

# Diagnostic and Prognostic Performance of MicroRNA-25, Carbohydrate Antigen 19-9, Carcinoembryonic Antigen, and Carbohydrate Antigen 125 in Pancreatic Ductal Adenocarcinoma

Yan Gong<sup>1</sup>, MD, PhD; Lele Song<sup>2</sup>, MD, PhD; Lei Ou<sup>1</sup>, MD, PhD; You-Yong Lu<sup>3</sup>, MD, PhD; Xianyong Huang<sup>1</sup>, MD, PhD; Qiang Zeng<sup>1</sup>, MD, PhD

<sup>1</sup>Health Management Institute, The Second Medical Center and National Clinical Research Center for Geriatric Disease, Chinese PLA General Hospital, Beijing, China;

<sup>2</sup>Division of Cancer Early Screening, Genetron Health (Beijing) Technology Co. Ltd., Beijing, China;

<sup>3</sup>Laboratory of Molecular Oncology, School of Oncology, Peking University, Beijing Cancer Hospital and Institute, Beijing, China

## Correspondence:

Qiang Zeng, MD, PhD;  
Chinese PLA General Hospital, 28 Fuxing Road, Postal code: 100853, Beijing, P.R. China

Tel: +86 10 68295751

Fax: +86 10 68295928

Email: zq301@126.com

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## What's Known

- micro-RNA-25 (miR-25) has recently attracted attention as a potential biomarker for pancreatic ductal adenocarcinoma (PDAC) detection.
- Serum proteins including carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), and carbohydrate antigen 125 (CA125) have been suggested as markers for PDAC detection. These markers could be potential indicators for PDAC prognosis.

## What's New

- Combining miR-25 and CA19-9 significantly increased the detection sensitivity of PDAC compared to any single marker.
- miR-25 could detect early-stage PDAC and is a potential marker for PDAC screening. Tumor stage, CA19-9, and CA125 levels are found to be independent risk factors for PDAC prognosis.

## Abstract

**Background:** Pancreatic cancer is a malignancy with high mortality due to the difficulties in early detection. We investigated and compared the diagnostic and prognostic performance of several blood biomarkers, including microRNA-25 (miR-25), carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), and carbohydrate antigen 125 (CA125).

**Methods:** A retrospective study was conducted at the Chinese People's Liberation Army General Hospital from May 2014 to September 2018. Serum specimens were collected, and miR-25 expression levels were measured using real-time quantitative polymerase chain reaction. Serum CA19-9, CEA, and CA125 levels were measured using enzyme-linked immunosorbent assay (ELISA). Statistical analyses including nonparametric test, receiver operator characteristic (ROC) curves, Kaplan-Meier analysis, and subsequent log-rank test were performed with PRISM 5.0 software. Univariate and multivariate analyses were performed with the R software.  $P < 0.05$  was considered statistically significant.

**Results:** A total of 250 individuals were recruited, including 75 with pancreatic ductal adenocarcinoma (PDAC), 75 with benign lesions, and 100 healthy controls. miR-25, CA19-9, CEA, and CA125 exhibited an area under the curve (AUC) of 0.88, 0.91, 0.81, and 0.76 with a sensitivity of 78.7%, 74.7%, 37.3%, and 35.7% and specificity of 91.5%, 97.0%, 98.2%, and 98.3%, respectively. The combination of miR-25 and CA19-9 further increased the sensitivity to 93.3% with a specificity of 88.5%. Stage-dependent sensitivity was observed with CA19-9, CEA, and CA125. miR-25 levels significantly stratified the prognosis by median level (4,989.97 copies/mL). CA19-9, CEA, and CA125 levels significantly stratified the prognosis by median levels. Univariate and subsequent multivariate analyses identified tumor (T) stage, CA19-9, and CA125 as independent risk factors for PDAC prognosis.

**Conclusion:** The combination of miR-25 and CA19-9 significantly enhanced the detection sensitivity of PDAC. T stage, CA19-9, and CA125 levels were independent risk factors for PDAC prognosis.

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**Keywords** • Pancreatic neoplasm • PDAC • Screening • microRNA • Adenocarcinoma

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common malignant tumors of the digestive system, exhibiting invasive and metastatic properties with poor prognosis. According to the American Cancer Society, the annual number of deaths from pancreatic cancer reached 45,750 in 2019<sup>1</sup> and is expected to be the second leading cause of death by 2030.<sup>2</sup> Cancer statistics in China estimated 90,100 new cases and 79,400 deaths due to pancreatic cancer in 2015, with an upward trend in morbidity and mortality.<sup>3</sup> At times, pancreatic cancer presents no obvious clinical symptoms and is difficult to differentiate from pancreatic benign lesions solely based on imaging. Therefore, only 7% of pancreatic cancer cases are diagnosed at an early stage.<sup>3</sup>

The United States Food and Drug Administration (FDA) has approved carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) as biomarkers for diagnosing pancreatic cancer.<sup>4</sup> CA19-9 levels have been shown to elevate two years prior to the diagnosis of pancreatic cancer.<sup>5</sup> However, its use for screening is nonspecific, since the levels can also rise due to benign pancreatic lesions. Moreover, 5% of the general population show elevated CA19-9 levels due to germline genetic variation, which further limits its application in screening and early diagnosis of pancreatic cancer.<sup>6</sup> As an alternative, microRNA (miRNA) has attracted great attention in cancer research.<sup>7</sup> Several stable miRNAs in human serum/plasma can be used for the diagnosis, treatment, and prognosis of pancreatic cancer.<sup>8, 9</sup> miR-25, one of the most studied and well-documented miRNAs, is produced by transcription of the microchromosome maintenance protein 7 (MCM7) gene with a length of 22 nucleotides. It is highly expressed in a variety of human tumor tissues, including brain tumors, gastric adenocarcinoma, lung cancer, and ovarian cancer.<sup>10-13</sup> Previous studies suggested that miR-25 is overexpressed in PDAC patients, and that it could be used as a potential biomarker for PDAC detection.<sup>13-15</sup> Pancreatic cancer can be detected by serum miR-25 with a sensitivity of 75.58% and specificity of 93.03%.<sup>15</sup> However, the performance of miR-25 in combination with existing protein biomarkers in pancreatic cancer detection has not been investigated. On the other hand, the prognostic potential of miR-25 in pancreatic cancer has not yet been fully studied. In this study, we investigated the diagnostic performance of miR-25 in PDAC patients and

compared it with the performance of CA19-9, CEA, and carbohydrate antigen 125 (CA125). We further studied the prognostic potential of these biomarkers in terms of clinicopathological factors in patients diagnosed with pancreatic lesions. We trust that our study will contribute to establishing a diagnostic and prognostic model for PDAC patients.

## Materials and Methods

A single-center retrospective cohort study was conducted at the Chinese People's Liberation Army General Hospital from May 2014 to September 2018. The target population was patients diagnosed with pancreatic lesions. The inclusion criteria were patients aged  $\geq 18$  years, confirmed diagnosis of PDAC or benign pancreatic diseases (e.g., chronic pancreatitis, ductal papillary myxoma, pancreatic intraepithelial neoplasia, and mucinous cystic tumor), complete demographic and clinicopathological information, willingness to participate in the study, and presence of written informed consent. The exclusion criteria were a history of any type of malignancy or receiving any form of therapy. In addition, healthy individuals with confirmed "no evidence of disease" (NED) and no family history of the disease were included as controls.

Sample size estimation was based on the following equation for known detection sensitivity:  $N = Z^2 \times [p(1-p)] / E^2$ , where  $Z$  is a statistical parameter ( $Z = 1.96$  with 95% confidence interval [CI]), and  $E$  represents the error (which was set at 10% in this study), and  $p$  represents the putative sensitivity. If the  $P$  value is 0.75 for miR-25 and CA19-9 or 0.25 for CEA and CA125, a minimal sample size of 72 PDAC patients will be required. A ratio of at least 1:1 was used for the number of healthy individuals with NED. Accordingly, a total of 250 individuals were recruited and assigned to three groups, namely patients with PDAC (PDAC group,  $n = 75$ ), patients with benign pancreatic diseases (benign group,  $n = 75$ ), and healthy controls (NED group,  $n = 100$ ) (table 1).

All blood samples were collected prior to any treatment, e.g., surgery, chemotherapy, and radiotherapy. Differential diagnosis between benign pancreatic disease and pancreatic cancer was performed by histological examination or fine needle aspiration cytology. Pancreatic cancer staging was determined based on the classification proposed by the Union for International Cancer Control (UICC). Diagnosis, pathological, and clinical stages of the cancer were confirmed by pathological examinations,

**Table 1: Demographic and clinicopathological characteristics of the participants**

Factors	Groups (n=250)		
	NED	Benign	PDAC
Participants (n%)	100 (40)	75 (30)	75 (30)
Age (years)*	49.28±8.53	42.26±16.31	58.49±9.83
Sex (n%)	Man	59 (23.6)	31 (12.4)
	Woman	41 (16.4)	44 (17.6)
Clinical stage	I		11
	II		18
	III		20
	IV		26
Smoking (n%)	Yes	39 (39%)	20 (26.6%)
	No	61 (61%)	55 (73.4%)
Average tumor size (cm, range)			4.14 (1-10)
Differentiation	Well/moderate		27 (64.29%)
	Poor		15 (35.71%)
DM	Yes	14 (14%)	7 (9.33%)
	No	86 (86%)	68 (90.67%)

\*Data expressed as mean±SD. NED: No evidence of diseases; PDAC: Pancreatic ductal adenocarcinoma; DM: Diabetes mellitus

ultrasound, dynamic computed tomography (CT), magnetic resonance imaging (MRI), angiography, and/or endoscopic ultrasound. For diagnosis, pathological examination and CT or MRI were used as the gold standard.

**Sample Processing and RNA Extraction**

Peripheral blood samples (5 mL) were obtained from patients and healthy volunteers at the time of diagnosis or prior to any therapy. Serum was collected using serum separation tubes and centrifuged (800 g for 10 min and 10,000 g for 15 min) to completely remove cell debris. The supernatant was collected and stored at -80 °C for analysis. The total RNA was extracted from 100 µL of serum through phenol/chloroform purification followed by centrifugation in isopropyl alcohol. Two microliters of total RNA were reverse-transcribed into cDNA using an avian myeloblastosis virus (AMV) reverse transcriptase kit (Takara, Dalian, China) and a stem-loop RT primer (Applied Biosystems, Foster City, CA, USA). The expression levels of miRNAs were normalized to the serum volume.

**RT-qPCR Detection of miR-25**

miRNA quantification was performed using real-time quantitative polymerase chain reaction (qPCR) hydrolysis probes (Applied Biosystems, Foster City, CA, USA). This detecting system is highly specific for targeted miRNA examination without detecting pre-processed precursors or other highly-homologous miRNAs. The analysis was performed using an ABI-7500 detection system (Applied Biosystems, Foster City, California, USA) and a serum miR-25 quantification kit (life codes, Jiangsu, China). Reverse transcription qPCR (RT-qPCR) was

performed simultaneously on test samples and reference samples. All reactions were performed in triplicate. The Ct values were determined based on fixed threshold settings. All technicians were blinded to the identities of the patients and controls, and samples were randomly assigned to the RT-qPCR plates to avoid batch effects.

**Serum ELISA Assay**

CA19-9, CEA, and CA125 levels were measured using the electrochemical luminescence method with corresponding assay kits (Roche Diagnostics, Shanghai, China). All three protein markers were tested using Roche E170 modular instruments (RLH, London, United Kingdom) in accordance with the routine protocol of the clinical biochemistry laboratory.

**Ethical Considerations**

The study was approved by the Ethics Committee of the Chinese People’s Liberation Army General Hospital (code: S2014-137-01) and conducted in accordance with the Declaration of Helsinki 2013.

**Statistical Analysis**

Analyses were performed, and figures were plotted using PRISM 5.0 software (GraphPad Software, Inc., La Jolla, CA 92037, USA). Expression levels of serum miR-25 were compared using the nonparametric test (Mann-Whitney test). Receiver operator characteristic (ROC) curves were established to evaluate the diagnostic performance of serum miR-25, CA19-9, CEA, and CA125. Kaplan-Meier analysis and subsequent log-rank tests were performed to plot and compare the difference between survival curves. Univariate and multivariate analyses

were performed with the R software (<https://cran.r-project.org/>, The R Foundation, Vienna, Austria).  $P < 0.05$  was considered a statistically significant difference.

## Results

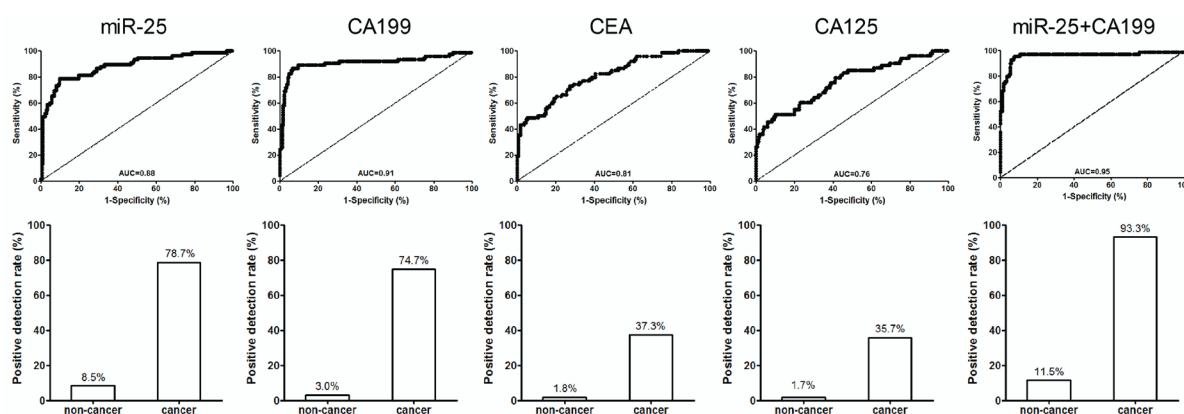
### Diagnostic Performance and Influencing Factors of miR-25, CA19-9, CEA, and CA125 in PDAC

The PDAC patients (n=75) included 11, 18, 20, and 26 patients with cancer stages I, II, III, and IV, respectively. Initially, the diagnostic performance of miR-25, CA19-9, CEA, and CA125 was investigated and compared. The ROC curves were plotted based on dichotomized grouping of cancer patients and non-cancer patients (including NED and benign groups). The threshold for miR-25 was determined at 3,333 copies/mL based on the Youden index calculation,<sup>13-15</sup> and for CA19-9, CEA, and CA125 in accordance with the kit manufacturer's instructions. CA19-9 and miR-25 exhibited the best diagnostic performance with an AUC of 0.91 (95% CI=0.86-0.96) and 0.88 (95% CI=0.83-0.93), respectively (figure 1). The sensitivity of CA19-9 and miR-25 reached 74.7% and 78.7%, with a specificity of 97% (positive detection rate (PDR) of 3% in non-cancer patients) and 91.5% (PDR of 8.5% in non-cancer patients), respectively. The performance of CEA and CA125 was inferior to that of CA19-9 and miR-25. The AUC for CEA and CA125 reached 0.81 (95% CI=0.75-0.87) and 0.76 (95% CI=0.68-0.84), respectively, and the sensitivity was 37.3% and 35.7% with a specificity of 98.2% (PDR of 1.8% in non-cancer patients) and 98.3% (PDR of 1.7% in non-cancer patients), respectively.

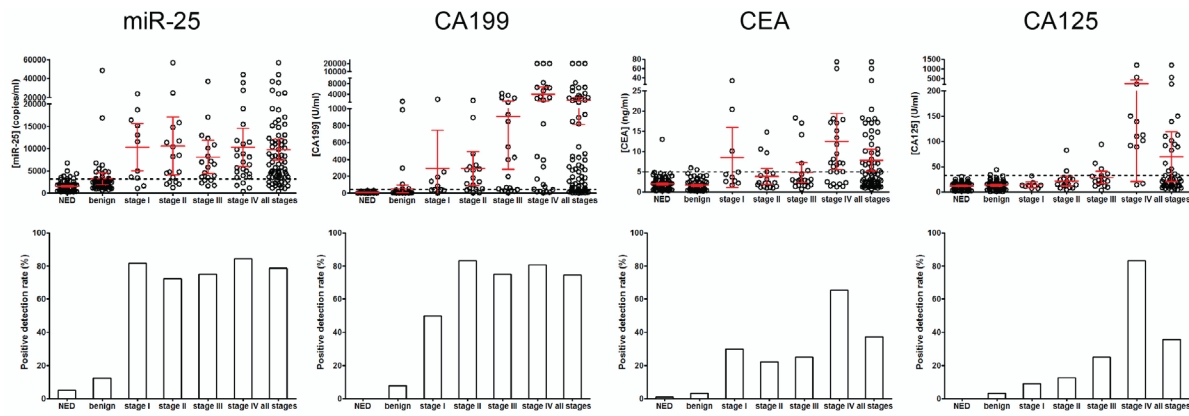
of 1.7% in non-cancer patients), respectively. Interestingly, the combination of the two best markers, CA19-9 and miR-25, achieved an AUC of 0.95 with a sensitivity of 93.3% and a specificity of 88.5% (PDR of 11.5% in the non-cancer group), indicating improved performance when combined.

The stage-related detection performance for the four markers is illustrated in figure 2. Scatter plots show the distribution of marker levels for the NED, benign, and PDAC groups. Overall, the PDAC group exhibited significantly higher serum marker levels than the benign and NED groups, while no significant difference was found between the benign and NED groups. Furthermore, CA19-9, CEA, and CA125 exhibited higher marker levels in higher cancer stages, while miR-25 expression levels were high across stages I to IV. The corresponding sensitivity (PDR) for all subgroups is also illustrated in figure 2. Stage-dependent detection sensitivity was found with CA19-9, CEA, and CA125, while miR-25 exhibited high sensitivity across all stages (I-IV).

Figure 3 shows that sex and age did not stratify the patient biomarker levels. No stratification for the biomarkers by cancer location, size, and differentiation was observed, except for the cancer location in CEA. Pancreatic body/tail cancer exhibited significantly higher CEA levels than pancreatic head cancer ( $P=0.01$ ). Similarly, no stratification for biomarkers was observed on the status of diabetes mellitus (DM) and smoking, except for smoking status in CEA where patients with a history of smoking exhibited significantly higher CEA levels than



**Figure 1:** microRNA-25 (miR-25) and carbohydrate antigen 19-9 (CA19-9) exhibited satisfactory sensitivity and their combination further enhanced the performance in pancreatic ductal adenocarcinoma (PDAC) detection. The receiver operating characteristic (ROC) curves for miR-25, CA19-9, carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA125), and combined miR-25/CA19-9 are shown. The area under the curve (AUC) for each ROC is labeled (upper row). miR-25 and CA19-9 showed higher AUC than CEA and CA125, while the combination of miR-25 and CA19-9 exhibited higher AUC than any single marker. The positive detection rate (PDR) for the non-cancer group and PDAC (cancer) group for each biomarker is shown in the lower row. miR-25 and CA19-9 showed significantly higher PDR than CEA and CA125, and a further increase in PDR can be observed with the combination of miR-25 and CA19-9. The normal range for miR-25, CA19-9, CEA, CA125 was 0~3,333 copies/ $\mu$ L, 0~40 U/mL, 0~5.0 ng/mL, 0~35 U/mL, respectively.



**Figure 2:** Carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), and carbohydrate antigen 125 (CA125) exhibited stage-dependent detection sensitivity and microRNA-25 (miR-25) exhibited high sensitivity for early-stage PDAC detection. The blood levels of miR-25, CA19-9, CEA, and CA125 for normal subjects (no evidence of disease; NED), patients with benign pancreatic diseases (benign) and stage I-IV pancreatic ductal adenocarcinoma (PDAC) are shown as scatter plots (upper row). The positive detection rate (PDR) for all patient groups is also shown (lower row). All markers, except for miR-25, exhibited a stage-dependent positive detection rate (PDR). miR-25 and CA19-9 exhibited significantly higher PDR than CEA and CA125 in stages I-III. Scale bars (in red) represent the median with a 95% confidence interval.

those without ( $P=0.03$ ). These observations suggested that miR-25 and CA19-9 are effective markers for PDAC detection and exhibited significantly better performance than CEA and CA125. The combination of miR-25 and CA19-9 further increased the detection sensitivity. No stratification for biomarker levels by sex, age, location, size, differentiation, DM, and smoking status was observed with miR-25 and CA19-9.

*Prediction of PDAC Prognosis by Clinicopathological Factors and Blood Biomarkers*

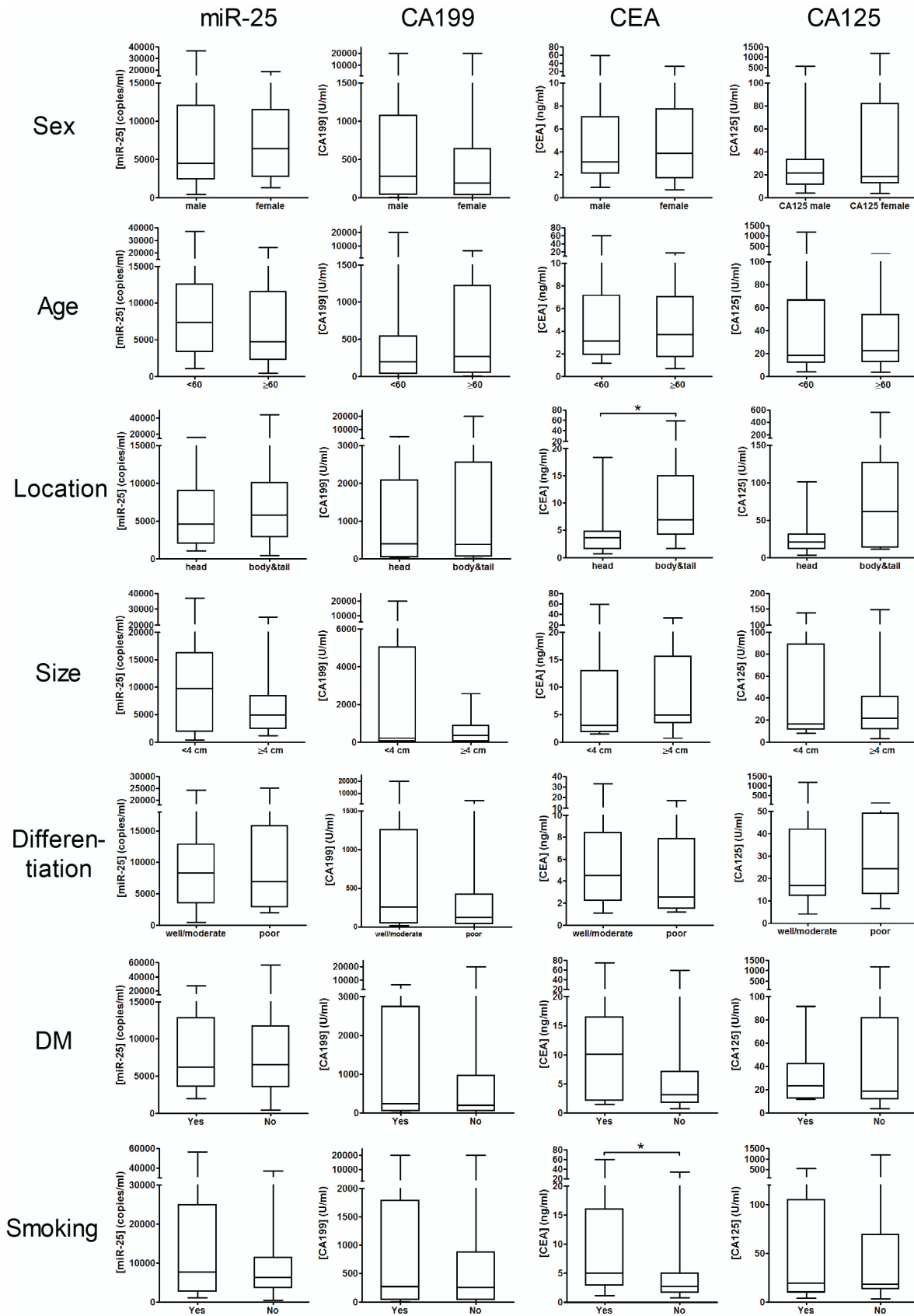
Initially, the influence of clinicopathological factors on PDAC prognosis was examined. All patients were followed up for 2,723 days (median at 240 days). Figure 4 shows the prognostic stratification of PDAC patients based on a series of clinicopathological factors. It was observed that clinical stage ( $P=0.013$ ), T stage ( $P=0.049$ ), N stage ( $P=0.027$ ), M stage ( $P=0.0002$ ), and tumor size (maximal diameter, stratified by median,  $P=0.050$ ), all significantly stratified patient prognosis. In contrast, cancer location (head vs. body/tail), age ( $<60$  vs.  $\geq 60$ ), sex (male vs. female), smoking (with vs. without smoking history), and DM status (with vs. without) did not significantly stratify patient prognosis.

Stratification with miR-25 was subsequently investigated. Patients were divided into two groups based on the detection threshold (3,333 copies/mL, negative vs. positive) (figure 5). No significant stratification was observed over the complete time span ( $P=0.398$ ). Similarly, no significant stratification was observed in year one ( $P=0.089$ ), year two ( $P=0.084$ ), year three ( $P=0.137$ ), year four ( $P=0.216$ ), and year five ( $P=0.363$ ). However,

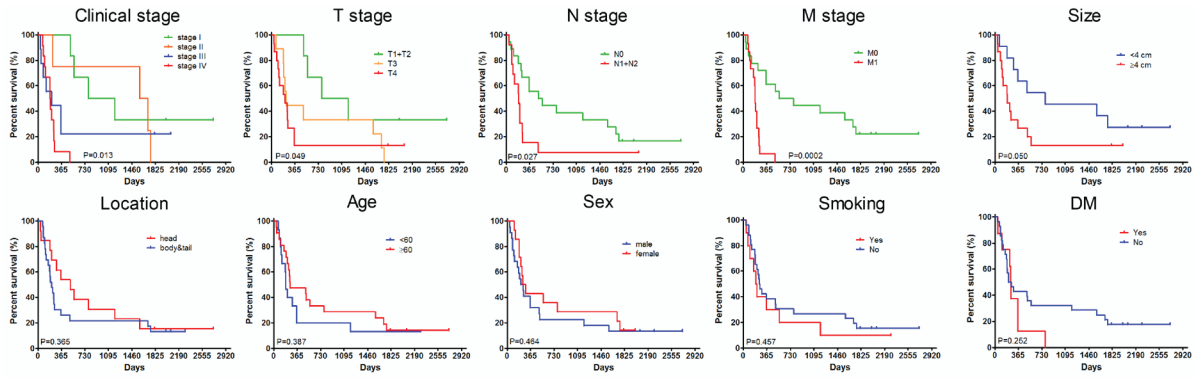
significant differences in miR-25 blood levels between living patients and dead patients at the end of year one ( $P=0.02$ ), year two ( $P=0.02$ ), and year three ( $P=0.05$ ) were observed, with living patients exhibiting significantly lower miR-25 levels than dead patients. When patients were divided into two groups (low or high) based on median miR-25 level (4,989.97 copies/mL) (figure 6), significant stratification was observed between survival rate and low and high miR-25 levels ( $P=0.009$ ). Similarly, stratification was significant at year one ( $P=0.032$ ), year two ( $P=0.015$ ), year three ( $P=0.021$ ), year four ( $P=0.030$ ), and year five ( $P=0.026$ ).

Stratification based on CA19-9, CEA, and CA125 serum biomarker levels was also investigated (figure 7). When patients were divided into two groups based on detection threshold (positive vs. negative), no significant stratification was observed with CA19-9 ( $P=0.152$ ) and CEA ( $P=0.130$ ), while significant stratification was observed with CA125 ( $P=0.027$ ). In contrast, when patients were divided into two groups based on median biomarker level (median at 393.5U/mL for CA19-9, at 4.85 ng/mL for CEA, and at 25.83 U/mL for CA125), CA19-9 ( $P=0.001$ ), CEA ( $P=0.050$ ), and CA125 ( $P=0.007$ ) all exhibited significant stratification.

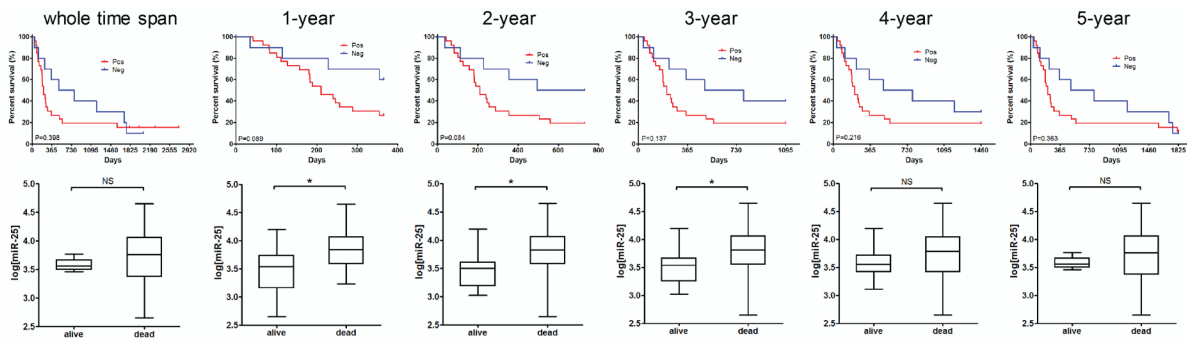
To further assess the risk factors for PDAC, univariate and multivariate analyses were performed. Univariate analysis showed that M stage, clinical stage, miR-25 levels, CA19-9 levels, and CA125 levels (high or low) were significant risk factors for patient prognosis (table 2). Multivariate analysis showed that T stage, CA19-9 levels, and CA125 levels were independent risk factors for PDAC patient prognosis (table 3).



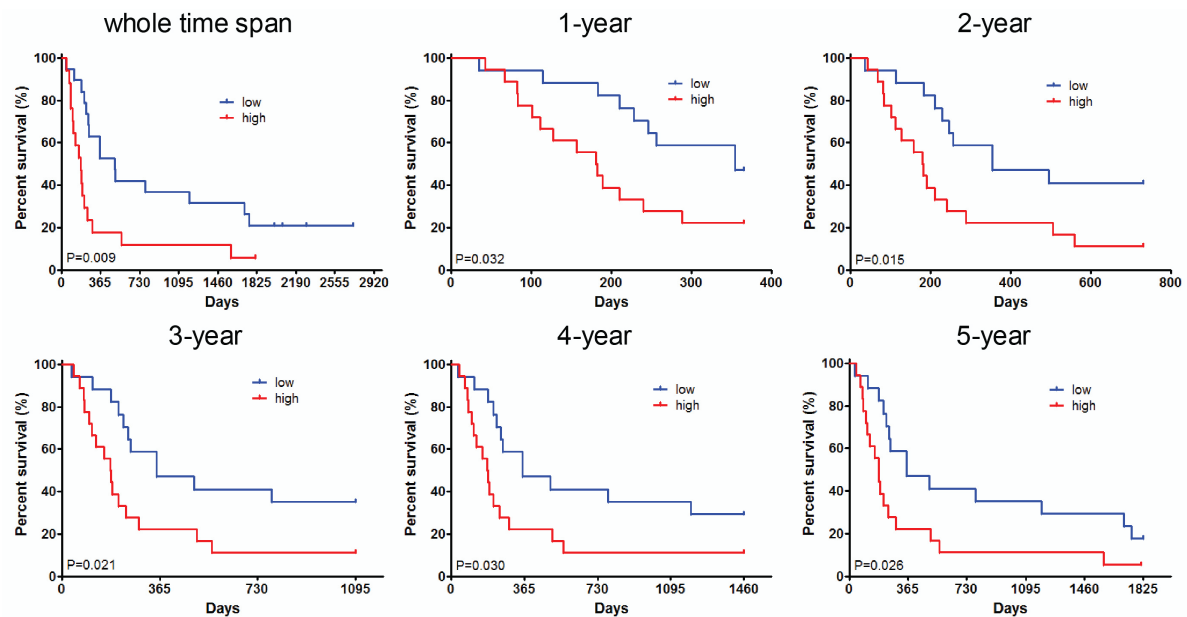
**Figure 3:** Comparison of blood microRNA-25 (miR-25), carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA) and carbohydrate antigen 125 (CA125) levels among different clinicopathological statuses showed that CEA levels exhibited significant differences among different locations and smoking status. The blood levels of the biomarkers at different sex, age, cancer location, cancer size, cancer differentiation, diabetes mellitus, and smoking status were compared. Significant blood level differences were only observed in CEA for cancer location and smoking status. \*P<0.05.



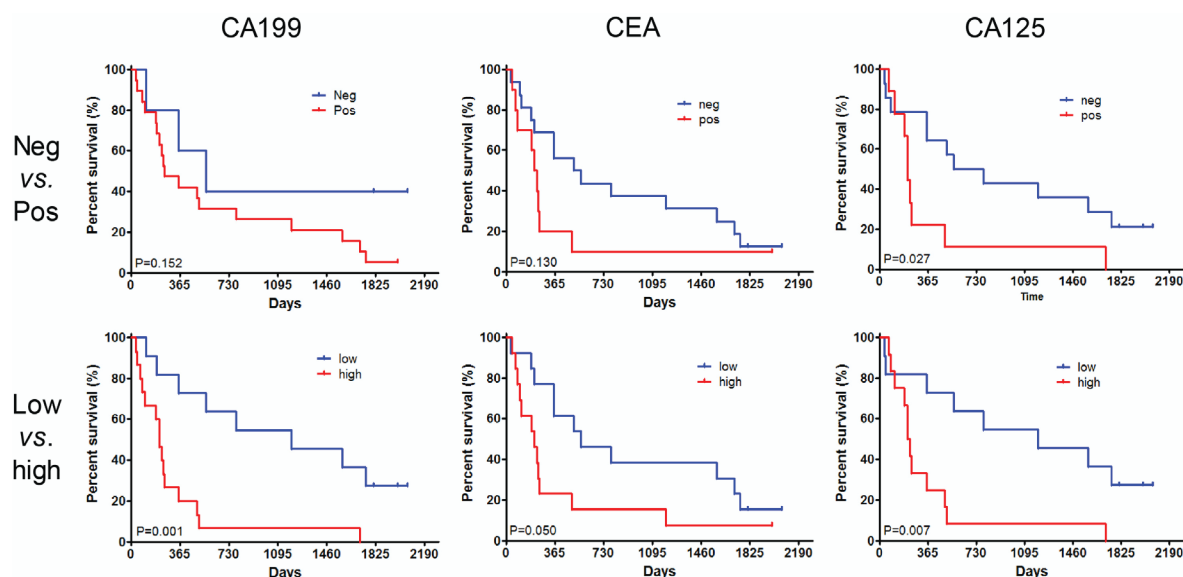
**Figure 4:** Kaplan-Meier survival analysis for pancreatic ductal adenocarcinoma (PDAC) patients with various clinicopathological statuses showed that stage and tumor size significantly stratified patient prognosis. Significant differences in patient survival were observed with different statuses of clinical stage, tumor (T) stage, lymph node (N) stage, metastasis (M) stage, and cancer size (maximal diameter), while no significant difference was observed with different status of cancer location, age, sex, smoking, and diabetes mellitus. P values for each clinicopathological factor were labeled in each panel.



**Figure 5:** Kaplan-Meier analysis for patients with different microRNA 25 (miR-25) status and corresponding blood miR-25 levels showed no significant stratification of patient prognosis by miR-25 detection threshold. Comparison of survival between patients with positive or negative miR-25 levels (threshold at 3,333 copies/ $\mu$ L) is shown for the complete time span, one-year, two-year, three-year, four-year, and five-year time points (upper row). No significant difference is observed in survival between patients with positive and negative miR-25 levels. The miR-25 levels between those alive and dead at each time point are also compared (lower row), and significant differences are observed at one-year, two-year, and three-year time points. NS: Not significant; \* $P < 0.05$ .



**Figure 6:** Kaplan-Meier survival analysis for patients with low or high blood microRNA 25 (miR-25) levels showed significant stratification at all time points. Patients were divided into two groups by miR-25 median (4,989.97 copies/ $\mu$ L). A significant difference in survival can be observed between patients with low and high miR-25 levels for the complete time span ( $P = 0.009$ ), one-year ( $P = 0.032$ ), two-year ( $P = 0.015$ ), three-year ( $P = 0.021$ ), four-year ( $P = 0.030$ ), and five-year ( $P = 0.026$ ) time points.



**Figure 7:** Kaplan-Meier survival analysis based on different thresholds for carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), and carbohydrate antigen 125 (CA125) showed distinct stratification between detection threshold and median. Upper row: comparison of survival (overall survival, OS) between patients with positive and negative biomarker levels. A significant difference was only observed in CA125 (P=0.027), while no significant difference in OS was observed between positive or negative CA199 or CEA groups. Lower row: comparison of OS between patients with high and low biomarker levels, divided by median. A significant difference in survival can be observed for all three markers, as indicated by the P values in each panel. The medians for CA19-9, CEA, and CA125 were 334.1U/mL, 4.85 ng/mL, and 30.16U/mL, respectively.

These analyses suggested that clinical stage (T, N, M), tumor size, levels of miR-25, CEA, CA19-9, and CA125 all significantly stratified patient prognosis, while T stage, CA19-9 levels, and CA125 levels were independent risk factors for PDAC patient prognosis.

### Discussion

Our results showed that miR-25 alone or CA19-9 alone provided sufficient potency for PDAC detection, while the combination of the two further increased detection performance. To our knowledge, this is the first study to show that a combination of miR-25 and CA19-9 significantly increased detection sensitivity in PDAC. More interestingly, it appeared that miR-25 had consistent detection sensitivity across stages I-IV PDAC, which compensated for the low detection sensitivity of stage I PDAC with CA19-9, thus making the detection of early-stage PDAC possible. Early detection of cancer is widely accepted as the critical step in detecting and treating cancer, when it is still curable and in reducing mortality.<sup>16</sup> Therefore, the combined capability of miR-25 and CA19-9 provided a sensitive and suitable solution for early PDAC detection. Furthermore, a correct distinction between stage I PDAC and benign pancreatic diseases using miR-25 and CA19-9 biomarkers enabled the differential diagnosis of benign and malignant pancreatic lesions, which may help design therapeutic strategies for different conditions.

Serum-circulating microRNAs have recently attracted attention as potential biomarkers for cancer detection. Their potential use is based on cancer-specific expression characteristics. In a previous study, 1,063 serum samples from 303 PDAC patients were collected, and their miR-25 expression levels were measured using real-time fluorescent-based qPCR.<sup>17</sup> It was shown that the AUC of miR-25 reached 0.915 (95% CI=0.893-0.937), which was significantly higher than that of serum CEA (AUC=0.725) and CA19-9 (AUC=0.844). These data suggested that serum miR-25 is a potential new biomarker for the detection of PDAC. In another study, TaqMan low-density arrays (TLDA) were used to identify 16 serum microRNAs that were significantly increased in PDAC-DM (cancer emerging from type 2 diabetes mellitus) samples. Six different microRNAs, including miR-25, were selected as biomarkers for PDAC-DM for accurate diagnosis and differentiation of PDAC-DM from healthy controls and non-neoplastic T2DM.<sup>18</sup> These studies suggested that miR-25 alone or in combination with other miRNAs had a good diagnostic ability for pancreatic cancer. Similar to previous studies,<sup>17, 18</sup> we suggest that serum miR-25 combined with CA19-9 has great potential as a novel marker combination for early detection of PDAC, and can be used as a tool for differential diagnosis. Since our study represented retrospective observations from a single center, further validation by prospective studies from multiple centers is recommended.



**Table 2:** Univariate analysis of clinicopathological factors and biomarker levels for patient prognosis

Factors	Categories	HR (95% CI)	P value
T stage	T1	Reference	
	T2	0.577 (0.059-5.601)	0.635
	T3	1.638 (0.205-13.098)	0.642
	T4	2.081 (0.266-16.307)	0.485
N stage	N0	Reference	
	N1	1.748 (0.570-5.361)	0.328
	N2	2.609 (0.320-21.299)	0.371
M stage	M0	Reference	
	M1	2.837 (1.292-6.232)	0.009
Clinical stage	I	Reference	
	II	1.794 (0.400-8.046)	0.445
	III	2.875 (0.737-11.222)	0.128
	IV	5.173 (1.435-18.652)	0.012
Maximal tumor diameter	<4 cm	Reference	
	≥4 cm	1.205 (0.903-1.609)	0.205
Location	Body and tail	Reference	
	Head	0.714 (0.337-1.515)	0.380
Differentiation	Low	Reference	
	Well/moderate	0.256 (0.067-0.972)	0.054
Age	<60 years	Reference	
	≥60 years	0.985 (0.947-1.024)	0.437
Sex	Man	Reference	
	Woman	1.313 (0.636-2.712)	0.462
Smoking	No	Reference	
	Yes	1.375 (0.613-3.084)	0.440
Diabetes mellitus	No	Reference	
	Yes	1.639 (0.709-3.790)	0.248
miR-25	High	Reference	
	Low	0.451 (0.219-0.929)	0.031
CA19-9	High	Reference	
	Low	0.266 (0.106-0.665)	0.005
CEA	High	Reference	
	Low	0.449 (0.193-1.044)	0.063
CA125	High	Reference	
	Low	0.270 (0.099-0.731)	0.010

HR: Hazard ratio; CI: Confidence interval; miR: microRNA; CA: Carbohydrate antigen; CEA: Carcinoembryonic antigen; T: Tumor; N: Lymph node; M: Metastasis; Univariate analysis was used to analyze the data. P<0.05 was considered as a significant difference.

The results showed that the detection performance of miR-25 and CA19-9 was not affected by sex, age, cancer location, cancer size, differentiation, DM status, and smoking. This may facilitate their use in the general population, although the benefits of screening specific high-risk groups were reported.<sup>19-21</sup> High-risk population of pancreatic cancer includes individuals aged 40 years and over with non-specific upper abdominal discomfort, a family history of pancreatic cancer (especially germline mutations), sudden onset diabetes (especially atypical diabetes), chronic pancreatitis, ductal papillary myxoma or familial adenomatous polyposis, patients undergoing distal gastrectomy for benign lesions (especially >20 years post-surgery), long-term smoking and/or heavy drinking history, and long-term exposure

to harmful substances. Current diagnostic strategies include invasive techniques, endoscopic ultrasound screening for selected high-risk populations, and extensive use of radiation-based screening methods.<sup>4, 5, 22, 23</sup> A non-invasive test using blood-based biomarkers could be very helpful in screening asymptomatic patients. In addition, sensitive blood-based biomarkers can be used for early diagnosis or follow-ups of suspected pancreatic cancer cases.<sup>4, 5</sup> One great potential of the miR-25 and CA19-9 combination is the screening of early-stage PDAC, which may help identify affected individuals within the high-risk population during physical examination.<sup>15, 17</sup>

In the present study, we also investigated the prognostic value of a series of clinicopathological factors along with miR-25, CA19-9, CEA, and CA125.

**Table 3:** Multivariate analysis of clinicopathological factors and biomarker levels for patient prognosis

Factors	Categories	HR (95% CI)	P value*
T stage	T1	Reference	
	T2	244.78 (4.41-13597.52)	0.007
	T3	49.26 (1.45-1668.92)	0.030
	T4	124.85 (2.14-7300.16)	0.020
N stage	N0	Reference	
	N1	0.17 (0.01-2.43)	0.192
	N2	9.44 (0.34-261.82)	0.186
M stage	M0	Reference	
	M1	0.37 (0.03-4.71)	0.442
miR-25	High	Reference	
	Low	4.32 (0.86-21.68)	0.076
CA19-9	High	Reference	
	Low	0.06 (0.01-0.49)	0.009
CEA	High	Reference	
	Low	7.30 (0.75-70.96)	0.086
CA125	High	Reference	
	Low	0.01 (0.00-0.19)	0.002

HR: Hazard ratio; CI: Confidence interval; T: Tumor; N: Lymph node; M: Metastasis; CA: Carbohydrate antigen; CEA: Carcinoembryonic antigen; Multivariate analysis was used to analyze the data. \*P<0.05 was considered as a significant difference.

We showed that the T stage (including cancer size), N stage, M stage, and their combination (clinical stage) all stratified patient survival. In contrast, cancer location, sex, age, smoking, and DM status did not significantly stratify patient survival. In line with our results, previous studies have reported the effect of cancer stages on patient prognosis.<sup>22, 23</sup> Some studies have reported a significant association of patient's personal habits (e.g., smoking) and DM with patient prognosis.<sup>24, 25</sup> In contrast, we did not observe significant stratification based on smoking and DM status, which could be due to the small sample size and the effect from other factors. Therefore, a complete collection of patient prognostic information and control of population heterogeneity is important for risk factor analysis.

Although the trend between negative miR-25 and better survival was clear in our analysis, no statistically significant stratification was observed at either the complete time span or year 1-5 time points. The results showed nearly significant P values at one-year and two-year survival analysis, but higher P values when the duration was extended, suggesting that miR-25 levels may be more sensitive in predicting survival status within two years rather than longer than three years. This was also reflected in the dichotomized analysis of miR-25 levels in dead patients and living patients at each time point, in which significant difference in miR-25 levels was only observed at years one, two, and three. It was interesting to find significant stratification of survival by miR-25 when dividing patients based on median level, whether at the

complete time span or at years 1-5 time points. Patients with lower miR-25 levels exhibited significantly better survival than those with higher levels. This observation suggested that the threshold for the patient grouping was important for prognosis prediction, since the diagnostic threshold for interpreting negative or positive results may not be appropriate as the prognostic threshold. Therefore, specific thresholds should be used for diagnosis and prognosis purposes. This observation was further confirmed by investigating PDAC prognosis with CA19-9, CEA, and CA125. Both CA19-9 and CEA did not stratify patient survival when a diagnostic threshold was used, while both exhibited significant stratification when the threshold was set at median level. In contrast, grouping by both diagnostic and prognostic thresholds of CA125 exhibited significant stratification, while grouping based on the prognostic threshold showed more significant difference in survival. These observations indicated that miR-25 and conventional protein biomarkers are all capable of stratifying patient long-term prognosis, provided that an appropriate threshold is chosen.

Based on survival analysis, we identified risk factors that may influence the patient's prognosis. Univariate analysis showed that distant metastasis (M stage and clinical stage IV) and three of the four blood markers were significant risk factors for prognosis, while multivariate analysis showed T stage, CA19-9, and CA125 as independent prognostic risk factors. T stage is a widely known independent risk factor for many cancer types, which is in close correlation with tumor size.<sup>26-29</sup> Not surprisingly, the T stage

was also an independent risk factor in Chinese PDAC patients. In terms of blood markers, it appeared that CA19-9 or CA125 levels can independently predict patient prognosis and should be determined prior to any therapy to provide diagnostic and long-term prognostic information. Previous studies on stratification of PDAC patient prognosis by CA19-9 and CA125 reported similar findings to our study.<sup>26-29</sup>

The main limitation of our study was the low sample size, especially for survival analysis. Large cohort studies should be conducted to confirm our findings. In addition, a prospective study should be performed to validate the performance of miR-25 and its combination with CA19-9 for the early detection of PDAC. It is recommended to develop a prognostic model for individual patients to quantitatively assess patient survival.

### Conclusion

miR-25 alone or CA19-9 alone provided sufficient potency for PDAC detection, while their combination significantly increased the detection sensitivity in PDAC patients. More interestingly, it appeared that miR-25 had consistent detection sensitivity across stages I-IV PDAC, which compensated for the low detection sensitivity of stage I PDAC with CA19-9, thus making the detection of early-stage PDAC possible.

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### Authors' Contribution

Y.Z, Q.Z, L.S: Study design. All authors collected the samples and clinical information. All authors performed the data analysis and wrote the paper. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Conflict of Interest:** None declared.

### References

- 1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin.* 2019;69:7-34. doi: 10.3322/caac.21551. PubMed PMID: 30620402.
- 2 Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res.* 2014;74:2913-21. doi: 10.1158/0008-5472.CAN-14-0155. PubMed PMID: 24840647.
- 3 Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer statistics in China, 2015. *CA Cancer J Clin.* 2016;66:115-32. doi: 10.3322/caac.21338. PubMed PMID: 26808342.
- 4 Ge L, Pan B, Song F, Ma J, Zeraatkar D, Zhou J, et al. Comparing the diagnostic accuracy of five common tumour biomarkers and CA19-9 for pancreatic cancer: a protocol for a network meta-analysis of diagnostic test accuracy. *BMJ Open.* 2017;7:e018175. doi: 10.1136/bmjopen-2017-018175. PubMed PMID: 29282264; PubMed Central PMCID: PMC5770961.
- 5 Nolen BM, Brand RE, Prosser D, Velikokhatnaya L, Allen PJ, Zeh HJ, et al. Prediagnostic serum biomarkers as early detection tools for pancreatic cancer in a large prospective cohort study. *PLoS One.* 2014;9:e94928. doi: 10.1371/journal.pone.0094928. PubMed PMID: 24747429; PubMed Central PMCID: PMC3991628.
- 6 Rahat B, Ali T, Sapehia D, Mahajan A, Kaur J. Circulating Cell-Free Nucleic Acids as Epigenetic Biomarkers in Precision Medicine. *Front Genet.* 2020;11:844. doi: 10.3389/fgene.2020.00844. PubMed PMID: 32849827; PubMed Central PMCID: PMC7431953.
- 7 Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends Mol Med.* 2014;20:460-9. doi: 10.1016/j.molmed.2014.06.005. PubMed PMID: 25027972.
- 8 Liu R, Chen X, Du Y, Yao W, Shen L, Wang C, et al. Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. *Clin Chem.* 2012;58:610-8. doi: 10.1373/clinchem.2011.172767. PubMed PMID: 22194634.
- 9 Zhang H, Zuo Z, Lu X, Wang L, Wang H, Zhu Z. MiR-25 regulates apoptosis by targeting

- Bim in human ovarian cancer. *Oncol Rep.* 2012;27:594-8. doi: 10.3892/or.2011.1530. PubMed PMID: 22076535.
- 10 Wu T, Hu H, Zhang T, Jiang L, Li X, Liu S, et al. miR-25 Promotes Cell Proliferation, Migration, and Invasion of Non-Small-Cell Lung Cancer by Targeting the LATS2/YAP Signaling Pathway. *Oxid Med Cell Longev.* 2019;2019:9719723. doi: 10.1155/2019/9719723. PubMed PMID: 31316723; PubMed Central PMCID: PMC6604298.
  - 11 Wang Z, Wang N, Liu P, Chen Q, Situ H, Xie T, et al. MicroRNA-25 regulates chemoresistance-associated autophagy in breast cancer cells, a process modulated by the natural autophagy inducer isoliquiritigenin. *Oncotarget.* 2014;5:7013-26. doi: 10.18632/oncotarget.2192. PubMed PMID: 25026296; PubMed Central PMCID: PMC4196180 to disclose.
  - 12 ZiaSarabi P, Sorayayi S, Hesari A, Ghasemi F. Circulating microRNA-133, microRNA-17 and microRNA-25 in serum and its potential diagnostic value in gastric cancer. *J Cell Biochem.* 2019;120:12376-81. doi: 10.1002/jcb.28503. PubMed PMID: 30861177.
  - 13 Zhang J, Bai R, Li M, Ye H, Wu C, Wang C, et al. Excessive miR-25-3p maturation via N(6)-methyladenosine stimulated by cigarette smoke promotes pancreatic cancer progression. *Nat Commun.* 2019;10:1858. doi: 10.1038/s41467-019-09712-x. PubMed PMID: 31015415; PubMed Central PMCID: PMC6478927.
  - 14 Abel Y, Rederstorff M. Stem-Loop qRT-PCR-Based Quantification of miRNAs. *Methods Mol Biol.* 2021;2300:59-64. doi: 10.1007/978-1-0716-1386-3\_6. PubMed PMID: 33792871.
  - 15 Yu Y, Tong Y, Zhong A, Wang Y, Lu R, Guo L. Identification of Serum microRNA-25 as a novel biomarker for pancreatic cancer. *Medicine (Baltimore).* 2020;99:e23863. doi: 10.1097/MD.00000000000023863. PubMed PMID: 33350781; PubMed Central PMCID: PMC7769376.
  - 16 Global Burden of Disease Cancer C, Fitzmaurice C, Allen C, Barber RM, Barreagar L, Bhutta ZA, et al. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study. *JAMA Oncol.* 2017;3:524-48. doi: 10.1001/jamaoncol.2016.5688. PubMed PMID: 27918777; PubMed Central PMCID: PMC6103527.
  - 17 Deng T, Yuan Y, Zhang C, Zhang C, Yao W, Wang C, et al. Identification of Circulating MiR-25 as a Potential Biomarker for Pancreatic Cancer Diagnosis. *Cell Physiol Biochem.* 2016;39:1716-22. doi: 10.1159/000447872. PubMed PMID: 27639768.
  - 18 Dai X, Pang W, Zhou Y, Yao W, Xia L, Wang C, et al. Altered profile of serum microRNAs in pancreatic cancer-associated new-onset diabetes mellitus. *J Diabetes.* 2016;8:422-33. doi: 10.1111/1753-0407.12313. PubMed PMID: 25991015.
  - 19 Rustgi AK. Familial pancreatic cancer: genetic advances. *Genes Dev.* 2014;28:1-7. doi: 10.1101/gad.228452.113. PubMed PMID: 24395243; PubMed Central PMCID: PMC3894408.
  - 20 Humphris JL, Johns AL, Simpson SH, Cowley MJ, Pajic M, Chang DK, et al. Clinical and pathologic features of familial pancreatic cancer. *Cancer.* 2014;120:3669-75. doi: 10.1002/cncr.28863. PubMed PMID: 25313458.
  - 21 Petersen GM. Familial Pancreatic Adenocarcinoma. *Hematol Oncol Clin North Am.* 2015;29:641-53. doi: 10.1016/j.hoc.2015.04.007. PubMed PMID: 26226902; PubMed Central PMCID: PMC4522044.
  - 22 McGuigan A, Kelly P, Turkington RC, Jones C, Coleman HG, McCain RS. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J Gastroenterol.* 2018;24:4846-61. doi: 10.3748/wjg.v24.i43.4846. PubMed PMID: 30487695; PubMed Central PMCID: PMC6250924.
  - 23 Ma ZY, Gong YF, Zhuang HK, Zhou ZX, Huang SZ, Zou YP, et al. Pancreatic neuroendocrine tumors: A review of serum biomarkers, staging, and management. *World J Gastroenterol.* 2020;26:2305-22. doi: 10.3748/wjg.v26.i19.2305. PubMed PMID: 32476795; PubMed Central PMCID: PMC7243647.
  - 24 Weissman S, Takakura K, Eibl G, Pandol SJ, Saruta M. The Diverse Involvement of Cigarette Smoking in Pancreatic Cancer Development and Prognosis. *Pancreas.* 2020;49:612-20. doi: 10.1097/MPA.0000000000001550. PubMed PMID: 32433397; PubMed Central PMCID: PMC7249487.
  - 25 Khadka R, Tian W, Hao X, Koirala R. Risk factor, early diagnosis and overall survival on outcome of association between pancreatic cancer and diabetes mellitus: Changes and advances, a review. *Int J Surg.* 2018;52:342-6. doi: 10.1016/j.ijsu.2018.02.058. PubMed

- PMID: 29535016.
- 26 Chang JC, Kundranda M. Novel Diagnostic and Predictive Biomarkers in Pancreatic Adenocarcinoma. *Int J Mol Sci.* 2017;18. doi: 10.3390/ijms18030667. PubMed PMID: 28335509; PubMed Central PMCID: PMC5372679.
- 27 Yang C, Cheng H, Luo G, Lu Y, Guo M, Jin K, et al. The metastasis status and tumor burden-associated CA125 level combined with the CD4/CD8 ratio predicts the prognosis of patients with advanced pancreatic cancer: A new scoring system. *Eur J Surg Oncol.* 2017;43:2112-8. doi: 10.1016/j.ejso.2017.07.010. PubMed PMID: 28802662.
- 28 Gu YL, Lan C, Pei H, Yang SN, Liu YF, Xiao LL. Applicative Value of Serum CA19-9, CEA, CA125 and CA242 in Diagnosis and Prognosis for Patients with Pancreatic Cancer Treated by Concurrent Chemoradiotherapy. *Asian Pac J Cancer Prev.* 2015;16:6569-73. doi: 10.7314/apjcp.2015.16.15.6569. PubMed PMID: 26434876.
- 29 Chen Y, Gao SG, Chen JM, Wang GP, Wang ZF, Zhou B, et al. Serum CA242, CA199, CA125, CEA, and TSGF are Biomarkers for the Efficacy and Prognosis of Cryoablation in Pancreatic Cancer Patients. *Cell Biochem Biophys.* 2015;71:1287-91. doi: 10.1007/s12013-014-0345-2. PubMed PMID: 25486903.