

Methylation of Tumor Suppressive miRNAs in Plasma from Patients With Pancreaticobiliary Diseases

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Abstract. *Background/Aim:* We previously reported the usefulness of aberrant methylation of tumor suppressive miRNAs in bile to discriminate pancreaticobiliary cancers (PBCs) from benign pancreaticobiliary diseases (BD). Here we performed a methylation analysis of plasma miRNAs to identify miRNAs specific for PBCs. *Patients and Methods:* Plasma was collected from 80 patients with pancreatic cancer (PC); 18 with biliary tract cancer (BTC) and 28 with BD. Sequences encoding 3 tumor suppressive miRNAs (miR-200a, -200b, and -1247) were PCR amplified and sequenced, and their methylation rates were determined. *Results:* The methylation rate of miR-1247 was significantly higher in patients with BTC than in those with BD, and tended to be higher in patients with PC than in those with BD. Furthermore, it was significantly higher in three patients with stages I/II BTC than in those with BD. *Conclusion:* Methylation of miR-1247 in plasma may be useful to distinguish BTC from BD.

Pancreatic cancer (PC) is one of the deadliest cancers having an exceptionally high mortality rate and is the fourth leading cause of cancer-related deaths in most developed countries (1). The overall five-year survival rate has been reported to be about 6% (ranging from 2% to 9%) (2). Furthermore,

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prognosis of biliary tract cancer (BTC) is poor. Five-year survival was 5% and 17% for intra and extrahepatic ducts cancer, respectively for cases diagnosed between 2000 and 2007 in Europe (3). Although tumor markers carbohydrate antigen (CA 19-9) and carcinoembryonic antigen (CEA) are commonly used, they are not recommended for diagnoses of early pancreaticobiliary cancers (PBCs) (1, 4-6).

microRNAs (miRNAs) are non-coding RNAs 20-25 nucleotides in length, which function in posttranscriptional regulation of gene expression. They are regulators of various cellular activities including cell growth, differentiation, development, and apoptosis (7, 8). Dysregulation of miRNAs has been reported to be associated with the initiation and progression of various cancers (9, 10). miRNAs have been reported to be up- or down-regulated in PC compared with normal pancreas and chronic pancreatitis tissue samples (11-14), and in BTC compared with benign biliary disease tissue samples (15, 16).

miRNAs have been analyzed in the serum and plasma from patients with PC (17-21) and those with BTC (22-26). Although miRNA abnormalities in bile have been reported in patients with PBCs (27-30), those in both serum/plasma and bile have seldom been examined (27, 29). We previously reported the usefulness of methylation of tumor suppressive miRNAs, miR-1247, -200a, and -200b in bile to distinguish PBCs from benign pancreaticobiliary diseases (BD) (31). Although methylation of miRNA in serum has been investigated in gastrointestinal cancers, including PC (32), epigenetic abnormalities of miRNAs have not been examined in bile and plasma in patients with PBCs. We employed methylation of tumor suppressive miRNAs in bile and plasma to identify miRNAs specific for PBCs.

Patients and Methods

Plasma collection and patient characteristics. Plasma was obtained from 126 patients with pancreaticobiliary diseases, including 80 with PC, 18 with BTC, and 28 with BD at Kanazawa University Hospital (Kanazawa Japan) from 2015 to 2021. The demographic and clinical characteristics of the patients are shown in Table I. Of

Table I. *Patients characteristics.*

	Pancreatic cancer (n=80)		Biliary tract cancer (n=18)		Benign pancreaticobiliary diseases (n=28)	
Age (median)	39-90 (69)		29-79 (69)		35-78 (63)	
Sex, M/F	51/29		10/8		14/14	
Location	Head	35	Bile duct (intrahepatic)	5	Chronic pancreatitis	12
	Body and/or tail	45	Bile duct (hilar)	3	Autoimmune pancreatitis	6
			Bile duct (distal)	5	Cholelithiasis	3
			Gallbladder	5	Gallbladder adenomyomatosis	2
					Acute pancreatitis	1
					Acute cholangitis	1
					Primary sclerosing cholangitis	1
					Biliary dyskinesia	1
					Pancreaticobiliary maljunction	1
Stage (UICC 8 th)	IA	5	I	1		
	IB	5	IIB	2		
	IIA	1	IIVA	2		
	IIB	2	IVB	13		
	III	17				
	IV	50				

the 80 patients with PC, 35 had tumors located in the pancreatic head and 45 in the pancreatic body and/or tail. According to their TNM classification, 8th Edition (33), five tumors were Stage IA, five were Stage IB, one was Stage IIA, two were Stage IIB, 17 were Stage III, and 50 were Stage IV. Of the 18 patients with BTCs, five had intrahepatic, three had hilar, and five had distal bile duct cancers, while five had gallbladder cancer. According to their TNM classification, 8th Edition, one tumor was Stage I, two were Stage IIB, two were Stage IVA and 13 were Stage IVB. The 28 patients with BD included 12 with chronic pancreatitis, six with autoimmune pancreatitis, three with cholelithiasis, two with gallbladder adenomyomatosis, and one each with acute pancreatitis, acute cholangitis, primary sclerosing cholangitis, biliary dyskinesia, and pancreaticobiliary maljunction.

DNA methylation analyses. Cell-free DNA samples were extracted from 500 µL aliquots of plasma using a Maxwell RSC cfDNA Plasma Kit (AS 1480; Promega, Madison, WI, USA), and treated with sodium bisulfite modification using an EZ DNA Methylation Lightning kit (Zymo Research, Irvine, CA, USA). Sequences of 3 tumor suppressive miRNAs (miR-200a, -200b, and -1247), in which methylation rates in bile were significantly higher in patients with PC and/or BTC than in those with BD in our previous report (31), were amplified by FastStart Taq DNA Polymerase (Roche, Basel, Switzerland). 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 the Ion PGM Hi-Q View Kit (Thermo Fisher Scientific). Templates were loaded onto the 318v2 chip and sequenced using the Ion PGM system, followed by signal processing and base calling 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 base sequences of the CpG islands of the 3

miRNAs analyzed in this study have been previously described (31, 34). 9, 12, and 8 sites of miR-200a, miR-200b, and miR-1247 were selected, respectively.

Statistical analysis. All statistical analyses were performed using Stata (StataCorp LLC, College Station, TX, USA) statistical software. Differences in methylation rates among the three groups of patients were determined by Kruskal-Wallis test. If *p*-Value was <0.10, Mann-Whitney *U*-test, subsequently Bonferroni correction was performed, with *p*<0.05 indicating statistical significance. Furthermore, the ability of each methylated miRNA to discriminate PBCs from BD was assessed by receiver operating characteristic (ROC) curve analysis. High, moderate, and low accuracy was defined as areas under the curve >0.9, 0.7-0.9, and <0.7, respectively. The relation between bile and plasma in DNA methylation was evaluated by regression line. Significant correlation was defined as correlation coefficients >0.4.

Ethical statement. This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The protocol of this study was approved by the Ethics Committee of Kanazawa University. Written informed consent was obtained from each patient.

Results

DNA methylation analyses in plasma. In plasma analysis in the present study, the medians of the methylation rate at one (No. 6) of eight CpG sites in the upstream region (hg38:chr14:101561474-101561607) of miR-1247 (34) were 20.7 % (ranging from 0.1 to 53 %), 21.3% (ranging from 10.2 to 62.7 %), 14.9 (ranging from 4.8 to 50.6 %) in patients with PC, BTC and BD, respectively. *p*-Value of methylation rates among the three groups of patients was

0.06 in Kruskal-Wallis test. The methylation rate was significantly higher in patients with BTC than in those with BD ($p=0.012$) and tended to be higher in patients with PC than in those with BD ($p=0.08$) in Mann-Whitney U -test (Figure 1a). In the bile analysis of our previous report, methylation rate at CpG site No. 6 in miR-1247 was also significantly higher in patients with BTC than in those with BD ($p<0.01$) (Figure 1b) (31). Significantly higher methylation rate was confirmed in patients with BTC than in those with BD following Bonferroni correction. Compared with the BD group, the AUC at CpG site No. 6 in miR-1247 was 0.72 in the BTC group (Figure 2). Methylation rate at CpG site No. 6 in miR-1247 was significantly higher in three patients with stages I and II BTC than in those with BD. No significant difference in methylation rate was identified for the other 7 CpG sites in miR-1247 among patients with PC, BTC, and BD. In addition, no significant difference in methylation rate was confirmed for 9 CpG sites in miR-200a and 12 in miR-200b among patients with PC, BTC, and BD.

Relationship between bile and plasma in DNA methylation. We compared methylation rate at CpG site No.6 in miR-1247 between bile and plasma in 27 patients with PC, BTC, and BD, in whom methylation rates of both bile and plasma were analyzed. However, no significant correlation of methylation rate between bile and plasma was observed ($R^2=0.05$) (Figure 3).

Discussion

Although CA 19-9 and CEA are commonly used for tumor markers in PBCs, they are not useful for detecting PBCs at early stages (1, 4-6). Recently, hematopoietic growth factors and various enzymes have been reported to be potential biomarkers for PC. They include macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF), macrophage inhibitory cytokine (MIC-1) and alcohol dehydrogenase. In addition, Kras mutation in serum is becoming more common and efficient. Kras mutation has been reported to occur in 47 to 100% of patients with PC, most commonly at codon 12 (1). On the other hand, promising circulating diagnostic and prognostic biomarkers, such as matrix metalloproteinase-7 (MMP-7), osteopontin, interleukin 6 (IL-6), have been reported in BTC (5). However, no effective method of sufficient diagnostic accuracy to detect PBCs at early stages has been reported. Abnormalities of miRNAs have been reported in serum and plasma from patients with PC (17-21) and BTC (22-26). Zou *et al.* have reported six significantly upregulated miRNAs (let-7b-5p, miR-192-5p, -19a-3p, -19b-3p, -223-3p, and -25-3p) in serum of PC (18). Cao *et al.* reported that an miRNA panel (miR-486-5p, -126-3p, -106b-3p, -938, -26b-3p, and -1285) had high accuracy in distinguishing PC from chronic

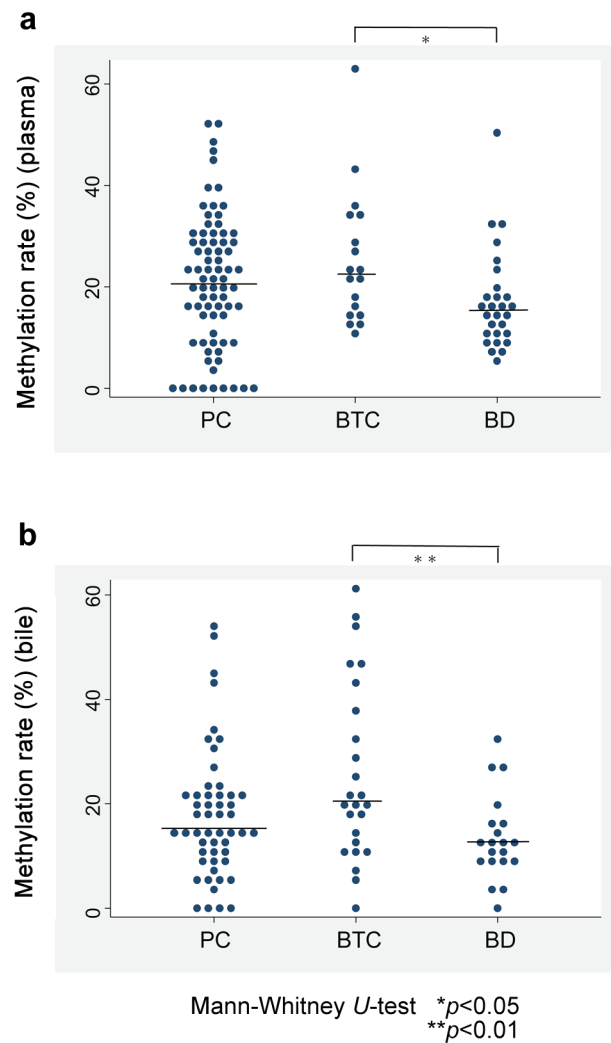


Figure 1. Methylation rate at CpG site No. 6 in miR-1247 in plasma (a) and bile (b) from patients with pancreaticobiliary diseases. Dot plots show each CpG island of miRNAs measured in plasma and bile. Medians are shown by horizontal bars. In plasma analysis, the methylation rate is significantly higher in patients with BTC than in those with BD and tends to be higher in patients with PC than in those with BD (a). In bile analysis, methylation rate is significantly higher in patients with BTC than in those with BD (b). PC, Pancreatic cancer; BTC, biliary tract cancer; BD, benign pancreaticobiliary diseases.

pancreatitis (CP) and the diagnostic value of the panel in discriminating PC from CP were comparable to that of CA 19-9 (19). On the other hand, Loosen *et al.* have reported that serum concentrations of miR-122, -192, -29b and -155 were significantly elevated in patients with BTC compared to healthy controls or patients with primary sclerosing cholangitis without malignant transformation, and a strong postoperative decline of miR-122 serum levels was significantly associated with favorable prognosis (23). Zhang

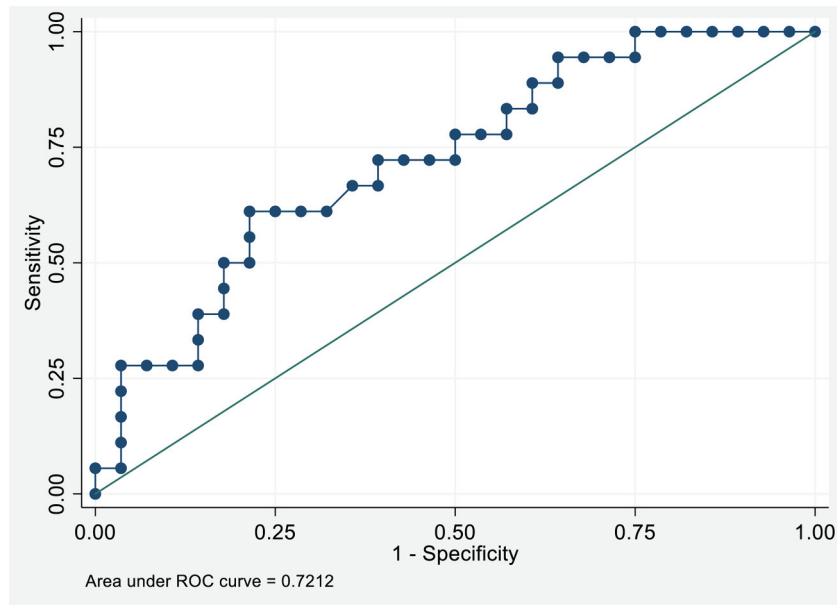


Figure 2. Accuracy of methylation at CpG site No. 6 in miR-1247 in plasma in diagnosing BTC. Receiver operating characteristic curves showing the performance of CpG island of miRNA in plasma distinguishing patients with BTC from those with BD.

et al. reported that both plasma and tissue miR-146a expression correlated with favorable overall survival in patients with intrahepatic cholangiocarcinoma (26).

In the present study, the methylation rate at one of eight CpG sites in miR-1247 was significantly higher in patients with BTC than in those with BD in plasma. However, no significant difference in methylation rate was observed for the other seven CpG sites in miR-1247, nine in miR-200a and 12 in miR-200b among patients with PC, BTC, and BD. On the other hand, in the bile analysis of our previous report, the methylation rates in miR-200a, -200b, and -1247 were significantly higher in patients with PC and/or BTC than in those with BD (31). There are two reports in which miRNA analysis was performed in both bile and plasma from patients with PBCs. In one report, the expression of seven (miR-10b, -30c, -106b, -155, -181a, -196a, and -212) of 10 miRNAs in plasma was concordant with that in bile from patients with PC (27). In the other report, different miRNAs were analysed between plasma and bile in patients with BTC (29). In this methylation analysis of miRNA, although the methylation rate at CpG site No.6 in miR-1247 in plasma was significantly higher in patients with BTC than in those with BD, no correlation of methylation rate was observed between bile and plasma in the same patient. miRNAs are estimated to leak easier from tumor tissue to bile than to plasma in patients with PBCs. The difference in methylation rate between bile and plasma may be due to the amount of miRNA leaking from the tumor. In addition, the time lag in sample collection between bile and plasma in some patients may be implicated.

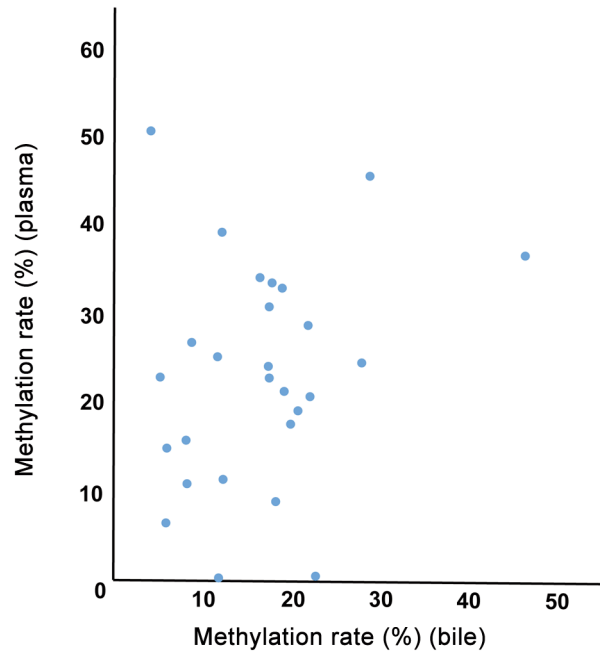


Figure 3. Methylation rate at CpG site No.6 in miR-1247 in bile and plasma. No significant correlation of methylation rates between bile and plasma was observed.

In this study, methylation rate at CpG site No. 6 in miR-1247 was significantly higher in three patients with stages I and II BTC than in those with BD in plasma. Therefore,

methylation analysis of miR-1247 in plasma may be useful to distinguish early stage BTC from BD. Further analysis in many samples is needed for early detection of BTC.

This study has several limitations. First, we analyzed methylation of miRNAs in fewer patients with BTC and BD (18 and 28 patients, respectively) than PC (80 patients). Second, we examined only 3 tumor suppressive miRNAs (miR-200a, -200b, and -1247), in which methylation rates were significantly higher in patients with PBCs than in those with BD in bile. Investigation of the methylation of additional miRNAs may identify miRNAs that can more accurately distinguish PBCs from BD. Third, we could not verify the reverse correlation between methylation and expression of miR-1247 in patients with PC, BTC, and BD.

In conclusion, hypermethylation of miR-1247 in plasma may be useful for distinguishing BTC from BD. In particular, it may be a biomarker for early stage BTC. Since hypermethylation of miR-1247 tends to be higher in patients with PC than in those with BD, future studies are warranted to confirm the usefulness of methylation of miRNAs in pancreatic juice from patients with PC for early detection of PC.

Conflicts of Interest

Author KO received a donation from Eli Lilly and Company.

Authors' Contributions

KO and KM contributed to study conception and design. KO and KY contributed to collection of samples. KM contributed to DNA methylation analyses and acquisition of data. KO and KM contributed to analyses and interpretation of data. KO and KM wrote the manuscript. SA, KF, CS, HK, AT, AN, SN, ST, and SY provided intellectual advice. All Authors have read and approved the final manuscript.

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