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Original research

Next-generation sequencing of bile cell-free DNA for the early detection of patients with malignant biliary strictures

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ABSTRACT

Objective Despite significant progresses in imaging and pathological evaluation, early differentiation between benign and malignant biliary strictures remains challenging. Endoscopic retrograde cholangiopancreatography (ERCP) is used to investigate biliary strictures, enabling the collection of bile. We tested the diagnostic potential of next-generation sequencing (NGS) mutational analysis of bile cell-free DNA (cfDNA).

Design A prospective cohort of patients with suspicious biliary strictures (n=68) was studied. The performance of initial pathological diagnosis was compared with that of the mutational analysis of bile cfDNA collected at the time of first ERCP using an NGS panel open to clinical laboratory implementation, the Oncomine Pan-Cancer Cell-Free assay.

Results An initial pathological diagnosis classified these strictures as of benign (n=26), indeterminate (n=9) or malignant (n=33) origin. Sensitivity and specificity of this diagnosis were 60% and 100%, respectively, as on follow-up 14 of the 26 and eight of the nine initially benign or indeterminate strictures resulted malignant. Sensitivity and specificity for malignancy of our NGS assay, herein named Bilemut, were 96.4% and 69.2%, respectively. Importantly, one of the four Bilemut false positives developed pancreatic cancer after extended follow-up. Remarkably, the sensitivity for malignancy of Bilemut was 100% in patients with an initial diagnosis of benign or indeterminate strictures. Analysis of 30 paired bile and tissue samples also demonstrated the superior performance of Bilemut.

Conclusion Implementation of Bilemut at the initial diagnostic stage for biliary strictures can significantly improve detection of malignancy, reduce delays in the clinical management of patients and assist in selecting patients for targeted therapies.

Significance of this study

What is already known on this subject?

- In spite of significant advances in imaging, endoscopic and pathological evaluation, distinguishing between benign and malignant biliary strictures remains a diagnostic challenge. This situation dramatically affects the identification, management and prognosis of patients with biliopancreatic tumours.
- Endoscopic retrograde cholangiopancreatography (ERCP) plays a key role in the evaluation of biliary strictures and enables the collection of bile samples.
- The genetic landscape of biliopancreatic tumours has been defined in recent years.

What are the new findings?

- We have selected a next-generation sequencing (NGS) panel open to clinical laboratory implementation and developed a mutational analysis of bile cell-free DNA (cfDNA) collected during ERCP, the Bilemut assay.
- Our results confirm the better performance of liquid biopsy strategies increasing the diagnosis sensibility and the number of mutations detected in bile compared with the corresponding paired tumours.
- We have tested the Bilemut assay in a prospectively collected cohort of bile cfDNA samples from patients undergoing first diagnostic ERCP. Our test alone was markedly superior to the initial pathological diagnosis, particularly for cases of strictures initially diagnosed as of benign or indeterminate origin.

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- Our findings highlight the remarkable diagnostic potential of bile cfDNA NGS-based analysis for patients undergoing ERCP. Implementation of Bilemut at the initial diagnostic stage for biliary strictures can significantly improve malignancy detection, reduce delays in clinical management of patients and assist in selecting patients for targeted therapies.

INTRODUCTION

The accurate aetiological diagnosis of biliary stenoses remains a clinical challenge. Strictures of the bile duct may have diverse origin,[1] and the discrimination between benign and malignant stenoses in their early stages has not been satisfactorily resolved yet.[2] Benign conditions include primary sclerosing cholangitis (PSC), chronic pancreatitis, choledocolithiasis and bile duct injury and infections, while malignant stenoses are mostly attributable to neoplasia arising from the biliary tree such as cholangiocarcinoma (CCA) or from the pancreas, like pancreatic ductal adenocarcinoma (PDAC).[2–5] CCAs and PDACs are very aggressive neoplasms, and therefore, their early diagnosis is essential for the application of potentially curative surgical procedures and/or pharmacological therapies.[5–7] Biliary cancers are technically difficult to biopsy,[7] and several multidisciplinary diagnostic tools are used to discriminate benign from malignant biliary strictures.[4 8] These include a range of non-invasive imaging techniques plus endoscopic retrograde cholangiopancreatography (ERCP). ERCP allows relief of biliary obstruction in patients with stenosis while providing high-resolution fluoroscopic images and tissue sampling by biliary brushings and endoluminal biopsies.[4] However, the sensitivity for detecting malignancy with ERCP, even when combined with brush cytology and fluorescent in situ hybridisation, plus the analysis of the circulating tumour biomarker carbohydrate antigen 19–9 (CA19-9), is still suboptimal, ranging from 14% to 60%.[4 9–11] Patients may often undergo repeated ERCP procedures, and critical therapeutic decisions can be delayed. Alternatively, a false diagnosis of malignant stricture may result in an unnecessary extensive surgery.[12] Therefore, the identification of robust markers that can allow early and reliable discrimination between benign and malignant biliary stenoses is very much needed.

Advances in the molecular characterisation of biliary tumour tissues have revealed their mutational landscape. In CCA, as well as in PDAC, next-generation sequencing (NGS) technologies have identified recurrent alterations in a relatively small number of oncogenes and tumour suppressor genes, including *TP53*, *KRAS*, *CDKN2A*, *SMAD4*, *PIK3CA*, *GNAS*, *ERBB2* and *FGFRs*, with some of these being amenable to pharmacological targeting.[7 13–20] A recent study demonstrated that a customised NGS analysis of ERCP-obtained bile duct biopsies improved the sensitivity of pathological evaluation in the detection of malignant strictures.[10]

Liquid biopsy strategies are actively pursued in all fields of oncology,[21 22] and the detection of mutations in blood cell-free DNA (cfDNA) holds promise for the diagnosis of patients with pancreatic and biliary carcinomas.[23 24] Of note, the ERCP procedure enables the collection of biliary fluid. The cfDNA isolated from bile may include DNA molecules originating from premalignant or malignant cells anywhere in the bile duct system. Therefore, cfDNA analysis may avoid the limited sensitivity of intraductal tissue brushings and biopsies and capture the genetic alterations found in biliopancreatic tumours.[10 25 26] Earlier studies evaluated the presence of specific *KRAS* and *TP53* mutations in bile DNA samples from patients with benign and malignant strictures.[27–30] These studies supported the potential of such analyses to improve diagnosis of malignant disease. Moreover, it was also realised that the detection of such mutations in bile samples from patients without malignancy could identify individuals with preneoplastic lesions and at risk of progressing to cancer.[28] However, the conventional sequencing technologies applied in these studies lacked the sensitivity of current amplification and sequencing tools, and the analyses were restricted to a few codons within *KRAS* and *TP53* genes, markedly reducing the overall performance of the assays.

In this prospective proof-of-concept pilot study, we evaluated if NGS mutational analysis of cfDNA in bile could represent a new type of liquid biopsy for the detection of malignancy. For a rapid translation of the expected results, we tested two commercially available NGS cancer gene panels open to clinical laboratory implementation.

MATERIALS AND METHODS

Patients

A cohort of 68 patients prescribed to undergo ERCP with a diagnosis of bile duct stenosis was prospectively accrued for the study from January 2017 to December 2020 at the Navarra University Hospital Complex, Pamplona, Spain. Their demographic and clinical characteristics are summarised in figure 1A. All patients were older than 18 years and provided written informed consent for the examination of their samples and the use of their clinical data. The initial diagnosis of the strictures at the time of bile sample collection was based on histological result from the first ERCP-bile duct brushing or biopsy, bile duct or pancreatic mass endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) or percutaneous needle biopsy. The selection of the diagnostic procedure was established according to patient and tumour characteristics and following standard clinical practice. Initial diagnosis of indeterminate stenosis was assigned when there was no sufficient sample to establish a diagnosis. The final clinical diagnosis was defined as in previous studies, based on histological evidence and clinical or radiographic follow-up.[4 8 10 31–34] We designated malignant stenosis when we found histological evidence of adenocarcinoma or clinical or radiographic malignancy progression after ≤12 months of follow-up or death determined to be due to a malignancy involving the bile duct. We designated benign stenosis based on the absence of clinical and radiographic progression or resolution of the bile duct stricture after ≤12 months follow-up or when surgically resected specimens confirmed benign cholangiopathy. In total, 50 ERCP-bile duct brushings, nine ERCP-bile duct biopsies, 19 EUS-FNA and three percutaneous needle biopsies were collected for pathological evaluation to define the initial diagnosis. Representative images of cytological preparations resulting in correctly diagnosed and misdiagnoses cases after follow-up are shown in online supplemental figure 1. In addition, 68 bile samples were collected. Diagnostic and clinical follow-up information for each patient is described in detail in online supplemental table 1. The mean time of follow-up was 15.5 months (range: 0–42 months). Informed consent was obtained from each patient. Patients and the public were not involved in the design, conduct, reporting or dissemination plans of this research. The study protocol was approved by the ethics committee of the Navarra

A

Characteristics of patients with biliary strictures (n=68)			
Gender n (%)	Female	27	(39.7)
	Male	41	(60.3)
Median age years (range)		72.5	(43-91)
Location of stricture n (%)	Intrahepatic	3	(4.4)
	Perihilar	17	(25)
	Distal	48	(70.6)
CA19-9 U/mL n (%)	<44	20	(30.3)
	≥44	46	(69.7)

B

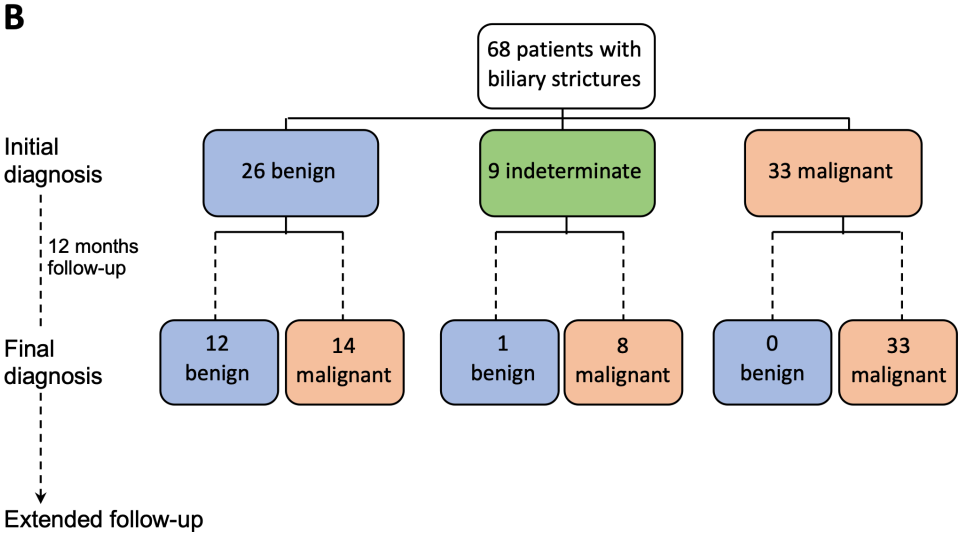

Figure 1 *Characteristics of the patients included in the study and their diagnosis*. (A) Demographic description, location of biliary strictures and serum carbohydrate antigen 19–9 (CA19-9) levels of patients included in this study at the time of initial diagnosis. (B) Flow chart indicating the initial and final clinical diagnosis of the patients.

University Hospital Complex, Pamplona, Spain (protocol number: 2016/91).

Additional details are provided as online supplemental material 1.

RESULTS

Diagnostic performance of conventional clinicopathological evaluation of patients with bile duct stenoses

A cohort of 68 patients undergoing ERCP for a bile duct stricture was prospectively accrued (figure 1A). At the time of the initial ERCP, EUS and pathological examination, patients were diagnosed and classified as having stenoses of benign (n=26), indeterminate (n=9) or malignant origin (n=33) (figure 1B). After a follow-up time of 12 months, the final clinical diagnosis resulted in 14 out of the 26 cases initially classified as benign resulting malignant and eight out of the nine indeterminate cases also being malignant, while the malignancy of the remaining 33 cases was confirmed (figure 1B and online supplemental table 1). Our initial pathological diagnosis was in agreement with the high specificity for detection of malignancy, 100% in our case, reported in current clinical practice.[10] Also in line with current standards, the sensitivity for malignancy in our initial clinical diagnosis was 60%.[1 4 8 10] The evaluation of CA19-9 serum levels at a threshold of >44 U/mL[10] yielded a sensitivity of 74% and specificity of 61% for detecting malignancy. These findings confirm the poor overall performance of the currently implemented clinicopathological evaluation of patients with biliary strictures in terms of sensitivity yield and further emphasise the need to develop complementary effective strategies.

Mutational analysis of bile cfDNA: methodological set-up and selection of best-performing NGS panel

As introduced above, we hypothesised that the evaluation of the mutational landscape of bile cfDNA might improve the limited sensitivity of the overall diagnostic procedure, including the pathological analysis of intraductal tissue brushing and biopsies, therefore better detecting the eventual presence of tumours. We extracted and purified cfDNA from 1 mL of bile aliquots, and for comparison, we also extracted cfDNA from the same volume of plasma. Remarkably, we found that the total amount of cfDNA in bile was about 20-fold of that obtained from the same volume of plasma (886.10±182.3 ng/mL *vs* 40.52±7.46 ng/mL, respectively, n=20). Most interestingly and consistent with a recent report that analysed bile cfDNA obtained by percutaneous transhepatic cholangial drainage,[35] we also observed that in contrast with cfDNA obtained from plasma, bile cfDNA was enriched in much larger DNA fragments. This can be appreciated in the electropherogram shown in figure 2A. Here, we provide direct confirmation of this difference by showing the PCR amplification of a large DNA fragment from a test gene (*TP53*) only in bile cfDNA, while a smaller DNA fragment from the same gene was amplified in cfDNA from both biofluids (figure 2B).

Among the commercially available NGS panels open to clinical laboratory implementation, the Oncomine Comprehensive Assay

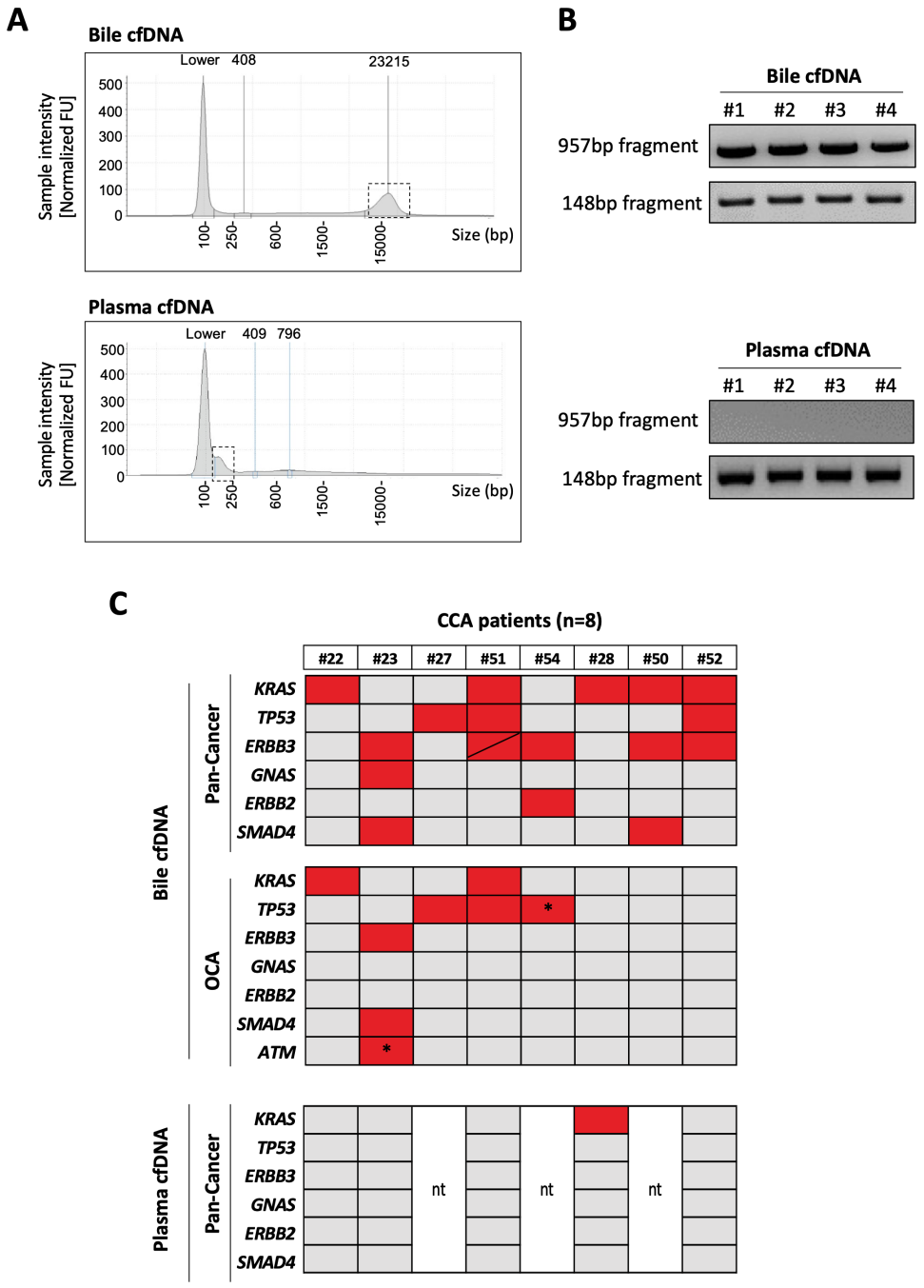

Figure 2 *Analysis of bile and plasma cell-free DNA (cfDNA) and comparison of Pan-Cancer and Oncomine Comprehensive Assay (OCA) panels' performance in bile cfDNA from patients with cholangiocarcinoma (CCA)*. (A). Representative electropherograms showing the size distribution of cfDNA obtained from human bile and plasma. (B) Representative images of agarose gels showing PCR-amplified fragments of *TP53* gene of high and low molecular weight from four independent samples of bile or plasma cfDNA. (C) Heat maps show the mutated genes detected by both panels in the same bile samples, and the mutations detected by the Pan-Cancer panel in plasma cfDNA. Asterisks indicate specific mutations that are not included in the Pan-Cancer panel. 'nt': not tested. BP, base pairs; FU, fluorescence units.

panel V.3 (OCA, Thermo Fisher) presents an extensive mutational coverage and works well with the relatively larger DNA fragments obtained from formalin-fixed paraffin-embedded (FFPE) tissues.[36] On the other hand, the Oncomine Pan-Cancer Cell-Free assay has been developed for the evaluation of the small and low abundant circulating cfDNA obtained from plasma[37] and also works well in other biological samples such as cerebrospinal fluid.[38] Therefore, we tested side by side the performance of the OCA and Pan-Cancer NGS panels in eight bile samples from patients with firmly established CCA diagnosis. As shown in figure 2C, the Pan-Cancer Cell-Free assay detected a total of 18 mutations in all eight cfDNA samples. However, the OCA panel, which has a threefold higher coverage for genetic alterations than the Pan-Cancer assay, failed to detect any mutation in three patients, identifying a total of eight mutations. There were two specific mutations, in *TP53* and *ATM* genes, marked with an asterisk in figure 2C, that were detected only with the OCA panel as they are not included in the Pan-Cancer panel design. As the Pan-Cancer panel is designed for the analysis of liquid biopsies, we also tested five plasma cfDNA samples from

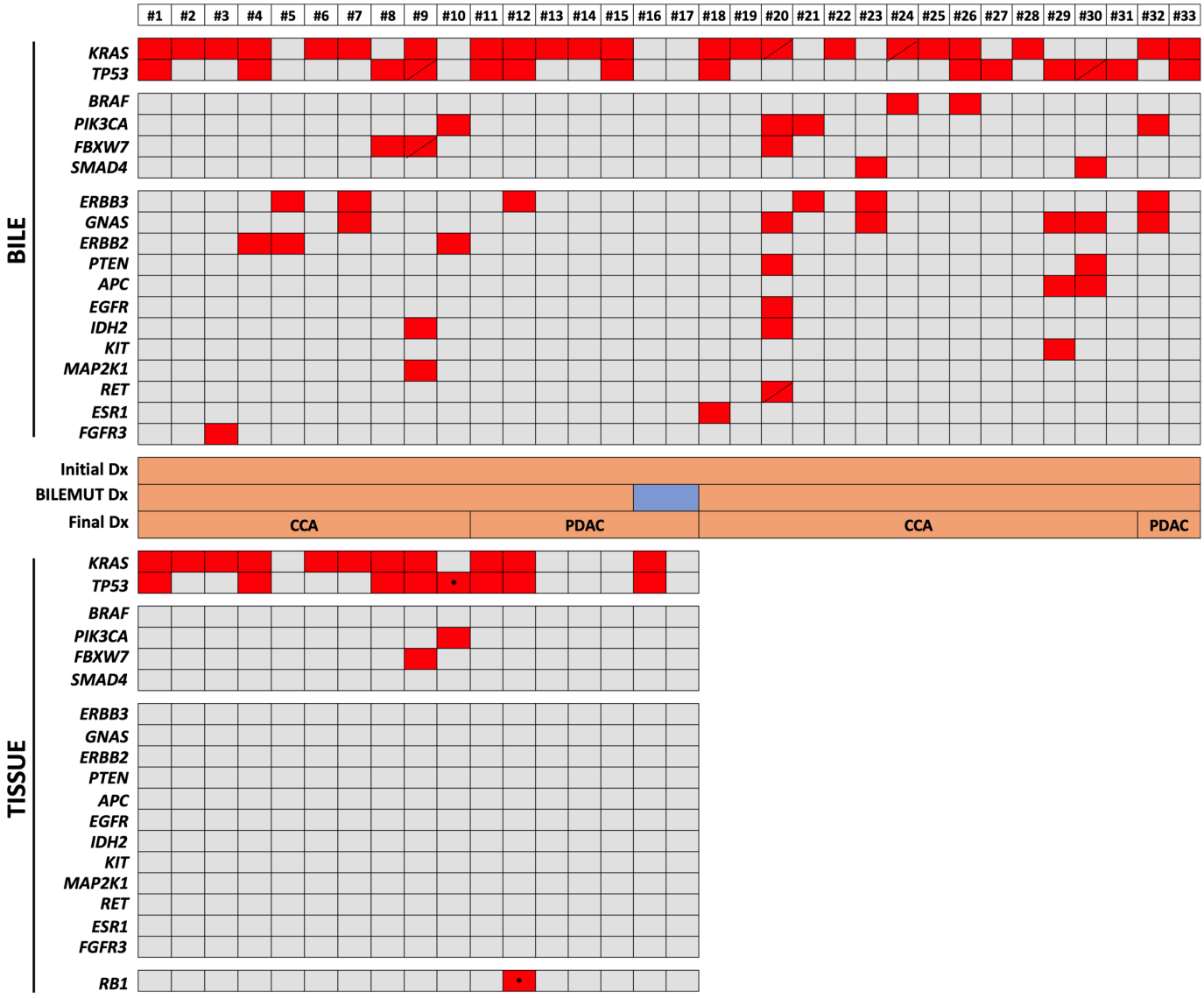

Figure 3 *Mutational profile of bile cell-free DNA (cfDNA) and paired tissue DNA samples from patients with malignant stenoses*. The heatmap in upper panel shows the mutations detected with the Pan-Cancer panel in bile cfDNA, and the heatmap in the lower panel shows mutations identified with the Oncomine Comprehensive Assay (OCA) panel in the available paired tissues. Asterisks indicate specific mutations that are not included in the Pan-Cancer panel. Diagonal lines indicate the detection of two different mutations in the corresponding gene. The initial diagnosis (Dx), Bilemut diagnosis and final clinical diagnosis, as well as the type of tumour, are indicated. CCA, cholangiocarcinoma; PDAC, pancreatic ductal adenocarcinoma.

this set of patients using this panel. As shown in figure 2C, only one of these samples tested positive in plasma, while mutations were found in all the paired bile samples. These observations indicated that the Pan-Cancer Cell-Free assay implemented in bile cfDNA samples, named here the Bilemut assay, has increased sensitivity for the detection of mutations and therefore was the optimal choice of our subsequent bile cfDNA screening.

Performance of the Bilemut assay in patients with malignant stenoses

We first assessed the performance of the Bilemut assay in the 33 bile samples collected at the initial ERCP from patients with a confirmed final clinical diagnosis of malignancy (24 CCAs and nine PDACs), from which 17 paired tumour tissue samples were also available. The Bilemut assay identified mutations in all but two bile samples (numbers 16 and 17) corresponding to patients diagnosed with PDAC (figure 3 upper panel). The OCA panel detected mutations in 12 of the 17 tumour tissues tested, with one CCA and four PDAC samples (including number 17) resulting negative (figure 3 lower panel). Mutations in *KRAS* and *TP53* genes were the most frequent alterations identified both in bile and tissues. However, the Bilemut assay captured mutations in a much wider range of genes, including *ERBB3*, *GNAS*, *FBXW7*, *ERBB2*, *IDH2*, *MAPK2K1* and *FGFR3*, across all samples compared with the OCA panel assay performed in tissues (figure 3). The sensitivity of the Bilemut assay in this set of samples was 93.9%. We also analysed plasma cfDNA from five patients diagnosed with PDAC (patient numbers 12–16). The Pan-Cancer panel did not detect mutations in any of them (data not shown). At this stage, we also tested six bile cfDNA samples collected from healthy living liver donors as negative controls for the Bilemut assay and did not find any genetic alterations (data not shown). Moreover, independent repeat analysis of two previously tested positive and negative bile cfDNA

samples yielded the same results, attesting to the reproducibility of the Bilemut assay (data not shown).

Performance of the Bilemut assay for the early diagnosis of patients with non-malignant bile duct strictures

Next, we implemented the Bilemut assay in the cohort of patients that received an initial diagnosis of stenosis of benign origin (figure 1B). Bile samples were collected at the time of first ERCP; cfDNA was analysed; and according to the absence or presence of any genetic alteration, patients were classified as having benign or malignant stenoses. For patients with an initial pathological diagnosis of benign stenosis (n=26), mutations were found in 18 of them. Therefore, considering the application of Bilemut assay at the moment of the initial diagnosis, of the 26 benign stenoses, 18 would be classified as malignant and eight as benign. On the other hand, the final clinical diagnosis resulted in 14 of the 26 patients having malignant stenoses. These results are graphically summarised in figure 4A. The Bilemut mutational landscape of all these patients is shown in figure 4B, which also indicates their classification into benign and malignant stenoses according to the final clinical diagnosis. In figure 4C, we show the timeline of the final clinical diagnosis of the 14 patients that ended up developing malignant stenoses. We were also able to analyse 10 paired tumour tissue samples. The results were similar to those shown in figure 3, with the Bilemut assay detecting a higher number of mutations than the OCA assay, which was negative for one tissue sample (number 53) (online supplemental figure 2A).

There were four patients with a final clinical diagnosis of benign stenosis that had mutations in their bile cfDNA and therefore would be considered as false-positive cases for the Bilemut assay. Importantly, extended follow-up of these patients beyond 1 year revealed that one of them was diagnosed with PDAC ten months later (patient number 45). This finding underscores the value of the information provided by Bilemut assay also for its purported false-positive cases.

Next, we implemented the Bilemut assay in bile samples from patients with an initial pathological diagnosis of stenosis of indeterminate origin (figure 1B). Of the nine patients studied, mutations were detected in eight of them. Importantly, the final clinical diagnosis resulted in these eight patients developing malignant stenoses, as graphically summarised in figure 5A. Their Bilemut mutational landscape is shown in figure 5B. No mutations were detected in the only patient that received a final diagnosis of benign stenosis (patient number 60). This patient remains tumour-free 22 months after the initial diagnosis and Bilemut assay. We were also able to analyse three paired tumour tissue samples. As observed before, the Bilemut assay identified a greater number of mutations than the OCA panel, which was negative for one tissue sample (number 67) (online supplemental figure 2B). The timeline of the final diagnosis of the eight patients that ended up having malignant stenoses is shown in figure 5C.

In the whole cohort of patients, the Bilemut assay had a sensitivity of 96.4% and a specificity of 69.2% for detecting malignancy. When considering patients with an initial diagnosis of stenosis of benign or indeterminate origin (n=35), the sensitivity of this assay for malignancy detection was 100%. In this same group of patients and considering the extended follow-up, elevated CA19-9 serum levels at a threshold of >44 U/mL[10] at the time of initial diagnosis yielded a sensitivity of 74% and a specificity of 50% for detection of malignancy, while Bilemut had a sensitivity and specificity of 100% and 80%, respectively.

Pathological molecular findings: mutational analyses

The most prevalent mutations identified with the Bilemut assay among positive bile samples were in the *KRAS* (71.9%), *TP53* (47.4%), *ERBB3* (22.8%), *GNAS* (15.8%), *ERBB2* (8.8%), *BRAF* (8.8%), *PIK3CA* (8.8%), *FBXW7* (7.0%) and *SMAD4* (7.0%) genes. The complete information of the different genes found in bile cfDNA is shown in online supplemental table 2. These data are in general agreement with the mutational landscape described for biliary and pancreatic tumours, considering the high proportion of CCAs of extrahepatic origin included in our cohort of patients.[13 23 39] When the 30 paired bile and tumour tissue samples were compared, a total of 66 mutations were detected by the Bilemut assay, while the OCA analysis of tissues only identified 43 alterations. There were seven tissue samples (23%) in which no mutations were found but that were positive in the Bilemut assay, while this test only missed one case that had mutations in its paired tissue. There were 32 mutations detected both in tissue and bile samples, while the Bilemut assay detected 34 additional mutations not found in the corresponding tissues. For *KRAS*, the most frequently mutated gene in our cohort of patients, 18 mutations were found in tissue samples, and all but two were also captured in bile cfDNA. However, bile cfDNA analysis identified five additional *KRAS* mutations that were not detected in paired tissues. For *TP53*, 13 mutations were found in tissue samples, two of them not analysed by the Bilemut assay and another not detected. However, four additional *TP53* mutations were found in bile cfDNA that were not detected in paired tissues. The concordance between the mutations detected in bile and tissue samples is described in online supplemental figure 2C. The identity of all mutations identified in bile cfDNA and tissue samples is provided in online supplemental table 3, and detailed information of the genes analysed by the Pan-Cancer and OCA panels is provided in online supplemental table 4.

The Pan-Cancer panel includes potentially actionable genetic alterations that are found in biliary and pancreatic malignancies, such as those involving the *FGFR* genes, *ERBB2* and *ERBB3*, *BRAF*, *IDH1* and *IDH2*, *PIK3CA*, *MET*, *RET* and *MAP2K1*[7 20 40]. Our study shows that mutations in potentially actionable genes were detected in 54% of samples that were positive for the Bilemut assay (online supplemental table 3).

DISCUSSION

In spite of recent and significant advancements in imaging, endoscopic and pathological approaches, the accurate aetiological diagnosis of biliary strictures is still far from satisfactory.[8 41] In this study, we demonstrate that the mutational analysis of bile cfDNA obtained at the first ERCP procedure in the evaluation of patients with suspected biliary strictures can transform the diagnostic pipeline. For a quick and widespread transfer to the clinic and after evaluating the concentrations and physical characteristics of cfDNA present in bile, we selected the Pan-Cancer Cell-Free assay, an NGS panel readily available for the clinical laboratory, which does not require specialised bioinformatic data interpretation.[37 38] With this assay, herein named the Bilemut assay, we first validated the high sensitivity for malignancy detection of bile cfDNA mutational analysis in a significant cohort of patients (n=33), which had a final clinical diagnosis of malignant strictures (75% of them were CCAs). Our findings are in agreement with recent reports that detected bile cfDNA mutations with high sensitivity in patients diagnosed with gall bladder cancer[35 42] and PDAC[43] or in two small groups of patients diagnosed with CCA (n=4 and 6).[35 43] We found that our NGS assay had a remarkably high sensitivity for detecting malignancy (96.4%) in comparison

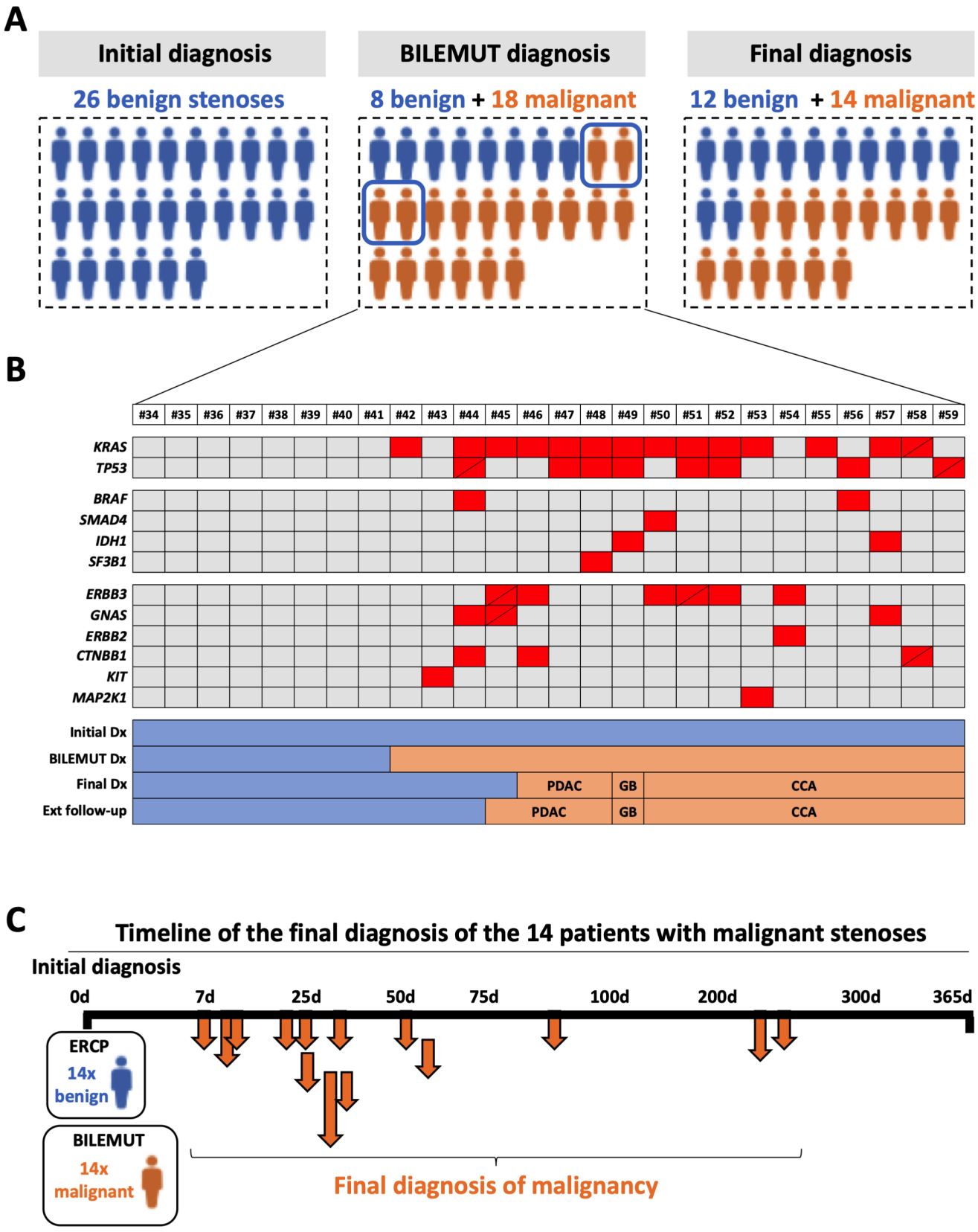

Figure 4 *Diagnostic performance of the Bilemut assay in patients with an initial diagnosis of benign stenosis*. (A) Schematic representation of the initial clinical diagnosis, the Bilemut assay diagnosis and the final clinical diagnosis of patients. The four Bilemut false-positive patients are encircled. (B) Heatmap showing the mutational profile of bile cell-free DNA (cfDNA), Bilemut assay, at the time of initial diagnosis. Diagonal lines indicate the detection of two different mutations in the corresponding gene. The initial diagnosis (Dx), Bilemut diagnosis, final diagnosis and extended follow-up diagnosis are indicated. The type of tumour diagnosed (pancreatic ductal adenocarcinoma (PDAC), cholangiocarcinoma (CCA) or gall bladder (GB)) is also indicated. (C). Chronology of malignancy detection during follow-up of patients with an initial diagnosis of benign stenosis. ERCP, endoscopic retrograde cholangiopancreatography.

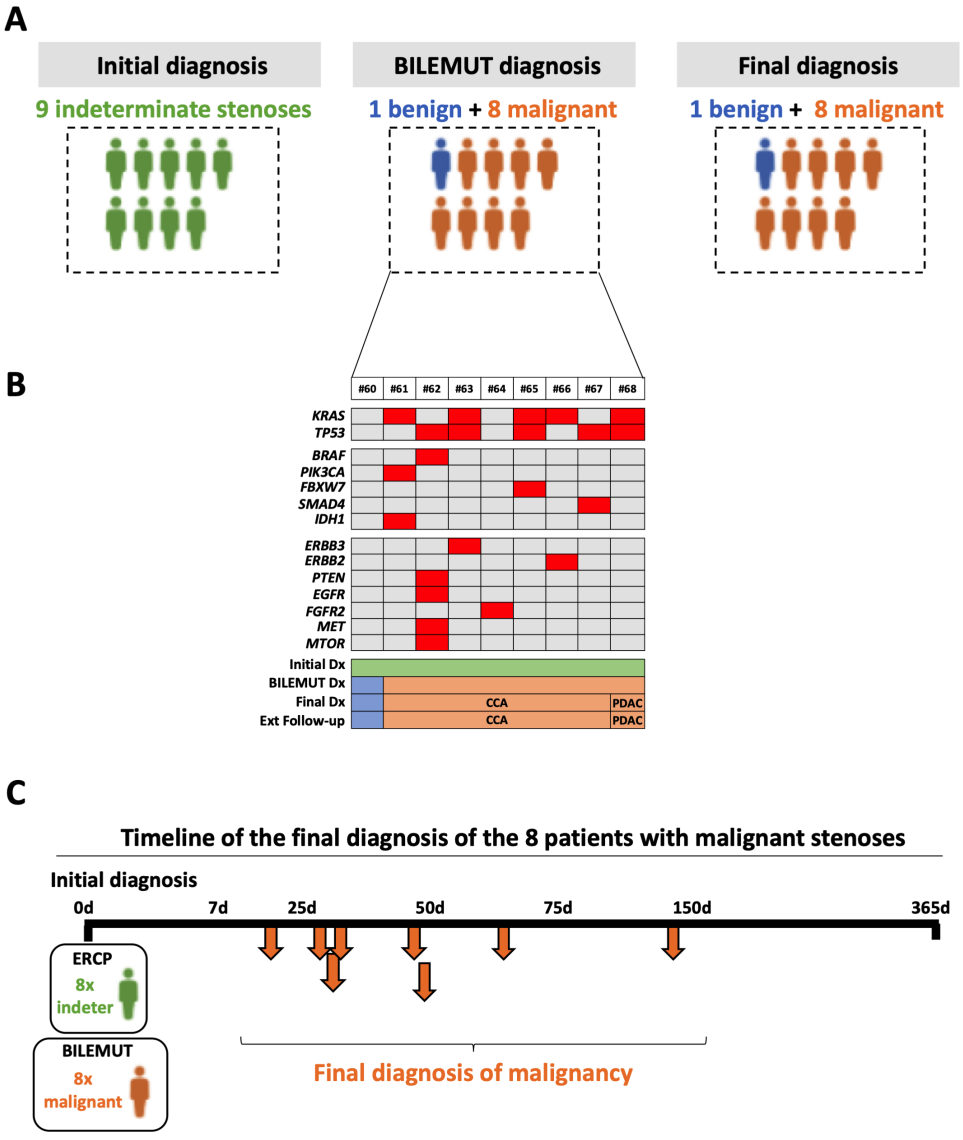

Figure 5 *Diagnostic performance of the Bilemut assay in patients with an initial diagnosis of indeterminate stenosis*. (A) Schematic representation of the initial clinical diagnosis, the Bilemut assay diagnosis and the final clinical diagnosis of patients. (B) Heatmap showing the mutational profile of bile cell-free DNA, Bilemut assay, at the time of initial diagnosis. The initial diagnosis (Dx), Bilemut diagnosis, final diagnosis and extended follow-up diagnosis are indicated. The type of tumour diagnosed (pancreatic ductal adenocarcinoma (PDAC) or cholangiocarcinoma (CCA)) is also indicated. (C) Chronology of malignancy detection during follow-up of patients with an initial diagnosis of indeterminate stenosis. ERCP, endoscopic retrograde cholangiopancreatography.

with that of the initial diagnosis (60%). Most importantly, our study demonstrated that the implementation of the Bilemut assay can be of particular value for patients that receive an initial diagnosis of stenosis of benign or indeterminate origin, in which this assay demonstrated a 100% sensitivity, considerably advancing in time the diagnosis of malignancy. A recent prospective study evaluated the performance of an NGS panel in DNA obtained from bile duct brushings and biopsies collected during ERCP for the detection of malignancy in patients with biliary stenosis.[10] Although this approach demonstrated an improved sensitivity over clinicopathological evaluation (73% *vs* 48%), the authors still reported a 25% of false-negative cases. The reason for this limited performance was attributed to inadequate sampling of strictures and/or low specimen tumour cellularity.[10] This relatively high failure to detect mutations in bile duct brushings and biopsies is consistent with previous works that performed NGS analyses on tissue samples from biliary cancers (see, for instance, [44]) and with our current study in which 23% of tumour tissues were negative for mutations. As we postulate here, this limitation can be circumvented by the analysis of bile cfDNA, since this fluid may contain genetic material released from tumorous cells anywhere along the biliary tract. The high sensitivity of the Bilemut assay also suggests that in patients undergoing ERCP, this diagnostic approach could be more informative than the mutational analysis of plasma cfDNA, given the known limited performance of the latter in the setting of early-stage disease,[22 45] and our present observations.

One apparent shortcoming of our study was the detection of mutations in bile cfDNA from patients that received a final diagnosis of benign disease, thus lowering the specificity of the assay. However, this issue should be interpreted with caution. An earlier work that evaluated the presence of *KRAS* mutations in free cells present in bile from patients with PSC showed that on follow-up, only those individuals having *KRAS* mutations developed CCAs

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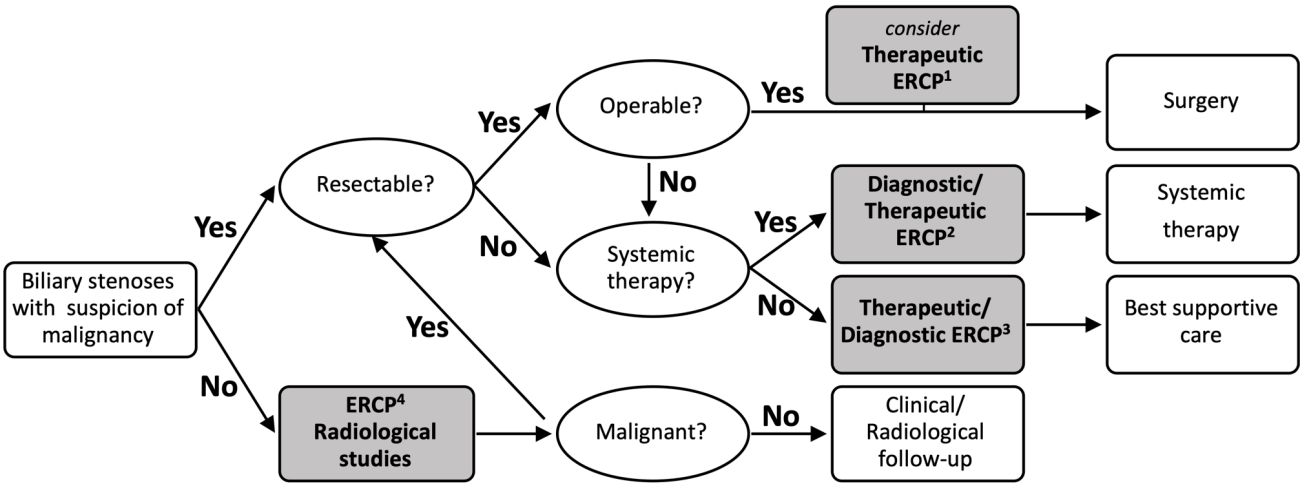

B

Advantages and limitations of Bilemut assay

Advantages

- It does not entail any additional risk for patients undergoing ERCP.
- It is based on an NGS platform open to clinical laboratory implementation.
- Its high sensitivity for malignancy can hasten diagnosis, avoiding additional and unnecessary diagnostic interventions and their associated complications.
- It provides more comprehensive mutational information than tissue analysis.
- It may guide patient selection for targeted therapies, particularly in unoperable cases needing systemic treatment from which no tissue is available for mutational profiling.

Limitations

- It can be only applied to patients undergoing ERCP or other diagnostic procedures in which bile can be obtained.
- The mutation analysis is limited to a defined panel of genes.
- False positives may occur, although these should be interpreted with caution.

Figure 6 (**A**) Implementation of the Bilemut assay in an algorithm for the management of patients with biliary stenosis. The four steps in which Bilemut could be applied are indicated by grey boxes. See text for details. This algorithm is based on the National Comprehensive Cancer Network guidelines. (B) Summary of the advantages and limitations of the Bilemut assay. ERCP, endoscopic retrograde cholangiopancreatography; NGS, next-generation sequencing.

or dysplasias.[28] These findings suggest that the appearance of mutations in bile cfDNA could indicate the existence of precancerous lesions or very early tumour stages, somewhere in the biliary tree. In fact, in our study, one of the four false-positive cases ended up developing PDAC when followed-up beyond the time established for the final clinical diagnosis. Therefore, those patients with a positive Bilemut assay and a final clinical diagnosis of benign disease may indeed benefit from closer follow-up surveillance.

Besides early cancer detection, tumour genotyping can detect the presence of mutations amenable to targeted therapies, which also occur in biliary and pancreatic malignancies.[7 18–20 40 46 47] Analysis of bile cfDNA also provides such information and, as previously discussed, even with better sensitivity than tumour tissue genomic profiling.

We acknowledge that the performance of the Bilemut assay for early detection of malignancy needs to be validated in an independent cohort of patients. Ideally, future studies should include patients with PSC who are at high risk of CCA development,[48] as well as patients with CCA, that were underrepresented in our cohort.

Nevertheless, in view of the extraordinary sensitivity of the Bilemut assay, we strongly believe that its implementation may leverage the diagnosis and management of patients with biliary stenosis and suspicion of malignancy. In figure 6A, we propose how Bilemut could be applied within an algorithm for the

management of these patients. For those patients with suspicion of malignant stenosis, susceptible of surgical resection and who need preoperative ERCP biliary drainage (case 1), Bilemut could improve presurgical diagnosis and prevent unnecessary surgery in cases of benign aetiology.[1 12] In fact, had Bilemut been applied in these patients, 100% of them would have gone to surgery with a confirmed diagnosis of malignancy, while that only occurred in 48% of such cases. For those patients not amenable to surgery that require systemic treatment (case 2), Bilemut could confirm the pathological diagnosis, avoid the need of additional diagnostic tests and interventions and also identify mutations to guide targeted therapies. In patients that are not amenable to surgical or systemic therapy and that need biliary drainage (case 3), Bilemut could also confirm the diagnosis of malignancy and thus inform on patients' prognosis. With the application of Bilemut, 100% of these patients would have received best supportive care with a confirmed diagnosis of malignancy, while this only occurred in 63% of these cases. Finally, in those patients with low suspicion of malignancy (case 4), Bilemut could complement the pathological diagnosis and reduce follow-up time and the number of tests performed. Moreover, as mentioned above, patients with a positive Bilemut that remain free of cancer on follow-up might still benefit from a closer clinical surveillance. Lastly, from a different perspective, although the costs of an NGS analysis such as Bilemut are still high, its application may avoid the need for repeat diagnostic procedures and other medical expenses, finally resulting in a positive benefit–cost ratio. The advantages and limitations of the Bilemut assay are summarised in figure 6B.

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Supplementary Fig. S1. A. Representative image of a preparation from a cytological brushing with negative diagnosis for malignancy (Papanicolaou stain, 400x). This patient developed PDAC. **B**. Representative image of a preparation from a cytological brushing with positive diagnosis for malignancy (Romanowsky stain, 400x). This patient developed CCA.

Supplementary Fig. S2. A. Heatmaps showing the mutational profile of paired bile cfDNA and tissue DNA samples from patients with an initial diagnosis of benign stenosis that ended up developing malignancy analyzed with the Pan-Cancer and OCA panels, respectively. Initial diagnosis (Dx), Bilemut diagnosis and final diagnosis are indicated. Asterisks indicate specific mutations that are not included in the Pan-Cancer Panel. **B**. Heatmaps showing the mutational profile of paired bile cfDNA and tissue DNA samples from patients with an initial diagnosis of stenosis of indeterminate origin that ended up developing malignancy analyzed with the Pan-Cancer and OCA panels, respectively. Initial diagnosis (Dx), Bilemut diagnosis and final diagnosis are indicated. Asterisks indicate specific mutations that are not included in the Pan-Cancer Panel. **C**. Analysis of the concordance of mutations detected in the paired bile and tissue samples included in this study.

Supplementary Materials and Methods

Bile collection and DNA extraction

Patients were fasted overnight and ERCPs were conducted by highly experienced endoscopists. During standard ERCP procedure, after cannulation of the bile duct, and in most cases before contrast injection (Omnipaque, iohexol), a bile sample of 2 to 6 ml from each patient was aspirated through the sphincterotome as we previously described [1].

A second group of patients (n=6) included healthy living liver donors from which gallbladder bile was collected at the time of surgery. These samples were collected at the Department of General, Transplant and Liver Surgery, Medical University of Warsaw, Warsaw, Poland, with the approval of the Institutional Ethics Committee (protocol# KB/49/2015). Informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

After collection, bile samples were maintained at 4°C, centrifuged for 10 min (4°C) at 3,500 g and stored in aliquots at -80°C in biobank facilities. All the process was performed in less than 2 hours. Prior to cfDNA isolation, bile was slowly thawed at 4°C and centrifuged for 10 min (4°C) at 13,000 g to ensure removal of impurities in the supernatant. Bile and, when available, plasma cfDNA were extracted with the Maxwell® RSC Automated cfDNA Plasma Kit (Promega, Madison, WI) using the Maxwell® Nucleic Acid Purification Instrument (Promega) according to the manufacturer's instructions. cfDNA was quantitated with the QuantiFluor® dsDNA Sample Kit (Promega) and cfDNA size distributions were analysed by Agilent 2100 Bioanalyzer (Agilent

Arechederra M, *et al. Gut* 2021;0:1–11. doi: 10.1136/gutjnl-2021-325178

Technologies, Santa Clara, CA). PCRs were performed to amplify different size fragments of the *TP53* gene in bile and plasma cfDNA. A 148bp fragment was amplified with primers located at exon 6 (forward: 5´-TGGGCCTGTGTTATCTCCTA-3'; reverse: 5'-GGCAAGTGGCTCCTGACCT-3') whereas a 957bp fragment was amplified with primers located at exon 5 (forward:5'-CCGCGCCATGGCCATCTACAAG-3') and exon 7 (reverse: 5'-GAGTCTTCCAGTGTGATGATGG-3') as we previously described [2].

For 30 patients paired bile and tumor tissue samples were available. Genomic DNA extracted from formalin-fixed and paraffin-embedded (FFPE) CCA and PDAC tissues, three pooled 5μm slices per sample, using the Maxwell® RSC DNA FFPE kit from Promega. All samples included in the study had an estimated tumor tissue content ≥ 40% based on histopathologic assessment.

Next Generation Sequencing DNA analyses

Coded bile and plasma cfDNA samples were blindly tested with the Oncomine™ Pan-Cancer Cell-Free Assay following the manufacturer's instructions (PanCancer, Thermo Fisher Scientific, Waltham, MA)[3]. This panel includes 52 genes enabling hotspot single nucleotide variation (SNV) and short indel as well as copy number variation (CNV) detection in key genes frequently mutated in multiple cancer types, including pancreatobiliary cancers. Four PanCancer libraries were manually prepared prior to be sequenced within an Ion 540 Chip in the Ion S5 system. Using an input of 50 ng of cfDNA, tagging individual DNA fragments with short random oligonucleotides called unique molecular identifiers and defining 2 as the minimum number of variants supporting functional families, this assay allowed variant detection as low as 0.02% given that on average ~8,000-10,000 functional families were identified

by sequenced base. A mutant allele frequency (MAF) value ≥ 0.15% was defined as presence of mutation.

cfDNA extracted from bile samples and DNA extracted from FFPE CCA tissues was analyzed using the Oncomine™ Comprehensive Assay panel v3 (OCA, Thermo Fisher), designed for FFPE tissue samples, following manufacturer's instructions. The OCA panel covers 161 genes enabling hotspot SNV and indel as well as CNV and fusion detection in cancer driver genes, also with extensive coverage of genes altered in pancreatobiliary cancers. Eight OCA libraries were automatically prepared by the Ion Chef Instrument prior to be sequenced within an Ion 540 Chip in the Ion S5 System. This assay allowed variant allele detection as low as 5% given that on average, 2000x sequencing depth was obtained per base (only variants with >500x were interpreted).

Statistical analyses

The sample size was determined according to previous studies addressing the same diagnostic issues [4]. Sensitivity and specificity were calculated using standard 2x2 contingency tables, essentially as described in previous similar studies [4].

Arechederra M, *et al. Gut* 2021;0:1–11. doi: 10.1136/gutjnl-2021-325178

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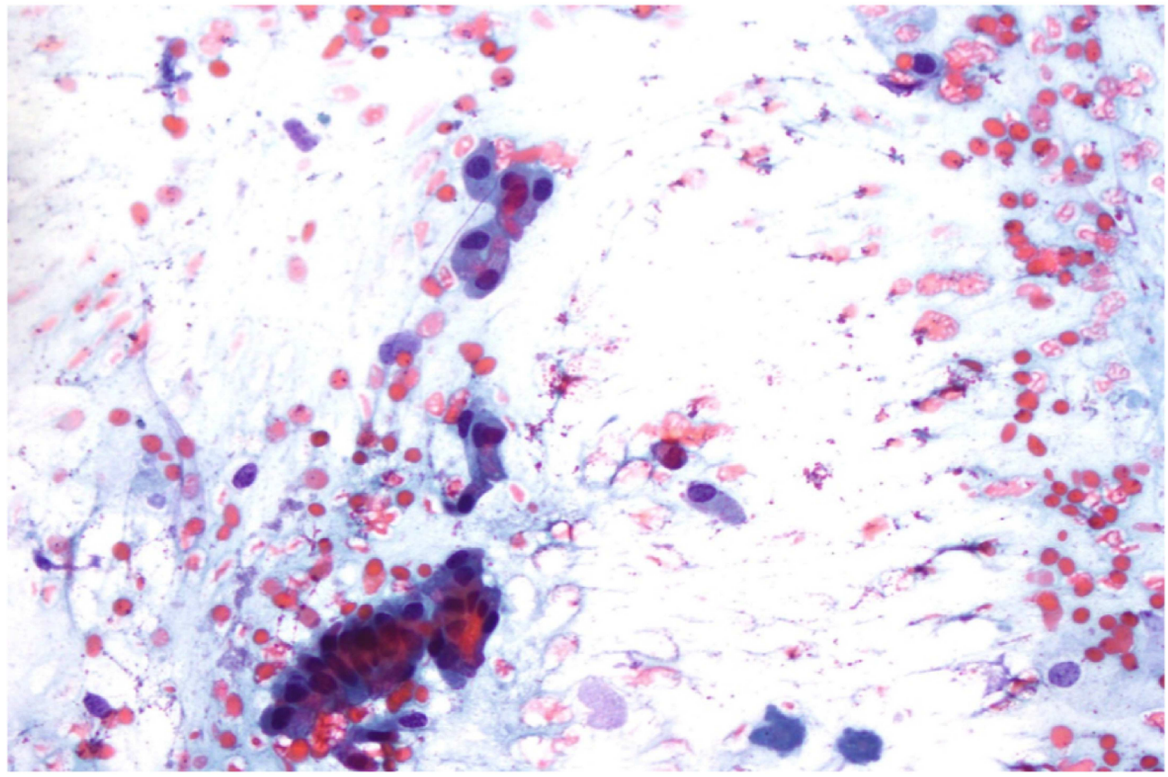
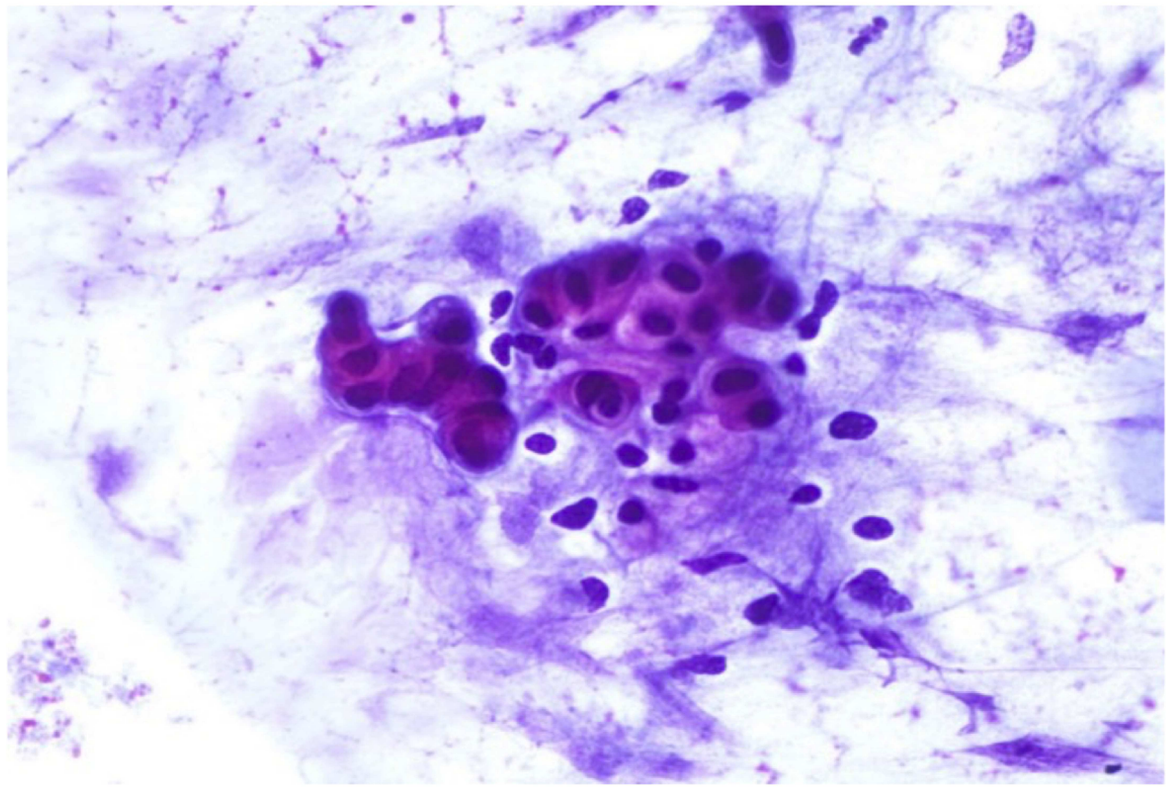
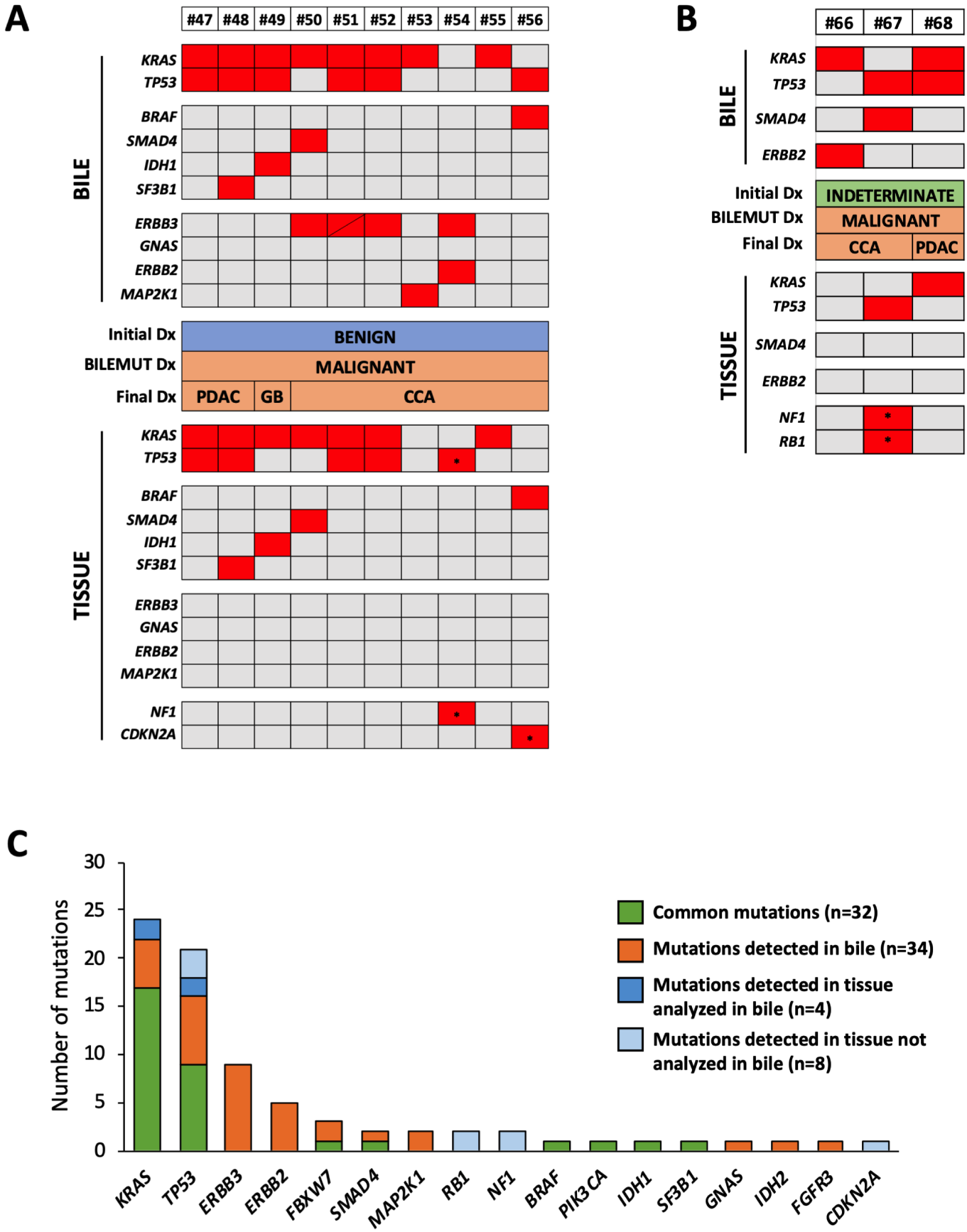
A



B



Supplementary Figure S1



Supplementary Figure S2

Supplementary Table S1. Detailed clinical, laboratory, pathologic and follow-up characteristics of the 68 patients submitted to Bilemut assay.

Patient ID	Gender	Age (years)	Serum CA 19-9 (U/ml)	Serum CEA (U/ml)	Location of stricture	Malignant* pathology of ERCP-bile duct brushing or biopsy/ EUS-FNA bile duct mass or pancreatic mass or percutnaeous biopsy	Initial diagnosis after first ERCP/EUS-FNA	Final diagnosis	Method of follow-up diagnosis	Diagnostic tests done to reach final diagnosis	Days of follow-up to reach final diagnosis	Status, Months of Follow-up
#1	Male	71	1692	91	Distal	Positive/Positive	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		3	Deceased, 18
#2	Male	68	2	3	Distal	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		4	Alive, 42
#3	Female	77	11	2	Distal	NS/Positive	Malignant	Cholangiocarcinoma	Bile duct mass EUS-FNA		0	Alive, 40
#4	Male	61	827	14	Intrahepatic	NS/Positive	Malignant	Cholangiocarcinoma	Bile duct mass EUS-FNA		4	Deceased, 6
#5	Male	70	41	2	Distal	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		2	Alive, 36
#6	Female	72	27	4	Distal	Positive/Positive	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		3	Deceased, 13
#7	Female	67	100	1	Distal	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		7	Deceased, 11
#8	Male	56	390	2	Distal	NS/Positive	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		1	Alive, 18
#9	Male	78	4	0	Distal	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		1	Alive, 10
#10	Male	73	373	2	Perihilar	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		0	Alive, 7
#11	Female	50	1061	1	Distal	Positive/Positive	Malignant	Pancreatic Ductal Adenocarcinoma	Pancreatic mass EUS-FNA		5	Alive, 18
#12	Female	59	2	4	Distal	NS/Positive	Malignant	Pancreatic Ductal Adenocarcinoma	Pancreatic mass EUS-FNA		5	Alive, 17
#13	Male	63	419	5	Distal	NS/Positive	Malignant	Pancreatic Ductal Adenocarcinoma	Pancreatic mass EUS-FNA		1	Deceased, 6
#14	Female	57	318	5	Distal	NS/Positive	Malignant	Pancreatic Ductal Adenocarcinoma	Pancreatic mass EUS-FNA		1	Deceased, 32
#15	Male	78	368	9	Distal	NS/Positive	Malignant	Pancreatic Ductal Adenocarcinoma	Pancreatic mass EUS-FNA		1	Deceased, 4
#16	Male	43	575	2	Distal	NS/Positive	Malignant	Pancreatic Ductal Adenocarcinoma	Pancreatic mass EUS-FNA		10	Deceased, 9
#17	Male	66	88	N/A	Distal	Negative/Positive	Malignant	Pancreatic Ductal Adenocarcinoma	Pancreatic mass EUS-FNA		0	Deceased, 16
#18	Male	62	6	2	Distal	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		4	Deceased, 10
#19	Male	56	1478	35	Perihilar	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		5	Deceased, 15
#20	Female	85	12000	176	Perihilar	Positive/Positive	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		4	Deceased, 2
#21	Male	84	12000	3	Distal	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		2	Deceased, 16
#22	Male	84	66	2	Distal	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		6	Lost, 1
#23	Male	81	88	3	Perihilar	NS/Positive	Malignant	Cholangiocarcinoma	Bile duct mass EUS-FNA		3	Deceased, 1
#24	Female	91	12000	24	Perihilar	NS/NS	Malignant	Cholangiocarcinoma	Clinicoradiographic impression		1	Deceased, <1
#25	Male	57	2	27	Perihilar	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		3	Deceased, <1
#26	Female	88	18015	13	Perihilar	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		2	Deceased, 1
#27	Female	77	11	2	Distal	NS/Positive	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		7	Deceased, <1
#28	Male	83	12000	4	Intrahepatic	NS/Positive	Malignant	Cholangiocarcinoma	Bile duct mass percutaneous biopsy		3	Deceased, 3
#29	Female	78	1347	3	Distal	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		6	Deceased, 12
#30	Male	60	243590	288	Perihilar	NS/Positive	Malignant	Cholangiocarcinoma	Bile duct mass percutaneous biopsy		0	Deceased, 1
#31	Female	82	112	8	Perihilar	Positive/NS	Malignant	Cholangiocarcinoma	ERCP bile duct pathology brushing or biopsy		2	Deceased, 1
#32	Male	74	1433	6	Distal	NS/Positive	Malignant	Pancreatic Ductal Adenocarcinoma	Pancreatic mass EUS-FNA		4	Alive, 33
#33	Male	66	12000	2	Distal	Positive/NS	Malignant	Pancreatic Ductal Adenocarcinoma	ERCP bile duct pathology brushing or biopsy		4	Alive, 4
#34	Male	57	12000	1	Perihilar	Negative/NS	Benign	Benign bile duct stricture	Resection		45	Deceased, 31
#35	Male	91	194	N/A	Distal	Negative/NS	Benign	Benign bile duct stricture	Clinicoradiographic impression		365	Alive, 31
#36	Female	74	N/A	N/A	Distal	Negative/NS	Benign	Benign bile duct stricture	Clinicoradiographic impression	2 ERCP	365	Alive, 28
#37	Male	59	35	5	Distal	Negative/NS	Benign	Benign bile duct stricture	Clinicoradiographic impression	2 CT	365	Alive, 38
#38	Male	66	61	N/A	Distal	Negative/Negative	Benign	Benign bile duct stricture	Clinicoradiographic impression	5 ERCP, 1 US and 1 CT	365	Alive, 37
#39	Male	80	231	2	Distal	Negative/NS	Benign	Benign bile duct stricture	Clinicoradiographic impression	1 ERCP	365	Alive, 25
#40	Male	66	684	N/A	Distal	Negative/NS	Benign	Benign bile duct stricture	Clinicoradiographic impression	1 EUS, ERCP and 2 CT	366	Alive, 20
#41	Female	66	10	N/A	Distal	Negative/NS	Benign	Benign bile duct stricture	Resection		27	Alive, 8
#42	Male	49	N/A	N/A	Distal	Negative/NS	Benign	Benign bile duct stricture	Clinicoradiographic impression	2 ERCP and 1 MR	365	Alive, 34
#43	Male	74	6	4	Distal	Negative/NS	Benign	Benign bile duct stricture	Clinicoradiographic impression	2 ERCP and 1 EUS	365	Alive, 25
#44	Female	87	15	8	Distal	Negative/NS	Benign	Benign bile duct stricture	Clinicoradiographic impression	2 ERCP	366	Alive, 19
#45	Female	68	26	1	Distal	Negative/Negative	Benign	Benign bile duct stricture	Clinicoradiographic impression	4 ERCP, 2 EUS, 4 CT and 2 MR	365	Alive, 31
#46	Female	56	617	9	Distal	NS/Negative	Benign	Pancreatic Ductal Adenocarcinoma	Subsequent pancreatic mass EUS-FNA	1 EUS-FNA, 1 CT and 1 MR	207	Deceased, 10
#47	Male	77	141	3	Distal	Negative/NS	Benign	Pancreatic Ductal Adenocarcinoma	Resection		12	Alive, 22
#48	Female	68	251	2	Distal	Negative/NS	Benign	Pancreatic Ductal Adenocarcinoma	Resection		23	Alive, 4
#49	Male	80	125	2	Perihilar	Negative/NS	Benign	Gallbladder cancer	Resection		16	Alive, 3
#50	Female	81	2	12	Distal	Negative/NS	Benign	Cholangiocarcinoma	Resection	1 ERCP and EUS	34	Deceased, 20
#51	Female	61	550	6	Distal	Negative/NS	Benign	Cholangiocarcinoma	Resection	1 ERCP	37	Alive, 42
#52	Male	48	483	7	Distal	Negative/NS	Benign	Cholangiocarcinoma	Resection		28	Deceased, 32
#53	Male	66	39	5	Perihilar	Negative/NS	Benign	Cholangiocarcinoma	Resection		83	Deceased, 36
#54	Female	81	35	2	Distal	Negative/NS	Benign	Cholangiocarcinoma	Resection		36	Deceased, 38
#55	Male	73	97	3	Distal	Negative/NS	Benign	Cholangiocarcinoma	Resection		28	Alive, 15
#56	Male	77	4650	1	Distal	Negative/NS	Benign	Cholangiocarcinoma	Resection	2 ERCP	57	Alive, 4
#57	Female	72	365	1	Distal	Negative/NS	Benign	Cholangiocarcinoma	Subsequent bile duct mass percutaneous biopsy	1 US and Percutaneous biopsy	10	Deceased, 5
#58	Female	84	878	6	Perihilar	Negative/NS	Benign	Cholangiocarcinoma	Clinicoradiographic impression		260	Deceased, 16
#59	Male	87	2	8	Perihilar	Negative/NS	Benign	Cholangiocarcinoma	Clinicoradiographic impression		50	Deceased, 1
#60	Female	87	2	N/A	Distal	NS/NS	Indeterminate	Benign bile duct stricture	Clinicoradiographic impression		366	Alive, 21
#61	Male	75	12000	3	Perihilar	Negative/NS	Indeterminate	Cholangiocarcinoma	Subsequent bile duct mass Percutaneous biopsy	1 US and Percutaneous biopsy	18	Deceased, 10
#62	Male	86	3079	2	Distal	NS/NS	Indeterminate	Cholangiocarcinoma	Clinicoradiographic impression		148	Deceased, 5
#63	Female	70	952819	6833	Intrahepatic	Negative/NS	Indeterminate	Cholangiocarcinoma	Clinicoradiographic impression		45	Deceased, 1
#64	Female	81	290	2	Distal	Negative/NS	Indeterminate	Cholangiocarcinoma	Clinicoradiographic impression		64	Deceased, 2
#65	Male	76	16	4	Perihilar	Negative/NS	Indeterminate	Cholangiocarcinoma	Subsequent US-paracentesis	1 US and paracentesis	49	Deceased, 2
#66	Female	65	124	1	Perihilar	Negative/NS	Indeterminate	Cholangiocarcinoma	Resection		38	Deceased, 16
#67	Male	75	250	3	Distal	NS/NS	Indeterminate	Cholangiocarcinoma	Resection		32	Alive, 8
#68	Male	58	335	3	Distal	Negative/Negative	Indeterminate	Pancreatic Ductal Adenocarcinoma	Resection		35	Deceased, 13

*Malignant biliary duct pathology was defined as at least high-grade dysplasia or at least suspicious for malignancy.

Abbreviations: N/A, not available; NS, not submitted; ERCP, Endoscopic Retrograde Cholangiopancreatography; FNA, Fine-needle aspiration; EUS, Endoscopic Ultrasound; CT: Computed Tomography; US: Abdominal Ultrasound; MR: Magnetic Resonance

Supplementary Table S2. List of mutated genes detected in the bile cfDNA of the 57 patients positive for Bilemut assay.

Mutated gene	Number of patients	% of Bilemut positive patients
KRAS	41	71.9
TP53	27	47.4
ERBB3	13	22.8
GNAS	9	15.8
BRAF	5	8.8
PIK3CA	5	8.8
ERBB2	5	8.8
FBXW7	4	7.0
SMAD4	4	7.0
IDH1	3	5.3
CTNBB1	3	5.3
PTEN	3	5.3
APC	2	3.5
EGFR	2	3.5
IDH2	2	3.5
KIT	2	3.5
MAP2K1	2	3.5
SF3B1	1	1.8
RET	1	1.8
ESR1	1	1.8
FGFR2	1	1.8
FGFR3	1	1.8
MET	1	1.8
MTOR	1	1.8

Supplementary Table S3. List of mutations identified in the paired bile cfDNA and tissue DNA (left) or bile cfDNA (right) of each patient. Asterisk (*) indicates the presence of actionable mutations.

PATIENT ID	GENE NAME	BILE MUTATIONS		TISSUE MUTATION
#1	*KRAS*	G12R		G12R
	TP53	V218E		V218E
#2	*KRAS*	G12D		G12D
#3*	*KRAS*	G12D		G12D
	FGFR3	F384L		nd
#4*	*KRAS*	G12D		G12D
	TP53	na	P151H	R337C
	ERBB2	S310F		nd
#5*	*ERBB3*	V104L		nd
	ERBB2	G776V		nd
#6	*KRAS*	G12R		G12R
#7*	*KRAS*	G12D		G12D
	ERBB3	V104M	G284R	nd
	GNAS	R201C		nd
#8	*KRAS*	nd		G12D
	TP53	nd	C135F	R248Q
	FBXW7	R465C		nd
#9*	*KRAS*	G13D		G13D
	TP53	723delC	R273H	723delC
	FBXW7	R465C	R689W	R465C
	IDH2	R140Q		nd
	MAP2K1	E203K		nd
#10*	*TP53*	na		673-2A>C
	PIK3CA	E524K		E542K
	ERBB2	S310F		nd
#11	*KRAS*	G12V		G12V
	TP53	903delA		903delA
#12*	*KRAS*	G12D		G12D
	TP53	662_663delAG		662_663delAG
	ERBB3	V104M		nd
	RB1	na		1850delG
#13	*KRAS*	G12D		nd
#14	*KRAS*	G12R		nd
#15	*KRAS*	G12D		nd
	TP53	H193R		nd
#16	*KRAS*	nd		G12D
	TP53	nd		H179Q
#47	*KRAS*	G12R		G12R
	TP53	780delC		780delC
#48	*KRAS*	G12D		G12D
	TP53	G262V		G262V
	SF3B1	K700E		K700E
#49*	*KRAS*	G12V		G12V
	TP53	H214R		nd
	IDH1	R132C		R132C
#50*	*KRAS*	G12D		G12D
	SMAD4	R361H		R361H
	ERBB3	V104M		nd
#51*	*KRAS*	G12D		G12D
	TP53	657delC		657delC
	ERBB3	G284R	T355I	nd
#52*	*KRAS*	G12R		G12R
	TP53	G154V		G154V
	ERBB3	V104M		nd
#53*	*KRAS*	G12C		nd
	MAP2K1	F53I		nd
#54*	*TP53*	na		1028_1029delAG
	ERBB3	D297Y		nd
	ERBB2	S310F		nd
	NF1	na		R711C
#55	*KRAS*	G12D		G12D
#56*	*TP53*	V173M		nd
	BRAF	G466A		G466A
	CDKN2A	na		Y129*
#66*	*KRAS*	G12D		nd
	ERBB2	V777L		nd
#67	*TP53*	S241F		S241F
	NF1	na		E1387G
	RB1	na		1332+2T>C
	SMAD4	R361C		nd
#68	*KRAS*	G12D		G12D
	TP53	R273H		nd

PATIENT ID	GENE NAME	BILE MUTATIONS	
#18	*KRAS*	G12R	
	TP53	R175H	
	ESR1	V392I	
#19	*KRAS*	G13D	
#20*	*KRAS*	G12V	G13D
	PIK3CA	Q546P	
	GNAS	R201H	
	FBXW7	R689W	R505C
	IDH2	R140Q	
	PTEN	R173C	
	EGFR	R451C	
	RET	R912W	R912Q
#21*	*PIK3CA*	E545K	
	ERBB3	D297Y	
#22	*KRAS*	G12V	
#23*	*SMAD4*	R361C	
	ERBB3	V104M	
	GNAS	R201C	
#24*	*KRAS*	G12V	Q61H
	BRAF	G596R	
#25	*KRAS*	G12V	
#26*	*KRAS*	G12V	
	TP53	R248W	
	BRAF	D594G	
#27	*TP53*	R282W	
#28	*KRAS*	G12V	
#29*	*TP53*	R248W	
	GNAS	R201C	
	APC	R1450	
	KIT	T670I	
#30	*TP53*	E285K	R282W
	SMAD4	R361C	
	GNAS	R201H	
	PTEN	R173C	
	APC	R1114*	
#31*	*TP53*	M246V	
#32	*KRAS*	G12V	
	PIK3CA	E545K	
	ERBB3	V104L	
	GNAS	R201H	
#33	*KRAS*	G12C	
	TP53	R248W	
#42	*KRAS*	G12R	
#43*	*KIT*	V530I	
#44*	*KRAS*	G12V	
	TP53	E285K	H179R
	BRAF	D594N	
	GNAS	R201H	
	CTNNB1	S45P	
#45*	*KRAS*	G12D	
	ERBB3	V104M	G284R
	GNAS	R201C	R201H
#46*	*KRAS*	G12V	
	ERBB3	V104M	
	CTNBB1	S45F	
#57*	*KRAS*	G12V	
	IDH1	R132G	
	GNAS	R201S	
#58	*KRAS*	G12D	Q61P
	CTNNB1	S45P	T41A
#59	*TP53*	S241F	V173L
#61*	*KRAS*	G12D	G13D
	PIK3CA	R93W	
	IDH1	R132H	
#62*	*TP53*	R282W	
	BRAF	V600E	
	PTEN	R173C	
	EGFR	R451C	
	MET	R988C	
	MTOR	R2217W	
#63*	*KRAS*	G12D	
	TP53	Y220C	
	ERBB3	V104M	
#64*	*FGFR2*	A648T	
#65	*KRAS*	G12D	
	TP53	R248Q	
	FBXW7	S582L	

Common mutations (n=32)
Mutations detected in bile (n=34)
Mutations detected in tissue analized in bile (n=4)
Mutations detected in tissue not analized in bile (n=8)

Supplementary Table S4

A

List of gene targets in the Oncomine Comprehensive Assay v3 (COL110993 0120)

Hotspot genes				Full-length genes			Copy number genes		Gene fusions (inter- and intragenic)		
AKT1	*ESR1*	*KIT*	*PDGFRB*	*ARID1A*	*FBXW7*	*PTEN*	*AKT1*	*FGFR4*	*AKT2*	*FGFR2*	*NUTM1*
AKT2	*EZH2*	*KNSTRN*	*PIK3CB*	*ATM*	*MLH1*	*RAD50*	*AKT2*	*FLT3*	*ALK*	*FGFR3*	*PDGFRA*
AKT3	*FGFR1*	*KRAS*	*PIK3CA*	*ATR*	*MRE11*	*RAD51*	*AKT3*	*IGF1R*	*AR*	*FGR*	*PDGFRB*
ALK	*FGFR2*	*MAGOH*	*PPP2R1A*	*ATRX*	*MSH6*	*RAD51B*	*ALK*	*KIT*	*AXL*	*FLT3*	*PIK3CA*
AR	*FGFR3*	*MAP2K1*	*PTPN11*	*BAP1*	*MSH2*	*RAD51C*	*AXL*	*KRAS*	*BRCA1*	*JAK2*	*PRKACA*
ARAF	*FGFR4*	*MAP2K2*	*RAC1*	*BRCA1*	*NBN*	*RAD51D*	*AR*	*MDM2*	*BRCA2*	*KRAS*	*PRKACB*
AXL	*FLT3*	*MAP2K4*	*RAF1*	*BRCA2*	*NF1*	*RNF43*	*BRAF*	*MDM4*	*BRAF*	*MDM4*	*PTEN*
BRAF	*FOXL2*	*MAPK1*	*RET*	*CDK12*	*NF2*	*RB1*	*CCND1*	*MET*	*CDKN2A*	*MET*	*PPARG*
BTK	*GATA2*	*MAX*	*RHEB*	*CDKN1B*	*NOTCH1*	*SETD2*	*CCND2*	*MYC*	*EGFR*	*MYB*	*RAD51B*
CBL	*GNA11*	*MDM4*	*RHOA*	*CDKN2A*	*NOTCH2*	*SLX4*	*CCND3*	*MYCL*	*ERBB2*	*MYBL1*	*RAF1*
CCND1	*GNAQ*	*MED12*	*ROS1*	*CDKN2B*	*NOTCH3*	*SMARCA4*	*CCNE1*	*MYCN*	*ERBB4*	*NF1*	*RB1*
CDK4	*GNAS*	*MET*	*SF3B1*	*CHEK1*	*PALB2*	*SMARCB1*	*CDK2*	*NTRK1*	*ERG*	*NOTCH1*	*RELA*
CDK6	*H3F3A*	*MTOR*	*SMAD4*	*CREBBP*	*PIK3R1*	*STK11*	*CDK4*	*NTRK2*	*ESR1*	*NOTCH4*	*RET*
CHEK2	*HIST1H3B*	*MYC*	*SMO*	*FANCA*	*PMS2*	*TP53*	*CDK6*	*NTRK3*	*ETV1*	*NRG1*	*ROS1*
CSF1R	*HNF1A*	*MYCN*	*SPOP*	*FANCD2*	*POLE*	*TSC1*	*EGFR*	*PDGFRA*	*ETV4*	*NTRK1*	*RSPO2*
CTNNB1	*HRAS*	*MYD88*	*SRC*	*FANCI*	*PTCH1*	*TSC2*	*ERBB2*	*PDGFRB*	*ETV5*	*NTRK2*	*RSPO3*
DDR2	*IDH1*	*NFE2L2*	*STAT3*				*ESR1*	*PIK3CB*	*FGFR1*	*NTRK3*	*TERT*
EGFR	*IDH2*	*NRAS*	*TERT*				*FGF19*	*PIK3CA*			
FRRR2	*JAK1*	*NTRK1*	*TOP1*				*FGF3*	*PPARG*			
ERBB3	*JAK2*	*NTRK2*	*U2AF1*				*FGFR1*	*RICTOR*			
ERBB4	*JAK3*	*NTRK3*	*XPO1*				*FGFR2*	*TERT*			
ERCC2	*KDR*	*PDGFRA*					*FGFR3*				

B

List of gene targets in the Pan-Cancer Cell-Free Assay (COL113397 1220)

DNA hotspots					Tumor suppressors	CNVs		Fusions	
AKT1	*EGFR*	*FLT3*	*KRAS*	*PDGFRA*	*APC*	*CCND1*	*ERBB2*	*ALK*	*FGFR3*
ALK	*ERBB2*	*GNA11*	*MAP2K1*	*PIK3CA*	*FBXW7*	*CCND2*	*FGFR1*	*BRAF*	*MET*
AR	*ERBB3*	*GNAQ*	*MAP2K2*	*RAF1*	*PTEN*	*CCND3*	*FGFR2*	*ERG*	*NTRK1*
ARAF	*ESR1*	*GNAS*	*MET*	*RET*	*TP53*	*CDK4*	*FGFR3*	*ETV1*	*NTRK3*
BRAF	*FGFR1*	*HRAS*	*MTOR*	*ROS1*		*CDK6*	*MET*	*FGFR1*	*RET*
CHEK2	*FGFR2*	*IDH1*	*NRAS*	*SF3B1*		*EGFR*	*MYC*	*FGFR2*	*ROS1*
CTNNB1	*FGFR3*	*IDH2*	*NTRK1*	*SMAD4*					
DDR2	*FGFR4*	*KIT*	*NTRK3*	*SMO*					