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# Evaluation of Exosome-derived Small RNAs as Potential Biomarkers for Pancreatic Ductal Adenocarcinoma Using Next-generation Sequencing

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**Background:** Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis and lacks clinical biomarkers. Exosomes are extracellular vesicles that facilitate cell–cell communication by distributing macromolecules, such as small RNAs (smRNAs). We assessed the potential of exosome-derived small RNAs (Ex-smRNAs) as PDAC biomarkers.

**Methods:** Peripheral blood was collected from 51 patients with PDAC and 15 control individuals. Exosomes were isolated using an aqueous two-phase system. Ex-smRNAs were analyzed using smRNA sequencing. smRNA-mediated target gene regulation was verified via The Cancer Genome Atlas analysis and *in vitro* transfection and wound-healing assays using PDAC organoids.

**Results:** The total Ex-smRNA count was substantially reduced in patients with PDAC compared with that in control individuals. The levels of microRNAs (miRNAs) miR-125a-5p, miR-30e-5p, miR-16-2-3p, miR-98-5p, and the let-7 family were significantly suppressed, whereas that of miR-6731-5p was significantly elevated. Let-7c-5p and miR-98-5p were found to interact with the long non-coding RNA OLMALINC to regulate their common target genes, *BACH1* and *CCND1*, thus controlling PDAC proliferation and migration. The expressions of CARS1-AS1 and miR-142-5p were upregulated in treatment-responsive patients. Multivariable Cox regression analyses, adjusting for potential prognostic factors such as sex, Eastern Cooperative Oncology Group performance status, and tumor size and stage, revealed that CARS1-AS1 (adjusted hazard ratio [HR] 0.33; 95% confidence interval [CI], 0.15–0.73; $P=0.0061$) and miR-142-5p (adjusted HR 0.79; 95% CI, 0.61–1.01; $P=0.0581$) were associated with improved overall survival.

**Conclusions:** We identified potential Ex-smRNA biomarkers involved in PDAC progression and prognosis that reflect key molecular alterations in PDAC and may serve as clinically relevant biomarkers for disease monitoring.

**Key Words:** Biomarker, Exosome, Pancreatic ductal adenocarcinoma, Small RNA

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## INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) represents one of the most lethal malignant tumors, with a 5-yr survival rate of approximately 10%, despite extensive efforts to improve management strategies [1]. Surgical resection at an early stage remains the only curative treatment; however, most patients with PDAC receive a diagnosis at an unresectable stage [2]. Research on predictive biomarkers is urgently required to enhance early-stage diagnostic efficacy, enable accurate prognostication, and identify *de novo* therapeutic targets.

Liquid biopsies comprise circulating tumor cells, circulating tumor DNA, and other tumor-derived components such as circulating cell-free RNA, exosomes, and tumor-educated platelets found in body fluids. Genomic and proteomic analyses of liquid biopsies have established new possibilities for disease detection, longitudinal monitoring, precision oncology, and resistance marker identification across various malignancies [3]. Although most liquid biopsy approaches utilize DNA-based biomarkers, plasma RNA analysis has also been applied for tumor state interrogation and molecular subtyping [4].

Exosomes carry various molecules, including DNA, RNA, bioactive lipids, and proteins, which can be transferred from donor cells to recipient cells and function as important physiological mediators [5]. Exosomes harbor messenger RNAs (mRNAs) and diverse non-coding RNAs, including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs, small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and piwi-interacting RNAs [6]. Non-coding small RNAs (smRNAs) in exosomes serve as genome regulators, and contribute to PDAC pathogenesis. Among these, lncRNAs (>200 nucleotides) and miRNAs (~22 nucleotides) modulate cell proliferation, migration, differentiation, and angiogenesis, and their dysregulation may drive tumorigenesis and cancer progression in either oncogenic or tumor-suppressive roles. Accordingly, smRNAs in exosomes derived from tumor tissues and body fluids have been extensively studied as candidate disease biomarkers, prognostic indicators, and therapeutic targets [7, 8].

We investigated the differential expression of exosome-derived smRNAs (Ex-smRNAs) in liquid biopsies from patients with PDAC and volunteer control individuals using smRNA sequencing (smRNA-seq) to identify novel biomarkers and molecular targets implicated in PDAC development, metastasis, and prognosis. We validated potential biomarkers via *in vitro* experiments with PDAC patient-derived organoids and cell lines, as well as using *in silico* The Cancer Genome Atlas (TCGA) dataset analysis.

## MATERIALS AND METHODS

### Study patients and samples

In total, 51 patients with PDAC and 15 control individuals were enrolled at Samsung Medical Center (Seoul, Korea) between December 2016 and July 2019. Blood samples (10 mL) were collected in accordance with the Institutional Review Board (IRB) regulations and under IRB approval (SMC 2020-05-174, SMC 2018-08-006, and SKKU 2019-04-004). All procedures followed the ethical standards of the responsible committee on human studies and complied with the Helsinki Declaration. Informed consent was obtained from all individuals.

Plasma was prepared by centrifuging the blood samples at 2,000×*g* at room temperature (20–25°C) for 10 min. Exosomes were extracted from plasma samples using an Exo2D exosome isolation kit (EXOSOME Plus, Suwon, Korea) based on an aqueous two-phase system [9]. Total RNA was extracted from the exosomes for smRNA-seq. RNA concentrations were determined using a Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Waltham, MA, USA). RNA integrity and size distribution were analyzed using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). RNA quality was evaluated using electrophoresis.

### RNA processing and sequencing

SmRNA libraries were prepared from 1–10 ng of total RNA using a SMARTer smRNA-seq kit (Takara Bio, San Jose, CA, USA). The libraries were generated in a ligation-free manner, ensuring that diverse smRNA species are represented with minimal bias. The libraries were sequenced on a HiSeq 2500 platform (Illumina, San Diego, CA, USA), generating 100-bp paired-end reads. Adapter sequences were eliminated from the raw reads using Cutadapt [10]. Reads matching at least the first 5 bp of the 3′-adapter sequence were identified as adapter sequence and were trimmed accordingly. Trimmed reads should be ≥18 bp for reliable analysis. The 3′-end of each read was trimmed based on a quality score threshold of 10. Reads that were trimmed or had no adapter sequences with an "N" base were considered low-quality reads and were filtered out. The smRNA-seq datasets used and/or analyzed during the current study are available from Gene Expression Omnibus under accession code GSE268771 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE268771).

### Bioinformatic analysis of miRNAs

MiRNAs were predicted using miRDeep2 (v2.0.1.2) [11]. Trimmed reads were processed and aligned to a human refer-


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ence genome (hg38) using the mapper.pl script running the bowtie aligner (v1.3.1) with the following options: bowtie -f -n 0 -e 80 -l 18 -a -m 5–best–strata. Known and novel miRNAs were identified using miRDeep2.pl, and expression levels (reads per million, RPM) were quantified using quantifier.pl. Precursor and mature miRNA reference sequences were obtained from miRbase v22.1 [12]. MiRNAs with a mean expression of >5 RPM were retained. Differentially expressed miRNAs (DEmiRNAs) were identified using DESeq2 [13] based on $|\log_2$ fold change$|$ >1 and adjusted $P<0.05$. DEmiRNA target genes were obtained from miRanda v3.3 [14], TargetScan v8.0 [15], and miRTarBase v8.0 [16] and subjected to gene set enrichment analysis (GSEA) using Enrichr [17]. Significantly enriched pathways in the Gene Ontology (GO) biological process category were identified (adjusted $P<0.05$).

### Bioinformatic analysis of lncRNAs

Trimmed reads were mapped to the GRCh38 human reference genome in GENCODE v.34 [18] using STAR v.2.7.3a. To map lncRNAs, we used the lncRNA gene annotation file in GENCODE v.34. The aligned reads were quantified using RSEM v1.3.1, and transcript per million (TPM) values were determined. LncRNAs with a mean expression of >5 TPM were used. Differentially expressed lncRNAs (DElncRNAs) were identified using DESeq2 [13] based on $|\log_2$ fold change$|$ >1 and adjusted $P<0.05$. DElncRNA target genes were identified using the LncTarD2.0 database [19] and subjected to pathway enrichment analysis using Enrichr [17]. Significantly enriched pathways in the GO biological process category were selected (adjusted $P<0.05$).

### Prognostic analysis of Ex-smRNAs

SmRNA expression profiles associated with prognosis were investigated in a subset of 32 patients with PDAC who received palliative chemotherapy. We identified significant DEmiRNAs or DElncRNAs between 20 responders and 12 non-responders. Differential expression and pathway enrichment analyses were conducted using DESeq2 [13]. Overall survival (OS) and progression-free survival (PFS) were analyzed using the Kaplan–Meier method. OS was defined as the time from diagnosis to death or last follow-up, and PFS as the time from treatment initiation to disease progression. To assess associations with survival, patients were divided into high- and low-expression groups according to the upper and lower quartiles of specific smRNA expression. Multivariable Cox proportional hazards models were used to adjust for potential confounders, including clinically significant variables such as sex, Eastern Cooperative Oncology Group (ECOG) performance status, tumor size, and tumor stage (8th American Joint Committee on Cancer [AJCC]). Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated to evaluate associations between variables and OS outcomes.

### miRNA–lncRNA interaction prediction

Potential interactions between DElncRNAs and DEmiRNAs were predicted using the RNAInter database [20] based on a confidence score >0.5. Interactions between lncRNAs and target mRNAs were evaluated using the LncRRIsearch web tool [21], which predicts local base-pairing interactions.

### TCGA data analysis

The GEPIA2 [22] webserver was used to identify differentially expressed genes between tumor and normal tissues and for survival analysis. For differential gene expression analysis, PDAC gene expression data were obtained from TCGA-PAAD (dbGaP accession No.: phs000178), and normal pancreatic tissue expression data were retrieved from the Genotype-Tissue Expression (GTEx) project. The TCGA and GTEx datasets were integrated for comparison between tumor and normal tissue transcriptomes.

### *In vitro* validation

Normal human pancreatic duct epithelial (HPDE) cells, the PDAC cell lines AsPC1 and CAPAN1, and PDAC patient-derived organoids [23] were transfected with smRNA mimics/inhibitors using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Waltham, MA, USA) and incubated for 1–3 days for quantitative reverse transcription PCR, western blotting, and wound-healing assays (Supplemental Data Table S1 and Supplemental Methods).

### Statistical analysis

Differences in counts or expression levels between the two groups were calculated using the Wilcoxon rank-sum test. Differential expression was analyzed using DESeq2, which adjusts *P*-values from the Wald test using the Benjamini–Hochberg method. For pathway enrichment analysis using Enrichr, *P*-values were calculated using Fisher's exact test and adjusted using the Benjamini–Hochberg method. The significance of differences between Kaplan–Meier survival curves was determined using the log-rank test. Multivariate Cox proportional hazards regression was performed to estimate HRs. Statistical significance was set to $P<0.05$. All statistical analyses were performed using R v.4.1.2 or GraphPad Prism v.8.0 (GraphPad Software, La Jolla,

CA, USA).

## RESULTS

### Patient characteristics

The baseline characteristics of the 51 patients with PDAC are provided in Table 1. The median age was 62 yrs (range, 36–79 yrs), and 28 patients were men (54.9%). The median carbohydrate antigen (CA)19-9 concentration at diagnosis was 8,836.1 U/mL (range, 3.1–140,000 U/mL). The median tumor size was 4.4 cm (range, 1.9–16 cm). Two patients (3.9%) had stage IB, four (7.9%) had stage IIB, 12 (23.5%) had stage III, and 33 (64.7%) had stage IV PDAC. Seven patients (13.7%) underwent surgery, and 32 (62.7%) received palliative chemotherapy. In total, 49 patients (96.1%) died during the study period, and the median OS was 10.1 months. The control group comprised six men (40%) and nine women (60%), with a median age of 58 yrs (range, 30–82 yrs) (Supplemental Data Table S2).

**Table 1.** Baseline characteristics of the patients with PDAC (N = 51)

| Characteristic | | Median (range) or N (%) |
|---|---|---|
| Age (yrs) | | 62.0 (36–79) |
| Male | | 28 (54.9) |
| Performance status (ECOG) | 0 | 26 (50.9) |
| | 1 | 24 (47.1) |
| | ≥2 | 1 (1.9) |
| Diabetes mellitus | Yes | 9 (17.6) |
| Primary mass location | Head | 31 (60.8) |
| | Body/tail | 18 (35.3) |
| | Multiple | 2 (3.9) |
| Total bilirubin (mg/dL) | | 0.6 (0.2–19.0) |
| CA19-9 (U/mL) | | 8,836.1 (3.1–140,000) |
| Tumor Size (cm) | | 4.4 (1.9–16) |
| AJCC stage, 8th | IB | 2 (3.9) |
| | IIB | 4 (7.9) |
| | III | 12 (23.5) |
| | IV | 33 (64.7) |
| Surgery | | 7 (13.7) |
| Palliative chemotherapy | | 32 (62.7) |
| Death | | 48 (94.1) |
| Overall survival (months) | | 10.1 (0.33–95.13) |

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; ECOG, Eastern Cooperative Oncology Group; CA19-9, carbohydrate antigen 19-9; AJCC, American Joint Committee on Cancer.

### SmRNA profiling

For smRNA profiling, we isolated exosomes from the blood of patients with PDAC and control individuals and performed smRNA-seq using the extracted Ex-smRNAs (Fig. 1A). The landscape and proportion of RNAs in patients with PDAC (N = 51) and controls (N = 15) are shown in Fig. 1B and C. The total smRNA count and most smRNA types, including lncRNAs and miRNAs, were significantly reduced in the PDAC group compared with those in the normal control group (Fig. 1D). The proportions of small Cajal body-specific RNAs (scaRNAs), small cytoplasmic RNAs (scRNAs), and snRNAs were significantly decreased, whereas those of mitochondrial (Mt)_rRNAs, Mt_tRNAs, and protein-coding biotypes were increased in PDAC (Supplemental Data Fig. S1).

### Evaluation of miRNAs and lncRNAs associated with PDAC progression

Among Ex-smRNAs, the expressions of 2,654 and 68 lncRNAs were upregulated and downregulated, respectively, in patients with PDAC compared with that in control individuals. Further, the expressions of 36 and 102 miRNAs were upregulated and downregulated, respectively, in the exosomes of patients with PDAC. These results indicated that the expressions of lncRNAs were mostly upregulated (97.5%) and those of miRNAs were mostly downregulated (73.9%) in PDAC (Fig. 2A). Volcano plots of the 10 most significantly upregulated or downregulated lncRNAs and miRNAs between patients with PDAC and control individuals are shown in Fig. 2B. The expressions of the lncRNAs ARMCX3-AS1 (adjusted $P = 8.3 \times 10^{-6}$), LHX5-AS1 (adjusted $P = 2.7 \times 10^{-4}$), and NECTIN3-AS1 (adjusted $P = 3.5 \times 10^{-4}$) were upregulated in PDAC. The expressions of the miRNAs miR-125a-5p (adjusted $P = 3.0 \times 10^{-9}$), miR-30e-3p (adjusted $P = 6.0 \times 10^{-6}$), miR-16-2-3p (adjusted $P = 1.8 \times 10^{-5}$), miR-98-5p (adjusted $P = 1.8 \times 10^{-5}$), and the let-7 family (adjusted $P < 0.05$) decreased, whereas that of miR-6731-5p (adjusted $P = 3.0 \times 10^{-9}$) increased in exosomes from patients with PDAC. DElncRNA and DEmiRNA target genes were significantly enriched in biological processes related to cell proliferation and regulation of transcription/translation in PDAC (adjusted $P < 0.05$) (Fig. 2C).

### Validation of the involvement of miRNAs, lncRNAs, and their target genes in PDAC progression

To validate the smRNA candidates related to PDAC progression, we investigate miRNA–lncRNA interactions and interactions with their target genes in PDAC. First, we identified DEmiRNAs and DElncRNAs between patients with PDAC and control individuals


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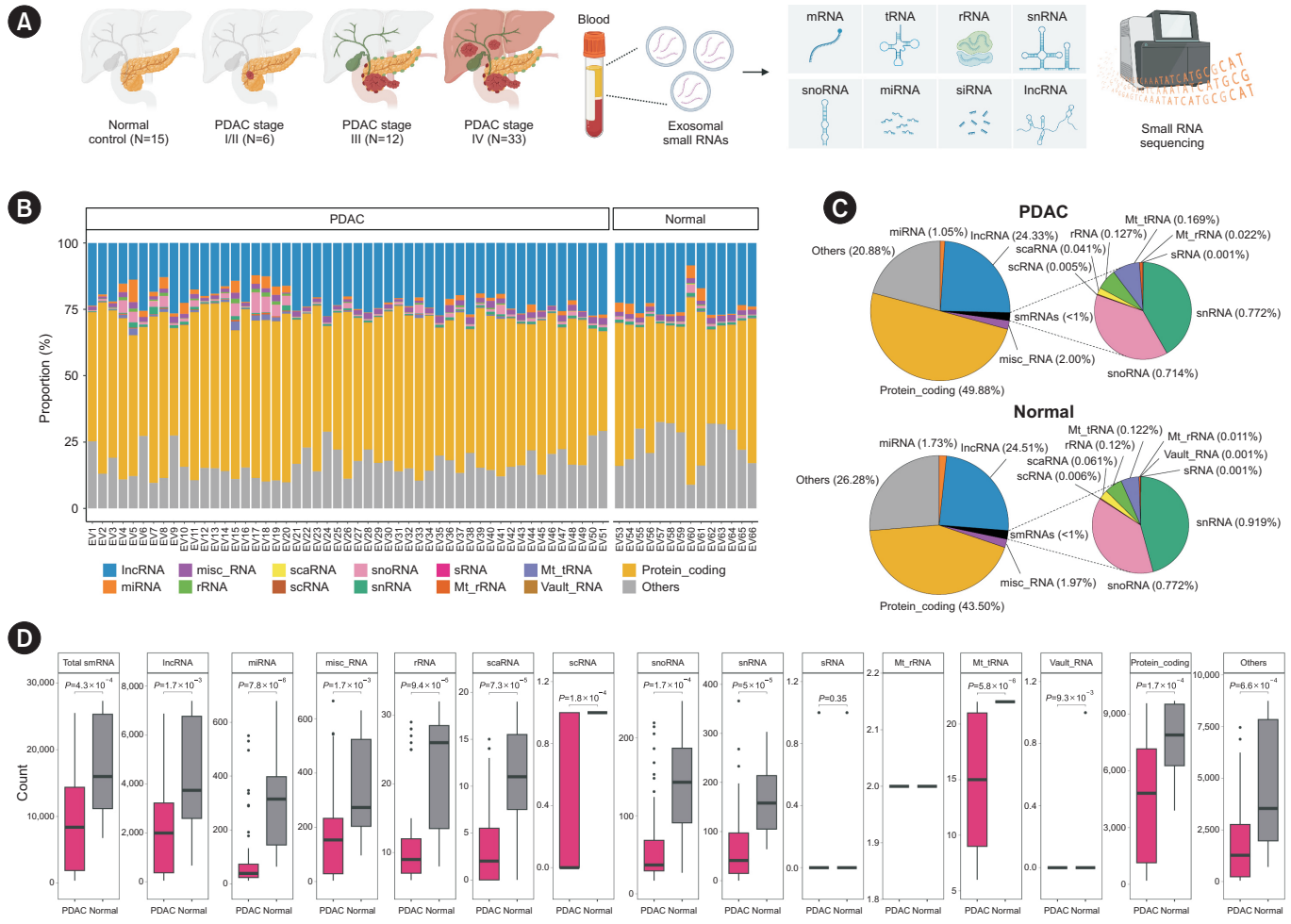


**Fig. 1.** Ex-smRNA landscape in patients with PDAC and control individuals. (A) Experimental study design. (B, C) Bar plot (B) and pie chart (C) showing the proportions of various types of smRNAs in patients with PDAC (N = 51) and control individuals (N = 15). (D) Comparison of the counts of various small RNA types between patients with PDAC and control individuals.

Abbreviations: Ex-smRNAs, exosome-derived small RNAs; PDAC, pancreatic ductal adenocarcinoma; lncRNA, long non-coding RNA; miRNA, microRNA; misRNA, miscellaneous RNA; rRNA, ribosomal RNA; scaRNA, small Cajal body-specific RNA; scRNA, small cytoplasmic RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; sRNA, bacterial small RNA; Mt_rRNA, mitochondrial ribosomal RNA; Mt_tRNA, mitochondrial transfer RNA.

and selected those with inverse expression patterns as candidate miRNA–lncRNA interaction pairs based on the RNAInter database (Fig. 3A). Let-7c-5p and miR-98-5p were the most strongly downregulated miRNAs ($\log_2$ fold change < –3) in PDAC exosomes, and the expression of the lncRNA OLMALINC, which was predicted to interact with both let-7c-5p and miR-98-5p, was significantly upregulated in PDAC (Supplemental Data Table S3).

In the screening of potential target genes of the candidate miRNAs, we focused on the top three pathways, including regulation of transcription by RNA polymerase II (GO:0006357), negative regulation of DNA-templated transcription (GO:0045892), and negative regulation of transcription by RNA polymerase II (GO:0000122). Among 126 targets, *BACH1* and *CCND1* were selected as common potential target genes of let-7c-5p and miR-98-5p using miRTarBase. The association of both target genes with OLMALINC was identified based on a local base-pairing interaction identified using the lncRNA–RNA interaction prediction web tool LncRRIsearch. *BACH1* and *CCND1* are involved in the progression of cancers, including PDAC [24, 25].

To verify the *in-silico* analysis results, we experimentally assessed the expression of miRNAs, interacting lncRNAs, and their target genes in two PDAC cell lines (AsPC1 and CAPAN1) and organoids. In line with the *in-silico* results, the expression of let-7c-5p and miR-98-5p was substantially decreased, whereas that of the lncRNA OLMALINC was increased in the two PDAC cell lines

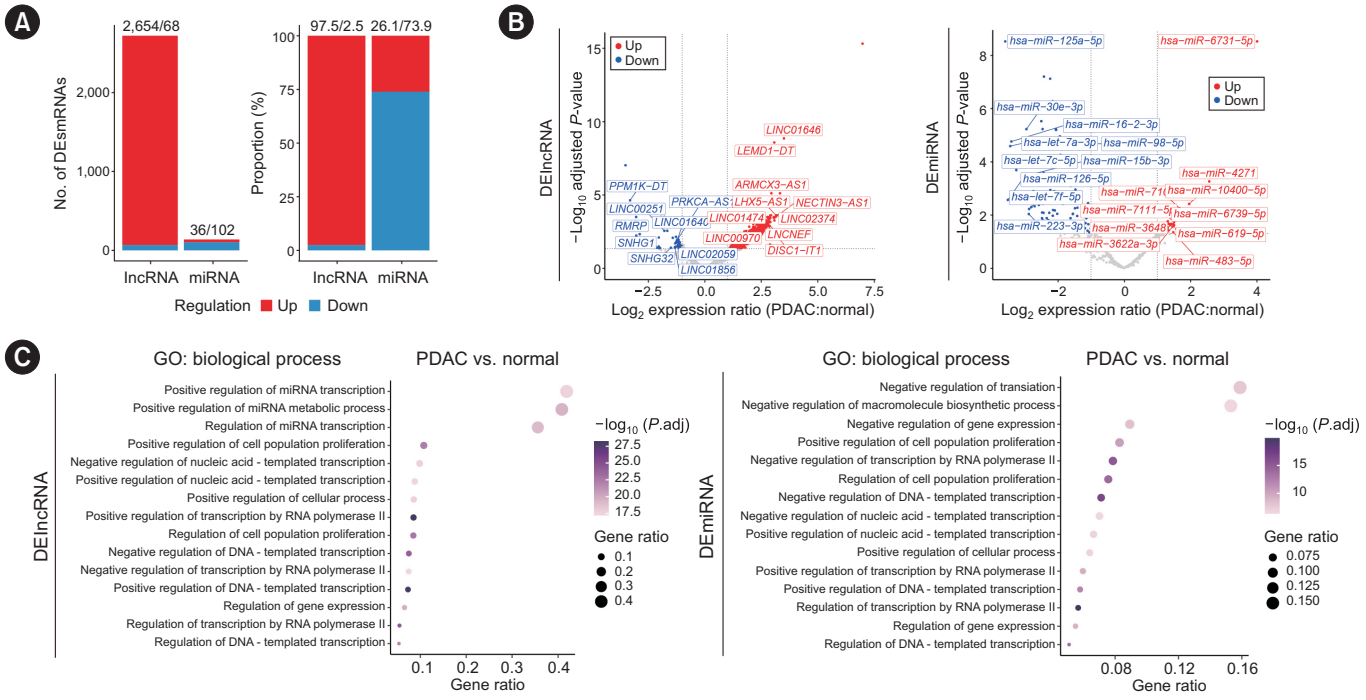


**Fig. 2.** Evaluation of Ex-smRNAs in PDAC progression. (A) Numbers and proportions of small RNAs, particularly miRNAs and lncRNAs, with upregulated and downregulated expressions in PDAC. (B) Volcano plots of DElncRNAs and DEmiRNAs between patients with PDAC and control individuals. The top 10 DEmiRNAs and DElncRNAs were annotated. (C) Functional enrichment analysis of DElncRNAs and DEmiRNAs in patients with PDAC vs. control individuals based on GO biological processes. The 15 most significantly enriched pathways are shown.
Abbreviations: Ex-smRNAs, exosome-derived small RNAs; PDAC, pancreatic ductal adenocarcinoma; DE, differentially expressed; miRNA, microRNA; lncRNA, long non-coding RNA; GO, Gene Ontology.

and organoids compared with the levels in HPDE normal control cells (Fig. 3B). The levels of putative target genes *BACH1* and *CCND1* were significantly elevated in PDAC (Fig. 3B), as validated using TCGA and GTEx data analysis using GEPIA2 (Fig. 3C).

We next transfected smRNA inhibitors or mimics into PDAC organoids to explore their interaction and regulation. Let-7c-5p and miR-98-5p inhibitors upregulated the expression of their interacting partner OLMALINC (Fig. 3D) and their putative target genes *BACH1* and *CCND1* (Fig. 3E). In contrast, let-7c-5p and miR-98-5p mimics and the OLMALINC inhibitor suppressed *BACH1* and *CCND1* expression (Fig. 3F, 3G), whereas the OLMALINC mimic enhanced their expression (Fig. 3H) in PDAC organoids. Furthermore, the let-7c-5p and miR-98-5p mimics significantly suppressed the proliferation and migration of AsPC1 and CAPAN1 cells (Fig. 3I).

The count or proportion of Ex-smRNAs did not significantly differ between metastatic (N = 33) and localized (N = 18) PDAC (Supplemental Data Fig. S2). Differential lncRNA analysis helped identify 689 DElncRNAs with upregulated expressions, including DDX59-AS1, SNHG25, and LYPLAL1-DT, and 381 DElncRNAs with downregulated expressions, such as MIR548A1HG, in metastatic PDAC. The expressions of the miRNAs miR-4454, miR-16-5p, let-7f-5p, miR320a-3p, and miR140-3p were upregulated, whereas those of miR-766-3p, miR-148b-3p, and miR-337-3p were downregulated in metastatic PDAC. GSEA based on GO biological processes using DElncRNA and DEmiRNA target genes showed that miRNA-mediated gene silencing, protein localization to the nucleus, and intracellular protein transport were significantly associated with metastatic PDAC (adjusted $P < 0.05$, Supplemental Data Fig. S3).

## Identification of exosomal miRNAs and lncRNAs related to the prognosis of PDAC

Thirty-two patients with PDAC received palliative chemotherapy, and their response to treatment was assessed using RECIST v1.1. We examined DElncRNAs and DEmiRNAs between responders (N = 20) and non-responders (N = 12) and identified 310 and 377 lncRNAs with upregulated and downregulated expressions, respectively, and six and three miRNAs with upregu-


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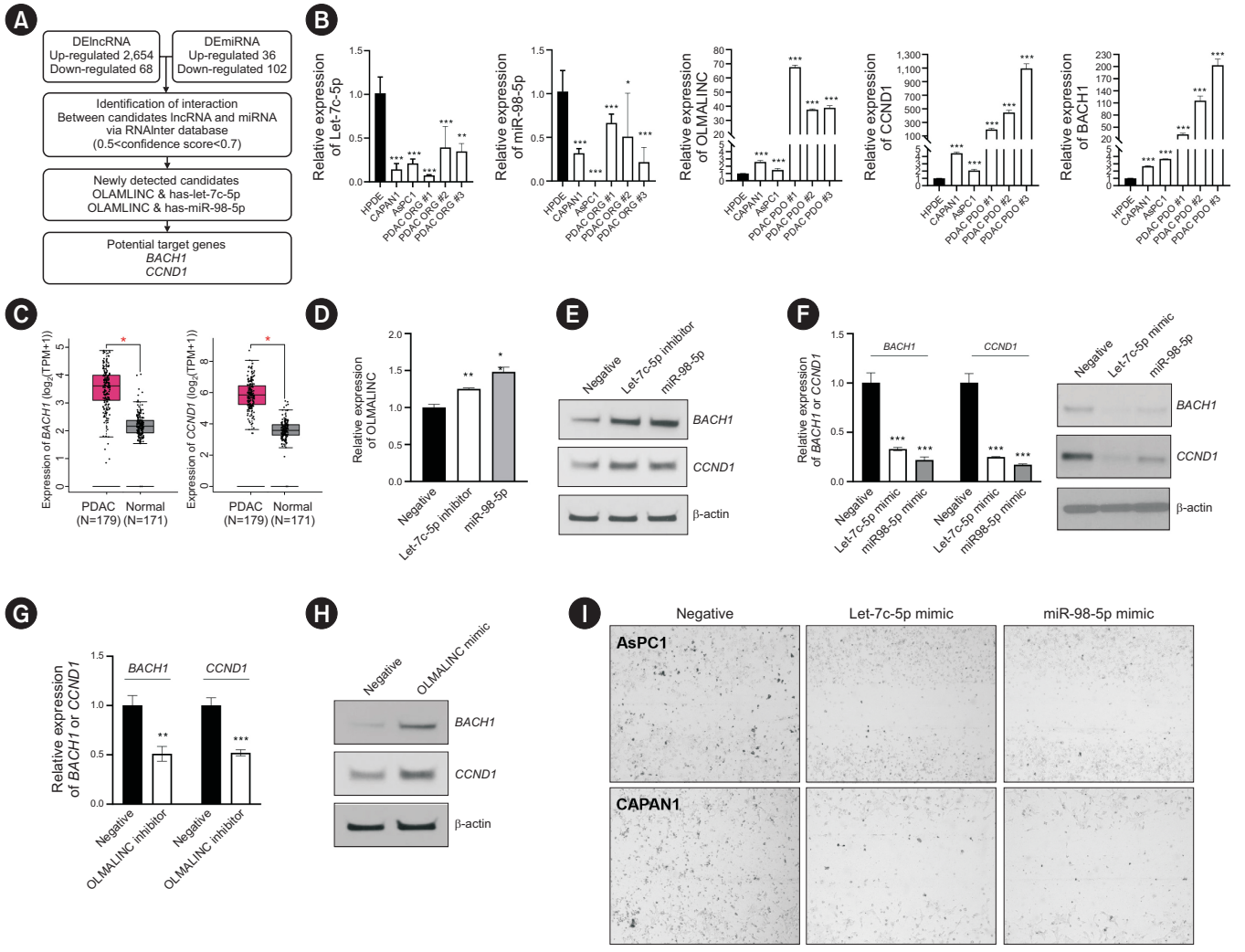


**Fig. 3.** Validation of Ex-smRNA regulation in PDAC progression. (A) Schematic flow for the identification of the miRNA-lncRNA-target gene interactions. (B) Relative expression levels of let-7c-5p and miR-98-5p, OLMALINC, *BACH1*, and *CCND1* in CAPAN-1 and AsPC-1 PDAC cells and PDAC patient-derived organoids based on RT-qPCR. HPDE cells were used as a normal control. (C) Differential expression of *BACH1* and *CCND1* in patients with PDAC (N = 179) vs. control individuals (N = 171) in TCGA and GTEx data. **P* < 0.05. After transfecting let-7c-5p or miR-98-5p inhibitors into PDAC organoids for 24 h, (D) the expression of OLMALINC was determined using RT-qPCR, and (E) that of *BACH1* and *CCND1* using western blotting. (F) After transfecting let-7c-5p or miR-98-5p mimics into PDAC organoids, the expression of *BACH1* and *CCND1* was determined at the mRNA and protein levels. After transfecting (G) an OLMALINC inhibitor or (H) OLMALINC mimic into PDAC organoids, *BACH1* and *CCND1* expression was assessed using RT-qPCR and western blotting. Negative mimics or inhibitors were used as controls. (I) AsPC1 and CAPAN1 cell proliferation and migration were examined using wound-healing assays after transfection with let-7c-5p and miR-98-5p mimics for 2 days.
**P* < 0.05, ***P* < 0.01, ****P* < 0.001.
Abbreviations: Ex-smRNAs, exosome-derived small RNAs; PDAC, pancreatic ductal adenocarcinoma; PDO, patient-derived organoid; RT-qPCR, quantitative reverse transcription PCR; HPDE, human pancreatic duct epithelial; TPM, transcript per million.

lated and downregulated expressions, respectively (Fig. 4A). In multivariable Cox regression analyses, after adjusting for potential prognostic factors, including sex, ECOG performance status, and tumor size and stage, the lncRNA CARS1-AS1 was significantly associated with improved OS (adjusted HR 0.33; 95% CI, 0.15–0.73; *P* = 0.0061), and miR-142-5p showed a potential association (adjusted HR 0.79; 95% CI, 0.61–1.01; *P* = 0.0581) (Fig. 4B). CARS1-AS1 expression was significantly upregulated in responders and was associated with improved PFS and OS at high expression levels with a quartile-based cut-off (Fig. 4C). High expression of miR-142-5p was also associated with favorable PFS and OS (Fig. 4D). Functional enrichment pathway anal-

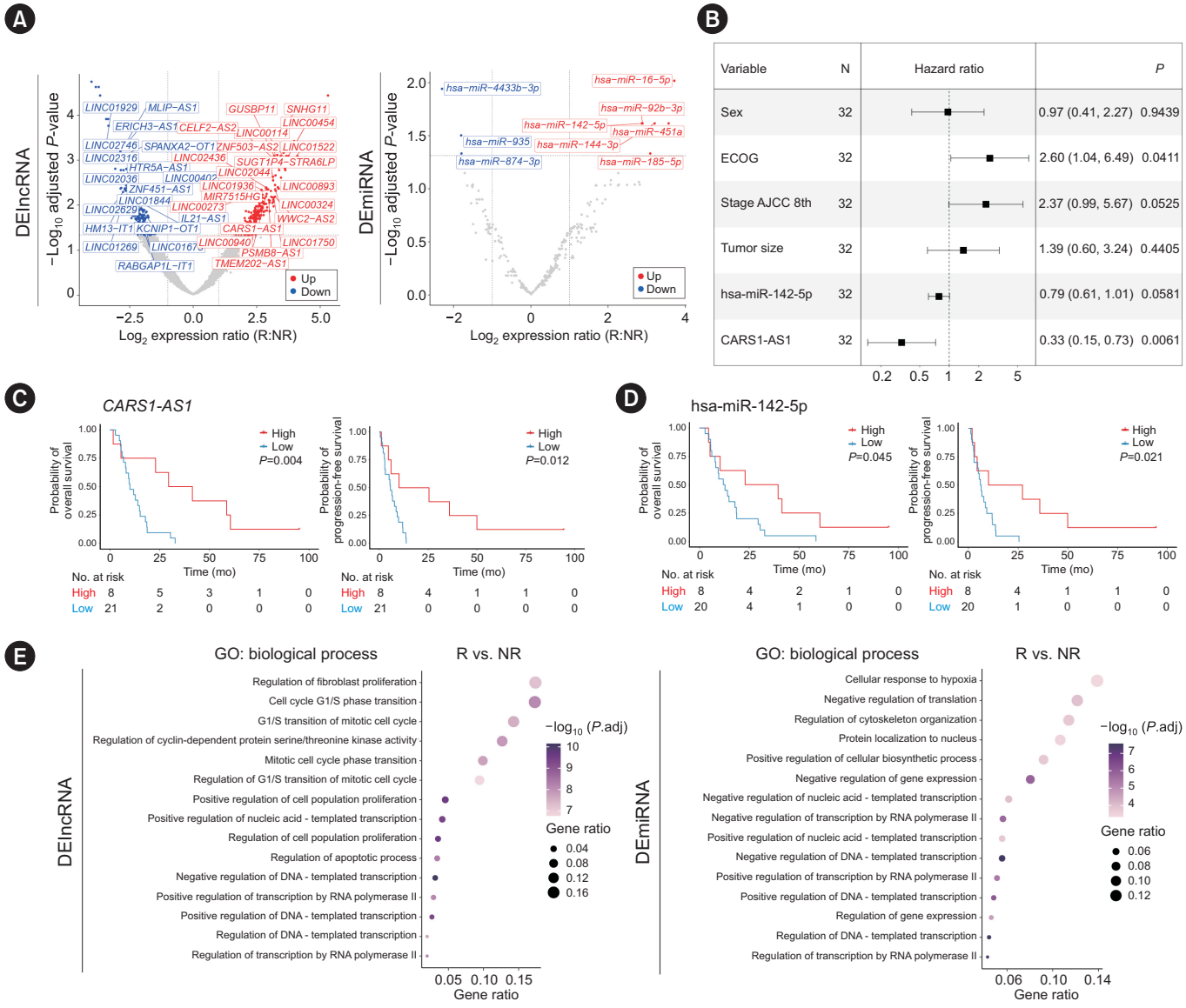


**Fig. 4.** Identification of smRNAs according to PDAC prognosis. (A) Volcano plots of DElncRNAs and DEmiRNAs between responders (R, N = 20) and non-responders (NR, N = 12) among patients with PDAC. The designated DElncRNAs with |log$_2$ fold change| > 2 and adjusted $P < 0.01$ were annotated. (B) Multivariable Cox regression analysis of OS in patients with PDAC. (C, D) Kaplan–Meier survival plots of CARS1-AS1 and miR-142-5p based on quartile expression (top 25% vs. bottom 25% expression). (E) Functional enrichment analysis of DElncRNAs (left panel) and DEmiRNAs (right panel) based on GO biological processes. The 15 most significant pathways are shown.

Abbreviations: lncRNA, long non-coding RNA; miRNA, microRNA; R, responder; NR, non-responder; ECOG, Eastern Cooperative Oncology Group, AJCC; American Joint Committee on Cancer; smRNA, small RNA; PDAC, pancreatic ductal adenocarcinoma; OS, overall survival; DE, differentially expressed; GO, Gene Ontology.

ysis revealed 15 most significantly enriched pathways, including regulation of vascular-associated smooth muscle cell proliferation, cell differentiation/proliferation, and metabolic process (adjusted $P < 0.05$) (Fig. 4E).

## DISCUSSION

We used next-generation smRNA-seq to characterize the RNA cargo of plasma exosomes in patients with PDAC compared with control individuals and evaluated the associations between Ex-smRNAs and PDAC. We thus identified promising novel biomarker candidates potentially involved in PDAC development,


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progression, and prognosis.

Several studies have indicated the widespread disruption of miRNA expression in cancer [26, 27]. Tumors often exhibit reduced expression of mature miRNAs because of miRNA dysregulation via various mechanisms, including miRNA gene amplification or deletion, abnormal transcriptional control of miRNAs, dysregulated epigenetic changes, and defects in the miRNA biogenesis machinery [27]. Previous studies primarily focused on individual smRNA species such as miRNAs, and comprehensive profiling of multiple types of smRNAs remains rare [28]. We explored all types of Ex-smRNAs in patients with PDAC according to the disease stage/metastasis, compared with those in control individuals. We analyzed not only the absolute abundance but also the relative abundance of each Ex-smRNA type. In addition to miRNAs, the expressions of most smRNA types, including lncRNAs, miscellaneous RNAs, rRNAs, scaRNAs, snoRNAs, snRNAs, Mt_tRNAs, and protein-coding biotypes, were significantly downregulated in PDAC exosomes. Further studies are needed to assess other Ex-smRNA types in PDAC.

Our findings highlight the potential of Ex-smRNAs as promising non-invasive biomarkers for PDAC, with miR-125a-5p, miR-30e-5p, miR-16-2-3p, miR-98-5p, miR-6731-5p, and the let-7 family being potentially useful for the assessment of PDAC development, and CARS1-AS1 and miR-142-5p for prognosis prediction. Some of these biomarkers, such as miR-125a-5p, miR-98-5p, and the let-7 family, have been previously reported to act as tumor suppressors, the downregulation of which contributes to drug resistance and tumor aggressiveness in PDAC [29, 30], which aligns with our results. MiR-6731-5p expression was prominently upregulated in patients with PDAC compared with that in control individuals. MiR-6731-5p serum levels are reportedly elevated in patients with chronic pancreatitis [31]; however, its role and expression pattern in PDAC remain to be fully elucidated. The Ex-smRNAs identified, particularly miR-142-5p and CARS1-AS1, showed strong associations with favorable treatment outcomes and prolonged survival. MiR-142-5p inhibits pancreatic cancer cell migration and invasion [32], which may have contributed to the improved prognosis. One of the key distinctions of our study lies in that we suggest blood-derived, particularly exosome-derived, smRNAs as potential biomarkers. These biomarkers may be developed into liquid biopsy-based assays to aid in early diagnosis, prognostic evaluation, and therapeutic outcome monitoring in PDAC. Our study complements previous findings while contributing novel insights into the functional and prognostic roles of Ex-smRNAs in PDAC. Incorporating Ex-smRNA profiling into clinical workflows may enhance personalized treatment planning and ultimately improve survival outcomes in patients with PDAC.

Several recent studies have reported exosome-based transcriptomic analysis in PDAC. Nakamura, *et al*. [7] conducted a multicenter study and elucidated the exosome-based transcriptomic signature of 13 cell-free and exosomal miRNAs to efficiently identify patients with PDAC, which proved to be better than CA19-9 measurement. Nishiwada, *et al*. [8] reported six exosomal miRNA signatures that could predict tumor recurrence in patients with PDAC with high efficacy. These promising results have instigated genome-guided prospective clinical randomized trials in PDAC patients. We also identified putative Ex-smRNAs and related pathways. Future studies are warranted to elucidate the transcriptomic signature based on each smRNA in PDAC exosomes, which requires validation in another cohort. These findings open new avenues for developing exosome-based biomarker panels that can be implemented in minimally invasive clinical settings.

We used an *in-silico* approach to match miRNAs with their interacting lncRNAs based on smRNA-seq data and to identify their target genes. We thus identified an interaction among let-7c-5p, miR-98-5p, and OLMALINC, regulating the expression of *BACH1* and *CCND1* in PDAC. The regulation of the let-7 family has been associated with poorly differentiated, aggressive tumors and epithelial-mesenchymal transition in PDAC [33]. Let-7c-5p expression is downregulated in pancreatic cancer, and its overexpression led to the inhibition of cancer proliferation [34]. The downregulation of miR-98-5p expression promotes PDAC proliferation and metastasis by reversely regulating the MAP4K4 pathway [35]. BACH1 is a transcription factor that regulates reactive oxygen species production, the cell cycle, and immunity. It is highly expressed in cancer tissues, and its expression level is inversely correlated with prognosis in patients with PDAC [36]. CCND1, a cell cycle regulator, is a downstream effector of KRAS, and increased expression is associated with poor prognosis in PDAC [37]. We functionally validated novel lncRNA-miRNA-mRNA regulatory axes, such as OLMALINC-miR-98-5p/let-7c-5p-BACH1/CCND1, and their participation in the proliferation and migration of PDAC. Our findings provide mechanistic insights into how smRNAs may influence tumor biology. The functional roles of let-7c-5p and miR-98-5p in regulating key oncogenes via interaction with OLMALINC suggest potential targets for future RNA-based therapeutic strategies.

This study had some limitations. The sample size of patients with PDAC and the number of control individuals were relatively small, and an independent validation cohort was lacking. The

reproducibility and statistical robustness of our findings may be, therefore, limited, although we attempted to mitigate this limitation with functional validation experiments and TCGA dataset analysis. The fact that this study was conducted at a single center limits the generalizability of our findings. To validate and extend the results, future multicenter studies involving diverse patient populations across different geographical regions are warranted. Furthermore, the study cohort consisted primarily of patients with advanced-stage PDAC, which limits extrapolation to earlier stages or other types of the disease. Lastly, despite previous reports implicating smRNAs in cancer metastasis, we observed no significant differences in Ex-smRNA counts or proportions between localized and metastatic PDAC, likely because of the limited sample size or the functional relevance of specific Ex-smRNAs not being reflected in their overall abundance. Further prospective studies with larger patient cohorts are warranted to generalize our results to the broader PDAC population and establish the clinical implications of the biomarkers.

Using smRNA-seq, we established the potential utility of Ex-smRNAs for exploring novel biomarkers for PDAC. Our findings suggest that Ex-smRNAs may predict PDAC progression and prognosis and demonstrate the feasibility of transcriptomic-based liquid biopsy platforms for precision oncology approaches from early detection to monitoring.

## SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.3343/alm.2025.0121.

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## AUTHOR CONTRIBUTIONS

Kim H contributed to data curation, formal analysis, funding acquisition, investigation and writing–original draft; Park S contributed to data curation, formal analysis, software and writing–original draft; Goh MJ was involved in data curation, methodology and writing–original draft; Choi JH, Kim M, and Choi YH contributed to data curation and writing–original draft; Kim JH contributed to data curation and formal analysis; Lee EM contributed to methodology; Lee SH was involved in funding acquisition and project administration; Lee KH and Lee KT contributed to project administration and resources; Lee JK contributed to conceptualization, resources, and supervision; Lee S contributed to formal analysis, funding acquisition, investigation, and supervision; Park JK was involved in funding acquisition, project administration, resources, supervision, and writing–original draft. All authors read and approved the final manuscript.

## CONFLICTS OF INTEREST

None declared.

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