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CLINICAL—PANCREAS

A MicroRNA Signature Identifies Pancreatic Ductal Adenocarcinoma Patients at Risk for Lymph Node Metastases

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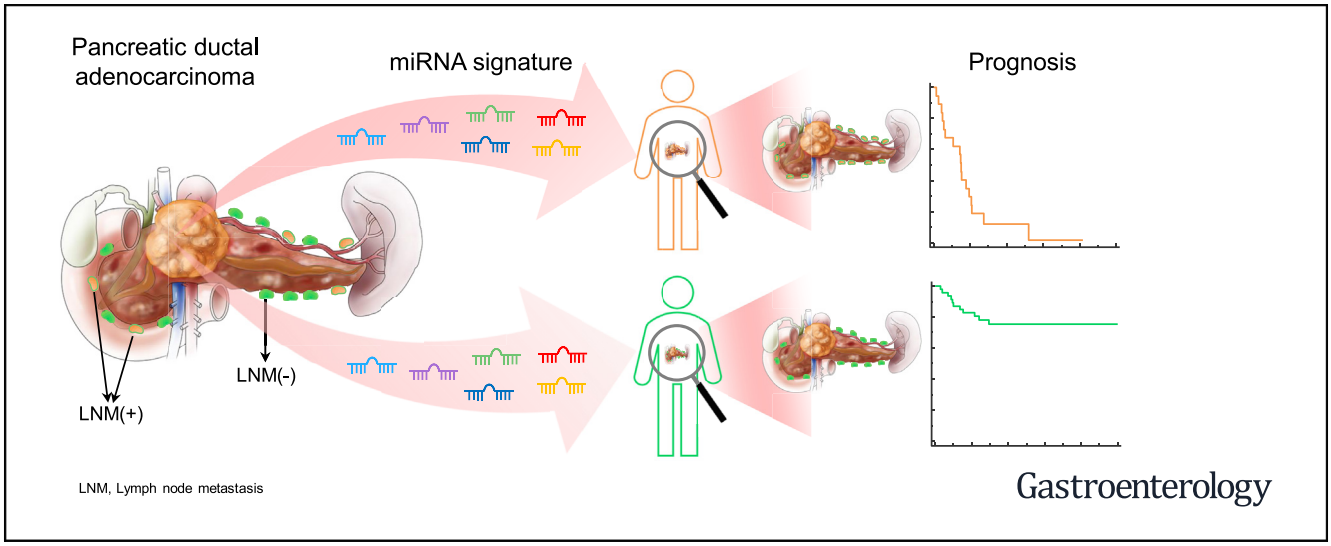

BACKGROUND & AIMS: Pancreatic ductal adenocarcinomas (PDACs) frequently metastasize to the lymph nodes; strategies are needed to identify patients at highest risk for lymph node metastases. We performed genome-wide expression profile analyses of PDAC specimens, collected during surgery or endoscopic ultrasound–guided fine-need aspiration (EUS-FNA), to identify a microRNA (miRNA) signature associated with metastasis to lymph nodes. **METHODS:** For biomarker discovery, we analyzed miRNA expression profiles of primary pancreatic tumors from 3 public data sets (The Cancer Genome Atlas, GSE24279, and GSE32688). We then analyzed 157 PDAC specimens (83 from patients with lymph node metastases and 74 without) from Japan, collected from 2001 through 2017, for the training cohort and 107 PDAC specimens (63 from patients with lymph node metastases and 44 without) from a different medical center in Japan, from 2002 through 2016, for the validation cohort. We also analyzed samples collected by EUS-FNA before surgery from 47 patients (22 patients with lymph node metastases and 25 without; 17 for the training cohort and 30 from the validation cohort) and 62 specimens before any treatment from patients who received neoadjuvant chemotherapy (9 patients with lymph node metastasis and 53 without) for additional validation. Multivariate logistic regression analyses were used to evaluate the statistical differences in miRNA expression between patients with vs without metastases. **RESULTS:** We identified an miRNA expression pattern associated with diagnosis of PDAC metastasis to lymph nodes. Using logistic regression analysis, we optimized and trained a 6-miRNA risk prediction model for the training cohort; this model discriminated patients with vs without lymph node metastases with an area under the curve (AUC) of 0.84 (95% confidence interval [CI], 0.77–0.89). In the validation cohort, the model identified patients with vs without lymph node metastases with an AUC of 0.73 (95% CI, 0.64–0.81). In EUS-FNA biopsy samples, the model identified patients with vs without lymph node metastases with an AUC of 0.78 (95% CI, 0.63–0.89). The miRNA expression pattern was an independent predictor of PDAC metastasis to lymph nodes in the EUS-FNA cohort (odds ratio, 17.05; 95% CI, 2.43–119.57) and in the pre-neoadjuvant therapy EUS-FNA cohort (95% CI, 0.65–0.87). **CONCLUSIONS:** Using data and tumor samples from 4 independent cohorts, we identified an miRNA signature that identifies patients at risk for PDAC metastasis to lymph nodes. The signature has similar levels of accuracy in the analysis of resected tumor specimens and EUS-FNA biopsy specimens. This model might be used to select treatment and management strategies for patients with PDAC.

Keywords: LNM; Cancer Progression; Prognostic Factor; Prognosis.

Pancreatic ductal adenocarcinoma (PDAC) is an extremely lethal malignancy and is projected to become the second leading cause of cancer-related deaths in the United States by 2030.[1–3] Although complete surgical resection for the localized tumors is the only treatment option available for a cure or long-term survival, 80%–90% of patients at initial diagnosis has unresectable or borderline resectable disease, leading to poor survival outcomes. Consequently, the 5-year survival rates in patients with PDAC after surgical resection is only 10% to 25% at best, because of the high risk of local and distant recurrence.[2,4–6] Considering that the surgery alone has minimal survival benefits, in the recent years, multidisciplinary treatment strategies for a more effective therapeutic targeting in patients with PDAC has aggressively been explored.[4,7–13] The National Comprehensive Cancer Network (NCCN) guidelines recommend neoadjuvant therapy (NAT) in patients with borderline resectable disease; however, in patients with PDAC with a resectable cancer, the guidelines suggest that NAT may be considered only in a limited subset of patients with specific high-risk features, including large regional lymph nodes (LNs), elevated carbohydrate antigen 19-9 (CA19-9) levels, large primary tumors, excessive weight loss, and extreme pain.

Among all of these risk factors, lymph node metastasis (LNM) status remains one of the most important predictors of survival in patients undergoing curative resection, and it is considered to be of tremendous clinical significance for risk stratification and therapeutic decision making in patients with PDAC.[10,14–21] Several recent studies have reported that patients with PDAC with potentially resectable cancers who underwent NAT followed by curative surgery exhibited improved survival and longer time to recurrence; especially those with LNM,[4,10,11,22–26] highlighting the fact that a pretreatment diagnosis of LNM is a critical determinant for developing a more personalized treatment strategy in patients with PDAC.[11,13,20] However, pretreatment diagnosis for the presence of LNM in patients with PDAC is clinically challenging. Currently, such diagnosis is often made by computed tomography, magnetic resonance imaging, endoscopic ultrasonography (EUS), and ^{18}F-fluorodeoxyglucose positron emission tomography. Unfortunately, however, all of these methodologies are inadequate for evaluating the LNM status in patients with PDAC because of their poor sensitivity and specificity,[20,27–30] highlighting the need to develop potential molecular biomarkers that can overcome the challenges of imaging-based methods for such diagnosis. Very limited research efforts have been made on this front. A couple of studies reported that the preoperative neutrophil-to-lymphocyte ratio and serum MMP7 levels could help predict LNM in PDAC,[20,31] but the accuracy of these biomarkers was insufficient for their clinical use.

Recent technological advances have now enabled innovative analysis of genomic and epigenomic profiling in various malignancies and have facilitated the identification of previously unrecognized molecular biomarkers.[32–40] MicroRNAs (miRNAs), which belong to the group of small noncoding RNAs, are single-stranded RNAs, 18–25 nucleotides long, that play key roles in posttranscriptional gene repression, oncogenesis, and tumor metastasis and are frequently dysregulated in various human cancers, including PDAC.[32,33,35–40] Importantly, because of their short length, miRNAs are emerging as important biomarker candidates by virtue of their ability to resist RNAase-mediated degradation and their intact expression in a variety of bodily fluids, as well as formalin-fixed, paraffin-embedded (FFPE) and biopsy tissues.[32,35,39–41] More specifically, several miRNAs, including miR-21, have been reported to be associated with early diagnosis and prognosis in PDAC. However, a systematic and genome-wide comprehensive analysis in multiple large, independent patient cohorts to determine the clinical significance of various miRNAs to serve as biomarkers to identify LNM preoperatively in PDAC has not been attempted.[32,33,38,39,42–49] The availability of such biomarkers will facilitate physicians in making more informed clinical decisions and developing individualized treatment strategies for improved treatment of patients with PDAC.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Pancreatic ductal adenocarcinomas (PDACs) frequently metastasize to the lymph nodes, but it is a challenge to identify patients at highest risk for development of lymph node metastases.

NEW FINDINGS

Using data and tumor samples from 4 independent cohorts, the authors identified a microRNA expression pattern that identifies patients at risk for PDAC metastasis to lymph nodes. The signature has similar levels of accuracy in analysis of resected tumor specimens and EUS-FNA biopsies.

LIMITATIONS

This was a retrospective study of data and samples from 4 cohorts. Larger, prospective studies are needed to test the prognostic ability of this miRNA expression pattern.

IMPACT

This model might be used to select treatment and management strategies for patients with PDAC. It might also be studied to identify therapeutic targets for pancreatic cancer.

CLINICAL PANCREAS

Abbreviations used in this paper: **AUC, area under the curve; CA19-9, carbohydrate antigen 19-9; CI, confidence interval; EUS, endoscopic ultrasonography; EUS-FNA, endoscopic ultrasound–guided fine needle aspiration; FFPE, formalin-fixed paraffin-embedded; LN, lymph node; LNM, lymph node metastasis; LNN, lymph node metastasis–negative; LNP, lymph node metastasis–positive; miRNA, microRNA; mRNA, messenger RNA; NAT, neoadjuvant therapy; NCCN, National Comprehensive Cancer Network; OR, odds ratio; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; RFS, relapse-free survival; ROC, receiver operator characteristic; TCGA, The Cancer Genome Atlas.**

Most current article

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Here, for the first time, we performed a genome-wide, systematic, and comprehensive biomarker discovery to identify and establish a novel miRNA expression signature for the detection of LNM in patients with PDAC. This signature was initially confirmed in multiple large, publicly available data sets, followed by rigorous validation and performance evaluation in 2 independent, large clinical cohorts. Finally, to translate our findings into a clinically -viable scenario, we were able to confirm the robustness of this biomarker signature in pretreatment EUS-guided fine needle aspiration (EUS-FNA) biopsy specimens, highlighting the clinical significance of these biomarkers for the treatment of patients with PDAC.

Materials and Methods

MicroRNA Biomarker Discovery

To perform a comprehensive biomarker discovery, we analyzed the miRNA expression profiling results of primary tumor tissues from 3 large, publicly available data sets (The Cancer Genome Atlas [TCGA], GSE24279, and GSE32688) to identify and establish an miRNA signature for the identification of LNM in patients with PDAC, as illustrated in Supplementary Figure 1. TCGA miRNA expression profiling data (level 3 miRNA-sequencing data) were downloaded from the University of California–Santa Cruz Xena Browser (https://xenabrowser.net). Likewise, GSE24279 and GSE32688 data sets (both normalized noncoding RNA profiling and clinical data) were downloaded from the Gene Expression Omnibus database in its processed form (https://www.ncbi.nlm.nih.gov/geo/) and from a previously published article.[50] The cases where patients had distant metastasis or insufficient pathologic LNM information, and those who received NAT (because pathologic LNM can be modified by such treatment) were excluded. In total, miRNA-expression profiling data from 269 patients with PDAC, including miRNA-sequencing data from the TCGA cohort (167 patients, 121 LNM positive [LNP] and 46 LNM negative [LNN]) and the GSE24279 (77 patients, 69 LNP and 8 LNN) and GSE32688 (25 patients, 17 LNP and 8 LNN) cohorts, were analyzed to identify an miRNA signature in the discovery and internal validation phases, respectively (Supplementary Table 1). To evaluate the diagnostic potential of the discovered miRNA signature, we first established a multivariate logistic regression model using the selected biomarkers and subsequently determined the area under the curve (AUC) values for each of the receiver operator characteristic (ROC) plots.[41,51]

Patient Cohorts

For the clinical training and validation of the identified miRNA signature, we analyzed biospecimens from 2 large independent patient cohorts. A total of 264 FFPE specimens were examined, which included a training cohort (n = 157; 83 LNP and 74 LNN) of patients with PDAC enrolled at the Nara Medical University between 2001 and 2017 and a validation cohort (n = 107; 63 LNP and 44 LNN) of patients enrolled at the Kumamoto University, Japan, between 2002 and 2016. None of these patients received preoperative cancer treatment, and all tumors were diagnosed as PDAC. Matched FFPE EUS-FNA biopsy samples from 47 patients (22 LNP and 25 LNN) in both cohorts (training cohort, 17; validation cohort, 30) were also obtained. Additionally, EUS-FNA biopsy samples from 62 patients with PDAC (9 LNP and 53 LNN) who received NAT followed by surgery were also collected for additional validation. These EUS-FNA biopsy specimens were obtained before the initiation of any treatment, and the specimens were collected and processed as per the standard diagnostic procedures by using endoscopic and cytologic techniques.[52,53] Tumors were classified according to the TNM staging system of the International Union Against Cancer, version 7. The LNM status was determined from histopathologic examination of resected LNs. The patients who had positive peritoneal washing cytology or para-aortic LNM without other distant metastases were included in this study.[54] Exclusion criteria included macroscopically incomplete resection or a tumor histology other than diagnosis of PDAC. All patients were followed until death or June 2018. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients, and the study was approved by the institutional review boards of all participating institutions.

RNA Extraction and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from 10-mm-thick FFPE surgical tissues and EUS-FNA biopsy specimens by microdissection to enrich for neoplastic cells by using the AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Synthesis of complementary DNA from total RNA was performed by using Taqman MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). Real-time quantitative reverse transcription polymerase chain reaction analysis was performed using the SensiFAST probe Lo-ROX Kit (Bioline, London, UK) on the QuantStudio 7 Flex Real Time PCR System (Applied Biosystems, Foster City, CA), and expression levels were evaluated with Applied Biosystems QuantStudio 7 Flex Real Time PCR System software. The relative abundances of target transcripts were evaluated and normalized to the expression levels of small nuclear RNA U6 as an internal control using the $2^{-\Delta DCt}$ method. Normalized values were further $\log_{10}$ transformed.[41,55]

MicroRNA Regulatory Network

The miRNA:messenger RNA (mRNA) regulatory network was constructed by using the validated miRNAs to elucidate pathways perturbed through data analysis by using miRWalk, version 3.0.[56–58] The miRNA:mRNA network was constructed, wherein the target genes were consistently expressed in at least 2 of the 3 sources—TargetScan, miRDB, and miRTarBase. The pathway enrichment analysis for the selected target genes was performed by using KEGG pathways and Gene Ontology.[41,59,60]

Statistical Analysis

The unpaired *t* test was used to evaluate the statistical differences in miRNA expression between LNP and LNN patients in the public data sets. Pearson correlation analysis was performed to test multicollinearity. Recursive feature elimination with the random forest method was performed to select

important features of miRNAs. For all cohorts, ROC curves and AUC values were determined using Medcalc statistical software, version 16.2.0 (Medcalc Software, Ostend, Belgium). Univariate and multivariate logistic regression were used to evaluate various clinicopathologic variables, including age, sex, CA19-9, tumor location, tumor size evaluated by computed tomography, and the miRNA signature for the detection of LNM status. The cutoff thresholds for continuous variables were divided by the median value in the total participants. The overall survival (OS) time and relapse-free survival (RFS) times were calculated from the date of surgery to the date of death from any cause or recurrence or the last follow-up date. We estimated OS and RFS by using the Kaplan-Meier method. We analyzed the primary endpoint with a stratified log-rank test.[7,61] The median follow-up was calculated by the reverse Kaplan-Meier method.[7,62] A multivariate Cox proportional hazard regression model was established, and a P value of $<.05$ was considered statistically significant. The statistical analyses were performed by using the Medcalc statistical software, version 16.2.0; GraphPad Prism, version 7.0 (GraphPad Software, San Diego, CA); and R, version 3.5.0 (R Development Core Team, https://cran.r-project.org/).

Results

Genome-Wide MicroRNA Expression Profiling Led to the Identification of a Novel 7- MicroRNA Signature for the Detection of Lymph Node Metastasis in Patients With Pancreatic Ductal Adenocarcinoma

We performed a genome-wide, unbiased, comprehensive biomarker discovery analysis in 3 independent miRNA expression profiling data sets (TCGA, GSE24279, and GSE32688) to identify an miRNA signature for the detection of LNM in patients with PDAC. We first compared the miRNA expression profiles between LNP and LNN patients in the TCGA and GSE24279 cohorts, which included patients who had undergone curative surgery without NAT, and identified 13 differentially expressed ($P < .05$) candidate targets with data availability in at least 50% of all cases, excluding highly correlated miRNAs, and with consistent expression profiles in both cohorts. The random forest–based recursive feature elimination with a 10-fold cross-validation on this data set reduced this to a signature of 10 miRNAs. Among these, 7 miRNAs exhibited consistent expression profiles in all 3 data sets: miR-155-5p, miR-196b-5p, miR-365a-5p, miR-629-5p, miR-675-3p, miR-92b-3p, and let-7d-5p. Finally, a logistic regression model with these 7 miRNAs in the TCGA cohort resulted in an AUC of 0.76 (95% confidence interval [CI], 0.66–0.82). Furthermore, the diagnostic ability of this 7-miRNA signature was significantly validated in 2 additional data sets (GSE24279: AUC, 0.75; 95% CI, 0.64–0.84; GSE32688: AUC, 0.92; 95% CI, 0.74–0.99) (Figure 1*A*–*C*), highlighting the diagnostic performance of this signature for the identification of LNM in patients with PDAC.

Clinical Training and Validation Resulted in the Establishment of a MicroRNA Signature for Detecting Lymph Node Metastasis in Patients With Pancreatic Ductal Adenocarcinoma

To confirm the diagnostic robustness of our discovered miRNA signature, we next performed training and validation of these biomarkers in 2 large, independent clinical cohorts (Table 1). First, the performance of the 7-miRNA signature was evaluated by real-time quantitative reverse transcription polymerase chain reaction assays in a training cohort of 157 patients with PDAC (83 LNP and 74 LNN). In the training cohort, we excluded 1 miRNA (let-7d-5p) because it exhibited inconsistent expression in the internal validation cohort, resulting in a final signature of 6 miRNAs (miR-155-5p, miR-196b-5p, miR-365a-5p, miR-629-5p, miR-675-3p, and miR-92b-3p). We successfully reconfirmed the diagnostic accuracy of this 6-miRNA model for its ability to detect LNM in patients with PDAC from the 3 public data sets (Supplementary Figure 2). Subsequently, we trained a 6-miRNA risk-prediction model by using logistic regression analysis in the training cohort, which robustly identified patients with PDAC with LNM (AUC, 0.84; 95% CI, 0.77–0.89) (Figure 2*A* and *C*). The risk score model was developed based on the coefficients of individual miRNAs and the

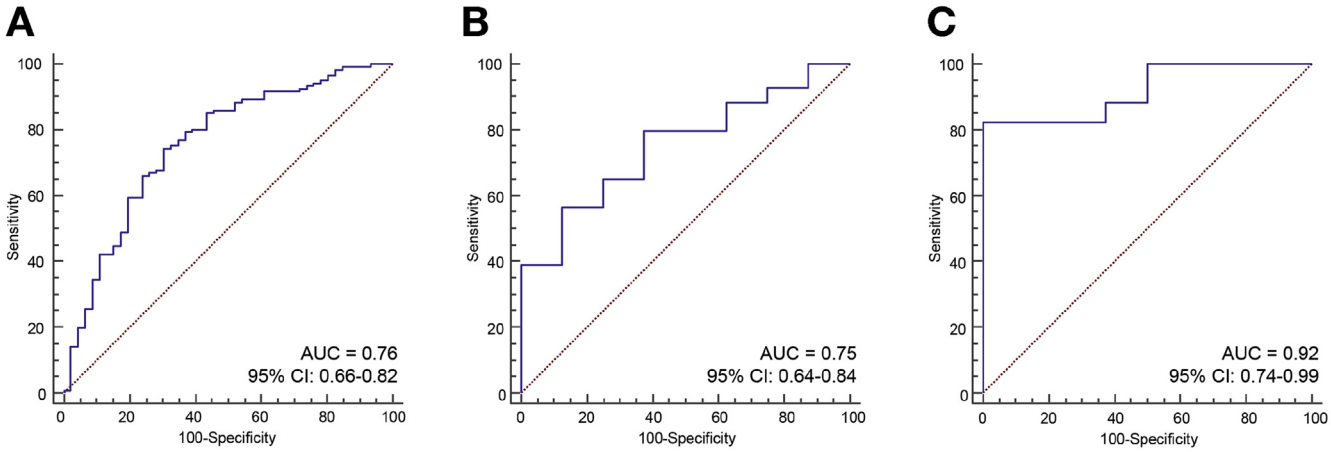

Figure 1. Genome-wide discovery and validation of a novel miRNA signature to detect LNM in patients with PDAC. (*A*–*C*) The ROC curves show the diagnostic performance of the 7-miRNA signature for distinguishing patients with LNM in the (*A*) TCGA, (*B*) GSE24279, and (*C*) GSE32688 cohorts.

Table 1. Clinicopathological Characteristics of Clinical Cohorts

Characteristics	Surgical specimens, n (%)		Matched FNA biopsy specimens, n (%)	Total participants
	Training cohort (n = 157)	Validation cohort (n = 107)	(n = 47)	(n = 264)
Gender				
Male	93 (59.2)	55 (51.4)	29 (61.7)	148 (56.1)
Female	64 (40.8)	52 (48.6)	18 (39.3)	116 (43.9)
Age (years)				
median (range)	70 (32-87)	70 (37-90)	69 (51-82)	70 (32-90)
Preoperative CA19-9 (U/mL)				
median (range)	93 (1-19296)	57.7 (0.1-3722)	60 (0.6-1714)	71.5 (0.1-19296)
Tumor location				
Ph	100 (63.7)	71 (66.4)	33 (70.2)	171 (64.8)
Pbt	57 (36.3)	36 (33.6)	14 (29.8)	93 (35.2)
Tumor size (mm)				
median (range)	27 (5-90)	30 (4-65)	26 (10-90)	28 (4-90)
T status				
T1-2	15 (9.6)	15 (14.0)	2 (4.3)	30 (11.4)
T3-4	142 (90.4)	92 (86.0)	45 (95.7)	234 (88.6)
Lymph node metastases				
Negative	74 (47.1)	44 (41.1)	25 (53.2)	118 (44.7)
Positive	83 (52.9)	63 (58.9)	22 (46.8)	146 (55.3)
UICC stage (ver.7)				
IA, IB	11 (7.0)	12 (11.2)	2 (4.3)	13 (4.9)
IIA	60 (38.2)	31 (29.0)	22 (46.8)	91 (34.5)
IIB	70 (44.6)	57 (53.3)	19 (40.4)	127 (48.1)
III	0 (0.0)	1 (0.9)	1 (2.1)	1 (0.4)
IV	16 (10.2)	6 (5.6)	3 (6.4)	22 (8.3)
Adjuvant therapy				
Yes	120 (76.4)	86 (80.4)	41 (87.2)	206 (78.0)
No	37 (23.6)	21 (19.6)	6 (12.8)	58 (22.0)

NOTE. Plus-minus values are means standard error of the mean.
FNA, Fine needle aspiration; UICC, International Union Against Cancer.

constant derived from this analysis as follows: 2.50737 + (0.50693 × miR-155-5p) + (0.082317 × miR-196b-5p) + (0.014458 × miR-365a-5p) + (1.74439 × miR-629-5p) + (2.71643 × miR-675-3p) + (–5.15058 × miR-92b-3p). Subsequently, we assessed the robustness and accuracy of this 6-miRNA signature by applying the same statistical model into a large, independent validation cohort (63 LNP and 44 LNN cases). Once again, our miRNA biomarkers exhibited remarkable diagnostic accuracy for the identification of LNM in patients with PDAC in this validation cohort as well (AUC, 0.73; 95% CI, 0.64–0.81) (Figure 2*B* and *D*), underscoring the clinical significance of our miRNA signature in the presence of LNM in patients with PDAC.

A Combination Signature of MicroRNA Biomarkers and Carbohydrate Antigen19-9 Levels Showed a Significantly Higher Accuracy for Detecting Lymph Node Metastasis in Patients With Pancreatic Ductal Adenocarcinoma

Because CA19-9 is a widely established and important biomarker in PDAC, we next examined whether a combination model of our miRNA signature and this glycoprotein level might further improve the diagnostic accuracy for detecting LNM in patients with PDAC. Interestingly, indeed, this new combination signature showed a significantly superior diagnostic accuracy for LNM in both training and validation cohorts (AUC, 0.85 and 0.76, respectively) (Figure 2*E* and *F*). Furthermore, this new combination signature also showed a significantly improved diagnostic accuracy compared to other classic preoperative clinicopathologic features, including tumor location and size (Figure 2*E* and *F*). We next categorized all patients into high- and low-risk groups using the cutoff thresholds derived by the Youden index from this 6-miRNA signature model and subsequently performed logistic regression for univariate and multivariate analyses.[63] Of interest, the multivariate analysis showed that our newly established developed signature emerged as an independent predictor of LNM in patients with PDAC, in both clinical cohorts (training cohort: odds ratio [OR], 19.93; 95% CI, 7.55–52.62; $P < .01$; validation cohort: OR, 10.05; 95% CI, 3.42–29.59 $P < .010$ (Table 2).

Prognostic Potential of the MicroRNA Signature for Patients With Pancreatic Ductal Adenocarcinoma in the Clinical Cohorts

Because LNM is often associated with poor patient survival in patients with PDAC, we next were curious to

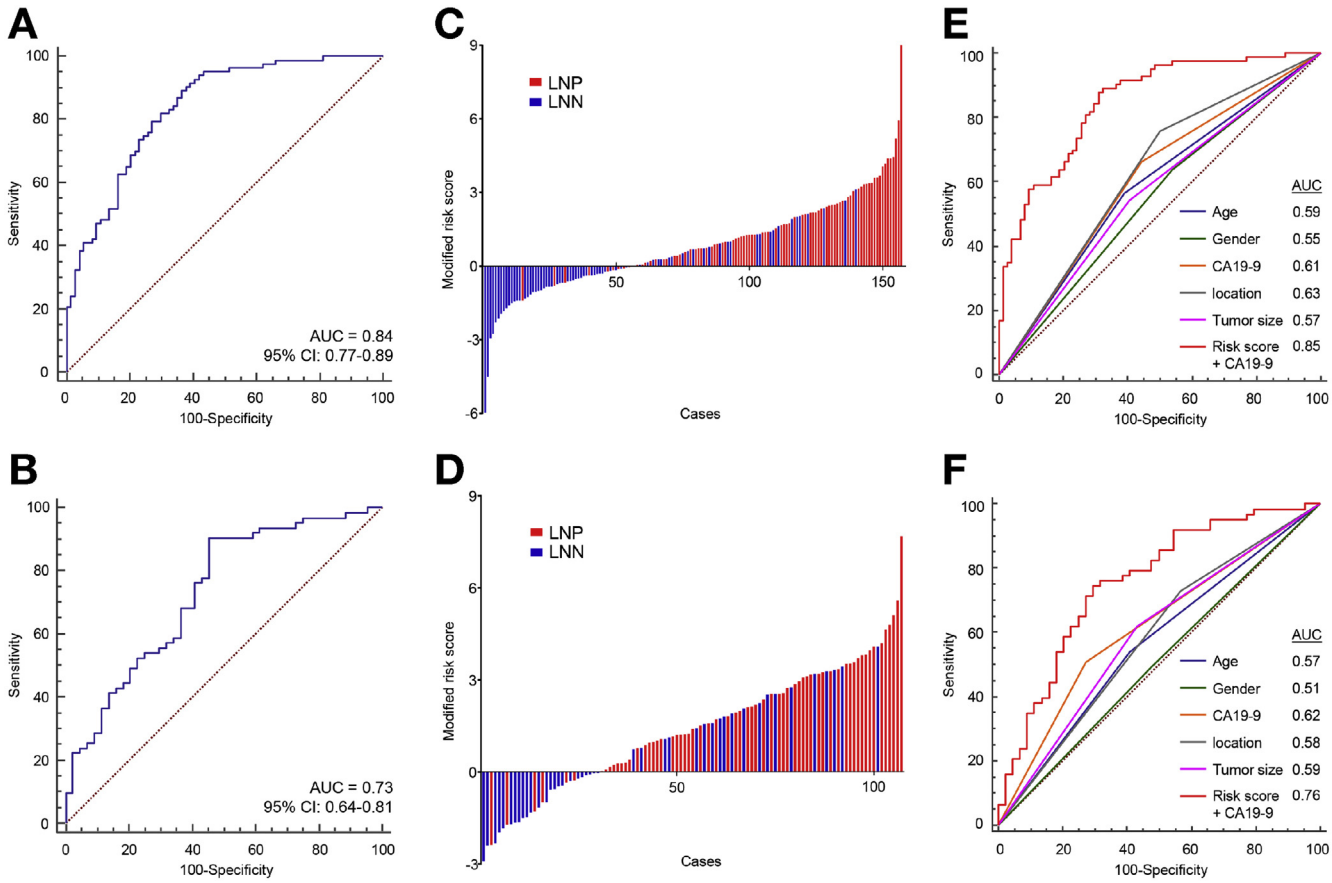

Figure 2. Training and validation of an miRNA signature for identifying LNM in patients with PDAC in 2 independent clinical cohorts. (*A*, *B*) An ROC curve of the 6-miRNA signature in the (A) training cohort (LNP = 83, LNN = 74, AUC = 0.84) and (*B*) validation cohort (LNP = 63, LNN = 44, AUC = 0.73). (*C*, *D*) Risk score distribution plot in the (*C*) training cohort and (*D*) validation cohort. Modified risk score was obtained from subtracting individual risk score from the Youden index value of the risk model. (*E*, *F*) The new combination model, miRNA signature and CA19-9, outperformed the detection accuracy in both the (*E*) training cohort and (*F*) validation cohort).

Table 2. Univariate and Multivariate Logistic Regression Analysis for Lymph Node Metastasis in Training and Validation Cohorts

	Univariate analysis			Multivariate analysis		
Characteristics	OR	95% CI	*P* value	OR	95% CI	*P* value
Training cohort (n = 157)						
Age (≥70 vs. <70 years)	0.55	0.29-1.04	.07	0.53	0.23-1.23	.14
Gender (Female vs. Male)	0.67	0.35-1.26	.21	0.45	0.19-1.06	.07
CA19-9 (≥ 71.5 vs. < 71.5 U/mL)	2.44	1.28-4.66	**<.01**	2.18	0.92-5.14	.08
Location (Pbt vs. Ph)	0.32	0.16-0.63	**<.01**	0.21	0.09-0.53	**<.01**
Tumor size (≥ 28 vs. < 28 mm)	1.75	0.91-3.38	**.01**	1.85	0.78-4.41	.17
6-miRNA signature (High vs. Low risk)	33.36	9.57-116.23	**<.01**	19.93	7.55-52.62	**<.01**
Validation cohort (n = 107)						
Age (≥70 vs. <70 years)	0.59	0.27-1.29	.19	1.00	0.39-2.60	.99
Gender (Female vs. Male)	0.94	0.44-2.04	.88	1.11	0.43-2.83	.83
CA19-9 (≥ 71.5 vs. < 71.5 U/mL)	2.75	1.20-6.30	**.02**	1.55	0.58-4.13	.38
Location (Pbt vs. Ph)	0.49	0.22-1.10	.08	0.50	0.19-1.29	.15
Tumor size (≥ 28 vs. < 28 mm)	2.14	0.98-4.68	.06	1.75	0.66-4.65	.26
6-miRNA signature (High vs. Low risk)	11.09	4.09-30.04	**<.01**	10.05	3.42-29.59	**<.01**

OR, odds ratio; CI, confidence interval.

inquire about the prognostic potential of our miRNA signature. Reassuringly, consistent with previous reports, the presence of LNM had significant impact on patients' prognosis in the training and validation cohorts (Supplementary Figure 3). To evaluate the prognostic potential of our miRNA biomarkers, we performed survival analysis for OS and RFS. The median follow-up times were 65.7 months (95% CI, 61.01–80.81) in the training cohort and 32.94 months (95% CI, 31.52–47.27) in the validation cohort. Importantly, patients with PDAC within the high-risk group showed a significantly worse prognosis in the training cohort (OS, $P < .01$; RFS, $P < .01$) and the validation cohort (OS, $P < .01$; RFS, $P = .03$) (Figure 3*A*, *B*, *D*, and *E*). In addition, in multivariate analysis using the Cox proportional hazard model along with other clinicopathologic factors, high-risk patients defined by the miRNA signature were associated with a significantly worse OS in both independent cohorts (training cohort: hazard ratio, 1.78; 95% CI, 1.16–2.73; $P < .01$; validation cohort: hazard ratio, 2.41; 95% CI, 1.08–5.40; $P = .03$) (Figure 3*C* and *F*). These results highlight that, in addition to the diagnostic ability of our signature in detecting LNM in patients with PDAC, it has a significant prognostic potential as well.

Higher-Order Validation of the MicroRNA Signature in Endoscopic Ultrasound–Guided Fine-Needle Aspiration Biopsy Specimens From Patients With Pancreatic Ductal Adenocarcinoma

Although the validation of biomarkers using surgically resected tissue specimens was necessary for constructing this LNM signature, we believe that validation of these biomarkers in pretreatment biopsy specimens would pave a path for an easier translation of our miRNA signature in clinical settings. The underlying rationale is that if such a validation in biopsy specimens is feasible, physicians would be able to make a more informed decision for offering NAT to patients categorized as high risk who are deemed to have otherwise resectable disease and improve their survival. Based on this hypothesis, we collected matched EUS-FNA biopsy specimens from a subset of 47

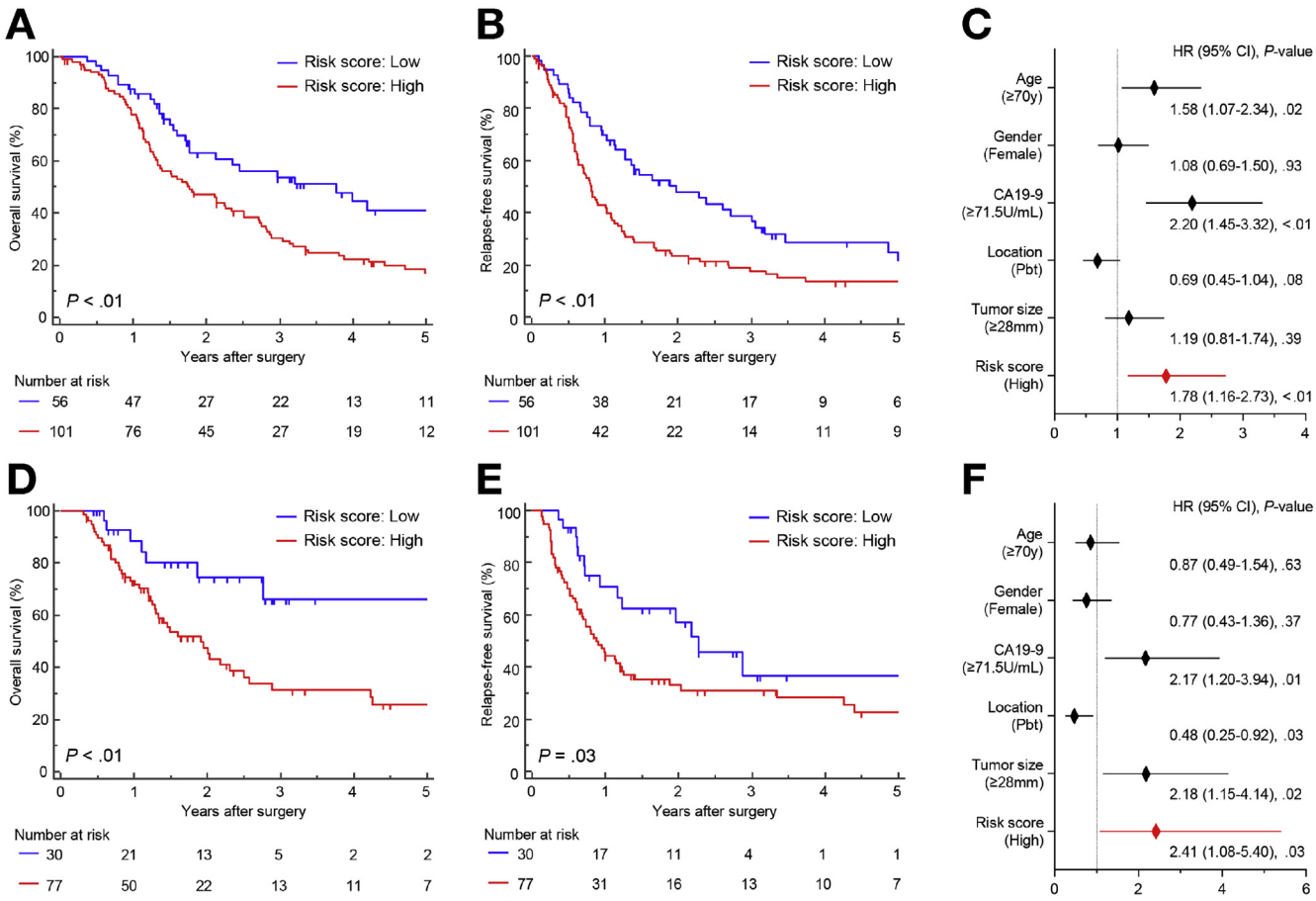

Figure 3. Prognostic potential of the miRNA signature for patients with PDAC in the clinical cohorts. (*A*, *B*) A comparison of (*A*) OS and (*B*) RFS between the high- and low-risk groups estimated by the 6-miRNA signature model in the training cohort. (*C*) Forest plot with hazard ratio of clinicopathologic variables and signature risk score status in multivariate Cox proportional analysis of OS in the training cohort. (*D*, *E*) A comparison of (*D*) OS and (*E*) RFS between the high- and low-risk groups estimated by the 6-miRNA signature model in the training cohort. (*F*) Forest plot with hazard ratio of clinicopathologic variables and signature risk score status in multivariate Cox proportional analysis of OS in the validation cohort. HR, hazard ratio.

patients with PDAC from the training and validation cohorts (Table 1). All 47 patients were classified as having resectable disease based on NCCN resectability status. We evaluated the diagnostic accuracy of our miRNA biomarkers in these biopsy specimens and were enthused to observe that we were successfully able to confirm that it yielded a satisfactory AUC value of 0.78 (95% CI, 0.63–0.89) for distinguishing LNM (Figure 4*A* and *B*). Consistent with the training and validation cohorts, the performance of this model was improved by combination with CA19-9 (AUC, 0.81; 95% CI, 0.67–0.91) and showed superior diagnostic potential than other clinicopathologic factors (Figure 4*C*). Furthermore, multivariate logistic regression analysis for LNM detection showed that our 6-miRNA signature was an exclusive significant detective marker in the FNA biopsy cohort ([OR], 17.05; 95% CI, 2.43–119.57) (Figure 4*D*).

Performance Validation of the MicroRNA Signature for Predicting Residual Nodal Involvement After Neoadjuvant Therapy in Endoscopic Ultrasound–Guided Fine-Need Aspiration Biopsy Specimens

Currently, multidisciplinary treatment strategies, including NAT, for patients with resectable and borderline-resectable PDAC are actively being explored and are becoming more common, especially in Western countries. Moreover, pathologic nodal involvement status, which is modified by the effect of NAT (ypN), the residual LNM status after NAT, is often considered as one of the most important prognostic factors in patients with PDAC undergoing NAT after surgery.[4,10,22–26] We hypothesized that our miRNA signature might also be able to identify ypN status before any treatment, which could potentially inform physicians for a more appropriate treatment selection based on the

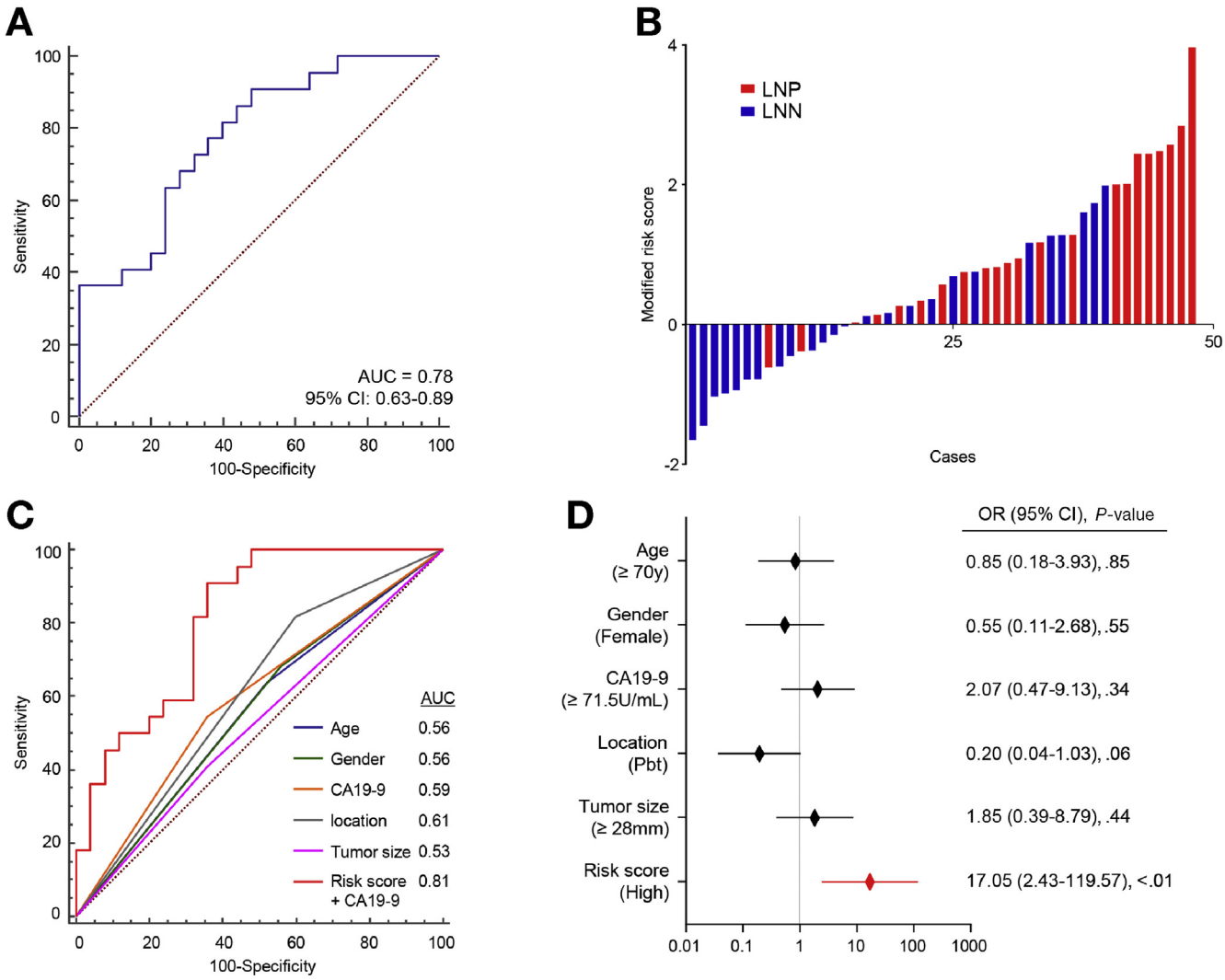

Figure 4. Higher-order validation of the miRNA signature in EUS-FNA biopsy specimens from patients with PDAC. (*A*) An ROC curve of the 6-miRNA signature in the EUS-FNA biopsy cohort (LNP, 22; LNN, 25; AUC, 0.78). (*B*) Risk score distribution plot in the EUS-FNA biopsy cohort. (*C*) The ROC curves of each clinicopathologic factor and the risk model constructed with 6-miRNA signature and CA19-9 (AUC, 0.81). (*D*) Forest plot with the ORs of clinicopathologic variables and signature risk score status in multivariate logistic regression analysis of LNM in the additional validation cohort.

preoperative LNM status. Accordingly, for an easier translation of our miRNA signature in clinical settings, we enrolled a cohort of 62 patients who underwent NAT followed by surgery (9 ypN positive and 53 ypN negative) (Supplementary Table 2) from whom pretreatment EUS-FNA specimens were available. Importantly, we applied the same statistical risk model to this pretreatment EUS-FNA cohort, which once again successfully confirmed the robustness of our risk stratification in identifying LNM-positive patients with an excellent AUC value of 0.78 (95% CI, 0.65–0.87) (Figure 5*A* and *B*). When we assessed the distribution of risk scores and ypN status, we observed that the patients who were ypN positive had significantly higher risk scores than those who were ypN negative ($P < .01$, Mann-Whitney test) (Figure 5*C*). Moreover, in multivariate analysis of the logistic regression model, our miRNA signature emerged as an independent feature for ypN prediction before treatment (OR, 18.54; 95% CI, 2.45–140.33; $P < .01$) (Figure 5*D*).

Discussion

As cancer treatment has entered a new era of precision medicine, the development of individualized treatment strategies is essential for patients with cancer. In the context of patients with PDAC, the presence of LNM is considered as

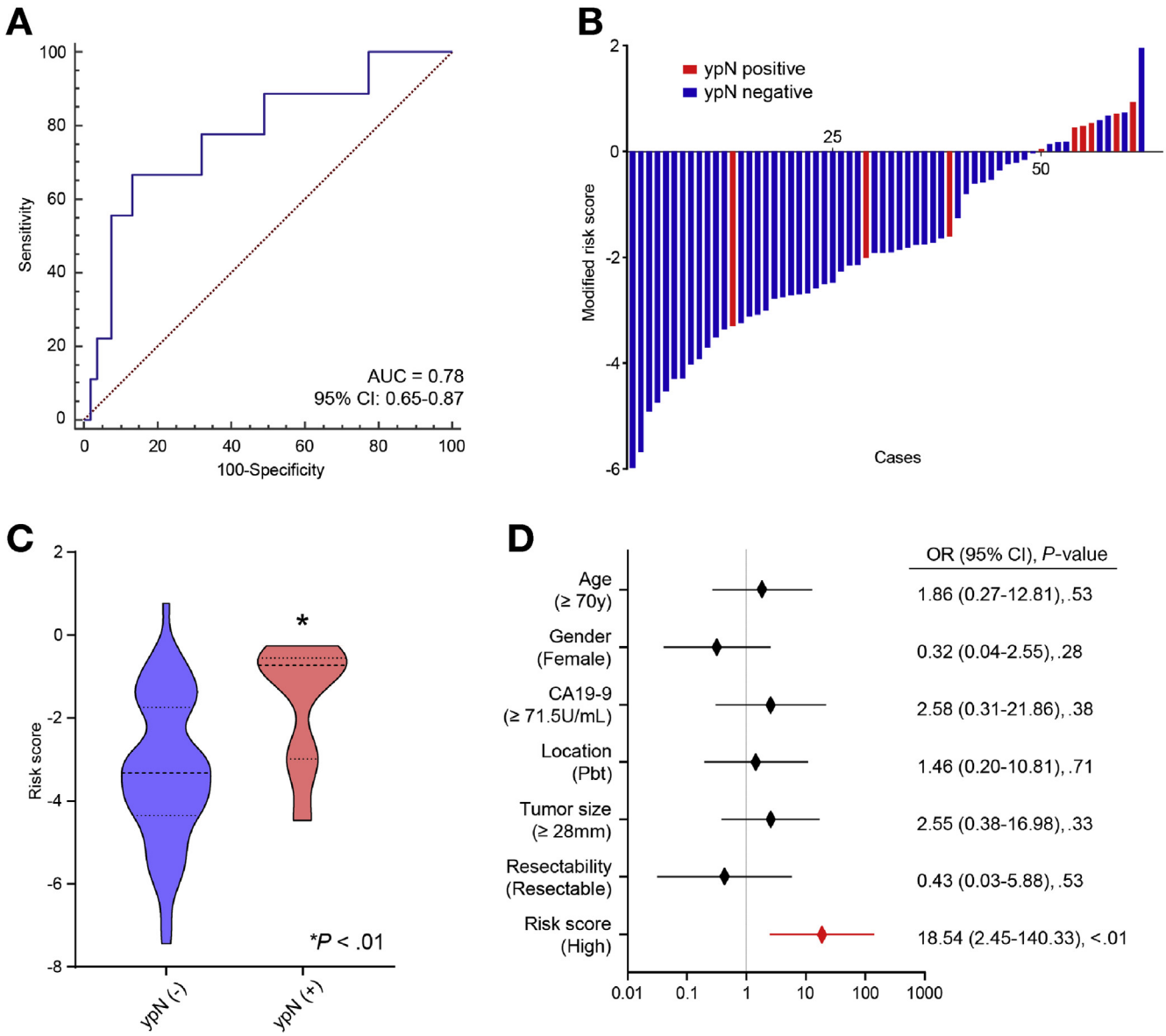

Figure 5. Additional validation of the performance of the miRNA signature for predicting residual nodal involvement after neoadjuvant therapy in EUS-FNA biopsy specimens. (*A*) An ROC curve of the 6-miRNA signature in the additional validation cohort (pre-NAT EUS-FNA biopsy specimens: ypN positive, 9; ypN negative, 53; AUC, 0.78). (*B*) Risk score distribution plots in an additional validation cohort. (*C*) The distribution of risk scores according to ypN status ($P < .01$, Mann-Whitney test). (*D*) Forest plots with ORs for clinicopathologic variables and risk scores in multivariate logistic regression analysis of ypN status in an additional validation cohort.

a high-risk feature; however, its diagnosis presents a clinical challenge. Our study was a step in this direction, wherein we undertook a systemic and comprehensive biomarker discovery and validation approach and successfully identified a novel miRNA signature that robustly identifies LNM in patients with PDAC. These findings were validated in resected tissue specimens from 2 independent clinical cohorts. Furthermore, we were able to confirm the diagnostic potential of this signature even in pretreatment EUS-FNA biopsy specimens, which was comparable to the performance of these biomarkers in surgically resected specimens; highlighting the potential significance of these findings for their clinical translation for improved risk assessment and survival in patients with PDAC. More interestingly, our miRNA signature was a robust predictor of the ypN status, which is considered as an important risk factor in patients who undergo NAT followed by surgery. These results highlight the potential clinical significance of our novel miRNA signature for the identification of LNM in patients with PDAC.

Several previous reports have favored the importance of multidisciplinary treatment, including NAT in patients with PDAC.[4,10–13,15,16,52] The NCCN guidelines indicate that NAT for resectable PDAC should be particularly considered in patients with high-risk features, with LNM being one such critical risk factor. On the other hand, in clinical settings, NAT has been actively explored and is becoming a common treatment option, regardless of resectability status in patients with PDAC. These findings highlight the need to develop robust biomarkers for LNM before any treatments, which offers superior risk stratification vis-à-vis other clinicopathologic factors in patients with PDAC. Our ability to successfully validate our miRNA signature in pretreatment biopsy specimens underscores its clinical significance for improved treatment strategies in patients with PDAC, especially those with LNM and, often, the worst survival outcomes. Several previous studies have similarly highlighted the clinical use of EUS-FNA biopsies for diagnostic purposes, as well as for drug response determination, in patients with PDAC; however, none of the previous studies directly used these for diagnosing LNM and ypN status, which can have a profound impact on the selection of treatment strategies.[52,53,64,65] Our findings for comparable performance of our biomarker signature in resected tissues and FNA biopsy specimen are in line with previous evidence[52,65] and highlight the clinical significance of such pretreatment specimens for the personalized treatment of patients with cancer.

TCGA Network performed miRNA clustering on the TCGA data set from 76 high-purity cases of PDAC. However, the scope of that study was very limited and did not include enough LNM cases. Nonetheless, among the 31 miRNAs reported in the consensus clustering, 1 of our identified miRNAs, miR-365a-5p, was reported to be down-regulated in clusters 1 and 3 and up-regulated in cluster 2.[66] Subsequently, we searched additional studies that have reported the clinical significance of our 6 candidate miRNAs. As illustrated in Supplementary Table 3, we observed that high expression of miR-155-5p and low expression of miR-92b-3p were associated with LNM in PDAC and gastric cancer, which is consistent with our study.[67,68] In addition, we constructed an miRNA:mRNA regulatory network for the 6 miRNAs and identified 176 gene targets that provide support to their mechanistic involvement in gene regulatory pathways (Supplementary Figure 4 and Supplementary Table 4). Subsequent pathway analysis of the validated downstream gene targets revealed significant biologically meaningful pathways associated with cancer cell biology (Supplementary Table 5), providing evidence for their involvement in key cancer-signaling pathways.

We would like to acknowledge a few potential limitations to our present study. First, the study had a retrospective design, and we analyzed our miRNA signature in a moderately sized clinical cohort; future, prospective studies with larger patient cohorts are required before consideration of these biomarkers in clinical settings. Second, molecular profiles from PDAC tissue specimens potentially have uneven tumor cellularity. To minimize this bias, we evaluated the performance of our miRNA signature by using independent multiple public data sets and clinical cohorts as well as surgically resected and EUS-FNA biopsy specimens. Finally, we did not have access to matched blood plasma specimens from the patient cohorts available to us, which otherwise would be a most ideal scenario for exploring the liquid biopsy approach for our discovered biomarkers. Nonetheless, our present study provides compelling evidence for the clinical significance of our miRNA signature for detecting LNM in patients with PDAC, and it is potentially an important major step toward the availability of robust molecular biomarkers for the risk assessment and management of a lethal malignancy such as PDAC.

In conclusion, using a genome-wide miRNA expression profiling effort, we have identified and developed a novel miRNA signature that was successfully validated in resected tissue specimens and EUS-FNA biopsy specimens for the identification of LNM in patients with PDAC. Pending validation in future prospective studies, our findings highlight the potential clinical impact of this signature in a more appropriate patient selection and the institution of improved individualized treatment strategies for patients with PDAC.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2020.04.057.

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CRediT Authorship Contributions
Satoshi Nishiwada, MD, PhD (Conceptualization: Lead; Data curation: Lead; Formal analysis: Lead; Methodology: Lead; Validation: Lead; Writing – original draft: Equal; Writing – review & editing: Equal). Masayuki Sho, MD, PhD (Formal analysis: Supporting; Validation: Supporting). Jasjit K. Banwait, PhD (Formal analysis: Equal; Methodology: Equal). Kensuke Ymamura, MD, PhD (Conceptualization: Equal; Methodology: Equal). Takahiro Akahori, MD (Resources: Supporting; Validation: Supporting; Writing – review & editing: Supporting). Kota Nakamura, MD, PhD (Resources: Supporting). Hideo Baba, MD, PhD (Funding acquisition: Supporting; Resources: Supporting). Ajay Goel, PhD (Conceptualization: Lead; Funding acquisition: Lead; Writing – review & editing: Lead).

Conflicts of interest
The authors disclose no conflicts.

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