Ph.D. Dissertation of Doo-Ho Lee

Prediction model for diagnosis of pancreatic cancer using a multi-biomarker panel

췌장암의 진단을 위한

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Abstract

Keyword: Diagnosis, Biomarker panel, Pancreatic ductal adenocarcinoma, Screening

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Background: Early diagnosis is essential to increase the survival rate of pancreatic ductal adenocarcinoma (PDAC), but at present, the tools for early diagnosis are insufficient. Recently, a retrospective study reported a multi-marker panel using triple-marker (LRG1, TTR, and CA19-9) validated in large-scale samples by multiple reaction monitoring-mass spectrometry and immunoassay has clinical applicability in the early detection of PDAC. The current study aimed to develop a prediction model for diagnosis of PDAC using the multi-marker panel (LRG1, TTR, and CA19-9) from large cohort of multi-centers as a diagnostic screening tool of PDAC.

Methods: A large multi-center cohort of 1,991 samples were collected from January 2011 to September 2019, of which 609 are normal (NL), 145 are other cancer (OC; colorectal, thyroid, and breast cancer), 314 are pancreatic benign disease (PB), and 923 are PDAC. The automated multi-biomarker Enzyme-Linked Immunosorbent Assay kit was developed using three potential biomarkers, LRG1, TTR, and CA 19-9. Using a logistic regression (LR) model trained on training data set, the predicted values for PDACs were obtained, and the result was classified into one of the three risk groups: low, intermediate, and high. The five covariates used to create the model were sex, age, and biomarkers TTR, CA 19-9, and LRG1. This multi-center study was approved by the institutional review boards of all participating

institutions (*SNUH, H-1703-005-835, YSH; 4-2013-0725, NCC; NCCNCS13818, SMC; 2008-07-065, AMC; 2013-1061, EUMC; 2018-05-030*). Bio-specimens were collected from participants who provided informed consent.

Results: Participants were categorized into four groups as normal (n=609), other cancer (n=145), pancreatic benign disease (n=314), and pancreatic ductal adenocarcinoma (n=923). The normal, other cancer, and pancreatic benign disease groups were clubbed into the non-pancreatic-ductal-adenocarcinoma group (n=1068). Significant differences were observed in age (non-PDAC; 55.5 ± 12.0 vs PDAC; 63.1 ± 9.9 years, $p < .001$), sex ratio (females: n = 474, 44.4% vs males: n = 561, 60.8%, $p < .001$), body mass index (23.6 ± 3.2 vs 22.9 ± 3.0 kg/m^2, $p = .001$), level of initial CA 19-9 (19.0 ± 98.6 vs 679.0 ± 1348.9 U/mL, $p < .001$), and level of LRG1, TTR, CA 19-9 in automated ELISA triple marker panel between the non-PDAC and PDAC groups. In the PDAC group, 39 (4.2%) patients were stage I, 618 (67.0%) patients were stage II, 52 (5.6%) patients were stage III, and 214 (23.2%) patients were stage IV. The mean of the four measures of the training data was 92.29, and the values of PPV, NPV, Sen, and Spe were 94.11, 90.40, 93.81, and 90.86, respectively. At threshold combinations of 0.22 and 0.88, the cut-off was 90%, and the number of samples in the high-, intermediate-, and low-risk groups were 306, 569, and 198, respectively.

Conclusions: This study demonstrates a significant diagnostic performance of the multi-marker panel in distinguishing PDAC from normal and benign pancreatic disease states, as well as patients with other cancers. The study indicates that the introduced multi-marker

panel prediction model for PDAC diagnosis can help guide medical decisions for patients, including patients with early stage PDAC or with normal levels of CA 19-9.

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Chapter 1. Introduction

1.1. Study Background

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal gastrointestinal malignancies and is the seventh leading cause of cancer-related deaths [1, 2]. Despite improving perioperative outcomes, PDAC has a poor prognosis, with a 5-year survival rate of only 8%–10% among patients [1-3]. Most patients are diagnosed in the advanced stages, and effective systemic therapies are lacking. Approximately 80% of patients with PDAC are detected at the unresectable or metastatic stage [1, 2].

Therefore, it is essential to improve early detection of PDAC to increase the survival rate. However, current imaging modalities, such as computed tomography (CT) or magnetic resonance imaging (MRI), are not suitable as screening tests for pancreatic cancer due to limitations of cost-effectiveness, time consumption, and capacity of inspection [4].

The only biomarker for pancreatic cancer that is clinically approved by the US Food and Drug Administration (FDA) is serum carbohydrate antigen 19-9 (CA 19-9) which has approximately 79% sensitivity and 82% specificity. However, CA 19-9 is ineffective for early diagnosis of PDAC in asymptomatic patients, and there is no individual marker that diagnoses PDAC with satisfactory sensitivity and specificity [5, 6]. Thus, there is an unmet need for a clinical method that can effectively differentiate pancreatic malignancy from normal, benign states, and other malignancies [6].

Increasing efforts to identify tumor-specific biomarkers have been made over the past few decades; however, the translation of these novel biomarkers into clinical practice has been very limited [7-9]. There are some assays that had been approved by the FDA for certain cancers, but none of these were introduced for pancreatic cancer except for CA 19-9 [10]. A microarray-based biomarker test (IMMray^{TM} PanCan-d) for PDCA was introduced and approved by the FDA; however, owing to its high cost, it may not be practical to be used as a screening tool [11].

Recently, two studies reported a multi-biomarker microarray, externally validated in a large cohort, using the serum of patients for early detection of PDAC [6, 11]. However, some requirements for an ideal screening test, such as cost-effectiveness and simplified usage were still lacking.

Park et al. reported that a multi-marker panel using a triple-marker, validated in large-scale samples by multiple reaction monitoring-mass spectrometry (MRM-MS) and immunoassay, has clinical applicability in the early detection of PDAC [6]. The panel including leucine-rich alpha-2 glycoprotein (LRG1), transthyretin (TTR), and CA 19-9 had a sensitivity of 82.5% and a specificity of 92.1%. The triple-marker panel exceeded the diagnostic performance of CA 19-9 by more than 10% (Area under ROC curve (AUC); $AUC_{CA19-9} = 0.826$, $AUC_{panel} = 0.931$, $p < 0.01$) in all PDAC samples and by more than 30% ($AUC_{CA19-9} = 0.520$, $AUC_{panel} = 0.830$, $p < 0.001$) in patients with a normal range of CA 19-9 [6].

1.2. Purpose of Research

For a diagnostic screening tool for PDAC, the multi-marker panel needs to be validated using cohorts from the normal population, benign pancreatic diseases, and other malignancies except PDAC. Hence, the current study aimed at developing a prediction model as a diagnostic screening tool for PDAC using a multi-marker panel in a large cohort including multiple centers.

Chapter 2. Material and Methods

2.1. Overview of study design

The automated multi-biomarker Enzyme-Linked Immunosorbent Assay (ELISA) kit was developed using three potential biomarkers, LRG1, TTR, and CA 19-9, that were identified in a previous study using MRM-MS, and for which external validation was performed at multiple centers [6].

To validate our proposed predictive model using the multi-marker ELISA kit, we collected a large amount of data including various types of data sets. Up to 1,991 samples were collected from various institutions. This included pancreatic benign disease (PB; chronic pancreatitis, low grade intra-ductal papillary mucinous neoplasm, well-differentiated neuroendocrine tumor, solid pseudopapillary neoplasm, mucinous cystic neoplasm, serous cystadenoma, pseudocyst, and pancreatolithiasis), other cancer (OC; breast, thyroid, and colorectal cancers), normal (NL), and PDAC. This was used for model development and verification of our proposed prediction model. The training data set was created by extracting 70% of NL and PDAC type data. The test data set was created by combining the remaining 30% of NL and PDAC, and all of the OC and PB data (Table 1). The NL and PDAC type data were randomly sampled at the same rate to prevent data skew. In addition, the values of the 3 markers used in the experiment were measured using the ELISA kit and log-transformed (Figure 1a-f).

Table 1. The number of samples of training and test data set

		NL	OC	PB	PDAC	Total
Data set	Training	426	0	0	647	1,073
	Test	183	145	314	276	918
	Total	609	145	314	923	1,991

NL, normal; OC, other cancer; PB, pancreatic benign; PDAC, pancreatic ductal adenocarcinoma.

Figure 1a. Box plots for expressions TTR. (NL; Normal control, OC; Other cancer, PB; Pancreatic benign disease, PDAC; Pancreatic ductal adenocarcinoma)

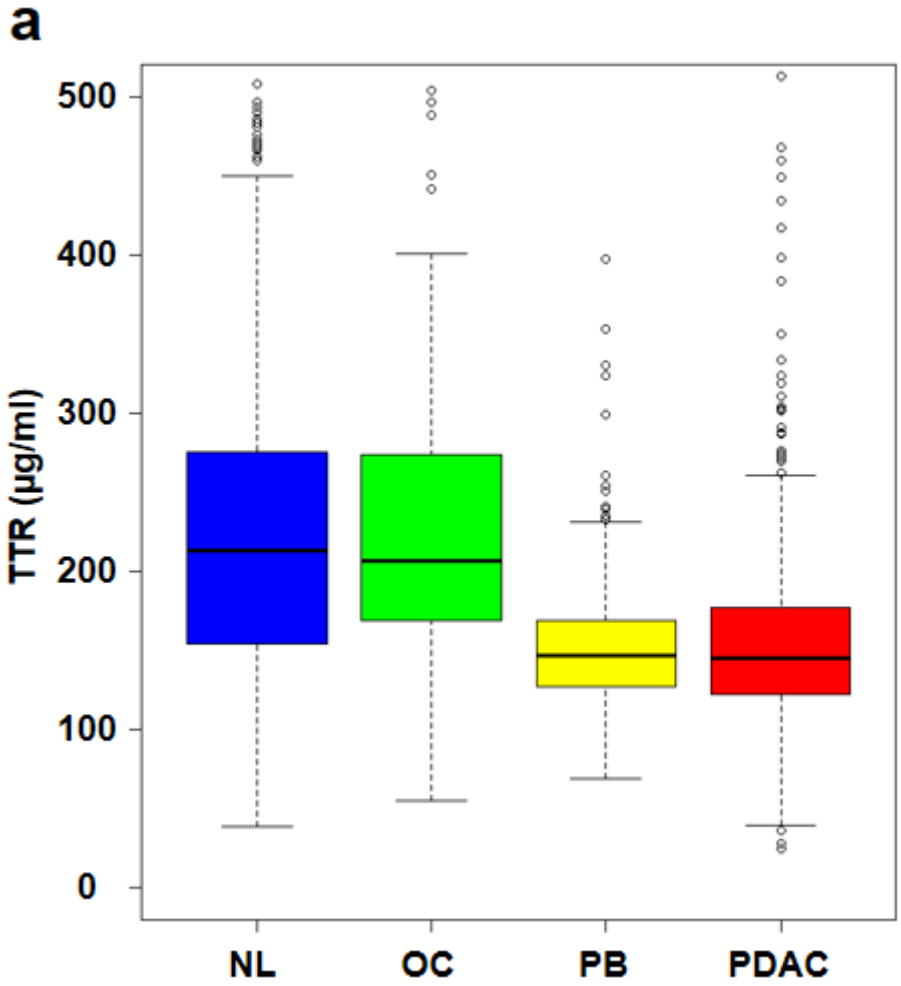


Figure 1b. Box plots for expressions CA 19-9. (NL; Normal control, OC; Other cancer, PB; Pancreatic benign disease, PDAC; Pancreatic ductal adenocarcinoma)

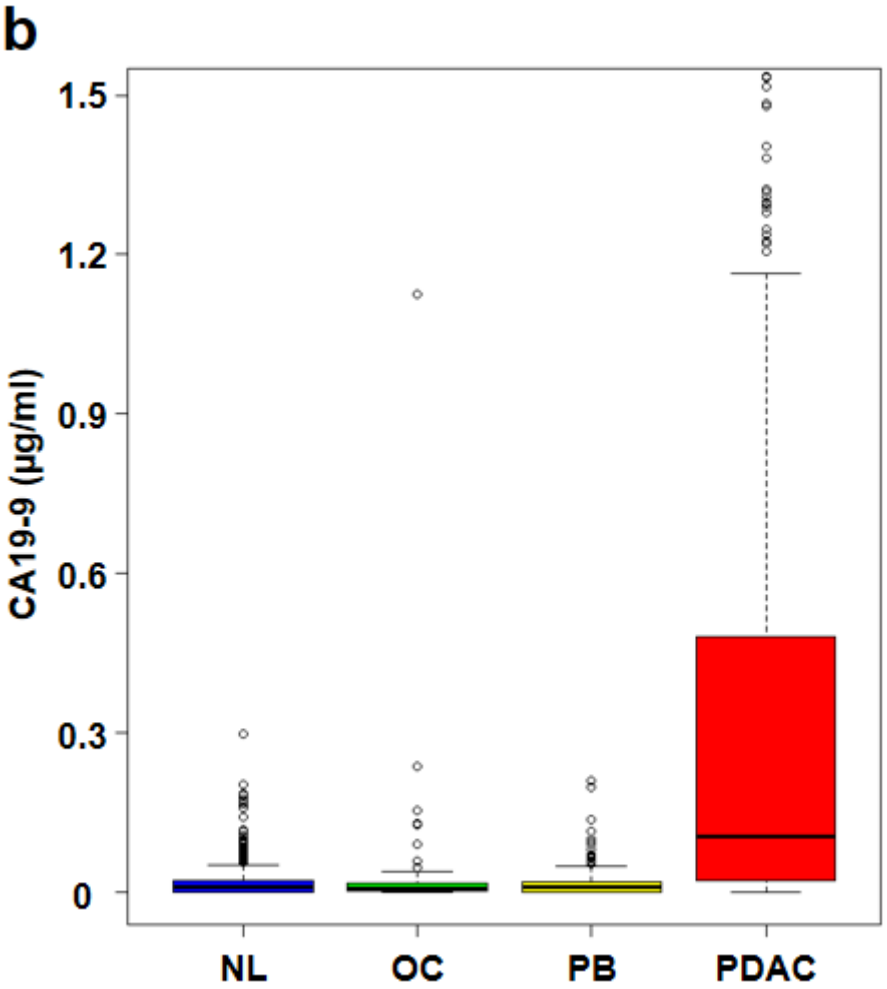


Figure 1c. Box plots for expressions LRG1. (NL; Normal control, OC; Other cancer, PB; Pancreatic benign disease, PDAC; Pancreatic ductal adenocarcinoma)

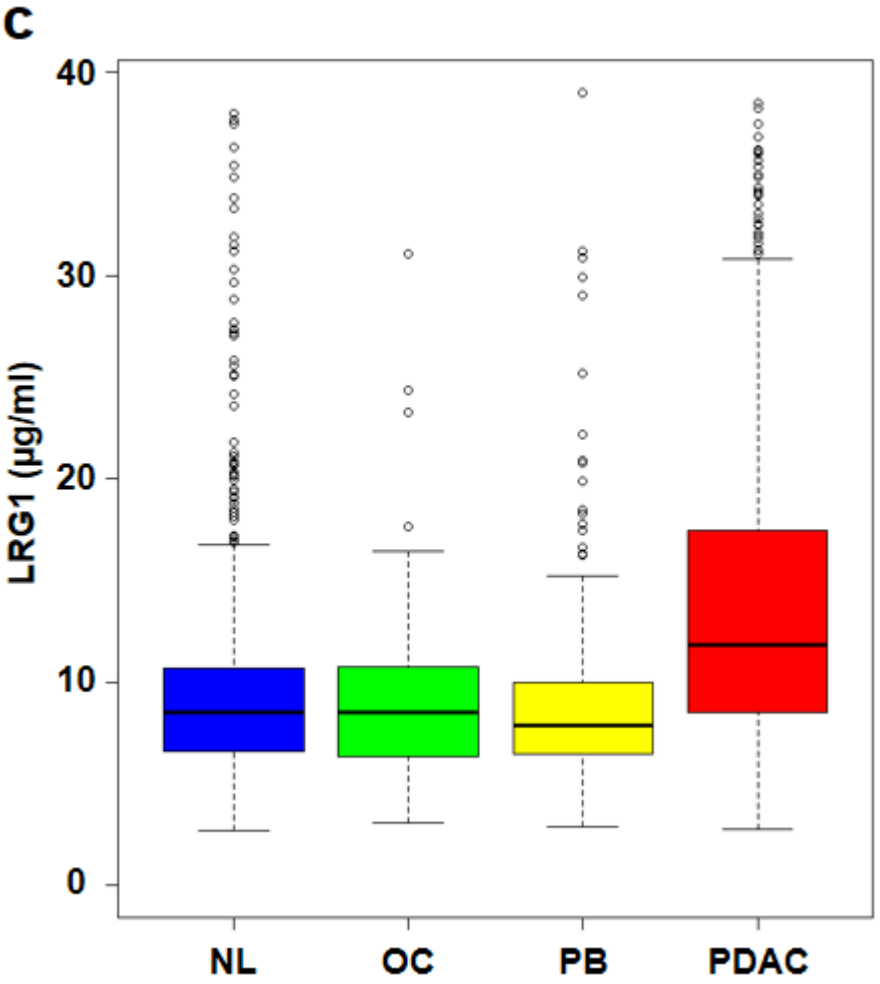


Figure 1d. Box plots for log-transformed of TTR. (NL; Normal control, OC; Other cancer, PB; Pancreatic benign disease, PDAC; Pancreatic ductal adenocarcinoma)

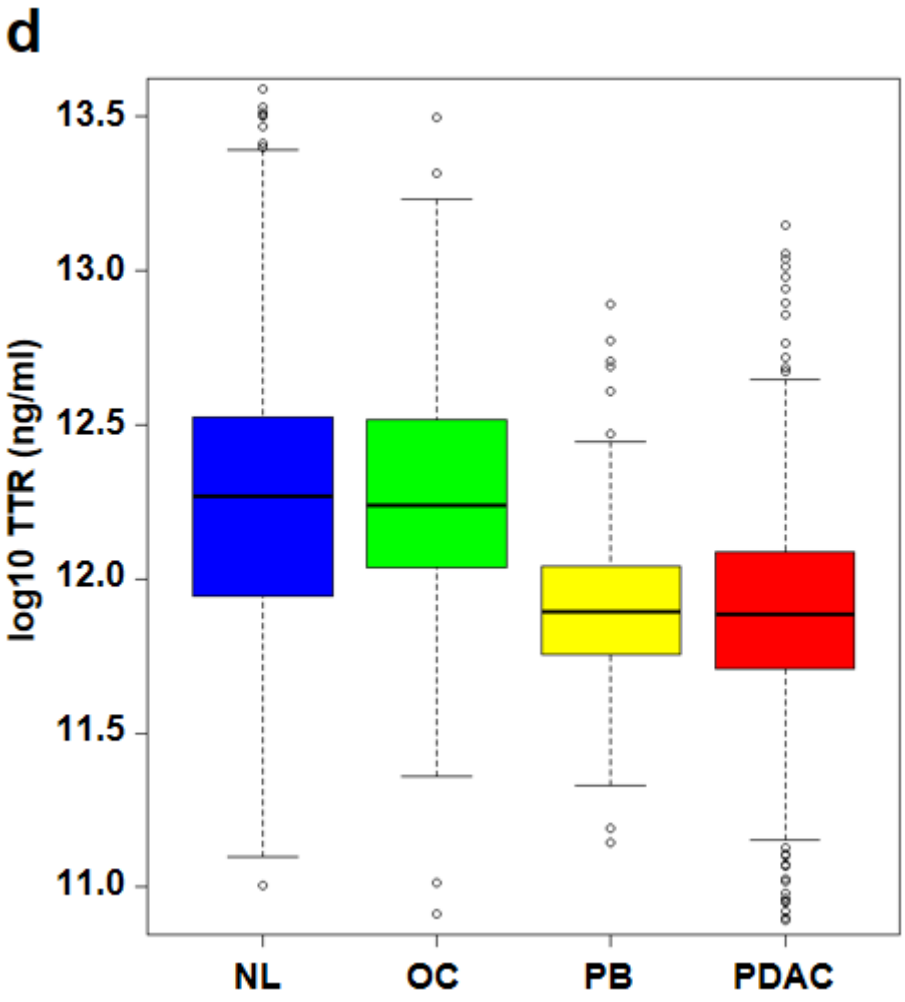


Figure 1e. Box plots for log-transformed of CA 19-9. (NL; Normal control, OC; Other cancer, PB; Pancreatic benign disease, PDAC; Pancreatic ductal adenocarcinoma)

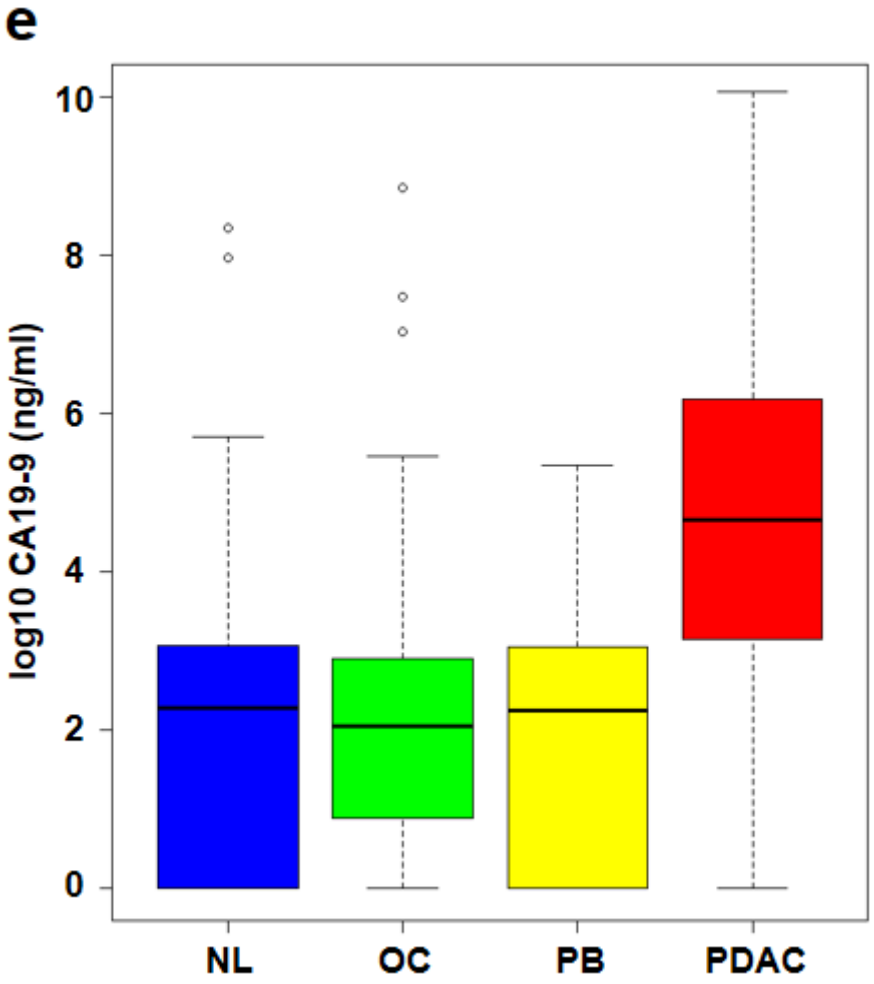
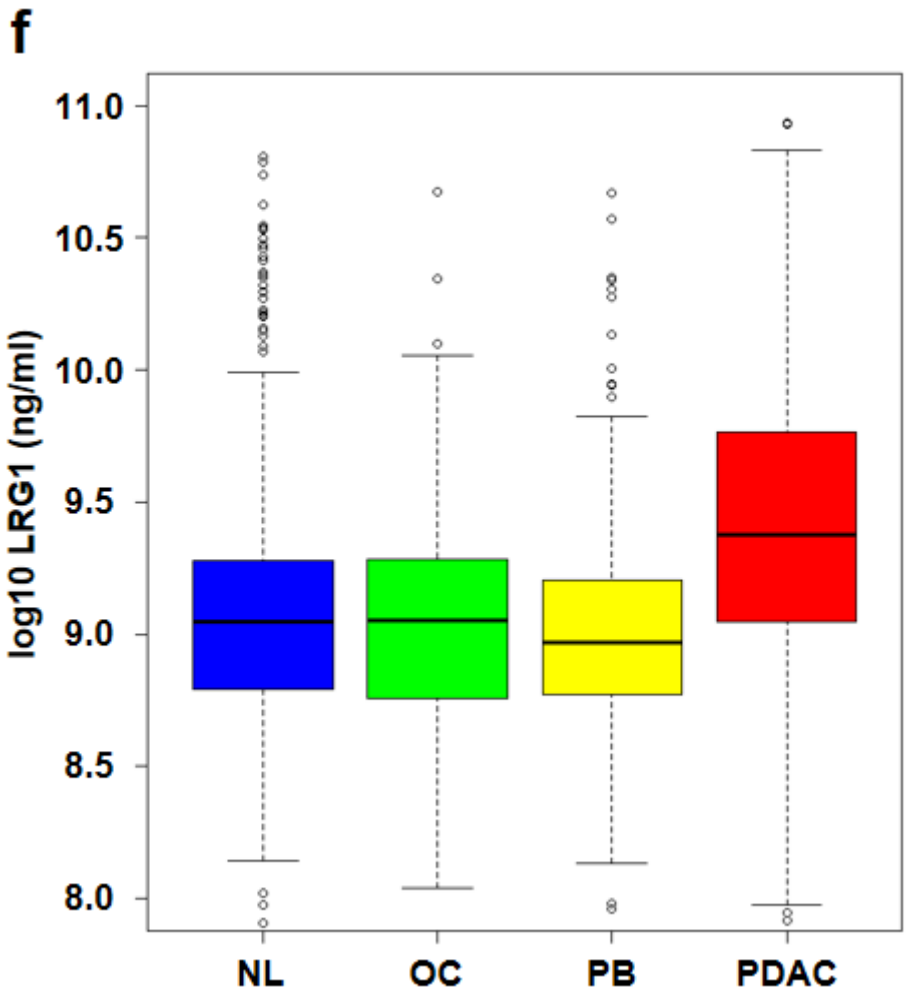


Figure 1f. Box plots for log-transformed of LRG1. (NL; Normal control, OC; Other cancer, PB; Pancreatic benign disease, PDAC; Pancreatic ductal adenocarcinoma)



Next, using the logistic regression (LR) method, a model developed on the training data set was used to obtain the predicted value for PDAC, and the result was classified into one of the three risk groups: low, intermediate, and high. To determine the risk groups, the predicted value for PDAC obtained through the model was divided using a combination of two thresholds. The optimal threshold was determined using four measures for the three risk groups: positive predictive values (PPV), negative predictive values (NPV), sensitivity (Sen), and specificity (Spe). The four measures were used as evaluation indicators for the predictive model. In this study, emphasis was placed on determining the cut-off value when the four measures attained a certain value at the same time.

Lastly, as the most important part of this study, it was confirmed that NL and PDAC data-based models showed a good performance in tests using OC and PB data. For this, model verification was conducted using large amount of data of various types including NL, PDAC, OC, and PB as test data sets. At this time, NL vs. PDAC, OC vs. PDAC, PB vs. PDAC, and non-PDAC (all test data set) vs. PDAC data set were each tested to compare and analyze the performance of the model for each type of data set.

2.2. Study population

A total of 1,991 samples were collected between January 2011 and September 2019, of which 609 were NL, 145 were OC, 314 were PB, and 923 were PDAC. Whole blood samples were collected in 10 ml syringe prior to surgery using standard blood collection technique, and they were stored in EDTA tubes. Samples were centrifuged at 3000 g for 5 min, after which supernatants were collected and stored at -80℃. Control blood samples were obtained from

609 healthy individuals who visited the hospital for medical check-ups and agreed to participate in the study. They have no evidence of pancreatic benign disease, acute inflammation in any organ, and malignant neoplasm including pancreatic cancer. These data were collected from seven centers in Korea including the Seoul National University Hospital (SNUH), Seoul National University Hospital Healthcare System Gangnam Center (SNUH HSGC), National Cancer Center (NCC), Asan Medical Center (AMC), Samsung Medical Center (SMC), Yonsei Severance Hospital (YSH), and Ewha Womans University Medical Center (EUMC) (Table 2).

Table 2. The number of samples by institute

Institute	NL	OC	PB	PDAC	All
Seoul national university hospital	563	145	40	294	1,042
Asan medical center	0	0	157	254	411
National cancer center	0	0	0	128	128
Samsung medical center	0	0	90	200	290
Yonsei severance hospital	0	0	27	47	74
Ewha womans university medical center	46	0	0	0	46
Total	609	145	314	923	1,991

NL, normal; OC, other cancer; PB, pancreatic benign; PDAC, pancreatic ductal adenocarcinoma.

This multi-center study was approved by the institutional review boards of all participating institutions *(SNUH, H-1703-005-835, YSH; 4-2013-0725, NCC; NCCNCS13818, SMC; 2008-07-065, AMC; 2013-1061, EUMC; 2018-05-030).* Bio-specimens were collected from participants who provided informed consent.

The development of the predictive model was carried out using the LR method using age, sex, and the three ELISA markers, TTR, CA 19-9, and LRG1 as covariates. The predicted values were classified into low-, intermediate-, and high-risk groups using a combination of two thresholds δ_1 and δ_2. The optimal threshold combination was the one that yielded the highest average for the four measures NPV, PPV, Sen, and Spe when all four measures exceeded the cut-off value simultaneously. The four measures were modified for the three risk groups by considering only the high- and low-risk groups for simplicity. The four modified measures were calculated without the intermediate group as follows:

$$NPV = \frac{n_{11}}{n_{11} + n_{21}}, PPV = \frac{n_{23}}{n_{13} + n_{23}}, Sen = \frac{n_{23}}{n_{21} + n_{23}}, Spe = \frac{n_{11}}{n_{11} + n_{13}}.$$

For NL, n_{11} represents the count of predicted probability smaller than δ_1, n_{12} the count between δ_1 and δ_2, and n_{13} the count larger than δ_2. For PDAC, n_{21}, n_{22}, and n_{23} represent the corresponding counts, respectively. Cut-off is defined as the value when all the four measures obtain a certain value, such as 90% at minimum. The mean of the four measures and the number of intermediates were determined by selecting a threshold combination. Since the four measures were calculated excluding the intermediate group, performance was highly dependent on the count of the intermediate group. In this study, the cut-off values ranging

85% to 95% were checked in increments of 1 unit following the various threshold combinations.

2.3. Statistical analysis

The demographic analysis was performed using R ver. 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria). It was also used to create the graphical representations. The categorical variables of the non-PDAC (NL, OC, and PB) and PDAC data groups were compared using the chi-square test. Continuous variables were summarized using the means and standard deviations or median with range (25-percentile to 75-percentile) and compared using the F-test and Student's/Welch's t-test. Two-sided p-values < 0.05 were considered significant.

Chapter 3. Results

3.1. Characteristics of the study population

Participants were categorized into four groups based on the diagnosis as NL (n=609, 30.6%), OC (n=145, 7.3%), PB (n=314, 15.7%), and PDAC (n=923, 46.4%). The NL, OC, and PB groups were together clubbed into the non-PDAC group (n=1068, 53.6%). Demographics of the participants in the non-PDAC and PDAC groups are summarized in table 3. Significant differences were observed in age (Non-PDAC; 55.5 ± 12.0 versus PDAC; 63.1 ± 9.9 years, $p < 0.001$), sex ratio (females: n=474, 44.4% vs. males: n=561, 60.8%, $p < 0.001$), body mass index (23.6 ± 3.2 versus 22.9 ± 3.0 kg/m^2, $p = 0.001$), level of initial CA 19-9 (19.0 ± 98.6 versus 679.0 ± 1348.9 U/ml, $p < 0.001$), and level of LRG1, TTR, CA 19-9 in automated ELISA triple marker panel between the non-PDAC and PDAC groups. Levels of LRG1 and CA 19-9 were higher in the PDAC group than in the non-PDAC group, whereas the level of TTR was lower in the PDAC group than in the NL and OC groups. In the PDAC group, 39 (4.2%) patients were stage I, 618 (67.0%) patients were stage II, 52 (5.6%) patients were stage III, and 214 (23.2%) patients were stage IV. There was no non-invasive intra-ductal papillary mucinous carcinoma and intra-ductal tumor in the PDAC group.

Table 3. Demographics of study population

	Total	Non-PDAC			Non-PDAC	PDAC	*p*-value[#]
		NL	OC	PB			
Number	1991 (100)	609 (30.6)	145 (7.3)	314 (15.7)	1068 (53.6)	923 (46.4)	
Age, years	59.0 ± 11.7	56.4 ± 11.0	55.2 ± 11.5	53.9 ± 13.8	55.5 ± 12.0	63.1 ± 9.9	< 0.001
Sex							< 0.001
Male	1035 (52.0)	322 (52.9)	37 (25.5)	115 (36.6)	474 (44.4)	561 (60.8)	
Female	956 (48.0)	287 (47.1)	108 (74.5)	199 (63.4)	594 (55.6)	362 (39.2)	
BMI, kg/m^2	23.3 ± 3.1	23.8 ± 2.9	23.3 ± 3.3	23.2 ± 3.8	23.6 ± 3.2	22.9 ± 3.0	0.001
Initial CA 19-9, U/ml		11.7 ± 33.3	61.7 ± 261.9	14.6 ± 23.7	19.0 ± 98.6	679.0 ± 1348.9	< 0.001
Initial CEA, ng/ml		1.2 ± 0.8	26.6 ± 137.2	2.0 ± 3.2	7.4 ± 67.2	29.6 ± 231.5	0.087
Automated ELISA triple marker panel							
LRG1, ng/ml[*]		11324 (8348-16281)	11427 (8531-15321)	9351 (7341-12585)	10697 (7909-15138)	16836 (11182-25638)	< 0.001
TTR, ng/ml[*]		286200 (154548-432460)	302800 (254720-342560)	151525 (127697-215568)	227910 (146600-365920)	150112 (123266-230000)	< 0.001
CA 19-9, U/ml[*]		11.0 (6.5-18.8)	10.6 (7.9-17.3)	10.8 (5.3-21.1)	10.8 (6.6-19.2)	118.6 (26.5-537.9)	< 0.001

PDAC, pancreatic ductal adenocarcinoma; NL, normal; OC, other cancer; PB, pancreatic benign; BMI, body mass index; CEA, carcinoembryonic antigen; CA, carbohydrate antigen;

ELISA, enzyme-linked immunosorbent assay; LRG, leucine rich alpha 2 glycoprotein; TTR, transthyretin.

Data are expressed as n (%) or mean ± standard deviation unless indicated otherwise.

*Values are expressed as median (range;25 percentile to 75 percentile).

#p-value was evaluated between the Non-PDAC and PDAC groups.

3.2. Diagnostic model and determining cut-off value

An LR-based prediction model was created using NL and PDAC data from an automated multi-panel ELISA kit. The five covariates used to create the LR model were sex, age, and the three biomarkers TTR, CA 19-9, and LRG1. The fitted LR model is represented as follows:

$$\log\left(\frac{P(PDAC)}{1-P(PDAC)}\right) = 51.03 + 0.04Age + 1.19(Sex\ M),$$

$$-5.12\log(TTR) + 0.61\log(CA19-9) + 0.80\log(LRG1).$$

Through the model thus obtained, the predicted value of PDAC incidence for each sample of the training data was obtained and classified into low, intermediate, and high-risk groups using two thresholds (Figure 2a and 2b). In order to establish the feasibility for clinical use along with better performance, the optimal threshold combination was evaluated. We checked the cut-off values from 85% to 95%. Considering the mean of the four measures, and the sample numbers of low-, intermediate-, and high-risk groups, the threshold combination of 0.22 and 0.88 when all four measure values exceeded 90% were selected. The mean of the four measures at this time was 92.29, and the values of PPV, NPV, Sen, and Spe were 94.11, 90.40, 93.81, and 90.86, respectively. At threshold combinations of 0.22 and 0.88, the cut-off was 90%, and the number of samples in the high-, intermediate-, and low-risk groups were 306, 569, and 198, respectively (Table 4).

Figure 2a. The box plot (a) shows that the predicted values from prediction model using training data set. The predicted value using data set by all types (c and d) and each type (e and f) are also shown. Sky blue and orange line indicate threshold value (δ_1, δ_2) = (0.22, 0.88) at cut-off 90%. (NL; Normal control, PDAC; Pancreatic ductal adenocarcinoma)

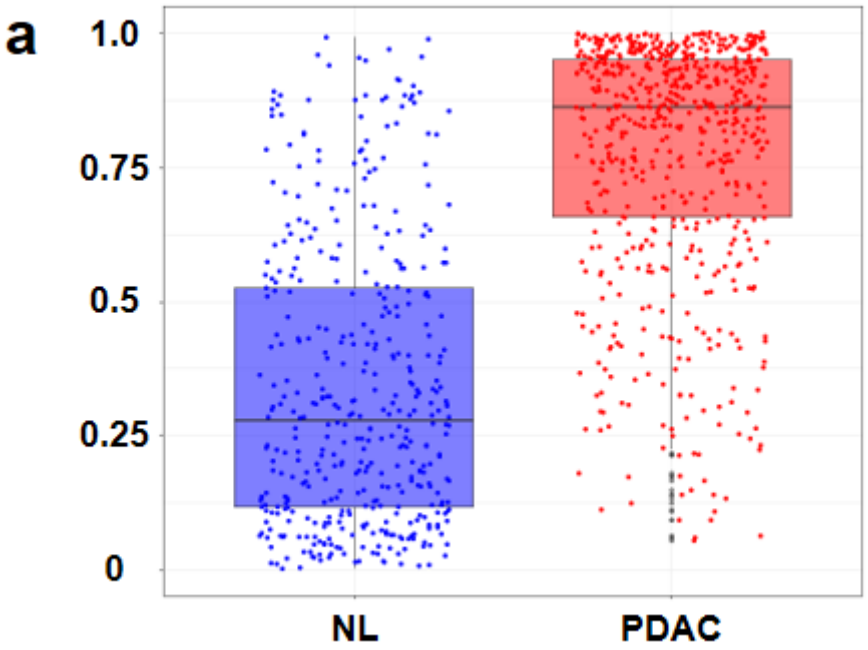


Figure 2b. The density plot (b) shows that the predicted values from prediction model using training data set. Sky blue and orange line indicate threshold value $(\delta_1, \delta_2) = (0.22, 0.88)$ at cut-off 90%. (NL; Normal control, PDAC; Pancreatic ductal adenocarcinoma)

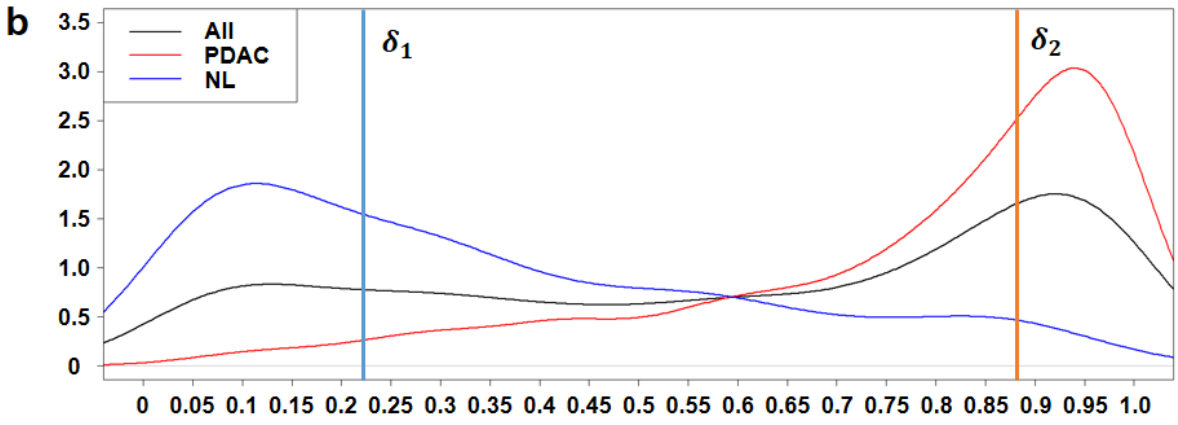


Table 4. Cut-off values of measures, threshold change and number of risk groups from training data set

Measures > cut-off	95%	94%	93%	92%	91%	**90%**	89%	88%	87%	86%	85%
δ_1	0.09	0.12	0.14	0.16	0.21	**0.22**	0.25	0.26	0.29	0.3	0.32
δ_2	0.96	0.94	0.92	0.89	0.89	**0.88**	0.87	0.86	0.82	0.8	0.79
PPV (percent)	97.72	96.27	96.62	95.84	95.84	**94.11**	92.63	92.63	91.93	91.37	91.11
NPV (percent)	96.47	94.06	93.43	92.15	91.5	**90.40**	89.23	88.93	87.00	86.09	85.51
Sensitivity (percent)	97.72	96.27	96.21	95.84	94.53	**93.81**	92.63	92.89	91.93	91.37	90.70
Specificity (percent)	96.47	94.06	94.11	92.15	93.54	**90.86**	89.23	88.54	87.00	86.09	86.12
Measures mean (percent)	97.09	95.17	95.097	94.00	93.87	**92.29**	90.93	90.75	89.46	88.73	88.36
High (n)	132	188	237	289	289	**306**	326	353	409	429	439
Intermediate (n)	856	767	699	631	594	**569**	524	494	410	378	351
Low (n)	85	118	137	153	190	**198**	223	226	254	266	283

PPV, positive predictive values; NPV, negative predictive values.

3.3. Verification using various types of test data sets

To evaluate the performance of the predictive model derived from the training data set, it was verified using the test data. During this, by using the PB and OC types data in addition to NL and PDAC, it was demonstrated that the model had reliable performance prediction ability even when various types of data were provided. First, during modeling using training data, four measure values at various threshold combinations and sample numbers of low -, intermediate -, and high-risk groups were identified. It was found that at the threshold combination of 0.22 and 0.88, which was the optimal threshold combination, the mean of the four measures was 89.68. This was not significantly different from 92.19 when training data was used ($p = 0.573$) (Table 5). The values of PPV, NPV, Sen, and Spe were 79.11, 97.13, 94.69, and 87.77, respectively, showing an overall good performance except for the PPV.

Table 5. Cut-off values of measures, threshold change and number of risk groups from test data set

Measures > cut-off	95%	94%	93%	92%	91%	**90%**	89%	88%	87%	86%	85%
δ_1	0.09	0.12	0.14	0.16	0.21	**0.22**	0.25	0.26	0.29	0.3	0.32
δ_2	0.96	0.94	0.92	0.89	0.89	**0.88**	0.87	0.86	0.82	0.8	0.79
PPV (percent)	84.28	86.66	83.62	80.28	80.28	**79.11**	76.78	75.97	72.97	71.48	70.23
NPV (percent)	99.07	98.60	98.24	98.43	97.00	**97.13**	96.76	96.79	95.51	95.63	94.97
Sensitivity (percent)	98.33	97.50	97.0	97.43	94.21	**94.69**	93.47	93.79	92.04	92.51	91.23
Specificity (percent)	90.67	92.15	89.83	87.09	89.01	**87.77**	87.33	86.34	83.24	81.64	81.06
Measures mean (percent)	93.09	93.73	92.17	90.81	90.13	**89.68**	88.59	88.22	85.94	85.32	84.37
High (n)	70	90	116	142	142	**158**	168	179	222	242	252
Intermediate (n)	740	685	631	584	542	**516**	472	458	384	355	328
Low (n)	108	143	171	192	234	**244**	278	281	312	321	338

PPV, positive predictive values; NPV, negative predictive values.

Next, we checked the accuracy of classifying NL, OC, PB, and PDAC into one of the three risk groups (Table 6). When 0.22 and 0.88 were selected as the threshold combination, the number of samples belonging to the high-, intermediate-, and low-risk groups for NL type was 8, 92, and 83, respectively. On the other hand, the corresponding numbers for PDAC type were 125, 144, and 7. Eight NL samples belonged to the high-risk group and the PDAC samples belonging to the low-risk group were seven. Therefore, we confirmed that our proposed prediction model has good prediction performance for PDAC. These results are demonstrated through the box plot and density plot (Figure 2c, 2d, 2e, and 2f).

Table 6. Number of samples in each risk group according to various cut-off values

Group	Low	Inter	High	Low	Inter	High	Low	Inter	High	Low	Inter	High
Cut-off		0			95%			94%			93%	
NL	21	160	2	42	138	3	52	127	4	63	115	5
OC	27	115	3	41	100	4	50	91	4	59	81	5
PB	12	302	0	24	286	4	39	271	4	46	259	9
PDAC	0	264	12	1	216	59	2	196	78	3	176	97
Cut-off		92%			91%			**90%**			89%	
NL	72	105	6	81	96	6	**83**	**92**	**8**	96	77	10
OC	63	74	8	72	65	8	**76**	**60**	**9**	82	53	10
PB	54	246	14	74	226	14	**78**	**220**	**16**	91	204	19
PDAC	3	159	114	7	155	114	**7**	**144**	**125**	9	138	129
Cut-off		88%			87%			86%			85%	
NL	97	75	11	105	65	13	108	59	16	113	54	16
OC	82	53	10	93	39	13	94	37	14	99	31	15
PB	93	199	22	100	180	34	105	170	39	109	161	44
PDAC	9	131	136	14	100	162	14	89	173	17	82	177

NL, normal; OC, other cancer; PB, pancreatic benign; PDAC, pancreatic ductal adenocarcinoma; Inter, intermediate.

Figure 2c. The predicted value using data set by all types (c) and each type are also shown. (PDAC; Pancreatic ductal adenocarcinoma)

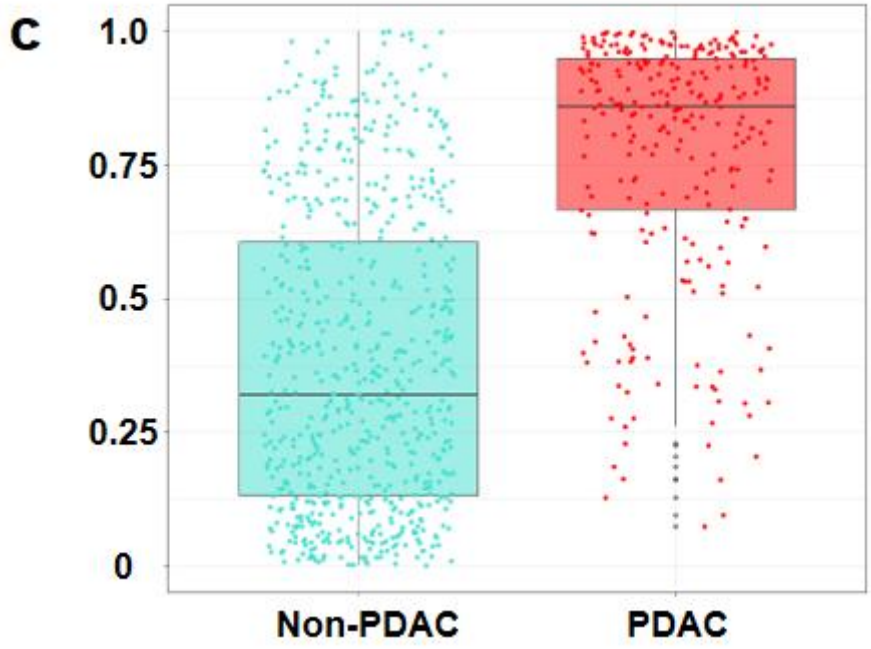


Figure 2d. The predicted value using data set by all types (d) and each type are also shown. Sky blue and orange line indicate threshold value $(\delta_1, \delta_2) = (0.22, 0.88)$ at cut-off 90%. (PDAC; Pancreatic ductal adenocarcinoma)

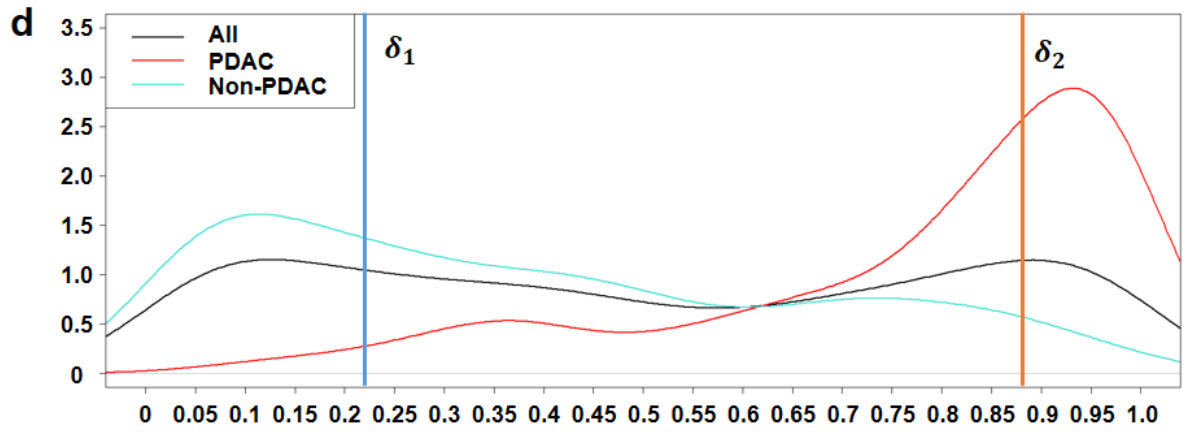


Figure 2e. The predicted value using data set by all types and each type (e) are also shown. (NL; Normal control, OC; Other cancer, PB; Pancreatic benign disease, PDAC; Pancreatic ductal adenocarcinoma)

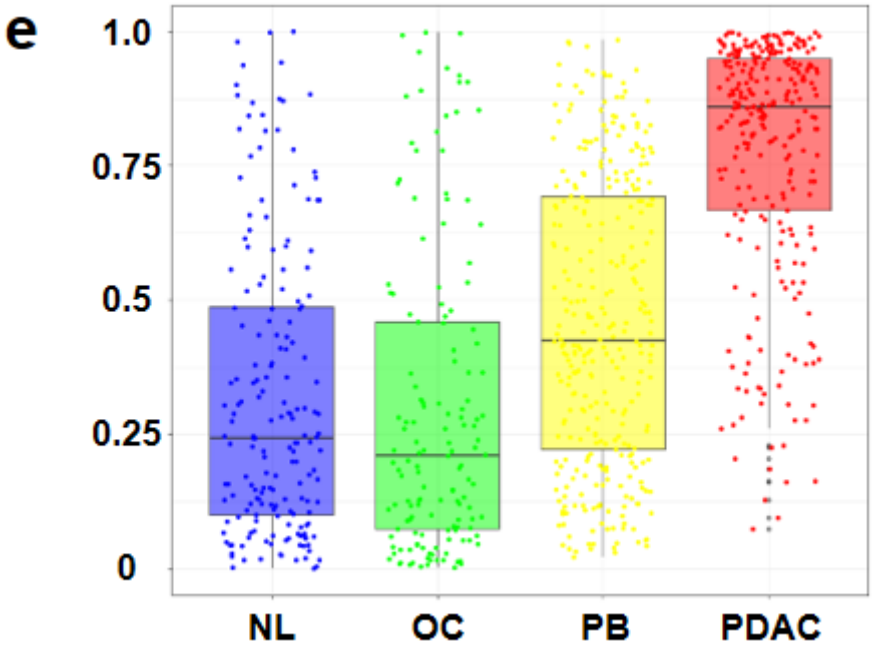
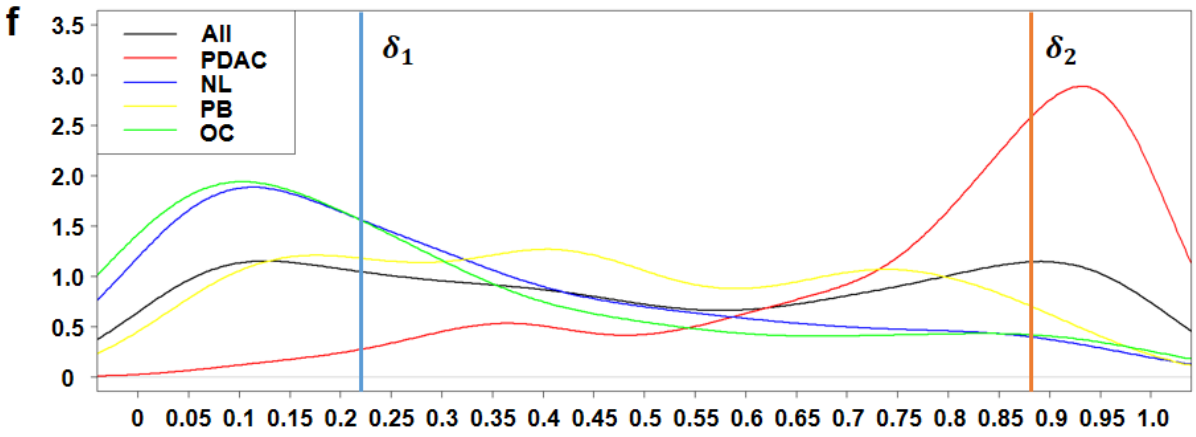


Figure 2f. The predicted value using data set by all types and each type (f) are also shown. Sky blue and orange line indicate threshold value $(\delta_1, \delta_2) = (0.22, 0.88)$ at cut-off 90%. (NL; Normal control, OC; Other cancer, PB; Pancreatic benign disease, PDAC; Pancreatic ductal adenocarcinoma)



Lastly, we compared the mean of the four measures by using test data of each data type (Table 7). The prediction model had been trained on NL and PDAC data, but we intended to check if the model had effective performance in other data categories such as PB and OC. For this purpose, the test data of each type were compared in pairs, such as NL vs. PDAC, OC vs. PDAC, PB vs. PDAC, and non-PDAC (NL, OC, and PB) vs. PDAC. The mean of the four measures was highest at 93.02 when verified with the test data set of NL and PDAC type combination. In contrast, the mean was the lowest at 89.52 when PB and PDAC type data were used. In case of non-PDAC vs. PDAC the mean was 89.68, showing a moderate performance between NL vs. PDAC and PB vs. PDAC. This result indicates that PB has intermediate properties between NL and PDAC, as shown in the above table, density plot, and box plot. Based on these results, it was confirmed that OC has characteristics close to NL and that PB does not belong to either NL or PDAC.

Finally, even when different types of data including NL, PDAC, OC, and PB were tested, the proposed prediction model demonstrated satisfactory performance.

Table 7. Performance measures of triple markers for discriminating various groups and number of corresponding risk groups. For all performance measures, the 90% cut-off value was used.

Performance Measure	Training data	Test data			
	NL vs PDAC	NL vs PDAC	OC vs PDAC	PB vs PDAC	non-PDAC vs PDAC
PPV (percent)	94.12	93.99	93.28	88.65	79.11
NPV (percent)	90.40	92.22	91.57	91.76	97.13
Sensitivity (percent)	93.81	94.7	94.7	94.7	94.7
Specificity (percent)	90.86	91.21	89.41	82.98	87.78
Measures mean (percent)	92.30	93.03	92.24	89.52	89.68
High (n)	306	133	134	141	158
Intermediate (n)	569	236	204	364	516
Low (n)	198	90	83	85	244

PPV, positive predictive values; NPV, negative predictive values; NL, normal; OC, other cancer; PB, pancreatic benign; PDAC, pancreatic ductal adenocarcinoma.

3.4. Comparison by stage of PDAC and normal range of CA 19-9 group

The proposed model showed similar performance in stage I or II of PDAC compared with all stage of PDAC in table 8. The values of PPV, NPV, Sen, and Spe were 91.75, 93.26, 93.68, and 91.21, respectively in stage I or II of PDAC. In addition, table 9 showed that even in the group of PDAC with normal range of CA 19-9, the performance of the triple-marker model was superior to that of the single marker model (The values of PPV, NPV, Sen, and Spe; 52.94, 94.32, 64.29, and 91.21 in triple markers versus 40.74, 77.33, 39.29, and 78.38 in single marker).

Table 8. Performance measures of triple markers for discriminating various stage of PDAC group and number of corresponding risk groups. For all performance measures, the 90% cut-off value was used.

Performance Measure	Training data	Test data		
	NL vs PDAC	NL vs PDAC	NL vs PDAC stage I or II	NL vs PDAC stage III or IV
PPV (percent)	94.12	93.99	91.75	81.82
NPV (percent)	90.40	92.22	93.26	98.81
Sensitivity (percent)	93.81	94.70	93.68	97.30
Specificity (percent)	90.86	91.21	91.21	91.21
Measures mean (percent)	92.30	93.03	92.48	83.91
High (n)	306	133	97	44
Intermediate (n)	569	236	189	130
Low (n)	198	90	89	84

PPV, positive predictive values; NPV, negative predictive values; NL, normal; PDAC, pancreatic ductal adenocarcinoma.

Table 9. Performance measures of triple and single marker for discriminating NL and PDAC with normal range of CA 19-9 (< 37 U/mL) along with number of corresponding risk groups. For all performance measures, the 90% cut-off value was used.

	Triple markers (LRG1, TTR, CA 19-9)			Single marker (CA 19-9)		
Performance Measure	Training data	Test data		Training data	Test data	
	NL vs PDAC	NL vs PDAC	NL vs PDAC with normal range of CA 19-9	NL vs PDAC	NL vs PDAC	NL vs PDAC with normal range of CA 19-9
PPV (percent)	94.12	93.99	52.94	91.14	91.16	40.74
NPV (percent)	90.40	92.22	94.32	76.40	77.33	77.33
Sensitivity (percent)	93.81	94.7	64.29	91.34	90.66	39.29
Specificity (percent)	90.86	91.21	91.21	75.93	78.38	78.38
Measures mean (percent)	92.30	93.03	75.69	83.70	84.38	58.93
High (n)	306	133	17	440	181	27
Intermediate (n)	569	236	169	472	203	172
Low (n)	198	90	88	161	75	75

PPV, positive predictive values; NPV, negative predictive values; NL, normal; PDAC, pancreatic ductal adenocarcinoma; CA 19-9, carbohydrate antigen 19-9.

Chapter 4. Discussion

In this large-scaled multi-center study, we introduced a prediction model for PDAC diagnosis using a multi-marker panel consisting of LRG1, TTR, and CA19-9 developed by conventional immunoassays and a high-throughput assay, followed by an advanced statistical machine-learning approach. The multi-marker panel was validated using bio-specimens of non-PDAC (NL, OC, PB), and PDAC in a large-scale cohort. Validated across various types of data including NL, OC, PB, and PDAC, the current proposed prediction model demonstrated satisfactory performance as a diagnostic screening tool for PDAC.

A multi-marker panel has been developed and used in the field of ovarian cancer prior to pancreatic cancer. Ova1 is a multivariate index assay approved by the FDA, comprising a 5-serum protein biomarker panel (CA125-II, transferrin, beta-2 microglobulin, apolipoprotein A-1, and transthyretin) for the triage of patients with pelvic mass into low- or high-risk ovarian cancer [12]. The Ova1 performance score produced 96% Sen at 35% Spe in 590 women slated for resection of ovarian tumor [13]. In addition, the FDA approved the next generation of Ova1, the Overa in 2016, exhibiting 91% Sen and 69% Spe [13]. However, there are no similar approved screening tools for the diagnosis of PDAC in clinical practice.

Several biomarkers for diagnosis of PDAC have been introduced through several studies. The CancerSEEK test using a PCR based assay showed an accuracy of 81-87%, a Sen of 70%, and a performance of 40% in the case of early stage of PDAC [14]. Glypican-1 showed 82.1% Sen and 76% Spe [15]. Of the 246 PDAC patients participating in the study, stage I was 4 and stage II was 187, and the control group included 26 patients with benign pancreatic

disease [15]. ApoA2 showed an AUC of 0.809 and did not show any difference from PDAC in patients with chronic pancreatitis and acute benign biliary obstruction [16]. Of the 131 PDAC patients who participated in this study, stage I was 2 and II was 10 [16]. The study using CEMIP involved 324 PDAC patients, of which 170 were stage IV [17]. The performance of CEMIP showed 92.9% Sen and 59.2% Spe [17]. The study using thrombospondin-2 showed an AUC of 0.832, 33.3% Sen, and 96.2% Spe in phase 2a involving 81 PDAC patients [18]. Phase 2b with 197 PDAC patients showed an AUC of 0.887, 58.3% Sen and 93.5% Spe [18].

The current triple marker panel consists of TTR, LRG1, and CA 19-9. TTR is synthesized by pancreatic islets and is involved in pancreatic β-cell death and insulin release [19]. Moreover, TTR is decreased in type 1 diabetes mellitus, yet is highly abundant in pancreatic juice because the pancreatic islets are destroyed, allowing proteins to leak into the pancreatic ductal system [20]. PDAC patients often experience malnutrition, lowering the levels of TTR, which is involved in energy intake, acute or chronic disease states, nutritional status, and inflammatory processes [21]. LRG1 levels are elevated in the blood of patients with non-small-cell lung cancer, ovarian cancer, colorectal cancer, and gliomas through transforming growth factor beta (TGF-β) signaling, which promotes endothelial cell proliferation and angiogenesis [22-26]. In addition, LRG1 is associated with endothelial dysfunction, arterial stiffness, and peripheral arterial disease in patients with type 2 diabetes [27]. LRG1 levels were elevated in the blood of patients with PDAC because of TGF-B signaling [28].

In the current study, the performance of triple-marker (LRG1, TTR, CA 19-9), double-marker (LRG1, CA 19-9), and single marker (CA 19-9) models was compared. The performance of

the model was superior in the order of triple-marker, double-marker, and single marker (Measures mean; 92.30 versus 86.29 versus 83.70). Due to the characteristics of TTR, there was a difference between the normal and PDAC groups, and the OC and PDAC groups, which may be the reason why the performance of the triple markers model is superior to that of the double or single marker models.

Recently, Mellby et al. reported a case-control study using a Scandinavian cohort, consisting of 16 patients with stage I, 132 patients with stage II, 65 patients with stage III, and 230 patients with stage IV PDAC, and 888 controls using an antibody microarray platform to identify the serum biomarker signature associated with early stage PDAC [11]. The biomarker signature could discriminate patients with stage I and II PDAC from controls in this independent patient cohort with an AUC of 0.96. However, the study had several limitations such as cost-effectiveness and lack of simplified usage. Moreover, the previous studies were not validated using patients with benign pancreatic disease and other malignancies excluding PDAC [11, 29]. In the current study, to ensure practical utility of biomarker panels, ELISA was used, making it minimally invasive and cost-effective as compared to MRM-MS. Through the use of ELISA, the platform is standardized, and can be used universally at a low cost and easier quality assurance.

Kim Y et al. reported a multi-center study to develop a diagnostic, multi-marker panel for PDAC using a targeted proteomics approach [30]. A total of 959 plasma samples from patients at multiple medical centers were used, and the multi-marker panel was developed using a training set comprised of 261 PDAC cases and 290 controls [30]. In this study, a multi-marker panel containing 14 proteins was developed. A multi-marker panel, consisting

of 14 protein markers [clusterin (CLU), complement C5 (C5), plasma kallikrein (KLKB1), platelet basic protein (PPBP), IFN-related developmental regulator 1 (IFRD1), IGF-binding protein 2 (IGFBP2), intercellular adhesion molecule 1 (ICAM1), C4b-binding protein alpha chain (C4BPA), receptor-type tyrosine-protein phosphatase eta (PTPRJ), extracellular matrix protein 1 (ECM1), vimentin (VIM), C4b-binding protein beta chain(C4BPB), plasma serine protease inhibitor (SERPINA5), transthyretin (TTR)], was selected, and the predicted probability of PDAC cases according to the multi-marker panel was calculated, as follows: Logit (p) = 0.1789 + (–1.6768 × CLU) + (1.5854 × C5) + (–1.9541 × KLKB1) + (2.0778 × PPBP) + (0.7881 × IFRD1) + (1.2657 × IGFBP2) + (1.3406 × ICAM1) + (1.5931 × C4BPA) + (–0.5218 × PTPRJ) + (–1.14 × ECM1) + (0.8387 × VIM) + (–1.2183 × C4BPB) + (0.6271 × SERPINA5) + (–1.0129 × TTR) [30]. The multi-marker panel achieved area under the curves (AUCs) of 0.977 and 0.953 for the training set and validation set, respectively. However, the control group used for the independent validation set consisted of patients with benign pancreatic disease; therefore, this validation set was unable to evaluate the discriminating performance of the panel to distinguish patients with PDAC from the healthy control group [30]. This study also mentioned the proposed multi-marker panel must be validated externally to determine its reproducibility across laboratories [30].

Kim H et al. reported a single center cohort study using six biomarkers (ApoA1, CA125, CA19-9, CEA, ApoA2, and TTR) to develop diagnostic algorithms and validate their performance in the diagnoses of PDAC [31]. Blood samples from 180 PDAC patients and 573 healthy controls were used. The AUC, specificity, and sensitivity were 0.992, 95%, and 96%, respectively, in the training set. Meanwhile, the measures were 0.993, 96%, and 93% in

the validation set. Because the PDAC patient group contains patients who had surgery for pancreatic cancer, only 29.5 % of total patients were stage 3 or 4. The authors mentioned a large-scale multi-center study is needed [31].

The current study used a diagnostic algorithm including age, sex, and triple markers to differentiate PDAC from non-PDAC. The algorithm for risk calculation provides a risk stratification to identify the actual likelihood of malignancy. At threshold combinations of 0.22 and 0.88, the cut-off was 90%, and the number of samples in the low-, intermediate-, and high-risk groups were 198, 569, and 306, respectively. Because of the existence of the intermediate group, the high-risk group and the low-risk group are more clearly distinguished, overcoming the shortcomings of a dichotomous classification, such as that using CA 19-9 alone. The intermediate-risk group can serve as a gray zone in the middle of the test values, allowing clinicians to diversify the basis for medical judgement. Therefore, when this multi-marker panel is used in actual clinical practice as a diagnostic screening tool for PDAC, proper management guidelines for each group should be prepared. Additional strategies for targeting each group should be addressed in further studies. The 'Intermediate-risk group' may require re-evaluation using multi-marker panel and/or additional imaging studies including abdominal CT or MRI to confirm the presence of pancreatic neoplasm. If participants are categorized in the high-risk group, they are highly suspicious of having PDAC and thus require more precise examination including abdominal CT or MRI or other interventions for confirming the diagnosis.

The current PDAC prediction model was created through logistic regression analysis. Recent study reported a multi-marker panel for diagnosis of PDAC containing 14 proteins using

MRM-MS [30]. In this study [30], a logistic regression analysis with stepwise selection was performed to build a multi-marker panel using the 44 biomarker candidates. During the stepwise selection procedure, a p-value of < 0.05 was required to allow a biomarker candidate into the model and remain in the model. Using such large-scale data, with various biological and patient-to-patient variations and different distributions in the raw states can challenge the development of an appropriate predictive model [30]. The transformation and normalization of data can reduce the skewness of data distribution, making biomarker candidates more comparable or normally distributed [30]. There was no difference between the logistic regression analysis and the machine learning method in terms of model performance. This study suggests that the preprocessing of large-scale quantitative data can improve the further development of multi-marker panels [30].

As a subgroup of this study, paired data analysis was performed by measuring the expression levels of triple markers (TTR, CA 19-9, LRG1) before and after surgery in 331 patients who underwent pancreatectomy after diagnosis of pancreatic cancer. The levels of triple markers before and after surgery were statistically significantly changed when the expression levels of TTR, CA 19-9, and LRG1 were compared before and after surgery, respectively. TTR and CA 19-9 were significantly decreased after surgery compared to before surgery, and LRG1 was significantly increased after surgery compared to before surgery. After surgery, it was confirmed that the triple marker values of patients change, and how this change affects the probability of diagnosing pancreatic cancer in the actual current prediction model was analyzed. When testing the current logistic regression model, it was confirmed the probability of diagnosing PDAC increased after surgery compared to before surgery. This means when

using the current prediction model, the probability of PDAC does not decrease when the patient's data after surgery is applied. This is because the distribution of markers in patients before surgery is different from that in patients after surgery. As an additional analysis, it was confirmed the distribution of patients before surgery and the distribution of patients after surgery were clustered through principal component analysis, which means the two distributions are different. In other words, the role of biomarker for monitoring recurrence of response to tumor burden after pancreatectomy is not suitable as the current prediction model. Postoperative patient monitoring requires the development and construction of biomarkers different from those used in this model. The TTR used in the current prediction model shows a statistically significant decrease after surgery, which increases the probability of diagnosing PDAC in this model.

The current prediction model had PPV, NPV, Sen, and Spe of 94.12, 90.40, 93.81, and 90.86, respectively, with the mean of the four measures being 92.29. Plasma LRG1 and TTR levels, with CA19-9, had a greater diagnostic value for PDAC than CA19-9 alone [5, 6]. Through this multi-marker panel, the ability to distinguish not only the NL group but also the OC and PB groups versus PDAC makes it more appropriate to be used for screening pancreatic cancer when compared to other studies [11, 29]. Although 10% to 15% of PDAC patients do not express CA19-9 due to the lack of Lewis A antigen, early stage pancreatic cancer can be distinguished by using the current multi-marker panel, thereby improving survival in patients with pancreatic cancer [5, 6]. Finally, the multi-marker panel could lead to early diagnosis, reduce the costs of screening and treatment, and lengthen survival. It could also improve the

quality of life of patients with PDAC because fewer invasive procedures would be performed and ineffective treatments would be withdrawn [6].

Chapter 5. Conclusion

The current study is the first multi-center and large-scale corroboration for constructing a prediction model to be used as screening tool for PDAC diagnosis using a multi-marker panel. This study demonstrates a significant diagnostic performance of the multi-marker panel in distinguishing PDAC from normal, benign pancreatic disease states, and patients with other cancers. The model satisfies the requirements of an ideal screening test, being simple to use, less expensive, and having a good diagnostic efficacy with NPV, PPV, Sen, and Spe, all greater than 90.0%. This model will be used for early diagnosis of pancreatic cancer, particularly in patients with early stage PDAC or patients with normal values of CA 19-9.

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국문 초록

주요어: 진단, 바이오마커 패널, 췌장암, 스크리닝

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췌장암의 장기 생존율을 높이기 위해서는 췌장암의 진단이 매우 중요하다. 하지만 현재까지도 췌장암의 진단을 위한 효과적인 진단 도구의 개발은 요원한 상태이다. 이에 본 연구의 목표는 다중 바이오마커 패널 (LRG1, TTR, CA19-9)을 이용하여 췌장암 진단을 위한 예측 모델을 개발하고 모델의 효용성을 평가하고자 한다.

2011년 1월 1일부터 2019년 9월 30일의 기간 동안 6개의 기관 (서울대학교병원, 국립암센터, 서울아산병원, 삼성서울병원, 연세대학교 세브란스병원, 이화여자대학교병원)으로부터 모집한 총 1991개의 혈액샘플을 사용한다. 이 중 609개의 정상인군, 145개의 기타암종군 (대장암, 갑상선암, 유방암), 314개의 췌장양성질환군, 923개의 췌장암군이다. 위 세 개의 다중 바이오마커 (LRG1, TTR, CA19-9)를 측정하기 위해 개발된 자동화 ELISA (Enzyme-Linked Immunosorbent Assay) kit를 사용하였다.

기존 연구에서는 정상인군과 췌장암군만을 이용하여 췌장암을 예측하는 모델을 만들었으나 본 연구에서는 췌장양성질환, 기타암종군을 추가하였으며 정상인군 및 췌장암군의 샘플 수를 더 추가하여 연구를 진행하였다. 이는 정상인군뿐만 아니라 췌장양성질환군과 기타암종군에 대해 본 모델이 췌장암에 대한 진단 능력을 평가하고자 함이다.

Training 데이터를 이용한 로지스틱 회귀분석 모델을 통해 췌장암 여부를 예측하는 값들(양성 예측률, 음성 예측률, 민감도, 특이도)을 구하였고, 이들 값에 대하여 low, intermediate, high risk 3개의 그룹으로 계층화 하였다. 로지스틱 회귀분석 모델에 사용된 인자로는 성별, 나이, LRG1, TTR, CA19-9 이다.

본 모델을 통하여 측정한 췌장암 진단 예측력은 양성 예측률 (Positive predictive value), 음성 예측률 (Negative predictive value), 민감도 (Sensitivity), 특이도 (Specificity)가 각각 94.12, 90.40, 93.81, 90.86 이다.

본 연구를 통하여 개발한 다중 바이오마커를 이용한 모델의 췌장암 진단 능력은 췌장암 진단을 위한 도구로 사용함에 있어 충분한 진단 능력을 보여준다. 또한 본 모델은 기존 연구에서는 시도되지 않았던 췌장 양성 질환 및 기타 암종을 구별할 수 있는 능력을 보여준다. 본 연구를 통해 개발된 모델을 이용하여 CA 19-9의 수치가 정상 범위를 보이거나 초기 병기의 췌장암 환자들을 포함한 환자들을 대상으로 췌장암의 진단을 위한 도구로 사용함으로써 실제 임상에서 의학적 결정을 내리는데 도움이 될 수 있다.