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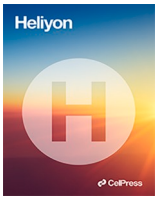

Research article

Combined ultrasound endoscopy-guided fine-needle aspiration with DNA methylation of SHOX2 and RASSF1A genes to enhance the auxiliary diagnostic precision of pancreatic cancer

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ABSTRACT

The purpose of this study was to assess the influence and the clinical effectiveness of the short stature homeobox 2 (SHOX2) and ras association domain family 1A (RASSF1A) genes by tissue sampling through ultrasound endoscopy-guided fine-needle aspiration (EUS-FNA) as auxiliary diagnostic tools for pancreatic cancer (PC). Methylation markers were detected in 96 patients using real-time fluorescence quantitative PCR (qPCR), and the performance of this diagnostic assay was compared with CA19-9, CEA, and puncture fluid-based exfoliative cytology using receiver operating characteristic curve (ROC) analysis. The PC group exhibited higher methylation rates for SHOX2, RASSF1A, and the combined assay of both genes compared to the control group (95.7 % vs. 54.0 %, 78.3 % vs. 36.0 %, and 73.9 % vs. 16.0 %, $P < 0.05$). The areas under the ROC curve (AUC) for CA19-9, CEA, liquid-based exfoliative cytology, SHOX2, RASSF1A, the combination of SHOX2 and RASSF1A, the combination assay with CEA, CA19-9, and liquid-based exfoliative cytology were 0.827, 0.692, 0.767, 0.770, 0.732, 0.870, 0.870, 0.933, and 0.900, respectively. Therefore, the methylation assay based on the combined SHOX2 and RASSF1A genes in EUS-FNA puncture fluid is more effective than using a single gene, liquid-based exfoliative cytology, or intravenous tumor markers for diagnosing PC. Combining the conventional marker CA19-9 enhances the diagnostic value, making it a promising approach to complement histology and cytology.

1. Introduction

Pancreatic cancer (PC) is an extremely aggressive disease with a low survival rate. Localized cases of PC only account for 9.7 % of all cases and contribute to 5 % of cancer-related deaths. The majority of tumors have already spread by the time of initial diagnosis [1].

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Due to the molecular characteristics of PC, early detection is challenging, and there is a lack of internationally standardized protocols for examining suspicious pancreatic masses, resulting in a high fatality rate. The limitations of current diagnostic procedures and the absence of effective biomarkers make it difficult to identify and diagnose PC in its early stages [2]. Early and accurate diagnosis is crucial for improving PC prognoses, and endoscopic ultrasound (EUS) is an important procedure for further examining pancreatic masses due to its high diagnostic accuracy and ability to collect tissue samples through fine needle aspiration (FNA). FNA is particularly useful for lesions smaller than 2 cm or when other methods fail to identify the mass and obtain a confirmatory biopsy in cases of suspected PC [3]. It can confirm PC diagnoses or further characterize solid focal lesions [4]. Currently, the six common tumor biomarkers for PC include CA19-9, CA242, CEA, CA125, microRNAs, and K-ras gene mutations [5]. However, studies have shown that the frequently used clinical biomarkers for PC, such as CA19-9, CEA, and CA125, lack accuracy in early PC detection [6–8]. CA19-9, for example, is expressed and excreted in various pancreatic, hepatobiliary, and other malignancies, making it less specific for diagnosing PC. Additionally, biliary tract infection, inflammation, or obstruction can lead to false positive results and low positive predictive values [9]. It has been discovered that simultaneous detection of serum CA19-9, CEA, CA125, and CA242 has greater sensitivity and specificity (90.4 % and 93.8 %, respectively) compared to using any single index alone. Some studies have paired CA19-9 with other serum biomarkers like CEA, CA125, and CA242, as well as new biomarkers, to detect tumors [10]. In recent years, serum tumor markers, cytology, and genomics have been combined for early PC identification [11]. Therefore, exploring new tumor markers and combining them with imaging techniques may be the preferred approach for PC screening. This study defines a positive co-test as the positive methylation of both SHOX2 and RASSF1A, emphasizing the importance of investigating new indicators and conducting combination testing for PC detection.

In order to achieve early detection and identification of cancers, an increasing number of methylation biomarkers are being identified. DNA methylation, a significant epigenetic modification that primarily occurs in CpG islands and plays a role in tumor formation during the early stages of cancer by inhibiting gene transcription, is involved in tumor development [12–14]. SHOX2 is an oncogene that regulates cell growth, apoptosis, and induces the epithelial-mesenchymal transition (EMT) [15]. Meanwhile, RASSF1A is a tumor suppressor gene (TSG) that controls the cell cycle, apoptosis, migration, and adhesion-related tumorigenesis. It is also an epigenetic potential marker for several cancer types, with methylation of RASSF1A playing a significant role [16]. Hypermethylation of SHOX2 and RASSF1A has been observed, with DNA methyltransferase 3B (DNMT3B) being the responsible agent [17]. The methylation tests for both SHOX2 and RASSF1A are essential for highly sensitive and specific screening and monitoring of tumors. These genes have also been found to support tumorigenesis and progression, and they serve as regulators or effectors of various cancer signaling pathways [18]. Based on these findings, we can confidently suggest that SHOX2 and RASSF1A methylation can be useful diagnostic tools for PC.

Despite extensive prospective screening trials demonstrating that multi-genomic testing can improve sensitivity and efficiency in cancer diagnostics, and help overcome challenges related to low tissue volume and limited puncture specimens, there has been limited attention given to the combination of SHOX2 and RASSF1A methylation detection in gastrointestinal tumors [19]. Further investigation is necessary to determine the prevalence of SHOX2 and RASSF1A methylation positivity in PC and its potential to enhance the pathological diagnostic accuracy of FNA specimens.

2. Materials and methods

Patients. This case-control research covers EUS-FNA patients with occupying lesions in Affiliated Hospital of Nantong University from July 2021 to March 2023. The EPK-i5000 and EG-3270UK linear-array echoendoscopes from PENTAX Medical were utilised for EUS-FNA under general anaesthesia. Patients with unequivocal pathology diagnoses, complete clinical data, detailed FNA records, and traceable follow-up were included. Patients with severe cardiac, cerebral, or pulmonary disease who could not tolerate endoscopic manipulation; pregnant women or mental illness patients who could not cooperate; patients on anticoagulants or with severe bleeding tendencies that could not be corrected; and patients with unclear clinical outcomes and incomplete follow-up data were excluded from this study. All patients obtained informed consent before enrolling. The Affiliated Hospital of Nantong University's ethical committee approved the current study, which followed the Helsinki Declaration (approval number 2019K055).

Endoscopic procedures. Boston Scientific Corp.'s 22G or 25G Expect™ EUS-FNA needles were used on each subject. An endoscopist under intravenous propofol anaesthesia performed the EUS-FNA after an anesthesiologist without cytopathology experience assessed it. A cytopathologist was not present for the puncture. Avoiding bile, pancreatic, and blood arteries, live ultrasonography guides the penetration spot. A syringe with negative pressure is used to withdraw the puncture needle core from the target lesion, then elevate and insert the needle more than 20 times. The endoscopist uses needle cores, regulates negative pressure, and fans the puncture according on the lesion's features, specimen retrieval, and personal expertise to establish the appropriate puncture technique and quantity. After each puncture, negative pressure is released, the needle is removed, the material is placed in a culture plate for histology, the residual cell fragments are sent for liquid-based cytology, and the puncture fluid is tested for DNA methylation.

Methylation detection. The commercial SHOX2 and RASSF1A methylation kits were purchased from Shanghai Turbine Life Technologies Ltd. According to the instruction of kits, after sulphite modification of both genes, ABI 7500 Realtime-PCR apparatus was used for the PCR amplification. The final standard curve was used to quantify the unknown template to derive the CT value, which is the amount of cycles of amplification required for the amplified product's fluorescence signal to reach a predetermined threshold. The PCR reaction system was supplemented with fluorescent groups, the PCR process was monitored by fluorescence signal acquisition, and the final standard curve was used to quantify the unknown template. $\triangle$Ct is equal to Ct_U - Ct_M, where U stands for methylation and M for non-methylation. $\Delta Ct \leq 9$ for SHOX2 is positive and $\Delta Ct \leq 12$ for RASSF1A is positive, with a smaller ΔCt indicating a higher methylation level.

Final diagnosis. The final diagnosis was reached in light of the patient's preoperative laboratory results, imaging findings, and clinical presentation. i) Pathological manifestations after FNA; ii) FNA-positive malignant tumor without surgical or pharmaceutical treatment and with a clinical course that supports the diagnosis of FNA; iii) Benign lesions with negative FNA or aspiration pathology and no worsening or spontaneous lesions on imaging after at least 6 months of follow-up monitoring remission. A puncture that reveals cancerous or tumor cells is considered FNA-positive, otherwise it is considered FNA-negative.

Statistical methods. The statistical analysis was done with IBM Corp.'s SPSS 26.0 application, and the continuous variables' normality test showed no normal distribution. For descriptive statistics, median (quartiles) and Mann-Whitney U tests were used. For categorical data, frequency or percentage was used, and the chi-square test was used to compare observation and control groups. Each assay's sensitivity, specificity, PPV, and NPV were computed, and Graphpad was used to compare groups. Prism 9 was utilised to plot ROC curves for SHOX2 and RASSF1A methylation, CEA, CA19-9, and liquid-based exfoliative cytology. The Jorden index calculated threshold values and the AUC assessed the diagnostic efficacy of each index, particularly the combination test. Univariate and multivariate logistic regression analyses were performed to confirm the impact of relevant risk variables. A P value of less than 0.05 was considered statistically significant.

3. Results

Patient and lesion characteristics. 96 patients with occupying lesions who underwent EUS-FNA were included based on the histopathological gold standard. Among them, 46 cases were categorized into the PC group while the remaining 50 cases were assigned to the comparison group. The comparison group consisted of 4 cases of stomach cancer, 2 cases of esophageal cancer, 5 cases of PC, 2 cases of rectum cancer, 2 cases of sigmoid colon cancer, 2 cases of gastrointestinal mesenchymal tumors, 6 cases of mediastinal lymph node masses associated with lung cancer, 6 cases of follicular lymphoma, 1 case of duodenal smooth muscle sarcoma, 1 case of hepatogastric interstitial space occupancy due to malignant ovarian tumor, and 1 case of cysto-rectal space swelling caused by malignant bladder tumor. A total of 23 cases of benign lesions were diagnosed through histopathology and clinical follow-up, including 6 cases of inflammatory mediastinal lymph node enlargement, 7 cases of benign pancreatic occupations, 4 cases of autoimmune pancreatitis, 3 cases of abdominopelvic masses, as well as 1 case each of intragastric ectopic pancreas, esophageal mass, and biliary ductitis. Table 1 displays the characteristics of the patients and lesions in both groups. Apart from age, there were no statistically significant differences in gender, age, number of punctures, or lesion size between the two groups.

Differences in positive rates of SHOX2 and RASSF1A methylation between PC and control groups. SHOX2 and RASSF1A's methylation rates in the PC group were 95.7 % and 78.3 %, respectively, and the positive rates of double and single gene methylation of SHOX2 and RASSF1A, respectively, were 73.9 % and 100.0 % ($P < 0.05$), which were statistically different from those of the control group (Table 2 and Fig. 1A and B). Moreover, we analyzed methylated expression of SHOX2 and RASSF1A genes and a clustered heatmap was created per sample. As presented, the two genes showed differential significance in the groups (Fig. 2). These results suggested that SHOX2 and RASSF1A gene methylation are involved in PC progression.

Correlation between SHOX2 and RASSF1A methylation levels and CA19-9. Our study investigated the correlation between the methylation levels and the traditional PC marker CA19-9 in order to verify the diagnostic performance of SHOX2 and RASSF1A. The results showed that the ΔCt values of both were negatively correlated with CA19-9 which indicated that the methylation level was positively correlated with CA19-9. In comparison, the correlation coefficient between the ΔCt values of SHOX2 and CA19-9 was -0.260 ($P = 0.011$), while that between RASSF1A and CA19-9 was -0.428 ($P < 0.001$) (Fig. 3A and B). We also examined the correlation between the two genes and cancer staging of TNM using Spearman correlation analysis (Fig. 4). There was no significant correlation between SHOX2 and RASSF1A ($P = 0.089$), however, there was a significant negative correlation between the ΔCt values of the two genes and TNM. In other word, the methylation level of the two genes was positively correlated with TNM (see Fig. 5).

Comparison of liquid-based cytology, tumor markers and methylation of SHOX2 and RASSF1A between the PC and control group. The findings of the investigation, which examined the clinical utility of serum tumor markers, puncture fluid-based cytology, and combination SHOX2 and RASSF1A methylation assays, are displayed in Table 3. When determining the diagnosis of PC, the positive rates for the serum tumor markers CEA and CA19-9 were 47.8 %, 78.3 %, and CA724 were 30.4 % ($P = 0.002$, 0.000, 0.709), respectively. Therefore, the difference between the two groups in the detection of CA724 was not statistically significant for the diagnosis of PC. By

Table 1
Comparison of patient and lesion characteristics between the two groups.

Characteristic	Pancreatic cancer group (n = 46)	Control group (n = 50)		P-value
		Other malignant tumors	Benign lesions	
Sex, n				1.000[a]
Male	23	15	10	
Female	23	12	13	
Median age (range), years	68 (64–74)	67 (58–73)	65 (50–68)	0.012[b]
Median number of punctures (range)	4 (3–4)	3 (3–4)	4 (3–4)	0.930[b]
Median lesion size (range), mm	34 (30–35)	35 (31–51)	30 (23–36)	0.549[b]

$P < 0.05$ was considered to indicate a statistically significant difference.
[a] $\chi 2$ test.
[b] Mann-Whitney *U* test.

Table 2
Comparison of positive methylation rate between the two groups.

Groups	SHOX2	RASSF1A	Double positive	Single positive
Pancreatic cancer group (n = 46)	44 (95.7 %)	36 (78.3 %)	34 (73.9 %)	46 (100.0 %)
Control group (n = 50)	27 (54.0 %)	18 (36.0 %)	8 (16.0 %)	37 (74.0 %)
Malignant lesions (n = 27)	18 (66.7 %)	7 (25.9 %)	3 (11.1 %)	22 (81.5 %)
Benign lesions (n = 23)	9 (39.1 %)	11 (47.8 %)	5 (21.7 %)	15 (65.2 %)
χ2	21.581	17.387	32.652	13.833
P-value	0.000	0.000	0.000	0.000

P < 0.05 was considered to indicate a statistically significant difference.

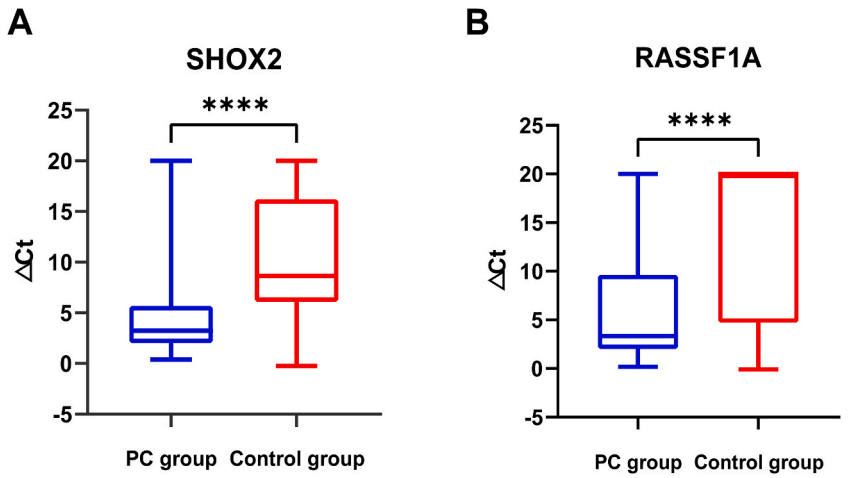

Fig. 1. Comparison of SHOX2 and RASSF1A between the two groups. (A) Differences in △Ct values of SHOX2 between the PC group and control group, P < 0.05. (B) Differences in △Ct values of RASSF1A between the PC group and control group, P < 0.05.

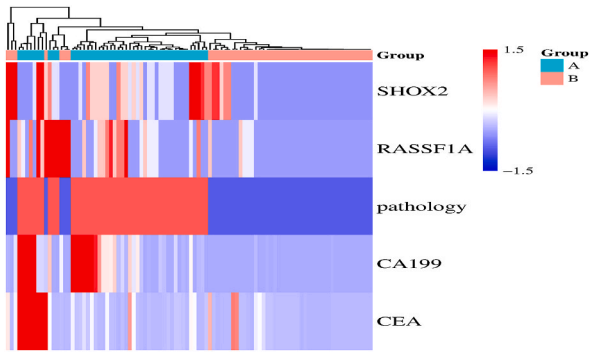

Fig. 2. Heatmap of the DNA methylation levels of SHOX2, RASSF1A and pathology, CA19-9, CEA, where the colors from red to blue represented alterations from high to low. A is the PC group. B is the control group.

using fluid-based cytology on puncture fluid, 93.5 % of cases of PC were detected (P < 0.05), which is statistically significant. In comparison to liquid-based cytology and the CA19-9 test, the combination SHOX2 and RASSF1A double gene methylation test had a lower positive rate for PC at 73.9 % (P = 0.000). Additionally, there are 2 cases which were clinically pancreatic cancer the positive in the combined assay and the negative of pathologic finding using tissue. It's not difficult to find the combined assay can be an important complementary aid in the diagnosis of PC when the pathologic finding using tissue is negative.

Comparative analysis of diagnostic value of serum tumor markers, liquid-based cytology, SHOX2 and RASSF1A methylation assays for pc diagnosis. ROC curves were plotted according to SHOX2, RASSF1A, serum tumor markers CEA, CA19-9, liquid-based exfoliative cytology, combined SHOX2 and RASSF1A, combined assay combined with CEA, combined assay combined with CA19-9 and combined

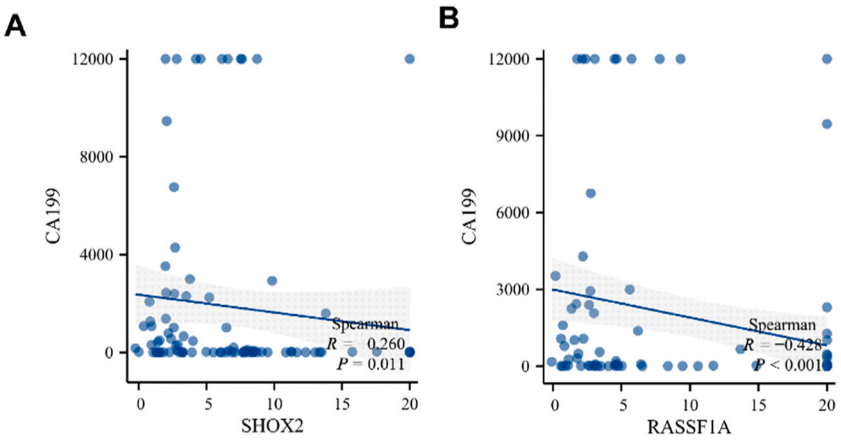

Fig. 3. Correlation between the ΔCt values of SHOX2 and RASSF1A and CA19-9.(A) The ΔCt value of SHOX2 was negatively correlated with CA19-9 with a correlation coefficient of −0.260 (P = 0.011). (B) The ΔCt value of RASSF1A was negatively correlated with CA19-9 with a correlation coefficient of −0.428 (P < 0.001).

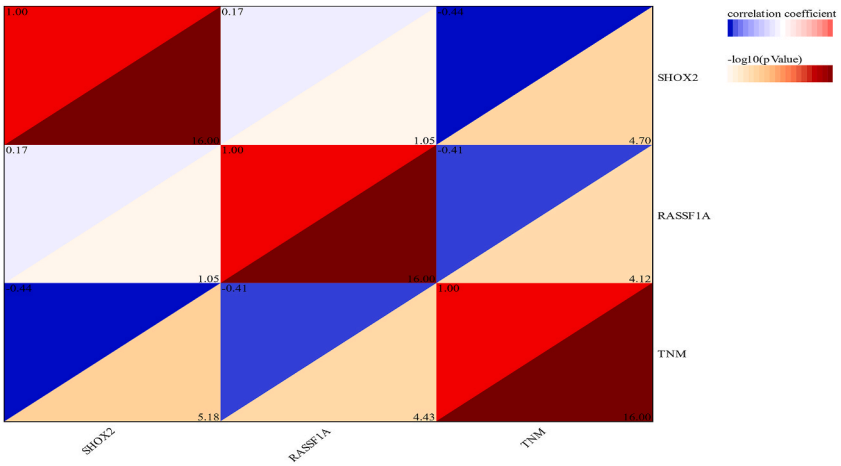

Fig. 4. Correlation between the two genes and cancer staging of TNM.

Table 3
Comparison of biomarkers, cytology and methylation between the two groups.

Groups	Double positive	CEA	CA19-9	CA724	cytology
Pancreatic cancer group (n = 46)	34 (73.9 %)	22 (47.8 %)	36 (78.3 %)	14 (30.4 %)	43 (93.5 %)
Control group (n = 50)	8 (16.0 %)	9 (18.0 %)	8 (16.0 %)	17 (34.0 %)	20 (40.0 %)
Malignant lesions (n = 27)	3 (11.1 %)	7 (25.9 %)	5 (18.5 %)	9 (33.3 %)	16 (59.3 %)
Benign lesions (n = 23)	5 (21.7 %)	2 (8.7 %)	3 (13.0 %)	8 (34.8 %)	4 (17.4 %)
χ2	32.652	9.748	37.409	0.139	21.581
P-value	0.000	0.002	0.000	0.709	0.000

P < 0.05 was considered to indicate a statistically significant difference.

assay combined with liquid-based exfoliative cytology (Fig. 5A–I), and the AUC and Jorden's index were calculated. As shown in Table 4, the combination test with CEA or liquid-based exfoliative cytology achieved the highest specificity of 0.957 while the highest sensitivity of 0.940 for the SHOX2 methylation assay. the AUC value calculated from the ROC curve showed that the combined assay combined with CA19-9 had the largest AUC value,which was 0.933 and the largest Jorden's index of 0.730.

The cut-off value for SHOX2 and RASSF1A. As demonstrated in Fig. 6A and B, the cut-off value for the DNA methylation of SHOX2

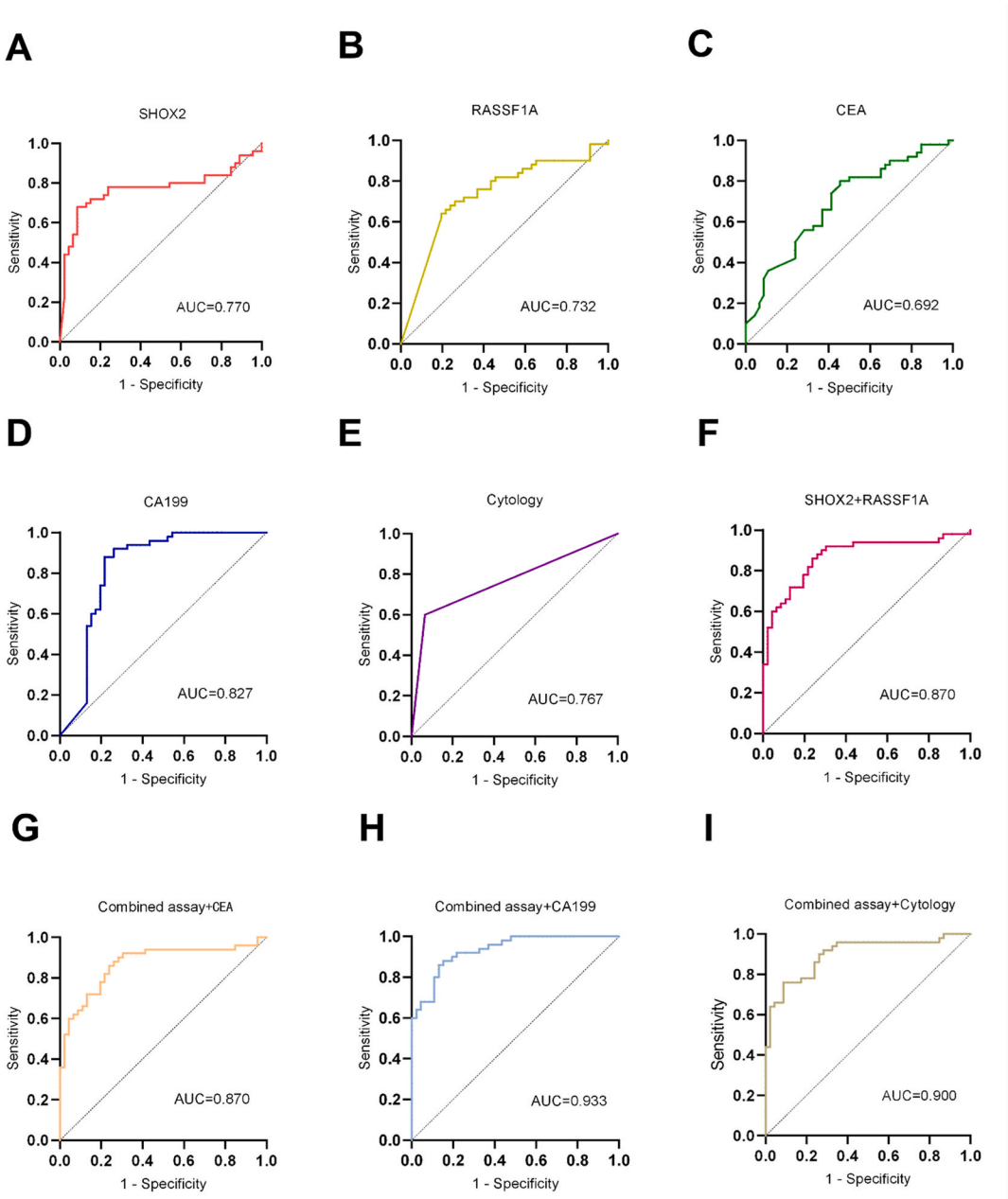

Fig. 5. ROC graph for each indicator. ROC, receiver-operating characteristic. (A–I): The ROC curves of SHOX2, RASSF1A, CEA, CA19-9, liquid-based exfoliative cytology, combined SHOX2 and RASSF1A, combined assay combined with CEA, combined assay combined with CA19-9 and combined assay combined with liquid-based exfoliative cytology, and the AUC value is 0.770, 0.732, 0.692, 0.827, 0.767, 0.870, 0.870, 0.933, 0.900, respectively.

gene (△Ct) was calculated to be 7.65, which represents a sensitivity of 91.3 % and specificity of 68.0 %, while the cut-off value for that of RASSF1A was 14.28, which represents a sensitivity of 80.4 % and specificity of 64.0 %.

Univariate and multivariate logistic regression analyses. Using regression analysis with univariate and multivariate, factors that could be linked to PC diagnosis were examined, which included genders, age, body mass index (BMI), lesion size, number of punctures, gallbladder disease, serum ferritin (SF), Cytokeratin 19 fragment (Cyfra21-1), SHOX2, RASSF1A, CA19-9, CEA and fluid-based exfoliative cytology. The outcomes of the statistical assessment are displayed in Table 5. Age, SHOX2, RASSF1A, CA19-9 and fluid-based exfoliative cytology were all linked with PC according to a univariate logistic regression analysis ($P < 0.05$). Analysis of multifactorial logistic regression suggested that SHOX2, RASSF1A, CA19-9, CEA, and fluid-based exfoliative cytology were associated

Table 4
Comparison of the sensitivity, specificity and diagnostic performance of different assays.

Diagnostic indicators	SEN	SPE	PPV	NPV	ACC	AUC	95%CI	P- value
SHOX2	0.957	0.460	0.620	0.920	0.698	0.770	0.668–0.872	0.000
RASSF1A	0.783	0.640	0.667	0.762	0.708	0.732	0.629–0.835	0.000
CEA	0.478	0.820	0.710	0.631	0.656	0.692	0.586–0.797	0.001
CA19-9	0.783	0.820	0.800	0.804	0.802	0.827	0.733–0.920	0.000
Cytology	0.935	0.600	0.683	0.909	0.760	0.767	0.670–0.865	0.000
SHOX2+ RASSF1A	0.739	0.840	0.810	0.778	0.792	0.870	0.795–0.944	0.000
Combined assay + CEA	0.348	0.940	0.842	0.610	0.656	0.870	0.795–0.944	0.000
Combined assay + CA19-9	0.587	0.920	0.871	0.708	0.760	0.933	0.888–0.979	0.000
Combined assay + Cytology	0.696	0.940	0.914	0.770	0.823	0.900	0.837–0.962	0.000

SEN, sensitivity; SPE, specificity; PPV, positive predictive value; NPV, negative predictive value; ACC, accuracy; AUC, areas under the ROC curve; 95%CI, 95%Confidence Interval. $P < 0.05$ was considered to indicate a statistically significant difference.

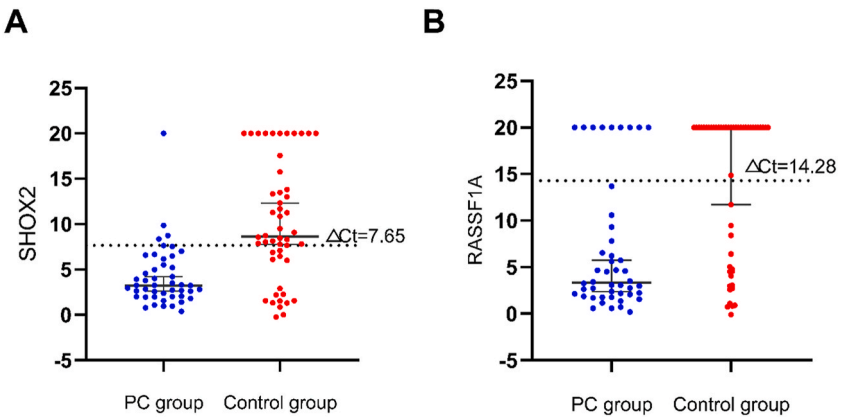

Fig. 6. SHOX2 and RASSF1A scatter plots. (A) The cut-off value for the DNA methylation of SHOX2 gene (△Ct) was 7.65. (B) The cut-off value for the DNA methylation of RASSF1A gene (△Ct) was 14.28.

with PC ($P < 0.05$) and could be modelled to predict PC with ORs of 0.530, 0.815, 1.002, 0.963, and 18.710, respectively. These five independent factors were used to construct the nomogram by using the rms package in R version 4.2.1 (http://www.r-project.org/) (Fig. 7). In a 2:1 ratio, 64 subjects were assigned to the training set and 32 were assigned to the validation set. On the nomogram, a worse prognosis was indicated by a higher overall number of points. It showed that the methylation of SHOX2 corresponded to the highest risk score (100 points). The performance was evaluated using the AUC and the C-index. The C-index of nomogram was 0.856 (95 % CI: 0.767–0.945) and 0.726 (95 % CI: 0.540–0.913) in the training and validation set, respectively (Fig. 8A and B), which indicated that the model had a good predictive discrimination.

4. Discussion

PC is one of the most common malignant tumors in clinical practice, with poor prognosis and high mortality rate and the 5-year overall survival rate is only about 10 %, recently. Therefore, early diagnosis and timely surgical intervention are currently effective means to improve the prognosis of PC patients. In addition to realizing early diagnosis based on clinical examination or biomarkers, it is very important to identify relevant risk factors and populations for those who are at high risk of PC [20,21]. In order to increase the precision of pathological diagnosis, our study investigated the diagnostic performance of novel molecular markers SHOX2 and RASSF1A methylation in conjunction with traditional biomarker CA19-9 by EUS-FNA for the identification of PC. And we assessed the diagnostic utility and clinical applicability of venous blood tumor markers, liquid-based exfoliative cytology, and SHOX2 and RASSF1A methylation for PC. According to our findings, the positive methylation rates of SHOX2 and RASSF1A in the PC group were considerably higher compared with those in the control group, and the diagnostic performance of the combined test was better than that of the conventional serum tumor markers CEA, CA19-9, and liquid-based exfoliative cytology alone, indicating that they may be potential PC biomarkers and can be an efficient complement to pathological diagnosis and cytological examination. Additionally, the diagnostic performance of PC detection is significantly enhanced when the combination test is used in conjunction with the conventional biomarker CA19-9. Furthermore, an original prognostic assessment model for patients was created by our research.

DNA methylation, a popular topic in tumor genetics, may give new ways to detect and assess tumors early [22]. Since aberrant DNA methylation occurs early in PC formation, epigenetics has become a promising biomarker for detection and prognosis [23]. Likewise,

Table 5
Univariate and multivariate logistic regression analysis associated with diagnosis of PC.

Characteristic	Univariate		Multivariate	
	OR (95%CI)	P-value	OR (95%CI)	P-value
Sex				
Male	1.000 (0.449–2.227)	1.000	0.081 (0.006–1.106)	0.060
Female	1		1	
Age, years	1.065 (1.019–1.113)	0.005	1.198 (0.998–1.438)	0.052
BMI	0.903 (0.806–1.011)	0.078	1.044 (0.781–1.396)	0.770
Size of lesions	0.981 (0.954–1.009)	0.182	0.955 (0.878–1.038)	0.280
Number of punctures	0.978 (0.660–1.448)	0.910	0.448 (0.157–1.276)	0.133
Gallbladder disease				
Yes	1.340 (0.591–3.039)	0.483	0.265 (0.030–2.352)	0.233
No	1		1	
SF	1.000 (0.999–1.001)	0.541	1.000 (0.996–1.004)	0.947
Cyfra21-1	1.065 (1.000–1.134)	0.051	0.952 (0.850–1.067)	0.400
SHOX2	0.788 (0.704–0.883)	0.000	0.530 (0.338–0.829)	0.005
RASSF1A	0.890 (0.842–0.941)	0.000	0.815 (0.702–0.946)	0.007
CA19-9	1.002 (1.001–1.004)	0.002	1.002 (1.000–1.004)	0.027
CEA	1.011 (0.999–1.023)	0.072	0.963 (0.932–0.996)	0.027
Cytology				
Positive	21.500 (5.860–78.888)	0.000	18.710 (1.067–327.990)	0.045
Negative	1		1	

BMI, Body Mass Index; SF, Serum ferritin; Cyfra21-1, Cytokeratin 19 fragment; SHOX2, short stature homeobox 2; RASSF1A, ras association domain family 1A; CA19-9, Carbohydrate antigen199; CEA, carcinoembryonic antigen. $P < 0.05$ was considered to indicate a statistically significant difference.

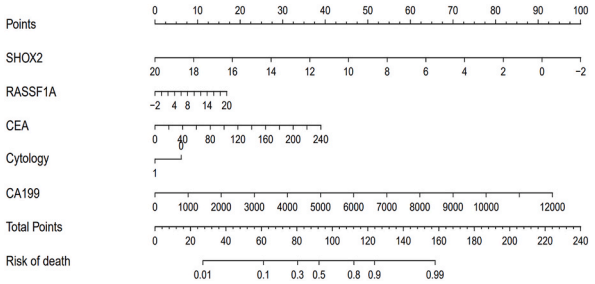

Fig. 7. Nomogram for the prediction of death. The points for each feature were summed to get the total point, and a vertical line was drawn on the total point to get the corresponding 'death risk'.

our investigation found that regardless of single or double positives, methylation of SHOX2 and RASSF1A was considerably higher in the PC group than those of the control group, indicating that SHOX2 and RASSF1A may participate in tumor growth. The findings are in accordance with those observed in earlier studies by Dammann et al. [24] that 29 of 45 primary adenocarcinomas (64 %), 10 of 12 endocrine tumors (83 %) and 8 of 18 pancreatitis cases (44 %) had RASSF1A hypermethylation, suggesting that it may be a potential early detection strategy for PC.

Investigating novel indicators in conjunction with combination assays is crucial for the diagnosis of PC, since multiple studies conducted in recent years have integrated cytology and genomes with serum tumor markers for the early identification of PC [11]. Our research indicates that the gene methylation assay in conjunction with CA19-9 had the best diagnostic efficacy, with an AUC of 0.933—much higher than that of either test alone or in combination with other assays. At this point, specificity increases significantly while sensitivity declines. Similar to our research, In a study of 346 patients with pancreatic ductal adenocarcinoma (PDAC) stages I–IV and 25 with chronic pancreatitis, Henriksen [25] found that plasma free DNA samples and serum CA19-9 show methylation of many genes, including RASSF1A. In addition, the AUC for the combination of gene methylation and serum CA19-9 in this study was 0.93, which was greater than that of serum CA19-9 alone, indicating its potential as a biomarker for the recognition of PDAC and for differentiating PDAC from chronic pancreatitis. The findings revealed that individuals with PDAC had more hypermethylated genes in their blood free DNA than those with chronic pancreatitis. One of the most often reported methylation inactivating oncogenes in PDAC is RASSF1, and the CpG island A of RASSF1A gets frequently methylated [26]. Pancreatic endocrine tumors (PET) are thought to undergo DNA methylation that silences the RASSF1A gene. Using Methylation-Specific PCR (MSP), Giorgio et al. [27] detected 80 % of PET and 65 % of normal pancreatic methylation, with 75 % of PET having higher mean methylation levels than normal ($P = 0.01$).

The initial application of the identification of SHOX2 and RASSF1A methylation patterns was for the detection of lung cancer. In bronchoalveolar lavage fluid (BALF), Sensitivity and specificity of the combined SHOX2 and RASSF1A promoter methylation assay

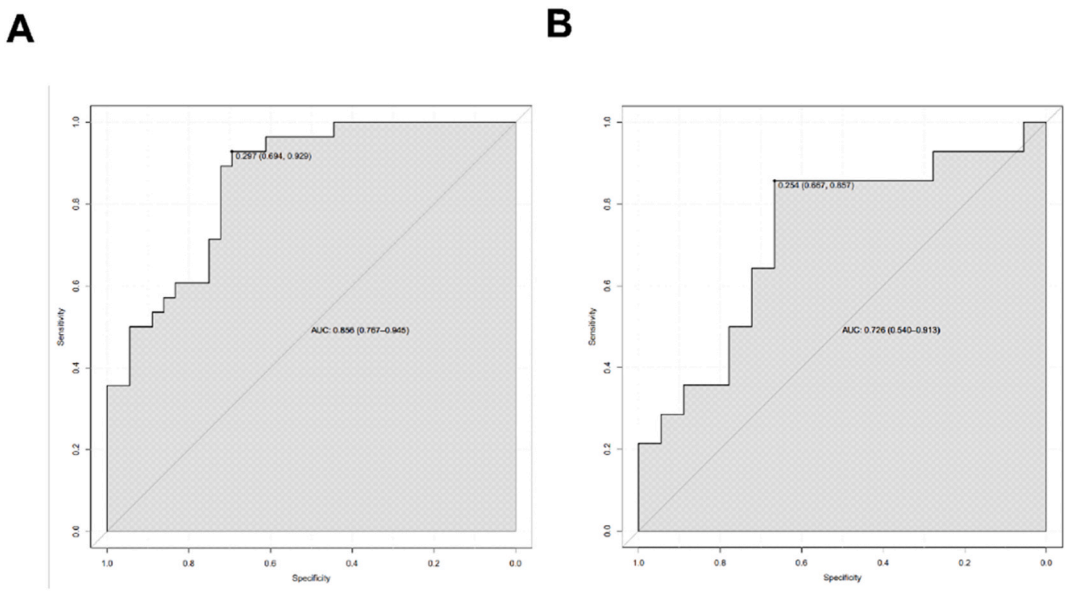

Fig. 8. ROC curves for the prediction of death in the training set and validation set. (A) the training set. (B) the validation set.

were 71.5–83.2 % and 90–97.4 %, respectively [28]. Our research revealed a positive correlation between the methylation of SHOX2 and RASSF1A and the conventional marker CA19-9. This suggests that there may be an inherent relationship between the methylation of these genes and the detection of PC. By using pyrophosphate sequencing and immunohistochemistry, Asano et al. [29]examined 46 anatomical regions from 33 cases of non-malignant intraductal papillary mucinous neoplasm (IPMN) in 2022 and discovered that potentially cancerous areas had higher rates of RASSF1A methylation indicating that it may be related to tumorigenesis, IPMN subtype, and prognosis. Thus, we developed predictive nomogram which cite SHOX2 and RASSF1A as predictors for outcome monitoring.

Unexpectedly, we discovered that while the specificity of SHOX2 and RASSF1A dual methylation positive detection of PC was higher than that of CA19-9, the sensitivity was lower. This difference might be attributed to the combination of EUS-FNA for tissue collection. The 2022 Clinical Practice Guidelines for Pancreatic Cancer (v1) [30], issued by the National Comprehensive Cancer Network (NCCN), contained advice for screening those who are at an elevated risk of developing PC as well as for thoroughly evaluating and adjusting for potential confounding variables. With a sensitivity, specificity, and accuracy of 87.6 %, 91.2 %, and 88.8 % for the diagnosis of probable PC, EUS-FNA was widely regarded as the most sophisticated and reliable approach in recent years [31]. Early PC screening has been proposed as a viable strategy that combines tumor markers and imaging techniques [32]. The merit of this study is the integration of EUS-FNA and new tumor markers for the initial assessment of potential pancreatic occupations by ultrasound endoscopy, followed by tissue collection using EUS-FNA in those with pancreatic lumps of unknown nature, with the histopathological presentation of the specimens obtained by aspiration as the gold standard, and sending for liquid-based exfoliative cytology and DNA methylation testing to aid diagnosis, in case of unsatisfactory EUS-FNA results or insufficient specimen volume. However, there are relatively few reports of exploringthe influence of DNA methylation of SHOX2 and RASSF1A genes with EUS-FNA of PC. Nevertheless, the purpose of our investigation was to evaluate the influence and the clinical effectiveness of both tests for PC.

The results of our study indicate that the SHOX2 gene methylation assay has the highest sensitivity, which is in line with the highest SHOX2 risk scores in the nomogram. However, there is little evidence in the literature linking SHOX2 to PC, and it is noteworthy that a commercial clinical assay based on the SHOX2 promoter methylation pattern has been developed as a companion diagnostic tool to help with patient assessment.

Our current study has limitations. First, the study's limited sample size and single-center design may bias patient selection and FNA diagnostic results. Due to PC's rarity, pathological classification is difficult. To eliminate selection bias, this study employed quality matching. Second, due to equipment and skill differences, a multicenter study with more participants may need more validation. Because our pathology department made all pathological diagnoses and it was impossible to assure that all specimens were assessed by the same doctor, we had to employ blinded approaches to eliminate differences.

In conclusion, SHOX2 and RASSF1A methylation testing should be clinically advantageous as an adjunctive diagnostic tool and a useful complement to cytological morphology, particularly in cases of insufficient histopathological volume and negative cytological testing, in order to prevent repeat puncture testing. Our findings may be useful in improving the adjuvant diagnostic value of PC.

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Data availability statement

The data analyzed during the current study are available from the supplementary data. And the corresponding author can provide all possible assistance to the requester of the original data.

Ethics approval and consent to participate

This study was reviewed and approved by the Ethical Committee of Affiliated Hospital of Nantong University with the approval number: 2019-K055. We certify that the study was performed in accordance with the 1964 dec laration of HELSINKI and later amendments.

All patients provided written informed consent and agreed to publish their data before participating the study. The informed consent informs the patient of the purpose of the study, the methodology of the study, and the possible benefits, risks and discomforts of participating in the study.

Patient consent for publication

Not applicable.

CRediT authorship contribution statement

Yangyang Shan: Writing – original draft, Visualization, Software, Methodology, Data curation. **Ying Teng:** Methodology, Investigation, Formal analysis. **Chengqi Guan:** Investigation, Data curation. **Zhenbiao Mao:** Supervision, Formal analysis. **Cuihua Lu:** Project administration, Conceptualization. **Weifeng Ding:** Writing – review & editing, Project administration, Funding acquisition. **Jianfeng Zhang:** Writing – review & editing, Validation, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Weifeng Ding reports financial support, administrative support, and equipment, drugs, or supplies were provided by National Natural Science Foundation of China. Weifeng Ding reports financial support, administrative support, and equipment, drugs, or supplies were provided by China Postdoctoral Science Foundation. Jianfeng Zhang reports financial support, administrative support, and equipment, drugs, or supplies were provided by Science and Technology Project of Nantong City. Jianfeng Zhang reports administrative support and equipment, drugs, or supplies were provided by Wu Jieping Medical Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34028.

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