absorption spectrophotometry (Spectra AA-300/400 Zeeman; Varian Corp., Sydney, Australia).

Chronologic laboratory data after R-PIHP Three of the12 animals underwent embolization of the puncture routes using coils and gelatin sponges after the conclusion of R-PIHP therapy. These three animals were kept alive for one week for serial laboratory testing.

Vivisection and histological examinations Vivisection was performed immediately (n=9) and seven days (n=3) after R-PIHP therapy. The liver, duodenum, small intestine, and colon were removed by laparotomy in 12 pigs, and the spleen was removed in 3 pigs 7 days after R-PIHP. All pigs were sacrificed after organ removal by an intravenous injection of potassium chloride. Tissues were fixed in 10 % buffered formalin and embedded in paraffin for histological examination. Sections were stained with haematoxylin and eosin. Other stains were used, if necessary, at the discretion of an experienced pathologist.

Statistical analyses

All results are expressed as mean and 95 % confidence interval (95 % CI). All the parameters related to haemodynamic changes and the pharmacokinetics of cisplatin were analysed with paired t-tests. SPSS version 21 software (SPSS Japan, Inc., Tokyo, Japan) was used for all statistical analyses. Values of $P<0.05$ were considered statistically significant.

Results

Percutaneous R-PIHP was successfully performed in all pigs. The measurement of systemic and arterial platinum (infusion site) concentrations in the hepatic circulation was also successful in all cases.

Effects of R-PIHP on haemodynamics

The systolic arterial blood pressure decreased by 14 mm Hg (13.9 %) on average (range, 8–20 mm Hg, 7.1 – 19.6 %; $P<0.001$), within 3 min of balloon occlusion of the IVC. Blood pressure remained stable at this level during R-PIHP and recovered immediately after deflation of the IVC balloon ($P<0.001$). Heart rates remained stable before, during, and after R-PIHP therapy.

Confirmation of the R-PIHP system

Retrograde-outflow isolated hepatic arteriography with rotary pumps (Fig. 2) confirmed that contrast media flowed into both the liver and the intrahepatic portal vein. The absence of opacification of the hepatic veins and IVC was confirmed in all pigs. Contrast opacification of the main portal trunk increased with time, confirming that the portal vein acted as an outflow tract. No collateral vessels to the surrounding organs were opacified during arteriography. In summary, these results suggest complete occlusion of the IVC and portal vein and verified the patency of the R-PIHP system in all pigs.

Pharmacokinetics of the serum platinum concentration

The systemic and hepatic circulatory serum platinum concentrations were measured in all pigs (Table 1). The mean C_{max} in the hepatic circulation was 86.3 mg/l (95 % CI, 73.8 – 98.7 mg/l) and the mean AUC was 1,330.8 mg·min/l (95 % CI, 1,128.2 – 1,533.4 mg·min/l). In contrast, the mean C_{max} in the systemic circulation was 2.2 mg/l (95 % CI, 1.6 – 2.9 mg/l) and the mean AUC was 44.6 mg·min/l (95 % CI, 29.5 –

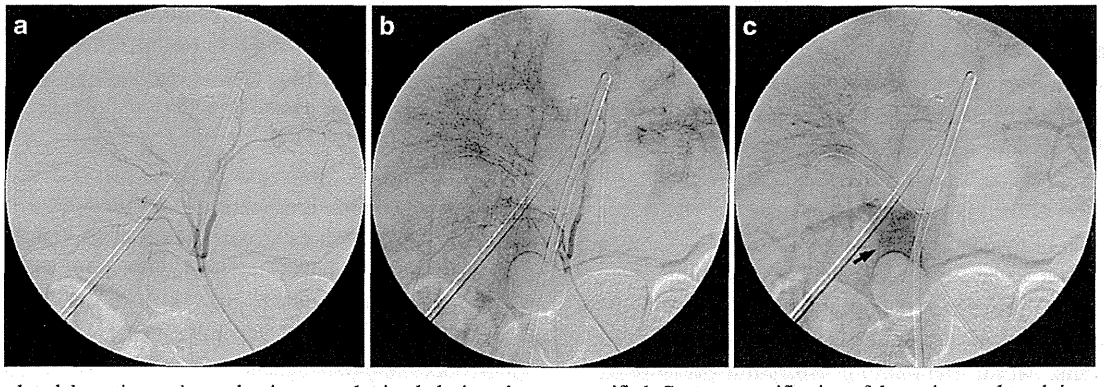

Fig. 2 Isolated hepatic angiography images obtained during the retrograde-outflow procedure. Contrast media flowed into the liver (A, early arterial phase; B, late arterial phase) and intrahepatic portal vein (C, portal phase). The hepatic veins and inferior vena cava were not opacified. Contrast opacification of the main portal trunk increased with time (C, arrow), confirming that the portal vein acted as an outflow tract. No collateral vessels to the surrounding organs were opacified

Table 1 C_{max} and AUC of platinum in the hepatic and systemic circulation

	HC		SC		HC/SC Exposure Ratio	
Pig	C_{max}	AUC	C_{max}	AUC	C_{max}	AUC
1	95.5	1500.7	1.1	13.6	85.3	110.5
2	78.5	1443.4	3.3	67.4	23.8	21.4
3	63.9	999.5	1.2	19.7	55.1	50.7
4	112.8	1802.1	2.0	37.7	57.3	47.8
5	51.8	1184.3	0.5	9.3	101.6	128.0
6	103.3	1305.2	3.3	81.9	30.9	15.9
7	92.6	863.5	2.0	44.3	47.0	19.5
8	105.2	1317.5	3.4	60.4	31.4	21.8
9	61.5	946.7	2.6	50.3	24.1	18.8
10	76.8	1450.8	1.5	24.5	50.5	59.3
11	90.4	1909.8	3.2	57.6	28.6	33.1
12	103.0	1246.5	2.8	68.6	37.1	18.2
Mean	86.3	1330.8	2.2	44.6	39.2	29.8
95% CI	73.8–98.7	1128.2–1533.4	1.6–2.9	29.5–59.7	32.1–63.3	21.5–69.3

The C_{max} is represented in mg/l, and the AUC for minutes 0–30 is indicated in mg·min/l.

C_{max} maximum platinum concentration,, *AUC* area under the concentration–time curve,, *CI* confidence interval,, *HC* hepatic circulation,, *SC* systemic circulation

59.7 mg·min/l). Platinum concentrations in the hepatic circulation were significantly higher ($P<0.01$) than those in the systemic circulation in all cases (Fig. 3). The hepatic-to-systemic circulation exposure ratio was 39.2 for C_{max} and 29.8 for the AUC.

Laboratory data

The laboratory data ($n=3$) are shown in Table 2 and Fig. 4. The levels of total protein, albumin, total bilirubin, and creatinine were within the normal range or were unchanged for one week after the procedure. Amylase, creatine phosphokinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels were increased after R-PIHP therapy. Each value peaked in one or three days after therapy, and then decreased with time until returning to the normal range or pre-therapy levels.

Vivisection and histological examinations

The liver, duodenum, small intestine, colon, and spleen had a normal appearance in all cases. No vascular obstruction, stenosis, or haemorrhage was observed in the liver or abdominal aorta. Histological assessment revealed no evidence of structural disorder, sinusoidal dilatation, or thrombi in the hepatic vessels. None of the examined organs in any of the pigs showed ischaemic or oedematous changes. No significant effect was observed in the liver parenchyma, or any other organs, due to the administration of cisplatin.

Discussion

Animal studies of IHP have demonstrated that this technique delivers intrahepatic concentrations of cytostatic agents that are up to five-fold higher than those delivered by TAI [27, 28].

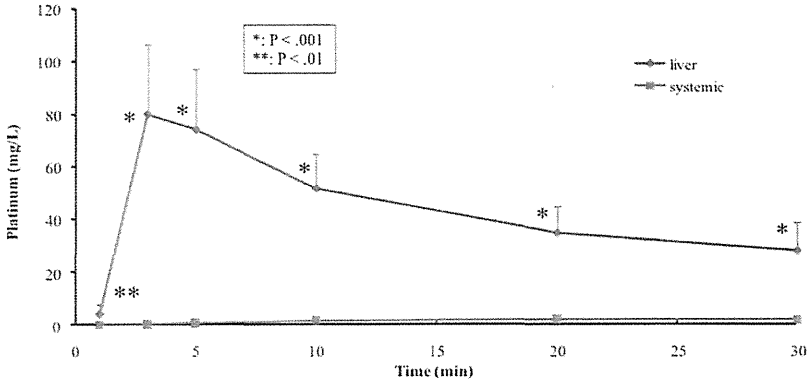

Fig. 3 Serum plasma platinum concentrations during retrograde-outflow isolated hepatic perfusion. All platinum concentrations in the hepatic circulation were significantly higher ($P<0.01$, paired t-test) than those in the systemic circulation.

Table 2 Chronologic laboratory data before and after the procedure

	Normal range	Pre-procedure	Post-procedure	1 day after	3 days after	5 days after	7 days after
TP	5.8–9.1 (g/dl)	5.1 (4.3, 6.0)	4.5 (2.7, 6.3)	5.8 (4.4, 7.2)	6.1 (5.0, 7.1)	6.1 (4.9, 7.4)	6.1 (4.7, 7.5)
Alb	3.2–4.8 (g/dl)	2.8 (2.6, 3.0)	2.5 (2.1, 2.8)	3.1 (2.3, 3.9)	3.2 (2.4, 3.9)	3.2 (2.0, 4.5)	3.1 (1.9, 4.4)
T-Bil	< 0.5 (mg/dl)	0.1 (0.0, 0.3)	0.1 (0.0, 0.3)	0.0 (0.0, 0.1)	0.0 (0.0, 0.1)	0.1 (0.0, 0.3)	0.0 (0.0, 0.1)
Cre	1.2–2.4 (mg/dl)	0.9 (0.3, 1.4)	1.1 (0.4, 1.8)	0.8 (0.7, 0.9)	0.9 (0.5, 1.3)	1.0 (0.6, 1.4)	1.0 (0.7, 1.3)
Amy	525–2105 (U/l)	1273.3 (390.7, 2155.9)	1398.3 (740.1, 2056.6)	2140.0 (1406.9, 2873.1)	1963.3 (521.0, 3405.6)	1731.0 (427.9, 3034.1)	1666.0 (492.1, 2839.9)
CK	679–3161 (U/l)	750.0 (453.1, 1046.9)	1281.0 (410.6, 2151.4)	1941.0 (1583.7, 2298.3)	4233.3 (1422.6, 7044.1)	1166.3 (199.4, 2133.3)	927.7 (14.6, 1840.8)
AST	< 44 (U/l)	26.7 (3.6, 49.7)	61.3 (15.4, 107.3)	95.3 (31.9, 158.8)	85.3 (13.9, 156.7)	44.7 (0.0, 99.2)	19.0 (0.2, 37.8)
ALT	< 58 (U/l)	29.3 (10.7, 48.0)	31.3 (5.2, 57.4)	73.3 (0.0, 171.2)	62.3 (37.0, 87.7)	47.7 (41.4, 53.9)	38.7 (15.6, 61.7)
LDH	577–1525 (U/l)	346.7 (268.0, 425.3)	399.0 (346.4, 451.6)	1111.0 (0.0, 3156.9)	986.3 (0.0, 2151.4)	736.7 (0.0, 1586.5)	520.0 (0.0, 1061.6)

Data in parentheses are 95% CIs.

TP total protein, *Alb* albumin, *T-Bil* total bilirubin, *Cre* creatinine, *Amy* amylase, *CK* creatine phosphokinase, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase

IHP also makes it possible to use perfusion drugs that do not have a high first-pass hepatic extraction rate, facilitating the prolonged exposure of the tumour to these drugs without concomitant systemic exposure. This advantage of IHP over TAI has yielded encouraging results in terms of prolonged survival in patients with metastatic liver tumours [10–13]. The use of anticancer drugs that demonstrate high systemic toxicity, such as tumour necrosis factor, might therefore become possible because the liver vascular bed can be cleared after perfusion. However, a feasible protocol for safe and repeatable percutaneous IHP is very desirable due to the associated major surgical trauma, morbidity, and the inability to repeat surgical IHP [10, 12–15].

The current orthograde percutaneous IHP techniques have been associated with significant leakage into the systemic circulation in clinical situations [16, 17, 29], contrary to results obtained in experimental settings [22]. The major reason is that the human suprahepatic vena cava is shorter than that of the pig, rendering its occlusion with a balloon difficult [19] and resulting in incomplete occlusion of the IVC or diaphragmatic veins. However, the IVC and some diaphragmatic veins can be occluded by clamping during an open surgical procedure without obstruction of the hepatic veins. In the present study, by using the R-PIHP technique, which allows outflow via the portal vein, we were able to overcome the anatomical disadvantage of the short human suprahepatic vena cava and

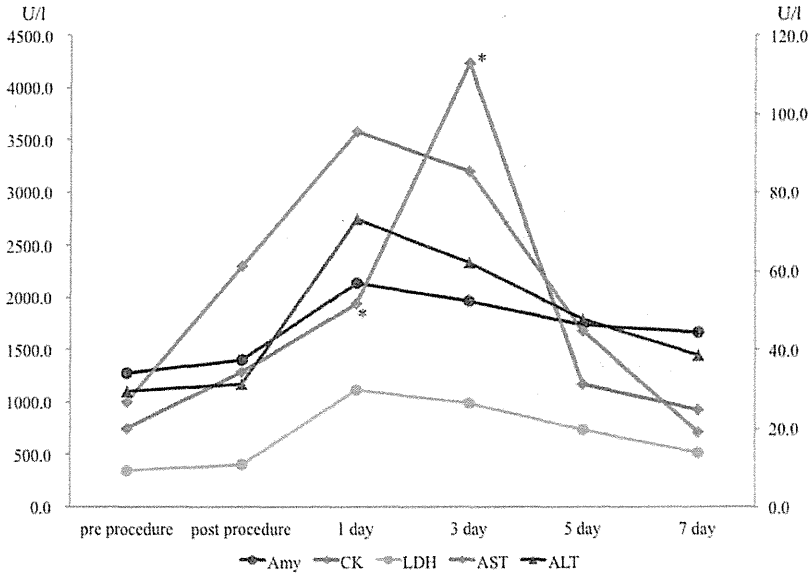

Fig. 4 Chronologic laboratory data before and after the procedure. Amylase, creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels increased after retrograde-outflow percutaneous isolated hepatic perfusion therapy. Each value peaked one or three days after therapy and then decreased until they returned to normal or pre-therapy values. *: $P<0.05$; Repeated analysis of variance and post-hoc analysis between the pre-procedural values and each measurement

the accompanying disadvantage of leakage via the IVC and diaphragmatic veins. The underlying principle is that occlusion of the IVC at the hepatic portion including the diaphragmatic veins provides an alternative to normal hepatic circulation [20, 21]. In the present study, isolated hepatic angiography clearly showed reversal of blood flow from the liver parenchyma into the portal vein, indicating the conversion of the portal vein from an inflow to an outflow vessel. This phenomenon occurs because of the high pressure generated in the periportal sinusoids by the continuous flow of blood draining into the sinusoids via the peribiliary plexus [26, 30]. The high pressure from the peribiliary plexus forces blood from the remaining sinusoids and hepatic veins to drain toward the lower pressure portal vein.

The pharmacokinetic data further demonstrated that R-PIHP is not inferior to orthograde percutaneous techniques or combined orthograde and surgical percutaneous IHP used in experimental pig studies [22]. In this context, it is relevant to consider whether percutaneous R-PIHP is a safe, efficacious, and feasible alternative to surgical IHP for hepatic malignancies. Although direct comparisons of this experimental study and previous clinical studies may not be precise, slightly more systemic leakage was observed in the present study compared with previously published orthograde surgical IHP methods. Surgical IHP has a low systemic leakage rate of approximately 3 % [19, 31–33] and a very high hepatic-to-systemic AUC ratio of 52.0 [32]. However, the hepatic-to-systemic circulation exposure ratio in 42 % (5/12) of the pigs in the present study was similar to that of other surgical IHP methods in terms of systemic leakage. These pharmacokinetic differences may be due to the techniques and instruments used, and further technical refinements may overcome this disadvantage of the R-PIHP system.

With regard to the safety of percutaneous procedures, the most common side effect has been transient hypotension, secondary to the reduction of venous return to the heart as a result of IVC occlusion [18]. Vasoactive cardiotropic agents are usually administered to maintain haemodynamic stability when the balloons are inflated, as venous return is compromised [18, 34]. In the present study, the aorta was occluded with a balloon just below the renal artery [22] to compensate for the decreased systemic venous return, creating a haemodynamically acceptable IHP system. Laboratory data showed a transient increase in values after R-PIHP therapy, but an eventual return to normal or pre-therapy levels. Histological assessment also revealed no evidence of abnormal findings in any of the pigs. Therefore, the R-PIHP technique appears to be a safe percutaneous approach to IHP therapy.

There were two main limitations to the present study. First, we used cisplatin as a reference drug; therefore, we could not directly compare drug leakage between the present study and previous studies that used alkylating agents such as melphalan, which is unavailable in Japan. Second, we did not use a tumour model because no such pig liver tumour model currently exists. Therefore, the efficacy of R-PIHP therapy was not confirmed. This was a pilot study that was designed to study the feasibility, pharmacokinetics, and potential benefits of R-PIHP.

In conclusion, our R-PIHP technique, wherein inflow was achieved via the hepatic artery and outflow was achieved via the portal vein, appears to be a feasible and safe percutaneous approach to IHP therapy.

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RESEARCH ARTICLE

MicroRNA Markers for the Diagnosis of Pancreatic and Biliary-Tract Cancers

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Data Availability Statement: All microarray data from this study are in agreement with the Minimum Information About a Microarray Experiment (MIAME) and are publicly available through the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/projects/geo/) under the accession number GSE59856.

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Abstract

It is difficult to detect pancreatic cancer or biliary-tract cancer at an early stage using current diagnostic technology. Utilizing microRNA (miRNA) markers that are stably present in peripheral blood, we aimed to identify pancreatic and biliary-tract cancers in patients. With "3D-Gene", a highly sensitive microarray, we examined comprehensive miRNA expression profiles in 571 serum samples obtained from healthy patients, patients with pancreatic, biliary-tract, or other digestive cancers, and patients with non-malignant abnormalities in the pancreas or biliary tract. The samples were randomly divided into training and test cohorts, and candidate miRNA markers were independently evaluated. We found 81 miRNAs for pancreatic cancer and 66 miRNAs for biliary-tract cancer that showed statistically different expression compared with healthy controls. Among those markers, 55 miRNAs were common in both the pancreatic and biliary-tract cancer samples. The previously reported miR-125a-3p was one of the common markers; however, it was also expressed in other types of digestive-tract cancers, suggesting that it is not specific to cancer types. In order to discriminate the pancreato-biliary cancers from all other clinical conditions including the healthy controls, non-malignant abnormalities, and other types of cancers, we developed a diagnostic index using expression profiles of the 10 most significant miRNAs. A combination of eight miRNAs (miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, and miR-4476) achieved a sensitivity, specificity, accuracy and AUC of 80.3%, 97.6%, 91.6% and 0.953, respectively. In contrast, CA19-9 and CEA gave sensitivities of 65.6% and 40.0%, specificities of 92.9% and 88.6%, and accuracies of 82.1% and 71.8%, respectively, in the same test cohort. This diagnostic index identified 18/21 operable pancreatic cancers and 38/48 operable biliary-tract cancers in the entire cohort. Our results suggest that the assessment of these miRNA markers is clinically valuable to identify patients with pancreato-biliary cancers who could benefit from surgical intervention.

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: At the time of the study, HS, JK, ST, SK and HN were full-time employees of Toray Industries, Inc. who provides marketed microarray products that were investigated in this study. However, this does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

Introduction

Pancreatic cancer is one of the most lethal cancers. Most pancreatic cancers do not accompany any particular clinical symptoms in the early stage, permitting the cancers to progress undetected. In addition, ambiguous radiological images of cancerous lesions and inflammatory conditions in the pancreas prevent pancreatic cancers from being correctly identified. Furthermore, the anatomical location of the pancreas, deep in a retroperitoneal space surrounded by many other organs, hinders the acquisition of a biopsy. All of these factors prevent the early detection of pancreatic cancer. The American Cancer Association estimated that 40,000 people would die of pancreatic cancer in 2014 in the United States [1]. The five-year survival rate for patients with exocrine pancreatic cancer is estimated at 14% for stage IA, but it drops to 1% for stage IV [1]. Because the most promising treatment for pancreatic cancer is surgical resection, detecting pancreatic cancer at surgically resectable stages is crucial for improving the survival rate of patients with pancreatic cancer. From this point of view, the screening of the early stages of pancreatic or biliary-tract cancers is imperative.

As a diagnostic screening method for pancreatic cancer, ultrasound is one of the most prevalent tests performed. However, this image analysis has its difficulty in differentiating non-malignant tissue from malignant tissue [2]. In addition, many tumor-associated antigens have been studied in connection with pancreatic cancer. The most validated and clinically useful biomarker is carbohydrate antigen (CA) 19–9; however, CA19-9 is known to be up-regulated in other inflammatory conditions, and its low positive predictive value makes it a poor biomarker for screening, limiting its current use mostly to the post-surgical monitoring of progressed pancreatic cancers [3, 4]. Today, there is no effective method to detect early, surgically resectable pancreatic cancers with sufficient diagnostic accuracy.

Recently, microRNAs (miRNAs) have been reported as potential biomarkers for various types of cancers. Using plasma samples from 50 cancer patients and ten healthy control subjects, as well as chemo-resistant pancreatic cell lines, Ali *et al.* suggested that serum miR-21 and other miRNAs could predict the aggressiveness of pancreatic cancer [5]. Ganepola *et al.* examined a dozen of plasma samples each from patients with and without pancreatic cancer, and concluded that three miRNAs, miR-642b, miR-885-5p, and miR-22, were more than 90% sensitive and specific in the diagnosis of pancreatic cancer [6]. Similarly, Li's group examined 20 or less serum samples each from patients with pancreatic cancer and control, respectively, as the biomarker discovery cohort, and found that multiple miRNAs including miR-1290 were useful in the early detection of pancreatic cancer [7]. More recently, researchers have analyzed more than 100 blood samples each from pancreatic cancer patients and controls, and some found miR-155, miR-181a, miR-181b and miR-196a [8], and others found miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191 [9] for blood miRNA markers. It should be noted that each of the previous studies suggested different circulating miRNA markers for pancreatic cancer. The discrepancies of previous studies could be attributed to various empirical factors that include the blood sample types (PBMC, plasma or serum), different detection technologies (PCR, microarray or sequencer), and heterogeneity of the sample cohorts. Especially, the sufficient size and the diversity of the sample cohorts are critical in biomarker research not only for the targeted group but also for the control group.

Here, we examined the expression profiles of comprehensive serum miRNAs from the largest cohorts of patients ever attempted: 100 patients with pancreatic cancer, 98 patients with biliary-tract cancer, 150 healthy control patients, 21 patients with non-malignant abnormalities in the pancreas or biliary tract, and 202 patients with other types of cancers. A highly sensitive microarray permitted the simultaneous analysis of more than 2,500 miRNAs that were recently updated in the miRBase (release 20), and serum samples from patients with the various clinical

conditions allowed us to evaluate a wide range of diagnostic specificity in the detection of pancreato-biliary cancer.

By combining eight miRNA markers, we were able to detect patients with pancreato-biliary cancers among those who were healthy, had non-malignant abnormalities or had other types of cancers, with a diagnostic accuracy of 91.6% and AUC of 0.953.

Methods

Ethics statement

This research on human subjects was approved by the National Cancer Center Hospital East Institutional Review Board (2010-096) and by the Human Tissue Samples Ethics Committee for R&D, Toray Industries Inc. (HC2013-4, 128 and HC2014-4). Written informed consent was obtained from each participant.

Clinical samples

Blood samples were obtained from a total of 421 patients who were admitted to the Japanese National Cancer Center Hospital East during the years 2010 to 2012. One hundred patients with pancreatic cancer, 98 patients with biliary-tract cancer, 50 patients with colon cancer, 50 patients with stomach cancer, 50 patients with esophageal cancer, 52 patients with liver cancer, and 21 patients with non-malignant pancreatic or biliary-tract diseases were registered in the Biobank and selected for use in this study. For pancreatic and biliary-tract cancers, patients with the following characteristics were excluded: (i) patients with intraductal papillary mucinous neoplasm, (ii) patients simultaneously or previously diagnosed with advanced cancer in another organ, (iii) patients with cancer uncertain to be either pancreatic or biliary, and (iv) patients with special histology other than adenocarcinoma. All the patients were histologically confirmed, and those with cancer were pathologically diagnosed as having adenocarcinoma. All blood samples from cancer patients were taken before any treatment except one pancreatic cancer case with ypStage I which had received therapeutic intervention. The detailed information about these patients is shown in Table 1B-D.

Control blood samples were obtained from healthy individuals recruited from the Japanese affiliate companies of Toray Industries Inc. in 2013. Inclusion criteria for healthy control individuals were age greater than 60 years, no history of any cancer, and no hospitalization during the last 3 months (Table 1A).

All peripheral blood samples were processed to serum within a day of acquisition, and serum biomarkers including CA19-9 and CEA were measured before the remaining serum samples were stored at -80°C for miRNA analysis.

Formalin Fixed Paraffin Embedded (FFPE) samples of pancreatic tumors and adjacent normal tissues were obtained from 10 randomly chosen patients with pancreatic cancer (one case of stage IIA, eight cases of stage IIB, and one case of stage III). In the FFPE sections with the size of 10×10μm, tumor area was marked by ink using the neighboring slides that were H.E. stained. The tumor area and the adjacent normal tissue were then macroscopically dissected.

miRNA expression analysis by microarray

For each clinical group, the miRNA expression analysis was performed simultaneously regardless of the training cohort or the test cohort. Total RNA was extracted from each 300-μL serum sample using "3D-Gene" RNA extraction reagent from a liquid sample kit (Toray Industries, Inc., Tokyo, Japan). Total RNA was also obtained from the FFPE sections using the "Arcturus" "Paradise" Extraction and Isolation kit (Life Technologies, Carlsbad, CA, U.S.A.) in accordance

Table 1. Demographics of healthy control individuals (A), patients with pancreatic cancer (B), patients with biliary-tract cancer (C), patients with non-malignant abnormalities (D), and (E) patients with other types of cancer.

A) Healthy control individuals		
Number of patients	-	150
Gender	Male	136
	Female	14
Age	median (range)	62 (60–69)
Serum CA19-9	median (range) U/mL	7.5 (0.5–98)
Serum CEA	median (range) ng/mL	1.9 (0.4–7.2)
B) Patients with pancreatic cancer		
Number of patients	-	100
Gender	Male	64
	Female	36
Age	median (range)	68 (33–81)
Tumor stage	pStage I	1
	pStage II	18
	cStage III	27
	cStage IV	54
Pathological location of tumor	Pancreatic head	46
	Pancreatic body	32
	Pancreatic tail	20
	Pancreatic body/tail	2
Serum CA19-9	median (range) U/mL	580 (0.1–971,000)
Serum CEA	median (range) ng/mL	5.5 (0.7–41.7)
Serum D-Bilirubin	median (range) ng/dL	0.2 (0–16.3)
C) Patients with biliary-tract cancer		
Number of patients	-	98
Gender	Male	63
	Female	35
Age	median (range)	67 (33–86)
Operability	Operable	48
	Inoperable	50
Pathological location of tumor	Intrahepatic bile duct	30
	Extrahepatic bile duct	22
	Gall bladder	29
	Hilar bile duct	9
	Ampulla of Vater	8
Serum CA19-9	median (range) U/mL	70.9 (0.1–68,120)
Serum CEA	median (range) ng/mL	3.2 (0.2–2,030)
Serum D-Bilirubin	median (range) ng/dL	0.2 (0.1–17.2)
D) Patients with non-malignant abnormalities in the pancreas or biliary tract		
Number of patients	-	21
Gender	Male	13
	Female	8
Age	median (range)	56 (33–76)

(Continued)

Table 1. (*Continued*)

Clinical condition	Chronic pancreatitis	9
	Chronic cholecystitis	5
	Abnormal conjunction of pancreatic duct and bile duct	2
	Intrahepatic stone	1
	High level of serum Dupan-2	1
	Bile-duct enlargement	1
	Abnormal pancreatic histology	1
	Acute weight loss	1
Serum CA19-9	median (range) U/mL	10 (0.1–255)
Serum CEA	median (range) ng/mL	1.9 (0.7–12.7)
E) Patients with other types of cancers		
E) -1 Colon cancer		
Number of patients	-	50
Gender	Male	33
	Female	17
Age	median (range)	63 (32–82)
Tumor stages	0	0
	I	18
	II	11
	III	16
	IV	0
	Undetermined	5
Serum CA19-9	median (range) U/mL	13.2* (0.1–3524)
Serum CEA	median (range) ng/mL	2.8 (0.1–268)
E)-2 Stomach cancer		
Number of patients	-	50
Gender	Male	31
	Female	19
Age	median (range)	69 (35–85)
Tumor stages	0	0
	I	35
	II	7
	III	8
	IV	0
	Undetermined	0
Serum CA19-9	median (range) U/mL	10.3 (0.1–77.4)
Serum CEA	median (range) ng/mL	2.7 (0.3–17.5)
E)-3 Liver cancer		
Number of patients	-	52
Gender	Male	40
	Female	10
Age	median (range)	69 (36–84)
Tumor stages	0	0
	I	24
	II	14
	III	10
	IV	2
	Undetermined	0
Serum CA19-9	median (range) U/mL	13.1* (0.1–105)
Serum CEA	median (range) ng/mL	3.2* (0.6–12.1)

(*Continued*)

Table 1. (*Contined*)

E)-4 Esophageal cancer		
Number of patients	-	50
Gender	Male	44
	Female	6
Age	median (range)	69 (47–87)
Tumor stages	0	0
	I	1
	II	5
	III	16
	IV	27
	Undetermined	1
Serum CA19-9	median (range) U/mL	-*
Serum CEA	median (range) ng/mL	3.1* (0.5–14.2)

*CA19-9 and/or CEA scores were not available in some cases.

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with the manufacturer's instructions. Comprehensive miRNA expression analysis was performed using a "3D-Gene" miRNA Labeling kit and a "3D-Gene" Human miRNA Oligo Chip (Toray Industries, Inc.), which was designed to detect 2,555 miRNAs registered in the miRBase release 20 (http://www.mirbase.org/).

Individual miRNAs were regarded as present if the corresponding microarray signals were more than (the mean + 2x standard deviation) of the negative control signals of which the top and bottom 5% ranked by signal intensity were removed. Once the miRNA was regarded as present, the miRNA signal was subtracted with the mean signal of the negative controls of which the top and bottom 5% ranked by signal intensity were removed. When the signal became a negative value (or was undetected) after the background subtraction, the value was replaced by the number that was the lowest signal intensity on the microarray minus 0.1 on a $\log_2$ scale. In order to normalize the signals across the different microarrays tested, quantile normalization was performed [10]. All microarray data from this study are in agreement with the Minimum Information About a Microarray Experiment (MIAME) and are publicly available through the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/projects/geo/) under the accession number GSE59856.

Statistical analysis

In this study, the miRNA expression profiles of the following five paired-clinical conditions were statistically compared: i) pancreatic cancer versus healthy control, ii) biliary-tract cancer versus healthy control, iii) pancreato-biliary cancer versus non-malignant abnormalities, iv) pancreato-biliary cancer versus other types of cancer, and v) pancreato-biliary cancer versus all other clinical conditions (healthy control, non-malignant abnormalities, and other types of cancer). For each condition, patients with pancreatic and/or biliary-tract cancer were regarded as the cancer group, and the other groups were regarded as the control.

After all microarray experiments were performed, the samples of each clinical condition were randomly divided into a training cohort (2/3 of the samples) and a test cohort (1/3 of the samples) by computation. The clinical characteristics of the training cohort and the test cohort was examined and confirmed that there was no significant difference in these two cohorts. The training cohort was used to select significant miRNA markers and to define their discriminant functions, and the test cohort which is independent from the training cohort was used to validate the diagnostic performance of the selected miRNA markers based on the same statistical

significance. The analyses of the training cohort and the test cohort were programmed in advance and sequentially performed without overcrossing those cohorts. In the selection process of miRNA markers, any two clinical groups were compared using two-sided Student's t-test, and a Bonferroni-corrected p-value of <0.01 was regarded as statistically significant. For FFPE analysis, since only eight miRNAs that were preselected was analyzed, Bonferroni correction was not applied to p-value. In order to obtain robust biomarkers, only miRNAs that showed a signal value of 2^6 in more than 50% in either clinical group being compared were selected for further analysis. Using these miRNAs, a Fisher's linear discriminant analysis was performed, and the resulting diagnostic sensitivity, specificity and accuracy were calculated for each miRNA marker or combination of miRNA markers.

When expressions of multiple miRNAs were used in the algorithm development, the discriminant functions were created with Fisher's linear discriminant analysis. The resulting values of the discriminant functions were called as diagnostic indices. Clinical samples that showed the indexed score over 0 were classified to pancreato-biliary cancer, and samples that showed the indexed score under 0 were classified to non-pancreato-biliary cancer (or other clinical conditions). As another statistical tool to analyze biomarkers' diagnostic performance, Receiver Operating Characteristic (ROC) analysis and its Area Under the Curve (AUC) values were also used.

All computations were performed using R version 3.0.2 (R Foundation for Statistical Computing, http://www.R-project.org), MASS package version 7.3–30 [11] and bee swarm package version 0.1.6 [12].

Results

Clinical characteristics of the healthy control individuals and the patients with pancreatic cancer, biliary-tract cancer, other types of cancer, or non-malignant abnormalities in pancreato-biliary tract

The characteristics of 150 healthy control individuals, 100 pancreatic cancer patients, 98 biliary-tract cancer patients, 21 patients with non-malignant abnormalities in those organs, and 202 patients with other types of cancers are presented in Table 1A-E. The median age of the healthy controls was slightly less (62 years of age) than that of the patients with pancreatic cancer (68 years of age) or with biliary-tract cancer (67 years of age). In addition, the healthy control group was clearly dominated by males (91%), while the patients with cancers were less so (64% male for both the pancreatic and biliary-tract cancer groups).

All 100 pancreatic cancer patients had pancreatic ductal adenocarcinoma. Twenty-one out of 100 patients with pancreatic cancer underwent surgical resection (1 ypStage I, 1 ypStage IIB, 17 pStage II, 1 cStage III and 1 cStage IV). All other patients with pancreatic cancer who did not undergo surgical resection were classified as cStage III or IV. On the other hand, biliary-tract cancer cases were classified simply by operable and unoperable cases, due to the complex TNM staging system and distinct biological identities across different primary sites (intrahepatic bile duct, extrahepatic bile duct, gall-bladder, etc.) within biliary tract.

Comparison of pancreatic cancer or biliary-tract cancer with healthy control

Using the training cohort, the patients with pancreatic cancer were statistically compared with the healthy control individuals, and 120 miRNAs showed a potential to separate those two clinical groups with Bonferroni-corrected p-value of less than 0.01 by Student's t-test. Out of the 120 miRNAs selected in the training cohort, 81 miRNAs were statistically validated in the test

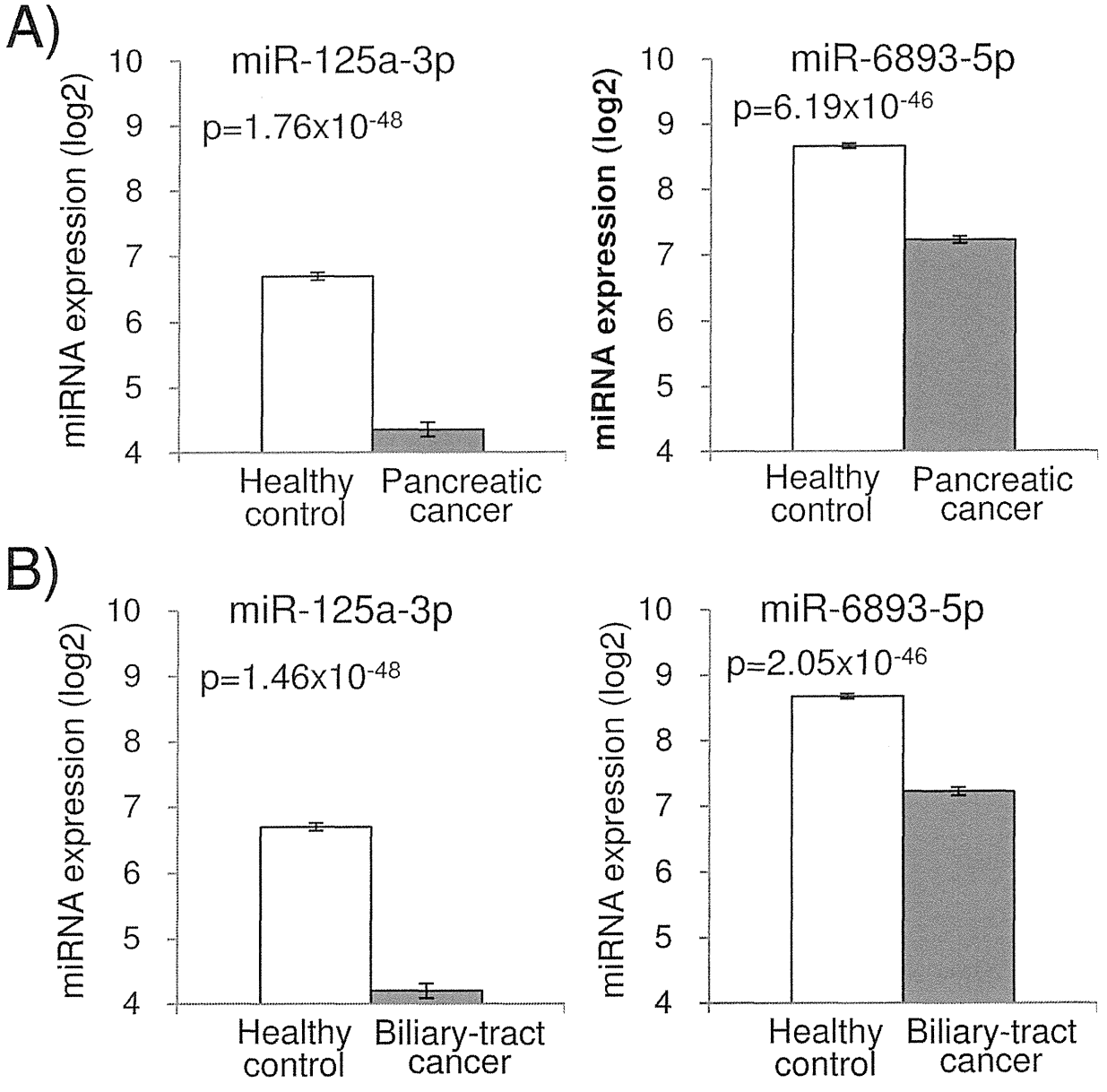

Fig 1. Expression signals of miR-125a-3p and miR6893-5p that showed the smallest p-values in comparison of pancreatic cancer and healthy control (A), or in comparison of biliary-tract cancer and healthy control (B) in the training cohort. The error bars indicate standard error. The p-values were Bonferroni-corrected.

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cohort ($p<0.01$). Among those, 40 miRNAs were up-regulated and 41 miRNAs were down-regulated in pancreatic cancer. The list of these selected miRNAs, their p-values, the expression levels and the diagnostic performance were provided in S1 Table. Fig. 1A shows expression signals of miR-125a-3p and miR-6893-5p which showed the smallest p-values in comparison of pancreatic cancer patients and the healthy control individuals in the training cohort, and were also validated in the test cohort.

The best marker, miR-125a-3p, was able to detect 30 of 33 (90.9%) pancreatic cancers in the test cohort. In contrast, the widely used blood biomarkers for pancreatic cancer, CEA and CA19-9, detected 52 (52.0%) or 77 (77.0%), respectively, out of 100 pancreatic cancers in the entire cohort. Overall, 40 of 81 validated miRNA marker candidates for pancreatic cancer showed sensitivities higher than that of CA19-9 in the test cohort (S1 Table).

Similarly, we performed a comparative analysis of the patients with biliary-tract cancer and the healthy control individuals. One hundred twenty-four miRNAs showed statistical significance ($p<0.01$) in the training cohort, and out of those 124 miRNAs, 66 miRNAs were validated in the test cohort (S1 Table). Among those, 30 miRNAs were up-regulated and 36 miRNAs were down-regulated in biliary-tract cancer. Expression signals of miR-125a-3p and miR-6893-5p which showed the smallest p-values in comparison of biliary-tract cancer and healthy control in the training cohort and also were validated in the test cohort were shown in Fig. 1B. Again, the best marker to detect biliary-tract cancer was miR-125a-3p, and was able to detect 32 of 33 (97.0%) in the test cohort. In contrast, CEA and CA19-9 detected 30 (30.6%) and 62 (63.3%), respectively, out of 98 biliary-tract cancers in the entire cohort. Overall, 61 of 66 validated miRNA marker candidates for biliary-tract cancer, showed sensitivity higher than that of CA19-9 (57.1%) in the test cohort (S1 Table).

We also found that, despite apparent histological differences, a number of miRNAs were selected as markers not only for pancreatic cancer but also for biliary-tract cancer; of the 81 miRNAs selected as marker candidates for pancreatic cancer, 55 (67.9%) were also selected as marker candidates for biliary-tract cancer. In contrast, when miRNAs that could discriminate pancreatic cancer from biliary-tract cancer were sought, none reached statistical significance. The miRNA that showed the smallest Bonferroni-corrected p-value (0.071) in comparison of pancreatic and biliary-tract cancer was miR-1227-5p; however, that miRNA was selected as a marker both for pancreatic cancer (ranked 78) and for biliary-tract cancer (ranked 46) (S1 Table). Those results led us to conclude that the differentiation of pancreatic cancer and biliary-tract cancer by serum miRNAs would be difficult.

This strong overlap in miRNA expression profiles between pancreatic cancer and biliary-tract cancer led us suspect that these miRNA markers were affected by jaundice, a common clinical condition over pancreas and biliary-tract. Therefore, we examined a jaundice marker, blood D-bilirubin, in pancreato-biliary cancer patients. Eighty-one of 100 pancreatic cancer patients and 78 of 98 biliary-tract cancer patients had blood D-bilirubin under the cutoff value of 0.8 mg/dL, and the median value of blood D-bilirubin concentration was 0.2 mg/dL both for pancreatic and biliary tract cancer patients, suggesting that a majority of these patients were non-jaundice. Furthermore, none of those 55 miRNA markers that overlapped in pancreatic cancer and biliary-tract cancer was correlated with D-bilirubin level (the highest Pearson's correlation was -0.37). These results confirmed that the common miRNAs selected above were not likely a marker for jaundice but a marker for pancreato-biliary cancer.

Based on these observations, we therefore decided to pursue the markers that would simultaneously detect both pancreatic and biliary-tract cancers.

Comparison of pancreato-biliary cancer with non-malignant abnormalities, or with other types of cancers

The ideal pancreato-biliary cancer biomarker should prove its specificity not only against healthy control but also against non-malignant disease conditions as well as other types of cancers. In order to test this thesis, we compared 198 pancreato-biliary cancer patients with 21 patients who had some abnormalities in either the pancreas or the biliary tract, but no trace of malignancy (Table 1D). We assumed that obtained miRNAs in this comparison should have

represented markers for malignancy, particularly in pancreato-biliary organs. Thirty-nine miRNAs showed significantly different expressions ($p<0.01$) in these two groups in the training cohort, and out of those 39 miRNAs, 4 miRNAs were validated in the test cohort. These 4 miRNAs (miR-6826-5p, miR-6757-5p, miR-3131 and miR-1343-3p) were unique and have not been identified in our previous analyses of pancreato-biliary cancer in contrast with healthy control (S1 Table). Their median expression levels in the pancreato-biliary cancer group and the non-malignant abnormality group were presented in Fig. 2A. The best marker, miR-6826-5p, correctly identified not only 77.3% pancreato-biliary cancers, but also identified 85.7% non-malignant abnormalities as negative control in the test cohort. In contrast, CEA and CA19-9 correctly identified 41.4% and 70.2% pancreato-biliary cancers, and 81.0% and 81.0% non-malignant abnormalities, respectively, in the entire cohort.

Furthermore, in order to examine organ-specificity of serum miRNA markers in malignant environment, a total of 202 serum samples obtained from patients with colon, stomach, esophagus, and liver cancers (Table 1E) were analyzed, and set as another control against pancreato-biliary cancer. This comparative analysis selected 193 miRNAs in the training cohort, and among them, 120 miRNAs were validated in the test cohort. Out of 120 validated miRNAs, 26 miRNAs have been identified in the previous analysis of pancreato-biliary cancer with healthy control (S1 Table), 4 miRNAs have been identified in the analysis of pancreato-biliary cancer with non-malignant abnormalities (Fig. 2A), and the rest of 90 miRNAs were unique. The expression of 4 miRNAs (miR-6880-5p, miR-6075, miR-4294, miR-187-5p) that were significantly different between pancreato-biliary cancer and other types of cancers were presented in Fig. 2B. These four miRNAs have been also identified in our previous analysis of pancreato-biliary cancer with healthy control (S1 Table), suggesting that they are highly specific markers for pancreato-biliary cancer. In terms of diagnostic performance, miR-6880-5p correctly identified not only 66.7% pancreato-biliary cancer, but also 85.7% other cancer types as negative control in the test cohort. In contrast, CEA and CA19-9 correctly identified 41.4% and 70.2% pancreato-biliary cancers, as stated above, and 82.0% and 84.4% other cancer types in the entire cohort.

The comparative analyses so far suggested that the resulting miRNA markers for pancreato-biliary cancers would largely affected by clinical characteristics of the control population. Fig. 3 shows how the significant levels of the miRNA markers that were obtained so far were distributed depending on the types of the control populations used.

Comparison of pancreato-biliary cancers with all other clinical groups as control that included healthy individuals, and patients with other types of cancers or non-malignant abnormalities in the pancreato-biliary organs

In order to obtain more robust and universal markers that could identify pancreato-biliary cancer patients from clinically heterogeneous population, the supposed-control: healthy individual, patients with non-malignant abnormalities, and patients with other cancer types, were all combined, and compared with pancrato-biliary cancer patients by discriminant analysis. The analysis identified 143 miRNAs that showed differential expression in the training cohort. Among them, 98 miRNAs were statistically validated in the test cohort. Ninety-one out of those 98 validated miRNAs were already identified in the previous analyses of pancreato-biliary cancer either with healthy control, with non-malignant abnormalities, or with other types of cancer. The 10 miRNAs that showed the smallest Bonferroni-corrected p-values are listed in Table 2. All 10 markers were previously selected as markers for detecting pancreatic cancer and biliary-tract cancer against the healthy control individuals (S1 Table). The best miRNA marker for detecting pancreato-biliary cancers among the other clinical conditions was miR-6075,

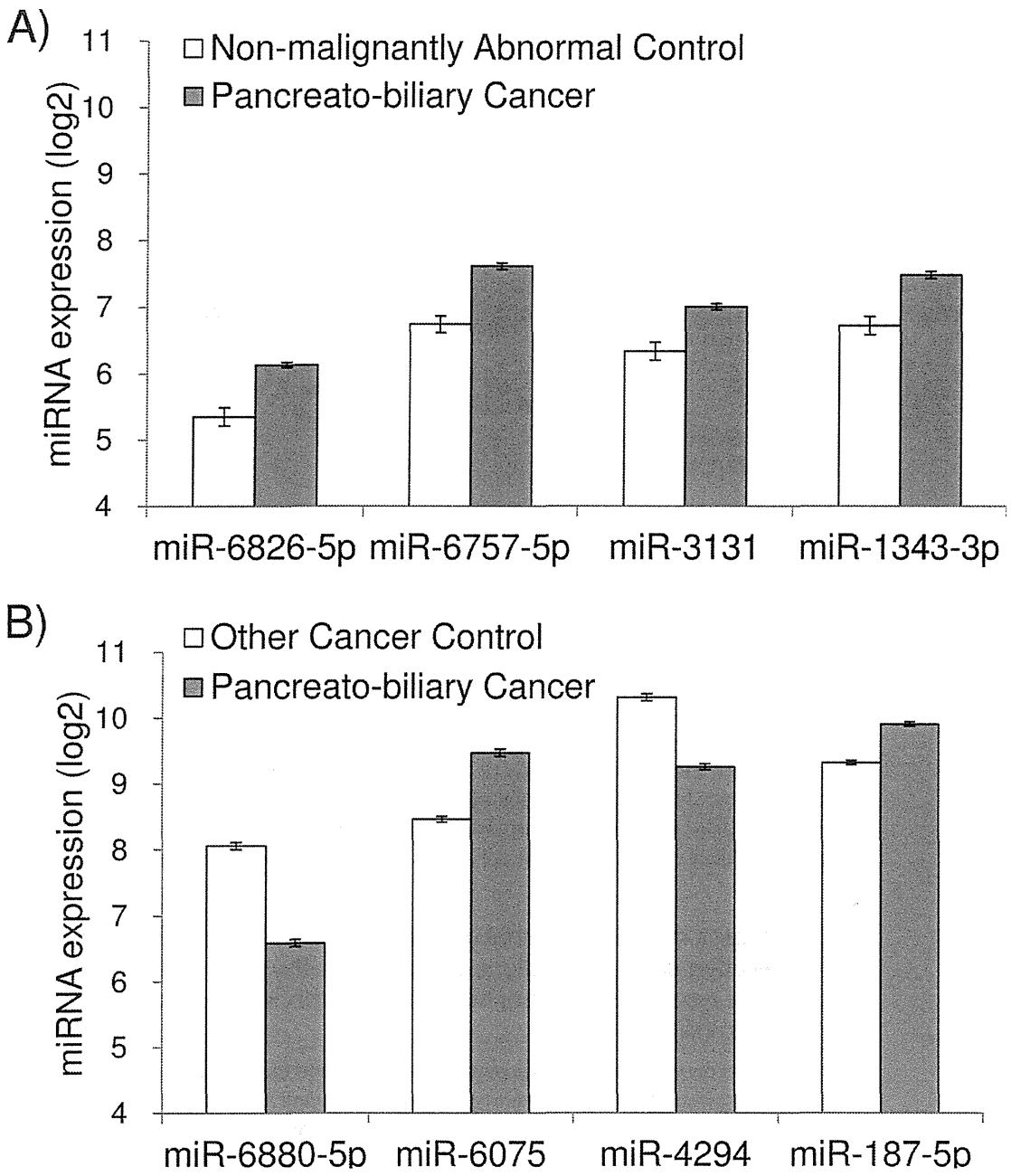

Fig 2. Expression signals of validated miRNAs that differentiated pancreato-biliary cancer from non-malignant abnormalities (A), or from cancers of other types (B). The error bars indicate standard error. The p-values were Bonferroni-corrected.

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which was ranked fourth among the markers for detecting pancreatic cancer and seventeenth among the markers for detecting biliary-tract cancer (S1 Table) against the healthy controls. miR-6075 showed a sensitivity, a specificity, and an accuracy of 63.6%, 93.5%, and 83.2%, respectively (Fig. 4A). Similarly, the second-best marker for detecting pancreato-biliary cancers against the other clinical conditions was miR-4294, which was ranked seventh among the

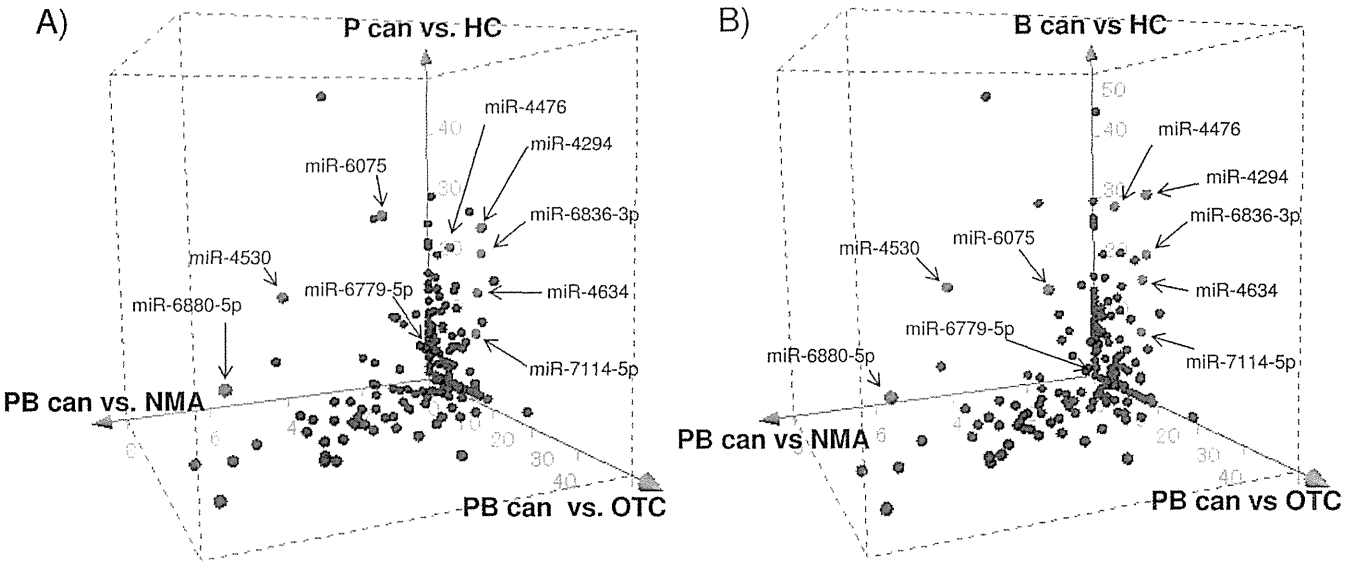

Fig 3. Plots of p-values in the analysis of pancreatic cancer (P can) (A) or biliary-tract cancer (B can) (B) with healthy control (HC) in z-axis, pancreato-biliary cancer (PB can) with non-malignant abnormalities (NMA) in y-axis, or pancreato-biliary cancer (PC can) with other types of cancer (OTC) in x-axis. The absolute values of the exponents of p-values on a $\log_{10}$ scale were plotted; therefore, miRNAs that were located in further outside are more statistically significant. The ten miRNA used in the diagnostic indices were specified.

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markers for detecting pancreatic cancer and fourth among the markers for detecting biliary-tract cancer (S1 Table). It turned out that miR-125a-3p, which was the best marker for detecting both pancreatic and biliary-tract cancers against healthy controls, also behaved similarly for the colon, stomach, liver, and esophageal cancers (Fig. 4B), resulting in a drop in its rank to fifth when the controls included the patients with non-malignant abnormalities and other cancer types (S1 Table and Table 2). This result indicated that miR-125a-3p was not a marker that is specific to pancreato-biliary cancers but was instead one that is effective for a broad range of cancer types.

A diagnostic miRNA index that differentiated pancreato-biliary cancers from all other clinical conditions including healthy controls and patients with other types of cancers or non-malignant abnormalities in the pancreato-biliary organs

According to the data above, a single serum miRNA achieved the detection of pancreatic and biliary-tract cancers with >80% accuracy. We aimed to achieve higher discriminant performance by combining multiple significant miRNAs. The 10 miRNAs (miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, miR-4634, miR-7114-5p, and miR-4476) listed in Table 2 were selected as potential markers in the training cohort, and their expression signals were utilized in developing diagnostic indices. Using the Fisher's linear discriminant analysis, all 1,023 combinations that included any one or more of the 10 miRNAs were calculated and validated in the test cohort. The miRNA combinations that showed the best accuracy with each number of miRNAs used (from 1 to 10) in this algorithm are listed in Table 3, and their discriminant functions are provided in S2 Table. It turned out that using only the single best miRNA, miR-6075, gave an accuracy of 84.7% in the detection of pancreato-biliary cancers in the test cohort; however, the accuracy increased when the number of miRNAs used in the algorithm increased, and it reached a maximum of 91.6% when four

Table 2. Top 10 validated miRNA markers that differentiated pancreato-biliary cancers from other clinical conditions including healthy controls, non-malignant abnormalities, and other types of cancers.

Rank	miRNA	Training cohort						Test cohort		
		p-value	Expression (median in log2)		Accuracy (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)	Sensitivity (%)	Specificity (%)
			Pancreato-biliary cancer	All other clinical groups						
1	miR-6075	8.12E-47	9.46	8.43	85.6	78.0	89.6	83.2	63.6	93.5
2	miR-4294	7.92E-42	9.25	10.39	78.7	77.3	79.5	76.8	66.7	82.3
3	miR-6880-5p	1.02E-33	6.60	7.63	78.2	78.8	77.9	83.2	75.8	87.1
4	miR-6799-5p	1.39E-30	7.88	8.24	79.3	83.3	77.1	78.4	69.7	83.1
5	miR-125a-3p	2.01E-28	4.08	6.02	75.6	82.6	71.9	78.4	81.8	76.6
6	miR-4530	4.55E-28	8.76	9.39	77.4	80.3	75.9	77.9	71.2	81.5
7	miR-6836-3p	1.09E-27	9.25	8.70	80.8	80.3	81.1	84.7	89.4	82.3
8	miR-4634	1.68E-26	10.08	9.85	76.4	81.8	73.5	74.2	75.8	73.4
9	miR-7114-5p	5.32E-25	6.59	6.91	76.6	77.3	76.3	70.0	63.6	73.4
10	miR-4476	1.53E-23	5.79	7.01	76.9	73.5	78.7	81.6	71.2	87.1

p-values were corrected for Bonferroni.

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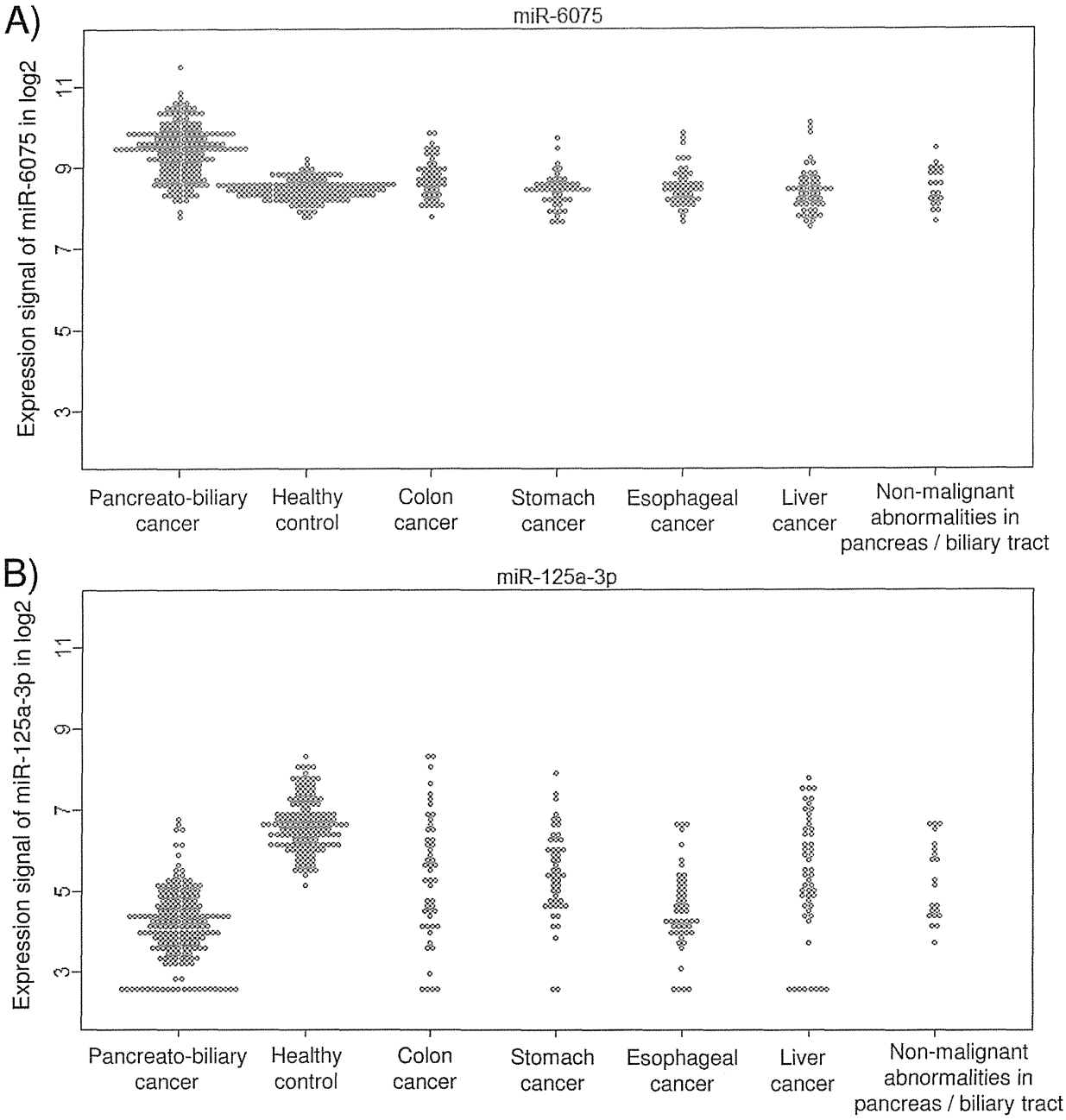

Fig 4. Plots of miR-6075 (A) and miR-125a-3p (B) expression signals on a log2 scale in patients with pancreato-biliary cancers, healthy control individuals, and patients with colon, stomach, esophageal, or liver cancer, or with non-malignant abnormalities in either the pancreas or the biliary tract.

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miRNAs, miR-6075, miR-6799-5p, miR-125a-3p and miR-6836-3p, were used. This combination also gave a sensitivity of 81.8% and a specificity of 96.8%. The discriminant function composed by these four miRNAs is: 1.20 x miR-6075-0.93 x miR-6799-5p—0.22 x miR-125a-3p + 0.71 x miR-6836-3p—8.55 (S2 Table), and the resultant discrimination of each clinical samples

Table 3. Combinations of miRNAs that showed the highest accuracy for each number of miRNAs used (one to ten) and their diagnostic performance in the test cohort.

Number of miRNAs used	Combinations of miRNAs	Accuracy (%)	Sensitivity (%)	Specificity (%)	AUC
1	miR-6836-3p	84.7	89.4	82.3	0.930
2	miR-6075, miR-6836-3p	89.5	75.8	96.8	0.941
3	miR-6075, miR-6836-3p, miR-4476	91.1	78.8	97.6	0.949
4	miR-6075, miR-6799-5p, miR-125a-3p, miR-6836-3p	91.6	81.8	96.8	0.949
5	miR-6075, miR-4294, miR-6799-5p, miR-125a-3p, miR-6836-3p	91.6	81.8	96.8	0.948
	miR-6075, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p	91.6	81.8	96.8	0.950
6	miR-6075, miR-4294, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p	91.6	81.8	96.8	0.948
	miR-6075, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-6836-3p, miR-4476	91.6	80.3	97.6	0.955
7	miR-6075, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, miR-4476	91.6	80.3	97.6	0.953
8	miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, miR-4476	91.6	80.3	97.6	0.953
9	miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, miR-7114-5p, miR-4476	90.0	80.3	95.2	0.967
	miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-6836-3p, miR-4634, miR-7114-5p, miR-4476	90.0	78.8	96.0	0.965
10	miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, miR-4634, miR-7114-5p, miR-4476	88.9	78.8	94.4	0.966

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is shown in Fig. 5. The same level of accuracy was maintained when the number of miRNAs used in the algorithm was increased up to eight, and it then started to decrease when the miRNAs ranked ninth (accuracy 90.0%) and tenth (accuracy 88.9%) were added. Beside accuracy obtained by Fisher's linear discriminatory analysis, ROC analysis gave slightly different result (Table 3); the AUC value started as 0.930 with the single best miRNA, miR-6075, and gradually increased with some fluctuation, and reached the maximum of 0.967 with a combination of the nine miRNAs (miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, miR-7114-5p, and miR-4476). Both analyses indicated that the diagnostic performance would reach to saturation when combinations of the ten miRNAs were used in the algorithm. The AUC of CEA and CA19-9 was 0.682 and 0.845, respectively, in the same test cohort (Fig. 6). No correlation was found between the expression of either CEA or CA19-9 and the expression of any of these ten miRNA markers used in the diagnostic indices.

We then focused on the algorithm that used the combination of four miRNAs: miR-6075, miR-6799-5p, miR-125a-3p, and miR-6836-3p, which was the combination with the least number of miRNAs that achieved the best accuracy calculated (Table 3). The number of positive and negative samples in each clinical group and cohort based on that miRNA discriminant, CEA or CA19-9 test are shown in Table 4. The discriminant index identified pancreato-biliary cancers with equivalent diagnostic power regardless of the tumor operability or location. Five (one ypStage I, 1 ypStage IIB, 2 pStage II and 1 cStage III) of 7 operable pancreatic cancers and 17 of 22 operable biliary-tract cancers in the test cohort were correctly detected by the index. When the entire cohort was included in the analysis, the index detected 18 of 21 (85.7%) operable pancreatic cancer and 38 of 48 (79.2%) operable biliary-tract cancer. It should be noted, however, that the discriminant performance of the index was not correlated with the tumor stage. Among the 22 CA19-9-negative patients with either pancreatic cancer or biliary-tract cancer in the test cohort, 20 (83.3%) were correctly detected by the discriminant index. Finally,