miR-3940-5p/miR-8069 ratio in urine exosomes is a novel diagnostic biomarker for pancreatic ductal adenocarcinoma

NAOHIKO YOSHIZAWA[1], KAZUSHI SUGIMOTO[1,2], MASAHIKO TAMEDA[1], YUJI INAGAKI[1], MAKOTO IKEJIRI[2], HIROYUKI INOUE[1], MASANOBU USUI[3], MASAAKI ITO[4] and YOSHIYUKI TAKEI[1]

[1]Department of Gastroenterology and Hepatology, Mie University Graduate School of Medicine; [2]Department of Central Laboratory, Mie University Hospital; Departments of [3]Hepatobiliary Pancreatic and Transplant Surgery, and [4]Cardiology and Nephrology, Mie University Graduate School of Medicine, Tsu, Mie 514-8507, Japan

Received July 16, 2019; Accepted November 11, 2019

DOI: 10.3892/ol.2020.11357

Abstract. Despite the development of several therapeutic options, the prognosis of pancreatic cancer remains poor. One reason for this is the difficulty of diagnosing the disease at an early stage. For example, carbohydrate antigen (CA) 19-9, which is the most widely used biomarker for pancreatic cancer, cannot be used to detect the disease at early stages. Some studies have attempted to find novel biomarkers for pancreatic cancer. The aim of the present study was to find a novel diagnostic biomarker for pancreatic ductal adenocarcinoma (PDAC) in urine exosomes. Exosomes were isolated from urine and serum samples of patients with PDAC and control subjects, or culture media of cancer cell lines. MicroRNAs (miRNAs) were purified from exosomes. Novel biomarker candidates for PDCA were identisfied from urine exosome miRNA using expression profiling, and validated in a larger number of samples using 3D digital PCR. The results of a preliminary analysis of nine PDAC and seven control subjects revealed that the miR-3940-5p/miR-8069 ratio in urine exosomes was elevated in the patients with PDAC. Experiments using cultured cancer cell lines revealed that the elevation of the miR-3940-5p/miR-8069 ratio was specific for PDAC. Furthermore, the elevation of the miR-3940-5p/miR-8069 ratio in exosomes tended to be higher in the urine than in the serum of patients with PDAC. Validation experiments on 43 PDAC, 12 chronic pancreatitis and 25 control subjects demonstrated that the miR-3940-5p/miR-8069 ratio in urine exosomes was elevated in PDAC at a relatively early stage of the disease. When this ratio was used in combination with CA19-9 for the diagnosis of PDAC, the sensitivity and positive predictive value improved to 93.0 and 78.4%, respectively, when either of them was positive. Additionally, the positive predictive value reached 100% when both were positive. The negative predictive value also improved to 89.7% when both were negative. The miR-3940-5p/miR-8069 ratio in urine exosomes may be useful as a tool for the diagnosis of PDAC, particularly when used in combination with CA19-9.

Correspondence to: Dr Kazushi Sugimoto, Department of Gastroenterology and Hepatology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan
E-mail: kazushi@clin.medic.mie-u.ac.jp

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; CP, chronic pancreatitis; CA19-9, carbohydrate antigen 19-9; PPV, positive predictive value; NPV, negative predictive value; miRNA, microRNA

Key words: pancreatic cancer, microRNA, urine, exosome, 3D digital PCR

Introduction

Pancreatic cancer still has an extremely poor prognosis. The number of deaths due to pancreatic cancer has been increasing worldwide. In fact, it was predicted that the total deaths from pancreatic cancer will become the second leading cause of cancer-related deaths in the United States by 2030 (1). In addition, National Cancer Center Japan (https://ganjoho.jp/reg_stat/statistics/stat/summary.html) reported that pancreatic cancer became the fourth leading cause of cancer deaths in Japan in 2016. There are several reasons for the low survival rate of pancreatic cancer. Among them, the most pressing problem that has been very hard to resolve is the difficulty of diagnosing the disease at an early stage. It is very difficult to detect pancreatic cancer by imaging studies performed in routine medical examinations, and by the time it is discovered due to worsening symptoms, such as jaundice or abdominal/back pain, it would have already progressed to an incurable phase. Another problem for its early diagnosis is the lack of a useful biomarker.

Many types of biomarkers for pancreatic cancer in patient serum, pancreatic juice, or stool have been proposed. Among these biomarkers, serum carbohydrate antigen (CA) 19-9 is the most commonly used in the usual clinical setting. However, several reports have suggested that the positive predictive value (PPV) of CA19-9 for pancreatic cancer was very low (0.5 to 0.9%) in asymptomatic patients (2,3). In addition, serum CA19-9 is elevated in many other types of cancers and benign diseases. As such, the utility of serum CA19-9 testing as a routine screening tool to find curable pancreatic cancer is

extremely limited. Although pancreatic cancers are found at an advanced stage in the majority of cases, Yachida *et al* (4) have shown that the progression of pancreatic cancer is relatively slow. According to their report, it takes at least 10 years for the initiated tumor cells to become parental non-metastatic founder cells, and at least 5 more years for the acquisition of metastatic ability. This suggests that if a potent diagnostic tool that can detect early stages of the disease was available, we may have a better chance than previously thought to find and treat pancreatic cancer before it becomes fatal.

MicroRNA (miRNA) is a small non-coding RNA that binds to complementary sequences in the 3' untranslated region of target messenger RNAs (mRNAs); this leads to the inhibition of their translation or their degradation, and thereby negatively regulate gene expression. Recent studies have revealed that many types of cancer cells produce specific miRNAs (5-7); therefore, the utility of specific miRNAs as biomarkers for cancers has been widely investigated. Many studies have shown that specific alterations in miRNA expression patterns are observable in the blood (8,9), pancreatic tissues (10,11), or pancreatic juice (12,13) of pancreatic cancer patients. For example, Abue *et al* (9) reported that miRNA-483-3p levels were elevated in the blood of pancreatic cancer patients when compared to intraductal papillary neoplasm patients and healthy controls, and it could be used to differentiate pancreatic cancer from intraductal papillary neoplasms with a sensitivity of 43.8%. In addition, Panarelli *et al* (10) showed that miR-21, miR-221, miR-155, miR-100, and miR-181b were overexpressed in resected pancreatic cancer tissues when compared to benign lesions.

One advantage of using miRNAs as biomarkers is their stability. Even though plasma has a high level of RNase activity, miRNAs are resistant to being degraded by them (14); they are protected from endogenous RNase activity because cells release the miRNAs by incorporating them into exosomes (15). Exosomes are membrane-bound particles of 40 to 100 nm that are released from many types of cells; they can be taken up by neighboring cells, and modulate the bioactivities of the recipient cells with their components, such as lipids, proteins, and nucleic acids (16). Exosomes are enriched with several hundred folds of mRNAs and miRNAs as compared to the donor cells, and the miRNAs in urinary exosomes have been widely explored as biomarkers, especially for cancers of the urinary tract (17-19). Moreover, some have raised the possibility that urine exosomes can be derived from diseases in organs other than the urinary tract (20,21).

Given these facts, in the present study, we investigated miRNAs specific for pancreatic cancer in urinary exosomes, and identified a novel biomarker candidate for this disease.

Materials and methods

Patients. Patients admitted to the Gastroenterology Department of Mie University Hospital between October 2015 and February 2017 with pancreatic ductal adenocarcinoma (PDAC) or chronic pancreatitis (CP), including autoimmune pancreatitis, were enrolled in this study. The final diagnoses of PDAC were confirmed by endoscopic ultrasound-guided fine-needle aspiration, endoscopic retrograde cholangiopancreatography, or endoscopic biopsy. The diagnoses of CP were made by endoscopic ultrasound-guided fine-needle aspiration and endoscopic retrograde cholangiopancreatography. The diagnoses of autoimmune pancreatitis were made from elevated serum IgG4 levels and positive staining for IgG4 by immunohistochemistry. Healthy control subjects, adjusted by age and sex, were also enrolled. All patients were newly diagnosed and treatment-naïve. Patients with a present or past history of any type of malignant neoplasm were excluded from this study.

This study was approved by the Ethics Committee of Mie University Hospital (reference no. 2833). Written informed consent was obtained from each patient included in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the *a priori* approval from the institution's human research committee.

Urine and serum sample collection. Urine samples (more than 10 ml) were collected from the PDAC, CP, and control subjects before highly invasive examinations, such as endoscopic procedures. The samples were kept at 4˚C, and centrifuged at 3,000 x g for 15 min within 8 h after collection. The supernatants were divided into 6-ml aliquots and kept at -30˚C until the analyses.

Serum was also collected from each subject before highly invasive examinations. The samples were kept at -80˚C until the analyses.

Cell lines. The human PDAC cell lines PANC-1 (RCB2095) and MIA PaCa-2 (RCB2094), human bile duct cell line HuCCT1 (RCB1960), human hepatocellular carcinoma cell line HuH-7 (RCB1942), human liver cancer cell line Hep G2 (RCB1886), and human gastric cancer cell line KATO III (RCB2088) were purchased from RIKEN BRC. The colon cancer cell line SW480 (ATCC CCL-228) was purchased from ATCC.

Cell cultures. All cell lines were cultured according to the manufacturers' instructions. Briefly, PANC-1 cells, SW480 cells, and HuH-7 cells were cultured at 37˚C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.). MIA PaCa-2 cells were maintained in DMEM with 10% FBS, 2.5% horse serum (Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin. HepG2 cells were maintained in modified Eagle's medium (Thermo Fisher Scientific, Inc.) with 10% FBS and 1% penicillin-streptomycin. Kato III cells and HuCCT1 cells were maintained in Roswell Park Memorial Institute medium 1640 (Thermo Fisher Scientific, Inc.) containing 10% FBS and 1% penicillin-streptomycin.

After incubation, the cells were washed with phosphate-buffered saline and incubated for 24 h in FBS-free medium. Then, the supernatants were centrifuged twice at 3,000 x g for 15 min and collected and divided into 6-ml aliquots and kept at -30˚C until the analyses.

Isolation of exosomes from urine samples and culture media. Exosomes were isolated from the urine samples and culture media using ExoQuick-TC (System Biosciences) according to

the manufacturer's protocol. Briefly, the frozen urine samples or culture media were thawed and centrifuged at 3,000 x g for 15 min to eliminate impure substances, such as cells and cell debris. Next, 5 ml of each supernatant and 1 ml of ExoQuick-Tc exosome precipitation solution were mixed well by flicking the tubes. The mixtures were allowed to settle overnight (for at least 12 h) at 4˚C and were then centrifuged at 1,500 x g for 30 min. The exosomes appeared as pellets and were isolated by aspiration of the supernatants.

Isolation of exosomes from serum samples. Exosomes were isolated from serum samples by using ExoQuick (System Biosciences) according to the manufacturer's instructions. The frozen sera were thawed and centrifuged at 3,500 x g for 20 min to eliminate impure substances, such as cells and cell debris. Next, 250 μl of each supernatant and 63 μl of Exo Quick exosome precipitation solution were mixed well by pipetting. The solutions were then allowed to settle for 30 min on ice and were then centrifuged at 1,500 x g for 35 min. The exosomes appeared as pellets and were isolated by aspiration of the supernatants.

Purification of the miRNA from exosomes. The miRNAs were purified from the exosomes by using a miRCURY RNA Isolation kit (Qiagen). Each exosome pellet was mixed with 350 μl of lysis solution by vortexing for over 15 sec and was confirmed to have completely dissolved. Subsequently, 200 μl of 99.5% ethanol was added to the mixture and vortexed for over 10 sec. The lysate was applied to a column assembled with a collection tube and centrifuged at 14,000 x g for 1 min, after which the flow-through was discarded. Next, 400 μl of wash solution was applied to the column, and centrifuged at 14,000 x g for 1 min, after which the flow-through was discarded. This washing procedure was repeated twice, and the column was dried by centrifugation at 14,000 x g for 2 min. Afterwards, 50 μl of eluent was applied to the column settled into an elution tube and centrifuged at 200 x g for 2 min, then 14,000 x g for 1 min. The extraction liquid was applied to the column again and centrifuged at 200 x g for 2 min, followed by 14,000 x g for 1 min. The purified samples were stored at -80˚C.

Quality checking of the miRNA. The quality of the extracted miRNA was examined using an Agilent Bioanalyzer 2100 and an RNA 6000 Small RNA kit (Agilent Technologies). All of the miRNA samples were confirmed to be suitable for further analyses.

miRNA expression profiling. The miRNA expression profiles were examined in nine PDAC patients and seven controls with SurePrint G3 Human miRNA Microarray, 8x60K Rel. 21.0, consist of 2549 human miRNA probes (Agilent Technologies) with 60 ng miRNA per sample according to the manufacturer's protocol. The hybridized chip was scanned using the G2539A Microarray Scanner (Agilent Technologies) and analyzed using Gene Spring GX software, version 13.1 (Agilent Technologies). Raw intensities were not normalized because the levels of miRNA expression were low.

Detection of miRNA. The levels of miRNA expression were validated in the culture media, patient sera, and a large number of urine samples using 3D digital PCR according to the results of the microarray.

Complementary DNA (cDNA) was synthesized from 6 μl of miRNA solution by using the miRCURY Universal cDNA Synthesis kit II (Qiagen) with the following conditions according to the manufacturer's instructions: 42˚C for 60 min, 95˚C for 5 min, and then held at 4˚C. Digital PCR was performed using a QuantStudio® 3D digital PCR (Thermo Fisher Scientific, Inc.). The reactions were prepared in a final volume of 18 μl, containing 9 μl of Mastermix QuantStudio® 3D digital PCR (Thermo Fisher Scientific, Inc.), 0.72 μl of cDNA, 1.8 μl of primers from the miRCURY LNA PCR Primer Set (Qiagen), and 1.8 μl of SYBR® Green I dye (Thermo Fisher Scientific, Inc.). The working solution of SYBR® Green I dye was diluted (1:1,000) with 100% dimethyl sulfoxide and stored in aliquots at -20˚C. The diluted SYBR® Green I dye was further diluted (1:20) with Tris-EDTA buffer (pH 8.0). The reaction mix (14.5 μl out of 18 μl) was loaded onto QuantStudio 3D digital PCR chips by using a QuantStudio 3D digital PCR chip loader. The amplification was performed with the following conditions: 96˚C for 10 min, 39 cycles of 60˚C for 2 min and 98˚C for 30 sec, 60˚C for 2 min, and then holding at 10˚C. The amplification results were analyzed with QuantStudio® 3D AnalysisSuite™ Cloud Software (Applied Biosystems). The software assessed the quality of the chip data and estimated the concentrations by counting the number of positive chambers.

Statistical analysis. All statistical tests were analyzed using SPSS software, version 24.0 (IBM Inc.). The Fisher exact test was applied to test the categorical data, and the Wilcoxon rank-sum test or Kruskal-Wallis test with a Dunn-Bonferroni post hoc test was applied to test the continuous data. The accuracy was investigated using receiver operating characteristic (ROC) curve analysis, the area under the curve (AUC), and accuracy measures for determining a suitable cutoff value. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of the miR-3940-5p/miR-8069 ratio as a novel PDAC biomarker. Firstly, as a preliminary study, we analyzed the expression of miRNA in urine exosomes by microarray in nine PDAC and seven control subjects, and the expression profiles were compared between these groups to find a candidate PDAC biomarker. Table I shows the characteristics of the subjects. There was no significant difference in the characteristics (age and sex) between the PDAC and control groups. In total, 38 miRNAs were upregulated by more than two-fold in the PDAC patients when compared to the controls. The five most upregulated miRNAs in the PDAC group are listed in Table II. Of note, the expression level of miR-3940-5p was significantly greater in all of the PDAC patients than in the controls (fold change: 3.047; $P=0.036$). On the other hand, miR-8069 was expressed at a similar level in all of the PDAC patients and controls (data not shown). Based on these results, we identified the miR-3940-5p/miR-8069 ratio in urine exosomes as a candidate novel biomarker for PDAC.

Table I. Patient characteristics analyzed by the microarray.

Subjects	Age, years	Sex	Size, mm	Tumor location	T classification (UICC 8th)	Clinical stage (UICC 8th)
Patients with pancreatic ductal adenocarcinoma	65	M	18	Head	T1	III
	83	M	22	Tail	T2	II
	76	F	22	Head	T4	III
	53	F	40	Head	T4	III
	73	F	30	Head	T4	IV
	84	M	16	Body-tail	T1	I
	67	M	20	Head	T2	I
	89	M	27	Head	T2	III
	59	F	46	Body-tail	T4	IV
Controls	78	M				
	62	M				
	76	F				
	68	F				
	57	M				
	81	F				
	76	M				

UICC 8th, the eighth edition of the International Union Against Cancer classification; M, male: F, female.

Table II. Top five most upregulated microRNAs in pancreatic ductal adenocarcinoma.

microRNA	Sequence	Fold change	P-value
hsa-miR-3940-5p	CAGAGCCCGCCC	3.047	0.036
hsa-miR-6085	TGTGCTCCCCCAGC	2.973	0.108
hsa-miR-4516	GCCCCGACCCTTC	2.662	0.120
hsa-miR-4298	CTGCCTCCTCCTCC	2.623	0.061
hsa-miR-6749-5p	GCTCCCCCAACCC	2.527	0.184

miR, microRNA.

Preliminary validation in cell lines. As the next step, we attempted to determine whether the increase in the miR-3940-5p/miR-8069 ratio was specific for PDAC by using seven different cancer cell lines. Because the expression levels of miRNA in the exosomes, especially those excreted into urine, were very low, it was difficult to quantify them accurately with the conventional quantitative PCR method; therefore, we measured them by 3D digital PCR. As shown in Fig. 1, the miR-3940-5p/miR-8069 ratio in exosomes from the culture media was significantly higher in the two PDAC cell lines (PANC-1 and MIA PaCa-2) than in the non-pancreatic cancer cell lines (HuH-7, HepG2, SW480, HuCCT1, and Kato III; P<0.001), suggesting that the increase in the miR-3940-5p/miR-8069 ratio was specific to PDAC.

Comparison of the expression levels in serum and urine exosomes. Next, we analyzed the miR-3940-5p/miR-8069 ratio in exosomes derived from the serum or urine of seven PDAC patients. Fig. 2 shows that the miR-3940-5p/miR-8069 ratio was greater in the urine exosomes than in the serum exosomes, although the difference did not reach a statistically significant level, perhaps due to the small sample size. These results suggested that urine samples are more suitable than serum samples for analyzing the miR-3940-5p/miR-8069 ratio as a biomarker for PDAC.

Validation in a larger number of samples. According to the results of the preliminary analyses, we attempted to further validate the usefulness of the miR-3940-5p/miR-8069 ratio in urine exosomes as a biomarker for PDAC in a larger number of samples. We examined a total of 43 PDAC, 12 CP, and 25 control subjects. Table III shows the characteristics of the patients. The mean tumor size of the PDAC cases was relatively large at 33.0±16.3 mm. According to the eighth edition of the International Union Against Cancer (UICC) classification system, 44.2% of the PDAC patients had T4 tumors, and 46.5% had clinical stage IV disease. As was mentioned above, the PDAC group in our study included advanced cases of PDAC.

The miR-3940-5p/miR-8069 ratio in the urine exosomes of each group is shown in Fig. 3. The median values of the control, CP, and PDAC subjects were 0.47, 0.54, and 1.07, respectively. The miR-3940-5p/miR-8069 ratio in urine exosomes was significantly higher in the PDAC group than in the control group (P<0.001) or the CP group (P<0.001).

To clarify the stage of PDAC at which the miR-3940-5p/miR-8069 ratio begins to increase, we analyzed the ratio according to T classification and clinical stage as defined by the eighth edition of the UICC classification (Fig. 4A and B). As shown in the graphs, the miR-3940-5p/miR-8069 ratio in the urine exosomes was significantly higher in the PDAC

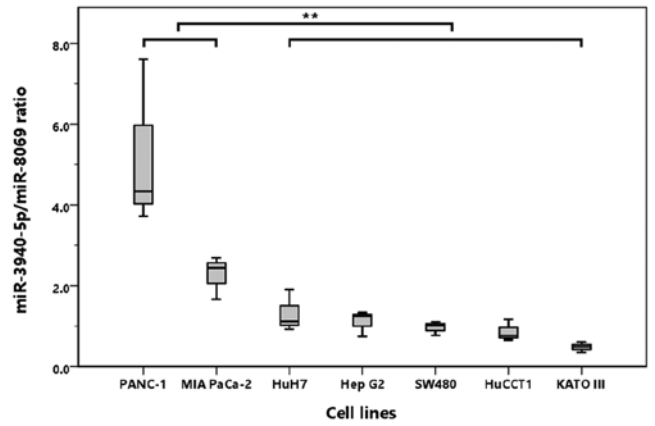

Figure 1. miR-3940-5p/miR-8069 ratio in exosomes from cancer cell lines. The levels of microRNAs in exosomes isolated from culture media were measured. The miR-3940-5p/miR-8069 ratio was significantly greater in pancreatic cancer cell lines than in other cancer cell lines. **P<0.01. miR, microRNA.

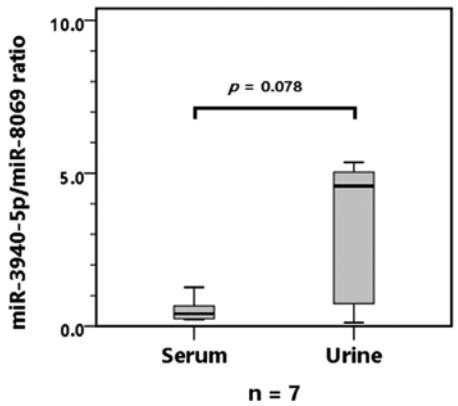

Figure 2. Comparison of the miR-3940-5p/miR-8069 ratio in exosomes derived from the serum and urine of patients with pancreatic ductal adenocarcinoma. The miR-3940-5p/miR-8069 ratio was greater in urine exosomes than in serum exosomes. miR, microRNA.

patients with T4 tumors or stage IV disease than in the control subjects. Interestingly, the ratio also tended to be elevated in T1 or stage I PDAC patients, although the differences did not reach a statistically significant level, perhaps due to the small sample sizes. Nevertheless, these results suggest the possibility that the miR-3940-5p/miR-8069 ratio in urine exosomes starts to increase from a relatively early stage of PDAC.

Diagnostic value of the miR-3940-5p/miR-8069 ratio for PDAC. The diagnostic accuracy of using the miR-3940-5p/miR-8069 ratio in urine exosomes for differentiating PDAC from CP and the controls was also evaluated using the receiver operating characteristic (ROC) curve (Fig. 5). The AUC was 0.732 (95% confidence interval: 0.621-0.843). The cutoff point established by the ROC curve was 0.939. By using this cutoff, the sensitivity, specificity, PPV, and negative predictive value (NPV) for PDAC were 58.1, 89.2, 86.2, and 64.7%, respectively (Table IV).

Diagnostic value of the miR-3940-5p ratio with CA19-9. Finally, we examined the utility of the miR-3940-5p/miR-8069 ratio in urine exosomes in combination with CA19-9 as biomarkers for differentiating PDAC. In our study subjects, the sensitivity, specificity, PPV, and NPV of CA19-9 (cutoff: 37 U/ml) were 79.1, 81.1, 82.9, and 76.9%, respectively (Table IV). After the miR-3940-5p ratio and CA19-9 were combined, when either of them was positive, the sensitivity and PPV reached 93.0 and 78.4%, respectively. In addition, the PPV reached 100% when both were positive. In contrast, when both were negative, the specificity and NPV were 70.3 and 89.7%, respectively (Table IV). These results clearly indicated that a combined analysis of the miR-3940-5p/miR-8069 ratio in urine exosomes and CA19-9 is more helpful for predicting or ruling out PDAC than when they are used alone.

Table III. Background characteristics of the 43 pancreatic ductal adenocarcinoma cases.

Characteristics	Number/mean ± SD	Percentage
Age, years	68.4±10.2	
Sex (male)	25	58.1
Tumor location		
Head	26	60.5
Body-tail	17	39.5
Tumor size, mm	33.0±16.3	
T classification (UICC 8th)		
T1	6	14.0
T2	13	30.2
T3	5	11.6
T4	19	44.2
Clinical stage (UICC 8th)		
I	12	27.9
II	3	7.0
III	8	18.6
IV	20	46.5

UICC 8th, the eighth edition of the International Union Against Cancer classification.

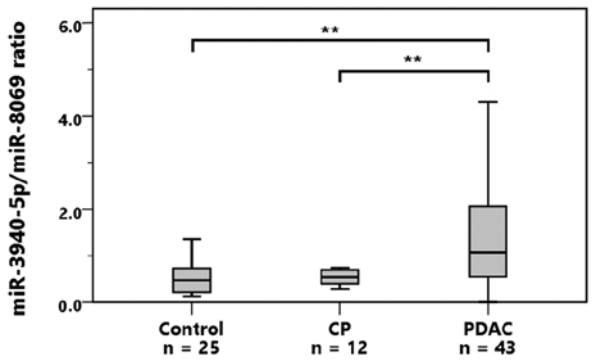

Figure 3. miR-3940-5p/miR-8069 ratio in the urine exosomes of the study subjects. The miR-3940-5p/miR-8069 ratio in urine exosomes was compared among 25 control, 12 CP and 43 PDAC samples. The ratio was significantly greater in the PDAC samples than in the control or CP samples. The mean values for the control, CP and PDAC samples were 0.47, 0.54 and 1.07, respectively. **P<0.01. CP, chronic pancreatitis; miR, microRNA; PDAC, pancreatic ductal adenocarcinoma.

Table IV. Diagnostic ability of the miR-3940-5p/miR-8069 ratio and CA19-9 for pancreatic ductal adenocarcinoma.

Parameters	miR-3940-5p/miR-8069	CA19-9	miR-3940-5p/miR-8069 + CA19-9
Sensitivity, %	58.1	79.1	93.0[a]
Specificity, %	89.2	81.1	70.3[b]
Positive predictive value, %	86.2	82.9	78.4[a] (100[c])
Negative predictive value, %	64.7	76.9	89.7[b]

[a]At least one of these parameters is positive; [b]Both parameters are negative; [c]Both parameters are positive. CA19-9, carbohydrate antigen 19-9; miR, microRNA.

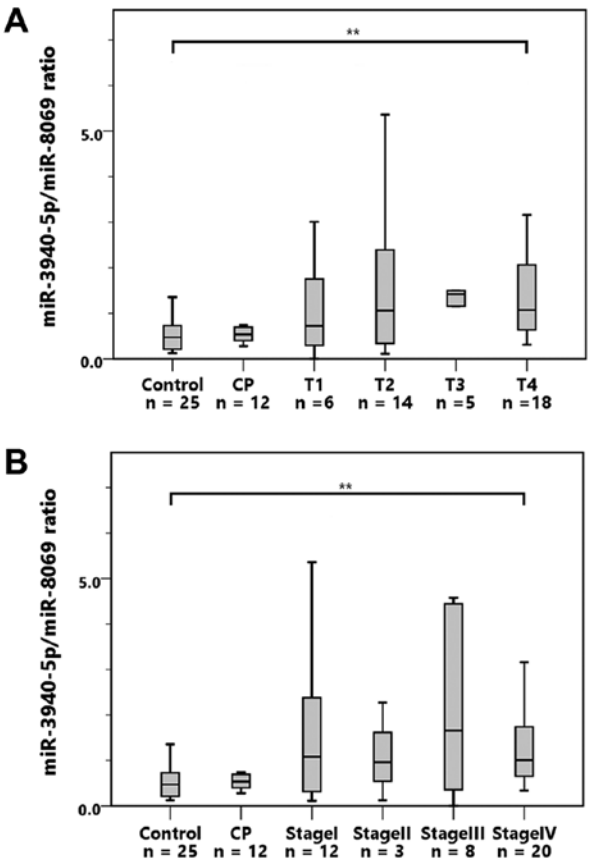

Figure 4. Alterations in the miR-3940-5p/miR-8069 ratio in urine exosomes at different stages of PDAC. The miR-3940-5p/miR-8069 ratio was analyzed according to (A) T classification and (B) clinical stage as defined by the eighth edition of the International Union Against Cancer classification. The miR-3940-5p/miR-8069 ratio tended to increase from the early stages of PDAC. **P<0.01. CP, chronic pancreatitis; miR, microRNA; PDAC, pancreatic ductal adenocarcinoma.

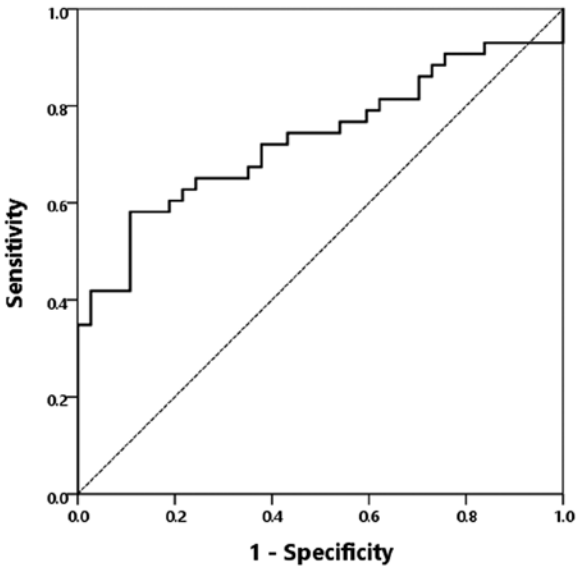

Figure 5. Receiver operating characteristic curve analysis of the miR-3940-5p/miR-8069 ratio. The area under the curve was 0.732 (95% CI, 0.621-0.843), and the cut-off established by the receiver operating characteristic analysis was 0.939. miR, microRNA.

Discussion

In the present study, we identified the miR-3940-5p/miR-8069 ratio in urine exosomes as a novel biomarker for PDAC. We also found that an elevation of this ratio was specific for PDAC in cultured cancer cell lines, and the ratio was greater in the urine exosomes than in the serum exosomes of PDAC patients. In addition, the diagnostic ability of the miR-3940-5p/miR-8069 ratio and CA19-9 value for identifying PDAC was much better when used in combination than when used alone.

Despite the development of several therapeutic options, the prognosis of PDAC is still poor. One of the biggest reasons for this is the difficulty of diagnosing the disease at an early stage. In addition to imaging studies, several biomarkers are currently used for the diagnosis of PDAC. Among them, CA19-9 is the most extensively studied biomarker, and it is known to correlate with prognosis (22); however, CA19-9 is also occasionally elevated in benign hepatobiliary diseases, pancreatitis, or other tumors, such as colorectal cancer and ovarian tumors (23,24), and the PPV in asymptomatic patients is extremely low (2,3). Therefore, many studies have attempted to find new diagnostic markers for PDAC in blood or pancreatic juice, and many studies have been focusing on miRNAs (8-13). In the present study, we explored miRNAs in urine exosomes, and identified the miR-3940-5p/miR-8069 ratio as a novel biomarker for PDAC. There were two major reasons why we focused on urine exosomes. First, urine is easy to obtain repeatedly from patients without any invasive procedures; as such, we believe more and more methodologies using urine samples will be developed hereafter for the diagnosis of many kinds of diseases. Indeed, recent studies have suggested the possibility that urine miRNAs can be biomarkers for several cancers, including PDAC (25,26).

The second reason is that miRNAs are more stable and concentrated in exosomes than in host cells or serum. The results of preliminary analyses using microarray data suggested that miR-3940-5p was upregulated in the urine exosomes of most PDAC patients when compared to the controls.

The precise mechanism by which exosomes derived from cells other than urinary tract are excreted into urine is not clear yet. However, several studies have actually shown that some disease-specific exosomes can be detected in urine, and they suggested that the miRNA contained in the exosomes can act as biomarkers for non-urologic diseases, including cancers (20,21,27). In addition, Weber *et al* (28) showed the differences of miRNA expression profiles in different human body fluids, and some miRNAs showed higher expression levels in urine compared to serum. However, the amounts of urinary exosomes and the miRNAs encapsulated in them are extremely low, so it has been difficult to obtain and assess a sufficient amount of them for use as biomarkers. To overcome this problem, Yasui *et al* (27) developed a new nanowire-based methodology to collect miRNAs encapsulated in urine extracellular vesicles, including exosomes, microvesicles, and apoptotic bodies. Using this method, they found that many miRNAs in urine extracellular vesicles were overexpressed or downregulated in lung, liver, and pancreatic cancers (27). In our present study, we used 3D digital PCR to accurately quantify the low levels of miRNA for validation in a large number of samples. Digital PCR, which is a third generation of PCR, has increased precision and sensitivity for detecting low amounts of target copies when compared to conventional quantitative real-time PCR (29,30). As such, it can be used for the detection and quantification of low levels of pathogens, rare genetic sequences, and cancer-related miRNAs (31-33). To more precisely compare the expression levels of miR-3940-5p in a large number of samples, we normalized the miR-3940-5p levels to the miR-8069 levels, because the results of microarray analysis indicated that miR-8069 was expressed at a similar level in all samples, including PDAC and control samples. We found that the miR-3940-5p/miR-8069 ratio in urine exosomes was significantly elevated in PDAC patients when compared to the controls or CP patients. We also found that this elevation could be observed at a relatively early stage of PDAC.

Sun *et al* (34) reported that the expression of miR-3940-5p was downregulated in non-small cell lung carcinoma (NSCLC) tissues when compared to normal lung tissues or tumor-adjacent tissues. They also found that miR-3940-5p was specifically reduced in nuclear cell proliferation antigen Ki-67-positive NSCLC cases (34). In addition, Ren *et al* (35) reported that miR-3940-5p may target cyclin D1 and ubiquitin specific peptide-28 to inhibit the growth of NSCLC. In the present study, the miR-3940-5p/miR-8069 ratio was increased in the PDAC group, and experimental results from studies using cultured cancer cell lines showed that this increase was specific to PDAC. However, the biological relevance of its upregulation and the reason for the discrepancy in results between PDAC and NSCLC remain to be elucidated. Furthermore, we found that the miR-3940-5p/miR-8069 ratio was greater in urine exosomes than in serum exosomes. The reason for this remains unclear, however, these results suggests that although the amount of PDAC-related exosomes that have a high miR-3940-5p/miR-8069 ratio in serum is extremely low, and a small proportion of them is excreted into the urine, the concentration of these exosomes is still higher in urine than in blood.

One of the most important findings of this study is that the miR-3940-5p/miR-8069 ratio and CA19-9 value as biomarkers can better predict PDAC when used in combination than when used alone. In fact, when we applied the cutoff point of 0.939, which was established from the ROC curve, for the miR-3940-5p/miR-8069 ratio, and the widely clinically used cutoff of 37 U/ml for CA19-9, the sensitivity and PPV improved to 93.0 and 78.4%, respectively, when either of them was positive. Moreover, the PPV reached 100% when they were both positive. Similarly, the NPV also improved to 89.7% when both were negative.

We acknowledge that this study has some limitations. First, it was carried out with a small number of samples. To improve the reliability of the findings of this study, more analyses with a larger number of patients is needed. Second, we did not clarify the relationship between the miR-3940-5p/miR-8069 ratio in urine exosomes and the prognosis of PDAC, nor possible changes in the ratio after therapy for PDAC. Although these points were beyond the scope of this study, further investigations focusing on these points are necessary as the next step in examining the miR-3940-5p/miR-8069 ratio in urine exosomes as a biomarker for PDAC.

In conclusion, the results of the present study showed that the miR-3940-5p/miR-8069 ratio in urine exosomes is elevated in PDAC patients, suggesting that it may be a potent diagnostic tool for PDAC, especially in combination with CA19-9.

Acknowledgements

The authors would like to thank Ms. Mina Tenpaku (Department of Gastroenterology and Hepatology, Mie University Graduate School of Medicine, Mie, Japan) for her technical assistance.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KS and MT conceived and designed the study. NY, HI and MU recruited the subjects, and collected and analyzed the data. NY, YI, MT and MIk performed experiments. KS, NY and MT wrote the manuscript. MIt and YT interpreted the data and supervised the study. MIk, HI, MU, MIt and YT revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Mie University Hospital (reference no. 2833). Written informed consent was obtained from each patient included in the present study.

Patient consent for publication

All participants provided consent for publication.

Competing interest

The authors declare that they have no competing interests.

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