

Plasma miR-21 is a novel diagnostic biomarker for biliary tract cancer

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Biliary tract cancer (BTC) has a generally poor prognosis. Furthermore, it is difficult to distinguish BTC from benign biliary disease (BBD) with commonly used modalities. Therefore, a novel biomarker to facilitate cancer detection is highly desirable. Recent studies have reported the use of circulating microRNAs (miRNAs) as biomarkers for cancers. The purpose of this study was to evaluate whether circulating miRNA-21 (miR-21) could be used as a biomarker for BTC. Plasma samples were obtained from 94 BTC patients, 50 healthy volunteers (HVs), and 23 BBD patients. miR-21 levels in the samples were measured by qRT-PCR. Plasma miR-21 levels in patients with BTC were significantly higher than in HVs or in patients with BBD ($P < 0.0001$ for both). Receiver-operator curve (ROC) curve analysis in differentiating BTC patients from HVs indicated that area under the curve (AUC), optimal sensitivity and specificity was 0.93, 85.1% and 100%, respectively, and those in differentiating BTC patients from BBD patients was 0.83, 72.3%, 91.3%, respectively. Validation of these results indicated that the negative predictive value, positive predictive value, sensitivity, specificity, and accuracy in differentiating BTC patients from HVs was 76.6%, 98.6%, 84.0%, 98.0%, and 88.9%, respectively, and those in differentiating BTC patients from BBD patients was 42.2%, 93.0%, 71.2%, 82.6%, and 72.6%, respectively. These sets of values were improved by combining miR-21 and CA19-9 measurements. Plasma miR-21 is a novel diagnostic biomarker for BTC, and may be useful in distinguishing between BTC and BBD patients. (*Cancer Sci* 2013; 104: 1626–1631)

Biliary tract cancer (BTC) is one of the most aggressive malignant tumors, and it is associated with local invasiveness and a high rate of metastasis.⁽¹⁾ Most BTC patients present with symptoms of biliary tract obstruction, similar to the symptoms presented by patients with benign biliary diseases (BBD).⁽²⁾ In addition, as a forming mass, BTC typically grows along the bile duct without projecting outward from the bile ducts; hence, commonly used diagnostic modalities, such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasonography, are often inadequate for revealing tumor lesions.⁽³⁾ Furthermore, the diagnostic yield for cytological diagnosis is not high.^(4–6) Additionally, it is difficult to distinguish between BTC and BBD by using standard tumor markers, such as carbohydrate antigen 19-9 (CA19-9), because they lack diagnostic accuracy.^(7,8) Therefore, novel biomarkers are urgently needed for cancer detection and clinical management. We focused on circulating microRNAs as potential diagnostic biomarkers for BTC.

MicroRNAs (miRNAs) are a class of noncoding small RNAs known to post-transcriptionally modulate gene expression by negatively regulating the stability or translational efficiency of their target mRNAs.^(9,10) MiRNAs play a crucial role in almost all cellular biological processes, including metabolism, survival, differentiation, and apoptosis. Several recent studies have

shown that miRNAs are stably detectable in plasma and serum.^(11,12) Mitchell *et al.*⁽¹³⁾ reported that circulating miRNAs can originate from cancer tissues and are protected from endogenous RNase activity. This finding raises the possibility that assaying miRNAs in plasma/serum could serve as a novel approach for the noninvasive blood-based detection of human cancers. The potential of circulating miRNAs as cancer biomarkers has been evaluated in some studies.^(14–21) Among the candidate miRNAs evaluated as cancer biomarkers, circulating miRNA-21 (miR-21) has been reported to be a useful biomarker for various types of cancers.^(14,19–21) miR-21 has been reported to contribute to cancer cell proliferation, migration, invasion, and chemoresistance, and to be secreted by cancer cells.^(22–24) Furthermore, miR-21 is overexpressed in human cholangiocarcinoma tissue and cell lines, and it regulates programmed cell death 4 (PDCD4) and tissue inhibitor of metalloproteinase 3 (TIMP3).^(25,26) To our knowledge, there are no reports on the significance of circulating miRNAs in patients with BTC. In the present study, we evaluated whether plasma miR-21 levels could be used as a diagnostic biomarker for BTC.

Materials and Methods

Patients and samples. Plasma samples were obtained from 94 patients with histologically proven BTC: 21 intrahepatic cholangiocarcinoma (ICC) patients, 48 bile duct cancer (BDC) patients, 14 gallbladder cancer (GBC) patients, and 11 ampulla Vater cancer (APC) patients. Samples were also obtained from 23 patients with BBD. All of the samples were obtained from patients who had undergone surgical resection at Osaka University Hospital in April 2004–January 2011. The diagnosis of these patients was based on histological assessment after surgical resection. Additionally, plasma samples were collected from 50 healthy volunteers (HVs). In HVs, tumor markers, including carcinoembryonic antigen (CEA), CA19-9, squamous cell carcinoma-related antigen (SCC), prostate specific antigen (PSA; in men), and carbohydrate antigen 15-3 (CA15-3; in women), were confirmed to be within the normal range. Healthy volunteers were also confirmed to be free of malignant disease for >2 years. The clinicopathological backgrounds of the patients and HVs are shown in Table 1. After sample collection, blood samples were centrifuged at 1700 g for 20 min at 4°C to spin down the blood cells. Plasma samples were then transferred into fresh collection tubes and stored at –80°C until further processing.

Study design. First, we examined whether plasma miR-21 expression levels are profoundly affected by the existence of cancer in the body and plasma miR-21 may be a biomarker for BTC. Plasma miR-21 expression was measured in the 10

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Table 1. Clinicopathological characteristics of patients with biliary tract cancer or benign biliary disease and healthy volunteers

Characteristics	BTC patients (n = 94)	BBD patients (n = 23)	HVs (n = 50)
Gender (men/women)	56/38	12/11	37/13
Age (years)†	66 ± 9	56 ± 14	61 ± 9
Disease type	Intrahepatic cholangiocarcinoma	21 Gallstone	3
	Biliary duct cancer	48 Cholecystitis	8
	Gallbladder cancer	14 Adenomyomatosis	1
	Ampulla of Vater cancer	11 Gallbladder polyp	2
		Cholangitis	1
		Choledochal cyst	3
		Malfusion of pancreaticobiliary duct	1
		Bile duct dysplasia	1
		Postoperative stenosis	1
CA19-9 (U/mL)†	161 ± 605	12.1 ± 9.8	3 ± 5
≤37	60	23	
>37	34	0	
Maximum tumor size (cm)†	3.2 ± 2.3		
Tumor number: single/multiple	91/3		
T factor: T1/T2/T3/T4	13/22/49/10		
Lymph node metastasis: negative/positive	50/44		
TNM staging: I/II/III/IV	15/35/27/17		
Curative/non-curative resection	69/25		

†Data are mean ± standard deviation. CA19-9, carbohydrate antigen 19-9.

BTC patients before and after curative resection. In addition, plasma miR-21 expression in the 94 BTC patients was compared to that of the 50 HVs. Then receiver-operator curve (ROC) curve analysis was conducted to evaluate the diagnostic power of plasma miR-21 to differentiate between BTC patients and HVs. In the next phase, the significance of plasma miR-21 in differentiating BTC patients from BBD patients was examined. Furthermore, we evaluated the utility of plasma miR-21 as a diagnostic biomarker compared to CA19-9.

The aim of the study was explained in detail to all patients, and written informed consent was obtained from the patients before enrollment in the study. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital.

RNA extraction. Total RNA was extracted from 400 µL of plasma with a mirVana PARIS Kit (Ambion, Austin, TX, USA) and eluted into 100 µL of preheated (95°C) Elution Solution according to the manufacturer's instructions. The concentration and purity of all RNA samples were evaluated by their absorbance ratio at 260/280 nm, as determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

Quantification of microRNA by quantitative real-time PCR. Amounts of miRNAs were quantified by quantitative real-time PCR (qRT-PCR) using the human TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA, USA). The reverse transcription reaction was carried out with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) in 15 µL containing 5 µL of RNA extract, 1.5 µL of 10× reverse transcription buffer, 0.15 µL of 100 mM dNTPs, 1 µL of MultiScribe reverse transcriptase, 0.19 µL of RNase inhibitor, 1 µL of gene-specific primer, and 4.16 µL of nuclease-free water. For synthesis of cDNA, the reaction mixtures were incubated with the PC818 thermal cycler (ASTECH, Fukuoka, Japan) at 16°C for 30 min, 42°C for 30 min, and 85°C for 15 min, and then held at 4°C. Then, 1.33 µL of cDNA solution was amplified by using 10 µL of TaqMan 2× Universal PCR Master Mix with No AmpErase UNG (Applied Biosystems), 1 µL of gene-specific primer, and 7.67 µL of nuclease-free

water. Quantitative PCR was performed on the 7900HT Fast Real-Time PCR system (Applied Biosystems), and reaction mixtures were incubated at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. The cycle threshold (C_t) values were calculated with SDS 2.4 software (Applied Biosystems).

In the present study, expression of the target miRNAs in plasma was normalized relative to the expression of the endogenous control miR-16. Data were analyzed by the ΔC_t method: $\Delta C_t = C_t$ value (miR-16) – C_t value (miRNA of interest). All data were represented by $2^{-\Delta C_t}$ value.

Statistical analysis. Differences between groups were assessed by Mann-Whitney *U*-test, Fisher's exact test, or chi-square-test, and Steel-Dwass test was performed for multiple comparisons. Paired samples were analyzed by Wilcoxon's single-rank test. The diagnostic value for differentiating between BTC patients and the control was assessed by calculating the area under the receiver-operator characteristic curve (AUC). Validation of ROC results was performed by the leave-one-out cross-validation method as described before.⁽²⁷⁾ In the validation, first, by using the subset of all but one sample, we built a ROC model, and defined the cut-off value in such a way that the sum of sensitivity and specificity was maximum. Then, using the cut-off value, the model was used to predict the left-out recorded samples. When this process was repeated for each sample, the prediction was obtained for every record in the dataset using a model that was blind to the predicted observation. All statistical analyses were performed with the use of JMP software (version 9.02; SAS Institute, Cary, NC, USA). A *P*-value of <0.05 denoted the presence of a statistically significant difference.

Results

Plasma miR-21 expression levels as a potential diagnostic biological marker. First, expression levels of plasma miR-21 from the 10 BTC patients who underwent curative surgical resection were measured before and after surgery by qRT-PCR. Plasma miR-21 expression levels were significantly lower after surgery than before surgery ($P < 0.05$; Fig. 1). Subsequently, plasma miR-21 expression levels were examined in the 94 patients

with BTC and in the 50 HVs by qRT-PCR. Expression levels in the patients with BTC were significantly higher than in HVs ($P < 0.0001$; Fig. 2). The ROC curve analysis in differentiating BTC patients from HVs indicated that the AUC for plasma miR-21 was 0.93. At a cut-off value of 0.17, the optimal sensitivity and specificity was 85.1%, and 100%, respectively (Fig. 3a). These results were validated by using the leave-one-out cross-validation method. The results indicated that the negative predictive value (NPV), positive predictive value (PPV), sensitivity, specificity, and accuracy in differentiating BTC patients from HVs was 76.6%, 98.6%, 84.0%, 98.0%, and 88.9%, respectively (Table 2). In the next phase, plasma miR-21 expression levels in the 23 BBD patients were measured by qRT-PCR. Expression levels in the patients with BTC were significantly higher than in patients with BBD ($P < 0.0001$; Fig. 2). Next, the significance of plasma miR-21 expression levels in differentiating BTC patients from BBD patients was also examined. The AUC was 0.83, and at a cut-off value of 0.23, the optimal sensitivity and specificity was 72.3%, 91.3%, respectively (Fig. 3b). The validation also indicated that NPV, PPV, sensitivity, specificity, and accuracy in differentiating BTC patients from BBD patients was 42.2%, 93.0%, 71.2%, 82.6%, and 72.6%, respectively (Table 2).

miR-21 expression in patients with each type of BTC was significantly higher than in the HVs and BBD patients. miR-21 expression levels in patients with each type of BTC and in BBD patients and HVs are summarized in Figure 4.

Combination of plasma miR-21 and CA19-9 as biomarkers for BTC. The use of a combination of plasma miR-21 and CA19-9 levels to differentiate BTC patients from HVs or BBD patients was investigated and compared to when either marker was used individually. The miR-21 expression levels and CA19-9 levels of the BTC patients, BBD patients, and HVs were plotted in Figure 5. The normal range of CA19-9 in the blood of healthy individuals is 0–37 U/mL. At a cut-off value of 37 U/mL for CA19-9, the NPV, PPV, sensitivity, specificity, and accuracy in differentiating BTC patients from HVs was 45.5%, 100%, 36.2%, 100%, 58.3%, and those in differentiating BTC patients from BBD patients was 27.7%, 100%, 36.1%, 100%, and 48.7%, respectively (Table 2). At the cut-off value identified by ROC curve analysis for plasma miR-21 and that of 37 U/mL for CA19-9, the diagnostic power of the combination of plasma miR-21 and CA19-9 levels was evaluated. The NPV, PPV, sensitivity, specificity, and accuracy for the combination in differentiating BTC patients from HVs was 84.7%,

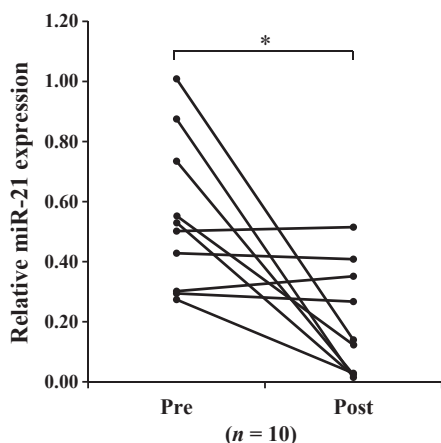


Fig. 1. Plasma miR-21 expression levels before and after surgery. miR-21 expression levels in patients with biliary tract cancer (BTC) were significantly lower after surgery than before surgery ($P = 0.0032$). $*P < 0.05$.

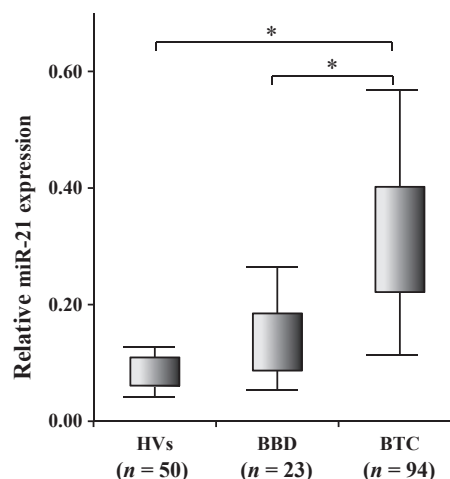


Fig. 2. Plasma miR-21 expression levels in biliary tract cancer (BTC) patients, benign biliary disease (BBD) patients, and healthy volunteers (HV). Plasma miR-21 levels in BTC patients ($n = 94$), BBD patients ($n = 23$), and in HVs ($n = 50$). Plasma miR-21 expression levels were significantly higher in patients with BTC than in both HVs and BBD patients ($P < 0.0001$). $*P < 0.05$.

100%, 90.4%, 100%, and 93.8%, respectively, and those in differentiating BTC patients from BBD patients was 52.5%, 97.4%, 79.8%, 91.3%, and 82.1%, respectively (Table 2).

Correlation between miR-21 expression levels and clinical and tumor-related factors. The correlation between miR-21 expression levels and various clinical and tumor-related factors was examined. These factors included gender, age, CA19-9, tumor size, T factor, lymph node metastasis, and TNM staging. Biliary tract cancer patients were divided into two groups according to their clinicopathological characteristics. As shown in Table 3, miR-21 expression was significantly higher in patients with Stage III/IV BTC compared to those with Stage I/II BTC. miR-21 expression increased with the progression of TNM staging, and miR-21 expression in patients at each stage was significantly higher than in patients with BBD or in HVs (Fig. 6a). Although there was a tendency for miR-21 expression to be higher in T3/4 patients compared to T1/2 patients, no significant difference in miR-21 expression was found between each other (Fig. 6b). There was no correlation between miR-21 expression and any of the other factors.

Discussion

Previous studies have shown that circulating miRNAs may be potential biomarkers for several disease conditions, including human cancer. Circulating miRNA is stably expressed in serum and plasma and can be readily detected by qRT-PCR. With this blood-based biomarker, it might be possible to diagnose various diseases without invasive procedures. In the present study, we focused on miR-21, which targets phosphatase and tensin homolog deleted from chromosome 10 (PTEN), programmed cell death 4 (PDCD4), and tissue inhibitor of metalloproteinase 3 (TIMP3) genes. miR-21 has been reported to be overexpressed in the bloodstream in the presence of several types of cancers. To our knowledge, there are no previous reports on the significance of circulating miR-21 in patients with BTC.

In the present study, the utility of plasma miR-21 as a diagnostic biomarker in differentiating BTC from BBD was evaluated by means of a two-phase study design. First, plasma miR-21 expression levels in BTC patients were significantly

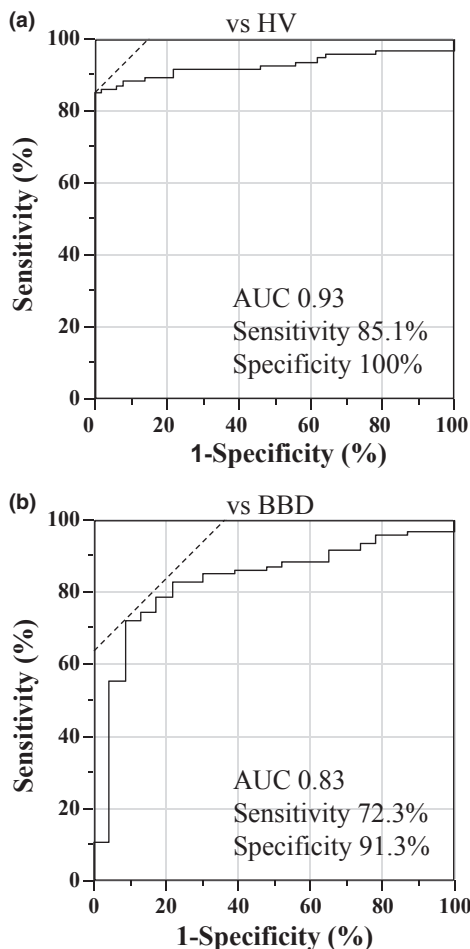


Fig. 3. Receiver–operator curve (ROC) curve analysis for biliary tract cancer (BTC) versus healthy volunteers (HV) and BTC versus benign biliary disease (BBD). (a) In differentiating BTC patients from HVs, the area under the curve (AUC) was 0.93, and at a cut-off value of 0.17, the optimal sensitivity and specificity was 85.1%, and 100%, respectively. (b) In differentiating BTC patients from BBD patients, the AUC was 0.83, and at a cut-off value of 0.23, the optimal sensitivity and specificity was 72.3%, 91.3%, respectively.

reduced after surgery compared to before surgery, and miR-21 level in patients with BTC were significantly higher than in HVs. Receiver–operator curve analysis in differentiating BTC patients from HVs indicated that AUC, optimal sensitivity and specificity was 0.93, 85.1% and 100%, respectively. These results suggest that plasma miR-21 expression levels are profoundly affected by the existence of cancer in the body, and

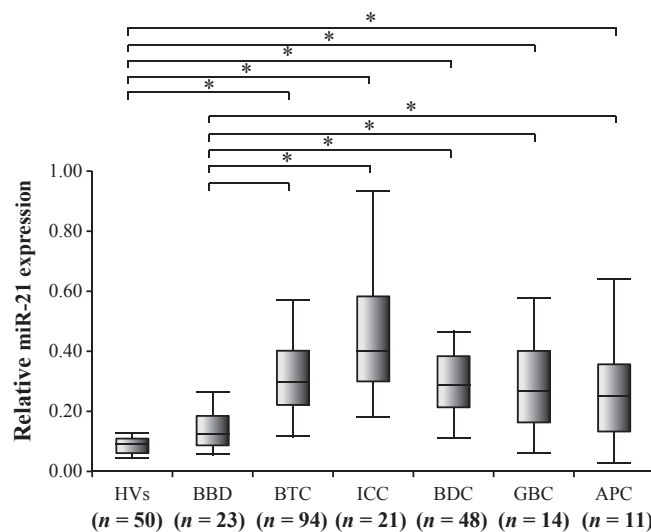


Fig. 4. Plasma miR-21 expression levels in each type of cancer. Bile duct cancer (BDC; $n = 48$), intrahepatic cholangiocarcinoma (ICC; $n = 21$), gallbladder cancer (GBC; $n = 14$), and ampulla vater cancer (APC; $n = 11$). Plasma miR-21 levels in patients with each cancer type were also significantly higher than in patients with benign biliary disease (BBD) or in healthy volunteers (HV). * $P < 0.05$.

that plasma miR-21 may be a useful biomarker for differentiating between patients with BTC and non-cancer patients. In the next phase, we examined the usefulness of miR-21 in differentiating BTC patients from BBD patients. Plasma miR-21 expression levels in patients with BTC were significantly higher than in patients with BBD or in HVs. Based on these results, we suggest that plasma miR-21 may be a useful biomarker in patients with very small tumors, since in these patients it may be difficult to distinguish between BTC and BBD.

CA19-9 has been used as a diagnostic biomarker for BTC, and elevated concentrations of serum CA19-9 (>37 U/mL) in BTC patients have been frequently reported.^(28,29) In the present study, serum CA19-9 was elevated in only 36.1% of BTC patients. CA19-9 levels are known to be elevated under conditions of biliary tract obstruction or inflammation, but they often decrease to within normal levels when these conditions are improved by drainage and antibiotics. Thus, CA19-9 levels may change depending on the conditions when samples are

Table 2. Diagnostic power of CA19-9, miR-21, and combination of CA19-9 and miR-21 in differentiating biliary tract cancer (BTC) patients from healthy volunteers and benign biliary disease (BBD) patients

Biomarkers	NPV (%)	PPV (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
BTC patients versus HVs					
CA19-9	45.5	100	36.2	100	58.3
miR-21	76.6	98.6	84.0	98.0	88.9
CA19-9 + miR-21	84.7	100	90.4	100	93.8
BTC patients versus BBD patients					
CA19-9	27.7	100	36.1	100	48.7
miR-21	42.2	93.0	71.2	82.6	72.6
CA19-9 + miR-21	52.5	97.4	79.8	91.3	82.1

CA19-9, carbohydrate antigen 19-9; HVs, healthy volunteers; miR-21, microRNA-21; NPV, negative predictive value; PPV, positive predictive value.

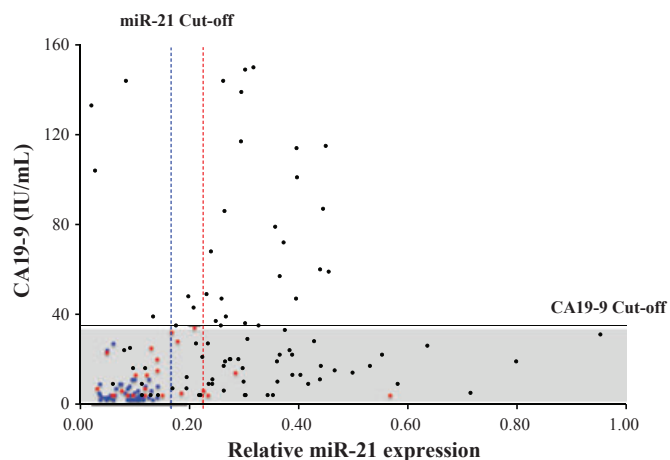


Fig. 5. Combination of CA19-9 and plasma miR-21 levels. Judging solely by CA19-9 levels, 60 biliary tract cancer (BTC) patients (63.8%) were within the normal range (marked by gray box). More BTC patients could be diagnosed by a combination of CA19-9 and miR-21 levels. Black circle, BTC patients; blue circle, healthy volunteers (HVs); red circle, benign biliary disease (BBD) patients; black line, cut-off value for CA19-9 (37 IU/mL); blue dotted line, cut-off value for miR-21 for differentiating BTC patients from HVs (0.17); red dotted line, cut-off value for miR-21 for differentiating BTC patients from HVs (0.23).

Table 3. Plasma miR-21 expression and clinicopathological characteristics

Characteristics	Patients	miR-21 expression (mean ± SD)	P-value
Gender			
Men	56	0.32 ± 0.17	0.5504
Women	38	0.37 ± 0.34	
Age (years)†			
<68	45	0.35 ± 0.20	0.6308
≥68	49	0.36 ± 0.35	
CA19-9 (U/mL)†			
≤37	60	0.36 ± 0.32	0.6118
>37	34	0.34 ± 0.22	
Tumor size (cm)†			
<3.0	46	0.34 ± 0.35	0.1652
≥3.0	48	0.36 ± 0.21	
T factor			
T1, T2	35	0.29 ± 0.16	0.0765
T3, T4	59	0.39 ± 0.34	
Lymph node metastasis			
Negative	50	0.30 ± 0.16	0.1679
Positive	44	0.40 ± 0.38	
TNM staging			
I, II	50	0.14 ± 0.14	0.0023**
III, IV	44	0.44 ± 0.37	

** $P < 0.01$. †Divided into two groups by median value. CA19-9, carbohydrate antigen 19-9; miR-21, microRNA-21.

collected. Because expression of miR-21 in patients with BBD, such as cholecystitis and cholangitis, was higher than in HVs, it is conceivable that miR-21 expression also increases under conditions of inflammation, similar to CA19-9. Previous reports have also shown that circulating miR-21 expression increase in inflammatory diseases, such as ulcerative colitis and chronic hepatitis.^(21,30) Nevertheless, in the present study, plasma miR-21 sufficiently distinguished BTC patients from BBD patients. In addition, the miR-21 expression in some patients did not change after surgical resection in the present results. In these patients, surgical stress and postoperative

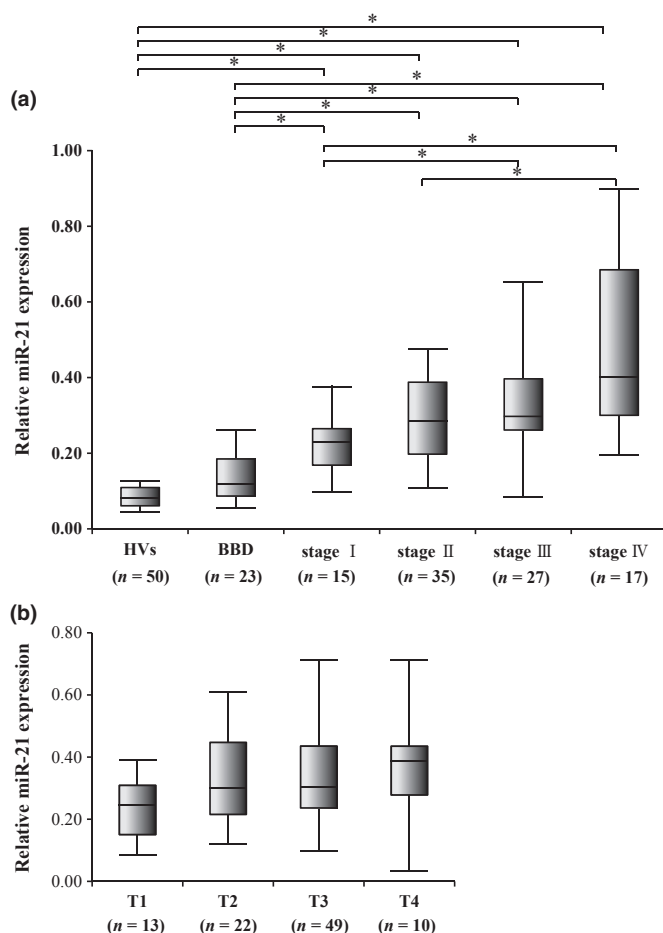


Fig. 6. Correlation between miR-21 expression levels and T factor, by TNM staging. (a) Mir-21 expression levels increased with the progression of TNM staging, and miR-21 expression in patients at each stage was significantly higher than in patients with benign biliary disease (BBD) or in healthy volunteers (HVs). (b) There was no significant difference in miR-21 expression between each other. * $P < 0.05$.

inflammatory response prolonged, which can induce the expression of miR-21, because the postoperative plasma samples were collected 7–21 days after surgery.

The utility of using a combination of plasma miR-21 and CA19-9 levels was compared to the use of either marker individually. According to our results, plasma miR-21 was a highly sensitive biomarker, while CA19-9 was a highly specific marker. Thus, a combination of plasma miR-21 and CA19-9 levels could ameliorate the weaknesses inherent in each marker when used individually.

Circulating miR-21 has been reported as a useful biomarker for other types of cancers, such as lymphoma, colorectal cancer, esophageal squamous cell carcinoma, and hepatocellular carcinoma.^(14,19–21) Thus, circulating miR-21 is not a specific biomarker for BTC, and miR-21 expression alone should not be relied upon for cancer detection. However, a combination of miR-21 and other diagnostic modalities may be useful in distinguishing BTC from benign diseases.

Circulating miRNAs are known to be encapsulated in micro-particles such as microvesicles, exosomes, and apoptotic bodies, and to be coupled with argonaute 2 (AGO2).^(24,31,32) Recent studies have shown that these microparticles are secreted actively from cancer cells and have important roles in cell–cell interactions.^(33–35) Kosaka *et al.*⁽³⁴⁾ reported that over-expression of miRNA in cells leads to increased secretion of

exosomes into the extracellular environment. Turchinovich *et al.* showed that miRNA/AGO2 complexes are byproducts of dead cells. Many miRNA/AGO2 complexes are thought to be released from dead or dying cells in tumor tissue.⁽²⁴⁾ Thus, overexpression of miRNAs in tumor tissue may be reflected by high expression in the bloodstream. In colorectal cancer, overexpression of miR-21 was observed in cancer-associated fibroblasts in tumor tissue.⁽³⁶⁾ Chen *et al.* have shown that miR-21 expression level in cancer cells was significantly higher than in normal cholangiocyte cells in ICC tumor.⁽²⁵⁾ Expression of miR-21 in cells that compose tumor tissue differs depending on each type of cancer. In the present study, we did not evaluate miR-21 expression in tumor tissues and the correlation between plasma miR-21 expression levels and miR-21 expression level in tumor tissues. Therefore, it is unclear which cell in tumor tissue is secreting miR-21. However, the results that plasma miR-21 expression level in the BTC patients were significant higher than in non-cancer patients, and that plasma miR-21 expression levels were significantly lower after surgery than before surgery suggests that circulating miR-21 in BTC patients is released in large amounts from tumor tissue. Further

analysis of miR-21 expression in tumor tissue is necessary to assess the source of circulating miR-21.

For the analysis of qRT-PCR for plasma miRNA expression levels, there is no current consensus on the use of an endogenous control. Several miRNAs, including miR-16, have been used in the normalization of circulating miRNAs. In the present study, expression of the target miRNAs in plasma was normalized relative to the expression of the endogenous control miR-16, which was confirmed in previous reports to exist abundantly and stably in plasma.^(14,16,21) In the present analysis, we could detect miR-16 abundantly in all samples.

In conclusion, the present study demonstrates that circulating miR-21 is a novel and noninvasive diagnostic biomarker for BTC that could be used along with other diagnostic modalities to detect early-stage disease. Further investigations are necessary to identify circulating miRNAs that can be used as specific biomarkers for BTC.

Disclosure Statement

The authors have no conflict of interest.

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