의학박사 학위논문

Diagnostic risk prediction model for pancreatic cancer using a multi-biomarker panel

바이오마커를 이용한 췌장암 진단 모델

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서울대학교 대학원

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바이오마커를 이용한 췌장암 진단 모델

지도 교수 장 진 영

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Diagnostic risk prediction model for pancreatic cancer using a multi-biomarker panel

Jin-Young Jang

Submitting a Ph.D. Dissertation of
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Graduate School of Medicine
Seoul National University
Surgery Major

Yoo Jin Choi

Confirming the Ph.D. Dissertation written by
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Vice Chair _____
Examiner _____
Examiner _____
Examiner _____

Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) has dismal survival rate due to late detection because of unspecific symptoms, rapid progression of tumor, and resistance to conventional therapies. The current diagnostic method for PDAC is imaging modalities, such as computerized tomography or magnetic resonance imaging, which also delay the early diagnosis due to high cost and invasiveness. Serum-based biomarkers have been used for early detection of cancers. Although many groups have discovered biomarkers for PDAC, biomarkers themselves cannot be applied to the real clinic. It needs a diagnostic model. Therefore, in this study, we developed an automated multi-marker enzyme-linked immunosorbent assay (ELISA) kit using 3 biomarkers (leucine-rich alpha-2-glycoprotein [LRG1], transthyretin [TTR], and CA 19-9) that were previously discovered and proposed a diagnostic model for PDAC based on this kit for clinical usage.

Methods: Individual LRG1, TTR, and CA 19-9 panels were combined into a single automated ELISA panel and tested on 728 plasma samples, including PDAC (n=381) and normal samples (n=347). The diagnostic model was developed using logistic regression according to the automated ELISA kit to predict the risk of pancreatic cancer (high-, intermediate-, and low-risk groups).

Results: The automated multi-marker ELISA kit showed

reproducibility and consistency. The proposed logistic regression model provided reliable prediction results with a positive predictive value of 92.05%, negative predictive value of 90.69%, specificity of 90.69%, and sensitivity of 92.05%, which all simultaneously exceed 90% cutoff value. The thresholds, delta 1 and delta 2, between low, intermediate and high were 32% and 60%.

Conclusion: This diagnostic model based on the triple marker ELISA kit could distinguish PDAC from normal samples well and showed better diagnostic performance than that of previous PDAC markers. It can give an information of the risk of pancreatic cancer, which, on that account, can be used as the diagnostic tool in the cancer screening. In the future, it needs external validation to be used in the clinic.

Keyword: Biomarkers, Enzyme-linked immunosorbent assay, Pancreatic intraductal neoplasms

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Chapter 1. Introduction

1.1. Study Background

Pancreatic cancer is the leading cause of cancer death, which mortality ranked the seventh in the world [1] and the least among major ten cancers in Korea [2]. Its 5-year survival rates were known to 2-9% [3]. The reasons that pancreatic cancer has the most lethal survival are late detection, rapid progression of tumor, and resistance to systemic therapies. Moreover, pancreas itself being close to major vessels which readily lead to vessel invasion and high recurrence rate are chief issues in management of pancreatic cancer. The reason for challenges for early diagnosis is that pancreatic cancer does not usually show specific symptoms at earlier stages, resulting in late diagnosis and advanced stages. About 80-85% of PDAC is unresectable at the time of diagnosis without any curative treatment modalities, which resulted in 5-year survival rate less than 5% [4]. Early diagnosis of any cancer is effective to improve survival rate and prognosis, especially for pancreatic cancer [5]. Although considerable advances have been made in diagnosis and management of PDAC to increase overall survival, there is no effective screening test or treatment other than surgical resection which is the only possible cure for PDAC so far. The current diagnostic modalities for pancreatic cancer are CT- or MRI-based or endoscopic ultrasound biopsy [6], which are not

cost-effective nor non-invasive. Also, only symptomatic patients or high-risk patients (e.g., familial pancreatic cancer) undergo imaging tests. Therefore, it is important to develop a screening test for the general population for early detection of PDAC so that diagnosed patients stand a better chance of survival after surgical resection of the tumor. This screening test should ideally be highly specific (minimizing false positive and negative), cost-effective, fast, simple and less invasive diagnostic modality.

Biomarkers or tumor markers detected in a simple blood test have provided increasing opportunities for screening, early diagnosis, prognosis, and monitoring therapy response for cancers [7,8]. Although many 'potential biomarkers' for PDAC have been discovered [9], the carbohydrate antigen 19-9 (CA 19-9) is the only one approved by the United States Food and Drug Administration (FDA) for pancreatic cancer so far. However, CA 19-9 has a sensitivity of 70-80% and a specificity of 82-90% for PDAC [9,10], and is absent in asymptomatic patients [12], 10-13% of pancreatic cancer patients [12,13] and is not tumor-specific [11]. These numbers are not high enough to be effective for early detection of PDAC. In line with this, there have been increasing efforts to combine some biomarkers to find a multi-marker panel with improved accuracy and higher sensitivity than CA 19-9 alone [14-17].

Despite effort to identify tumor-specific biomarkers, translation of these novel biomarkers into clinical practice has been very limited. To successfully bridge the gap between the laboratory

and clinic, we need precise proteomic quantitative technologies and good analytical performance of the quantitation [18,19]. There are some assays that had been approved by the FDA for certain cancers [20], but none of these were introduced for pancreatic cancer except for CA 19-9. Recently, two studies developed a serum multi-biomarker microarray for the early detection of PDAC that went through external validation on a large cohort [21,22]. However, they were still missing some requirements for an ideal screening test, such as cost-effectiveness and simplified usage.

1.2. Purpose of Research

For early diagnosis of pancreatic cancer, we developed a screening system to be used in the real clinic. First, to satisfy the qualification of the effective screening test, we manufactured a triple-marker ELISA kit which combined three biomarkers, LRG1, TTR, and CA 19-9 into one ELISA kit and generate it under the automated ELISA device for simple usage; this is called the automated triple-marker ELISA kit. We also developed the diagnostic model using the logistic regression model according to the ELISA values from the kit. Furthermore, we classified the prediction rate from the model to three risk groups (low, intermediate, and high) to finally predict the risk of pancreatic cancer.

Chapter 2. Materials and Methods

2.1. Study design

The automated multi-biomarker ELISA kit was developed using three potential biomarkers, leucine-rich alpha-2-glycoprotein (LRG1), transthyretin (TTR), and CA 19-9, which were discovered in the previous study [21]. Park et al. identified them using multiple reaction monitoring-mass spectrometry (MRM-MS), and for which external validation was done at multiple centers [21].

The diagnostic model using the values from the triple-marker ELISA kit was constructed with the logistic regression (LR) method. The numbers that were given from this model were considered as predicted rate of risk of pancreatic cancers. Then, we verified the consistency of the predictors between the automated multi-panel ELISA kit and the individual-marker ELISA panels of the previous study. The correlation between the two datasets was analyzed with the Pearson correlation method.

From the LR diagnostic model, three risk groups were classified using two thresholds. The conditions for finding the optimal combination of two thresholds were evaluated by introducing four measures, negative predictive values (NPV), positive predictive values (PPV), sensitivity, and specificity.

This study was approved by the institutional review boards of all participating institutions (SNUH surgery H-0901-010-267,

SNUH internal medicine H-0412-138-005 and H-0412-138-006, SNUH HSGC H-1305-573-489 and C-1301-095-458, YSH 4-2013-0725, NCC NCCNCS13818, SMC 2008-07-065, AMC 2013-1061) and biospecimens were collected from participants who provided informed consent.

2.2. Study population

A total of 728 samples were collected between January 2011 and December 2013, including 347 NL and 381 PDAC from multiple centers in Korea (Seoul National University Hospital or Seoul National University Hospital Healthcare System Gangnam Center, National Cancer Center, Asan Medical Center, Samsung Medical Center, and Yonsei Severance Hospital). The normal samples were defined by participants who were healthy or those with gallstones or cholecystitis without severe inflammation. They did not possess any malignancies or other serious health conditions. All PDAC were evaluated before they underwent any treatment. Age, gender, BMI, smoking and alcohol history were considered for all samples. All stages of PDAC were included and the stage of the disease was classified as per the seventh edition of American Joint Committee on Cancer. The numbers of data that were collected from different hospitals are listed in the Table 1.

Table 1. Numbers of data sets in individual panel and multi-panel ELISA kit

	NL	PDAC					
Institute	SNUH	SNUH	AMC	NCC	SMC	YMC	Total
Individual panel	348	50	75	128	96	47	396
Multi-panel	347	50	75	112	97	47	381
Common	346	50	75	112	92	47	376

Individual panel and multi-panel ELISA kit data set for experiment consistency. Common population data was used to calculate the correlation of values. SNUH; Seoul National University Hospital; AMC: Asan Medical Center; NCC: National Cancer Center; SMC: Samsung Medical Center; YMC: Yonsei severance Hospital.

2.3. Development of the automated multi-panel ELISA kit and validation of reproducibility

ELISA was used for quantitative analysis of proteins in serum samples. Conventionally, each three biomarkers had to be detected individually in different ELISA experiments, which would take much time and require many steps. Thus, instead of generating three panels individually in different ELISA wells and combining the results in an additional process, we developed an automated multi-panel ELISA kit that included LRG1, TTR and CA 19-9 panels in one ELISA microwell plate. This one microwell could have the functionality to screen for all 3 biomarkers at once and make the test faster.

The kit was tested for all 728 plasma samples (Human Pancreatic Cancer Trio ELISA kit, Abfrontier, Seoul, Republic of Korea) using the Dynex-DS2 (Dynex Tech. Inc. Chantilly, VA, USA). Dynex-DS2, designed as a fully automated system, is an automated ELISA machine that includes transfer, dispensing, washer, incubator, reader and analysis systems, all in one machine. It can generate several different assays easily and quickly at the same time. Most users can easily control the system and maintain the device. It provides users with highly accurate results.

The test was performed according to the manufacturer' s recommendations. Briefly, LRG1, CA 19-9 and TTR were diluted 2,000, 4- and 10,000-fold, respectively, using the designated

solutions. The standard, control reagents, and plasma samples (each 100 μL) were loaded onto assigned wells. The standard and control reagents were duplicated. The wells were incubated at room temperature for 2 hours. After the wells were washed three times, the Conjugate (100 μL) was added and incubated for one hour at room temperature. Again, after the wells were washed three times, the Substrate solution (100 μL) was added and incubated at room temperature for 30 min. The Stop solution (100 μL) was added to cease the reaction. The optical density was measured at 540 nm or 570 nm. The concentration was obtained by 4-parameter logistic curve fit, multiplied by the dilution factors.

The reproducibility of the experiments was confirmed by three times repeated experiments with the same sample. We applied a cutoff of 10% coefficient of variance (CV) in triplicate analyses of the verification. After about 200 existing pancreatic cancer samples were randomly assigned, variation of the ELISA value was checked and internal validation was confirmed with the Dynex-DS2.

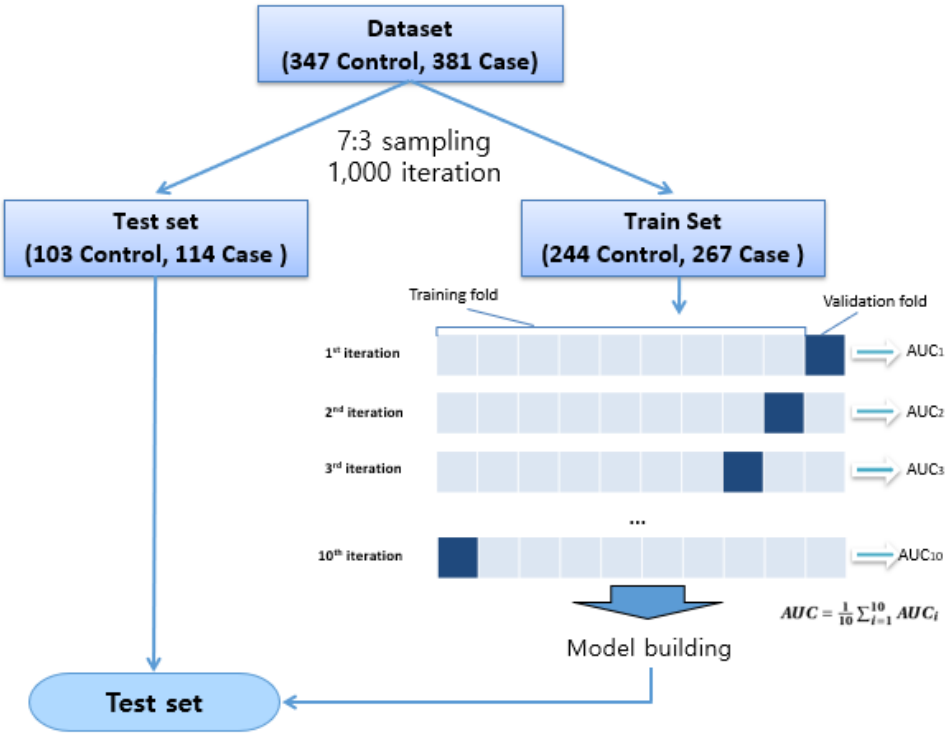
2.4. Logistic regression model development

Using ELISA values of the automated triple marker kit, a model that could suggest the probability of pancreatic cancer as a score was developed. The logistic regression (LR) was selected for model development because it is simple to interpret and commonly used to analyze a binary outcome according to multiple influencing

factors [23]. We used five variables that included age, sex, LRG1, TTR, and CA 19-9. Age and sex were selected from the multivariate analysis of risk factors. In addition, since age and sex are routinely and easily available by general practitioners in any clinics, we decided to use them in our model. The odds ratios obtained from the multivariate analysis were used for coefficient of the logistic regression model (Table 2).

The entire data was divided into a 7:3 ratio of training dataset (n=511) and test dataset (n=217) by random sampling (Figure 1). Normal (NL) and PDAC data were randomly distributed into the training and test datasets in the same ratio. The data for each category were divided at a constant rate so that data of a specific category would not be concentrated when the data were divided. The training set was then divided into 10 folds, in which there were one test fold and 9 train folds. The 10-fold cross validation method was used to increase the accuracy of the area under the curve (AUC) to be used to measure the performance of the model. In addition, the cross validation was repeated 1000 times to compare the performance in each situation using the final AUC.

Figure 1. Division of dataset for model building



Model development was done with the training set. Validation of the model was done with the test set.

Table 2. Comparison model coefficients by individual/multi-panel

	Individual panel		Multi-panel	
	Coefficient	p-value	Coefficient	p-value
Intercept	29.10003	9.50e-06 (***)	44.20993	1.22e-11 (***)
CA 19-9	1.17570	< 2e-16 (***)	0.86761	< 2e-16 (***)
LRG1	1.39415	3.59e-08 (***)	1.20293	9.84e-05 (***)
TTR	-3.84373	< 2e-16 (***)	-4.92254	< 2e-16 (***)
Age	0.03068	0.0196 (*)	0.03885	0.0021 (**)
Sex (M)	1.35949	7.56e-07 (***)	1.03061	3.67e-05 (***)

Comparison of coefficients of logistic regression (LR) models developed using individual panel and multi-panel ELIZA kit data set. *: $p < 0.05$; **: $p < 0.005$; ***: $p < 0.001$.

2.5. Consistency of predictors between individual vs. multi marker panel datasets

The triple-marker ELISA test results were compared with the individual ELISA test results based on the three markers, LRG1, TTR, and CA 19-9, generated in the previous study [21]. At this time, only common data from the same patient were used to check the experimental consistency between the single-panel and multi-panel ELISA kits. The Pearson and Spearman correlation coefficients were calculated for checking consistency between two ELISA datasets. The log-transformed observed values of each marker were investigated first and then the values predicted by the prediction model were considered.

2.6. Classification of low, intermediate, and high-risk groups

The diagnostic or risk prediction model was developed using the LR model. Based on the predicted probability of developing pancreatic cancer from the LR model, the patients were classified into low, intermediate and high-risk groups by two thresholds δ_1 and δ_2. In order to choose the values of δ_1 and δ_2 systematically, we considered four measures: NPV, PPV, sensitivity, and specificity. Note that from NL and PDAC, the classification model results in

three risk groups (low, intermediate, and high). For simplicity, we used modified versions of NPV, PPV, sensitivity, and specificity by considering only the high and low risk groups. For NL, let n_{11} represent 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, let n_{21}, n_{22}, and n_{23} be the corresponding counts, respectively. The four modified measures are calculated without the intermediate group as follows:

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

In order to choose the optimal values of δ_1 and δ_2, we changed these cut-off values from 0.01 to 0.99 by 0.01. We found the optimal combinations which yielded the highest average for the four measures under the conditions that all four measures exceeded the cut-off values such as 85%, 90%, and 95%, respectively. Since four measures are calculated excluding the intermediate group, performance is highly dependent on the count of the intermediate group.

2.7. Statistical analysis

The demographic analysis and graphical work were performed using R ver. 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). Categorical variables of the NL and PDAC groups were

compared via the chi-square test. The continuous variables were summarized using the means and standard deviations and compared via the Student's t-test. AUC values using receiver operating characteristic (ROC) curve were accessed with 95% confidence interval. DeLong's test was used to compare AUC values. Two-sided p-values < 0.05 were considered to be significant.

Chapter 3. Results

3.1. Clinical characteristics of patients

Clinical characteristics of PDAC patients and normal controls are shown in Table 3. Age and levels of initial CEA and initial CA 19-9 were significantly higher in PDAC group, whereas BMI was significantly lower in PDAC than NL group, all of which were consistent with the characteristics of PDAC. The rate of drinking alcohol was significantly lower in PDAC group. Also, the levels of automated ELISA kit of each markers were significantly higher in PDAC than NL, which well-discriminated the pancreatic cancer and normal. The levels of CA 19-9 and LRG 1 by the automated ELISA kit increased, and TTR decreased in PDAC (Figure 2).

Patients who were within the normal range of CA 19-9, which was less than 37 U/ml in our center, were also evaluated (Table 4). In this evaluation, the levels of initial CA 19-9 and each levels of the triple markers in the automated panel had significant differences. The pattern of the differences in all markers were equivalent to the whole data set; LRG1 and CA 19-9 were higher and TTR lower in PDAC than the normal.

Table 3. Demographics of study population

	Total (N=728)	PDAC (N=381)	NL (N=347)	P-value
Age (Mean±SD)	59.4 ± 9.6	61.6±10.3	56.9 ± 8.1	< 0.001
Sex (male %)	58.5	63.3	55.9	0.04
BMI (kg/cm^2) (Mean±SD)	23.4 ± 3.0	22.9 ± 3.0	23.8 ± 3.0	< 0.001
Alcohol (%)	425 (58.4)	158 (41.5)	267 (76.9)	< 0.001
Smoking (%)	287 (39.4)	146 (38.3)	141 (40.6)	0.523
Initial CEA (ng/ml)	20.2 ± 291.3 (n=526)	31.6 ± 368.7 (n=328)	1.2 ± 0.8 (n=198)	0.046
Initial CA19-9 (U/ml)	2,024.5 ± 10,140.8 (n=578)	3,073.3 ± 12,383.1 (n=380)	11.5 ± 33.1 (n=198)	< 0.001
Stages of PDAC (%)				
I	–	20 (5.2)	–	–
II		228 (59.8)		
III		30 (7.9)		
IV		100 (26.2)		
Automated ELISA triple marker panel				
LRG 1 (ng/ml)	10,260.4 ± 6,361.6	12,421.6 ± 7,656.9	7,887.4 ± 3,139.3	< 0.001
TTR (ng/ml)	237,267.2 ± 118,740.2	181,175.1 ± 60,287.7	298,855.3 ± 135,514.9	< 0.001
CA 19-9 (U/ml)	465.0 ± 1,755.8	875.9 ± 2,354.2	13.8± 19.8	< 0.001

PDAC, pancreatic ductal adenocarcinoma; NL, normal; SD, standard deviation; BMI, body mass index; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; ELISA, enzyme-linked immunosorbent assay; LRG, leucine rich alpha 2 glycoprotein; TTR, transthyretin

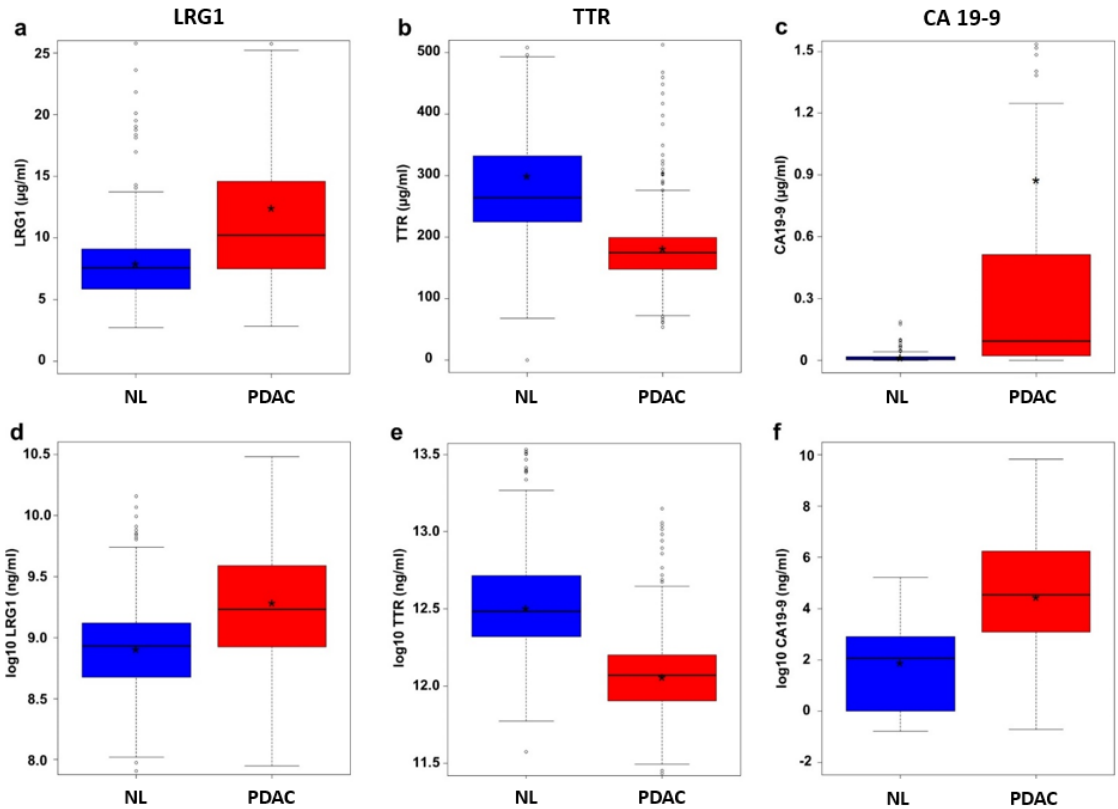
Table 4. Automated ELISA triple marker panel of samples with normal initial CA 19-9

	Total (N=317)	PDAC (N=192)	NL (N=125)	P-value
Initial CA 19-9 (U/ml)	9.8 ± 9.2	12.5 ± 10.7	8.1 ± 7.5	< 0.001
LRG 1 (ng/ml)	9,315.0 ± 5,912.6	11,921.3 ± 8,096.5	7,618.2 ± 2,817.7	< 0.001
TTR (ng/ml)	255,156.6 ± 139,888.7	180,943.0 ±57,069.7	303,472.8± 155,916.8	< 0.001
CA 19-9 (U/ml)	29.8 ± 182.7	55.8 ± 289.3	12.8 ± 13.1	0.040

ELISA, enzyme-linked immunosorbent assay; PDAC, pancreatic ductal adenocarcinoma; NL, normal; CA, carbohydrate antigen; LRG, leucine rich alpha 2 glycoprotein; TTR, transthyretin

Figure 2. Levels of LRG1, TTR, and CA19-9

Comparison of NL and PDAC levels of (a) LRG1, (b) TTR and (c) CA 19-9 from multi-panel ELISA kit. The levels of log-transformed NL and PDAC of (d) LRG1, (e) TTR and (f) CA 19-9 were also shown. The asterisk represented the arithmetic mean of each NL and PDAC data.



3.2. Diagnostic model development

The diagnostic model for PDAC was developed using LR based on the multi-panel ELISA kit for two categories of NL/PDAC. A total of five variables including covariates, sex and age, and three biomarkers CA 19-9, LRG1, and TTR, were selected to construct an LR model for diagnostics. The actual ELISA values of CA 19-9, LRG1, and TTR were used in the model. The fitted LR model is given as follows:

$$\log\left(\frac{P(PC)}{1-P(PC)}\right) = 51.03 + 0.04Age + 1.19(Sex\ M)$$
$$-5.12\log(TTR) + 0.61\log(CA19-9) + 0.80\log(LRG1)$$

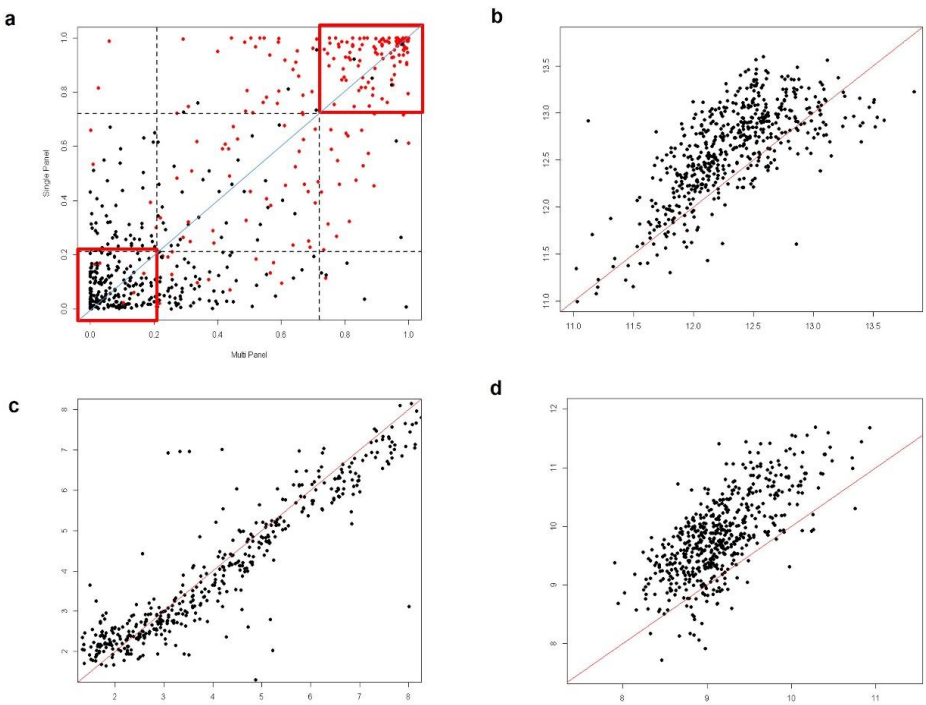
3.3. Consistency of predictors between multi-marker ELISA panel and individual marker ELSIA panel

Consistency of predictors from the LR model was confirmed along with the comparison between the individual biomarker ELISA kit data identified in the previous study [21] and the multi-panel ELISA kit in this study. Pearson correlation of predicted values by the LR prediction model showed a high correlation of 0.865 between the single panel and the multi-panel datasets (Figure 3a). In the scatter plot of predicted values, plots were appeared to be clustered

at the very low and the very high values, which demonstrated that the distribution of predicted values for PDAC was divided into three groups, and any two threshold values for dividing them can be identified. Moreover, correlations of log-transformed three-marker LRG1, CA19-9 and TTR to individual and multi marker data set were also high as predicted value (Figure 3b-d).

Figure 3. The relationship between individual panels and multi-panel ELISA kit datasets

X axis is multi panel and Y axis is single panel. (a) The scatter plot of predication values from the individual panels and multi-panel ELISA kit datasets. The red box indicates common regions of low and high risk groups using two thresholds. The level of log-transformed (b) LRG1, (c) TTR, and (d) CA19-9 were measured by individual and multi-panel ELISA kits.



3.4. Diagnostic performance by two classes

The AUCs of the triple-marker kit were calculated by applying the predictors from the LR model; the AUCs of CA 19-9 was calculated with initial CA 19-9 values (Figure 4). The ROC curves were generated for the triple-marker ELISA kit and CA 19-9, which were compared by DeLong' s test. Figure 4 represents the training datasets. The general performance, AUC, of the triple ELISA kit was 0.912 and CA 19-9 alone was 0.851 (P=0.001). The cut-off value the triple ELISA panel was 0.502 with sensitivity of 0.893 and specificity of 0.850.

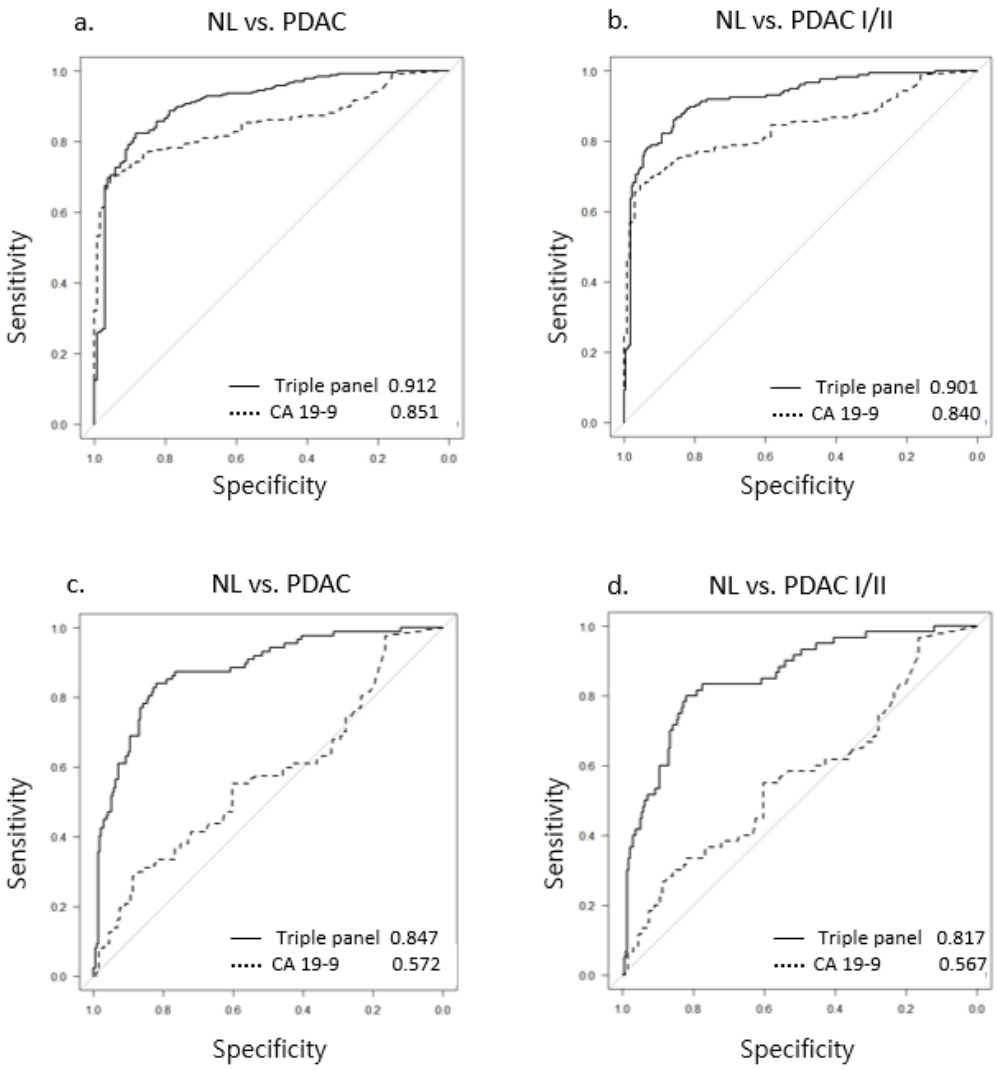
Our investigation of the performance of initial CA 19-9 alone towards differentiating pancreatic cancer from normal revealed the following estimates: AUC, 0.851; sensitivity 0.699; and specificity 0.956. The AUC was similar to those of prior studies, which reported AUCs of 0.842 - 0.886 [24,25]. The sensitivity and specificity of CA 19-9 alone were also in the range reported by previous studies, which were 57-80% and 80-90%, respectively [9,10,14-16,21].

In comparison between surgically operable and early-stage PDAC and the normal, the triple-marker ELISA kit and CA 19-9 alone had AUC of 0.901 and 0.840, respectively, with cut-off value for the triple ELISA panels of 0.434. In the normal CA 19-9 group, the AUC of the triple ELISA panel was 0.847 and that of CA 19-9 alone was 0.572. Similar results were shown in the early stage

PDAC. All of AUC interpretations were verified in the test datasets with similar results.

Figure 4. Receiver operating characteristic (ROC) curves for the triple-marker panel and CA 19-9

(a) General performance between normal and PDCA. Cut-off value was 0.502. (b) Performance between normal and stage I/II PDAC. Cut-off value was 0.434. (c and d) ROC curves for patients with < 37 U/ml CA 19-9. Cut-off values of both c and d were 0.361.



3.5. Optimizing threshold combination and prediction performance

For the optimal combination of δ_1 and δ_2, Table 5 shows the four evaluation measures and the numbers of risk groups for the given the cut-off values. As the cut-off values decrease, the numbers of high and low risk groups increase, while that of the intermediate group decreases. When the cut off value was 95%, for example, there were 216 subjects in the intermediate group, and the number of intermediate groups became 68 and 1 as the cut off values were reduced to 90% and 85%. For a real clinical application, it would be important to have enough numbers of high and low risk groups in the prediction results. Unfortunately, the greater the size of high and low risk groups, the smaller the evaluation measures. As a compromised solution for practical application, the 90% cut-off value was chosen which provided the optimized threshold values $(\delta_1, \delta_2) = (0.32, 0.60)$. For these thresholds, the values of NPV, PPV, sensitivity, specificity were 90.69, 92.05, 92.05 and 90.69, respectively and its mean was 91.37 (Table 5, Figure 5a, b).

In order to evaluate the performance of the proposed diagnostic model, we applied this model to the test dataset using the same optimal threshold value $(\delta_1, \delta_2) = (0.32, 0.60)$. For these thresholds, the values of NPV, PPV, sensitivity, specificity were 91.57, 90.48, 93.14 and 88.37, respectively and its mean was 90.89, as shown in the last column of Table 5.

The performance of the diagnostic model was also evaluated by stages, comparing early and late stages of PDAC (Table 6). The proposed model also showed high diagnostic performance in both stage I/II and stage III/IV, similar to the performance in all stages. Moreover, when applied to the model, both early and late stages were effectively classified into low, intermediate and high-risk groups (Figure 5c to f).

When optimized thresholds 0.32 and 0.6 were applied to patients with normal CA 19-9 levels (Figure 6), the values did not distinguish the three risk groups, compared to our results shown in Figure 5. The box plot showed that some members of the PDAC group were included in the intermediate group. However, the two peaks shown in the densitogram demonstrate the ability of the marker to distinguish between normal and pancreatic cancer samples, to some extent.

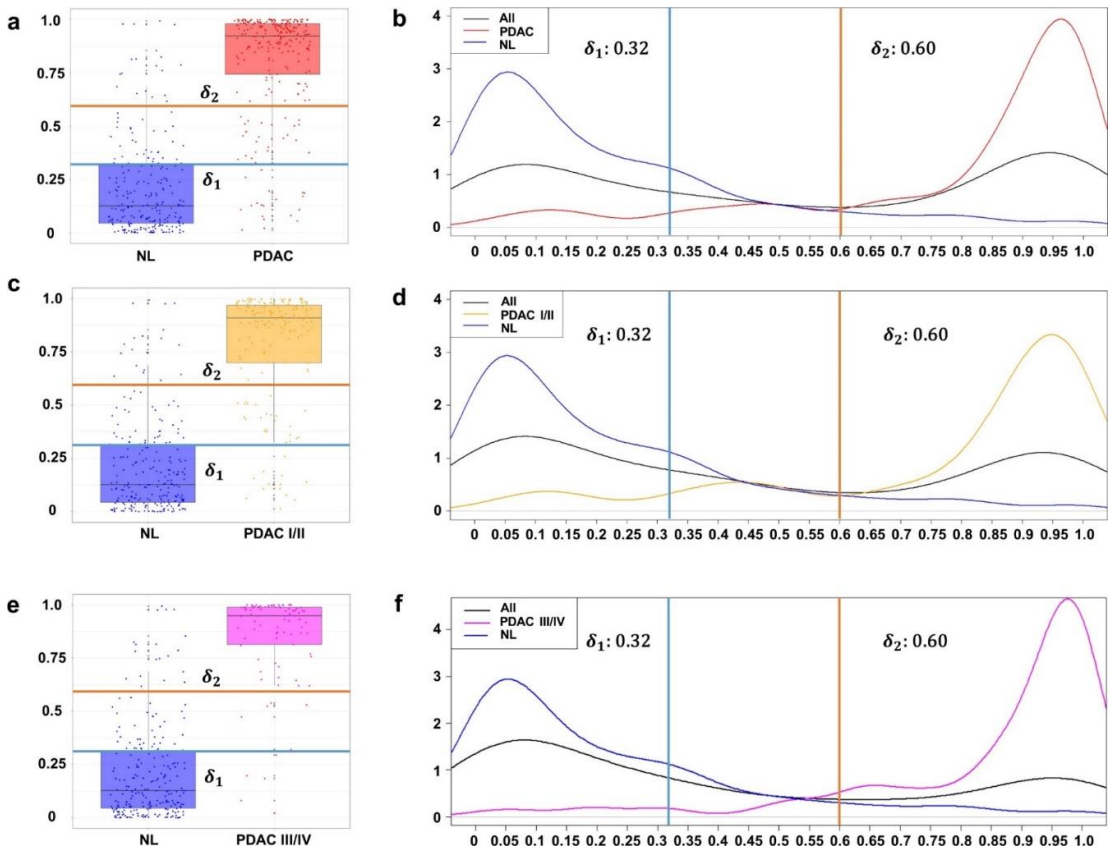
Table 5. Optimization of thresholds

Data set		Training Dataset			Test Dataset
Cut-off (%) for evaluation measures		95	90	85	90
Four evaluation measures	NPV	95.2	90.7	85.4	91.6
	PPV	97.4	92.1	87.8	90.5
	Sen	97.4	92.1	86.5	93.1
	Spe	95.2	90.7	86.8	88.4
Mean of measures		96.3	91.4	86.6	90.9
Number of risk group	Low (n)	105	204	247	83
	Inter. (n)	216	68	1	29
	High (n)	190	239	263	105
Thresholds	δ_1	0.08	0.32	0.46	0.32
	δ_2	0.83	0.6	0.47	0.60

Performance of the predicted model was compared with various cut-off values of evaluation measures and verified with training and test data set. The thresholds that satisfied high diagnostic evaluation measures and the lowest number of intermediate groups at the same time were selected.

NPV, negative predictive values; PPV, positive predictive values; Sen, sensitivity; Spe, specificity.

Figure 5. Optimized threshold combination for the ELISA triple-marker prediction model



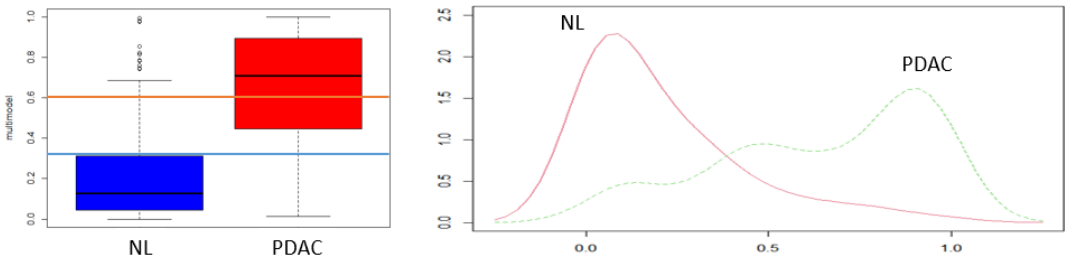
The box plot (a), and density plot (b) for all stages showed that the high risk group had a predicted value close to 1 and the low risk group has a value close to 0 using automated ELISA triple-marker kit. The intermediate group was in between δ_1 and δ_2. The diagnostic model was evaluated for the early stage (c, d) and the late state (e, f).

Table 6. Comparison of performance between NL vs PDAC early stage and NL vs PDAC late stage patients

Data set		Training	Test	Training	Test	Training	Test
		(NL vs PDAC all)		(NL vs PDAC I/II)		(NL vs PDAC III/IV)	
Four evaluation measures	NPV	90.7	91.6	93.0	93.8	97.4	97.4
	PPV	92.1	90.5	87.8	85.5	81.4	78.3
	Sen	92.1	93.1	90.7	92.2	94.3	94.7
	Spe	90.7	88.4	90.7	88.4	90.7	88.4
Mean of measures		91.4	90.9	90.6	90.0	90.9	89.7

Performance was compared between NL vs PDAC stage I/II (early) and NL vs PDAC stage III/IV (late) patients.

Figure 6. Distribution of normal and PDAC in subjects with normal CA 19-9 levels



The boxplot and density plot represent the distribution of normal individuals and patients with pancreatic cancer. In the boxplot, there is an overlapping region in the intermediate zone between 0.32 and 0.6. However, the two peaks that are present in the density plot demonstrate some discriminating ability.

3.6. Proportions of three risk groups

Table 7 represents proportions of three risk groups according to two thresholds, 0.32 and 0.6. For the training dataset, 75.82% of NL subjects were classified into the low risk group, while 82.40% of PDAC patients into the high-risk group. On the other hand, for the test dataset, 73.79% of NL subjects were classified into the low risk group, while 83.33% of PDAC patients into the high risk group. The predicted percentages of intermediate risk groups were 16.39% and 10.49% for NL and PDAC groups, respectively for the training dataset.

Characteristics of the intermediate-risk group are shown in Table 8. There were no significant differences in most variables between patients with PDAC and normal individuals. This demonstrates that PDAC and the normal subjects were not distinguished well.

Table 7. Proportions of NL and PDAC subjects in three risk groups

Risk group	Subject	
	PDAC	NL
Training dataset		
Low	185 (75.8)	19 (7.1)
Intermediate	40 (16.4)	28 (10.5)
High	19 (7.8)	220 (82.4)
Total	244 (100.0)	267 (100.0)
Test dataset		
Low	76 (73.8)	7 (6.1)
Intermediate	17 (16.5)	12 (10.5)
High	10 (9.7)	95 (83.3)
Total	103 (100.0)	114 (100.0)

Proportions of NL and PDAC subjects are distributed into the predicted low, intermediate, and high-risk groups according to the predicted thresholds. Values are presented as the number of individuals (%).

PDAC, pancreatic ductal adenocarcinoma

Table 8. Clinical characteristics of the intermediate risk group

	Total (N=97)	PDAC (N=40)	NL (N=57)	P-value
Age (Mean±SD)	58.3 ± 10.1	59.5 ± 10.0	57.5 ± 10.2	0.345
Sex (male %)	61 (62.9%)	24 (60.0%)	37 (64.9%)	0.622
BMI (Mean±SD)	23.2 ± 2.7	22.4 ± 2.7	23.8 ± 2.6	0.011
Alcohol (%)	51 (52.6%)	17 (42.5%)	34 (59.6%)	0.096
Smoking (%)	32 (33.0%)	13 (32.5%)	19 (33.3%)	0.932
Initial CEA (ng/ml)	4.0 ± 11.1 (n=526)	6.8 ± 15.3 (n=38)	1.3 ± 0.7 (n=38)	0.03
Initial CA19-9 (U/ml)	29.3 ± 64.8 (n=578)	43.0 ± 87.2 (n=40)	14.8 ± 17.0 (n=38)	0.054
Stage of PDAC I II III IV	(7th AJCC)	3 (7.5%) 30 (75.0%) 1 (2.5%) 6 (15.0%)		
LRG1	7,911.9 ± 3,119.7	8,915.2 ± 4,160.3	7,207.8 ± 1,845.6	0.019
TTR	215,962.8 ± 39,635.5	208,324.8 ± 46,915.8	221,322.9 ± 33,004.2	0.112
CA 19-9	25.7 ± 57.5	35.7 ± 84.8	18.6 ± 23.5	0.152

Chapter 4. Discussion

4.1. Discussion

In this multicenter biomarker study, we developed the automated triple marker kit using ELISA assay and the diagnostic risk prediction model through a machine-learning approach, using values from the ELISA kit. The diagnostic model with two thresholds, which distinguish three risk groups, had better diagnostic performance (all of NPV, PPV, sensitivity, and specificity over 90%) than known performance of CA 19-9 alone from previous studies (sensitivity and specificity 70~90%). In addition, the proposed model was well adopted even in the early stages of PDAC and in PDAC with normal range of CA 19-9, which were usually hard to detect in the clinic.

A blood-based cancer detection test is minimally invasive, less expensive than imaging diagnostic tools, and somewhat simple and convenient. For this reason, cancer-specific biomarkers have emerged as an important screening tool [26]. The elevated levels of markers, specific to certain cancers, should be stably reproducible at any stages and the performance of the markers should be validated in large cohort to be applied to the clinic.

In addition, the multi-biomarker panels have worked as better alternatives to single biomarker ones due to better diagnostic performance [27]. There are several studies [14-16,22,28],

including the study previous to this one [21], that introduced multi-marker panels for pancreatic cancer and demonstrated superior sensitivity and specificity to that of CA 19-9 (Table 9). However, most panels have been verified at a single institution. The triple biomarker panel (LRG1, TTR, and CA 19-9) that we chose to develop the diagnostic model for clinical translation is significant because it was demonstrated multi-institutional external validation from the previous biomarker discovery study [21]. Moreover, this triple marker panel demonstrated better performance than that for CA 19-9 alone for distinguishing PDAC from normal (NL), other cancers (breast, thyroid, and colorectal cancers), and benign pancreatic disease.

Table 9. Multi-marker panels from previous studies

Study	Multi-marker panel	Sen	Spe	CA 19-9		
				Sen	Spe	
Chang, 2009 [14]	CA 19-9, OPN, CHI3L1	93%	81%	80%	80%	In stage II/III patients
Brand, 2011 [15]	CA 19-9, ICAM-1, OPG	78%	94%	57.2%	90%	Discrimination from the normal
Nolen, 2014 [16]	CA 19-9, CEA, Cyfra 21-1	32.4%	95%*	25.7%	95%*	Significantly improved performance over CA 19-9
Gu, 2015 [28]	CA 19-9, CA 242, CA 125, CEA	90.4%	93.8%	82.7%	58.6%	Discrimination from the normal
Park, 2018 [21]	CA 19-9, LRG1, TTR	82.5%	92.1%	73%	89%	Discrimination from the normal
Mellby, 2018 [22]	IMMray™ PanCan-d microarray	95%	94%	-	-	Normal vs. stage I/II

* Specificity was set at 95%

Sen, sensitivity; Spe, specificity

Once potential biomarkers are identified, the next step is to develop a model for diagnostic accuracy, which would eventually be used in routine clinical practice [20,26]. Currently, there are biomarker-based models for certain cancers, approved for clinical practice [5,20,27,29]. For example, in ovarian cancer, OVA1 is an example of a successful translation of multi-biomarker panel to clinical use that has been cleared by the FDA [30]. OVA1, consisting of CA125, transthyretin, apolipoprotein A1, beta 2 microglobulin, and transferrin, demonstrated a sensitivity of 96% and negative predictive value of 98% for identifying high risk ovarian tumors [31]. There are no prediction models for pancreatic cancer yet in a clinical setting. But most recently, a microarray-based biomarker test (IMMray^{TM} PanCan-d), which achieved external validation, was introduced and was about to be approved by the FDA and marketed [22]. However, due to its high cost, it may not be practical to be used as a screening tool. Therefore, the PDAC diagnostic model with only three biomarkers, CA 19-9, LRG1, and TTR, described in this study would confer the advantage of being less expensive and more practical.

The algorithm for risk calculation needs risk stratification to identify actual likelihood of malignancy. To discriminate NL and PDAC, we classified risks into three groups, low, intermediate, and high, instead of a binary discrimination. We included the intermediate group for a specific reason. For example, if risk is 40%, it would be ambiguous to know whether there is a low or high risk to get pancreatic cancer. Inclusion of the intermediate group would

differentiate the low and high-risk groups incontestably. If individuals are positioned in the high-risk group, they are highly suspicious of having PDAC and thus need more precise examination or other interventions for treatment. If they are placed in the intermediate group, which implies moderate risk or above, they need further radiologic examination or follow up tests to ascertain any possibility of cancer. This may result in early detection and treatment planning for pancreatic cancer. The low risk groups may not need further checkups.

The levels of tumor markers are known to be varied by stages of cancer with usually higher detection rate at late stage [30]. Thus, it is important to know if our ELISA kit and model can discriminate the cancer and the normal even at early stages. When we analyzed NPV, PPV, sensitivity, and specificity for early and late stages separately, those values of both conditions showed similar evaluation measures as all stages. However, PPV of both conditions showed somewhat lower values than all stages, which could be explained by decreased sample sizes as we divided them. Moreover, our model was nicely applied to both early and late stages, discriminating the normal and the cancer.

It is also important to consider PDAC with low CA 19-9 levels as well as early-stage PDAC since patients with advanced PDAC sometimes show low CA 19-9 levels. CA 19-9 is not usually elevated in patients with asymptomatic PDAC or at very early stages. Moreover, CA 19-9 is a Lewis A antigen, and 10 - 15% of the Caucasian population have the Lewis-negative genotype [13],

and thus, do not express CA 19-9 and will not have elevated CA 19-9 levels [12,13]. On the other hand, CA 19-9 is elevated in other benign diseases, such as non-malignant obstructive jaundice and chronic pancreatitis [33,34], and in other cancers, such as colon cancer. Thus, we also checked the diagnostic performance of the triple marker ELISA kit in patients with PDAC with normal CA 19-9. Our results showed that discrimination between the three risk groups was not well defined in the cohort of patients with a normal range of CA 19-9. Since CA 19-9 is a strong marker for predicting pancreatic cancer, without high CA 19-9 expression, the number of individuals in the intermediate-risk group would increase. However, since the densitogram showed two peaks, for the normal and PDAC groups, our risk prediction model can be considered to be able to discriminate patients into three risk groups.

In biomarker studies for screening test, assay development should not only concentrate on diagnostic and clinical performance but also on time- and cost-effectiveness. It is desirable for assays to be precise, less time consuming, inexpensive, and have the ability to profile large amounts of proteins at a time [35,36]. The selection of a method may be dependent on the government healthcare support system, laboratory capacities and other factors. In this study, ELISA was used to quantitate the amount of biomarker proteins in serum samples instead of MRM-MS, which was formerly used in the study by Park et al. [21] The MRM-MS is a high throughput and sensitive protein-quantitating method that was also cost-effective and fast for the validation of the triple

marker panel [37]. The MRM-MS requires only small sample volumes, about 20㎕, and has no limitation on the number of markers in the multi-marker panel [37]. However, since this innovative device cannot analyze CA 19-9 and is not yet available in general clinics but only equipped in only laboratories, we needed a compromise between a real life and ideal setting. Furthermore, to make a panel kit simpler, we made an effort to combine three biomarker-panels into one microwell. In this way, we could achieve a faster and less expensive assay. For the practical use of the biomarker panels, ELISA technique was used to build the diagnostic model, making it minimally invasive and cost-effective.

In spite of the high diagnostic performance of the model, there were some limitations. First, samples were collected retrospectively due to the retrospective nature of the research. Moreover, since multi-institutional samples were limited within Korea only, the model might not be applicable to the general population elsewhere. Also, the training sets and test sets for construction of the model were separate portions of the same dataset. Ideally, models should be trained, tested and validated with different sets of data. However, due to the low incidence of PDAC, there were limitations in collecting samples that were large enough in size. Another limitation of the study was that diagnostic performance was only evaluated between PDAC and NL. Nevertheless, we need further experimentation to discriminate PDAC from other groups, such as other cancers and benign pancreatic disease, for which we are already preparing in another

study. Since the purpose of this study was to analyze the diagnostic performance of the automated ELISA kit and the development of the models, we will focus on a large-scale validation trial with the automated ELISA kit and the diagnostic model to prove its safety and efficacy in the next study.

The triple-ELISA diagnostic prediction model in this study satisfied the requirements of an ideal screening test, of being simple to use, being less expensive, having reduced turnaround time, and more importantly, showing high diagnostic performance with NPV, PPV, sensitivity and specificity, all greater than 90%. We demonstrated the performance of the diagnostic model for more than 700 samples collected from multiple centers in South Korea. This study, thus, proposes a model that could predict risk of pancreatic cancer (low, intermediate, and high) fort general population and could potentially replace the previous tumor marker CA 19-9 for diagnosing pancreatic cancer. However, it needs external validation and further investigation.

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국문초록

바이오마커를 이용한 췌장암 진단 모델

서울대학교 대학원

의학과 외과학 전공

최유진

배경: 췌장암은 초기에 비특이적인 증상으로 조기 발견이 어려워 발견 시 수술적 절제가 가능한 환자는 20% 내외에 불과하며 재발과 전이가 빈번하고 항암제에 잘 듣지 않는 종양 자체의 생물학적 특징이 있기 때문에 생존율이 낮다. 현재 진단 방법으로는 컴퓨터 단층 촬영(CT)나 자기공명영상(MRI)과 같은 방법 뿐이며, 이는 높은 비용과 침습성으로 인해 조기 진단을 지연시킨다. 암 진단 바이오 마커는 생존율을 증가시키기 위해 암의 조기 검출에 사용되어 왔고, 여러 췌장암에 대한 바이오마커를 발견했지만 아직까지 실제 임상에 적용할 수가 없다. 따라서, 이 연구에서는 췌장암 조기 진단을 위해 automated triple marker enzyme-linked immunosorbent assay (ELISA) 키트를

개발하고, triple marker 키트에 따라 췌장암 진단 모델을 개발하는 것이다. 또한 이 진단 모델의 높은 진단 성능을 달성하여 췌장암의 위험을 예측하는 것을 목표로 한다.

방법: 개별 LRG1, TTR 및 CA 19-9 panel을 한 개의 kit로 만들어 췌관선암 (n=381)과 정상 (n=347) 샘플을 포함한 728개의 plasma sample에서 검사를 진행하였다. 이전 개별 ELISA 값과 이번에 개발된 자동화 triple marker ELISA kit의 일관성 확인을 위해 두 data의 predictor에 대해 Pearson Correlation으로 비교하였다. 로지스틱 회기 방법을 이용하여 췌관선암 진단 모델을 개발하고 저, 중등도, 고 위험군으로 나눌 수 있는 위험도 예측 모델을 개발하였다.

결과: 이전 개별 ELISA 값과 triple marker 값 사이의 피어슨 상관계수는 0.865로 일관성이 있음을 확인하였다. 로지스틱 회귀 모델은 양성예측도 92.05%, 음성예측도 90.69%, 특이도 90.69%, 및 민감도 92.05%로 신뢰할 수 있는 예측 결과가 나왔으며 CA 19-9보다 더 나은 진단 성능을 보여주었다(AUC: 0.851 vs. 0.912, P=0.001). 이를 바탕으로 위험도 예측을 위하여 환자군을 저, 중등도, 고 위험도로 나누기 위한 두 개의 최적화된 threshold는 0.32와 0.6이었다.

결론: 이 연구에서 개발된 자동화 triple-marker ELISA kit는 혈액 기반 테스트로, 최소 침습적이며, 사용하기 편리하고, 영상 진단 도구들보다 저렴하다. 또한 이 kit를 기반으로 개발한 췌장암 진단 모델은 췌장암과 정상을 잘 구별할 수 있으며 췌장암의 위험도를 저, 중, 고 위험도로 나눌 수 있다. 따라서 췌장암의 선별검사로서 췌장암 고위험군의 환자식별에 사용될 수 있고 췌장암 조기 진단율을 높여

환자들이 수술적 치료를 받게 하여 생존율을 높일 수 있다. 자동화 triple-marker ELISA kit를 기반으로 개발된 췌장암 진단 모델은 이전 마커보다 우수한 진단 성능을 보여주길 기대한다. 앞으로는 실제 임상에서 사용하기 위해 이번 모델의 외부 검증이 필요하다.

주요어: 췌장암, 바이오마커,

학 번: 2019-35099