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

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Abstract

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 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 datasets (TCGA, 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 a 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-

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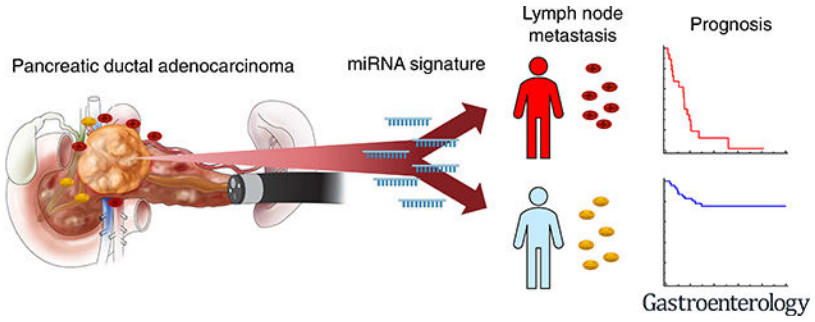
FNA before surgery from 47 patients (22 patients with lymph node metastases and 25 without; 17 for the training cohort and 30 from the validation cohort), and 62 specimens prior to any treatment from patients who received neoadjuvant chemotherapy (9 patients with lymph node metastasis and 53 without) 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 a 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 training cohort; this model discriminated patients with vs without lymph node metastases with an area under the curve (AUC) of 0.84 (95% 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 biopsies, 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 node in the validation cohort (odds ratio, 17.05; 95% CI. 2.43–119.57), and in the EUS-FNA cohort (95% CI. 0.65–0.87).

Conclusions: Using data and tumor samples from 3 independent cohorts, we identified a miRNA signature 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. This model might be used to select treatment and management strategies for patients with PDAC.

Graphical Abstract



LAY SUMMARY:

The authors identified genes that are expressed at higher levels in pancreatic tumors from patients with vs without metastases. This pattern of gene expression might be used to identify patients at higher risk for pancreatic tumor metastasis.

Keywords

LNM; cancer progression; prognostic factor; prognosis

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is an extremely lethal malignancy, and is projected to become the second leading cause of cancer-related deaths in the US by 2030[1–3]. While 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 an unresectable or borderline resectable disease- leading to poor survival outcomes. Consequently, the 5-year survival rates in PDAC patients following 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 PDAC patients 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 PDAC patients with a resectable cancer, the guidelines suggest that NAT may only be considered in a limited subset of patients with specific high-risk features, including: large regional lymph nodes (LNs), elevated CA-19–9 levels, large primary tumors, excessive weight loss, and extreme pain.

Among all these risk factors, lymph node metastasis (LNM) status remains one of the most important predictors of survival in patients undergoing curative resection, and is considered to be of tremendous clinical significance for risk stratification and therapeutic decision-making in PDAC patients[10, 14–21]. Several recent studies have reported that PDAC patients 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 pre-treatment diagnosis of LNM is critical determinant for developing a more personalized treatment strategy in PDAC patients[11, 13, 20]. However, pre-treatment diagnosis for the presence of LNM in PDAC patients is clinically challenging. Currently, such diagnosis is often made by computed tomography (CT), magnetic resonance imaging (MRI), endoscopic ultrasonography (EUS), and 18F-fluorodeoxyglucose positron emission tomography (PET). Unfortunately however, all of these methodologies are inadequate for evaluating the LNM status in PDAC patients due to their poor sensitivity and specificity[20, 27–30]; highlighting the need to develop potential molecular biomarkers that can overcome challenges of imaging-based methods for such diagnosis. Very limited research effort has been made on this front. A couple of studies reported that 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 identification of previously unrecognized molecular biomarkers[32–40]. MicroRNAs (miRNAs), which belong to the group of small non-coding RNAs, are 18 to 25 nucleotides long, single-stranded RNAs, that play key roles in post-transcriptional gene repression, oncogenesis and tumor metastasis, and are frequently dysregulated in various human cancers including PDAC[32, 33, 35–40]. Importantly, due to 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, formalin-fixed paraffin-embedded (FFPE), as well as biopsy tissues[32, 35, 39–41]. More specifically, several miRNAs, including miR-21, have been reported to associate with early diagnosis and prognosis in PDAC. However, the clinical significance as various miRNAs to serve as biomarkers to identify LNM pre-operatively in PDAC, through a systematic and genomewide comprehensive analysis in large, multiple independent patient cohorts has not been attempted[32, 33, 38, 39, 42–49]. Availability of such biomarkers will facilitate physicians in making more informed clinical decision-making and developing individualized treatment strategies for improved management of PDAC patients.

Herein, 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 PDAC patients. This signature was initially confirmed in multiple, large, publicly-available datasets, followed by rigorous validation and performance evaluation in two independent, large, clinical cohorts. Finally, in order to translate our findings into a clinically-viable scenario, we were able to confirm the robustness of this biomarker signature in pre-treatment endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) biopsy specimens, highlighting the clinical significance of these biomarkers for the management of PDAC patients.

MATERIALS AND METHODS

MiRNA biomarker discovery

To perform a comprehensive biomarker discovery, we analyzed miRNA expression profiling results of primary tumor tissues from three, large, publicly-available datasets (TCGA, GSE24279, and GSE32688), to identify and establish a miRNA signature for the identification of LNM in PDAC patients, as illustrated in supplementary figure S1. TCGA miRNA expression profiling data (level 3 miRNA-sequencing data) were downloaded from the UCSC Xena Browser (https://xenabrowser.net, accessed on July 12, 2018). Likewise, GSE24279 and GSE32688 datasets (both normalized non-coding RNA profiling and clinical data) were downloaded from the GEO database in its processed form (https://www.ncbi.nlm.nih.gov, accessed on July 12, 2018) and from a previously published article (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0034151)[50]. The cases where patients had distant metastasis, insufficient pathological LNM information, and those who received NAT (because pathological LNM can be modified by such treatment) were excluded. In total, miRNA-expression profiling data from total of 269 PDAC patients, including miRNA-sequencing data from TCGA cohort (167 patients, 121 LNM-positive [LNP] and 46 LNMnegative [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 (Table S1). 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 two large, and independent patient cohorts. A total of 264 FFPE specimens were examined, which included a training cohort (n = 157; 83 LNP and 74 LNN) of PDAC patients 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 PDAC patients (9 LNP and 53 LNN) who received NAT followed by surgery were also collected for additional validation. These EUS-FNA biopsy specimens were obtained prior to initiation of any treatment, and the specimens were collected and processed as per the standard diagnostic procedures using endoscopic and cytological techniques[52, 53]. Tumors were classified according to the TNM staging system of the International Union Against Cancer (UICC) 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. A 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 RT-qPCR

Total RNA was isolated from 10-mm-thick FFPE surgical tissues and EUS-FNA biopsy specimens by microdissection to enrich for neoplastic cells, using AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Synthesis of complementary DNA from total RNA was performed using Taqman MicroRNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA). Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) 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 abundance of target transcripts was evaluated and normalized to the expression levels of snRNA U6 as an internal control using the $2^{-\Delta DCt}$ method. Normalized values were further log10 transformed[41, 55].

MicroRNA Regulatory Network

The miRNA:mRNA regulatory network was constructed using the validated miRNAs to elucidate pathways perturbed, through data analysis 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 three sources -TargetScan, miRDB, and miRTarBase. The

pathway enrichment analysis for selected target genes was performed using KEGG pathways and Gene Ontology[41, 59, 60].

Statistical analysis

Unpaired t-test was used to evaluate the statistical differences in miRNA expression between LNP and LNN patients in the public datasets. Pearson's correlation analysis was performed to test multicollinearity. Recursive feature elimination using random forest method was performed to select important features miRNAs. For all cohorts, ROC curves and AUC values were determined using Medcalc statistical software V.16.2.0. Univariate and multivariate logistic regression were employed to evaluate various clinicopathological variables, including age, gender, carbohydrate antigen 19–9 (CA19–9), tumor location, tumor size evaluated by CT, and the miRNA signature for the detection of LNM status. The cutoff thresholds for continuous variables were divided by 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 last follow-up date. We estimated OS and RFS 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 < 0.05 was considered statistically significant. The statistical analyses were performed using the Medcalc statistical software V.16.2.0 (Medcalc Software bvba, Ostend, Belgium), GraphPad Prism V7.0 (GraphPad Software, San Diego, CA), and R (3.5.0, R Development Core Team, https://cran.r-project.org/).

RESULTS

Genome-wide miRNA expression profiling led to the identification of a novel 7-miRNA signature for the detection of lymph node metastasis in PDAC patients

We performed a genome-wide, unbiased, comprehensive biomarker discovery analysis in three independent miRNA expression profiling datasets (TCGA, GSE24279 and GSE32688) to identify a miRNA signature for the detection of LNM in PDAC patients. We first compared miRNA expression profiles between LNP and LNN patients in the TCGA and GSE24279 cohort, which included patients who had undergone curative surgery without NAT and identified 13 differentially expressed (P < 0.05) candidate targets with data availability in at least >50% of all cases, excluded 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 dataset reduced this to a signature of 10 miRNAs. Among these, 7 miRNAs exhibited consistent expression profiles in all three datasets: 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 two additional datasets (GSE24279: AUC = 0.75; 95% CI = 0.64–0.84, GSE32688: AUC = 0.92; 95% CI = 0.74–0.99; Fig. 1A–C); highlighting the diagnostic performance of this signature for the identification of LNM in PDAC patients.

Clinical training and validation resulted in the establishment of a miRNA signature for detecting Lymph node metastasis in PDAC patients

In order 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 RT-qPCR assays in a training cohort of 157 PDAC patients (83 LNP and 74 LNN). In the training cohort, we excluded one miRNA (let-7d-5p) as 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 PDAC patients from the three public datasets (Fig. S2). Subsequently, we trained a 6-miRNA risk-prediction model using logistic regression analysis in the training cohort, which robustly identified PDAC patients with LNM (AUC = 0.84, 95%CI = 0.77–0.89, Fig. 2A, C). The risk score model was developed based on the coefficients of individual miRNAs and the 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*miR629–5p) + (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 PDAC patients in this validation cohort as well (AUC = 0.73, 95%CI = 0.64–0.81, Fig. 2B, D); underscoring the clinical significance of our miRNA signature in identifying presence of LNM in PDAC patients.

A combination signature of miRNA biomarkers and CA-19–9 levels demonstrated a significantly higher accuracy for detecting LNM in PDAC patients

Since, 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 levels might further improve the diagnostic accuracy for detecting LNM in PDAC patients. Interestingly, indeed this new combination signature demonstrated a significantly superior diagnostic accuracy for LNM in both training and validation cohorts (AUC= 0.85 and, 0.76, respectively, Fig. 2E, F). Furthermore, this new combination signature also demonstrated a significantly improved diagnostic accuracy compared to other classic preoperative clinicopathological features, including tumor location and size (Fig. 2E, F). We next categorized all patients into high- and low-risk groups using the cutoff thresholds derived by Youden's index from this 6-miRNA signature model, and subsequently performed logistic regression for univariate and multivariate analyses[63]. Of interest, the multivariate analysis revealed that our newly established developed signature emerged as an independent predictor of LNM in PDAC patients, in both clinical cohorts [training cohort: odds ratio (OR) = 16.97; 95% CI = 6.78–42.50; $P < 0.01$, validation cohort: OR = 9.95; 95% CI = 3.49–28.34; $P < 0.01$, Table 2).

Prognostic potential of the miRNA signature for PDAC patients in the clinical cohorts

Since LNM is often associated with poor patient survival in PDAC patients, we next were curious to inquire 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 (Fig. S3). In order 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, PDAC patients within the high-risk group demonstrated a significantly worse prognosis in the training cohort (OS [$P < 0.01$]; RFS [$P < 0.01$]) and the validation cohort (OS [$P < 0.01$]; RFS [$P = 0.03$], Fig. 3A, B, D and E). In addition, in multivariate analysis using the Cox's proportional hazard model along with other clinicopathological factors, high-risk patients defined by the miRNA signature associated with a significantly worse OS in both independent cohorts [training cohort: Hazard ratio (HR) = 1.78; 95% CI = 1.16–2.73; $P < 0.01$, validation cohort: HR = 2.41; 95% CI = 1.08–5.40; $P = 0.03$, Fig. 3C and F]. These results highlight that in addition to the diagnostic ability of our signature in detecting LNM in PDAC patients, it has a significant prognostic potential as well.

Higher-order validation of the miRNA signature in EUS-FNA biopsy specimens from PDAC patients

While the validation of biomarkers using surgically resected tissue specimens was necessary for constructing this LNM signature, we believe that validation of these biomarkers in pre-treatment 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, the physicians are able to make a more informed decision for offering NAT to high-risk patients categorized that are deemed otherwise 'resectable' and improve their survival. Based on this hypothesis, we collected *matched* EUS-FNA biopsy specimens from a subset of 47 PDAC patients from the training and validation cohorts (Table 1). All 47 patients were classified as resectable 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 it yielded a satisfactory AUC value of 0.78 (95% CI = 0.63–0.89) for distinguishing LNM (Fig. 4A 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 demonstrated superior diagnostic potential than other clinicopathological factors (Fig. 4C). Furthermore, multivariate logistic regression analysis for LNM detection revealed that our 6-miRNA signature was an exclusive significant detective marker in FNA biopsy cohort (OR = 17.05, 95% CI = 2.43–119.57, Fig. 4D).

Performance validation of the miRNA signature for predicting residual nodal involvement following neoadjuvant therapy in EUS-FNA biopsy specimens

Currently, multidisciplinary treatment strategies including NAT for resectable and borderline-resectable PDAC patients are actively being explored and becoming more common, especially in western countries. Moreover, pathological nodal involvement status

which is modified by the effect of NAT (ypN), the residual LNM status following NAT, is often considered as one of the most important prognostic factors in PDAC patients undergoing NAT following 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 upon their pre-operative 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, Table S2), from whom pre-treatment EUS-FNA specimens were available. Importantly, we applied the same statistical risk-model to this pre-treatment 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, Fig. 5A and B). When we assessed the distribution of risk scores and ypN status, we observed that the patients with ypN positive had significantly higher risk scores than those who were ypN negative ($P < 0.01$, Mann Whitney test, Fig. 5C). Moreover, in multivariate analysis of 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; $P < 0.01$, Fig. 5D).

DISCUSSION

As cancer treatment has entered a new era of precision-medicine, development of individualized treatment strategies is essential for cancer patients. In the context of PDAC patients, presence of LNM is considered as high-risk feature; however, its diagnosis presets 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 PDAC patients. These findings were validated in resected tissue specimens from two independent clinical cohorts. Furthermore, we were able to confirm the diagnostic potential of this signature even in pre-treatment 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 PDAC patients. 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 identification of LNM in PDAC patients.

Several previous reports have favored the importance of multidisciplinary treatment including NAT in PDAC patients[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 PDAC patients. These findings highlight the need to develop robust biomarkers for LNM prior to any treatments, which offer superior risk stratification vis-à-vis other clinicopathological factors in PDAC patients. Our ability to successfully validate our miRNA signature in pre-treatment biopsy specimens underscores its clinical significance for improved treatment strategies in PDAC patients, especially the ones with LNM, and often

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 PDAC patients; however, none of the previous studies have directly used these for diagnosing LNM and ypN status which can have profound impact in the selection of treatment strategies[52, 53, 64, 65]. Our findings for comparable performance of our biomarker signature in resected tissues and FNA biopsies in line with previous evidence[52, 65], and highlights the clinical significance of such pre-treatment specimens for personalized treatment of cancer patients.

The Cancer Genome Atlas Research Network performed miRNA clustering on the TCGA dataset from high-purity 76 PDAC cases. However, the scope of this study was very limited and did not include enough LNM cases. Nonetheless, among the 31 miRNAs reported in the consensus clustering, one of our identified miRNAs, miR-365a-5p, was reported to be downregulated in clusters 1 and 3 and upregulated in cluster-2[66]. Subsequently, we searched additional studies that have reported the clinical significance of our 6 candidate miRNAs. As illustrated in Table S3, we observed that high-expression of miR-155–5p and low-expression of miR-92b-3p associated with LNM in PDAC and gastric cancer; which are consistent with our study[67, 68]. In addition, we constructed a miRNA:mRNA regulatory network for the 6 miRNAs and identified 176 gene targets that provide support to their mechanistic involvement in gene regulatory pathways (Table S4). Subsequent pathway analysis of the validated downstream gene targets revealed significant biologically meaningful pathways associated with cancer cell biology (Table S5), providing an 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 was a retrospective design and we analyzed our miRNA signature in a moderately-sized clinical cohort; and future, prospective studies using larger patient cohorts are required before consideration of these biomarkers in clinical settings. Second, molecular profiles from PDAC tissue specimens potentially possess uneven tumor cellularity. In order to minimalize this bias, we evaluated the performance of our miRNA signature using independent multiple public datasets and clinical cohorts, surgically resected as well as EUS-FNA-biopsy specimens. Lastly, 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 PDAC patients, is potentially an important major step towards availability of robust molecular biomarkers for the risk-assessment and management of a lethal malignancy such as PDAC.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AUC	Area under the curve
CT	Computed tomography
EUS-FNA	Endoscopic ultrasound-guided fine needle aspiration
FFPE	Formalin-fixed paraffin-embedded
LNs	lymph nodes
LNM	Lymph node metastasis
LNN	Lymph node metastasis-negative
LNP	Lymph node metastasis-positive
miRNA	MicroRNA
MRI	Magnetic resonance imaging
NAT	Neoadjuvant therapy
NCCN	The National Comprehensive Cancer Network
OS	Overall survival
PDAC	Pancreatic ductal adenocarcinoma
PET	18F-fluorodeoxyglucose positron emission tomography
RFS	Relapse-free survival
ROC	Receiver operator characteristic
RT-qPCR	Real-time quantitative reverse transcription polymerase chain reaction
UICC	The International Union Against Cancer

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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 3 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 3 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.

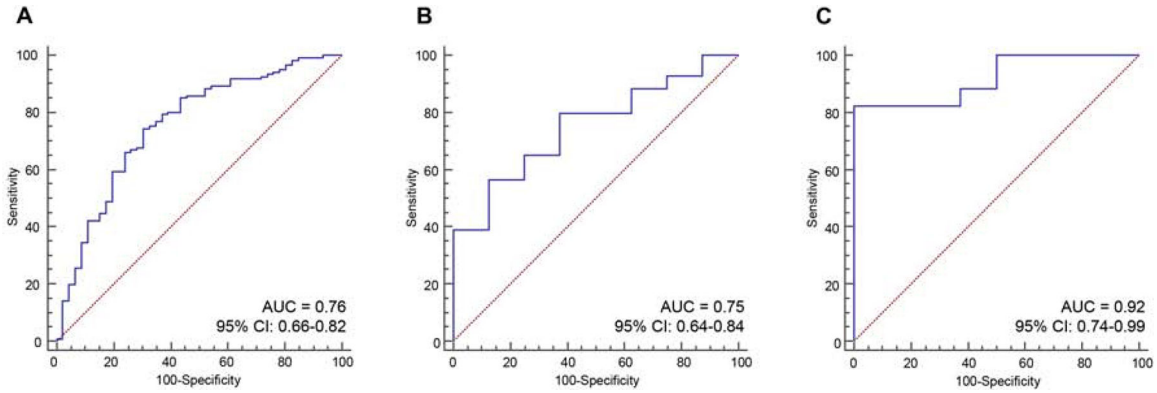

Figure 1.

Genome-wide discovery and validation of a novel miRNA signature to detect lymph node metastasis in PDAC patients.

(A-C) The ROC curves demonstrate the diagnostic performance of the 7-miRNA signature for distinguish the patients with lymph node metastasis in (A) TCGA, (B) GSE24279 and (C) GSE32688 cohorts.

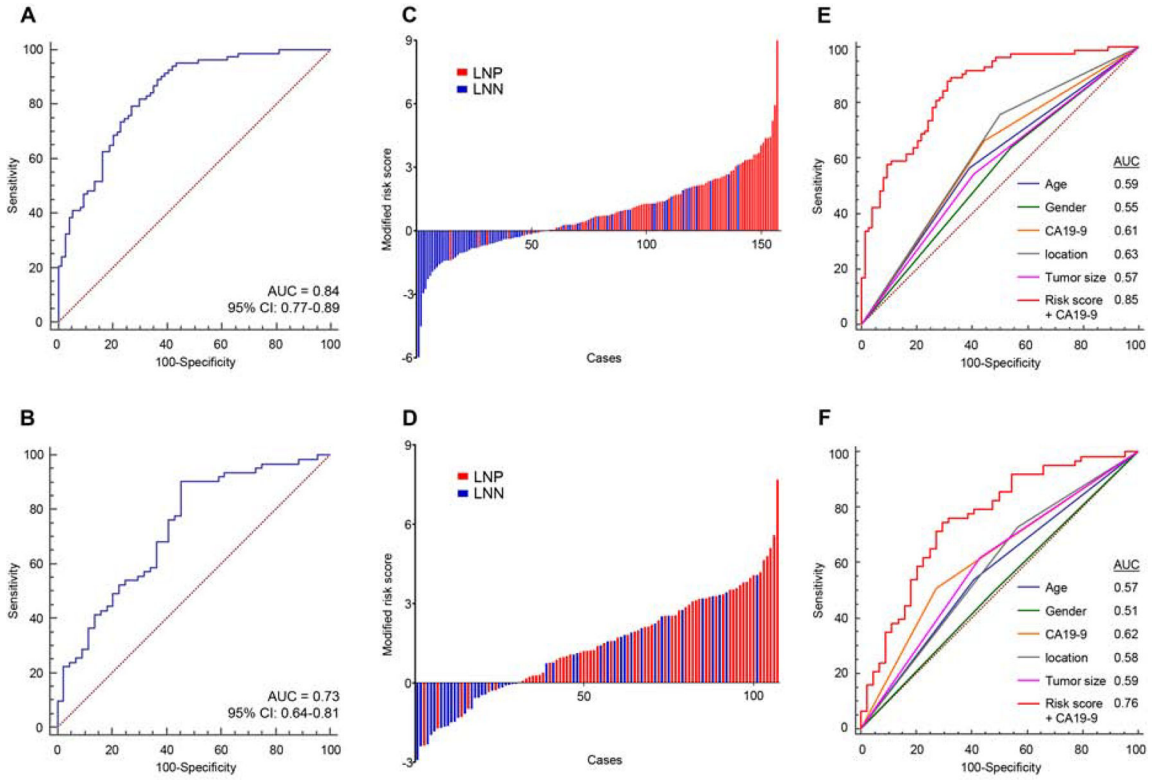

Figure 2.

Training and validation of a miRNA signature for identifying Lymph node metastasis in PDAC patients in two independent clinical cohorts.

(A, B) A 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 (C) training cohort and (D) validation cohort. Modified risk score was obtained from subtracting individual risk score from Youden's index value of risk model. (E, F) The new combination model, miRNA signature and CA19–9, outperformed the detection accuracy in both clinical cohorts (E: training cohort, F: validation cohort). LNN: lymph node metastasis negative, LNP: lymph node metastasis positive.

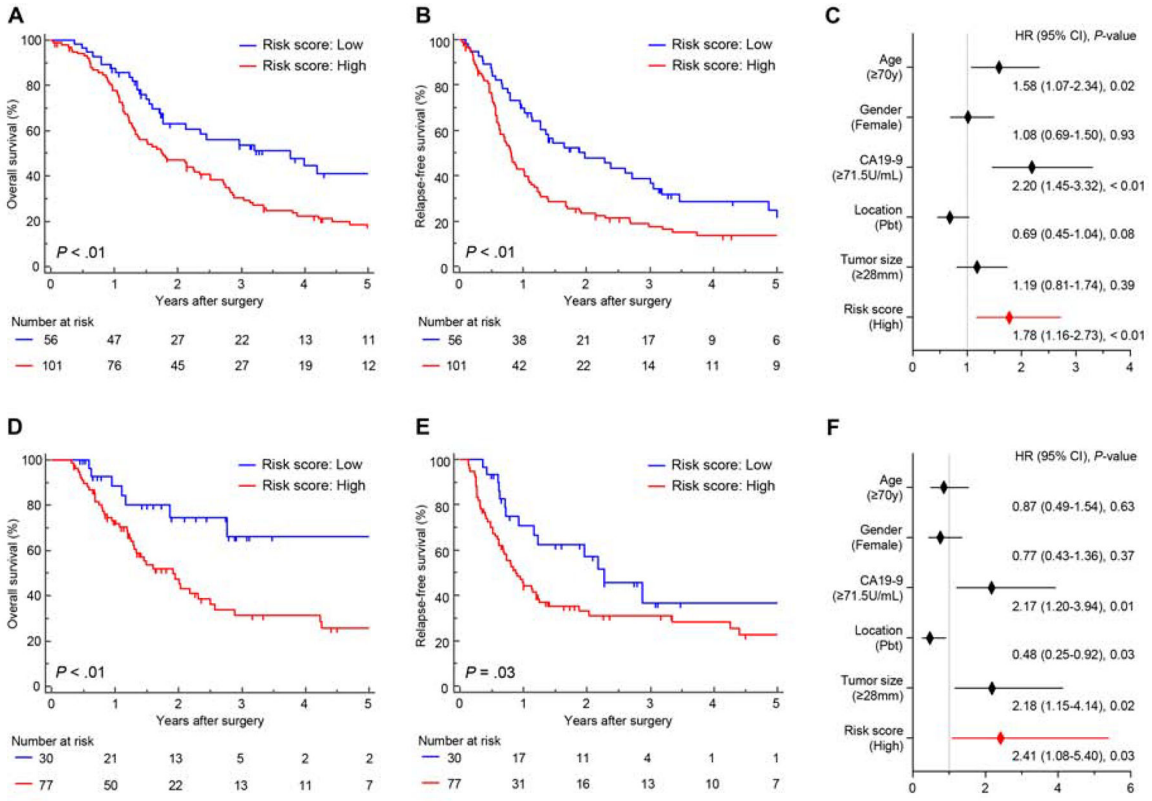

Figure 3.

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

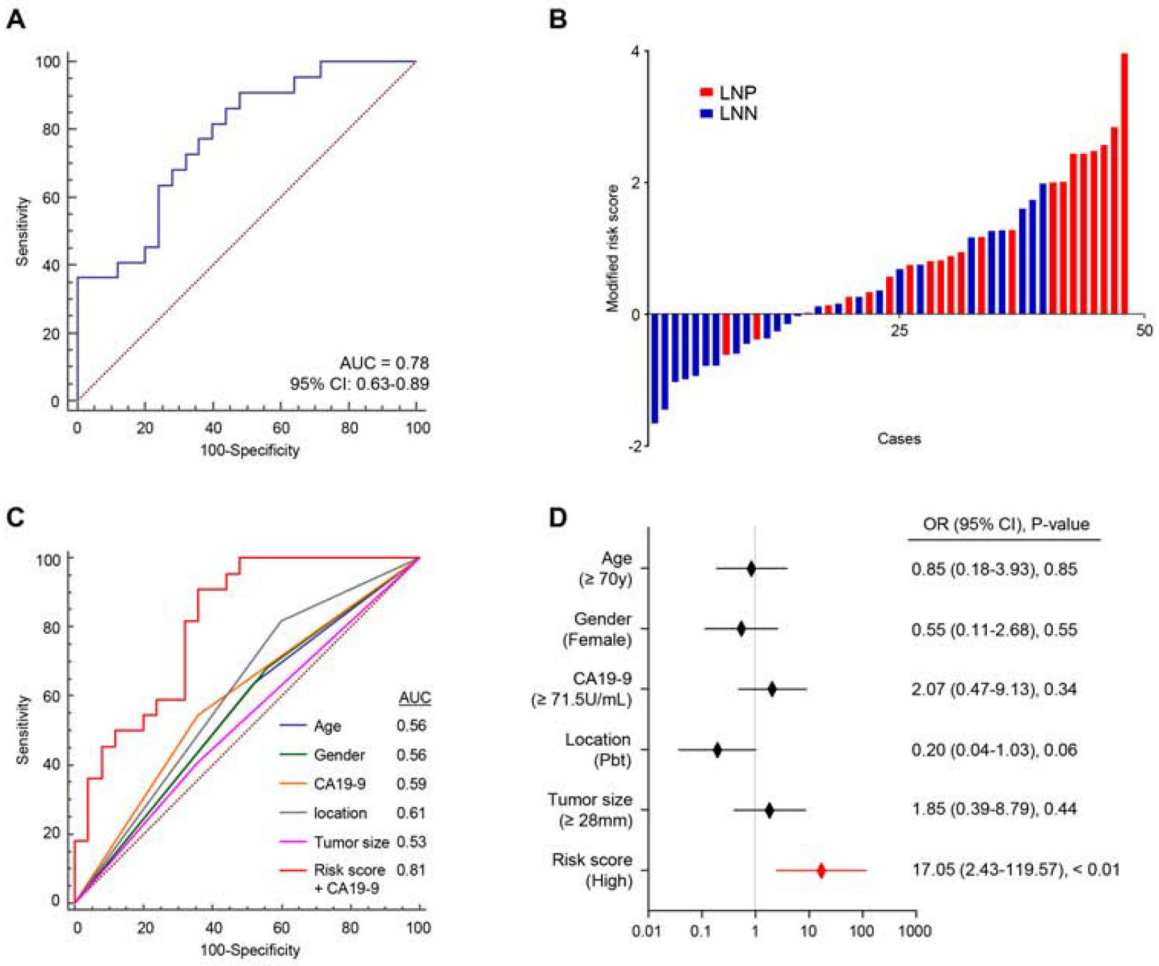

Figure 4.

Higher-order validation of the miRNA signature in EUS-FNA biopsy specimens from PDAC patients.

(A) A ROC curve of the 6-miRNA signature in EUS-FNA biopsy cohort (LNP = 22, LNN = 25, AUC = 0.78). (B) Risk score distribution plot in EUS-FNA biopsy cohort. (C) The ROC curves of each clinicopathological factors and the risk model constructed with 6-miRNA signature and CA19–9 (AUC = 0.81). (D) Forest plot with odds ratio of clinicopathological variables and signature risk score status in multivariate logistic regression analysis of LNM in additional validation cohort. LNN: lymph node metastasis negative, LNP: lymph node metastasis positive.

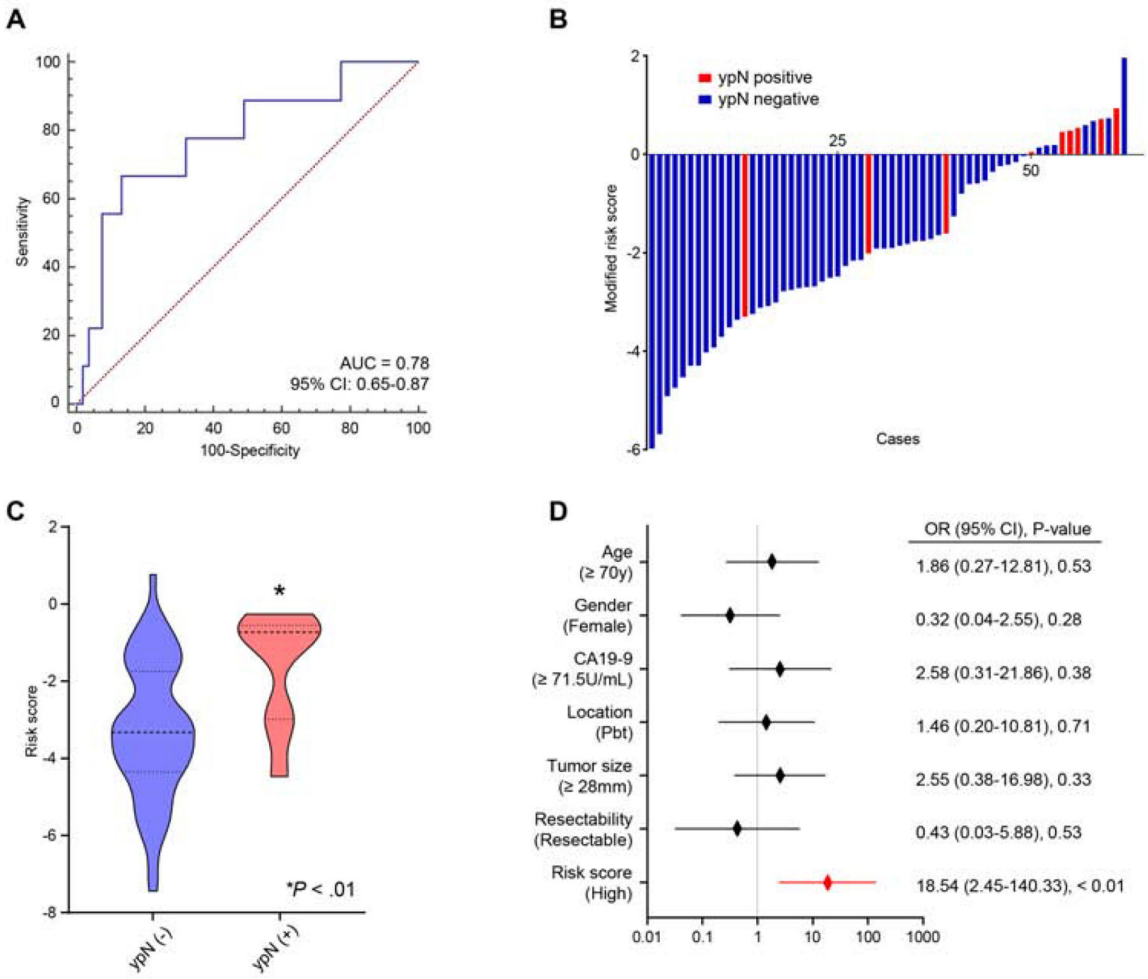

Figure 5.

Additional validation of performance of the miRNA signature for predicting residual nodal involvement following neoadjuvant therapy in EUS-FNA biopsy specimens.

(A) A ROC curve of the 6-miRNA signature in additional validation cohort (pre-NAT EUS-FNA biopsies; 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 ($P < 0.01$, Mann Whitney test. (D) Forest plots with odds ratio for clinicopathological variables and risk scores in multivariate logistic regression analysis of ypN status in an additional validation cohort.

Table 1:

Clinicopathological characteristics of clinical cohorts

Characteristics	Surgical specimens, n (%)		Matched FNA biopsy specimens, n (%) (n = 47)	Total participants (n = 264)
	Training cohort (n = 157)	Validation cohort (n = 107)		
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)

FNA, Fine needle aspiration; *UICC*, International Union Against Cancer

*Plus-minus values are means standard error of the mean.

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	0.07	0.53	0.23–1.23	0.14
Gender (Female vs. Male)	0.67	0.35–1.26	0.21	0.45	0.19–1.06	0.07
CA19–9 (≥ 71.5 vs. < 71.5 U/mL)	2.44	1.28–4.66	**< 0.01**	2.18	0.92–5.14	0.08
Location (Pbt vs. Ph)	0.32	0.16–0.63	**< 0.01**	0.21	0.09–0.53	**< 0.01**
Tumor size (≥ 28 vs. < 28 mm)	1.75	0.91–3.38	**0.01**	1.85	0.78–4.41	0.17
6-miRNA signature (High vs. Low risk)	33.36	9.57–116.23	**< 0.01**	19.93	7.55–52.62	**< 0.01**
Validation cohort (n = 107)						
Age (≥70 vs. <70 years)	0.59	0.27–1.29	0.19	1.00	0.39–2.60	0.99
Gender (Female vs. Male)	0.94	0.44–2.04	0.88	1.11	0.43–2.83	0.83
CA19–9 (≥ 71.5 vs. < 71.5 U/mL)	2.75	1.20–6.30	**0.02**	1.55	0.58–4.13	0.38
Location (Pbt vs. Ph)	0.49	0.22–1.10	0.08	0.50	0.19–1.29	0.15
Tumor size (≥ 28 vs. < 28 mm)	2.14	0.98–4.68	0.06	1.75	0.66–4.65	0.26
6-miRNA signature (High vs. Low risk)	11.09	4.09–30.04	**< 0.01**	10.05	3.42–29.59	**< 0.01**

OR, odds ratio; *CI*, confidence interval