Exploration of Exosomal Micro RNA Biomarkers Related to Epithelial-to-Mesenchymal Transition in Pancreatic Cancer

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Abstract. *Background/Aim:* Epithelial-to-mesenchymal transition (EMT) plays important roles in cancer progression. This study aimed to identify the exosomal miRNA (exo-miRNA) profiles related to the EMT status in pancreatic cancer (PC). *Materials and Methods:* Comprehensive exo-miRNA-expression profiles in the culture media of PC cell lines were analyzed through microarray technology. The identified miRNAs were analyzed to investigate their clinical implication using The Cancer Genome Atlas (TCGA) dataset and clinical samples. *Results:* We prioritized 291 exo-miRNAs differentially expressed between epithelial and mesenchymal cell types. Among them, survival analysis based on the TCGA dataset revealed that mir-196b and mir-204 significantly stratify the prognosis of PC cases. In addition, analysis of cell lines indicated miR-196b-3p as a mesenchymal marker and miR-204-3p as an epithelial marker. Finally, we demonstrated that miR-196b-3p and miR-204-3p in serum exosomes were differentially expressed among intraductal papillary mucinous neoplasms, mucinous cystic neoplasms, and PC. *Conclusion:* Serum exo-miRNA biomarkers potentially identify the pancreatic tumor status through less-invasive methods.

Pancreatic cancer (PC), comprising mainly pancreatic ductal adenocarcinoma, is one of the deadliest malignancies worldwide and the seventh leading cause of cancer-related mortality with an estimated 432,000 deaths in 2018 (1).

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PC's poor clinical prognosis is generally attributed to the high frequency of local invasiveness and distant metastasis (2). Epithelial-to-mesenchymal transition (EMT) has important roles in cancer cell invasion and metastasis (3). We previously reported that the EMT status of PC, as defined by immunohistochemistry for E-cadherin and vimentin, was associated with PC prognosis and the mesenchymal type had higher invasive features such as peritoneal invasion, portal vein invasion, and lymph node metastasis (4). For these reasons, we hypothesized that the clinical behavior of PC, whether locally invasive or metastatic, would be related to the EMT status and the understanding of the EMT status of PC can contribute to development of a novel therapy for PC patients.

Micro RNAs (miRNAs) have been identified as important regulators of gene expression in tissue-specific physiological pathways, in response to environmental cues and in various diseases including human malignancies (5, 6). The mature form of miRNAs consist of 21 to 25 base pairs and is capable of inhibiting transcription by inducing degradation of the target mRNAs (5). Although the functional roles of miRNAs in tumor biology are incredibly complicated and all their functions have not been fully understood, we expect that circulating blood miRNA could predict the clinical behavior of PC.

Exosomes are cell-derived microvesicles that are present in different kinds of fluids such as blood, saliva, urine, and also in the culture medium of cell lines (7). Accumulating evidence supports that cancer exosomes play an important role in cell-to-cell communication leading to cancer progression (8). In addition, as exosomes released from cancer cells contain several RNA types including miRNAs, exosomal miRNA (exo-miRNA) could reflect the miRNA signature of the parental cancer cell and can thus inform us on the future behavior of cancer cells (9).

The aim of this study was to identify the exo-miRNA profiles related to the EMT status of PC patients through genome-wide microarray technology. In addition, we attempted to clarify the clinical implication of the selected

Table I. The TaqMan® assays used in this study.

Assay name	AssayID	miRBase (v21) accession number	Mature miRNA sequence
hsa-miR-196b*	121219_mat	MIMAT0009201	UCGACAGCACGACACUGCCUUC
hsa-miR-636	002088	MI0003651	UGUGCUUGCUCGUCCCGCCGCA
hsa-miR-204-3p	463101_mat	MIMAT0022693	GCUGGGAAGGCAAGGGACGU
hsa-miR-3648	464401_mat	MIMAT0018068	AGCCGCGGGGAUCGCCGAGGG
hsa-miR-16	000391	MI0000070	UAGCAGCACGUAAUAUUGGCG

exo-miRNA biomarkers in clinical specimens derived from patients. Through this approach, we aimed to gain a deeper understanding of the relationship between EMT status and PC that could lead to personalized medicine for PC patients.

Materials and Methods

Cell lines used in the study. PC cell lines (PANC-1, MIA PaCa-2, HPAF-II, Capan-1, Capan-2, BxPC-3, COLO357, CFPAC-1, AsPC-1, SW1990, TU-8902, KP1-NL, and MPanc96) used in this study were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM or RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies Corp., Grand Island, NY, USA). All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. EMT status of each PC cell line was determined according to our previous report (4).

Patients and sample collection. This study and all procedures were approved by the Institutional Review Board at Nagoya University and all patients provided written informed consent. All clinical investigations were conducted in accordance with the principles of the Declaration of Helsinki (10). The pancreatic tissues were collected from 16 PC patients undergoing pancreatectomy at the Department of Gastroenterological Surgery, Nagoya University Hospital. All surgically obtained tissue samples mounted in O.C.T. compound (Sakura Finetek, Tokyo, Japan) were immediately frozen in the liquid nitrogen and stored at -80°C until further analysis. We collected serum samples from patients with PC (n = 44), intraductal papillary neoplasm (IPMN, n=15), and mucinous cystic neoplasm (MCN, n=4), from which we extracted the circulating exosomes.

Isolation of exosomes from culture media and nanoparticle tracking analysis. Exosomes were isolated from the culture media of four PC cell lines. To remove cells, collected culture media were centrifuged for 5 min at 900 × g, followed by another centrifugation for 1 h at 10,000 × g to remove remaining debris. To remove all particles larger than 200 nm, the centrifuged medium was filtered through 0.2 µm pore filters (Sigma-Aldrich, St. Louis, MO, USA). To concentrate the exosomes, the filtrate was passed through a Vivacell 100 (Sartorius AG, Goettingen, Germany) during a 30 min centrifugation at 4°C and 400 × g. Ten microliters of the concentrated supernatant were passed through a Sepharose CL-2B (GE Healthcare, Munich, Germany) and 1 ml fractions were eluted. The number of nanoparticles in whole culture-media-derived

exosomes was measured using NanoSight NS300 nanoparticle characterization system (NanoSight Ltd., Amesbury, UK). Samples were diluted to 1/2000 and injected into the 405 nm laser chamber with a constant output controlled by a syringe pump. Three recordings were performed for each sample. Nanoparticle Tracking Analysis software (NanoSight Ltd., Amesbury, UK) was used to measure the size and the concentration of nanoparticles. The Batch Process included in the software was used to integrate the three technical measurements of each sample.

Comprehensive analysis of exo-miRNA expression. To identify an exo-miRNA signature for discriminating the EMT status of PC, comprehensive miRNA- expression profiles of exosomes in culture media from two epithelial cell types and two mesenchymal cell types included interrogation of 2,565 probes, using the 3D-Gene® Human miRNA Oligo Chip ver.21 (TORAY, Kanagawa, Japan). For extraction of exosomes, four cell lines, PANC-1, BxPC-3, MIA PaCa-2, and Capan-2, were cultured and culture medium from each cell line was collected. Total RNA was extracted from the resuspended exosomes using 3D-Gene® RNA extraction reagent from a liquid sample kit (TORAY, Kanagawa, Japan). Comprehensive miRNA expression analysis was performed using a 3D-Gene® miRNA Labeling kit (TORAY, Kanagawa, Japan) and a 3D-Gene® Human miRNA Oligo Chip (TORAY, Kanagawa, Japan) as previously reported (11). All microarray data of the study were in agreement with the Minimum Information About a Microarray Experiment (MIAME) guidelines (12).

Publicly available dataset. Normalized Illumina-HiSeq data for pancreatic adenocarcinoma from The Cancer Genome Atlas (TCGA) were downloaded from the Broad GDAC Firehose (<http://gdac.broadinstitute.org/>, accessed on June 1st, 2018). This dataset includes 185 PC cases, including 138 cases with information on recurrence-free survival (RFS). This dataset also includes the sequencing data of four solid normal pancreatic tissues. We also downloaded the normalized microarray data of GSE24279 from Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>, accessed on June 1st, 2018) for selecting candidate exo-miRNAs (13).

Extraction of exosomes and exo-miRNA from serum specimen. To isolate exosomes from the patients' sera, MagCapture™ Exosome Isolation Kit PS (Wako, Osaka, Japan) was used according to the manufacturer's instructions. Subsequently, total RNA was extracted from collected exosomes with the use of the microRNA Extractor SP Kit (Wako, Osaka, Japan) according to the manufacturer's instructions.

qPCR analysis. For miRNA-based quantitative PCR (qPCR) analysis, 10 ng of total RNA was reverse transcribed using the

TaqMan® Micro RNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a total reaction volume of 15 µl. qPCR was performed with MicroRNA Assay Kits (Applied Biosystems, Foster City, CA, USA) and TaqMan® Universal MasterMix II, no UNG (Applied Biosystems, Foster City, CA, USA) as previously reported (11). The mean Ct values of each sample were determined from duplicate reactions. The relative expression level of each miRNA examined was expressed as ΔCt , which was defined as the subtraction of the Ct value of the target miRNA from the Ct value of the internal control miRNA-16 (14). The TaqMan® Assays used in the study are shown in Table I.

Statistical analysis. Continuous variables were compared using Wilcoxon signed-rank test or student *t*-test, as appropriate. Categorical variables were compared using the χ^2 or Fisher's exact tests, as appropriate. RFS rates were estimated using the Kaplan-Meier method and compared using a log-rank test. All statistical analyses were performed using R version 3.4.3 (<https://www.r-project.org/>) and GraphPad Prism 7.03 (Graph Pad Software, San Diego, CA, USA).

Results

Exosomes collected from the culture media of PC cell lines. To investigate differentially expressed exo-miRNAs between the mesenchymal type of PC and the epithelial type, we first analyzed using microarrays the exo-miRNA expression profile in the culture media of four PC cell lines: PANC-1, BxPC-3, MIA PaCa-2, and Capan-2. Based on the expression levels of E-cadherin and vimentin, PANC-1 and MIA PaCa-2 comprised the mesenchymal cell type, while BxPC-3 and Capan-2 the epithelial cell type. From the collected culture media of each cell line, exosomes were purified using ultracentrifugation and the morphology was confirmed by electron microscopy (Figure 1A). The sizes of the extracellular vesicles including the exosomes were also measured with the nanoparticle tracking analysis and particles with diameters from 30 to 200 nm were regarded as exosomes (Figure 1B).

Genome-wide analysis of exo-miRNA from the culture media of PC cell lines. As a result of comprehensive exo-miRNA profiling using microarray technology, a heatmap of the differentially expressed exo-miRNAs between mesenchymal and epithelial PC cell types was generated (Figure 2A) (absolute log₂ fold change >0.58). Of the total of 2,565 miRNAs, 67 miRNAs were expressed at higher levels in exosomes from the mesenchymal type than from the epithelial type [log₂ fold change (mesenchymal/epithelial) >0.58, Figure 2B]. To filter out the miRNAs from non-cancerous cells, to reduce the number of target miRNAs and, therefore, to increase their practical future utility in the clinic, the microarray data of healthy pancreatic tissue (n=22) and pancreatic ductal adenocarcinoma tissue (n=136) from GSE24279 was also analyzed. There were 193 highly expressed miRNAs in the PC tissues compared with the normal pancreatic tissues in this dataset. Consequently, we

selected the two miRNAs, miR-196b-3p and miR-636, in the intersection of 67 miRNAs from our microarray analysis and the 193 miRNAs from GSE24279 (Figure 2B). In addition, with the analysis of our microarray data, three down-regulated exo-miRNAs in the mesenchymal type, miR-204-3p, miR-3648, and miR-4497, were identified by the following criteria: log₂ fold change (mesenchymal/ epithelial) <2 and *p*<0.05 derived from student *t*-test (Figure 2C). Finally, we selected five miRNAs for further analyses. To understand the putative function of these five miRNAs, mirPath v.3 analysis was performed that revealed the pathways related to miR-204-3p and miR-3648, but no pathways were associated with the other three miRNAs (Figure 2D) (15).

Analysis with publicly available datasets. As the exosomes related to cancer cell communication are mainly released by cancer cells or surrounding cancer associated cells such as fibroblasts (16), we analyzed the expression of selected miRNAs in PC tissues using the TCGA dataset. Because expression levels of the mature type of selected miRNAs were basically low and the purpose of this study was to elucidate exo-miRNAs related to the EMT status of PC, we downloaded pre-miRNA sequence data from Broad GDAC FIREHOSE instead of mature miRNA sequence data. Based on the normalized RNA-sequencing data of TCGA, four miRNAs except for mir-4497 were available for further analyses. The comparisons between PC tissue and solid normal tissue in the TCGA dataset revealed that the expression levels of mir-196b in PC tissues were relatively higher than those in normal tissues and the expression levels of mir-204 in PC tissues were lower than those in normal tissues (mir-196b, *p*=0.18; mir-204, *p*=0.33, Figure 3A). Furthermore, PC cases were divided into two groups according to each miRNA expression levels in PC tissues (Figure 3B). As a result, the RFS of the PC cases with high mir-196b expression was significantly worse than that of PC cases with low mir-196b expression (*p*=0.0003, Figure 3B) and the RFS of the PC cases with low mir-204 expression was significantly worse than that of PC cases with high mir-204 expression (*p*=0.0014, Figure 3B).

Expression of selected miRNAs in PC cell lines. Before using specimens derived from patients, we confirmed the expression levels of the identified miRNAs in seven PC cell lines: HPAF-II, Capan-1, Capan-2, BxPC-3, COLO357, CFPAC-1, and AsPC-1. The expression levels of E-cadherin and Vimentin in each cell line are based on our previous study (4). The comparisons of the normalized miRNA expression levels among PC cell lines are provided in Figure 4. Collectively, miR-196b-3p was regarded as a mesenchymal marker and miR-204-3p as an epithelial marker.

Confirmation of the expression levels of selected miRNAs in PC tissue and patient's serum. To investigate the selected

Figure 2. Identification of exosomal micro RNAs (exo-miRNAs) associated with epithelial to mesenchymal transition in pancreatic cancer (PC) cell lines. (A) A heatmap of the differentially expressed exo-miRNAs between mesenchymal and epithelial PC cell lines. There were 291 miRNAs differentially expressed between the two types of PC cells. (B) A Venn diagram of exo-miRNAs highly expressed in the mesenchymal type from in-house microarray data and miRNAs highly expressed in pancreatic ductal adenocarcinoma from publicly available microarray dataset (GSE24279). MiR-196b-3p and miR-636 were in the intersection of the two circles. (C) Exo-miRNAs were expressed less in the culture media of mesenchymal PC cell lines. p-Value was derived from student's t-test. (D) Analysis of the putative pathways related to identified miRNAs differentially expressed between exosomes of epithelial and mesenchymal types.

Figure 3. Analysis of identified miRNAs using The Cancer Genome Atlas (TCGA) dataset. (A) Comparisons of expression levels of the selected miRNAs between pancreatic cancer (PC) tissue and non-cancerous pancreatic tissue. Sequencing data of mir-4497 was not available in this dataset. (B) Recurrence free survival (RFS) of PC patients in TCGA stratified by the expression levels of the selected miRNAs.

Figure 4. Expression analysis of identified miRNAs in pancreatic cancer cell lines. Expression analysis of identified miRNAs; miR-196b-3p, miR-636, and miR-204-3p was performed by quantitative PCR. (A) Normalized expression levels of miR-196b-3p. (B) Normalized expression levels of miR-636. (C) Normalized expression levels of miR-204-3p. (D) Normalized expression levels of miR-3648.

epithelial and mesenchymal exo-miRNA markers in clinical specimens, we extracted total RNA from resected PC tissues embedded with the O.C.T. compound and compared the expression levels in PC tissue and matched adjacent normal tissue. The expression levels of miR-196b-3p were significantly higher in PC tissue than in the paired normal

pancreatic tissue ($p=0.009$, Figure 5A), while the expression levels of miR-204-3p did not differ significantly ($p=0.85$, Figure 5A). In addition, to evaluate the potential utility of these two exo-miRNAs as less-invasive biomarkers, exo-miRNAs in the sera from patients with PC, IPMN, and MCN were extracted and used for quantification of exosomal miR-196b-

Figure 5. Analysis of miR196b-3p and miR-204-3p in clinical samples. (A) Normalized expression of miR-196b-3p and miR-204-3p in resected pancreatic cancer tissues and matched normal pancreatic tissues. (B) Normalized expression of miR-196b-3p and miR-204-3p in serum specimens derived from patients with intraductal papillary mucinous neoplasm, mucinous cystic neoplasm, and pancreatic cancer. PC: Pancreatic cancer; N: normal pancreatic tissue; IPMN: interpapillary mucinous neoplasm; MCN: mucinous cystic neoplasm; PC-CT: pancreatic cancer treated with chemotherapy.

3p and miR-204-3p. Expression of miR-196b-3p in serum exosomes -from PC patients was higher than that in IPMN patients with marginal significance ($p=0.14$, Figure 5B, while there was no significant difference between MCN and PC patients ($p=0.59$, Figure 5B). The expression of miR-204-3p

was significantly higher in exosomes derived from the serum of MCN patients than that from IPMN patients ($p=0.02$, $p=0.007$, Figure 5B), while there was marginally significant difference between MCN and PC patients ($p=0.24$, Figure 5B). Furthermore, the comparison between serum exosomes from

non-treated PC patients and PC patients treated with chemotherapy indicated that both exo-miRNAs were significantly lower in PC patients treated with chemotherapy (miR-196b-3p, $p=0.007$; miR-204-3p, $p=0.04$, Figure 5B).

Discussion

In this study, we performed a comprehensive miRNA microarray-based expression profiling analysis of culture media from four different PC cell lines, to identify an exo-miRNA biomarker for the EMT status of an original PC cell. We subsequently evaluated the expression of selected miRNAs in publicly available datasets and PC cell lines to understand the significance of the expression levels of these miRNAs in PC tissues and cells. Finally, we demonstrated that miR-196b-3p and miR-204-3p were differentially expressed in serum exosomes from IPMN, MCN, and PC. Namely, serum exo-miRNA biomarkers potentially characterize the pancreatic tumor status through less-invasive methods.

PC is one of the lethal diseases due to the fact that it easily spreads through the perineural spaces surrounding abdominal arteries (17, 18). In addition, the pancreas is anatomically juxtaposed to arteries such as the superior mesenteric artery, the hepatic artery, and the celiac artery and poses a difficulty in removing the locally advanced PC with sufficient resection margins. While some PCs extensively invade surrounding tissues through the perineural space, other PCs are diagnosed as completely resectable, and distant metastasis such as liver and peritoneal metastasis occurs after resection. Thus, advanced PCs comprise different types of PC, and have a different progression behavior.

EMT, the capability of cells to switch between epithelial and mesenchymal type, has been essential for the generation of complex body patterns and is the mechanism used by cancer cells to disperse throughout the body (19). We have previously reported that the EMT status is one of the independent prognostic factors for surgically resected PCs. The expression of lysyl oxidase homolog 2 (LOXL2), one of the important regulators of EMT, was significantly related to PC patients' survival and it is one of the putative therapeutic targets (4, 20). In addition, evidence supports that EMT may mediate chemoresistance in PC and defining the EMT status of each PC patient might contribute to suitable multidisciplinary therapy (21, 22). However, clinically useful biomarkers for distinguishing the EMT status of PC have not been identified.

Cancer cells secrete various types of humoral factors. Exosomes are small membranous vesicles that differ in their cellular origin and contain mRNA, DNA, proteins, and miRNA (23). Extracellular vesicles including exosomes and microvesicles from cancer cells have been found in the blood of cancer patients and therefore, provide a source of cancer-related molecules (24). In addition, several studies have shown that exo-miRNAs promote metastases and enhance

endothelial cell migration (25, 26), and cancer-associated fibroblasts can also secrete exosomes, which can regulate the survival and proliferation of cancer cells (16). Therefore, as exosomal molecules can inform us about the type and nature of the original tumor cell, we analyzed the expression of exo-miRNAs using a microarray-based technology.

In the current study, miR-196b-3p and miR-636 were found to be upregulated in the exosomes from mesenchymal PC cells. MiR-196b-3p is a human myometrial miRNA that is modulated by exogenous oxytocin and its expression has been shown to be downregulated in a rat model of lipopolysaccharide-induced acute lung injury (27, 28). Park *et al.* have revealed that the intrinsic constitutive signaling circuit composed of I κ B α /NF- κ B(p65), miR-196b-3p, Meis2, and PPP3CC is formed during the emergence of castration-resistant prostate cancer and this circuit controls the expression of stem cell transcription factors that drive high tumorigenicity (29). Schultz *et al.* have reported two microRNA panels in whole blood for the detection of PC using the combination of four or 10 miRNAs including miR-636 (30). In another study, miR-636 has also been identified as commonly down regulated miRNA in recurrent prostate cancer compared with non-recurrent prostate cancer by meta-analysis of six publicly available datasets (31). We also examined the expression levels of miR-204-3p in resected PC tissue and sera from patients with IPMN, MCN, and PC. In several kinds of human malignancies, miR-204 acts as tumor suppressor but the role of miR-204 in cancer development remains controversial (32, 33). Roldo *et al.* investigated miRNA expression profiles in normal pancreas tissue and pancreatic tumors including insulinomas, and they found miR-204 is expressed primarily in insulinomas and correlates with immunohistochemical expression of insulin (34). Besides, in the study for discovering a urine biomarker for early detection of PC with microarray analysis, miR-204 showed statistically higher levels in Stage I compared to Stages II, III, and IV (35). In our analysis of serum specimens, the expression of miR-204-3p in MCN patients was significantly higher than that in IPMN and PC patients. Thus, the expression of miR-204 may be downregulated in advanced PC and may be relatively high in early stage of PC or other low-malignant pancreatic tumors.

The recent advancements in broad genomic and transcriptomic analysis using microarray or high-throughput sequencing procedures have resulted in molecular characterization of several cancer types (36-38). However, to our best knowledge, this study is the first to extract miR-196b-3p and miR-636 from exosomes of PC cells and to identify the association with the EMT status of PC. Although, our results indicate that the discovered exo-miRNAs are differentially expressed among exosomes derived from IPMN, MCN, and PC, there were some inherent limitations to this study. First, in biomarker discovery, we should consider the potential difference between cell lines and heterogeneous human tissue. We employed four PC cell lines to elucidate differentially expressed miRNAs in exosomes

from epithelial and mesenchymal cell types because the heterogeneity of the clinical specimens diminishes the difference between the two types. Second, the clinical specimens were very limited and from a single institution. To confirm the practical utility of the discovered biomarkers, evaluation of prospectively corrected specimens should be conducted. Finally, as we cannot analyze the relation between the selected exo-miRNAs and clinical features such as chemo-sensitivity and survival, we are now collecting blood samples from PC patients undergoing neoadjuvant chemotherapy, adjuvant chemotherapy, and chemotherapy for unresectable or metastatic PCs for a future analysis of the identified exo-miRNA biomarkers.

Conclusion

In conclusion, we identified novel exo-miRNAs associated with the EMT status of PC using genome-wide exo-miRNA profiling in exosomes from the culture media of PC cell lines. Our findings may contribute in the identification of less-invasive biomarkers for EMT status of PC patients and improve the decision for PC therapy.

Conflicts of Interest

The Authors declare no potential conflicts of interest associated with this manuscript.

Authors' Contributions

Conception and design: FS; SY, Financial support: SY; YK, Administrative support: YK, Provision of study materials and patients: HT; MT; MK; CT; DK; GN; MK; MF, Collection and assembly of data: FS; ST; MH; MS; YS, Microarray data analysis: ST; FS, Public data analysis: FS, qPCR data analysis and interpretation: FS; MH; MS; YS, Manuscript writing: FS; ST; SY, Final approval of manuscript: all Authors.

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