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A Tissue-based Biomarker Risk Score for Predicting Survival in Pancreatic Ductal Adenocarcinoma

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Abstract

Background/Aim: Pancreatic cancer is a highly aggressive disease, with limited prognostic tools available for risk stratification. This study aimed to evaluate the prognostic significance of nine tissue biomarkers and develop a biomarker-based risk score for predicting patient survival.

Patients and Methods: Tumor samples from 141 resected patients with pancreatic cancer were analyzed with tissue microarrays and immunohistochemistry to assess the expression levels of CA 19-9, CA 50, CA 242, CA 724, GDF15, MMP7, MUC2, TFF1, and THBS2. A Lasso-Cox regression model was used to develop a prognostic risk score and the performance of the risk score was assessed using Kaplan–Meier survival analysis and receiver operating characteristic (ROC) curves.

Results: Among the nine biomarkers, CA19-9, CA50, CA242, CA724, and THBS2 were identified as significant predictors of survival in univariable analyses. A prognostic model was constructed and included CA19-9, CA724, THBS2, tumor location, resection margin status, grade, and American Joint Committee on Cancer stage. The prognostic risk score effectively stratified patients into high- and low-risk groups, demonstrating a significant difference in median survival (14.8 *vs.* 36.0 months) and 5-year survival (5.9% *vs.* 26.0%) (p<0.001). The model achieved good predictive performance for long-term survival with an AUC of 0.704.

Conclusion: This study identifies several tissue biomarkers associated with survival and introduces an integrative risk model to stratify pancreatic cancer patients by outcomes. The model shows good discriminatory ability and may provide a basis for more personalized risk assessment and treatment planning, although additional validation is required.

Keywords: Pancreatic cancer, biomarkers, prognosis, risk model, survival analysis, risk stratification.

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Introduction

Pancreatic ductal adenocarcinoma is a highly lethal disease with a 5-year survival below 5% (1) and a global mortality of 467000 deaths per year (2). The majority of patients are diagnosed at an advanced stage, with only approximately 15-20% of patients amenable to surgical resection. The death rate is increasing and pancreatic cancer is predicted to become the second leading cause of cancer death in the United States in 2030 (3).

The identification of prognostic factors is crucial for patient management by offering insights into tumor biology, aiding in risk stratification and enabling the development of precision medicine approaches. Conventional histopathological factors, such as tumor size, grade, stage, vascular invasion and resection margin status (4), as well as clinical risk scores (5-7), have prognostic utility, but may not accurately account for the observed variability in patient survival.

Molecular subtyping of tumor specimens may enhance prognostic stratification. In recent years, genomic and transcriptomic profiling of pancreatic cancer has revealed distinct molecular subtypes with differing therapeutic vulnerabilities and clinical outcomes (8-13). While molecular subclassification of pancreatic cancer may yield valuable information, the cost of genomic sequencing and the volume of raw data generated has made its translation into routine clinical practice challenging (14).

Immunohistochemistry (IHC) is a widely used method in routine pathology and clinical practice, enabling the assessment of biomarker expression, while also providing details into tissue and cellular architecture (15). In solid tumor pathology, most current prognostic markers are evaluated using immunohistochemistry. Previous studies have identified a plethora of prognostic immunohistochemical markers for pancreatic cancer (16, 17). Despite these efforts, no individual biomarker has yet reached clinical applicability, and the combination of multiple biomarkers may be necessary to provide comprehensive prognostic information.

The aim of this study was to evaluate the prognostic utility of established and investigational immunohistochemical biomarkers in pancreatic cancer. By expanding on previous research and considering the interplay between molecular factors and clinicopathological parameters, a prognostic model is generated that may guide patient management.

Patients and Methods

Patient cohort. The patient cohort consisted of consecutive patients with pancreatic ductal adenocarcinoma who underwent curative intent pancreatic surgery from 1995 to 2017 at Skåne University Hospital, Lund and Malmö, Sweden, for whom archival formalin-fixed, paraffin-embedded tumor tissues were available. The cohort has been previously described in detail (18). Clinical and histopathological data were obtained from hospital and pathology records. Ethical permission for the study was granted by the Ethical Committee at Lund University (Ref 2010/684, 2012/661, 2015/266, 2015/833, 2017/320) and the Swedish Ethical Review Authority (Ref 2022-02371-01). This study followed STROBE (19) and REMARK guidelines (20).

Tissue microarray construction. Areas representative of cancer were marked on hematoxylin and eosin stained slides and tissue microarrays (TMAs) were constructed as previously described (18). In brief, a set of four cores with a diameter of 2 mm were extracted from each specimen using an automated tissue array device (Minicore® 3, Alphelys, Plaisir, France) and fixed into a new paraffin block.

Immunohistochemistry. For immunohistochemical analysis, 3 µm TMA-sections were automatically pre-treated using the PT-link system (Dako, Agilent Technologies, Glostrup, Denmark). The individual TMA-slides were incubated with respective primary antibodies as stated in Table I. After a washing step, the sections were treated with biotinylated horse anti-mouse or goat anti-rabbit secondary antibodies (dilution 1:200, Vector Laboratories, Burlingame, CA, USA). Avidin–biotin–peroxidase complex (Vectastain Elite ABC-HRP Kit, Vector Laboratories) was applied for signal amplification. The color was developed using chromogen diaminobenzidine

Table I. *Primary antibodies.*

Primary antibody	Company	Cat no	Dilution
CA19-9	Abcam, Amsterdam, the Netherlands	ab289665	1:500
CA50	LSBio, Newark, CA, USA	LS-C77472	1:100
CA242	Innovex Biosciences, Richmond, CA, USA	MAB560C	1:200
CA724	Abcam, Amsterdam, the Netherlands	ab199002	1:200
GDF15	Atlas Antibodies, Bromma, Sweden	AMAb90687	1:100
MMP7	SCBT, Dallas, TX, USA	sc-80205	1:100
MUC2	SCBT, Dallas, TX, USA	sc-7314	1:200
TFF1	SCBT, Dallas, TX, USA	sc-271464	1:75
THBS2	Abcam, Amsterdam, the Netherlands	Ab112543	1:100

(DAB) (Vector Laboratories). The nuclei were counter stained with hematoxylin.

Imaging and quantitation. Imaging and image processing. Immunolabeled TMA slides were scanned using a Hamamatsu S210 microscope slide scanner (Hamamatsu, Hamamatsu City, Japan). The slides contained 0-4 labeled tissue sections for each patient. The TMA tissue sections selected for analysis were annotated using the NDPview software (Hamamatsu). Artefacts, such as folds, necrotic or other inapplicable regions were excluded from the sections. A Python script was used to extract the x and y coordinates of the region of interest (ROI). ImageJ script was then used to extract each TMA ROI as a jpg file and for color deconvolution to separate each image into two images, one immunolabelled (DAB-brown) and one hematoxylin (blue) stained image.

Digital analyses and quantitation. The DAB labeling intensity was determined employing three thresholds: high, medium and low (3 to 0). The distribution of labeling was recorded as percentage of the total area (0-100%).

H-score: H-score was calculated using the formula: 3 × % area + 2 × % area + 1 × % area (where 3 represents high intensity, 2 represents medium intensity and 1 represents low intensity), resulting in a total H-score for each section and marker between the range 0 and 300.

Lasso-Cox regression and survival analysis: Correlation between biomarker expression and clinical variables was determined using the Mann–Whitney *U* and Kruskal–Wallis tests. The R packages "survival" and "glmnet" were used for Lasso–Cox analysis. The seed was set to 100, and 10-fold cross-validation was used to evaluate model performance. The "maxstat" package was used to calculate the optimal cutoff risk score value. The Kaplan–Meier survival curve was generated using the "survfit" function and visualized with the "ggsurvplot" package. The ROC curves for 1-year, 3-year, and 5-year survival were calculated using the "timeROC" package and visualized with "ggplot". Missing values were imputed using the "mice" R package. The effect of the imputations was evaluated by comparing the distribution of the data and the results of univariable Cox analysis before and after imputation. All analyses were carried out using the R software, version 4.5.1 (The R Foundation, Vienna, Austria).

Results

Following antibody optimization and staining, biomarker expression was evaluated in 144 of the 146 (98.6%) tumors represented in the TMA. Three patients had to be excluded from further analyses due to lack of clinical and follow-up information and the final patient cohort included 141 patients. A detailed list of patient characteristics is reported in Table II. The median follow-up was 2.1 (range=0.1-19.8) years. Representative IHC images are shown in Figure 1A. CA19-9 displayed the highest median H-score among the markers (Figure 1B).

Table II. *Baseline patient characteristics.*

Variables	All patients (N=141)
Age, years	67 (63-73)
Female sex	68 (48.2%)
Tumor location (head)	119 (84.4%)
Tumor size (cm)	3 (2.5-4)
Resection margins	
Negative	85 (60.3%)
Positive	56 (39.7%)
Tumor grade	
Well/moderately differentiated	57 (40.4%)
Poorly differentiated	83 (58.9%)
Unknown	1 (0.7%)
AJCC stage	
1	24 (17.0%)
2	62 (44.0%)
3	54 (38.3%)
Unknown	1 (0.7%)
Adjuvant chemotherapy	
None	21 (14.9%)
Received	107 (75.9%)
Unknown	13 (9.2%)

AJCC: American Joint Committee on Cancer. Qualitative data are presented as number (%) and quantitative data as median (interquartile range).

Table III. *Univariable Cox analysis.*

	HR	95%CI	*p*-Value
Biomarkers			
CA 19-9	1.003	1.000-1.005	0.022
CA 50	1.007	1.002-1.012	0.003
CA 242	1.004	1.001-1.006	0.020
CA 724	1.008	1.004-1.013	<0.001
GDF15	1.002	0.998-1.006	0.465
MMP7	1.006	0.992-1.019	0.423
MUC2	1.003	0.992-1.015	0.549
TFF1	1.005	0.989-1.021	0.541
THBS2	1.006	1.000-1.013	0.048
Clinicopathological parameters			
Age, years	1.000	0.980-1.021	0.972
Female sex	0.826	0.588-1.161	0.271
Tumor location (body/tail)	1.361	0.860-2.155	0.188
Tumor size (cm)	1.051	0.923-1.196	0.451
Resection margin (positive)	1.283	0.909-1.813	0.156
Tumor Grade (poorly differentiated)	1.448	1.020-2.055	0.038
AJCC Stage			
1	1		
2	1.207	0.749-1.943	0.440
3	1.318	0.811-2.142	0.265
Adjuvant chemotherapy	0.685	0.427-1.099	0.117

Association between biomarker expression and clinicopathological parameters. As illustrated in Figure 2, CA242 was significantly associated with tumor location (p=0.032). There were no other significant correlations between biomarker expression and clinicopathological parameters.

Prognostic value of individual biomarkers and clinicopathological parameters. Univariable Cox regression analysis demonstrated that CA19-9 (p=0.022), CA50 (p=0.003), CA242 (p=0.020), CA724 (p<0.001), and THBS2 (p=0.048), were significant prognostic biomarkers (Table III). Among the clinicopathological parameters, only tumor grade (p=0.038) was significantly associated with prognosis.

Construction of a prognostic model. Lasso-Cox analysis was used to construct a prognostic model based on biomarker and clinicopathological data (Figure 3). The minimal lambda was chosen as the optimal lambda value, which was 0.08553922.

The model was constructed as follows: Risk Score = 0.001030577 * CA19-9 + 0.001061149 * THBS2 + 0.005908164 * CA724 + 0.151973069 * (Tumor location) + 0.045138433 * (Resection margin) + 0.210925412 * Grade + 0.063698671 * [American Joint Committee on Cancer (AJCC) Stage].

The risk score of each sample was calculated using the prediction model. The optimal cutoff value (1.07) was determined using the "maxstat" package. The samples were divided into "high-risk" and "low-risk" groups according to this cutoff value. The Kaplan–Meier plot showed that overall survival was significantly worse in the "high-risk" group compared to the "low-risk" group (p<0.0001; Figure 4). We performed Cox proportional hazards regression analysis to assess the association between the two risk groups and survival outcomes. The hazard ratio (HR) for the "high-risk" group compared to the "low-risk" group was 2.23 (95%CI=1.52-3.29; p<0.001). The median survival in high-risk group was 14.8

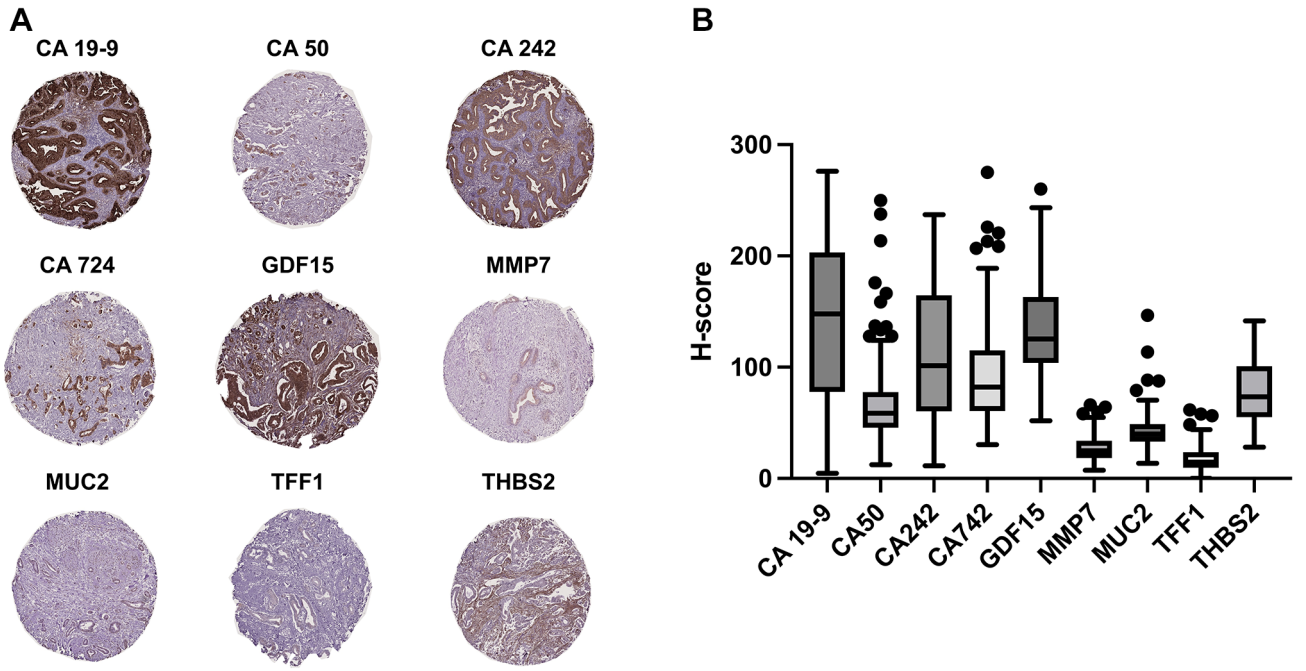

Figure 1. *Expression of CA 19-9, CA 50, CA 242, CA 724, GDF15, MMP7, MUC2, TFF1, and THBS2 in pancreatic cancer tissue microarrays. A) Representative immunohistochemical staining images. B) H-scores for each biomarker.*

months, compared to 36.0 months in the low-risk group, and with a 5-year survival of 5.9% *vs.* 26.0% ($p<0.001$). The predictive ability of the model was evaluated using ROC curve analysis. The AUC was 0.671 for 1-year survival, 0.713 for 3-year survival, and 0.704 for 5-year survival (Figure 5).

Discussion

In this study, we evaluated the prognostic value of selected immunohistochemical biomarkers. Yet, relying on individual biomarkers may not provide sufficient predictive accuracy. By combining CA19-9, THBS2 and CA724 and clinicopathological variables a robust survival model was constructed. We identified high-risk and low-risk groups, which demonstrated a clear difference in median and 5-year survival. The model had an AUC of 0.704, which indicates good discriminatory ability.

Several of the biomarkers assessed in this study, can also be measured in the circulation. Compared to serum-based prognostic models, a tissue-based model is considered less influenced by potential comorbidity. Serum measurements could be affected by other conditions, such as biliary obstruction, metabolic syndrome, age and sex (21-25).

CA19-9 is a sialylated oligosaccharide expressed on the surface of many types of cancer, particularly pancreatic cancer. Importantly, CA19-9 is not just a passive marker but actively involved in cancer progression and metastasis. CA19-9 interacts with endothelial selectins to promote the adhesion of cancer cells to the vascular endothelium, which facilitates cancer dissemination (26). Furthermore, CA19-9 may be involved in the pronounced fibroinflammatory response in pancreatic cancer (27), and may also contribute to immunosuppression by its ability to inhibit the proliferation of activated T-cells (28). CA19-9 is overexpressed in pancreatic cancer tissue compared to normal pancreas (29), but the prognostic role of tissue CA19-9 expression has not been extensively explored. Elevated serum CA19-9 levels are often seen in patients

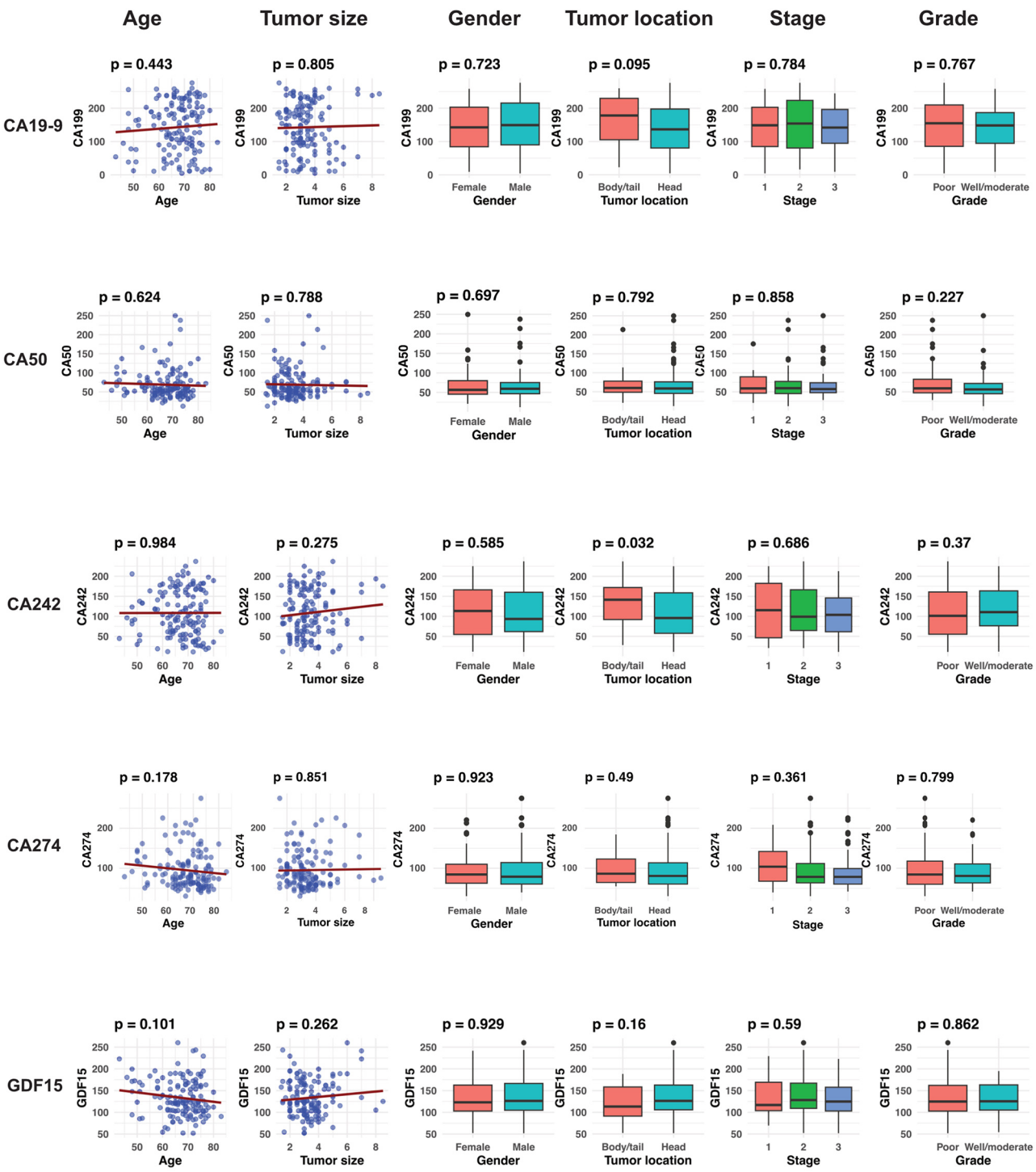

Figure 2. *Continued*

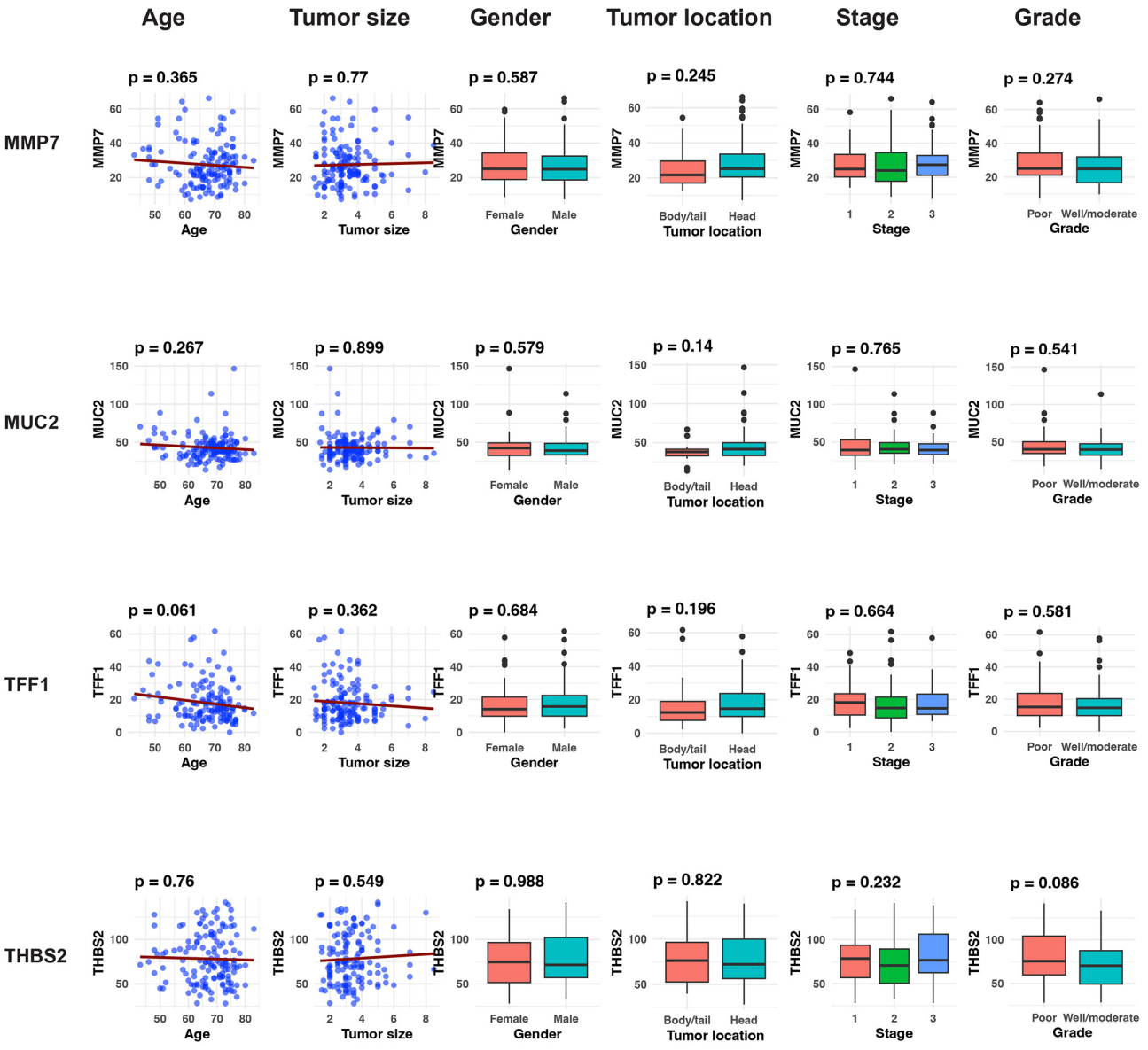

Figure 2. *Correlation between biomarker levels and clinicopathological parameters.*

with pancreatic cancer and correlate with tumor burden and poor survival (30).

CA724, tumor-associated glycoprotein 72 (TAG-72), is a mucin-like glycoprotein expressed by many cancer types (25). CA724 is highly expressed in pancreatic cancer tissue (31), but prognostic data are limited. Higher serum levels of CA724 have been associated with unresectable pancreatic tumors, indicating its potential role in assessing tumor resectability (32).

THBS2 is a glycoprotein expressed by cancer associated fibroblasts (33). It is upregulated by TGF-β1 and is thought to be involved in cancer cell adhesion through interactions with integrin $\alpha_v\beta_3$ and CD36 (33). In colorectal cancer, THBS2 is thought to cause oxaliplatin

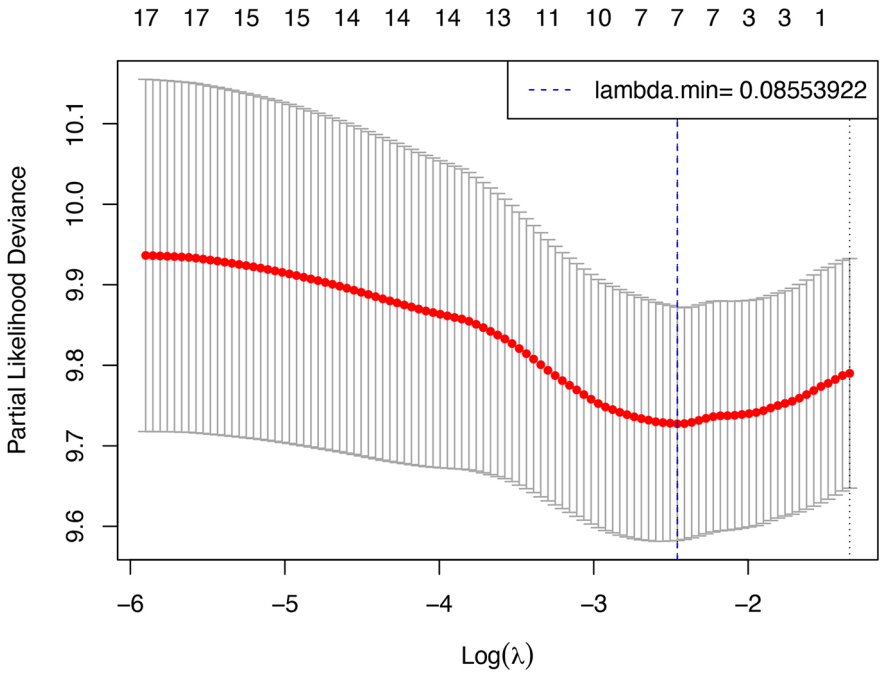

Figure 3. *Construction of a prognostic model using Lasso-Cox regression analysis.*

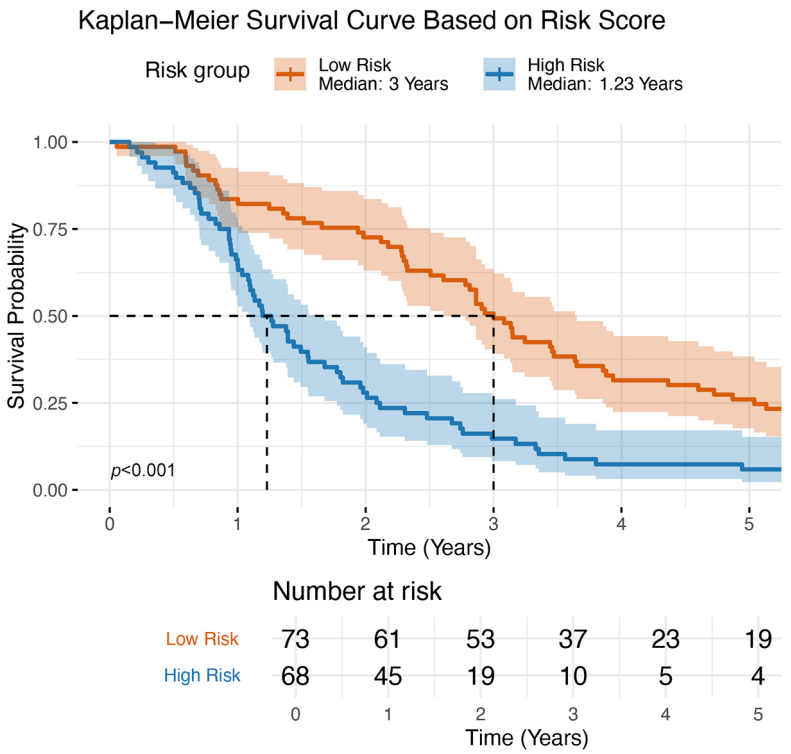

Figure 4. *Kaplan–Meier survival curves stratified according to risk score value.*

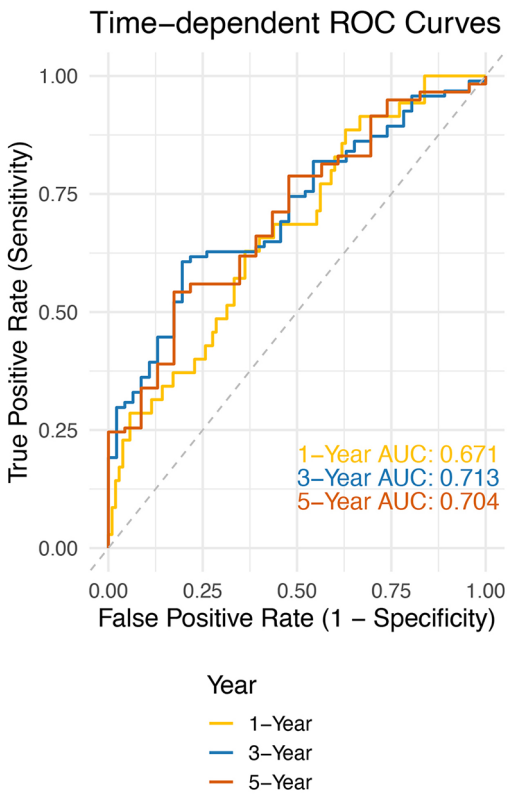

Figure 5. *Predictive ability of the model (area under the curve, AUC) at 1, 3, and 5 years.*

resistance (34). Notably, oxaliplatin is part of the FOLFIRINOX chemotherapy regimen used in pancreatic cancer (35). In a previous study, immunohistochemical analysis of THBS2 revealed significantly higher expression pattern in pancreatic cancer compared to normal tissue. The increased levels correlated with metastasis and worse prognosis (33), which is in agreement with our results.

In the correlation analysis, we found that CA242 is significantly overexpressed in tumors located in the body/tail of the pancreas. Furthermore, CA242 emerged as a prognostic factor, aligning with the well-documented observation that patients with body/tail tumors generally have poorer outcomes compared to those with tumors in the head of the pancreas (36). While the causal relationship between elevated CA242 expression and the unfavorable prognosis in body/tail tumors remains to be elucidated, this finding underscores the heterogeneity of pancreatic cancer and warrants further investigation.

Study limitations include the small training cohort; a larger one would have led to a more reliable model with better discriminatory ability. The lack of an external validation cohort naturally limits the generalizability of our research and is needed to evaluate its clinical utility.

In conclusion, we evaluated the tissue expression of immunohistochemical biomarkers in pancreatic cancer and constructed a novel biomarker-based prognostic model to aid in risk stratification and development of personalized treatment strategies.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

Daniel Kriz: Investigation; Methodology; Writing – Original Draft; Lizhi Lin: Methodology; Formal analysis; Ragnar Norrsell: Writing – Review & Editing; Monika Bauden: Investigation; Methodology; Katarzyna Said Hilmersson: Investigation; Methodology; Roland Andersson: Conceptualization; Resources; Funding acquisition; Supervision; Writing – Review & Editing; Daniel Ansari: Conceptualization; Resources; Funding acquisition; Supervision; Writing – Review & Editing.

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Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools, including large language models or machine learning software, were used in the preparation, analysis, or presentation of this manuscript.

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