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Aberrant Methylation of Tumor Suppressive miRNAs in Bile from Patients With Pancreaticobiliary Diseases

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Abstract. *Background/Aim: Epigenetic abnormalities in microRNAs (miRNAs) have not been analyzed in samples other than pancreaticobiliary tissues in patients with pancreaticobiliary cancer (PBC). To identify miRNAs specific for PBC, the present study analyzed the methylation of tumor-suppressive miRNAs in bile from patients with pancreaticobiliary diseases. Materials and Methods: Bile was collected endoscopically or percutaneously from 52 patients with pancreatic cancer, 26 with biliary tract cancer, and 20 with benign pancreaticobiliary diseases. Sequences encoding 16 tumor-suppressive miRNAs were amplified by polymerase chain reaction and sequenced, and their methylation rates were determined. Results: The methylation rates of miR-1247 and miR-200a were significantly higher in patients with pancreatic cancer, and biliary tract cancer than in those with benign diseases, and the methylation rate of miR-200b was significantly higher in patients with pancreatic cancer than in those with benign diseases. Conclusion: Methylation of miR-1247, miR-200a, and miR-200b in bile may be useful for distinguishing PBC from benign diseases.*

MicroRNAs (miRNAs) are non-coding RNAs 20-25 nucleotides in length that play important roles in cell survival, proliferation, differentiation and apoptosis, as well as in angiogenesis (1, 2). Dysregulation of miRNAs has been associated with the development of various cancer types (3, 4). This dysregulation may be caused by the gain, loss or translocation of chromosomal regions; aberrant expression of miRNAs; activation of transcriptional factors; epigenetic alterations; and changes in miRNA processing (5).

Aberrant expression of miRNAs has been observed in pancreatic cancer (PC) and biliary tract cancer (BTC). For example, miRNAs have been reported dysregulated in PC samples compared to samples of normal pancreas and chronic pancreatitis tissue (6-10), and in BTC tissues compared to samples of benign biliary disease tissue (11, 12). miRNAs can be classified as oncogenic or tumor-suppressive (13), with tumor progression enhanced by increased expression of oncogenic miRNAs or reduced expression of tumor-suppressive miRNAs.

Tumor-suppressor genes in various types of cancers are silenced by epigenetic mechanisms, including DNA hypermethylation and histone acetylation or hypermethylation (14, 15). Hypermethylation has been observed in tumor-suppressor genes, such as *p16* (16) and Ras-association domain family 1 (*RASSF1A*) (17) in PC and adenomatous polyposis coli (*APC*) and Src homology region 2 domain-containing phosphatase-1 (*SHP1*) (18) in BTC. Silencing of tumor-suppressive miRNAs due to epigenetic abnormalities promotes the development and progression of cancer. In addition, aberrant methylation of miRNAs has been reported in PC (19-24) and BTC (25).

To date, miRNAs have been analyzed in samples of pancreatic tissues (6-10), pancreatic juice (26, 27), serum (28, 29), and plasma (30, 31) from patients with PC, and in biliary tissues (32), serum (33), and plasma (34) from patients with BTC. However, miRNAs have rarely been analyzed in bile samples from patients with pancreaticobiliary cancer (PBC) (35-37). Moreover, to our knowledge, epigenetic abnormalities of miRNAs have not been analyzed in samples other than pancreaticobiliary tissues in patients with PBC. In order to identify miRNAs specific for PBC and detect PBC at an early stage, the present study analyzed the methylation

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Key Words: miRNA, methylation, bile, pancreatic cancer, biliary tract cancer.

Table I. *Patient characteristics*.

Characteristic		Pancreatic cancer (n=52)		Biliary tract cancer (n=26)		Benign pancreaticobiliary disease (n=20)	
Age, years	Mean (range)	66.6 (44-94)		70.5 (52-90)		61.0 (23-82)	
Gender	Male/female	28/24		17/9		12/8	
Location, n		Head	45	Bile duct (intrahepatic)	3	Chronic pancreatitis	2
		Body/tail	7	Bile duct (hilar)	4	Autoimmune pancreatitis	4
				Bile duct (distal)	9	Hepatocholithiasis	1
				Gallblader	8	Choledocholithiasis	3
				Ampulla	2	Choledochocystolithiasis	1
						Gallbladder polyp	1
						Gallbladder adenomyomatosis	1
						Cholangitis	2
						IgG4-related sclerosing cholangitis	1
						Xanthogranulomatous cholecystitis	2
						Benign biliary stricture	1
						Pancreaticobiliary maljunction	1
Stage*, n		IA	3	IA	1		
		IB	6	IIB	7		
		IIB	4	IIIA	4		
		III	10	IIIB	4		
		IV	29	IVA	3		
				IVB	7		

*Union for International Cancer Control (38).

of tumor-suppressive miRNAs in bile samples from patients with pancreaticobiliary diseases.

Materials and Methods

Bile collection and patient characteristics. Bile was obtained from 98 patients with pancreaticobiliary diseases, including 52 with PC, 26 with BTC, and 20 with benign pancreaticobiliary diseases (BD) by endoscopic retrograde cholangiopancreatography or percutaneous transhepatic biliary drainage at the Kanazawa University Hospital, Japan, from 2012 to 2018.

Of the 52 patients with PC, 45 had tumors located in the pancreatic head and seven in the pancreatic body/tail. According to their classification by TNM [eighth edition (38)], the majority of tumors (39/52, 75%) were stage III or higher at the time of bile collection. Of the 26 patients with BTCs, three had intrahepatic, four had hilar, and nine had distal bile duct cancer; eight had gallbladder cancer; and two had ampullary cancer. According to their TNM classification, the majority of tumors (18/26, 69%) were stage III or higher at the time of bile collection. The 20 patients with BD included four with autoimmune pancreatitis and three with choledocholithiasis. The demographic and clinical characteristics of these patients are shown in Table I.

The protocol of this study was approved by the Ethics Committee of Kanazawa University (approval number 2351-3) and conformed to the Declaration of Helsinki. Written informed consent was obtained from each patient.

DNA methylation analyses. Cell-free DNA was extracted from 300 μl aliquots of bile by Maxwell RSC (Promega, Madison, WI, USA) using a Maxwell RSC ccfDNA Plasma Kit (AS 1480; Promega). The cell-free DNA samples were treated with sodium bisulfite modification using an EZ DNA Methylation Lightning kit (Zymo Research, Irvine, CA, USA). Sequences of 16 tumor-suppressive miRNAs (*miR-26a1, miR-29c, miR-30d, miR-31, miR-34bc, miR-96, miR-126, miR-130b, miR-145, miR-192, miR-200a, miR-200b, miR-345, miR-615-5p, miR-1247* and *miR-1254-1*), all of which have been reported to be down-regulated in tumor relative to normal tissue (7, 21, 24, 39-49), were amplified by FastStart Taq DNA Polymerase (Roche, Basel, Switzerland). The primers for bisulfite polymerase chain reaction (PCR) were designed using MethPrimer (http://www.urogene.org/methprimer/) as shown in Table II. For next-generation sequencing, amplicon libraries were generated by an Ion Plus Fragment Library Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The libraries were re-loaded into the Ion Chef instrument, and templates were prepared using Ion PGM Hi-Q View Kit (Thermo Fisher Scientific). Templates were loaded onto the 318v2 chip and sequenced on PGM, followed by signal processing and base calling were performed using Torrent Suite 5.0.2 (Thermo Fisher Scientific). Methylation analysis was performed using a Methylation Analysis_Amplicon plug-in v1.3 (Thermo Fisher Scientific). The amplicon containing the three miRNAs (*miR-200a, miR-200b,* and *miR-1247*) analyzed in this study are shown in Figure 1. The base sequences of the CpG islands of the three miRNAs as above are also shown in Figure 1. Eight, nine, and 12 sites of *miR-1247, miR-200a,* and *miR-200b*, respectively, were selected.

Immunohistochemistry of regulator of chromosome condensation 2 (RCC2). Pancreatic carcinoma tissue samples were obtained at diagnosis of PC by endoscopic ultrasound guided fine-needle aspiration (EUS-FNA), fixed in formalin and embedded in paraffin. Four-micrometer-thick sections were cut for immunohistochemistry.

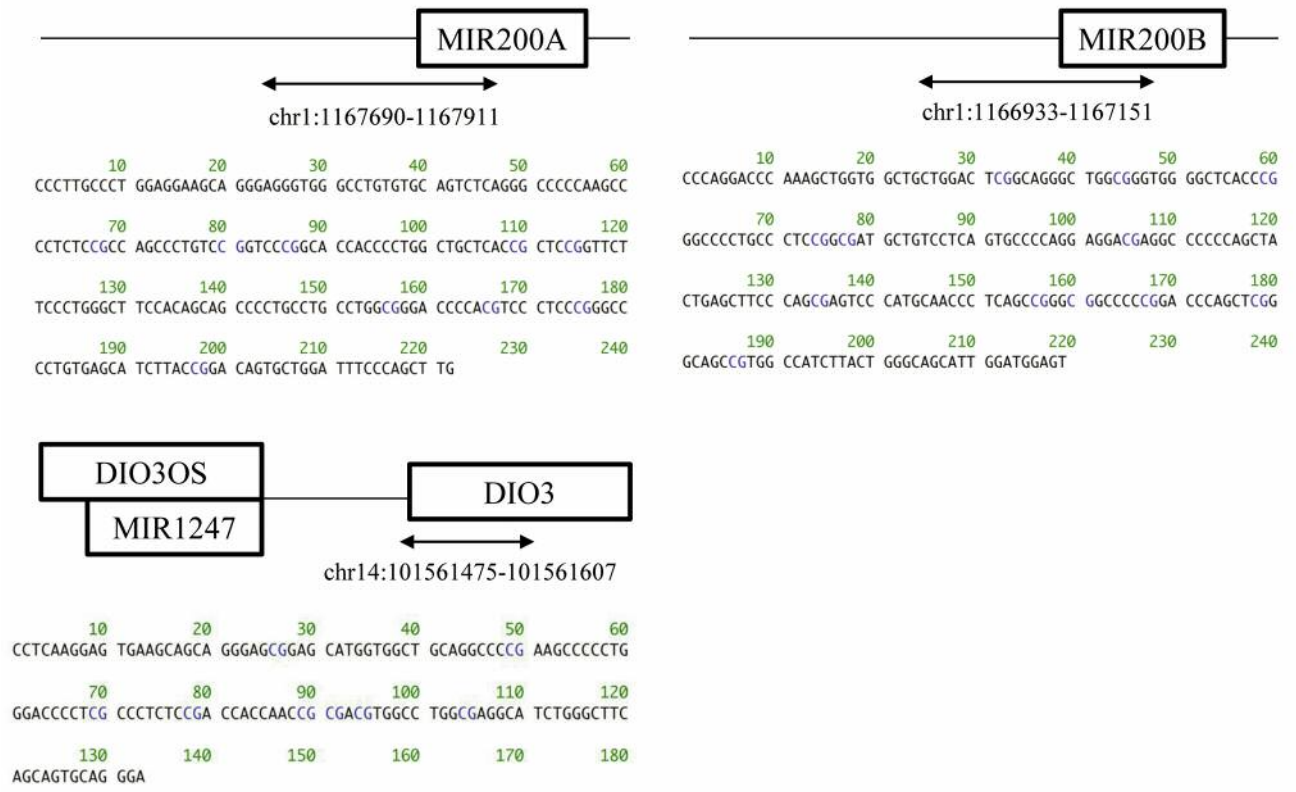

Figure 1. *Base sequences indicate amplicons of CpG islands of miR-200a, miR-200b and miR-1247. CpG islands are indicated by blue characters. PC: Pancreatic cancer; BTC: biliary tract cancer; BD: benign pancreaticobiliary disease.*

Table II. *Primer sequences of miRNAs.*

	miR-	Forward primer	Reverse primer	Chromosome no.	bp	CpG no.
1	26a1	TATTGTTTAGGTTTTTTTTAGTTA	AACCAAAAACTCAAATTATTC	3	140	11
2	29c no. 1	TTGGAGTTTTTTTTAGAGATAGGGT	TCCCAAAATTTAACCATAAACTTAC	1	116	7
	29c no. 2	GGGTAGGGGAGAGGGTAGTATATT	ATCAACCTATATAAAAAATAAACCAATAAT	1	186	6
3	30d	GTTTATTTTTGGTTTTTATTTAGA	ACAATTACAAAAAACTCTACCCTC	8	161	11
4	31	GGTTGAGGATAGGAGAGAGGTTATT	AATCCACCTACAAAAACCAAAACT	9	209	8
5	34bc	GAGAGAGTTAGTTTTAGGGTTTGGG	AAAAATACCAAACCTCCCCTTC	11	164	8
6	96	GTTTATTTTAAGAGTTATTGGGTTT	ACAACCTCCACCACTACTC	7	188	10
7	126 no. 1	GTTATGTGGTTTTAGAGGAGATTTG	AACCCTTTACTAACTTTCAAACCC	8	195	12
	126 no. 2	GTTTTATATTAGTTAAGAAGGTAGAAGTGT	AAAAAATCAAAACTAAAATCTCAAC	8	212	19
	126 no. 3	GGTTTATTTTTTTATTTTTAAGTTTA	ACTCAACACAAAATCCAATCCTAC	8	208	13
8	130b	ATTTAGTGTAGGGTAAGGGGTTAGG	CCCAAACTAAAAAATTATTCCAATC	22	257	23
9	145	TGGTAGGAGATTGGGGAATATATAT	ACCCCATCTATAACAACCAAAATAA	5	184	7
10	192 no. 1	AGAGGTAGATGGGGTTTAGTTTGAT	CCCTAAACCCAAAACCTTTTAAA	11	174	6
	192 no. 2	GGGTTATGAGTAGAAGGGGTTG	CCCCAAACTCAATCTTAAACACTAT	11	201	16
11	200a	TTTTTGTTTTGGAGGAAGTAGG	CAAACTAAAAAATCCAACACTATCC	1	222	9
12	200b	TTTAGGATTTAAAGTTGGTGGTTGT	ACTCCATCCAATACTACCCAATAAA	1	219	12
13	345	TTTGGATTGGGTTGTAGAGTG	AATATCAAAAACTCCTAAAAAACC	14	209	18
14	615 no. 1	GAAAGATTTTAAAATGAAAAGTAAAGAGGT	AAAACCCAATACTCCAACACCTAC	12	155	9
	615 no. 2	AGGTTTTTTTGGGTTTTTTG	CCTAAATAATACTTCTTTACTAATCTTTC	12	203	14
15	1247	TTTTAAGGAGTGAAGTAGTAGGGAG	TCCCTACACTACTAAAACCCAAATAC	14	133	8
16	1254-1	GATGGGGTTTTATTATGTTAGTTAGTATG	CCCACCCTATATCCAAATATTCTC	10	216	12

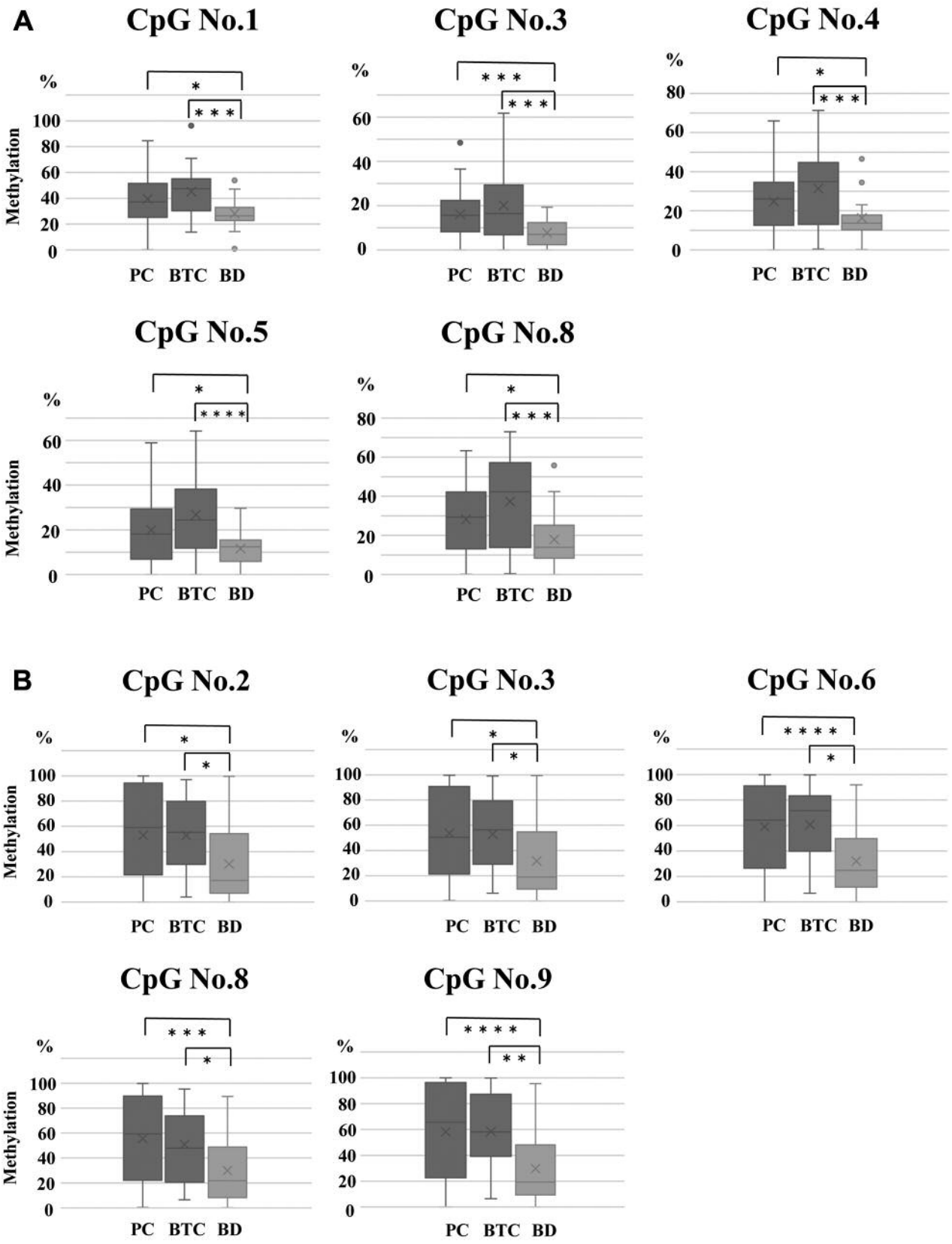

Figure 2. *Continued*

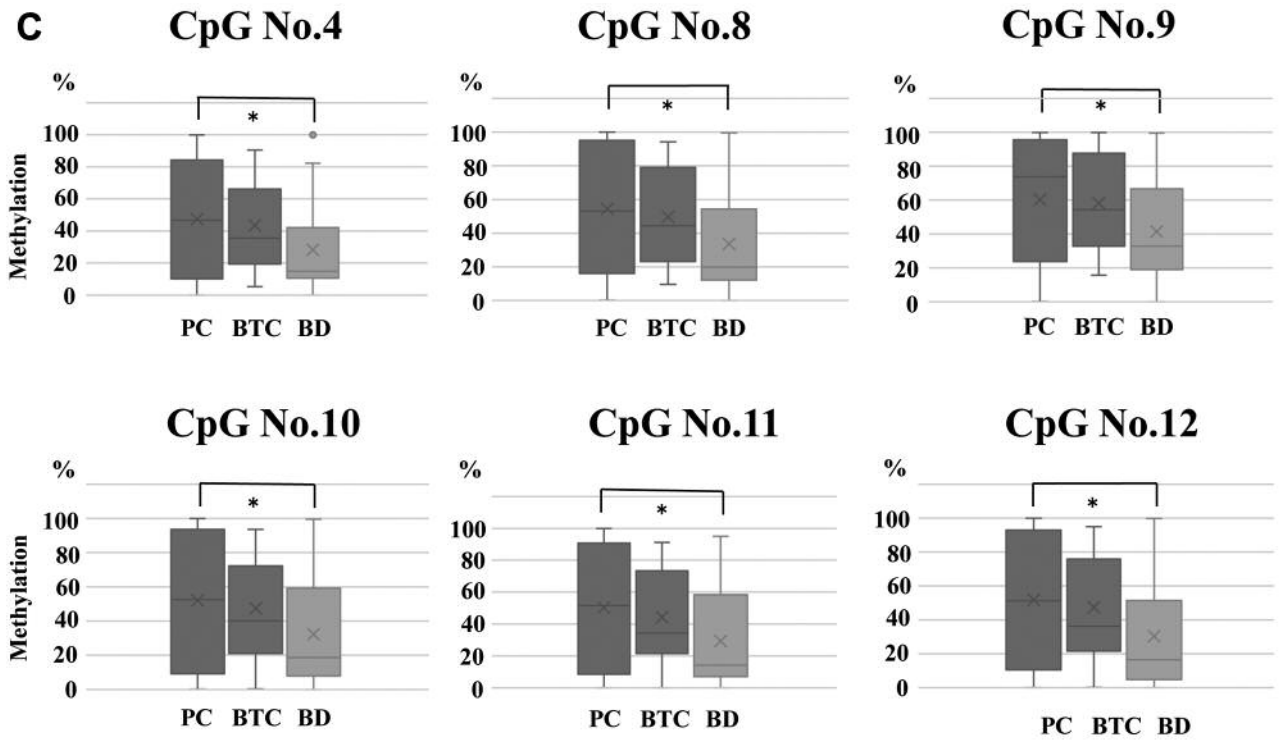

Figure 2. *Methylation rates of miR-1247 (A), miR-200a (B), and miR-200b (C) in bile from patients with pancreaticobiliary diseases. Box plots show methylator of each CpG island of miRNAs measured in bile. Rates of miR-1247 and miR-200a methylation were significantly higher in patients with pancreatic cancer (PC) and biliary tract cancer (BTC) than in patients with benign pancreaticobiliary disease (BD), whereas the rate of miR-200b methylation was significantly higher in patients with PC than in patients with BD. Significantly different at: *p<0.05, **p<0.01 ***p<0.005 and ****p<0.001.*

Immunohistochemical staining of RCC2 was performed as described elsewhere (48), using a rabbit polyclonal antibody to RCC2 (Abcam, Cambridge, UK) as primary antibody. Staining for RCC2 was evaluated as the percentage of cancer cells with positive nuclear staining (0-100%) compared with the staining of the internal control (surrounding lymphoid and acinar cells). Samples were evaluated independently by two investigators (KO, KN) blinded to clinical information, with differences resolved by consensus.

Statistical analyses. All statistical analyses were performed using Stat Mate version IV (ATMS, Tokyo, Japan) and Stata (StataCorp LLC, College Station, TX, USA) statistical software. Differences in methylation rates among the three groups of patients were determined by two-sided *t*-tests, with $p<0.05$ indicating statistical significance. When statistical significance was observed, the ability of each methylated miRNA to distinguishing PBC from BD was assessed by receiver operating characteristic (ROC) curve analysis. High, moderate, and low accuracy were defined as areas under the curve (AUC) of >0.9, 0.7-0.9, and <0.7, respectively.

Results

Methylation analyses. The methylation rates at five (numbers 1, 3, 4, 5, and 8) out of eight CpG sites in *miR-1247* were significantly higher in patients with PC and BTC than in those with BD (Figure 2A). In addition, methylation rates at the other three CpG sites (numbers 2, 6 and 7) in *miR-1247* were significantly higher in patients with BTC than in those with PC and BD (data not shown). Methylation rates at five (numbers 2, 3, 6, 8, and 9) out of the nine CpG sites in *miR-200a* were also significantly higher in patients with PC and BTC than in those with BD (Figure 2B), and methylation rates at six (numbers 4 and 8-12) of the 12 CpG sites in *miR-200b* were significantly higher in patients with PC than in those with BD (Figure 2C). In contrast, evaluation of *miR-126* showed methylation rates at nine out of the 13 CpG sites were significantly higher in the BD than in the PC and BTC groups, and that methylation rates at two other CpG sites were significantly higher in the BD than in the PC group (data not shown). Sequencing of *miR-615-5p* showed that methylation rates at one of 13 CpG sites were significantly higher in the BTC than in the PC and BD groups, that methylation rates at a second CpG site were significantly higher in the BTC than in the PC group, and that methylation rates at a third CpG site were significantly higher in the BD than in the PC group (data not shown). However, in other 11 miRNAs, no significant differences of methylation rates were not observed among PC,

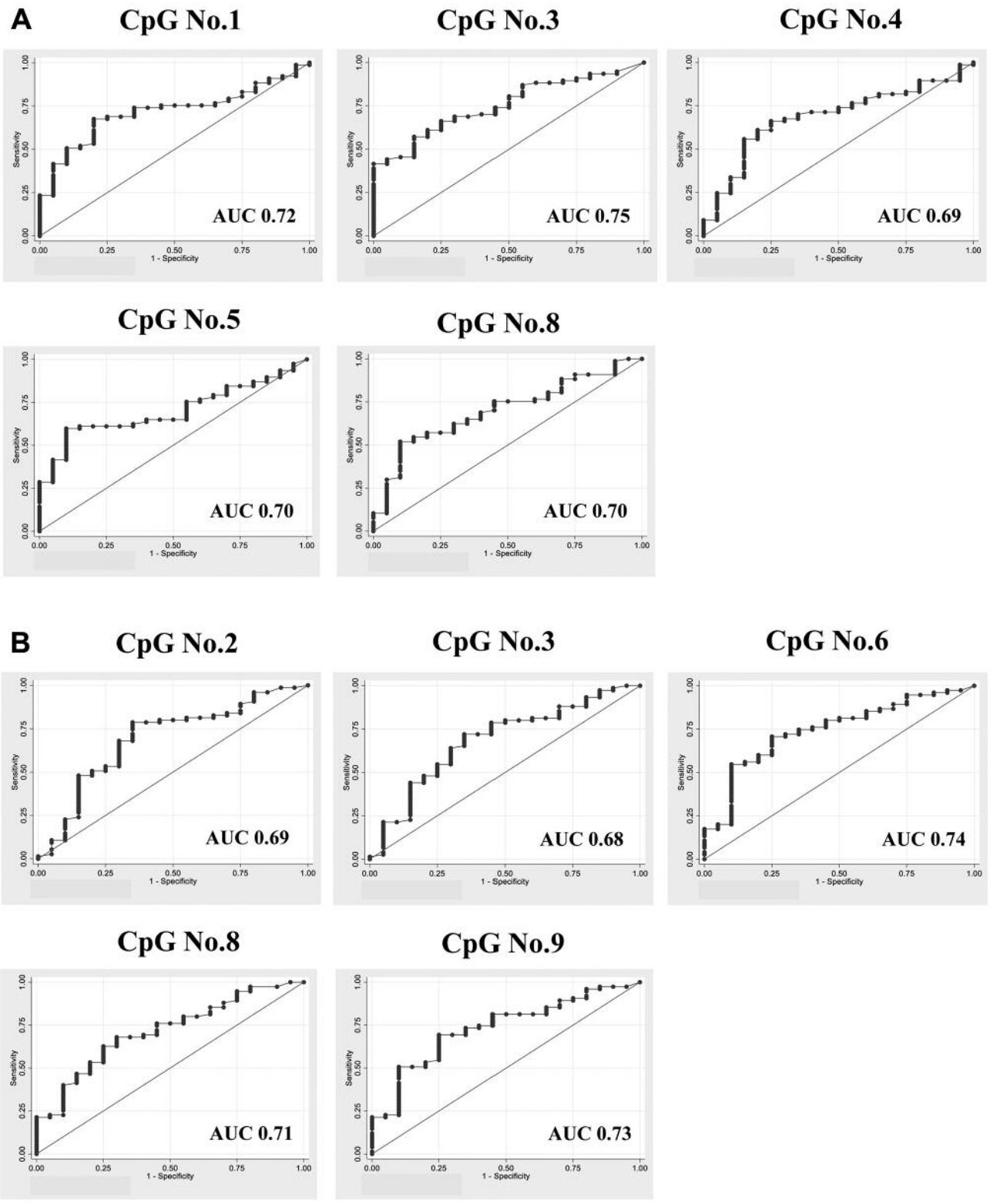

Figure 3. *Continued*

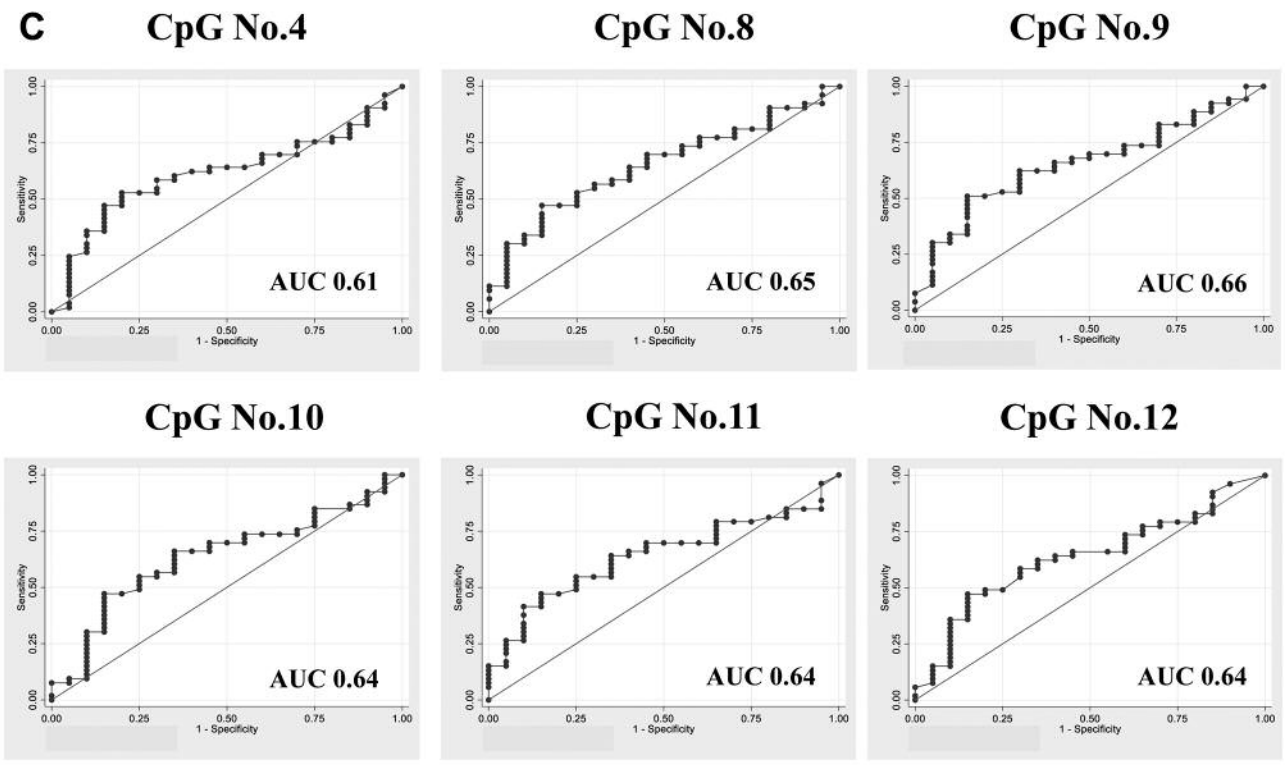

Figure 3. *Receiver operating characteristic curves showing the performance of each site of CpG island methylation of miR-1247 (A), miR-200a (B), and miR-200b (C) in bile distinguishing patients with pancreaticobiliary cancer from patients with benign pancreaticobiliary diseases. AUC: Area under the receiver operating characteristics curve.*

BTC, and BD. Because methylation rates of *miR-1247, miR-200a* and *miR-200b* were significantly higher in patients with stages I and II PC/BTC than in patients with BD, this study focused on these three miRNAs.

ROC curves of the methylation rates of these miRNAs significantly differed among the three groups of patients. Compared with the BD group, the AUCs at four CpG sites (numbers 1, 3, 5, and 8) in *miR-1247* were >0.7 in the PBC groups (*i.e.* those with PC and BTC groups; Figure 3A), with the maximum AUC being 0.75 for CpG site 3. Although an AUC >0.7 was observed at one CpG site in *miR-1247* (number 6) in BTC relative to the BD group, AUCs >0.7 were not observed at three CpG sites (numbers 2, 6, and 7) in BTC relative to the PC group (data not shown). Compared with the BD group, the AUCs at three CpG sites in *miR-200a* (numbers 6, 8, and 9) were >0.7 in the PBC groups (Figure 3B), with the maximum AUC being 0.74 at CpG site 6. In contrast, none of the AUCs at six CpG sites in *miR-200b* (numbers 4 and 8-12) were >0.7 when comparing the PC and BD groups (Figure 3C). Taken together, these results suggest that *miR-1247* and *miR-200a* may be biomarkers distinguishing PBCs from BD.

Expression of RCC2 in pancreatic carcinoma tissues obtained by EUS-FNA. miR-1247 has been reported to reduce the expression of RCC2, a component of chromosomal passenger complex, at both the mRNA and protein levels in PC cells (48). To confirm the association between RCC2 expression and PC, tumor specimens were obtained by EUS-FNA from patients with PC whose bile samples had previously been used in methylation analyses of miRNAs. Immunostaining showed that the level of RCC2 expression in these eight tumor specimens ranged from 30 to 80%, although surrounding lymphoid and acinar cells were negative (Table III, Figure 4).

The maximum AUC for *miR-1247* in bile was observed at CpG site 3. Because hypermethylation was defined as a methylation rate >15%, the specificity of hypermethylation of *miR-1247* in bile was 85%. Of the eight tumor specimens analyzed, five, from patients 1 and 5-8, showed hypermethylation of CpG site 3 in *miR-1247* (Table II).

Discussion

The prognoses of patients with PBC have been reported to be dismal (50, 51), with patients with PC having a 5-year overall survival rate of only 7% (50). Poor prognosis has been associated with advanced stage at diagnosis, aggressive tumor growth, and resistance to most anticancer drugs. Early

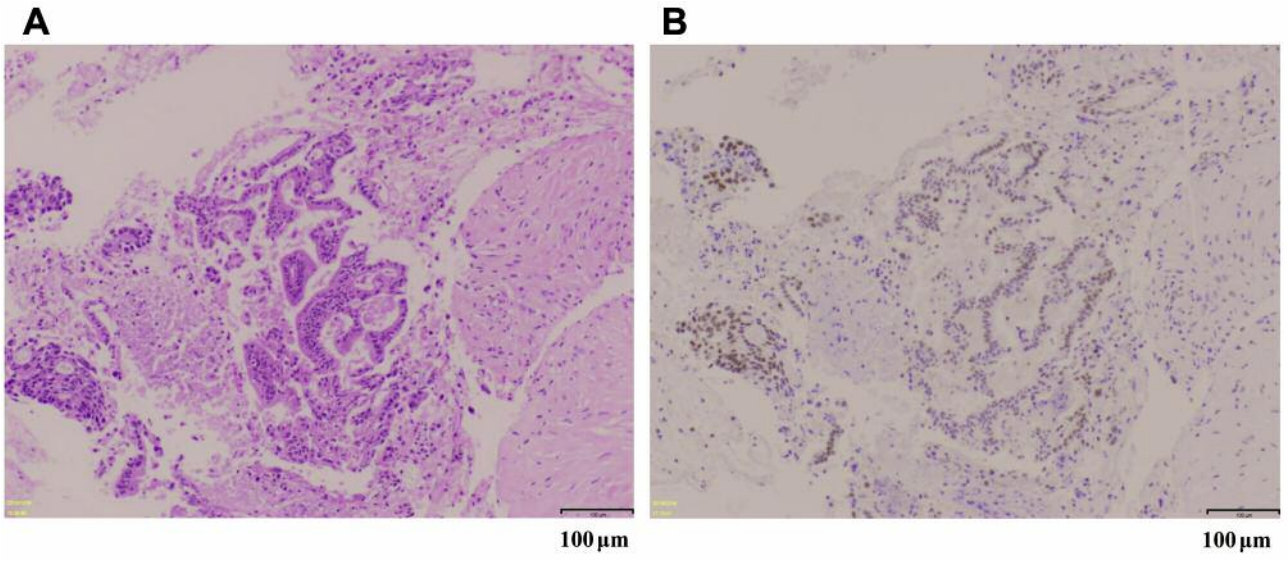

Figure 4. *Expression of regulator of chromosome condensation 2 (RCC2) in pancreatic carcinoma tissues obtained by endoscopic ultrasound guide fine-needle aspiration. Hematoxylin and eosin staining (A) and RCC2 immunostaining (B) are shown for case 6 (see Table III). About 80% of the area of this pancreatic tumor was positive for RCC2.*

detection may therefore improve prognosis for these patients.

Abnormalities in miRNA expression have been reported not only in pancreaticobiliary tissues from patients with PBC (6-12, 32), but also in the serum (28, 29, 33) and plasma (30, 31, 34). These miRNAs may be genetic biomarkers, distinguishing PBC from BDs. Few studies, however, have analyzed epigenetic abnormalities in miRNAs of patients with PBC (19-25). Reduced expression of *miR-132* was reported to correlate negatively with *miR-132* methylation status in 16 out of 20 PC tissues (21). Furthermore, *miR-124* was reported to be more highly methylated in PC than in non-cancerous tissues from the same patients, with hypermethylation mediating the silencing of *miR-124* (22). *miR-192* has been reported to be down-regulated by promoter methylation in tissue samples from patients with PC and chronic pancreatitis, with the latter being a major risk factor for the development of PC (24).

Hypermethylation of miRNAs may also be useful as epigenetic biomarkers distinguishing PBC from BD in samples other than pancreaticobiliary tissues. In this study, we initially attempted to assess the methylation of tumor-suppressive miRNAs in pancreatic tissues from patients with PC. However, it was not possible to perform methylation analyses due to the inadequate quantity of DNA in these pancreatic tissue samples. Therefore, we analyzed bile from patients with pancreaticobiliary diseases.

Several genetic biomarkers, including *KRAS* and *p53* mutations, have been reported to distinguish PBC from BD in bile (52-55). Although several studies have analyzed miRNAs in bile from patients with PBC (35-37), to our knowledge, epigenetic analyses have never been performed in bile samples from such patients. Epigenetic analyses of miRNAs in bile may distinguish PBC from BD, allowing the detection of PBC at an early stage. The present study therefore assessed the methylation of tumor-suppressive miRNAs in bile from patients with pancreaticobiliary diseases, finding that the methylation rates of *miR-1247* and *miR-200a* were significantly higher in patients with PC and BTC than in those with BD. In addition, the methylation rate of *miR-200b* was significantly higher in patients with PC than in those with BD. The ROC curves of methylation rate suggested that *miR-1247* and *miR-200a* may be promising biomarkers distinguishing PBC from BD with moderate

Table III. *Immunohistochemistry of regulator of chromosome condensation 2 (RCC2) in pancreatic carcinoma tissues obtained by endoscopic ultrasound guided fine-needle aspiration.*

Case	Age, years	Gender	Methylation rate of *miR-1247* (%)*	RCC2 staining (%)
1	72	Female	21.5	30
2	48	Female	1.6	60
3	44	Male	1.4	60
4	69	Female	0	30
5	76	Male	22.6	50
6	60	Female	18.1	80
7	66	Male	25.5	80
8	62	Female	23.0	70

*CpG site 3.

accuracy. Further analyses, involving additional samples, are necessary to validate the usefulness of hypermethylation of these miRNAs.

RCC2, a component of the chromosomal passenger complex, is a crucial regulator of chromosomes, the cytoskeleton, and membrane dynamics through mitosis (56, 57). *RCC2* knockdown induced cell-cycle arrest at pro-metaphase in HeLa cells (58). *miR-1247*, which has been reported to have tumor-suppressive activity in PC, was shown to reduce the expression of *RCC2* mRNA and protein in PC cells (48).

This study analyzed RCC2 protein expression in tissue samples by immunostaining rather than analyzing *RCC2* mRNA expression or the expression of relevant miRNAs. RCC2 was found to be expressed in all eight pancreatic carcinoma specimens from patients with PC. Bile samples from five out of these eight patients showed hypermethylation of *miR-1247* (CpG site 3) in bile, the CpG site providing maximum AUC. The hypermethylation of *miR-1247* in these specimens may be responsible for their increased expression of RCC2. The discrepancy between *miR-1247* methylation status and RCC2 expression in the other three patients with PC may have been due to the heterogeneity of these EUS-FNA specimens.

This study had several limitations. Firstly, bile is generally collected only after patients with pancreaticobiliary diseases develop obstructive jaundice. The restricted availability of bile may have introduced a selection bias. Secondly, we examined only 16 tumor-suppressive miRNAs. Investigation of the methylation of additional tumor-suppressive miRNAs may identify miRNAs that can more accurately distinguish PBC from BD. Thirdly, we did not confirm the actual expression of *miR-1247, miR-200a* and *miR-200b* in patients with PBC showing significantly higher methylation rates than BD.

In conclusion, hypermethylation of *miR-1247, miR-200a* and *miR-200b* in bile may be useful for distinguishing PBC from BD. Future studies should assess the methylation of these miRNAs in pancreatic juice and plasma from patients with PBC to confirm that these miRNAs are useful biomarkers for early detection of PBC.

Ethical Statement

This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Acknowledgements

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Conflicts of Interest

Author KO received a donation from Eli Lilly and Company.

Authors' Contributions

Study conception and design: KO and KM. Collection of bile: KO and KY. DNA methylation analyses: KM. Suggestion of study design: SA, KF, NY, CS, SO, YA, AT, AN, KY and ST. Evaluation of immunohistochemistry: KN. Statistical analyses: KY. Article writing: KO and KM. Coordination of the team and final corrections: SY. All Authors read and approved the final article.

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