Development and Multiple Validation of the Protein Multi-Marker Panel for Diagnosis of Pancreatic Cancer

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Translational Relevance

Pancreatic ductal adenocarcinoma (PDAC) is a deadly disease that is difficult to detect early and has an overall 5-year survival rate of less than 10%. Carbohydrate antigen 19-9 levels, which are the current gold standard for diagnosing PDAC, lack the desired sensitivity and specificity required for early detection. Therefore, less-invasive, blood-based biomarkers should be developed to improve the early detection of PDAC. In this study, we developed a protein-based multi-marker panel that provides superior PDAC detection with sufficient diagnostic performance. The multi-marker panel showed improved diagnostic abilities for the classification of patients with PDAC compared with healthy controls or those with benign pancreatic diseases. The diagnostic performance of the multi-marker panel was validated in several cohorts, each consisting of a substantial number of samples. For the early detection of PDAC, the potent biomarkers identified in this study may play an important role in improving the outcomes for patients with PDAC.

Abstract

Purpose: To develop and validate a protein-based, multi-marker panel that provides superior pancreatic ductal adenocarcinoma (PDAC) detection abilities with sufficient diagnostic performance.

Experimental Design: A total of 959 plasma samples from patients at multiple medical centers were used. To construct an optimal, diagnostic, multi-marker panel, we applied data preprocessing procedure to biomarker candidates. The multi-marker panel was developed using a training set comprised of 261 PDAC cases and 290 controls. Subsequent evaluations were performed in a validation set comprised of 65 PDAC cases and 72 controls. Further validation was performed in an independent set comprised of 75 PDAC cases and 47 controls.

Results: A multi-marker panel containing 14 proteins was developed. The multi-marker panel achieved areas under the curve (AUCs) of 0.977 and 0.953 for the training set and validation set, respectively. In an independent validation set, the multi-marker panel yielded an AUC of 0.928. The diagnostic performance of the multi-marker panel showed significant improvements compared with carbohydrate antigen (CA) 19-9 alone (training set AUC = 0.977 vs. 0.872, $P < 0.001$; validation set AUC = 0.953 vs. 0.832, $P < 0.01$; independent validation AUC = 0.928 vs. 0.771, $P < 0.001$). When the multi-marker panel and CA 19-9 were combined, the diagnostic performance of the combined panel was improved for all sets.

Conclusions: This multi-marker panel and the combined panel showed statistically significant improvements in diagnostic performance compared with CA 19-9 alone and has the potential to complement CA19-9 as a diagnostic marker in clinical practice.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers, responsible for 432,242 patient deaths worldwide annually (1). PDAC is the fourth leading cause of cancer-related mortality, with a five-year survival rate of approximately 10% (2). Because of its poor prognosis, mortality (n = 432,232) coincides with incidence (n = 458,918) (1). According to recent studies, the incidence of new PDAC has been gradually increasing over time, and PDAC is projected to become the second leading cause of cancer-related mortality by 2030 (3). Although surgical resection remains the most effective treatment, only 15% to 20% of diagnosed patients are eligible to undergo this process because the vast majority of patients are diagnosed at later stages (locally advanced or metastatic PDAC) (4). Consequently, improvements in early detection are integral to allowing PDAC patients to access existing curative therapies.

Current efforts to develop a diagnostic test for PDAC have primarily focused on blood or urine biomarkers due to their minimally invasive natures (5). Carbohydrate antigen 19-9 (CA 19-9), which is the sole blood-based biomarker approved by the Food and Drug Administration (FDA) for the clinical management of PDAC, has been thoroughly studied and utilized in clinical applications. However, CA 19-9 demonstrates inadequate sensitivity and specificity, which has limited its diagnostic performance for general screening (6). Previous studies have reported that the sensitivity and specificity of CA 19-9 levels for PDAC diagnosis ranged from 70% to 80% and 80% to 90%, respectively (7). Moreover, CA19-9 is irrelevant to the approximately 10% of PDAC patients who do not express Lewis blood group antigens (7). Therefore, the development of a biomarker with increased sensitivity and specificity that is applicable to all PDAC patients remains necessary.

Previous studies have suggested the addition of alternative biomarkers to compensate for the limitations of CA 19-9 for PDAC diagnosis (8). Considering the heterogeneity of cancer development and progression and the complexity of bio-fluids, such as serum or plasma, single biomarkers are likely to be insufficient to achieve the sensitivity and specificity required for cancer diagnosis (9). Therefore, recent biomarker studies have shifted to a multi-parametric analysis. The combination of multiple biomarkers has been reported to be a useful strategy in several studies, providing increased diagnostic accuracy, sensitivity, and specificity (10). Using multiple markers, both the sensitivity and specificity reached relatively high values for the discrimination of PDAC patients from healthy individuals. Recent studies have reported the notable diagnostic performance of various combinations of proteins (11), metabolic biomarkers (8), and microRNAs (12) for PDAC diagnosis.

Protein biomarkers have been utilized in numerous clinical applications, including diagnosis, disease prognosis, therapeutic evaluations, and drug efficacy assessments (13). The evaluation of human plasma proteins can reflect various aspects of patient physiology, including tumor growth and disease conditions. Consequently, blood-based protein biomarkers represent a promising source of disease indicators that can be evaluated through less-invasive diagnostic tests (14). Proteomic analysis by mass spectrometry (MS) has been widely applied to biomarker development in recent years. In particular, MS-based, targeted proteomic approaches [e.g., multiple reaction monitoring-mass spectrometry (MRM-MS)] have been applied for their robust and high-throughput capabilities, which enable protein measurements in the range of hundreds of proteins while retaining high sensitivity, selectivity, and reproducibility (15). Further, MRM-MS is largely unaffected by matrix

effects and other contaminants because it specifically detects peptides of interest and their corresponding fragments. These characteristics of the MRM-MS assay indicate its potential to serve as a complementary method to antibody-based assays for biomarker development (16).

Using a large scale data set containing protein biomarker candidates that were discovered and verified in our previous study (17), we aimed to develop a protein-based, multi-marker panel for PDAC diagnosis and to compare the performance of this panel against that for CA 19-9, both alone and in conjunction with other biomarkers. In addition, the protein-based, multi-marker panel was validated using multiple independent data sets.

Materials and Methods

Study design

This study used a targeted proteomic (MRM-MS) dataset that was obtained from a previous study (17) and contained quantitative data from 1,008 plasma samples. Briefly, clinical plasma samples were obtained from recruited patients with PDAC, benign pancreatic disease, other cancers, other benign diseases, and healthy controls. Patients were recruited from five medical centers in Seoul, Korea [National Cancer Center (NCC, n = 128), Seoul National University Hospital or Seoul National University Hospital Healthcare System Gangnam Center (SNUH, n = 553), Samsung Medical Center (SMC, n = 131), Asan Medical Center (AMC, n = 122), and Yonsei Severance Hospital (YSH, n = 74)], between January 2011 and December 2013. The total cohort comprised 401 patients with PDAC, 109 patients with benign pancreatic disease, 300 healthy controls, 149 patients with other cancers, and 49 patients with other benign diseases. The distribution of tumor stages in PDAC patients was 248 early-stage cases [stage IA (n = 8), IB (n = 12), IIA (n = 86), and IIB (n = 142)] and 153 late-stage cases [stage III (n = 31) and IV (n = 122)]. Tumor stages for PDAC were classified according to the 7th edition of the American Joint Committee on Cancer (AJCC) (18). Detailed information regarding patient characteristics is presented in Table 1 and Supplementary Table 1.

To discriminate PDAC, the data set was reconfigured to define PDAC as the case group and healthy controls and benign pancreatic diseases as the control groups. The total clinical

samples were divided into 4 sets for developing and validating the multi-marker panel. Samples from four medical centers (NCC, SNUH, SMC, and YSH) were integrated and randomly divided to form a training set and a validation set, with the training set consisting of 261 PDAC cases (148 early-stage and 113 late-stage) and 290 controls and the validation set consisting of 65 PDAC cases (27 early-stage and 38 late-stage) and 72 controls. An independent validation set, consisting of 75 PDAC cases (73 early-stage and 2 late-stage) and 47 controls, was obtained from the AMC. To perform subgroup analyses, an additional dataset was constructed containing other cancers cases (breast cancer, n = 52; colon cancer, n = 45; and thyroid cancer, n = 52), in addition to PDAC cases. The additional set consisted of 50 PDAC cases and 149 other cancer cases, which were all obtained from SNUH. To minimize any variations that might have resulted from using samples that originated from different institutions, several PDAC cases from the training and validation sets were reused. Because PDAC was set as the case group while healthy controls or benign pancreatic diseases were set as the control group, the other benign disease group was not used in this study.

This study was conducted in accordance with the guiding principles of the Declaration of Helsinki. The study protocols were approved by the corresponding 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 written informed consent was obtained from all participants who contributed biospecimens.

The identified biomarker candidates

In previous studies, we identified 54 proteins (68 peptides) as biomarker candidates using a large-scale, systematic process. Through the biomarker discovery phase in the previous study, several sources, including databases, traditional cancer markers, and microarray analysis data from resected PDAC tissue, were used to obtain 1,000 protein biomarker candidates for the diagnosis of PDAC using proteomic analysis (database and literature: 508 proteins; traditional cancer markers: 22 proteins; microarray analysis: 456 proteins; known mutated protein: 14 proteins). Of 1,000 initial candidates, 205 proteins with AUC values > 0.6, when evaluated as single markers, were identified as targets that can be detected by MS, based on 134 plasma samples (50 PDAC cases and 84 controls) obtained from SNUH. Among 205 proteins, 176 proteins (corresponding to 217 peptides) were selected as unique. MRM-MS quantitative analysis using stable isotope-labeled standard peptide (SIS-peptide) resulted in the identification of 54 differentially expressed proteins between PDAC and healthy controls, which were chosen as the final MRM-MS quantification targets. During the verification phase, a total of 54 proteins (68 peptides) were measured by MRM−MS in 1,008 plasma samples. The biomarker discovery, verification, and sample preparation methods and the MRM−MS procedure were detailed in a previous study (17).

Data preprocessing

The data preprocessing consisted of two steps. The first step was the selection of high-intensity proteins to reduce analytical variations. Using raw data from the MRM-MS analysis, which was imported into Skyline (McCoss Lab, University of Washington, WA, USA), those with peak intensities below the following criteria were removed (peak intensity < 1,000). A total of 11 biomarker candidates were removed, leaving 57 biomarkers. The second step was to transform the data type, to improve the normality of the distribution. Many statistical procedures assume that the variables are normally distributed. If this assumption is unmet, the development of an erroneous multi-marker panel will occur. To ensure the normal distribution of each marker, 3 different types of transformation were performed: (1) raw; (2) log ($x+10^{-10}$); and (3) square root. Then, the skewness value for each marker was calculated using the skewness function in the e1071 package of R (19). When the skewness value was calculated following data transformation for 57 biomarker candidates, including CA 19-9, the skewness values ranged from 0.18 to 29.89.

The type of data transformation was selected, in which the average of the absolute values of the skewness of the case group and the control group is least. The data type for each marker was transformed using the selected transformation method: 11 log ($x + 10^{-10}$), 42 square roots, and 5 raw. The skewness value of these optimized transformations ranged from 0.1 to 1.96, and the normality of all 58 biomarker candidates was improved (20). After transformation, all biomarker candidates were standardized to a normal distribution, with a mean of 0 and a standard deviation of 1. From proteins associated with multiple peptides, only a single peptide was selected based on AUC and *P*-values, to ensure that each peptide corresponded to a single protein. Throughout

this process, 13 peptides were removed, and 44 biomarker candidates remained. Data that were pertinent to the 58 biomarker candidates are included in Supplementary Table 8.

Data Analysis

Differences in the protein expression levels between two groups were compared using the Mann-Whitney U test. 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. The multi-marker panel was trained to classify patients with PDAC against healthy controls or patients with benign pancreatic disease. To provide a more accurate indication of how well the multi-marker panel generalizes to unseen data, the multi-marker panel was validated using the corresponding validation set and an independent validation set. The diagnostic performance of the multi-marker panel for the discrimination of patients with PDAC from healthy controls or patients with benign pancreatic diseases was assessed using receiver operating characteristic (ROC) curve analysis, particularly area under the ROC curve (AUC) and 95% confidence intervals (CIs). The AUC CIs were calculated using the DeLong method and integrated within the pROC package (21). Sensitivity and specificity were calculated at the optimal cut-off value, which was determined by the Youden index (22) for the training set. The threshold for statistical significance was set at 0.05. All reported *P*-values are two-sided. Statistical analyses were performed using R (version 3.6.0).

Results

Description and Preprocessing of the Data Set

We previously identified and verified 68 peptides (corresponding to 54 proteins) as PDAC biomarker candidates from 1,000 PDAC-related initial protein candidates (17). The 1,000 candidate proteins were selected as potentially PDAC-related proteins by compiling databases, traditional cancer markers, and microarray analysis data from resected PDAC tissue. Of these 1,000 candidate proteins, 205 were detectable by MS in plasma samples. Among these individual 205 protein markers, 68 peptides associated with 54 proteins had AUCs greater than 0.60 for the discrimination of PDAC cases from healthy controls and were subsequently selected for the quantification analysis using a large-scale cohort. These selected targets were then quantified by MRM-MS in a large-scale, plasma sample cohort, comprising PDAC patients (n = 401), benign pancreatic disease patients (n = 109), healthy controls (n = 300), other cancer patients (n = 149), and other benign disease patients (n = 49), which were obtained from five major medical centers (NCC, SNUH, SMC, YSH, and AMC). Using this large-scale, quantification dataset, we developed a proteome-based predictive model.

To improve the protein-based predictive model, data preprocessing procedures were applied, considering two contributing factors: 1) the robustness of quantified markers and 2) the distribution of the dataset. Regarding the robustness of the markers, 11 peptides with low intensities (lower than 1,000) in the chromatogram were excluded from the list of 68 biomarker

candidates because low intensities may affect analytical reproducibility and variable predictive performance.

In addition, large-scale quantitative data from biological sources tend to be skewed in distribution. To reduce these differences and strengthen the classification of PDAC cases, the distribution of each biomarker candidate was explored to calculate its skewness. The skewed distribution of peptides was addressed by transforming the distribution of each peptide using common data transformations: raw, square root, and logarithmic transformations [log $(x + 10^{-10})$]. Skewness values were calculated for each case and control group because differences in the expression levels of these markers were observed for each case (PDAC) and control (healthy control and benign pancreatic disease) group. The optimal transformation was performed for each peptide to minimize the average absolute values of skewness for both groups (case and control). A total of 53 skewed candidates were transformed with log or square root transformations to mimic normal distribution. No transformation was required for 5 peptides with minimally skewed raw data. The square root transformation was performed for 42 peptides, and 10 peptides and CA 19-9 were transformed by log $(x + 10^{-10})$ transformation. After performing data transformations, the skewness values for all protein candidates were lower than 2, and the distribution largely resembled a normal distribution, resulting in more symmetrical data, suitable for subsequent statistical analyses (Supplementary Figure 2). After data transformation, all biomarker candidates were standardized to a mean of 0 and a standard deviation of 1.

Prior to the development of the multi-marker panel, proteins associated with multiple peptides were matched by selecting the best peptide so that single peptides corresponded to

single proteins. To match a single peptide to a single protein, the *P*-values and AUC values of all peptides associated with a single protein were compared. A single peptide was selected for 13 proteins that were each associated with 2 peptides. Consequently, 44 peptides (corresponding to 44 proteins) were selected as biomarker candidates to establish a predictive model. The overall study design is presented in Figure 1 and Supplementary Figure 1.

Development of a Protein-Based Multi-Marker Panel

We constructed a multi-marker panel from selected biomarker candidates to improve the diagnostic performance for PDAC. To distinguish PDAC from healthy controls and non-cancerous benign pancreatic diseases, the case group was set to PDAC patients, and the control group was set to healthy controls and benign pancreatic disease patients. In a large-scale data set, samples from four medical centers were integrated to form training and validation data sets, consisting of 261 PDAC cases (148 early-stage and 113 late-stage) and 290 controls in the training set and 65 PDAC cases (27 early-stage and 38 late-stage) and 72 controls in the validation set (Table 1, Supplementary Table 1). A multi-marker panel was developed using the 44 selected biomarker candidates described in the preprocessing procedures. To construct a multi-marker panel with optimal predictive performance, we applied logistic regression analysis to the training set, with stepwise selection, using *P*-values. Using stepwise selection, a multi-marker panel, consisting of 14 protein markers [Clusterin (CLU), Complement C5 (C5), Plasma kallikrein (KLKB1), Platelet basic protein (PPBP), Interferon-related developmental regulator 1 (IFRD1), Insulin-like growth factor-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). All markers in the multi-marker panel selected by the stepwise selection procedure were significantly associated with the prediction of PDAC (P-value < 0.05, Supplementary Table 2).

We evaluated the diagnostic performance when distinguishing PDAC cases from controls using the multi-marker panel and compared the results of the multi-marker panel against the diagnostic performance of CA 19-9 alone. The optimal cutoff value that the multi-marker panel predicted for PDAC patients was 0.576, as determined by the Youden index. In the training set, the developed multi-marker panel yielded an AUC of 0.977 (95% CI = 0.967 to 0.987), which was significantly greater than the AUC for CA19-9 alone (AUC = 0.872, 95% CI = 0.838 to 0.907; P < 0.001, DeLong's test). The panel yielded a sensitivity of 90.4% and a specificity of 95.9%, which was higher than the sensitivity of CA 19-9 alone (69.7%) but with slightly lower specificity (98.6%, Table 2 and Figure 2A).

In the validation set, the panel also returned a significantly greater AUC value than CA 19-9 alone (0.953, 95% CI = 0.914 to 0.991 vs. 0.832, 95% CI = 0.754 to 0.910; P < 0.01, DeLong's test). The sensitivity was 84.6%, and the specificity was 94.4%, demonstrating a

great improvement in sensitivity for the multi-marker panel compared with CA 19-9 alone (66.2%) while retaining slightly lower levels of specificity (98.6%, Table 2 and Figure 2B). These results indicated that the multi-marker panel achieved improved diagnostic performance in the new set.

The diagnostic performance of CA 19-9 showed lower sensitivity and higher specificity than the multi-marker panel for both the training set and the validation set. Considering these characteristics, we examined the ability of the multi-marker panel to complement CA 19-9 by investigating whether the combination of the multi-marker panel and CA 19-9 could improve the diagnostic performance. The classification performance for the combination of the multi-marker panel and CA19-9 achieved the best AUC value for both the training and validation sets (AUC = 0.989, 95% CI = 0.981 to 0.996 in training set, AUC = 0.972, 95% CI = 0.944 to 0.999 in validation set). The sensitivity values were 92.7% and 86.2%, respectively, and the specificity values were 98.3% and 97.2%, respectively. These results indicated that the inclusion of CA19-9 in the multi-marker panel improved the AUC and sensitivity compared with using CA19-9 alone for distinguishing between PDAC patients and healthy controls or benign pancreatic disease patients (Table 2 and Figure 2C-D).

Validating the diagnostic performance using an independent data set

The further validation of the multi-marker panel was performed using an independent validation set, consisting of 75 PDAC cases (73 early-stage and 2 late-stage) and 47 controls obtained from a single medical center (AMC, Table 1, Supplementary Table 1). In the independent validation set, the multi-marker panel had an AUC of 0.928 (95% CI = 0.885 to 0.971) for the discrimination of PDAC cases from controls, with a sensitivity of 81.3% and a specificity of 89.4%. The AUC for the multi-marker panel was significantly greater than that for CA 19-9 alone (0.771, 95% CI = 0.687 to 0.856; $P < 0.001$, DeLong's test). The multi-marker panel outperformed CA 19-9 for sensitivity, although specificity was lower (Table 3 and Figure 3A). Moreover, the multi-marker panel that included CA 19-9 yielded an AUC of 0.952 (95% CI = 0.918 to 0.985), which was significantly higher than that of CA 19-9 alone ($P < 0.001$, DeLong's test). The multi-marker panel combined with CA 19-9 also had higher sensitivity than CA 19-9 alone (89.3% vs. 61.3%) but had lower specificity (87.2% vs. 95.7%, Table 3 and Figure 3B).

Subgroup analysis

The major limitation of CA 19-9 is that it may be elevated in patients with other malignancies, such as ovarian, liver, and colorectal cancers (23). This non-specific expression of CA 19-9 makes CA 19-9 inadequate for the reliable diagnosis of PDAC. Therefore, we evaluated the ability of the multi-marker panel to distinguish PDAC cases

from other cancer cases. We procured an additional data set consisting of PDAC cases and other cancer cases (50 PDAC cases and 149 other cancer cases) from SNUH. The other cancer cases included 52 breast cancer cases, 45 colon cancer cases, and 52 thyroid cancer cases (Supplementary Table 3).

The results from this additional data set indicated that the multi-marker panel had a higher AUC of 0.946 (95% CI = 0.916 to 0.976) compared to an AUC of 0.886 (95% CI = 0.820 to 0.952) for CA 19-9 alone, with sensitivity improving from 76% to 92%. However, specificity was lower for the multi-marker panel than for CA 19-9 alone (79.2% vs. 92.6%, Supplementary Table 4 and Supplementary Figure 3A). The combined panel, including CA 19-9, showed greater improvements than the multi-marker panel without CA 19-9, as demonstrated by an AUC of 0.968 (95% CI = 0.945 to 0.991). The sensitivity was 98%, and the specificity improved to 82.6% (Supplementary Table 4 and Supplementary Figure 3B). Details on the subgroup analysis between PDAC and cancer type can be found in Supplementary Materials, Supplementary Figure 5, and Supplementary Table 5.

Discussion

The development of an effective biomarker that can be used to screen and provide an earlier diagnosis of PDAC will allow more PDAC patients to be identified early enough to undergo resection and ultimately improve survival rates (24). The ideal diagnostic biomarkers for effective screening are those that can be obtained in a minimally invasive manner and can distinguish PDAC from healthy individuals or other benign pancreatic diseases with satisfactory sensitivity and specificity.

In this study, a 14-protein multi-marker panel was developed to diagnose PDAC and tested in 2 validation sets to investigate the clinical applicability of the MRM-MS assay. The multi-marker panel demonstrated significant improvements over the CA 19-9, as evidenced by an AUC value of 0.977, with a sensitivity 90.4% and a specificity 95.9% for the training set. The multi-marker panel showed a high diagnostic performance AUC value of 0.953, with a sensitivity of 84.6% and a specificity of 94.4% for the validation set. When evaluated using the independent validation set, the multi-marker panel displayed a high AUC value of 0.928, with a sensitivity of 81.3% and a specificity of 89.4%. Although CA 19-9 alone consistently had high specificity, the multi-marker panel outperformed CA 19-9 for both AUC and sensitivity. The discrimination performance of the multi-marker panel for the training set was consistent with the results of the validation set and the independent validation set. The multi-marker panel yielded significantly improved discrimination performance relative to CA 19-9 alone and showed high diagnostic discrimination power when used for three different sets. In particular, the multi-marker panel showed high diagnostic performance for the independent validation set that consisted of early-stage

PDAC, indicating the possibility of achieving early PDAC diagnosis. Moreover, adding CA 19-9 to the multi-marker panel increased the performance, suggesting that the multi-marker panel can complement CA 19-9 and result in the increased power to distinguish PDAC cases from controls. The combined panel showed a better AUC value than the multi-marker panel alone, for all three data sets, and showed improvements in both sensitivity and specificity. These results indicated that the AUC and sensitivity of the combination panel are higher than that of CA 19-9 alone, representing a pronounced improvement over the results for a single marker. In the subgroup analysis, the multi-marker panel showed a higher AUC and sensitivity than CA 19-9 for distinguishing between PDAC and other cancers, suggesting that the multi-marker panel complemented the properties of CA 19-9 when CA 19-9 was expressed non-specifically (23) and was more effective for distinguishing PDAC from other carcinomas than CA 19-9 alone.

In our previous study, an MS-based targeted proteomic approach was used to select potential PDAC biomarkers for development of an ELISA (enzyme-linked immunosorbent assay). In the current study, using the same method, the multi-marker panel that we established was designed with the express purpose of identifying PDAC cases at an early stage for clinical use. Our study features the following advantages: 1) the relatively large number of samples (401 patients with PDAC and 558 controls, including healthy controls, benign pancreatic disease patients, and patients with other cancers), which were obtained from five medical centers, increase the credibility of our results; 2) the multi-marker panel was tested on two independent validation sets, including one whose samples were obtained from a single medical center; and 3) the use of a targeted proteomics approach (MRM-MS) to assess biomarker candidates. Although new cancer-related biomarker candidates have been discovered recently, through many biomarker studies, only a very small number of these biomarkers have been used in clinical

practice (25). To develop a meaningful candidate PDAC biomarker with the potential for clinical application, we have tested these candidates using a large number of samples from multiple medical centers, and these biomarkers exhibited reproducible predictive performances in various data sets. To verify the diagnostic abilities of the multi-marker panel with confidence, the use of an independent validation process with a different population is recommended (26). The training set that was used to build the multi-marker panel may not accurately reflect all of the characteristics of the underlying populations of interest (26). By subjecting the multi-marker panel to an independent validation set, especially one that features a distinct population from a single medical center, the general applicability of the panel and its robustness can be assessed. If the multi-marker panel performs well in several independent sets, it is more likely to be viable for clinical applications (27).

The targeted proteomics approach (MRM-MS), which produces robust measurements with a high degree of quantitative and analytical reproducibility while increasing throughput compared with conventional antibody-based methods (28), represents an alternative strategy that can overcome the limited multiplex capabilities inherent to methods such as ELISA (29). Based on these characteristics, the MRM-MS assay is a viable alternative to conventional assays, and several assays are already being tested in clinical trials (30). One particular MRM-MS assay that measures thyroglobulin, a biomarker for monitoring thyroid carcinoma, is being used in 6 clinical laboratories throughout North America (31). Another study has attempted to predict overall survival by measuring HER2 expression in breast cancer patients by MRM-MS assay (32), and a separate clinical trial was conducted to

differentiate benign and malignant nodules in the lung by measuring 2 proteins (33). These impressive cases demonstrate that the MRM-MS assay is applicable to clinical practice.

Challenges in the clinical application of the MRM-MS assay include the establishment of standard operating procedures (SOPs) for sample preparation and the confirmation of the analytical validity of the MRM-MS assay. The latter can be obtained by applying the validation guidelines for bioanalytical methods (e.g., calibration curve, precision, accuracy, reproducibility) of the FDA (34) and European Medicines Agency (EMA) (35). Our group validated the analytical method of the MRM-MS assay according to guidelines in a previous study (36).

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 (37). In particular, skewed distributions are indicative of bias, which may be reflected in the developed model. The transformation and normalization of data can reduce the skewness of data distribution, making biomarker candidates more comparable or normally distributed (20). By transforming the scale of each biomarker candidate, we have not only reduced natural biological variations but also impacted the potential performance of the multi-marker panel. This result suggests that the preprocessing of large-scale quantitative data can improve the further development of multi-marker panels.

The 14 biomarkers in our multi-marker panel, CLU, C5, KLKB1, PPBP, IFRD1, IGFBP2, ICAM1, C4BPA, PTPRJ, ECM1, VIM, C4BPB, SERPINA5, and TTR, represent proteins that are significantly differentially expressed between patients with PDAC and healthy control groups or benign pancreatic disease patients, based on the entire data set (Supplementary Figure 4). The

capacity of our l4 markers for comparing PDAC and non-PDAC is described in Supplementary Materials and Supplementary Table 6. These biomarkers were identified as being associated with PDAC in previous studies. TTR, a major circulating thyroxine-binding protein, was reported to be elevated in PDAC patients compared with healthy controls (38). IGFBP2, which belongs to the family of IGFBP (Insulin-like growth factor binding proteins), is also known to be associated with PDAC. IGFBP2 levels have been shown to increase with increasing PDAC stages, suggesting that this protein may serve as a biomarker for disease progression (39). PPBP (also called CXCL7) belongs to the angiogenic CXC chemokine family and functions to promote angiogenesis. In a proteomic study, the level of PPBP was found to significantly decreased in patients with PDAC (40). Complement C5 is produced by cancer cells to promote immunosuppression and allow tumor growth in lung cancer. C5 is also significantly elevated in PDAC patients, particularly in the presence of jaundice, compared with healthy controls (41). CLU has been associated with tumorigenesis and apoptosis and was overexpressed in PDAC tissue (42). ICMA 1 is known to modulate adhesion (cell-to-cell and cell-to-matrix) and has been well-studied in inflammation. The enhanced expression of serum ICAM 1 in PDACs can discriminate PDAC patients from healthy controls with high sensitivity (43). C4b-binding protein, which is involved in the regulation of the classical complement pathway, has two major isoforms, C4BPA and C4BPB. The serum C4BPA levels in PDAC patients were significantly increased compared with healthy control and pancreatitis patients (44). C4BPB has been reported to belong to the PDAC subtype associated with intermediate survival among PDAC patients (45). PTPRJ plays a regulatory role in angiogenesis, cell proliferation, and migration and may serve as a promising candidate for PDAC gene therapy

(46). ECM1 is involved in cell proliferation, angiogenesis, migration, and metastasis and demonstrates significantly increased expression in PDAC tissues, associated with poor prognosis in patients with PDAC (47). VIM is a cytoplasmic intermediate filament protein associated with tumor development and progression. The proteomic analysis of VIM revealed the increased expression of VIM under conditions of high PDAC cell invasion (48). KLKB1 is a glycoprotein associated with the surface-dependent activation of blood coagulation, fibrinolysis, kinin generation, and inflammation. KLKB1 was differentially expressed in PanNET (pancreatic neuroendocrine tumors), accounting for 1%–2% of all pancreatic tumors (49). SERPINA5 is loosely associated with PDAC, but some studies have indicated that this protein is overexpressed in PDAC or subtypes of PDAC (45). To our knowledge, IFRD1 has not been explored in PDAC studies. However, IFRD1 expression in colon cancer is associated with poorer patient prognosis (50).

The limitation of our study was that the control group used for the independent validation set consisted of benign pancreatic disease patients; therefore, this validation set was unable to evaluate the discriminating performance of the panel to distinguish PDAC patients from the healthy control group. Validating the panel using an independent data set that includes a healthy control group will further increase the credibility of the diagnostic power of the panel. For clinical application of the multi-marker panel, its performance must be validated in a separate cohort, because the cohort that was used in this study was homogeneous with regard to ethnicity. A future study on samples that originate from several ethnic and genetic backgrounds will confirm the multi-marker panel as a generalizable model. Also, the multi-marker panel must be validated externally to determine its reproducibility across laboratories. If the MRM-MS assay meets analytical validation standards across facilities, the marker panel can be deemed to be

reproducible. We expect other researchers who wish to apply our work using their own data to follow the detailed methods in Supplementary Materials and Supplementary Table 7.

In conclusion, using a targeted proteomics approach (MRM-MS), this study developed and validated a diagnostic, multi-marker panel for PDAC that significantly outperformed CA 19-9 alone for distinguishing of patients with PDAC from healthy individuals or benign pancreatic disease patients in several data sets. These results suggested that the developed multi-marker panel has the potential for clinical application, serving a complementary role to CA 19-9 screening tests.

Acknowledgments

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Tables

Table 1. Demographics and clinical characteristics of the study

		Training set			Validation set			Independent validation set	
Characteristic		PDAC (n=261)	Healthy control (n=241)	Pancreatic benign (n=49)	PDAC (n=65)	Healthy control (n=59)	Pancreatic benign (n=13)	PDAC (n=75)	Pancreatic benign (n=47)
Sex, n (%)									
	male	167 (64)[a]	138 (57.3)	19 (38.8)	38 (58.5)	33 (55.9)	9 (69.2)	46 (61.3)	16 (34)
	female	94 (36)	103 (42.7)	30 (61.2)	27 (41.5)	26 (44.1)	4 (30.8)	29 (38.7)	31 (66)
Age (years)									
	Mean (SD)	62.1 (10.5)	56.6 (7.6)	54.1 (15.5)	59.9 (9.4)	59.1 (6.0)	57.2 (13.1)	62.2 (10.6)	50.6 (12.9)
	<50	30	30	19	11	2	4	10	18
	50-60	76	141	12	26	36	1	19	16
	61-70	97	56	9	17	19	7	30	12
	71-80	53	14	9	11	2	1	14	1
	>80	5	0	0	0	0	0	2	0
AJCC-Stage									
	IA	7			1			0	
	IB	2			0			10	
	IIA	55			11			20	
	IIB	84			15			43	
	III	20			10			1	
	IV	93			28			1	
CA 19-9 (U/mL)									
	≤ 36	73	239	48	21	58	13	29	45
	> 36	188	2	1	44	1	0	46	2
Body mass index (kg/m^2)									
	< 25	199	161	35	53	45	10	55	34
	≥ 25	62	80	14	12	14	3	20	13
Risk factors									
Smoking, n (%)	Yes	109	106	12	17	33	5	29 (38.7)[a]	16 (34)
	No	151	104	37	47	13	8	46 (61.3)	31 (66)
	Unknown	1	31	0	1	13	0	0	0
Alcohol, n (%)	Yes	111	209	16	16	51	4	39 (52)[a]	20 (42.6)
	No	149	0	33	48	0	9	36 (48)	27 (57.4)
	Unknown	1	32	0	1	8	0	0	0
Pancreatic benign									
	NET			14			2		5
	SPT			2			0		7
	IPMN			14			5		5
	MCN			4			0		10
	SCN			3			1		5
	Pancreatitis			4			2		12
	Pseudocyst			0			0		3
	SPN			8			3		0

Abbreviations: PDAC, Pancreatic ductal adenocarcinoma; SD, standard deviation; NET, neuroendocrine tumor; SPT, solid pseudopapillary neoplasm; IPMN, Intraductal papillary mucinous neoplasm with low-grade dysplasia; MCN, mucinous cystic neoplasm; SCN, serous cystadenoma; SPN, Solid-pseudopapillary neoplasm; CA19-9, carbohydrate antigen 19-9.

[a] Percentage between two groups

Table 2. Result of diagnostic performance of multi-marker panel, combined panel, and CA 19-9 in training and validation set.

	Training set				Validation set			
	AUC (95% CI)	**Sensitivity (%)**	**Specificity (%)**	***P*-value**[a]	**AUC (95% CI)**	**Sensitivity (%)**	**Specificity (%)**	***P*-value**[a]
CA 19-9	0.872 (0.838-0.907)	69.7	98.6		0.832 (0.754-0.910)	66.2	98.6	
Multi-Marker Panel	0.977 (0.967-0.987)	90.4	95.9	< 0.001	0.953 (0.914-0.991)	84.6	94.4	< 0.01
Multi-Marker Panel + CA19-9	0.989 (0.981-0.996)	92.7	98.3	< 0.001	0.972 (0.944-0.999)	86.2	97.2	< 0.001

[a] Comparisons between AUC values of CA 19-9 and Multi-Marker Panel or Combined Panel (*P*-value from DeLong's test).

Abbreviations: AUC, area under the curve; CI, confidence interval.

Table 3. Result of diagnostic performance of multi-marker panel, combined panel and CA 19-9 in an independent validation set

	Independent validation set			
	AUC (95% CI)	**Sensitivity (%)**	**Specificity (%)**	***P*-value[a]**
CA 19-9	0.771 (0.687-0.856)	61.3	95.7	
Multi-Marker Panel	0.928 (0.885-0.971)	81.3	89.4	< 0.001
Multi-Marker Panel + CA19-9	0.952 (0.918-0.985)	89.3	87.2	< 0.001

[a] Comparisons between AUC values of CA 19-9 and Multi-Marker Panel or Combined Panel (*P*-value from DeLong's test).

Abbreviations: AUC, area under the curve; CI, confidence interval;

FIGURE LEGENDS

Figure 1. Overall Study design. The large-scale, quantitative, proteomic data set consisted of 1,008 plasma samples collected from five medical centers. The training/validation sets consisted of samples from multiple medical centers (NCC, SNUH, SMC, and YSH), whereas the independent validation set consisted of samples from a single medical center (AMC). The multi-marker panel was developed through the training set was first validated using the validation set and then validated using the independent validation set. To confirm the ability to classify PDAC from other cancers, an additional set was configured. All additional sets consisted of samples from SNUH. Abbreviations: NCC, National Cancer Center; SNUH, Seoul National University Hospital; SMC, Samsung Medical Center; YSH, Yonsei Severance Hospital; AMC, Asan Medical Center; PDAC, Pancreatic ductal adenocarcinoma.

Figure 2. Diagnostic performance of the multi-marker panel, the combined panel, and CA 19-9 alone for the training and validation sets. Receiver operating characteristic (ROC) curves for the multi-marker panel, the combined panel, and CA19-9 were compared in the (A, C) training set and (B, D) validation set. The AUC values of the multi-marker panel were 0.977 (95% CI:0.967–0.987) and 0.953 (95% CI:0.914–0.991) for the training set and validation set, respectively. The combined panel yielded AUC values of 0.989 (95% CI:0.981–0.996) and 0.972 (95% CI:0.944–0.999) for the training set and validation set, respectively. Both the multi-marker panel and the combined panel showed statistically

significant improvements in AUC values compared with CA 19-9 alone ($P < 0.001$ in A, C, and D and $P < 0.01$ in B; Delong's test). Abbreviations: AUC, the area under the curve; CI, confidence interval.

Figure 3. Diagnostic performance of the multi-marker panel, the combined panel, and CA 19-9 in the independent validation set. Receiver operating characteristic (ROC) curves for the multi-marker panel, the combined panel, and CA19-9 in the independent validation set. (A) The multi-marker panel had an AUC of 0.928 (95% CI:0.885–0.971), and (B) the combined panel showed an AUC of 0.952 (95% CI: 0.918–0.985) for the independent validation set. The multi-marker panel and the combined panel showed significant improvements in AUC values compared with the value for CA 19-9 alone ($P < 0.001$ in A and B; Delong's test). Abbreviations: AUC, the area under the curve; CI, confidence interval.

Figure 1

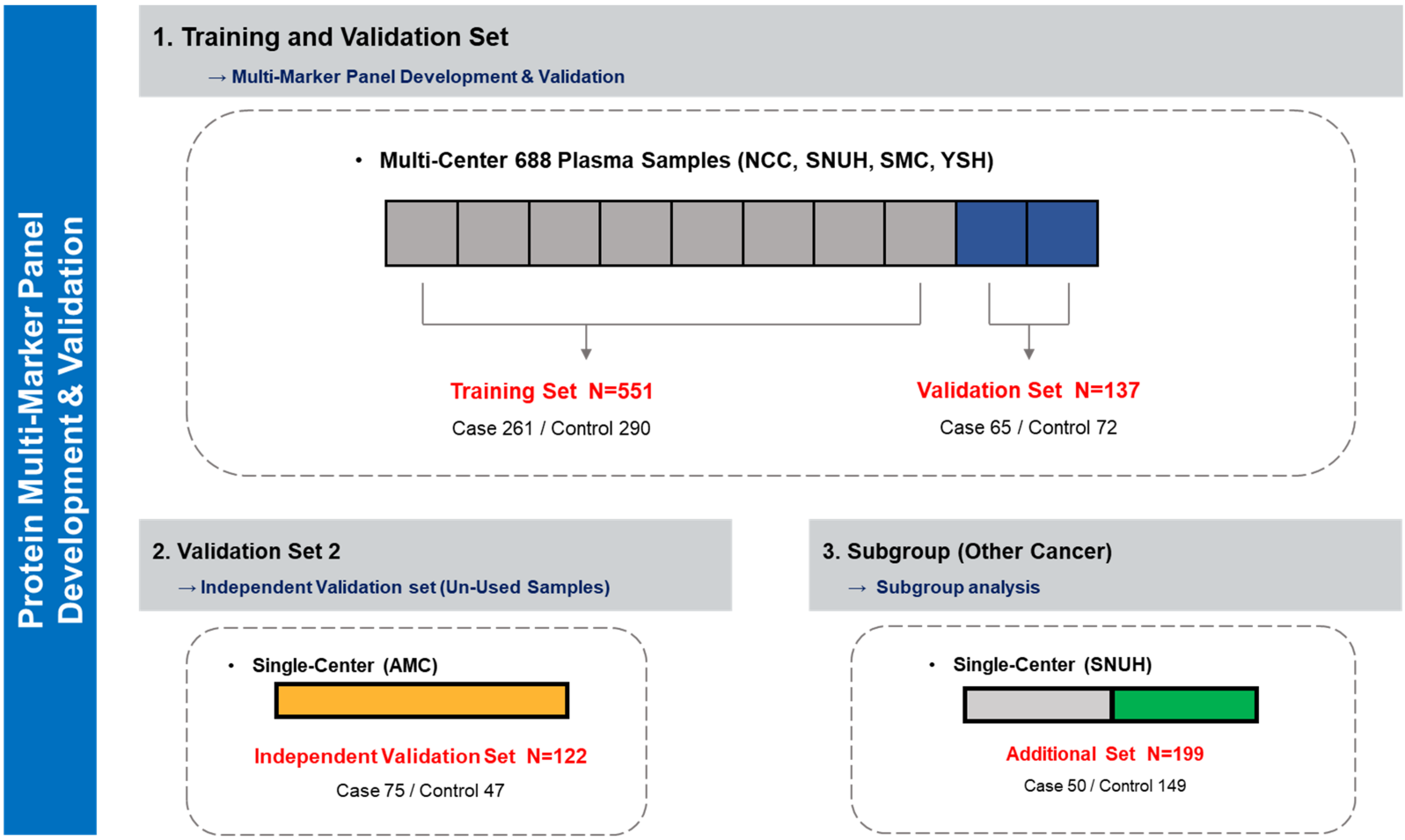

Protein Multi-Marker Panel Development & Validation
1. Training and Validation Set
→ Multi-Marker Panel Development & Validation
• Multi-Center 688 Plasma Samples (NCC, SNUH, SMC, YSH)
Training Set N=551
Case 261 / Control 290
Validation Set N=137
Case 65 / Control 72
2. Validation Set 2
→ Independent Validation set (Un-Used Samples)
• Single-Center (AMC)
Independent Validation Set N=122
Case 75 / Control 47
3. Subgroup (Other Cancer)
→ Subgroup analysis
• Single-Center (SNUH)
Additional Set N=199
Case 50 / Control 149

Figure 2

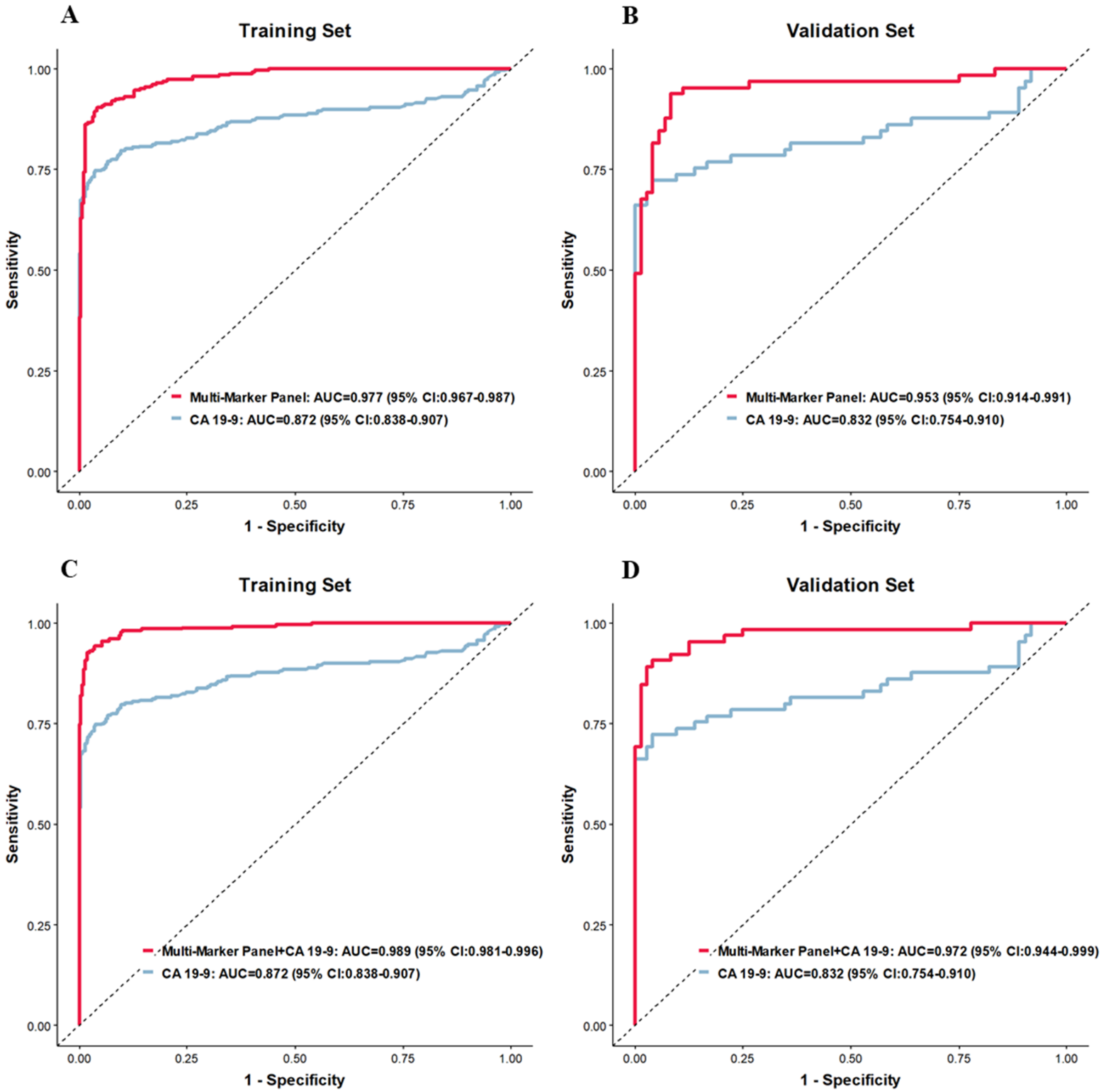
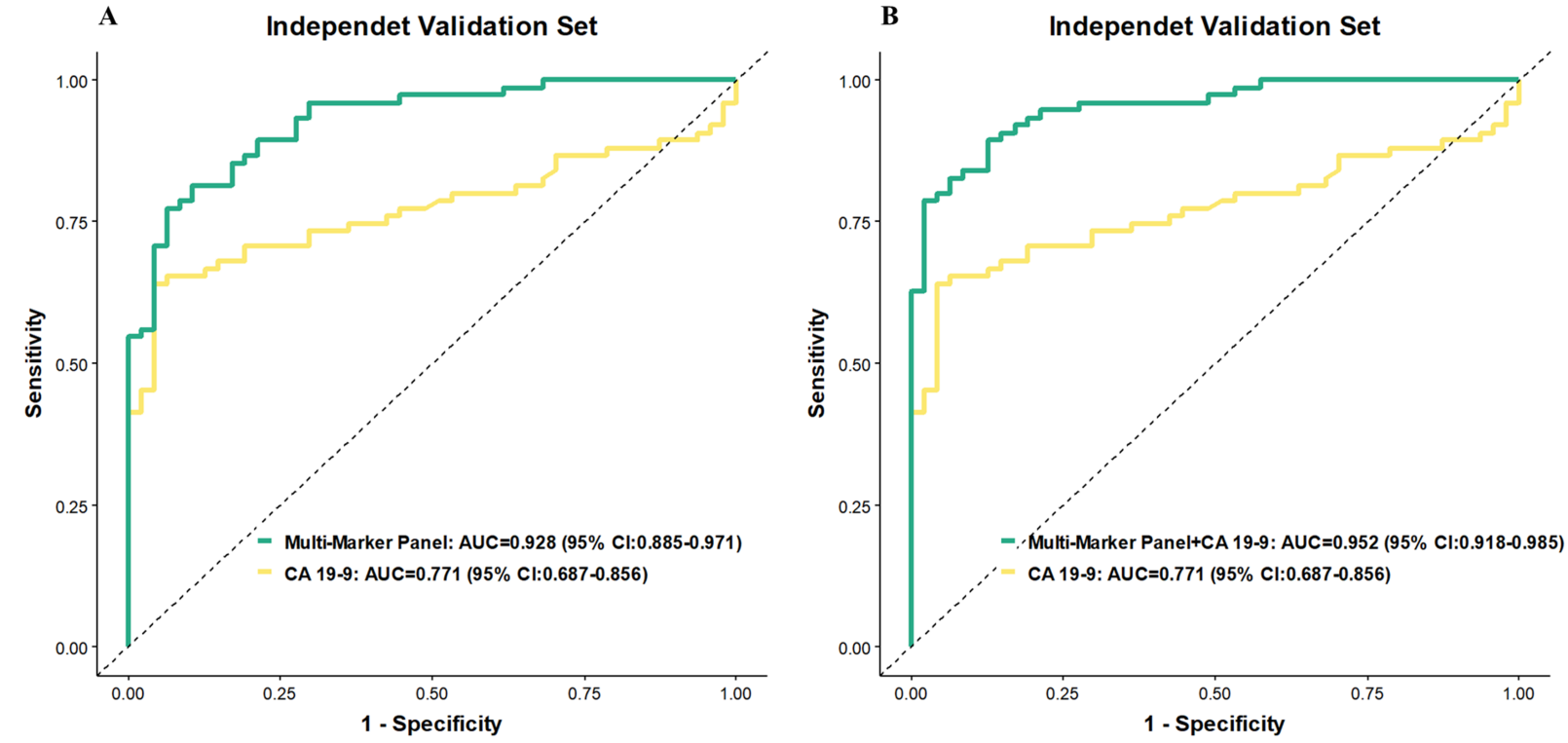

A
Training Set
Sensitivity
1 - Specificity
Multi-Marker Panel: AUC=0.977 (95% CI:0.967-0.987)
CA 19-9: AUC=0.872 (95% CI:0.838-0.907)
B
Validation Set
Sensitivity
1 - Specificity
Multi-Marker Panel: AUC=0.953 (95% CI:0.914-0.991)
CA 19-9: AUC=0.832 (95% CI:0.754-0.910)
C
Training Set
Sensitivity
1 - Specificity
Multi-Marker Panel+CA 19-9: AUC=0.989 (95% CI:0.981-0.996)
CA 19-9: AUC=0.872 (95% CI:0.838-0.907)
D
Validation Set
Sensitivity
1 - Specificity
Multi-Marker Panel+CA 19-9: AUC=0.972 (95% CI:0.944-0.999)
CA 19-9: AUC=0.832 (95% CI:0.754-0.910)

Figure 3


A
Independet Validation Set
Sensitivity
1 - Specificity
0.00
0.25
0.50
0.75
1.00
Multi-Marker Panel: AUC=0.928 (95% CI:0.885-0.971)
CA 19-9: AUC=0.771 (95% CI:0.687-0.856)
B
Independet Validation Set
Sensitivity
1 - Specificity
Multi-Marker Panel+CA 19-9: AUC=0.952 (95% CI:0.918-0.985)
CA 19-9: AUC=0.771 (95% CI:0.687-0.856)