Molecular diagnosis of pancreatobiliary tract cancer by detecting mutations and methylation changes in bile samples

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Summary

Background Patients with pancreatobiliary tract cancer usually have a poor clinical outcome, with a 5-year overall survival rate below 20%. This is mainly associated with the late diagnosis. In addition, the standard-of-care for patients with malignant biliary stenosis involves a major surgery, the Whipple procedure. An accurate preoperative diagnosis, including differentiation from benign diseases, is critical to avoid unnecessary treatment. Here we developed BileScreen, a sensitive detection modality for the diagnosis of pancreatobiliary tract cancer based on massively parallel sequencing mutation and methylation changes in bile samples.

Methods A total of 338 patients, from five hospitals in China, with pancreatobiliary system disorders were enrolled in this study between November 2018 and October 2020, and 259 were included for the analysis of BileScreen. We profiled 23 gene mutations and 44 genes with methylation modifications in parallel from bile samples, and set up a model for the detection of malignancy based on multi-level biomarkers.

Findings We applied the BileScreen assay in a training cohort (n = 104) to set up the model and algorithm. The model was further evaluated in a validation cohort (n = 105), resulting in 92% sensitivity and 98% specificity. The performance of BileScreen was further assessed in a prospective test cohort (n = 50) of patients diagnosed with suspicious or negative pathology by endoscopic retrograde cholangiopancreatography and were confirmed in follow-up. BileScreen yielded 90% sensitivity and 80% specificity, and outcompeted serum carbohydrate antigen 19-9 in detecting pancreatobiliary tract cancer in all three cohorts, especially in terms of specificity.

Interpretation Taken together, BileScreen has the ability to interrogate mutations and methylation changes in bile samples in parallel, thus rendering it a potentially sensitive detection method to help in the diagnosis of pancreatobiliary tract cancer in a safe, convenient and less-invasive manner.

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Bile tract cancer (BTC) includes cholangiocarcinoma (CCA), gallbladder cancer (GBC) and ampullary cancer. There is a rapid increase in the global incidences of BTC (CCA), gallbladder cancer (GBC) and ampullary cancer. Although the specificity of pathological evaluation of ERCP-obtained biopsies and brushings for the diagnosis of malignant biliary strictures is 99%, the sensitivity is only 45–48%. Thus, a negative result does not exclude the possibility of malignancy. Therefore, a sensitivity and reliable detection modality could help improve the diagnosis of pancreatobiliary tract cancer to provide timely and appropriate treatment for each patient.

**Added value of this study**
Herein we developed a next-generation-sequencing-based assay parallel profiling both genetic alterations and methylation changes, BileScreen, to detect malignancies in the pancreatobiliary tract system from bile samples. We applied the BileScreen assay in a training cohort (n = 104) to set up the model and algorithm. The model was further evaluated in a validation cohort (n = 105), resulting in 92% sensitivity and 98% specificity, which was superior to conventional solutions. The performance of BileScreen was further assessed in a prospective test cohort (n = 50) of patients with suspicious diagnosis of malignancy as determined with ERCP. We compared the readout of BileScreen with the follow-up results. BileScreen showed 90% sensitivity and 80% specificity. Thus, BileScreen may improve the detection and diagnosis of pancreatobiliary tract malignancies.

**Implications of all the available evidence**
To the best of our knowledge, BileScreen is the first detection method to complete the parallel interrogation of mutations and methylation changes from bile samples with sensitivity and specificity significantly better than the standard-of-care methods. BileScreen could be a reliable, safe, convenient and less-invasive supplementary diagnosis method for the diagnosis of pancreatobiliary tract cancer.

**Introduction**
Bile tract cancer (BTC) includes cholangiocarcinoma (CCA), gallbladder cancer (GBC) and ampullary cancer. There is a rapid increase in the global incidences of BTC in recent years.3,4 Meanwhile, pancreatic cancer is one of the most common and lethal cancers in humans. It is the seventh leading cause of cancer death worldwide.5 Therefore, strategies specific for the detection and treatment of pancreatobiliary tract cancer may improve the quality of life and survival of affected individuals.

Diagnosis of pancreatobiliary tract cancer is however challenging. Most patients are diagnosed at advanced stages, due to the nonspecific symptoms or even the absence of symptoms at early stages.6,7 Patients with bile duct strictures and jaundice may have CCA, GBC or pancreatic cancer, but the distinction between malignancies and benign strictures, such as iatrogenic bile duct injuries, primary sclerosing cholangitis (PSC) and choledocholithiasis, is difficult.8 In general, late diagnosis results in poor prognosis for BTC patients, and the 5-year overall survival rate is less than 20%.4 Pancreatobiliary tract cancer is typically diagnosed through a combination of modalities, including clinical examination, radiographic imaging (computed tomography, magnetic resonance imaging, magnetic resonance cholangiopancreatography [MRCP]), endoscopic procedures (endoscopic ultrasonography [EUS], with fine-needle aspiration [EUS], and endoscopic retrograde cholangiopancreatography [ERCP]), pathological evaluation (histopathology and cytology) and biochemical tests (carbohydrate antigen 19-9 [CA19-9]).6,7 Nevertheless, these approaches have some limitations, in particular unsatisfactory sensitivities and specificities. For instance, ERCP is the gold standard for diagnosing pancreatobiliary tract cancer as the procedure is used both to detect the malignant bile duct strictures and to acquire bile brushings and forceps biopsies for further pathological evaluation. However, the
sensitivities of brush cytology and pathological evaluation of forceps biopsies to detect malignancies can be rather low (45–48%).14,15 and are even less in patients with PSC.16 ERCP inevitably fails to detect many malignant tumors, especially those at early stages, which leads to more aggressive treatment but poorer survival. The sensitivity of conventional brush cytology has been shown to be improved with ancillary approaches, including mutation analysis by PCR and fluorescence in-situ hybridization, but is still overall less than 70%.16–18 Conversely, serum CA19-9 has a relatively high sensitivity of ~80%, but its specificity varies significantly, from 63% to 98%.7,10,15 At this level of sensitivity and specificity, some patients may be overtreated, for example, with surgery. Indeed, it has been reported that ~15–24% of patients undergoing surgery for malignant biliary strictures are eventually diagnosed with benign disease.16,17 Moreover, CA19-9 is not applicable to patients who are Lewis-antigen negative (7% of the general population).17 Lastly, EUS-FNA has a high risk of tumor dissemination.19 Therefore, it is of urgent need to develop a safe detection modality for diagnosis of pancreaticobiliary tract cancer with high sensitivity and specificity.

Next-generation sequencing (NGS) is a highly sensitive technique used to profile genetic or epigenetic mutations at the level of the whole genome/exome or a panel of specific gene targets.1,9,19–22 A number of genes have been found to be frequently mutated in pancreaticobiliary malignancies, such as KRAS, TP53, CDKN2A, SMAD4 and ARID1A.12,13,17–25 Tumors also have aberrant DNA methylation patterns in CpG islands of tumor suppressor genes,26 which could be adopted to both detect tumors and identify the tissue of origin.27 For pancreaticobiliary malignancies, some genes have been reported to be highly methylated, like CDO1, CNRIP1, SEPT9, ZSCAN18 and 3-OST-2.28–31 Indeed, some studies have explored the detection of pancreaticobiliary malignancies using gene mutations or DNA methylation by molecular techniques including NGS. However, their clinical utility remains unclear as the sensitivity or specificity was not high,9 the patient number was small,20 or invasive tissue sampling only suitable for retrospective studies was used.11

Herein we present an NGS-based method of parallel profiling 23 gene mutations and 44 genes with methylation changes from bile samples using the Mutation Capsule technology.21 We applied the assay, BileScreen, to detect malignancies in the pancreaticobiliary tract system, and compared its performance with conventional methods, in particular ERCP-based pathological evaluation. The main goal of the study was to investigate whether the combination of mutations and methylation changes specific to pancreaticobiliary tract cancers might improve diagnostic capabilities in the clinic, especially with regard to undetected cases, relative to the current standard-of-care, pathological evaluation of ERCP-obtained samples. Our analysis demonstrated that this combination in fact showed greater sensitivity than either mutations or methylation changes alone, although specificity was similarly high for both independently. BileScreen also outperformed serum CA19-9.

**Methods**

**Patients and study design**

A total of 338 patients with pancreaticobiliary tract system disorders were enrolled in this study between November 2018 and October 2020, and they were from five hospitals in China, i.e. National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital (Chinese Academy of Medical Sciences), Dazhou Central Hospital of Sichuan Province, Beijing Chaoyang Hospital (Capital Medical University), Dongfang Hospital (Beijing University of Chinese Medicine), and Gucheng County Hospital of Hebei Province. Of these patients, 259 were included for the molecular testing analysis. Bile samples were taken from all patients. Most (70%) of the bile samples were collected through ERCP, and the samples taken from the 78 cholelithiasis patients were collected during surgery. The cases were divided into three cohorts for this study: training, validation and test.

The training and validation cohorts included the retrospective cohort of 209 cases with confirmed malignant or benign pancreaticobiliary diseases (malignant, n = 116, and benign, n = 93). The inclusion criteria for these two cohorts were the following: 1) patients with pancreaticobiliary malignancies diagnosed through ERCP-based pathology; 2) patients with benign diseases detected through standard pathological diagnosis of surgical specimens, or absence of malignancy during the minimum 12-month follow-up period. Benign diseases included cholelithiasis in patients (n = 78) who underwent surgical removal of the gallbladder and pathological diagnosis revealed chronic cholangitis but no malignancy, and the absence of malignancies during follow-up of at least 12 months in patients (n = 15) who received ERCP to remove biliary stones.

The test cohort refers to a prospective cohort. Patients (n = 50) with suspicious malignancies in the pancreaticobiliary tract system were recruited and grouped into an independent test cohort. The inclusion criteria for this cohort were the following: 1) patients with suspicious results as assessed in the initial ERCP-based pathological evaluation;22 and 2) patients with negative results as assessed in the initial ERCP-based pathological evaluation but with evidence of malignancy based on clinical detection of modalities and symptoms, as ERCP-based pathology does not completely rule out biliary cancers due to its low sensitivity (45–48%).6 In the test cohort, the final
judgement of malignancy depended on subsequent pathological diagnosis, or the patient’s status and clinical detection of modalities during the follow-up period. Patients were confirmed as benign if the resolution or stability of prior abnormalities were detected on repeat imaging, or they showed no clinical symptoms of malignancies based on follow-up by telephone of at least 12 months. Malignancy was also determined based on pathological diagnosis of resected or biopsy specimens clinical/radiographic diagnosis of the combination of a mass on radiographic imaging and clinical or radiographic progression after follow-up of at least 12 months, or clinical death due to pancreatobiliary malignancies. For pathology of resected and biopsy specimens, diagnoses were defined based on standard histomorphological criteria. In the test cohort, 40 malignant cases were confirmed during follow up based on pathological evaluation (surgical specimens, percutaneous needles or ERCP-obtained biopsies/brushings, n = 21), radiographic imaging (n = 5), or clinical criteria (n = 4), radiographic imaging (n = 1) or clinical criteria (n = 5) after a follow-up period of at least 12 months.

The exclusion criteria of the three cohorts were the following: 1) existence of non-pancreatobiliary carcinoma (n = 9); 2) unclear diagnosis due to lack of follow up (n = 14) or insufficient follow-up time to confirm benign status (n = 28); and 3) insufficient (n = 23, including 6 malignant, 13 benign, and 4 suspicious cases) or low levels of human DNA in bile samples (n = 5, including 4 benign and 1 suspicious cases). Patient enrollment and the study design are illustrated in Fig. 1.

Ethics statement
All patients provided written informed consent, and the study was approved by the ethics review committees of the abovementioned five hospitals (ID: NCC2018JJ-001; 22/378-3580).

Sample preparation
Bile fluid specimens were obtained either during ERCP prior to any treatments in 181 patients or during surgery in 78 cholelithiasis patients undergoing surgical removal of the gallbladder. Samples were centrifuged at 12,000 rpm for 10 min to separate supernatants and pellets. DNA was extracted from the pellets of bile fluids with the TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China). DNA quality was assessed with RQ-PCR for human GAPDH gene using Taqman probe. In addition to the bile fluids, paired ERCP-obtained biopsies or brushings were also available to perform NGS test for 34 and nine patients, respectively, with genomic DNA extracted using QIAamp DNA Mini Kit (Qiagen, USA). Serum CA19-9 was also determined for 197 patients, using electrochemiluminescence technology on the E601 system (Roche Diagnostics, Switzerland).

Profiling of mutations and methylation changes using BileScreen
Twenty-three gene mutations and 44 genes with methylation modifications were selected for sequencing in BileScreen (Table S1). Gene mutations in hepatobiliary and pancreatic cancers, such as KRAS, TP53, SMAD4 and CDKN2A, were selected from the published data sets at cbioPortal.org. Methylated genes in BTC and pancreatic cancer/cysts, such as SOX17, 3-OST-2, SEPT9 and TERT, were selected from previous studies. DNA (400 ng) was fragmented with ultrasonication, and then end repaired. The mutation and methylation status were analyzed with the Mutation Capsule (MC) technology. Briefly, DNA fragments were digested with the methylation-sensitive restriction enzyme HhaI (R0139S, New England Biolabs, MA, USA), which cleaves unmethylated, but not methylated DNA at the 4 bp restriction site GCCG. A customized adapter with DNA barcodes was ligated to the digested DNA and the adapter-ligated DNA was amplified to generate a whole genome library (MC library). The target regions covering the mutation and methylation sites of interest were amplified in a RACE-like manner with a universal primer matching the adapter sequence and a gene specific primer near the mutation site or the HhaI digestion site. Two rounds of amplification with nested primers generated the sequencing library for NGS.

Data process and mutation/methylation detection
Sequencing data was processed as previously described. Briefly, tags were extracted and adapter sequences were removed from sequencing reads, and the cleaned reads were mapped to the hg19 genome. Reads with the same tags, and the same start and end coordinates were grouped into Unique Identifier (UID) families. UID families containing at least two reads with at least 80% of the reads being the same types were defined as Effective UID (EUID) families. The frequency for each mutation was calculated by dividing the number of alternative EUID families by the sum of alternative and reference families. Mutations were further manually reviewed in IGV. The candidate variations were annotated with Ensembl Variant Effect Predictor (VEP).

Mutations were required to have at least four EUID families. For recurrently mutated oncogenes with hotspots, including KRAS (G12, G13, Q61 and A146), the limitation of detection (LOD) was set to 0.5%. For other mutated genes, including common tumor suppressor genes without hotspots (hence de novo mutations may be detected), including TP53, SMAD4 and uncommon...
mutations, the LOD was set to 1% to reduce the possibility of false positivity. Since there was no DNA from matched white blood cells to exclude germline mutations, mutations detected in samples were filtered with germline and somatic mutation databases to identify germline mutations of the highest likelihood. Mutations found in germline mutation databases (1000 AF, ESP6500 AA/EA and ExAC AF) with frequencies ≥0.1% were excluded as germline mutations. The passed mutations were further filtered. Mutations with high
frequencies (≥40%) occurring in less than 10 samples in the COSMIC database were likely to be germline mutations and hence further excluded.

For the methylation analysis, clusters of reads ending up with the GGCG restriction site are unmethylated sequences, and molecules that pass through at least one GGCG restriction site with ends beyond the restriction site, are regarded as methylated sequences. The methylation ratio for each target is defined as the ratio of the number of methylated molecules to the sum of the numbers of methylated and non-methylated molecules.

**Construction of BileScreen diagnosis model**
We profiled the bile samples from the training cohort (n = 104). After comparison of the distribution of the mutations among the 52 case and 52 control samples, we found that samples with mutations in most of the genes, including KRAS, TP53, SMAD4, and CDKN2A, were highly enriched in malignant samples. The only exceptions were CTNNB1 and GNAS mutations, which were detected in three benign samples and had no significant association with malignancy status. This result was consistent with previous reports that these two mutations were detected in precancerous lesions.43–45 Therefore, we regarded samples with at least one mutation, except CTNNB1 and GNAS, as mutation positive. Out of the 44 methylation markers, five, including SOX17, 3-OST-2, NXPH1, SEPT9, and TERT, were selected through the penalized logistic regression method in a stepwise manner, for the diagnosis model using the training cohort (Table S2). A penalized logistic regression was subsequently constructed from the training cohort with these five methylation markers, using leave-one-out cross validation to evaluate the model performance with area under the receiver operating characteristic (ROC) curve (AUC), sensitivity and specificity. The cutoff for methylation was optimized based on maximizing the resulting Youden’s index (sensitivity + specificity – 1) in ROC analysis. Integration of mutation and methylation, with positivity defined as either of the two being positive, was named the BileScreen model. The model’s performance was further evaluated in an independent validation cohort as well as a prospective test cohort. In the training and validation cohorts, benign cases were cholelithiasis patients, who tended to be younger and female compared with patients with malignancy. To exclude any potential influence of such artificial bias on the diagnosis prediction, we allocated 52 malignant and 52 benign age- and sex-matched patients to the training cohort. Consequently, the distributions of age and gender in the validation cohort were uneven (Table 1). However, all mutations and methylations were not significantly correlated with age or sex (correlation coefficient <0.5, or Wilcoxon rank sum test P > 0.05), so this imbalance should not significantly affect further analyses.

In the training and validation cohorts, the combination of mutation and methylation in our BileScreen model would sacrifice the specificity of profiling mutation alone (i.e. 100%). In this case, we tried a prioritized combination method to maintain the 100% specificity. However, this approach would decrease the sensitivity (Table S3), hence was not adopted.

**Statistical analysis**
ROC analysis (R package “pROC”) and Wilcoxon rank sum test were used to assess the performance of individual mutation or methylation markers in predicting disease status. The penalized logistic regression method (R package “glmnet”) was adopted to select markers for the diagnosis model. In the training cohort, ROC curves were also used to determine optimal cutoffs for methylation alone using Youden’s index, with the original scores as input. ROC analysis was also used to compare performances of different methods, with binary input determined by the corresponding cutoffs. Sensitivity and specificity were calculated using standard 2 × 2 contingency tables. All R package related analysis was based on the R software (V.3.6.3).

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The funders of the study had no role in study design, data collection, data analysis, data interpretation, or the writing of this report. Authors were not precluded from accessing data in the study, and they accept responsibility to submit for publication.

**Results**

**Development of BileScreen model with mutations and methylation alterations**
We profiled the BileScreen assay in a training cohort of 104 patients including 52 malignancies identified by ERCP-based diagnostic pathology and 52 benign diseases confirmed by surgical pathology or a follow-up of at least 12 months in patients with biliary stones. The clinical characteristics are summarized in Table 1. We profiled the DNA from bile samples with the Mutation Capsule technology to detect mutations in 23 genes as well as methylation changes in 44 genes with methylation sites that have been previously reported to be associated with biliary malignancies.23,24,33–37 The most frequently mutated genes in cancers were TP53 (50%) and KRAS (46%) (Fig. 2, Table S4). However, some mutations, including CTNNB1 and GNAS, were detected in both cancer and benign patients. These mutations had no significant association with malignancy status, which was consistent with other studies.33–41 Thus, they were excluded from the BileScreen model. Therefore, mutation positivity was defined as having at least one mutation detected in
Table S6. All together, BileScreen showed 92% sensitivity and 98% specificity in the validation cohort with an AUC of 0.95 (Table 2, Figure S1B). If only gene mutations were adopted, the sensitivity and specificity are 78% and 100% respectively. Profiling of methylation alone yielded a sensitivity and specificity of 81% and 98%, respectively (Tables 2 and S6). Overall, the sensitivity and specificity of BileScreen in the training and validation cohorts were 93% and 98%, respectively.

Performance of BileScreen in a prospective cohort of suspicious malignancy
We further validated BileScreen in a test cohort of 50 cases where ERCP-based pathology did not yield a clear diagnosis or exclusion of cancer (“suspicious malignancy” or “unable to exclude cancer”). According to the recommendations, these cases were followed up for at least 12 months unless malignancies were confirmed through repeated ERCP or other clinical approaches. After the follow-up, 40 out of the 50 cases were found to be malignant. The remaining 10 cases did not develop detectable cancer by the end of the follow-up and were thus diagnosed as benign. Thirty-six of the 40 malignant cases and 2 of the 10 benign cases were test-positive by KRAS, TP53 and other genes, excluding CTNNB1 and GNAS. Mutation alone discriminated pancreatobiliary malignancies from noncancer cases with 81% sensitivity, 100% specificity and an AUC of 0.90 (Table 2, Figure S1A). For the methylation changes, five markers, SOX17, 3-OST-2, NXPH1, SEPT9, and TERT, were selected for the prediction model through the penalized logistic regression method in a stepwise manner (Fig. 2, Tables S2 and S5). With the leave-one-out method, methylation alone robustly identified pancreatobiliary malignancies from noncancer cases with 88% sensitivity, 98% specificity and an AUC of 0.93 (Table 2, Figure S1A). The cutoff for the methylation score was 0.422, yielding the maximum Youden’s index (Figure S2). Finally, when mutation and methylation activity was de

| Gender | Total (n = 259) | Training set (n = 129) | Validation set (n = 50) | Test set (n = 50) |
|--------|----------------|------------------------|-------------------------|------------------|
|        | Negative (n = 54) | Positive (n = 50) | Negative (n = 45) | Positive (n = 60) | Negative (n = 12) | Positive (n = 38) |
| Female | 134 | 30 | 26 | 0.716 | 41 | 15 | <0.001 | 4 | 18 | 0.393 |
| Male   | 125 | 24 | 24 | 0.716 | 4 | 45 | <0.001 | 8 | 20 | 0.032 |
| Mean age (range), years<sup>a</sup> | | | | | | | | | | |
| <50    | 39.2 (17-49) | 44.2 (37-49) | 44.5 (36-49) | 0.951 | 33.2 (17-49) | 46.5 (41-49) | 0.002 | 36 (30-44) | 43.8 (32-48) | 0.082 |
| ≥50    | 63.8 (50-89) | 62.8 (50-82) | 63.0 (50-81) | 0.888 | 64.9 (50-87) | 64.5 (50-89) | 0.883 | 65 (54-72) | 64.1 (51-88) | 0.489 |
| HBV infection status<sup>b</sup> | | | | | | | | | | |
| HBV infection cases | 10 | 5 | 2 | 0.517 | 1 | 0 | 0.454 | 0 | 2 | 1.000 |
| Non-HBV infection cases | 236 | 48 | 46 | 43 | 53 | 11 | 35 | 0.000 |
| CA19-9<sup>c</sup> | | | | | | | | | | |
| >27 U/mL | 135 | 16 | 36 | <0.001 | 10 | 41 | <0.001 | 8 | 24 | 0.644 |
| ≤27 U/mL | 62 | 28 | 5 | 19 | 4 | 2 | 4 | 0.001 |
| Disease type | | | | | | | | | | |
| Intrahepatic cholangiocarcinoma | 2 | 0 | 0 | <0.001 | 0 | 0 | <0.001 | 0 | 2 | 0.001 |
| Panin hilar cholangiocarcinoma | 30 | 1 | 13 | 0 | 11 | 1 | 4 |
| Distal cholangiocarcinoma | 67 | 2 | 19 | 2 | 26 | 1 | 17 |
| Pancreatic carcinoma | 30 | 0 | 5 | 1 | 10 | 2 | 12 |
| Ampulla of Vater carcinoma | 18 | 0 | 9 | 2 | 6 | 0 | 1 |
| Gallbladder adenocarcinoma | 4 | 0 | 1 | 0 | 3 | 0 | 0 |
| Duodenal papilla carcinoma | 5 | 0 | 2 | 0 | 3 | 0 | 0 |
| Benign cholangiopathy | 103 | 51 | 1 | 40 | 1 | 8 | 2 | 0.000 |

BTC, bile tract cancer; CA19-9, carbohydrate antigen 19-9; HBV, hepatitis B virus. <sup>a</sup>Age was divided into two groups based on the average age of BTC. <sup>b</sup>HBV infection data were available for 246 of 259 patients. <sup>c</sup>CA19-9 data were available for 197 of 259 patients. *Calculated by all malignancies versus benign diseases.

Table 1: Clinical characteristics of three cohorts based on the results of BileScreen.
BileScreen, leading to a sensitivity of 90% and specificity of 80% (Fig. 3, Table S7, Figure S4). The positive predictive value (PPV) was 95%. In this case, BileScreen precisely predicted the clinical outcome of the patients with an unconfirmed diagnosis through ERCP-based pathology, the current standard-of-care ($P < 0.001$, Chi-square test with continuity correction). Mutation alone discriminated cancers from benign lesions with sensitivity of 75%, specificity of 90%, PPV of 97%, and an AUC of 0.83. Methylation alone showed 80% sensitivity, 80% specificity, 94% PPV and an AUC of 0.8 (Tables 2 and S7, Figure S1C).

Table 2: Performance of BileScreen in the three cohorts.

| Parameter         | Sensitivity | Specificity | PPV    | NPV    | AUC     |
|-------------------|-------------|-------------|--------|--------|---------|
| **Training set (n = 104)**                          |
| Methylation score | 88.66%      | 98.08%      | 97.87% | 89.47% | 0.9327  |
| Mutation score   | 80.77%      | 100.00%     | 100.00%| 83.87% | 0.9038  |
| BileScreen score | 94.23%      | 98.08%      | 98.00% | 94.44% | 0.9615  |
| **Validation set (n = 105)**                         |
| Methylation score | 81.25%      | 97.56%      | 98.11% | 76.92% | 0.8941  |
| Mutation score   | 78.13%      | 100.00%     | 100.00%| 74.55% | 0.8906  |
| BileScreen score | 92.19%      | 97.56%      | 98.33% | 88.89% | 0.9487  |
| **Training plus validation set (n = 209)**            |
| Methylation score | 84.48%      | 97.85%      | 98.00% | 83.49% | 0.9117  |
| Mutation score   | 79.31%      | 100.00%     | 100.00%| 79.49% | 0.8966  |
| BileScreen score | 93.10%      | 97.85%      | 98.18% | 91.92% | 0.9548  |
| **Test cohort (n = 50)**                              |
| Methylation score | 80.00%      | 80.00%      | 94.12% | 50.00% | 0.8000  |
| Mutation score   | 75.00%      | 90.00%      | 96.77% | 47.37% | 0.8250  |
| BileScreen score | 90.00%      | 80.00%      | 94.74% | 66.67% | 0.8500  |

AUC, area under curve; NPV, negative predictive value; PPV, positive predictive value.
Comparison between CA19-9 and BileScreen in the three cohorts

Serum CA19-9 data were available for 85 and 74 patients in the training and validation cohorts, respectively (Fig. 2, Table S6). We performed direct comparison of serum CA19-9 and BileScreen results in these patients. Serum CA19-9, using \( \geq 27 \) U/mL as the cutoff according to the manufacturer’s protocol, distinguished malignant cases from benign cases with an AUC of 0.78 and 0.81 respectively in these cohorts (Figure S3A and B). An elevated serum CA19-9 delivered sensitivities of 88\% and 91\%, and specificities of 67\% and 70\%, in the two cohorts, respectively (Tables 3 and S6). In comparison, BileScreen possessed sensitivities of 93\% and 94\%, and specificities of 98\% and 96\%, respectively. The sensitivity and specificity of CA19-9 were both inferior to those of BileScreen in the validation cohort. Therefore, BileScreen outcompeted serum CA19-9 in detecting biliary cancers, especially in terms of specificity. When BileScreen and serum CA19-9 were combined together, where positivity was defined as either of the two being positive and a cutoff of 150 U/mL was adopted for serum CA19-9 (to obtain the maximum Youden’s index), sensitivities were further increased to 95\% in the training cohort and 98\% in validation, although the specificities were not improved compared with BileScreen alone (Table 3).

Comparison of mutations in bile samples and paired ERCP-obtained biopsies or brushings

In the three cohorts, ERCP-obtained biopsy samples were available for 34 patients and brushing samples for 9 patients. We profiled the mutations in the bile and tissue samples to make a direct comparison. Of the 43 cases, 70 mutations were present in both sample types, while 5 mutations were detected exclusively in bile samples and 9 exclusively in tissues (Fig. 4, Table S8). In other words, 93\% (70/75) of mutations detected in bile samples were also identified in the matched tissues, with some additional mutations found only in bile samples. 89\% (70/79) of tissue-derived mutation were detected in bile samples. Mutations were also detected in at least one type of sample in 36 of the 43 paired samples. In 34 (94\%) of these 36 samples at least one shared mutation between the two sample types was detected. Therefore, the concordance of mutation status for patients between bile samples and tissues is 95\% (41/43).
Comparison of mutations in bile fluid and paired blood samples

To compare the performance in detection of mutations, we collected paired biles and plasma samples from 20 patients with pancreatobiliary tract cancer. As expected, mutation frequencies in plasma were significantly lower than those in biles (Figure S5A). Moreover, there were less mutations detected in plasma than in biles (Figure S5B). Therefore, the cutoff for mutation in plasma was set to 0.05%. Based on mutations in plasma, the sensitivity for discriminating malignancies was 75% (15/20), while the sensitivity of biles was 95% (19/20).

| Parameter       | Sensitivity | Specificity | PPV   | NPV   | AUC  |
|-----------------|-------------|-------------|-------|-------|------|
| Training set (n = 85) |             |             |       |       |      |
| CA19-9          | 88.37%      | 66.67%      | 73.08%| 84.85%| 0.7752|
| Methylation score| 86.05%      | 97.62%      | 97.37%| 87.23%| 0.9183|
| Mutation score  | 81.40%      | 100.00%     | 100.00%| 84.00%| 0.9070|
| BileScreen score| 93.02%      | 97.62%      | 97.56%| 93.18%| 0.9512|
| CA19-9 and BileScreen score | 95.35% | 95.24% | 95.35%| 95.24%| 0.9529|
| Validation set (n = 74) |             |             |       |       |      |
| CA19-9          | 91.49%      | 70.37%      | 84.31%| 82.61%| 0.8093|
| Methylation score| 80.85%      | 96.30%      | 97.44%| 74.29%| 0.8857|
| Mutation score  | 76.60%      | 100.00%     | 100.00%| 71.05%| 0.883 |
| BileScreen score| 93.62%      | 96.30%      | 97.78%| 89.66%| 0.9496|
| CA19-9 and BileScreen score | 97.87% | 96.30% | 97.87%| 96.30%| 0.9708|
| Training plus validation set (n = 159) |             |             |       |       |      |
| CA19-9          | 90.00%      | 68.12%      | 78.64%| 83.93%| 0.7906|
| Methylation score| 83.33%      | 97.10%      | 97.40%| 81.71%| 0.9022|
| Mutation score  | 78.89%      | 100.00%     | 100.00%| 78.41%| 0.8944|
| BileScreen score| 93.33%      | 97.10%      | 97.67%| 91.78%| 0.9522|
| CA19-9 and BileScreen score | 96.67% | 95.65% | 96.67%| 95.65%| 0.9616|
| Test cohort (n = 38) |             |             |       |       |      |
| CA19-9          | 83.87%      | 14.29%      | 81.25%| 16.67%| 0.5902|
| Methylation score| 74.19%      | 85.71%      | 95.83%| 42.86%| 0.7995|
| Mutation score  | 74.19%      | 85.71%      | 95.83%| 42.86%| 0.7995|
| BileScreen score| 87.10%      | 85.71%      | 96.43%| 60.00%| 0.8641|
| CA19-9 and BileScreen score | 93.55% | 57.14% | 90.63%| 66.67%| 0.7535|

AUC, area under curve; CA19-9, carbohydrate antigen 19-9; NPV, negative predictive value; PPV, positive predictive value. *When combined, the cutoff of serum CA19-9 was 150 U/mL.

Table 3: Performance of serum CA19-9 and BileScreen in subpopulations of three cohorts.
among these 20 cancer patients (Figure S5C). In summary, the performance in mutation detection from plasma is worse than that from bile samples.

Discussion
In this study we profiled genetic alterations and DNA methylation in parallel in bile samples, and demonstrated an improved performance in distinguishing pancreatobiliary tract malignancies from benign diseases. The combination of mutation and methylation increased the sensitivity from 79 to 84% (mutation or methylation alone) to 93% in the overall training and validation cohorts. The performance was superior to previous studies when single types of biomarkers were profiled.\(^4,5,6,47\) To the best of our knowledge, this study is the first integrating mutation and methylation to detect pancreatobiliary tract cancer in a large cohort of patients. The mutant genes and methylation sites were selected from previous publications/databases mainly developed from western populations. The promising performance in a Chinese population in this study demonstrates the importance of genetic and epigenetic alterations as diagnostic biomarkers for human pancreatobiliary tract cancer in general. The BileScreen model might provide consistent performance on western populations and other populations, although new training might be necessary to determine the detailed parameters of an algorithm for western populations.

Currently, the diagnostic pathology of ERCP-obtained biopsies/brushings is the gold standard for the diagnosis of malignancies in pancreatobiliary tract system when it gets a positive result suggesting malignancy. However, when the pathology is negative or suspicious, the possibility of malignancy cannot be excluded. In fact, the sensitivity of ERCP-obtained biopsies/brushings for the diagnosis of malignant biliary strictures is only 45–48%, although the specificity is as high as 99%.\(^8\) The limited sensitivity of ERCP-based pathological evaluation may be explained by the inadequate sampling due to the highly desmoplastic, paucicellular nature of CCA and the inaccessible anatomical location.\(^8\) According to the expert consensus,\(^4,5,6,47\) when patients with suspected malignant biliary strictures undergo ERCP examination but the pathological results do not support malignancy, multiple examinations (e.g., intraductal ultrasonography [IDUS], EUS-FNA, image examination) under the guidance of a multi-disciplinary team should be considered to confirm the diagnosis. However, the accuracy of these diagnosis methods is still not satisfying (IDUS, 66% sensitivity; IDUS plus CT, 82% sensitivity and 82% specificity; EUS-FNA, 74% sensitivity).\(^4,5,6,47\) Thus, the optimal time for treatment might be missed during the follow-up period. It is imperative to enhance the diagnosis accuracy for these patients.

In this study, BileScreen showed promising diagnostic value in a prospective cohort (test cohort) of 50 cases without a clear diagnosis based on the pathology of ERCP-obtained biopsies/brushings. The sensitivity was 90% among the malignant cases for which a confirmed diagnosis was not achieved through ERCP-based pathology. One possible explanation is that, the bile sample contains the tumor cells and DNA released from the whole pancreatobiliary system, including the locations difficult for ERCP to access.\(^4,5,6,47\) More importantly, the BileScreen assay also showed a high PPV of 95%. This result strongly suggests that once patients test positive based on BileScreen, they may be appropriately treated after consideration of all clinical evidence, including the BileScreen test. Such patients would receive treatment earlier possibly when the tumor load might still be low, rather than later after the tumor becomes clinically detectable during the follow-up period. Early intervention thus has the possibility of improving outcomes.

There are limitations to the study. First, the sample size of the prospective cohort in this study, especially the benign cases, was limited. Further validation in additional cases and clinical trials for intervention study would be needed to confirm the benefit of BileScreen in the early detection and treatment of the cases without a clear diagnosis from ERCP pathology. A second limitation in this study is the imbalance in age and sex in the validation cohort. As the ratio of malignancy was rather high, there were a limited number of benign cases that could be used as controls in our cohort of biliary strictures (training and validation cohorts). In this case, we collected bile samples from some cholelithiasis patients as benign controls for the training and validation cohorts. This led to bias in age and sex between the malignant and benign cases. To exclude the potential influence of age and sex bias in the prediction model, we allocated 52 malignant and 52 benign age- and sex-matched patients to the training cohort. As a consequence, the distributions of age and sex in the remaining cases (validation cohort) were uneven between cases and controls. Unfortunately, we did not have additional cases to build an age- and sex-matched validation cohort. However, we found that the mutations and methylations were not significantly correlated with age or sex. Age and sex were also not directly included in the algorithm. In this case, the imbalance in age and sex might not severely affect the performance of the validation cohort. More importantly, we have also tested the BileScreen model in a prospective test cohort without this issue and got promising results in the detection of malignancy. Furthermore, as the samples were collected mainly from patients undergoing ERCP, the majority of the subtypes were extrahepatic CCA, so that intrahepatic CCAs were rare in this study. Therefore, the distribution of the subtypes differed from the real world frequencies of BTC. Finally, BileScreen could only be used in patients with available bile samples containing sufficient DNA. Thus, BileScreen is practical for patients with
extrahepatic BTC, but not for some patients with intrahepatic BTC and pancreatic disorders.

In this study, the BileScreen assay showed high sensitivity and specificity. Furthermore, the sample availability of bile from decompensation routinely drawn in patients undergoing ERCP renders the assay widely applicable. Finally, the assay may help to improve the diagnosis and management of patients with pancreato-biliary tract diseases, by preventing unnecessary resection in benign cases and initiating treatment earlier in malignant cases.

Contributors
H.Z., Y.J., J.C. and G.W. directed, conceived the project and supervised the research. S.H., F.Z., H.Y., P.W., Y.B., Q.S. wrote the manuscript and analyzed the data. J.C., Z.H., Y. L., H.L., Q.C., L.L., J.Z., H.H., X.L., T.L. performed the experiments and critical review of the manuscript.

Data sharing statement
All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Declaration of interests
Y.J. is one of the cofounders, has owner interest in Genetron Holdings, and receives royalties from Genetron. Y.J. and H.Z. have filed patents/patent applications based on the technology and data generated from this work. The remaining authors declare that they have no competing interests.

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Appendix A. Supplementary data
Supplementary data related to this article can be found at https://doi.org/10.1016/j.eclinm.2022.101736.

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