of these mutant variants were also present in serum. Bile from case Bil49 contained neither GAT nor GTT *KRAS2*, but a minimal level of AGT *KRAS2* (<0.1%). No mutant *KRAS2* DNA was detected in the corresponding serum. Bile *KRAS2* mutation was not identified in case Bil52, but the corresponding serum contained a GAT mutant (1.2%). In case Bil73, we identified two mutants, GAT and GTT in bile, whereas only the GAT *KRAS2* mutant was present in serum.

Discussion

In the present study, we analyzed mutant *KRAS2* genes by LigAmp assay in bile or serum from patients with biliary tract carcinoma, and compared this to corresponding clinical samples obtained from individuals with benign pancreatobiliary disorders. Our data showed the presence of mutant *KRAS2* in bile from majority of the cancer patients, and in a minor subset of benign pancreatobiliary diseases. However, the levels of *KRAS2* mutations in bile of the cancer group as compared with the benign group were significantly higher, consistent with our previous findings in pancreatic cancer.[14] As noted previously, one of the higher mutant *KRAS2* levels in the "benign" category was obtained from a patient with PSC, an established cholangiocarcinoma precursor lesion. In addition, we demonstrated that we were able to detect *KRAS2* mutations in serum from majority of the cancer patients. Detection of *KRAS2* mutations in bile and/or serum might serve as, at least, an important ancillary test for diagnosis of biliary tract carcinoma. In addition, this data indicates the possibility of being able to monitor therapeutic responses in patients with biliary tract carcinoma by quantitative analysis of *KRAS2* mutations in serum using LigAmp.

We detected multiple *KRAS2* mutations in bile and serum of most cases. Multiple mutations in a single tumor have been reported previously by several groups, including our own.[14,20,21] This could be attributed to either multiple independent clones existing in the cancer,[22] or in theory due to the presence of precursor lesion(s) with different *KRAS2* mutation(s).

When comparing the results from bile to those from serum of the same patients, we observed, in one case, a *KRAS2* mutation is present in the serum, but not in the bile. This can be explained by following mechanisms: 1) little if any tumor DNA shed into the bile because of its extremely scirrhous nature; 2) extraluminal development of cancer; or 3) obstruction of the bile duct or cystic duct in the case of gallbladder carcinoma. Indeed, this is a case with a gallbladder carcinoma, one would imagine, with a completely obstructed cystic duct, no tumor cells from the gallbladder could access to the bile duct. In another case, two mutations were detected in the bile, while only one mutation was present in the serum. The mutation present in both bile and serum could be derived from the cancer, whereas the mutation only present in bile could be in theory from a coexisting precursor lesion which harbored a different mutant from the cancer.

Several candidate molecular markers in serum have been described for detection of biliary tract carcinoma.[23,24] However, the role of these markers in early detection or diagnosis is uncertain. Currently, the most commonly used serum marker for biliary tract carcinoma is CA19-9. Serum CA19-9 has been shown to be increased in 77.9% of patients with biliary tract carcinoma, but it is also raised in patients with colorectal, gastric, and gynecological malignancies, as well as in other benign conditions such as cholangitis and cholestasis.[24,25] In patients with PSC, serum CA19-9 has an even lower sensitivity and specificity for diagnosis of cholangiocarcinoma. In addition, only a small portion of cholangiocarcinoma patients with increased serum CA19-9 are surgically resectable. However, there are some promising serum markers currently being studied, including tumor-associated antigen receptor-binding cancer antigen expressed on SiSo cells, the cytokeratin 19 fragment CYFRA 21-1, and MUC5AC.[24]

Detecting tumor markers in bile might be an alternative approach to finding biliary tract carcinoma at an early stage. Proteomic analyses of biliary tract carcinomas have identified overexpressed proteins in tumor cells,[24,26] and these can be shed from tumor tissues into bile during tumor cell death. Secreted proteins derived from biliary tract carcinoma should be at higher local concentrations in bile. Kristiansen et al have identified several malignancy-related proteins in bile from patients with biliary tract carcinoma using a liquid chromatography and tandem mass spectrometric approach.[27] The same group further demonstrated that one of the identified proteins, Mac-2-binding protein, is a promising diagnostic marker for patients with biliary carcinoma.[28]

Detection of mutated genes in bile might be another approach for early detection of biliary tract carcinoma. In the current study, *KRAS2* mutations were detected in bile from 87.5% patients with biliary tract carcinoma. Therefore, analysis of *KRAS2* mutations in bile could be a potential early detection technique. Sensitivity might be further increased by including additional lower frequency *KRAS2* mutations (G12C, G12R, G12A, and G13 mutations), which our assay is currently not designed to de-

tect. However, *KRAS2* mutations have been reported to be present in benign pancreatobiliary conditions too, such as chronic pancreatitis and primary biliary cirrhosis.[5,29,30] The mutant cells in these lesions could be shed into the fluid, and mutant DNA could be detected in pancreatic juice or bile. This is supported by our previous and current studies.[14] However, the mutation levels in these conditions were significantly lower than those detected in the patients with biliary tract carcinoma. In 28 controls of this study, only two bile samples contained more than 2% mutant *KRAS2*, one of them was from a patient with PSC. PSC is a major risk factor for biliary tract carcinoma (specifically, cholangiocarcinoma) in the west. An estimated 8% to 40% of patients with PSC develop cholangiocarcinoma. Kubicka and colleagues analyzed *KRAS2* mutations in bile from patients with PSC, and found that 30% of patients had *KRAS2* mutant DNA in their bile fluid.[31] In addition, the presence of *KRAS2* mutations in bile of these patients was associated with an increased risk for development of cholangiocarcinoma. Therefore, dynamic monitoring of *KRAS2* mutant DNA in bile fluid might be helpful for early detection of malignant transformation in patients with PSC.

LigAmp analysis of serum *KRAS2* mutations appears promising for diagnosis of biliary tract carcinoma. We were able to detect *KRAS2* mutations in serum of more than 80% of cancer patients. In addition, serum *KRAS2* mutations should be, in theory, more specific for cancerous diseases than that of other body fluids including bile because of a unique vascular invasive characteristic of malignant cells. Use of *KRAS2* testing in practice will ultimately require correlating *KRAS2* cutoff values below the size of cancers that we want to detect and above premalignant lesions that can sometimes harbor these mutations (eg, benign PanIN1 lesions in the pancreas). The patient will then need to be scanned to discover the source of mutant *KRAS2*, and if that is unsuccessful, may require endoscopic retrograde cholangiopancreatography sampling or even angiographic sampling of venous return from organs most likely to be the source. In addition, periodic analysis of serum *KRAS2* mutations in these patients could help monitor therapeutic responses to non-invasive modalities like systemic chemotherapy or radiation, where direct access to tissues is not available. Currently, the majority of patients with biliary tract carcinomas are diagnosed at an advanced stage and they are not considered as candidates for curative surgery. An effective monitoring system would greatly facilitate the measurement of patients' responses to existing, as well emerging, therapies. LigAmp may be ideal for this purpose because of its high sensitivity and accurate quantification.

In conclusion, while LigAmp analysis of *KRAS2* mutations in bile might be a sensitive assay for an early detection of biliary tract carcinoma, analysis of mutant *KRAS2* in serum could be more specific for cancer. By dynamic monitoring *KRAS2* mutations in serum of cancer patients, we may be able to use LigAmp to determine patients' response to anticancer treatment and detect minimal residual disease.

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