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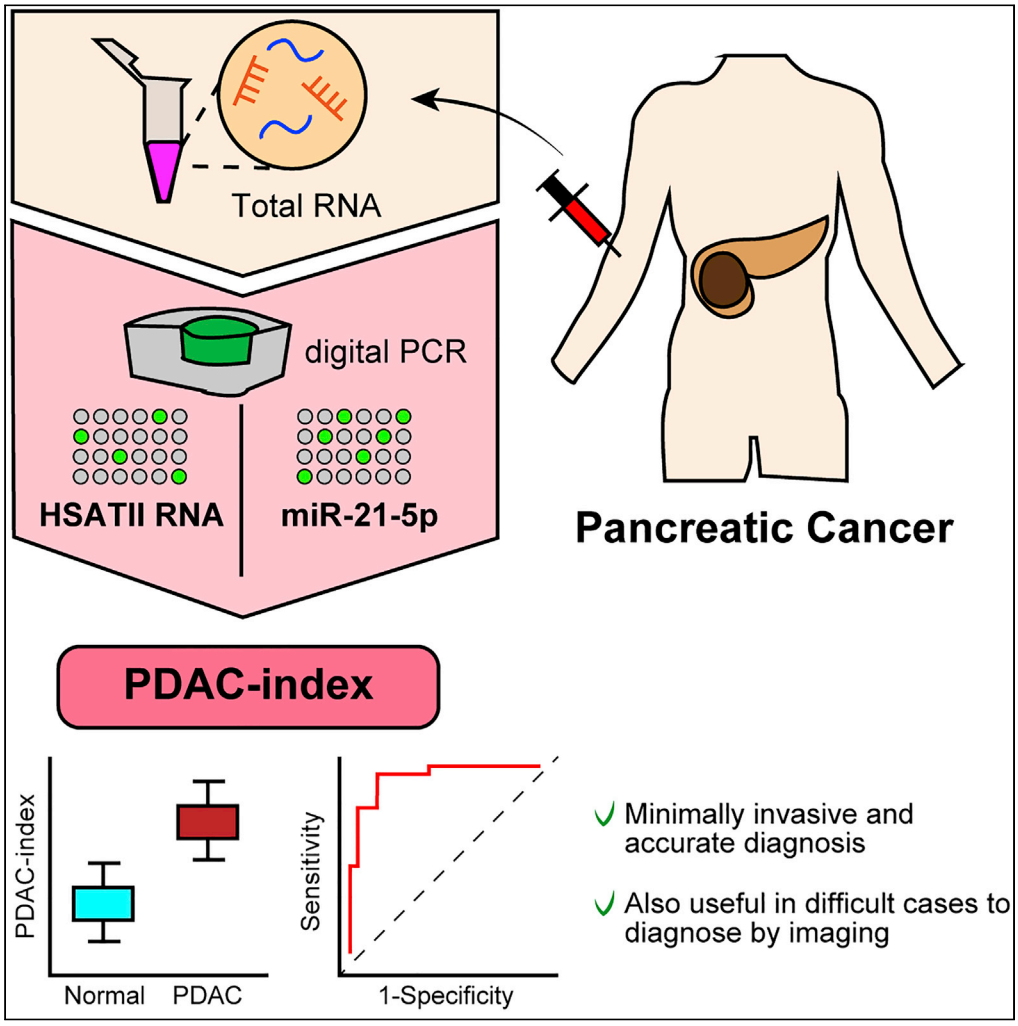

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Highlights

PDAC-Index was constructed using serum HSATII RNA and miR-21-5p levels

The PDAC-Index discriminates patients with PDAC with high accuracy

The PDAC-Index is useful as a supportive test in difficult-to-diagnose cases

Seimiya et al., iScience 26, 106021
February 17, 2023
https://doi.org/10.1016/j.isci.2023.106021

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Combination of serum human satellite RNA and miR-21-5p levels as a biomarker for pancreatic cancer

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SUMMARY

Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis due to the difficulty of its diagnosis. Because human satellite II (HSATII) RNA, a satellite repeat RNA, is highly and specifically expressed in human PDAC, the serum HSATII RNA level may be a biomarker of PDAC. To measure the serum HSATII RNA level with high sensitivity and reproducibility, we previously developed a convenient method, tandem repeat amplification by nuclease protection (TRAP) combined with droplet digital PCR (ddPCR). Here, we refined the original method by simultaneously measuring the serum miR-21-5p level to enhance the detection of PDAC. The resulting PDAC-Index, constructed using serum HSATII RNA and miR-21-5p levels, discriminated patients with PDAC with high accuracy. We verified the clinical usefulness of the PDAC-Index as a supportive test in difficult-to-diagnose cases. The PDAC-Index has satisfactory diagnostic performance and may routinely be applied for detecting PDAC.

INTRODUCTION

Pancreatic cancer, or pancreatic ductal adenocarcinoma (PDAC), has one of the worst prognoses of any malignancy.[1] PDAC is estimated to cause as many deaths (~432,000) as new cases (~459,000) worldwide each year, and is the seventh-leading cause of cancer-related death.[2] Most patients are diagnosed at the advanced stage due to the lack of early symptoms, such that the opportunity for curative resection is missed. Because the 5-year survival rates of localized and metastatic pancreatic cancer are 34% and 3%, respectively,[1] precise diagnosis and treatment at an early clinical stage are crucial to improve the overall prognosis.

In addition, differential diagnosis of pancreatic cancer and other non-malignant lesions is problematic in some cases, even by pathological examination of samples obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). Serum carbohydrate antigen 19-9 (CA19-9), a biomarker of pancreatic cancer, is not suitable for early detection and diagnosis of pancreatic carcinoma because of its poor sensitivity, false-negative results in Lewis-negative patients, and false-positive results in the presence of obstructive jaundice.[3,4] Therefore, better biomarkers are required for diagnosis of PDAC; this would, in turn, improve the prognosis.

Aberrant expression of satellite RNAs, particularly human satellite II (HSATII) RNA, was detected in pancreatic cancer tissues but not normal tissues.[5] Based on this, we developed a highly sensitive, convenient, and reproducible method, the tandem repeat amplification by nuclease protection followed by droplet digital PCR (TRAP-ddPCR) method, to measure the serum HSATII RNA level for detecting PDAC.[6] However, there remain demands to improve the diagnostic performance of the original method; there were some limitations of the previous analyses as they were performed using a relatively small number of patients from a single institute.

Here, we evaluated the diagnostic utility of the serum HSATII RNA level, as measured by the TRAP method, in combination with the microRNA-21-5p (miR-21-5p) level, another highly expressed circulating biomarker

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https://doi.org/10.1016/j.isci.2023.106021

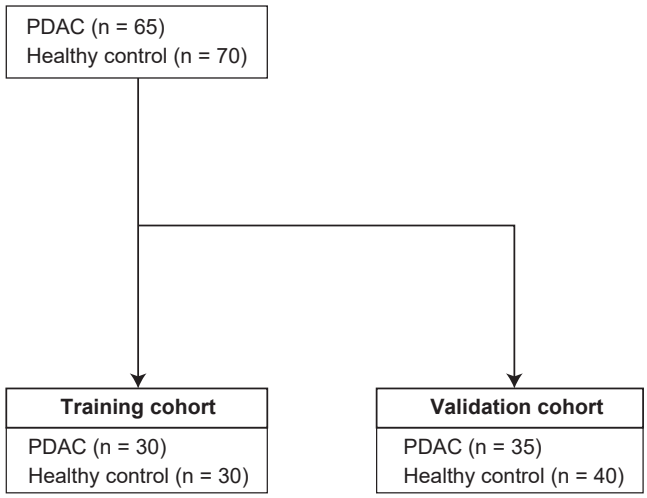

Figure 1. Flowchart of the cases enrolled in this study

Serum samples were obtained from 65 patients with pancreatic ductal adenocarcinoma (PDAC) and 70 healthy controls. These samples were randomly divided into training and validation cohorts.

of various cancers, including PDAC.[7,8] We examined serum samples obtained from patients with PDAC and healthy controls with no apparent malignancies, from two institutions. We developed a convenient diagnostic index based on the HSATII RNA and miR-21-5p levels for discriminating PDAC, which has better diagnostic performance and can be routinely applied for the detection of PDAC.

RESULTS

Patient characteristics and study overview

The patient characteristics and an overview of the study are presented in Figure 1 and Table S1. A total of 135 patients were enrolled in this study, including 65 patients with pancreatic cancer (31 localized and 34 metastatic). As a control, serum samples from 70 patients with no evidence of pancreatic or malignant disease were enrolled. The samples were collected consecutively and stored frozen until measurement in a single laboratory.

Measurement of the serum HSATII RNA level using TRAP-ddPCR (Figure S1A) is useful for screening for PDAC.[6] Circulating microRNAs are also recognized as promising biomarkers for the diagnosis of various cancers.[9–12] To improve the sensitivity and specificity for PDAC, we developed a refined method involving measurement of the serum HSATII RNA and microRNA levels. We subjected miR-21-5p to further analysis, because miR-21-5p was the most highly upregulated microRNA in mouse pancreatic cancerous tissues in our comprehensive evaluation (Figure S1B). In addition, it is frequently upregulated in various cancers including PDAC.[7,8]

We measured serum HSATII RNA and miR-21-5p levels in 135 cases (Figures 1 and S1C). HSATII RNA and miR-21-5p levels were significantly higher in patients with PDAC, irrespective of localized or metastatic lesions, than in healthy controls (Figures S2A and S2B). There were no significant differences in miR-21-5p levels between female and male patients with PDAC and healthy controls (Figures S2C and S2D), while in healthy controls, HSATII RNA levels were significantly higher in males than in females (Figure S2E). In patients with PDAC, there were no significant differences in HSATII RNA levels between males and females (Figure S2F). In addition, patient age was not correlated with miR-21-5p or HSATII RNA levels (Figures S2G and S2H).

Establishment of the PDAC-Index based on the training cohort

To develop a model for discrimination between pancreatic cancer and non-cancer samples using serum HSATII RNA and miR-21-5p levels, we randomly assigned the 135 cases into training and validation cohorts. In the training cohort, serum HSATII RNA and miR-21-5p levels were significantly higher in the patients with

PDAC than the healthy controls (Figures 2A and 2B). HSATII RNA showed moderate diagnostic performance [area under the curve (AUC): 0.87 (95% confidence interval (CI): 0.78–0.97); sensitivity: 0.80; specificity: 0.87] (Figure 2C). miR-21-5p also showed moderate diagnostic performance [AUC: 0.87 (95% CI: 0.77–0.98); sensitivity: 0.76; specificity: 1.00] (Figure 2D). To further increase the diagnostic performance, we developed the PDAC-Index as a combination of the HSATII RNA and miR-21-5p levels using Fisher linear discriminant analysis [$y = 1.01 \times \log10$ (HSATII RNA level) $+ 1.43 \times \log10$ (miR-21-5p level) $- 0.95$]. This formula returns numeric scores, and a positive score was regarded as indicative of PDAC. The PDAC-Index could discriminate PDAC from healthy controls accurately [AUC: 0.90 (95% CI: 0.83–0.99); sensitivity: 0.80; specificity: 1.00] in the training cohort (Figure 2E). Although there was no significant difference between the AUC of the PDAC-Index and that of HSATII RNA ($p = 0.39$), it was suggested that the PDAC-Index may better discriminate between patients with PDAC and healthy controls.

The PDAC-Index can discriminate patients with PDAC

Based on the results of the training cohort, we next examined the HSATII RNA and miR-21-5p levels, and the PDAC-Indices, of the validation cohort. The serum HSATII RNA and miR-21-5p levels were significantly higher in patients with PDAC than in the healthy controls (Figures 3A and 3B). HSATII RNA showed moderate diagnostic performance [AUC: 0.82 (95% CI: 0.72–0.91); sensitivity: 0.86; specificity: 0.65] (Figure 3C); miR-21-5p also showed moderate diagnostic performance [AUC: 0.90 (95% CI: 0.83–0.97); sensitivity: 0.94; specificity: 0.75] (Figure 3D). The PDAC-Index provided significantly better discrimination in the validation cohort [AUC: 0.92 (95% CI: 0.86–0.99); sensitivity: 0.94; specificity: 0.85] than HSATII RNA alone (AUC: 0.92 vs. 0.82, $p = 0.004$) (Figure 3E). Furthermore, in the age- and sex-matched subgroup in the validation cohort, the PDAC-Index accurately discriminated patients with PDAC from healthy controls [AUC: 0.99 (95% CI: 0.96–1); sensitivity: 1.00; specificity: 0.94) (Figure 3F, Table S2). These results suggest that the combination of HSATII RNA and miR-21-5p in serum may be useful in the diagnosis of pancreatic cancer.

To test the diagnostic performance of the PDAC-Index in case of pancreatic benign lesions, we examined the PDAC-Index in 10 cases with chronic pancreatitis (CP) and 24 patients with intraductal papillary mucinous neoplasm (IPMN). The PDAC-Index showed negative values in 8 of 10 CP cases and 23 of 24 IPMN cases, while serum CA19-9 levels were elevated (cutoff: 37 U/mL) in 3 CP cases and 5 IPMN cases (Figure S3). These results suggest that the PDAC-Index may be able to discriminate CP and IPMN from PDAC more accurately than CA19-9.

To test the diagnostic performance of the PDAC-Index in other types of cancer, we examined the PDAC-Index in 21 patients with colorectal cancer (CRC). In CRC cases, HSATII RNA and miR-21-5p levels were significantly higher than healthy controls and the PDAC-Index was also significantly higher than healthy controls (Figures S4A–S4C). When comparing CRC and PDAC cases, HSATII RNA and miR-21-5p levels were not significantly different between these groups. However, the absolute values from the PDAC-Index were significantly higher in PDAC than CRC cases (Figures S4D–S4F). These results suggest that although the PDAC-Index can be positive in cancers other than PDAC, it is still a promising biomarker for pancreatic cancer, especially for cases in which the prior probabilities of PDAC are clinically high.

PDAC-Index with the conventional marker CA19-9 for diagnosis of PDAC

While the PDAC-Index enabled discrimination of PDAC cases, we also tested its diagnostic performance in combination with the CA19-9 level. In the validation cohort, 52 cases (35 patients with PDAC and 17 healthy controls) were tested for CA19-9. Four patients with PDAC did not show an elevated serum CA19-9 level (cutoff, 37 U/mL), consistent with the fact that 5%–10% of patients with PDAC do not show an elevated CA19-9 serum level, partly because they are Lewis-negative.[13] Strikingly, among the four patients with a false-negative CA19-9 serum level, three showed positive PDAC-Index values (Figure 4A). The combination of the serum CA19-9 level and the PDAC-Index had a sensitivity of 100%, superior to that of CA19-9 alone (91%).

We verified the usefulness of the PDAC-Index in difficult-to-diagnose cases. A 39-year-old male patient with a space-occupying lesion in the pancreatic head was scrutinized due to suspicion of PDAC. However, irrespective of repeated tissue acquisition by ERCP and EUS-FNA, a pathological diagnosis of PDAC could not be obtained. At around the time when PDAC was finally diagnosed by the third EUS-FNA, the serum CA19-9 level was only 8 U/mL, but the PDAC-Index (0.28, a positive value) indicated a low probability of

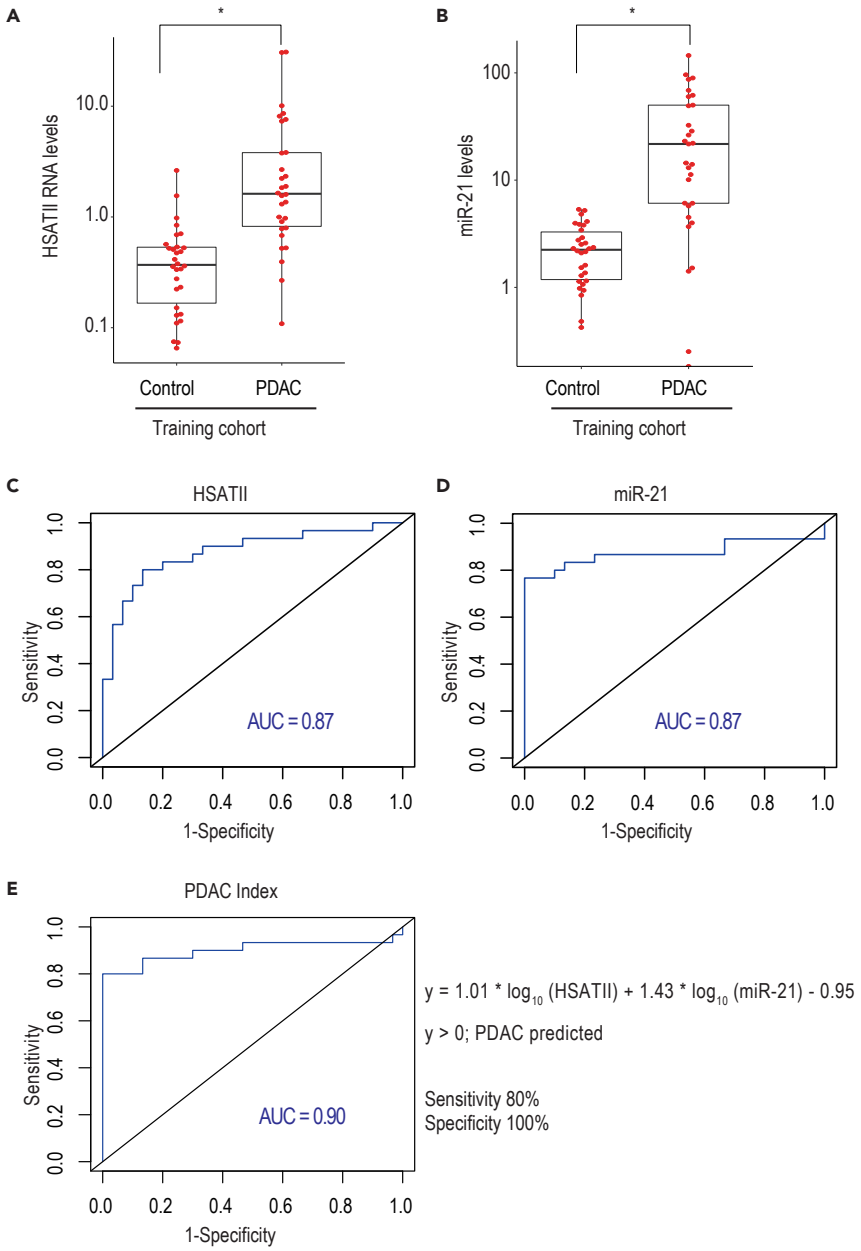

Figure 2. Development of a diagnostic model using human satellite II (HSATII) RNA and miR-21-5p based on the training cohort data

(A and B) HSATII RNA level (A) and miR-21-5p level (B) in the serum of patients with PDAC and healthy controls (Control) in the training cohort. Dot and boxplots are shown. The horizontal line in the middle of each box indicates the median, and the top and bottom borders of the box mark the 75th and 25th percentiles, respectively. The p values were calculated by Welch's *t*-test. *, $p < 0.05$.

(C and D) Receiver operating characteristic (ROC) curves for discriminating patients with PDAC from healthy controls based on the HSATII RNA (C) and miR-21-5p (D) levels in the training cohort. AUC, area under the curve.

(E) ROC curve plotted based on the new diagnostic index (PDAC-Index; y = 1.01 × log10 (HSATII RNA level) + 1.43 × log10 (miR-21-5p level) − 0.95; y > 0 indicates possible PDAC) based on the HSATII RNA and miR-21-5p levels in the training cohort.

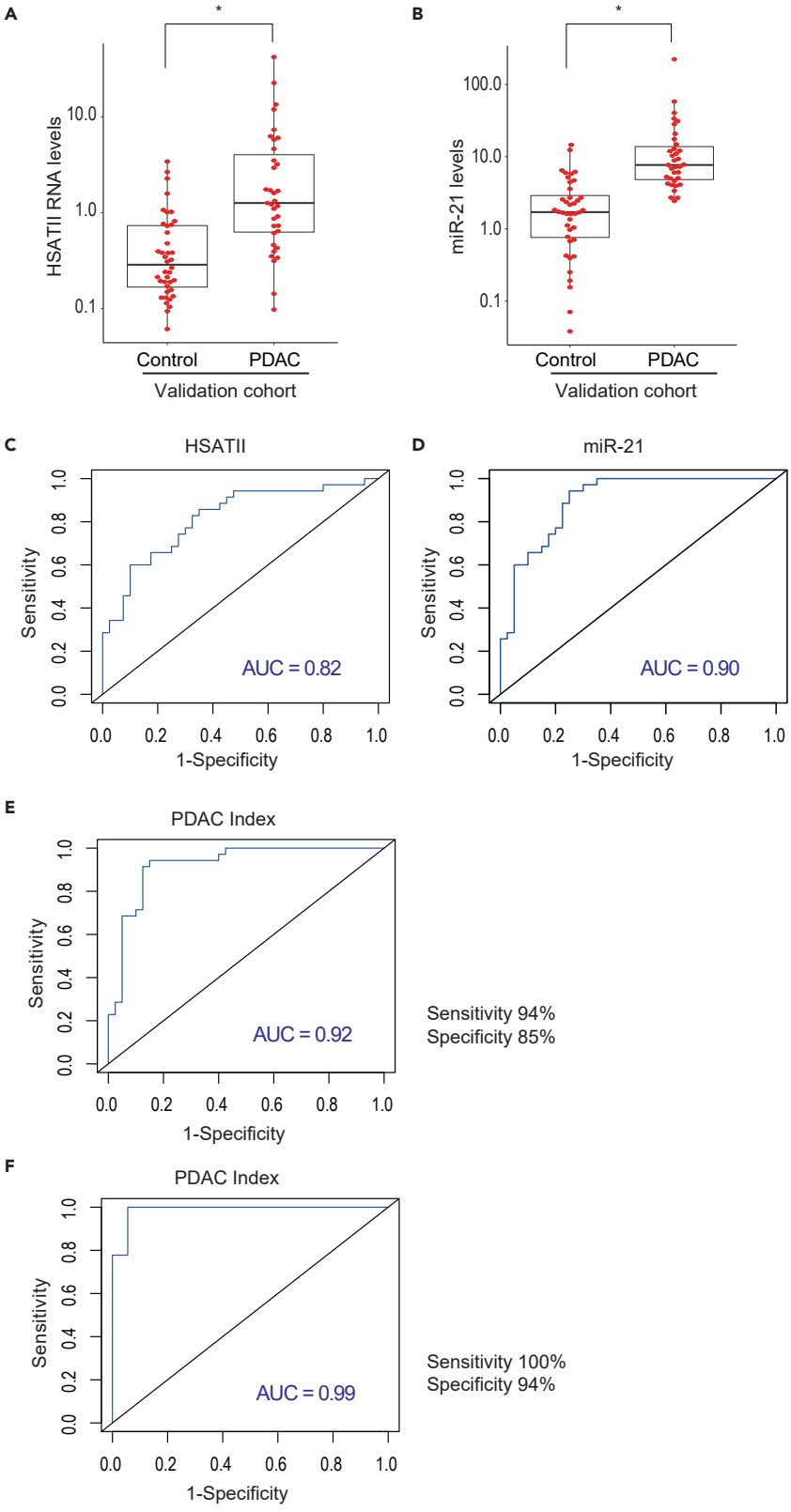
A
*
HSATII RNA levels
10.0
1.0
0.1
Control
PDAC
Validation cohort
B
*
miR-21 levels
100.0
10.0
1.0
0.1
Control
PDAC
Validation cohort
C
HSATII
Sensitivity
1-Specificity
AUC = 0.82
D
miR-21
Sensitivity
1-Specificity
AUC = 0.90
E
PDAC Index
Sensitivity
1-Specificity
AUC = 0.92
Sensitivity 94%
Specificity 85%
F
PDAC Index
Sensitivity
1-Specificity
AUC = 0.99
Sensitivity 100%
Specificity 94%

Figure 3. Validation of the utility of the newly developed diagnostic model

(A and B) HSATII RNA (A) and miR-21-5p (B) serum levels of patients with PDAC and healthy controls (Control) in the validation cohort. Dot and boxplots are as described in the legend to Figure 2. *, $p < 0.05$, by Welch *t*-test.

(C and D) ROC curves for discriminating patients with PDAC from healthy controls based on the HSATII RNA (C) and miR-21-5p (D) levels in the validation cohort. AUC, area under the curve.

(E) ROC curve based on the PDAC-Index in the validation cohort.

(F) ROC curve based on the PDAC-Index in the age- and sex-matched subgroup in the validation cohort.

a non-PDAC mass (Figure 4B). The patient underwent surgery and a final pathologic diagnosis of PDAC was obtained based on the resected tissues.

In another case, a 68-year-old male patient with a space-occupying lesion in the pancreatic body accompanying main pancreatic duct dilation was scrutinized due to suspicion of PDAC. Although the serum CA19-9 level (29 U/mL) was below the cutoff value, the PDAC-Index (0.40, a positive value) accurately predicted PDAC (Figure 4C). Indeed, a pathological diagnosis of PDAC was obtained by EUS-FNA. Although larger cohort studies are needed, these results suggest that the PDAC-Index may be useful as a supportive test for the diagnosis of PDAC, particularly in difficult-to-diagnose cases.

DISCUSSION

To discriminate patients with PDAC, we developed a new and convenient scoring system comprising the serum HSATII RNA and miR-21-5p levels. We refined our original TRAP-ddPCR method to measure the serum HSATII RNA and miR-21-5p levels to increase the diagnostic utility.

Aberrant ncRNA expression from the highly repetitive satellite DNA was detected in pancreatic tumors; also, a subset of satellite RNAs, particularly HSATII RNA, is specifically expressed at a higher level in human PDAC compared to normal tissues.[5] We recently developed a procedure, the TRAP-ddPCR method, for detecting repetitive RNAs in serum by specifically amplifying tandem repeat transcripts. The TRAP-ddPCR method enables highly sensitive detection and quantification of the serum HSATII RNA level of patients with PDAC.[6] In this study, we obtained samples different from the previously published ones and measured serum HSATII RNA and miR-21-5p levels. The serum HSATII RNA levels of patients with PDAC were significantly higher than those of healthy controls, demonstrating the reproducibility of the diagnostic performance of HSATII RNA. Moreover, the PDAC-Index, a combination of the HSATII RNA and miR-21-5p levels in serum, significantly improved the diagnostic performance (AUC of 0.92, compared to 0.82 by HSATII RNA alone in the validation cohort).

The PDAC-Index was also significantly elevated in patients with CRC compared to healthy controls. However, PDAC-Index levels were significantly higher in patients with PDAC than in patients with CRC, whereas neither HSATII RNA nor miR-21-5p alone was significantly different between patients with CRC and PDAC. These results suggest that the PDAC-Index is a potent biomarker for PDAC.

Although we used an external spike-in control to normalize nucleic acid levels, an internal control is preferable to compensate for sample-to-sample variation in RNA quality and quantity. Although some microRNAs have been proposed as internal controls, there is still no consensus as to which microRNAs are optimal for these kinds of assays. Further studies are needed to determine appropriate internal control settings.

Another critical issue for clinical testing in general, extrapolated from this study, is the formulation of standard operating procedures (SOPs) for sample handling. In this study, the samples of healthy controls were derived from a single health center, which is affiliated to the university hospital. In contrast to our previous report,[6] in which all samples were derived from a single university hospital, the serum HSATII RNA levels of the healthy controls were generally higher in this study. Nonetheless, patients with PDAC were efficiently discriminated, indicating the robustness of diagnosis based on the serum HSATII RNA level. However, it was considered that SOPs for sample preparation, such as standardization of sample storage methods between facilities, are critical for obtaining reproducible results.

The PDAC-Index had high sensitivity and specificity for the discrimination of PDAC. Indeed, the PDAC-Index, but not CA19-9, discriminated PDAC in a highly suspicious case that needed repeated invasive tests

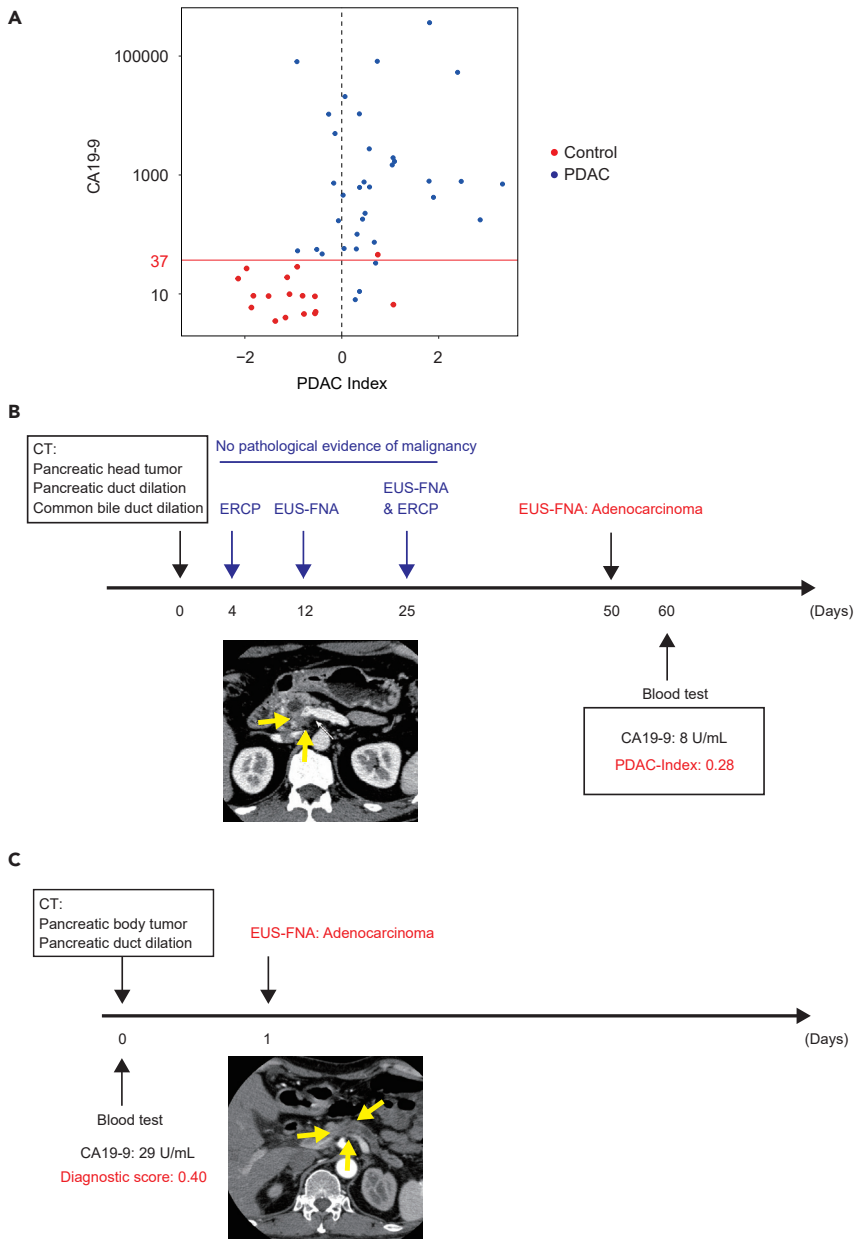

Figure 4. Diagnostic performance for PDAC of CA19-9 and the PDAC-Index

(A) Scatterplot for CA19-9 and the PDAC-Index in the validation cohort. Red dots, healthy controls (Control); blue dots, patients with PDAC; red line, CA19-9 cut-off.

(B) Clinical course of a patient in whom the diagnosis of PDAC was difficult. The CA19-9 and PDAC-Index values and a representative computed tomography image (yellow arrows; pancreatic mass) are shown.

(C) Clinical course of another patient with suspected pancreatic cancer. The CA19-9 and PDAC-Index values and a representative computed tomography image (yellow arrows; pancreatic mass) are shown.

to confirm pathological diagnosis of PDAC. In a meta-analysis of the diagnostic utility of adding *KRAS* mutation testing to the pathological examination of EUS-FNA specimens, the sensitivity was increased by 8% compared to EUS-FNA alone.[14] Therefore, combining the PDAC-index with pathological examinations may reduce the false-negative rate.

Although the PDAC-Index has high sensitivity and specificity, it may not be suitable for screening of PDAC in the general population, because of the low prevalence of PDAC (*e.g.*, 0.013% in Japan and 0.012% in the

US),[15,16] which may lead to a low positive predictive value. Therefore, it may be more realistic to use the index as supportive testing for difficult-to-diagnose cases or as a screening method among PDAC high-risk cases, such as patients with CP, IPMN, diabetic mellitus, and genetically susceptible patients.[17] To increase the clinical efficiency, stratification of patients at high risk of PDAC is required.

In summary, we refined our original TRAP-ddPCR method for screening PDAC by newly introducing the PDAC-Index, which is based on serum HSATII RNA and miR-21-5p levels. It may be useful as a supportive diagnostic test for difficult-to-diagnose patients with PDAC.

Limitations of the study

The PDAC-Index may not be suitable for screening of PDAC in the general population because of the low prevalence of PDAC. Prospective studies with larger cohorts, including stratified high-risk groups, are further required to evaluate the clinical utility of this index.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106021.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (22K15390, #22H02828, #22K15958, #21K15916, and # 21H02893) (to T.S., M.O., K.I., R.I., and T.K.), by, and by the grants from Japan Agency for Medical Research and Development, AMED (JP22ck0106557 to M.O.) and from JST CREST (#JPMJCR19H5 to M.O.). We thank Dr. Abe and Dr. Kidani, Medical & Biological Laboratories (Nagoya, Japan), for their technical assistance and critical discussion.

AUTHOR CONTRIBUTIONS

T.Seimiya and M.O. planned and designed the research. T.Seimiya, T.Suzuki, T.I., T.K., K.S., C.S., K.I., and R.I. conducted the experiments. H.F., H.O., S.K., T.Sato, Y.N., M.M., and R.N. acquired the data. H.I. established the gene-modified mice. T.Seimiya, M.O., and K.K. analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Received: April 26, 2022
Revised: December 26, 2022
Accepted: January 16, 2023
Published: January 19, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
miRNeasy Serum/Plasma Advanced Kit	Qiagen	Cat#217204
RNeasy MinElute Cleanup Kit	Qiagen	Cat#74204
TaqMan MicroRNA Reverse Transcription Kit	Thermo Fisher Scientific	Cat#4366596
Deposited data		
MicroRNA microarray data	This paper	GEO: GSE221449
Experimental models: Organisms/strains		
Mouse: C57BL/6-Ptf1a$^{cre/+}$; LSL-Kras$^{G12D/+}$; Tgfbr2$^{flox/flox}$	Ijichi et al.[18]	N/A
Oligonucleotides		
Arabidopsis thaliana (ath-)miR159a mirVana microRNA mimic	Thermo Fisher Scientific	Cat#4464066
HSATII protecting RNA probes: 5′-AUCAUCGAAUGGAAUC GAAUG-3′	Eurofins Scientific	N/A
Software and algorithms		
QuantaSoft analysis software	Bio-Rad	Cat#1864011
R software (version 3.5.2)	R Foundation for Statistical Computing	https://www.R-project.org
MASS R package (version 7.3-56)	W. N. Venables and B. D. Ripley[19]	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Motoyuki Otsuka (otsukamo-tky@umin.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- MicroRNA microarray data have been deposited at GEO and publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Serum samples

Serum samples were obtained from PDAC (male:female = 32:33; median age: 70 years old, range: 39–86), CP (male:female = 10:0; median age: 62.5 years old, range: 31–86), IPMN (male:female = 7:17; median age: 72.5 years old, range: 41–89), and CRC (male:female = 13:8; median age: 65 years old, range: 31–90) patients at the University of Tokyo Hospital, from February 2018 to June 2020, and from healthy controls (male:female = 43:27; median age: 55 years old, range: 25–84) with no evidence of pancreatic or malignant disease who attended the outpatient clinic of Omiya City Clinic from May to July 2018. The PDAC diagnosis was confirmed pathologically with tissue obtained by EUS-FNA.

Mice

To generate a genetically modified pancreatic cancer mouse model, Tgfbr2$^{flox/flox}$, Ptf1a$^{cre/+}$ and LSL-Kras$^{G12D/+}$ mouse lines were intercrossed to generate Ptf1a$^{cre/+}$; LSL-Kras$^{G12D/+}$; Tgfbr2$^{flox/flox}$ (KrasG12D+Tgfbr2$^{-/-}$) in a >95% C57BL/6 background. These mice developed spontaneous pancreatic cancer, as reported previously.[18] Male mice at 7 weeks of age were subjected to microRNA microarray analysis.

Study approval

Written consent for serum sampling was obtained from all patients; the study protocols were approved by the Local Ethics Committees of The University of Tokyo (approval number 11712-(7)) and the Institute of Medical Science of The University of Tokyo (approved number 28-19-0907).

The mouse experimental protocols were approved by the Internal Ethics Committee for Animal Experimentation (approval number H19-132) and the experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Medicine, University of Tokyo (Tokyo, Japan).

METHOD DETAILS

Microarray analysis

Pancreatic cancerous tissues from gene-modified mice and normal pancreas tissues from control mice were frozen in liquid nitrogen immediately after resection and stored at −80°C. Frozen tissues were crushed without thawing using the SK mill (Tokken, Chiba, Japan) and immediately immersed in ice-cold Isogen reagent for RNA extraction (Nippon Gene, Tokyo, Japan). MicroRNA microarray analysis was performed using microRNA microarrays (CosmoBio, Tokyo, Japan).

Blood processing and extraction of RNA from serum

Approximately 15 mL of whole peripheral blood was acquired from each subject, and the serum remaining after routine clinical testing was frozen immediately for downstream processing. Serum samples were centrifuged at 2,000 g for 10 minutes, and the supernatants were filtered through a 450-μm nylon mesh to exclude cell debris. As a spike-in outer control, *Arabidopsis thaliana* (ath-)miR159a mirVana microRNA mimic (Thermo Fisher Scientific, Waltham, MA, USA) was added to the filtered serum to a final concentration of 5 pM. Total RNA was extracted from 200 μL of the serum using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Tandem repeat amplification by nuclease protection method

To render the serum HSATII RNA level measurable, the TRAP method was used according to the original protocol[6] with several modifications. Briefly, isolated total RNA was mixed with 0.2 pmol HSATII protecting RNA probes (5′-AUCAUCGAAUGGAAUCGAAUG-3′) (Eurofins Scientific, Luxembourg, Luxembourg) in a buffer provided by Medical & Biological Laboratories (MBL, Nagoya, Japan), followed by denaturing at 90°C for 3 minutes and incubation at 60°C for 2 hours for hybridization. Subsequently, 10 μg of RNase A, 100 U of RNase T1, and 5 U of exonuclease T in 50 μL of 1× NE Buffer 4 (New England Biolabs, Ipswich, MA, USA) were added and incubated at 37°C for 30 minutes to degrade unhybridized single-stranded RNAs, followed by purification of the protected RNAs using the RNeasy MinElute Cleanup Kit (Qiagen) in 14 μL of RNase-free water.

Droplet digital PCR analysis of HSATII RNA level

To measure the HSATII RNA level in RNA samples processed by the TRAP method, reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), according to the manufacturer's instructions with some modifications. Briefly, 5 μL of protected RNA template and 3 μL of 5× RT primer for HSATII RNA were incubated at 85°C for 5 minutes, followed by 60°C for 5 minutes and 4°C for 5 minutes. The reverse transcription reagent mix was added and incubated at 16°C for 30 minutes, followed by 42°C for 30 minutes and 85°C for 5 minutes.

The ddPCR was conducted using the QX100 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, 3.3 μL of template cDNA with 20 × primer and a TaqMan probe set were partitioned into approximately 20,000 droplets by the QX100 Droplet Generator for amplification. The cycling conditions

were 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute, and a final 10-minute incubation at 98°C. The droplets were subsequently read automatically by the QX10 droplet reader. The data were analyzed with QuantaSoft analysis software (ver. 1.3.2.0; Bio-Rad).

Measurement of microRNAs

To measure the miR-21-5p level in serum and the ath-miR159a level in the spike-in control, 5 and 2 μL of RNA isolated from serum samples were used, respectively. TaqMan probes for miR-21-5p and ath-miR159a were purchased from Applied Biosystems (Foster City, CA, USA). Reverse transcription and quantitation by ddPCR were conducted according to the manufacturer's instructions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Samples were randomly divided into the training and validation cohorts. The training cohort was used to construct, and the validation cohort to validate, discriminant models. HSATII RNA and miR-21-5p levels were normalized to the spike-in control level. The diagnostic index was generated by Fisher linear discriminant analysis using the MASS R package (version 7.3-56).[19] Receiver operating characteristic (ROC) curves were used to determine the sensitivity and specificity of the method. Statistical analyses were performed using R software (version 3.5.2; R Foundation for Statistical Computing, Vienna, Austria). Significant differences between groups were determined by Welch's *t*-test or Fisher's exact test. p-values < 0.05 were considered indicative of statistical significance. The AUC values were statistically compared by DeLong's method.[20]