ORIGINAL ARTICLE

Novel prognostic markers within the CD44–stromal ligand network in pancreatic cancer

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Abstract

The dense stroma in pancreatic cancer tumours is rich in secreted extracellular matrix proteins and proteoglycans. Secreted hyaluronan, osteopontin and type IV collagen sustain oncogenic signalling by interactions with CD44s and its variant isoform CD44v6 on cancer cell membranes. Although well established in animal and *in vitro* models, this oncogenic CD44–stromal ligand network is less explored in human cancer. Here, we use a pancreatic cancer tissue microarray from 69 primary tumours and 37 metastatic lymph nodes and demonstrate that high tumour cell expression of CD44s and, surprisingly, low stromal deposition of osteopontin correlate with poor survival independent of established prognostic factors for pancreatic cancer. High stromal expression of hyaluronan was a universal trait of both primary tumours and metastatic lymph nodes. However, hyaluronan species of different molecular mass are known to function differently in pancreatic cancer biology and immunohistochemistry cannot distinguish between them. Using gas-phase electrophoretic molecular mobility analysis, we uncover a shift towards high molecular mass hyaluronan in pancreatic cancer tissue compared to normal pancreas and at a transcriptional level, we find that hyaluronan synthesising HAS2 correlates positively with CD44. The resulting prediction that high molecular mass hyaluronan would then correlate with poor survival in pancreatic cancer was confirmed in serum samples, where we demonstrate that hyaluronan >27 kDa measured before surgery is an independent predictor of postoperative survival. Our findings confirm the prognostic value of CD44 tissue expression and highlight osteopontin tissue expression and serum high molecular mass hyaluronan as novel prognostic markers in pancreatic cancer.

Keywords: pancreatic cancer; CD44; hyaluronan; type IV collagen; osteopontin; biomarkers

Introduction

Pancreatic ductal adenocarcinoma (pancreatic cancer) is generally diagnosed at a late metastatic stage and patients often die just months after their first symptoms appear [1,2]. Even patients diagnosed at an early stage have a median survival of less than 2 years despite surgery and chemotherapy with curative intent [3,4]. The tumours are characterised by an abundant desmoplastic stroma rich in extracellular matrix components [5]. These include fibrillar collagens (type I and type III collagen) and hyaluronan but also basement membrane proteins including type IV collagen and matricellular proteins such as osteopontin [5–7]. These secreted extracellular matrix components promote proliferation, migration and survival through cell adhesion receptors [6,8].

The cell adhesion receptor CD44 is widely expressed on both normal and malignant cells. Upon ligand binding, CD44 recruits receptor tyrosine kinases, which in turn promotes pancreatic cancer cell survival and migration *in vitro* and in mouse studies

[8–10]. In human pancreatic cancer, high expression of CD44s and the variant isoform 6 (CD44v6) have been associated with poor survival [9,11–14].

Hyaluronan promotes cancer cell survival and migration in various cancer types through CD44 interactions [15]. Synthesis and degradation produce hyaluronan species of different sizes. Low molecular mass hyaluronan promotes pancreatic cancer cell migration [16], while high molecular mass hyaluronan acts as an osmotic space occupant causing blood vessel compression and reduced chemotherapy delivery in pancreatic cancer mouse models [17,18]. In pancreatic cancer patients, high tumour expression of hyaluronan has been associated with poor survival [19,20]. Other stromal CD44 ligands include type IV collagen [21] and osteopontin [22]. Both type IV collagen and osteopontin promote pancreatic cancer cell invasion *in vitro* [23,24] and are increased in the circulation in pancreatic cancer patients [25].

In this study, we hypothesise that the CD44 receptor and its stromal ligands constitute a tumour-promoting network in pancreatic cancer and aim to find prognostic markers within this context.

Materials and methods

Ethics statement

All subjects provided written informed consent. The study was conducted in accordance with the Helsinki Declaration of 1975 and was approved by the regional research ethics board of northern Sweden (Dnr. 09-175M/2009-1378-31).

Tissue samples

A previously described tissue microarray was used for the tissue studies [26]. The tissue microarray has core areas of 1 mm in diameter selected by an experienced pathologist (WW). It includes three cores from each primary tumour ($n = 69$) and one to three cores from metastatic lymph nodes ($n = 32$) (Table 1). Due to sample loss during sectioning, the number of analysed tumour cores varied between 65 and 68 for primary tumours and between 24 and 32 for metastatic lymph nodes. Normal pancreatic tissue was collected from patients undergoing pancreatic surgery for non-malignant conditions ($n = 4$). The staining analysis was performed under blinded conditions.

Table 1. Clinical characteristics of the tissue microarray cohort and the cases and controls included in serum hyaluronan measurements

Variable (*n*, %)	Tissue cases (*n* = 69)	Serum cases (*n* = 44)	Serum controls (*n* = 22)
Gender			
Male	33 (47.8%)	24 (54.5%)	10 (45.5%)
Female	36 (52.2%)	20 (45.5%)	12 (55.5%)
Age (years)			
Mean (95% CI)	64.4 (62.4–66.5)	66.9 (64.5–69.2)	60.8 (57.1–64.5)
Tumour grade			
Grade 1	17 (24.6%)	9 (20.5%)	
Grade 2	42 (60.9%)	18 (40.9%)	
Grade 3	8 (11.6%)	14 (31.8%)	
NA	2 (2.9%)	3 (6.8%)	
Resection margin			
R1–2	44 (63.8%)	36 (81.8%)	
R0	20 (29.0%)	8 (18.2%)	
NA	5 (7.2%)	–	
TNM stage			
Stage I	16 (23.2%)	12 (27.3%)	
Stage II	37 (53.6%)	29 (70.3%)	
Stage III	4 (5.8%)	–	
Stage IV	1 (1.5%)	–	
NA	11 (16.0%)	1 (2.3%)	
Neoadjuvant treatment			
None	59 (85.5%)	44 (100%)	
Chemotherapy	2 (2.9%)	–	
Radiotherapy	1 (1.5%)	–	
Preop IP 5-FU*	7 (10.1%)	–	
Adjuvant treatment			
Yes	23 (33.4%)	23 (52.3%)	
No	39 (56.5%)	11 (25.0%)	
NA	7 (10.1%)	10 (22.7%)	
Survival (months)			
Median (range)	12.4 (0.5–140.9)	24.5 (0.5–79)	

Intraperitoneal 5-fluorouracil given as a single bolus the day before surgery

Immunohistochemical staining

5 μm tissue microarray sections were stained for CD44s, CD44v6, osteopontin and type IV collagen expression using an automated Ventana Benchmark staining machine (Ventana Medical Systems, Tucson, AZ, USA). The following antigen retrieval methods were applied: (1) citrate buffer at pH 6.0 for 8 min at 94 °C for CD44s, (2) EDTA buffer for 4 min at 100 °C for CD44v6 and (3) none for type IV collagen and osteopontin stainings. See supplementary material, Table S1 for more information on the primary antibodies and concentrations used. Hyaluronan was stained using a biotinylated hyaluronan-binding protein, isolated from bovine nasal cartilage (Corgenix, Broomfield, CO, USA), according to a previously described protocol [27]. Tissues serving as negative controls were incubated with hyaluronidase

from *Streptomyces hyalurolyticus* (H-1136; Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37 °C to ensure assay specificity. In the final steps, all stains were developed with 3,3-diaminobenzidine and counterstained with Mayer's haematoxylin. Human skin served as positive controls.

Scoring of tissue expression

CD44s and CD44v6: The percentages of positive cancer cells were measured using the cell counter tool in ImageJ software (version 2.0). The core with the highest percentage of positive cells represented the score for each individual patient.

Osteopontin, hyaluronan and type IV collagen: Stromal expression was scored semi-quantitatively by two independent observers. Each observer scored individually and cores that were scored differently by the two observers were re-scored by each observer. Persistent differences after the second round were discussed between the investigators and a final score was obtained by consensus. The mean score of the three primary tumour cores represented the score for each individual patient.

Osteopontin and hyaluronan: An intensity score (0 = negative, 1 = positive, 2 = strong staining) multiplied by a distribution score (0 = 0–10%, 1 = 10–50%, 2 = 50–90%, 3 > 90% positive) was used. Osteopontin scores were divided into high/low using a score of 2 as a cut-off. Hyaluronan scores were divided into high/low using a score of 3 as a cut-off.

Basement membrane expression of type IV collagen has been suggested to be disrupted or lost and accumulates in the desmoplastic stroma in pancreatic cancer tissue [28]. Hence, type IV collagen expression was scored by two methods. In primary tumour and lymph nodes, basement membrane structures were scored as 0 = negative, 1 = positive but not fully circumferential around tumour clusters and 2 = positive and fully circumferential around tumour clusters. The stromal expression was scored as: 0 = negative, 1 = positive or 2 = strong.

Cut-off values for all markers were determined by testing different cut-off values in relation to survival in a subset of cases (n = 21). If an 'optimal cut-off' was found that divided patients into groups with different survival rates, that cut-off was used in the analysis of the entire cohort (n = 69). If no such optimal cut-off was found, the cut-off in primary tumours was determined as the score closest to the median score using the entire cohort and in lymph nodes, the cut-off was determined as positive/negative.

mRNA data from The Cancer Genome Atlas

We extracted clinical data and mRNA expression data from all the available primary pancreatic cancer tissue samples in The Cancer Genome Atlas (n = 146), https://cancergenome.nih.gov/ (March 2017). mRNA expression data were generated by first aligning RNA-Seq reads with a reference genome followed by normalisation using the fragments per kilobase of transcript per million mapped reads, the upper quartile method [29,30]. The data were log transformed. Two patients were excluded from all survival analyses (one with missing data on survival time and one with survival <1 month). Two patients were excluded from analyses involving hyaluronan synthase 1 and one patient was excluded from analysis of *TP53* mutational status due to missing data. The cohort was divided into high versus low expression by quartiles of mRNA expression levels.

Serum samples

Serum samples were obtained from a biobank collecting tissue and blood samples from patients undergoing pancreatic surgery at Umeå University Hospital. We included patients with a histopathologically verified diagnosis of pancreatic ductal adenocarcinoma between August 2009 and July 2014. Postoperative follow-up samples (≥28 days) were included when available. Patients with a history of malignant disease, systemic inflammatory disease or liver disease were excluded. Clinical characteristics and serum bilirubin levels were obtained from medical records. Patients undergoing surgery for non-malignant conditions or those who had an endoscopic examination without pathological findings served as controls. None of the controls had a previous history of malignant disease.

Measurements of serum hyaluronan

Serum hyaluronan levels were measured with two hyaluronan-binding protein (HABP)-based ELISA-like kits: (1) a competitive assay (HA-ELISA K1200; Eschelon Biosciences, Salt Lake City, UT, USA) and (2) a sandwich assay (HA Test Kit; Corgenix, Broomfield, CO, USA), according to the manufacturers' instructions. The kits differ in that the sandwich assay is molecular mass restricted and unable to detect low molecular mass hyaluronan (<27 kDa) but is more accurate in detecting high molecular mass hyaluronan compared to the molecular mass promiscuous competitive assay [31]. All samples were run in duplicate and coefficient of variation percentage (CV%) <10% was considered acceptable. Four samples were run on all

plate assays and were used to normalise for inter-plate variation. Absorbance was measured on a Thermo Multiskan Ascent (Thermo Fisher Scientific, Waltham, MA, USA) and plotted against the concentration of the standard curve using four-parameter logistic regression.

Hyaluronan molecular mass profile analysis

Hyaluronan was isolated from normal pancreas tissue ($n = 2$) and cancer tissue ($n = 2$) using a previously described method [32] with slight modifications. Frozen tissue samples were sectioned (100 times 20 μm) in a cryostat followed by drying using a vacuum rotary evaporator. Before the first and after the last section used, 5 μm cryostat sections were H&E stained and analysed to confirm tissue morphology (see supplementary material, Figure S1). Then, proteins were digested with Proteinase K (Sigma–Aldrich) at 55 °C overnight. Next, nucleic acids were digested using benzonase nuclease (Sigma–Aldrich) for 5 h at 37 °C. Next, chondroitin was digested using Chondroitinase ABC (Sigma–Aldrich) for 10 min at 37 °C. At the end of each day, chloroform was added to each sample and the aqueous phase was dialysed against 0.1 M NaCl using Amicon Ultra 3K concentration units (Millipore, MA, USA) followed by precipitation overnight in 99% ethanol. Samples were loaded on anion exchange mini-spin columns (Thermo Fisher Scientific) and centrifuged to wash out sulfated glycosaminoglycans and any remaining non-hyaluronan contaminants based on NaCl binding. Finally, the sample was dialysed against 20 mM ammonium acetate (pH 8.0) in Amicon Ultra 3K concentration units to remove salt.

Hyaluronan molecular mass profiles were quantified using gas-phase electrophoretic mobility molecular analysis (GEMMA) (TSI Corp., Shoreview, MN, USA) [33]. Hyaluronan standards of 30 and 1000 kDa (Hyalose LLC, Oklahoma, OC, USA) were used as reference samples.

Statistics

Statistical analysis was performed using STATA 12.1 (StataCorp, College Station, TX, USA) and Prism 5 (GraphPad Software, La Jolla, CA, USA). Continuous variables were analysed with two-tailed Student's *t*-test (parametric data), Wilcoxon rank sum test (nonparametric data), or Wilcoxon signed rank test (paired non-parametric data). Categorical variables were compared using chi-squared test or Fisher's exact test when appropriate. Correlations were analysed using Spearman's rank test or Pearson's correlations when appropriate. Survival analysis was performed with Kaplan–Meier curves and log-rank and Wilcoxon–Breslow estimates. The Cox proportional hazard method was used for multivariable survival analysis. Patients still alive at analysis and patients who died within 1 month after surgery due to surgical complications were censored. Ordinal regression was performed to adjust for age in the serum-hyaluronan results. $p < 0.05$ was used as a cut-off for significance testing. The optimal cut-off in receiver operating characteristics (ROC) analysis was calculated as the maximal value for sensitivity + specificity − 1. Combined ROC curves were constructed using the predicted probability from a logistic regression model.

Results

High CD44 expression is associated with poor survival

Normal pancreatic acinar cells stained strongly for CD44s in cell membranes and weakly for CD44v6 in the cytoplasm (see supplementary material, Figure S2A, B). Interestingly, while normal acinar and ductal cells stained for CD44v6 in the cytoplasm (see supplementary material, Figure S2B), cancer cells showed a marked membranous staining pattern (Figure 1B). Stromal cells stained positively for CD44s in cancerous tissue (Figure 1A).

We divided the cohort into high/low expression groups for both CD44s and CD44v6 using the 75th percentile as a cut-off (Figure 1A,B). High expression of both CD44 isoforms was associated with shorter median survival (8.1 versus 16.6 months for CD44s and 8.1 versus 14.9 months for CD44v6) (Figure 1A, B). Similarly, high *CD44* mRNA expression in primary tumours was associated with shorter median survival (16.0 versus 21.1 months) (Figure 1C). We found a significant correlation between expression of the two *CD44* isoforms ($r = 0.52$, $p < 0.0010$) (Figure 1D). We then compared *CD44* mRNA levels in tumour suppressor *TP53* mutant versus wild-type tumours because previous reports have suggested that CD44 expression is negatively regulated by p53 [34]. However, we found no correlation between *CD44* mRNA levels and *TP53* status (Figure 1E).

Survival associations for stromal CD44 ligands

We then investigated survival associations with the stromal CD44 ligands type IV collagen, osteopontin and hyaluronan.
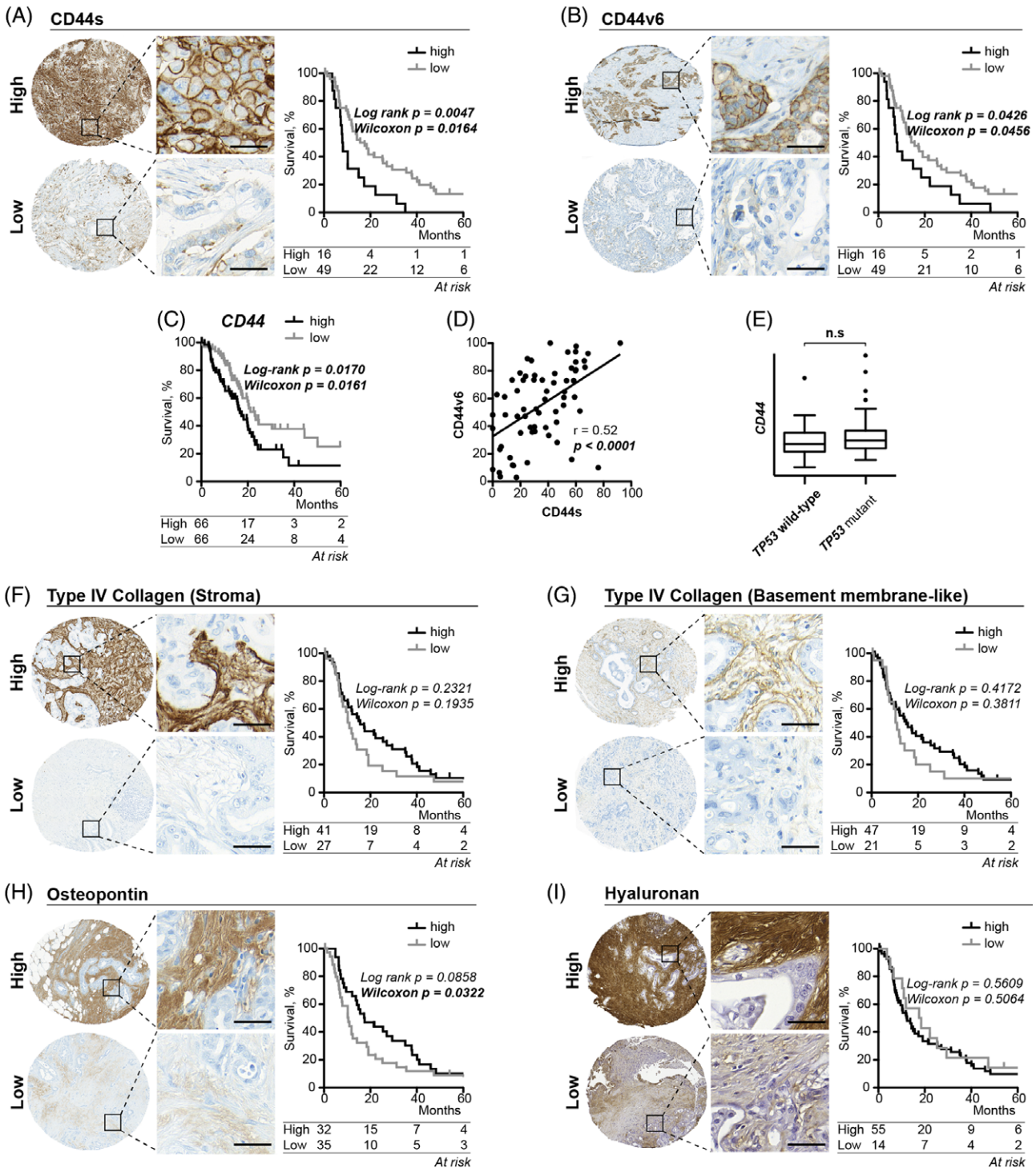
Figure 1. High versus low expression and associated Kaplan–Meier estimates for the primary tumour expression of (A,B) CD44 receptors, (F–G) type IV collagen, (H) osteopontin and (I) hyaluronan. Tissue microarray cores are 1 mm in diameter. Scale bar = 50 μm in highlighted areas. *Compared using Wilcoxon–Breslow estimates. (C) Kaplan–Meier survival estimates for patients with high versus low expression of CD44 mRNA transcripts. (D) Plot showing the correlation between CD44s and CD44v6 expression in primary tumours. (E) Box plots of *CD44* mRNA transcripts in *TP53* wild type and *TP53* mutant primary tumours.

In normal pancreas tissue, type IV collagen stained positively in basement membranes around acini and in connective tissue around ducts (see supplementary material, Figure S2C). In tumours, we detected type IV collagen-positive basement membranes in 69.1% of the cases (47/68) and type IV collagen-positive stroma in 73.1% (49/68) of the cases (Figure 1F,G). Retained basement membrane expression correlated significantly with the stromal expression (see supplementary material, Figure S3A). However, we found no significant association between either of the type IV collagen expression patterns and patient survival (Figure 1F,G). The same was also true at the transcriptional level (see supplementary material, Figure S3B).

In normal pancreas tissue, osteopontin localised in ductal connective tissue and, to a varying degree, in connective tissue around acini (see supplementary material, Figure S2D). We detected stromal osteopontin in 93% of the tumours (62/67) but with large variations in intensity and staining distribution. We found a significant association between low osteopontin expression and shorter postoperative survival (median survival 10.3 versus 17.4 months) when using Wilcoxon–Breslow estimates but not when using Log-Rank estimates (Figure 1H). This suggests a protective role of stromal osteopontin protein in pancreatic cancer tumours only in the initial years after surgery as Wilcoxon–Breslow estimates put more weight on early time points. Osteopontin mRNA expression was not associated with postoperative survival (see supplementary material, Figure S3C).

Hyaluronan stained sparsely in normal pancreatic ducts (see supplementary material, Figure S2E). By contrast, all tumours stained positively for hyaluronan in the stroma and the majority (53/67) stained strongly. Similarly, all metastatic lymph nodes stained positively and most (20/28) of them stained strongly. We found no association between the degree of stromal hyaluronan expression and patient survival (Figure 1I).

We detected no significant survival correlations to metastatic lymph node expression for any analysed markers, including the CD44 receptors (see supplementary material, Figure S4).

High molecular mass hyaluronan in PDAC tissue

As the molecular mass of hyaluronan has been reported to influence pancreatic cancer cell behaviour [16], we reasoned that hyaluronan mass profiles could be more informative than total hyaluronan amount. We therefore assessed the molecular mass profiles from two pancreatic cancer tissue samples and two normal pancreatic tissue samples. Interestingly, the two pancreatic cancer tissue samples were more abundant in high molecular mass hyaluronan compared to normal pancreatic tissue samples (Figure 2A).

By investigating three hyaluronan-synthesising proteins, hyaluronan synthases (HAS) 1–3 and two hyaluronan-degrading enzymes, hyaluronidases (HYAL) 1 and 2, we found that high *HAS1* mRNA levels (above the 75th percentile) and high *HYAL1* levels (above the 25th percentile) were associated with longer survival (24.6 versus 17.7 months and $p = 0.036$ for *HAS1* and 20.5 versus 16.6 months and $p = 0.0386$ for *HYAL1*) (Figure 2B,C). Interestingly, although mRNA levels of *HAS 2* were not associated with survival, a positive correlation with CD44 expression was found (Figure 2D).

Pancreatic cancer patients have increased levels of serum hyaluronan

Next, we hypothesised that the high tissue expression of hyaluronan would be reflected in the circulation to serve as a non-invasive biomarker. To test this, we compared serum hyaluronan levels in 44 pancreatic cancer patients (including 42 preoperative and 18 postoperative samples) and 22 healthy controls (Table 1) using two hyaluronan-binding protein-based ELISA assays. The assays differ in that the sandwich assay is restricted to detect high molecular mass hyaluronan (<27 kDa), while the competitive assay detects hyaluronan of any molecular mass (high molecular mass promiscuous) [31].

Independent of assay, preoperative serum hyaluronan levels were significantly higher in patients than in healthy controls (Figure 3A,B). The area under the curve (AUC) was 0.84 for both hyaluronan measurements and 0.91 for Ca 19-9 (Figure 3C,D). The combination of hyaluronan and Ca 19-9 measurements increased the AUC to 0.92 for the molecular mass promiscuous assay and 0.94 for the high mass restricted assay (Figure 3C,D). Postoperative serum hyaluronan levels were also significantly elevated compared to healthy controls but did not differ compared to preoperative levels (Figure 3A).

The pancreas patients were significantly older than controls ($p = 0.0047$) (Table 1) and as serum hyaluronan levels increased with age in control samples (Figure 3E,F), we suspected that age might have confounded our results. However, after adjusting for age, hyaluronan remained significantly increased in patients in both assays (see supplementary material, Table S2). However, serum hyaluronan measured with the molecular mass promiscuous assay did not increase with age
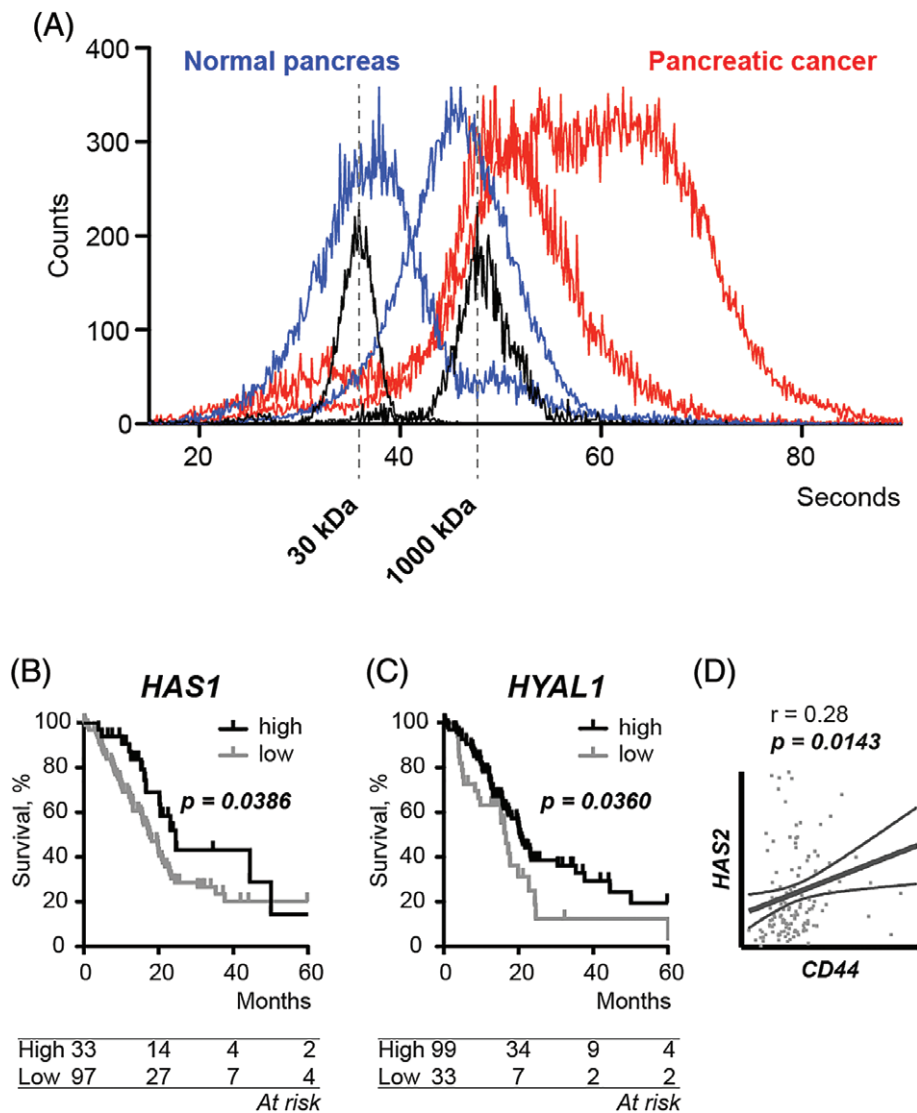
Figure 2. (A) Hyaluronan molecular mass profiles in two normal pancreas tissue samples (blue) and two pancreatic cancer tissue samples (red) detected by gas-phase electrophoretic molecular mobility analysis. With increasing time (*x*-axis), hyaluronan molecules of increasing molecular mass are counted (*y*-axis). Hyaluronan standards (black) indicate 30 and 1000 kDa. (B,C) Kaplan–Meier survival estimates for high versus low mRNA levels of (B) hyaluronan synthase 1 (*HAS1*) and (C) hyaluronidase 1 (*HYAL1*). P = log-rank test. (D) Correlation between *CD44* and hyaluronan synthase 2 (*HAS2*) mRNA levels. r = Pearson's r. The thin lines indicate confidence intervals.

in patients and the regression lines for patients and controls intersected at 80 years of age when including an interaction term between age and group designation in the ordinal model (see supplementary material, Table S3). This was not the case for serum hyaluronan measured with the high molecular mass restricted assay. Similar relationships were evident when plotting the linear correlation between hyaluronan levels and age in the two assays (Figure 3E,F). In addition, hyaluronan levels correlated significantly with bilirubin in both assays (Figure 3G,H), suggesting that bile duct

obstruction might also influence serum hyaluronan levels.

High mass serum hyaluronan, CD44 and OPN are independent predictors of PDAC survival

To adjust for clinical variables not accounted for in Kaplan–Meier analysis, we performed multivariable Cox regression including the following variables: CD44s protein expression, osteopontin protein expression, gender, age, tumour grade, TNM stage, R1
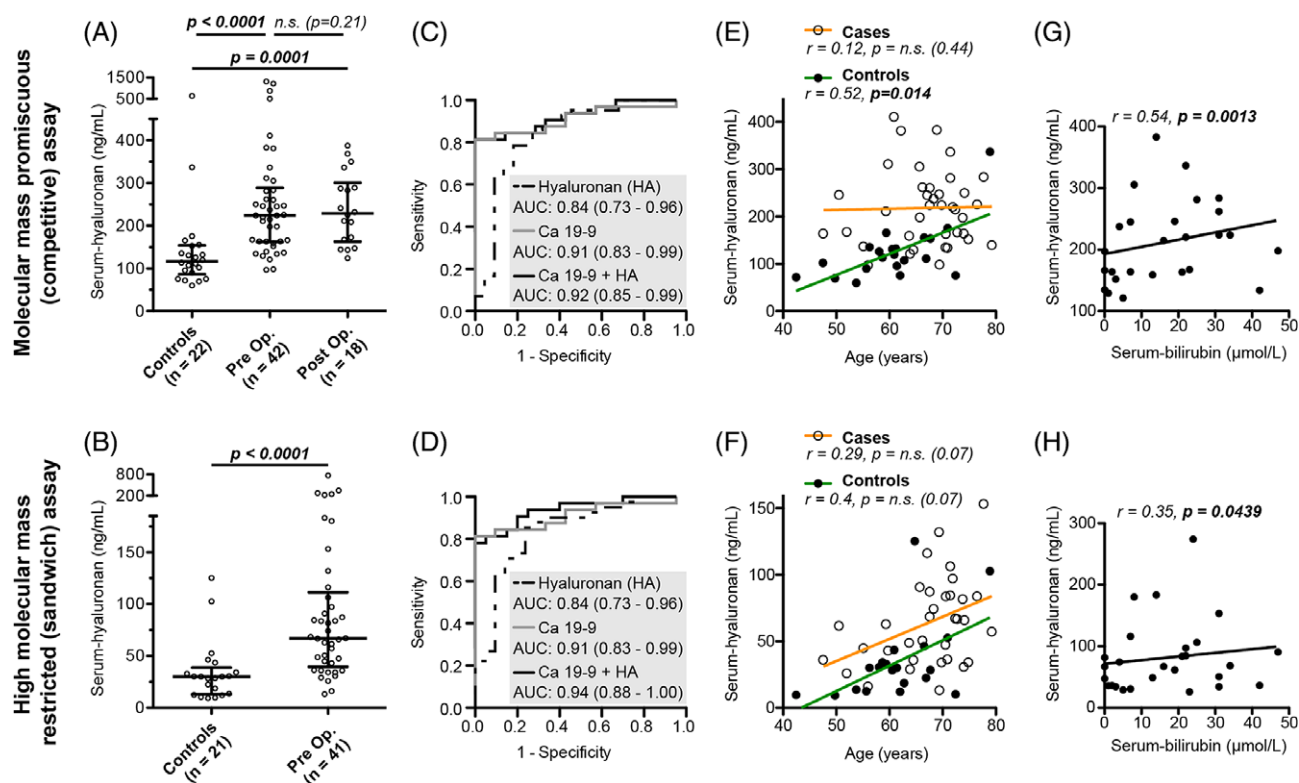
Figure 3. (A,B) Serum-hyaluronan measurements in pancreatic cancer patients and healthy controls measured with (A) a molecular mass promiscuous (competitive) assay and (B) a high molecular mass restricted (sandwich) assay. (C,D) Receiver operating characteristics curves discriminating preoperative pancreatic cancer patients from healthy controls with serum-hyaluronan, Ca 19-9 and Hyaluronan + Ca 19-9. AUC = area under the curve. 95% CI is within brackets. (E, F) Scatterplot showing the correlation between age and serum-hyaluronan and linear regression lines for cases and controls measured with (E) the molecular mass promiscuous assay and (F) the high molecular mass restricted assay. Outliers (serum-hyaluronan >500 ng/ml) are removed from the figure (not in the statistical analysis). The linear regression lines reflect that the effect of age on serum-hyaluronan is different between cases and controls and intercept at ~80 years of age when measured with the molecular mass promiscuous assay (E) but not when measured with the high molecular mass restricted assay (F). (G,H) The correlation between serum-hyaluronan and serum-bilirubin. Outliers are removed from the figure (not in the statistical analysis).

resection, neoadjuvant chemotherapy and adjuvant chemotherapy. In the multivariable model, high CD44s and low osteopontin independently predicted poor survival, as did poor differentiation, higher TNM stage, receiving preoperative intraperitoneal 5-FU and not receiving adjuvant chemotherapy (Table 2).

We then performed a separate multivariable Cox regression model including high molecular mass serum hyaluronan, measured with the high molecular mass restricted assay. High preoperative levels of high molecular mass hyaluronan predicted poor postoperative survival independent of age, gender, grade, TNM stage, R1 resection and adjuvant treatment (Table 3). By contrast, neither preoperative nor postoperative serum-hyaluronan levels measured with the molecular mass promiscuous assay were associated with survival (see supplementary material, Tables S4 and S5).

Table 2. Multivariable Cox regression model including the tissue expression of CD44s and OPN

Variable	Hazard ratio (95% CI)	p
CD44s score (per 25% positive cells)	1.9 (1.13–3.22)	**0.015**
Osteopontin score	0.8 (0.62–0.99)	**0.046**
Male	1.2 (0.63–2.42)	0.548
Age (per 10 years)	0.9 (0.55–1.59)	0.803
Tumour grade		
Grade 2	1.3 (0.61–2.92)	0.465
Grade 3	18.2 (3.91–84.6)	**<0.0001**
Non-radical resection	1.1 (0.53–2.46)	0.733
Stage II–IV	3.14 (1.27–7.79)	**0.013**
Neoadjuvant treatment		
Preop IP 5-FU	4.4 (1.13–17.3)	**0.033**
Chemotherapy	0.3 (0.10–3.03)	0.488
Adjuvant treatment	0.3 (0.11–0.73)	**0.009**

Significant P values are shown in bold underlined font.

Table 3. Cox regression models including preoperative S-HA levels measured with the high molecular mass restricted (sandwich) assay

Variable	Hazard ratio (95% CI)	p
Serum-hyaluronan (per 100 ng/ml)	1.3 (1.02–1.77)	**0.033**
Male	1.2 (0.44–3.20)	0.744
Age (per 10 years)	1.3 (0.64–2.70)	0.452
Tumour grade		
Grade 2	6.8 (1.54–29.6)	**0.011**
Grade 3	21.0 (4.63–95.1)	**<0.0001**
TNM stage II	8.5 (2.51–28.7)	**0.001**
Non-radical resection	8.0 (1.96–32.2)	**0.004**
Adjuvant treatment	0.1 (0.03–0.32)	**<0.0001**

Significant P values are shown in bold underlined font.

Discussion

The present study focuses on the CD44 receptor and its stromal ligands with the aim to find prognostic markers of postoperative survival in PDAC. We show that high expression of CD44s in pancreatic cancer cells and low stromal osteopontin expression independently predict poor postoperative survival, while hyaluronan and type IV collagen have limited potential as tissue-based biomarkers. On the other hand, we show that hyaluronan is highly expressed in almost all pancreatic ductal adenocarcinomas and is significantly increased in the serum of pancreatic cancer patients. In addition, we uncover a shift towards a high molecular mass profile of hyaluronan in pancreatic cancer that can be detected in the circulation, where increased levels of high molecular mass hyaluronan predict poor postoperative survival.

The association between CD44s expression and survival is in line with several previous reports [9,11–14]. We found that high expression of CD44, both at the protein and mRNA levels, is associated with worse patient outcome. This highlights CD44 as a potential therapeutic target in pancreatic cancer. However, while preclinical studies support this notion [10,35], a phase I clinical trial evaluating an antibody interfering with the hyaluronan-binding domain of CD44 had to be terminated due to lack of clinical response [36]. This trial, however, included 65 patients with 20 different tumour forms and such a liberal inclusion criterion might have obscured clinical responses pertinent to certain cancer forms. In breast cancer, CD44 is negatively regulated by the tumour suppressor p53 [34]. No such correlation was, however, found in our material and hence, other pathways must be critical in CD44 regulation in pancreatic cancer.

We found that high osteopontin expression is an independent predictor of longer survival. Collins *et al*

found similar results when comparing osteopontin-positive versus negative pancreatic cancer, although they scored cancer cells instead of stromal expression [37]. However, we found no association between osteopontin mRNA levels and survival, which is likely due to the high degree of post-translational regulation of osteopontin [38].

We have previously shown that type IV collagen is expressed in pancreatic cancer tissue in basement membrane-like structures close to cancer cells and that it stimulates cancer cell proliferation, migration and survival *in vitro* [23,39]. The lack of association with survival in the present study pinpoints a discrepancy between tumour-residing and -circulating type IV collagen with regard to prognostic value. Hence, type IV collagen leakage rather than production appears to be a malignant trait of pancreatic cancer.

High hyaluronan expression in pancreatic cancer was associated with shorter postoperative survival in two previous studies [19,20]. However, the study by Cheng *et al* relied on an antibody-based method to detect hyaluronan and as hyaluronan is a non-immunogenic molecule [40], the specificity of antibody-based staining is highly questionable [19]. The other study used the ratio between hyaluronan staining and total tumour area to divide tumours into high versus low expression [20]. That scoring method is inevitably affected by sample cellularity. Here, we used a HABP staining method, which specifically stains hyaluronan and scored the amount of positive stroma. In our hands, hyaluronan was strongly expressed in almost all primary tumours and metastases, with little variation and no correlation with any clinical parameter, including survival. This suggests that hyaluronan deposition is a universal trait of the desmoplastic pancreatic cancer stroma. Depletion of stromal hyaluronan is a promising therapeutic strategy in preclinical studies [18,41]. If stromal hyaluronan depletion is proven beneficial in human pancreatic cancer, our results suggest that such a therapy could aid a majority of patients owing to the ubiquitous expression of hyaluronan in the tumours. The strong expression also suggests that CD44-hyaluronan signalling is limited by receptor expression rather than ligand availability.

In pancreatic cancer, high HAS2 and low HYAL1 expression has been associated with shorter survival [19]. Similarly, we find that low *HYAL1* and also low *HAS1* mRNA expression in tissue correlate with shorter survival, suggestive of protective effects from a high HA turnover. But importantly, HAS and HYAL enzyme activities are extensively modulated post-transcriptionally and such layers of hyaluronan

biogenesis are beyond the scope of this manuscript. Nevertheless, our results suggest that the hyaluronan molecular mass profile in pancreatic cancer might be of greater importance than the total amount of hyaluronan expression. Indeed, we find that high molecular mass hyaluronan may be more abundant in pancreatic cancer tissue than in normal tissue, which calls for more extensive hyaluronan size profiling in pancreatic cancer.

We show that serum hyaluronan is significantly higher in pancreatic cancer patients compared to healthy controls. We also show that the AUC is higher when adding hyaluronan measurements to Ca 19-9. Interestingly, while both assays demonstrated that serum hyaluronan is elevated in pancreatic cancer, our result suggests that hyaluronan measured with the molecular mass promiscuous assay cannot be adjusted for age in the elderly. Bile duct obstruction might confound serum hyaluronan as we found a correlation between serum hyaluronan and bilirubin. Sinusoidal endothelial cells in the liver degrade >90% of circulating hyaluronan [42] and bile duct obstruction is associated with impaired hepatic sinusoidal cell function [43]. Importantly, elevated serum hyaluronan has been demonstrated in other cancer forms and in liver cirrhosis [44,45] and hence, this finding is not specific for pancreatic cancer. Thus, its potential to discriminate disease and predict outcome must be evaluated in a larger cohort with the confounders presented here considered.

In summary, tissue expression of both CD44 receptors and stromal CD44 ligands confers prognostic information in pancreatic cancer. High cancer cell expression of CD44s and low stromal expression of osteopontin are independent markers of poor prognosis, underscoring CD44 as a potential treatment target and the need to revisit the role of osteopontin in pancreatic cancer progression and metastasis. The CD44 ligand hyaluronan is strongly expressed in the stroma in almost all pancreatic ductal adenocarcinomas and lymph node metastases and is elevated in serum from pancreatic cancer patients. In addition, high preoperative serum high molecular mass hyaluronan (>27 kDa) predicts poor postoperative survival independent of other, established survival predictors.

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Author contributions statement

OF, OB, DÖ, UH and MS participated in planning the study and the study design. OF, DÖ and AB participated in immunohistochemistry scoring. OF, DÖ and WW participated in the planning and construction of the tissue microarray. OF, OB and CH participated in planning, extraction and analysing mRNA data. UH performed hyaluronan mass distribution analysis. OF and OB performed data analysis. OF, OB, DÖ, UH and MS wrote the manuscript. The final text was read and critically reviewed by all authors. All authors gave their final approval of the text.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1 Tissue sections before the first and after the last sections used in the hyaluronan molecular mass profile analysis

Figure S2 Protein expression in normal pancreatic tissue

Figure S3 Correlation and survival data for type IV collagen and osteopontin

Figure S4 Kaplan–Meier survival estimates comparing metastatic lymph node expression of CD44s, CD44v6, type IV collagen, osteopontin and hyaluronan

Table S1 Primary antibodies used for immunohistochemistry

Table S2 Ordinal regression to adjust serum-hyaluronan (dependent variable) for age differences in patients and controls

Table S3 Ordinal regression to adjust serum-hyaluronan (dependent variable) for age differences in patients and controls and the interaction term between age and patient/control designation

Table S4 Cox regression models including preoperative serum-hyaluronan levels measured with the molecular mass promiscuous (competitive) assay

Table S5 Cox regression models including postoperative S-HA levels measured with the molecular mass promiscuous (competitive) assay