Original Article

Multiplex screening of 422 candidate serum biomarkers in bladder cancer patients identifies syndecan-1 and macrophage colony-stimulating factor 1 as prognostic indicators

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Background: Serum protein biomarkers that correlate with urothelial bladder cancer (UBC) stage and outcome could accelerate and improve clinical management, but this requires thoroughly validated high–performance biomarker(s). Unbiased discovery of serum biomarkers by mass spectrometry is challenging due to their low abundance in a complex sample, and candidate-based discovery is slow and expensive due to the number of immunoassays required. We have utilised a novel multiplex platform to assay disease-associated proteins in the sera of bladder cancer patients with the aim of identifying novel staging and prognostic biomarkers.

Methods: All sera were collected as part of the Bladder Cancer Prognosis Programme. We randomly selected 10 non-UBC, 10 G1pTa, 10 G3pTa, 30 G3T1 and 30 G3T2+ UBC cases. Serum levels of proteins were determined with Proseek multiplex immunoassays (http://www.olink.com/). Multivariate linear regression analysis was used to identify significant associations between protein levels and bladder cancer stage and grade. Kaplan-Meier analyses and log-rank tests were used to identify associations between protein levels and disease-specific survival.

Results: There were no significant differences in age and gender between the groups. 422 proteins were successfully measured in the sera of the 10 non-cancer controls and 80 UBC patients. Linear regression identified 5 proteins significantly associated with UBC. In order of statistical significance (lowest P value first) these were nectin-4, syndecan-1, T-cell immunoglobulin mucin receptor 1, macrophage colony-stimulating factor 1 and matrilysin. Although none of these showed clear discrimination between stages of disease, high levels of syndecan-1 and macrophage colony-stimulating factor 1 were significantly associated with worse UBC-specific survival.

Conclusions: We have studied the relationship between UBC and the serum concentrations of over 400 proteins. Those which reach statistical significance include known biomarkers and new candidates that may warrant further investigation. Although bladder cancer does cause many biologically plausible changes in the serum proteome none of the proteins studied appears to be suitable for accurate non-invasive staging of bladder cancer. However, syndecan-1 and/or macrophage colony stimulating factor-1 (CSF-1) might prove useful as prognostic indicators.

Keywords: Bladder cancer; serum; biomarker; prognosis; syndecan-1

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Introduction

Urothelial bladder cancer (UBC) is the 9th most common cancer world-wide (1). Approximately three-quarters of new cases present as non-muscle invasive disease (NMIBC) and the remainder present as muscle-invasive disease (MIBC). MIBC is life-threatening and patients require radical treatment with chemotherapy followed by cystectomy or radiotherapy (2). NMIBC patients are stratified into low-, intermediate- and high-risk groups according to clinicopathological parameters and are treated accordingly: transurethral resection (TUR) and intravesical mitomycin C for low risk, and TUR and intravesical BCG for intermediate- and high-risk NMIBC (3). Radical cystectomy is also considered an option for high risk NMIBC. UBC is initially detected by flexible cystoscopy, and NMIBC patients are subjected to regular long-term surveillance with this burdensome procedure. Urine or serum biomarkers that could detect and characterise UBC non-invasively could improve management of bladder cancer patients in several ways, e.g.,

(I) Detecting primary tumours—to facilitate the triage of haematuria patients into those at high or low risk of having UBC;
(II) Detecting recurrent tumours—to reduce the reliance on cystoscopy for NMIBC surveillance;
(III) Prognosis—to improve existing clinicopathological prognostication and select more appropriate treatment regimens for individual patients;
(IV) Prediction—to predict which patients will benefit from which therapeutic agents;
(V) Staging—to determine which patients have MIBC at presentation and move directly to cross-sectional imaging and definitive radical therapies.

Considerable effort has been expended on identifying urinary biomarkers for detecting the presence of bladder cancer with many urine markers proposed, ranging from FDA-approved assays (e.g., NMP22, BTA) to numerous markers reported in single studies (4). Most of the proposed markers are proteins, and none retain both high (clinically useful) sensitivity and specificity upon large scale validation. More recently, DNA-based urine biomarkers have shown considerable promise and have re-awakened the hope that non-invasive disease detection with clinically useful sensitivity and specificity may be achievable (5-8).

Most prognostic studies have measured biomarkers directly on tumour tissue, although there are reports of biomarker levels in urine and plasma/serum providing prognostic information. Changes in DNA methylation, gene expression profiles and individual protein levels have shown promise as prognostic indicators in research studies [reviewed in (9)]. As with detection, no prognostic biomarkers have yet been widely accepted into clinical practice. In addition to detection and prognostic roles, predictive biomarkers are likely to find a place in the management of bladder cancer as new targeted therapeutic agents are adopted.

A biomarker test that assisted in non-invasive staging of bladder cancer could facilitate the fast-tracking of MIBC patients to cross-sectional imaging and definitive treatment, circumventing delays and possible disease dissemination due to TUR. An experienced urologist can distinguish between low-grade papillary NMIBC and high-grade (HG) solid tumours. However, discrimination between HG T1 NMIBC and MIBC is difficult to achieve at cystoscopy (10) and hence staging TUR is undertaken—an invasive and possibly detrimental procedure that could be avoided if there was an alternative for detecting muscle-invasion. Whilst a pinch biopsy of tumour may be collected during cystoscopy for biomarker analysis, for HG tumours mutation and gene expression profiles appear to traverse stages (11): a discernible "molecular switch" which enables (and hence indicates) muscle invasion has not yet been identified. Levels of molecules released directly from tumours or by tissue degradation into body fluids during invasion might better identify disease stage. Levels of urinary biomarkers are typically higher in MIBC then NMIBC patients; however, the NMIBC subgroup is comprised of low-grade and pTa disease in addition to HG T1 disease. The link between grade and biomarker concentration usually appears much stronger than the link between stage and biomarker concentration, and hence discrimination between HG T1 and MIBC disease based on urinary biomarkers is challenging. We hypothesised that non-invasive detection of MIBC might more effectively be achieved using serum/plasma biomarkers. Recently, levels of both circulating tumour cells and plasma ctDNA have shown promise as staging and prognostic markers (12-14). Nonetheless, the extremely low levels of these biomarkers in the circulation make them challenging to measure, whereas proteins are easier and faster to measure, potentially enabling point-of care testing. Additionally, only the "tip-of–the-iceberg" of the plasma proteome has been explored in bladder cancer patients to date, leaving plenty of potential for biomarker discovery. A literature search identified just over 20

Table 1 Patient characteristics. All patient samples were collected as part of BCPP as described in the methods section. Serum was collected from patients with incident primary bladder cancer. All numbers except mean age are numbers of patients

Grade and stage	Number of patients	Gender (male, female)	Age [mean, (SD)]	Age (≤60, 61–70, 71–80, >80)	Tumour size* (≤3 cm, >3 cm)	No. tumours* (single, multiple)	CIS (no, yes)
Non-UBC	10	8, 2	84.7 (9.6)	0, 4, 1, 6	NA	NA	NA
G1pTa	10	7, 3	76.7 (10.8)	0, 6, 2, 1	7, 3	8, 2	10, 0
G3pTa	10	9, 1	80.6 (10.2)	1, 3, 3, 3,	4, 5	4, 5	9, 1
G3T1	30	27, 3	79.3 (9.4)	4, 9, 15, 2	14, 15	13, 16	20, 10
G3pT2-4	30	24, 6	79.6 (10.1)	3, 8, 15, 4	9, 20	19, 10	23, 7

*, indicates that this data was not available for three patients. UBC, urothelial bladder cancer; CIS, carcinoma *in situ*.

proteins that have been investigated as serum markers for bladder cancer and that show a stage dependent increase in concentration. However, none substantially increase between HG NMIBC and MIBC; the largest increase is typically seen in stages T3 and T4 [e.g., MMP7 and HNPs1-3 (15,16)], most likely reflecting the largest increase in tumour burden. Indeed, levels of some of these serum biomarkers are highly prognostic and can predict non-organ-confined disease (17), but there are no convincing reports of serum biomarkers distinguishing between NMIBC and MIBC.

We reasoned that since none of the protein biomarkers reported to date looks highly promising for non-invasive staging, then future analyses should be broader to include more proteins. Mass spectrometry-based "shotgun" proteomics is the method of choice for identifying and quantitating large numbers of proteins in biological and clinical specimens (18). This approach is limited in terms of sample throughput, but more so in ability to detect low abundance proteins in serum: potentially relevant cytokines and tumour leakage products may be 10^9 fold more dilute than abundant serum proteins and so may not be detectable. Conversely, individual protein assays such as ELISAs would be prohibitively expensive and time-consuming if a large number of proteins were to be considered. We therefore utilised a novel multiplex assay which enables simultaneous measurement of 92 proteins in 90 samples using "proximity extension" and requiring only 1 µL of sample. In this method, a pair of oligonucleotide-conjugated antibodies is used for each analyte. When both antibodies bind to an analyte molecule the close proximity of the oligonucleotides enables ligation, extension and amplicon generation. The amplicons are subsequently analysed by qPCR (http://www.olink.com/) giving relative quantitation of all analytes across all samples.

Methods

Patient samples

All sera were collected as part of the Bladder Cancer Prognosis Programme between 2004 and 2011 (UK ethics ref: 06/MRE04/65). Full details of this multi-centre biospecimen collection have been published elsewhere (19). Briefly, patients whose diagnostic cystoscopy indicated primary bladder cancer were recruited to the study, and blood collected into serum tubes prior to TUR. The blood was left to clot for 90–150 min, centrifuged at 3,500 rpm for 10 min and the sera stored at –80 ℃. Ultimately, some of the patients were diagnosed with non-malignant conditions and these serve as non-cancer controls. All patients were followed for at least 3 years following initial diagnosis. Patient information is summarised in *Table 1*. Age and gender did not differ significantly between the patient groups.

Assays

Protein concentrations in patient sera were analysed using five Proseek Multiplex panels (Oncology, Inflammation, Neurology, Cardiovascular II and III) at the Proseek Multiplex Analysis Laboratory (http://www.olink.com/). Each Proseek assay measures 92 proteins. Briefly, a pair of oligonucleotide-conjugated antibodies to each protein are added to 1 µL of serum. When an antibody-protein-antibody sandwich is formed both antibodies are in close proximity, the oligonucleotides hybridize, and an extension reaction forms a unique sequence. These sequences are then quantitated by microfluidic qPCR.

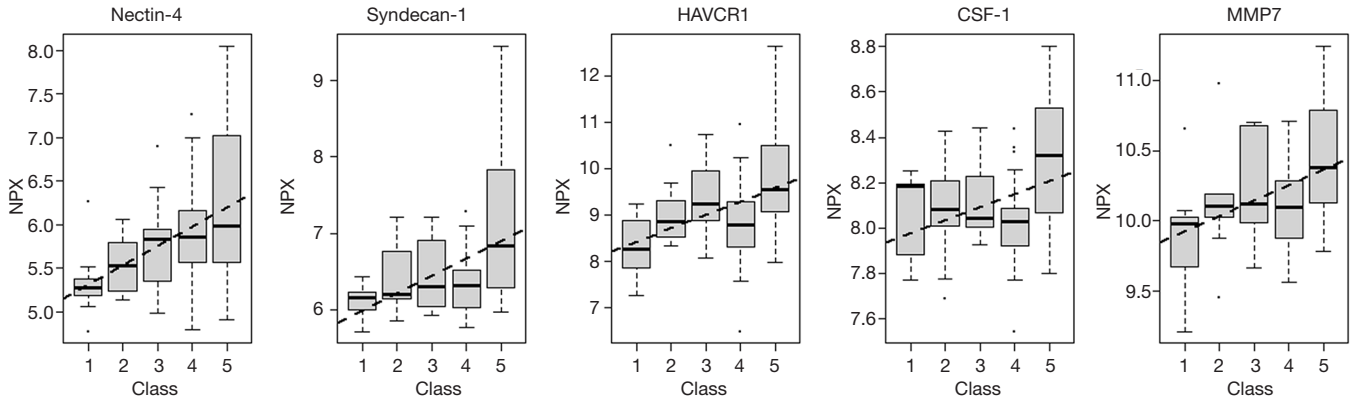

Figure 1 Box and whiskers plots of serum levels of proteins significantly associated with UBC. Data are shown as normalised protein expression (NPX) for five groups of patients (1= non-UBC, 2= G1pTa, 3= G3pTa, 4= G3pT1 and 5= G3pT2+). HAVCR1, T-cell immunoglobulin mucin receptor 1; CSF-1, colony stimulating factor-1; UBC, urothelial bladder cancer.

Data analysis

All data were analysed as normalised protein expression (NPX on a log2 scale). The patients were separated into five classes of increasing stage and grade: non-UBC, G1pTa, G3pTa, G3T1 and G3T2+. Associations between serum protein concentrations and stage/grade groups were tested using multivariate linear regression analysis with age and gender as covariates using R statistical software version 3.2.5. Multiple testing corrections were done with the Benjamini-Hochberg method and an adjusted P<0.05 was considered significant. Significant proteins were further investigated using ROC analyses and *t*-tests to compare NMIBC and MIBC patients and Kaplan-Meier analyses and log rank testing to study survival using SigmaPlot 12.5. For survival analyses the 80 UBC patients were divided into "high" and "low" according to whether their biomarker concentration was above or below the median value for the UBC patients (control subjects excluded).

Results

Assay performance

Sera from 90 patients were analysed on five Proseek multiplex immunoassay panels (92 analytes each). Patient characteristics are shown in *Table 1*. Quality control criteria for the datasets were considered good with 97–99% of the samples meeting QC criteria across the five Proseek panels and 94% of proteins being above the limit of detection in 100% of the samples. Due to redundancy between panels and some analytes not meeting QC criteria, reportable results were obtained for 422 unique proteins. In instances of proteins being measured on multiple panels good agreement between data from the panels was observed (data not shown). A list of all 422 analytes is provided in Supplemental Information.

Proteins associated with UBC

The concentrations of proteins in the sera of the non-UBC, G1pTa, G3pTa, G3pT1 and G3pT2+ UBC patients were compared using multivariate linear regression. Whilst 80 proteins appeared to be significantly associated with bladder cancer (P<0.05), this was reduced to five following Benjamini-Hochberg multiple testing correction (Supplemental Information). The five proteins were (in order of statistical significance, lowest P value first) nectin-4, syndecan-1, T-cell immunoglobulin mucin receptor 1, macrophage colony-stimulating factor 1 and MMP7. Boxplots of the serum concentrations of these proteins in the five patient groups are shown in *Figure 1*.

Protein associations with UBC stage

The concentrations of all five UBC associated proteins show a positive correlation with disease stage and all five of the UBC associated proteins were significantly more concentrated in the sera of patients with MIBC than NMIBC (P<0.05, *t*-test). *Table 2* shows the relative concentrations of these proteins in NMIBC (n=50) and MIBC patients (n=30). Despite being significantly elevated in MIBC patients relative to NMIBC patients,

Table 2 Serum levels of bladder cancer associated proteins in NMIBC and MIBC patients. The data shown are: change in mean protein concentration between the NMIBC and MIBC patient groups and the p-value and area under the ROC curve for this comparison and the Proseek panel used to measure each protein

Protein	Fold-change (MIBC/NMIBC)	P value (*t*-test)	Area under ROC curve	Panel
Nectin-4	1.33	0.0368	0.609	ONC
Syndecan-1	1.60	0.0013	0.708	ONC
T-cell immunoglobulin mucin receptor 1 (HAVCR1)	1.67	0.0043	0.683	CVD2
Macrophage colony stimulating factor-1 (CSF-1)	1.18	<0.001	0.741	INF
Matrilysin (MMP7)	1.21	0.0087	0.671	CVD2

NMIBC, non-muscle invasive disease; MIBC, muscle-invasive disease.

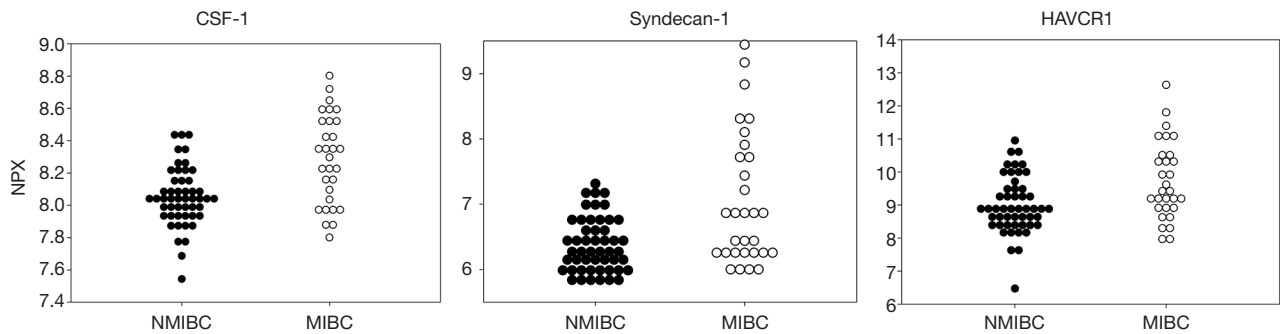

Figure 2 The distributions of the serum concentrations of CSF-1, syndecan-1 and HAVRC1 in patients with NMIBC or MIBC (n=50 and n=30 respectively). CSF-1, colony stimulating factor-1; NMIBC, non-muscle invasive disease; MIBC, muscle-invasive disease.

the concentration changes are modest and the areas under the ROC curves below 0.75. The distributions of the three most discriminatory proteins in the NMIBC and MIBC patients are shown in *Figure 2* and corresponding ROC curves are shown in *Figure 3*. The heat-map shown in *Figure 4* shows the distribution and heterogeneity of the 5 significant proteins' concentrations in the patient sera. Approximately half of the MIBC cases have elevated levels of several or all of these proteins, but there are also MIBC cases where none of the biomarkers are elevated. Thus, whilst high-levels of these proteins are highly indicative of MIBC, it would not be possible to devise a sensitive test for MIBC using any combination of these proteins.

Protein associations with outcome

We investigated the relationship between the levels of the five UBC associated proteins and bladder cancer specific survival. With all five proteins there was a trend towards poorer outcome with high biomarker levels; this approached statistical significance in the case of MMP7 and was highly significant in the cases of syndecan-1 and CSF-1 (*Figure 5*). Syndecan-1 does not reach significance if we only consider NMIBC patients (P=0.081, 50 patients, 7 UBC-specific deaths) but is highly significant in the MIBC patient group (P<0.001, 30 patients, 16 UBC-specific deaths).

Discussion

We have used multiplex immunoassays to measure the concentrations of 422 proteins in the serum of bladder cancer patients. The relationship between the serum concentrations of many of these proteins and UBC have not been previously reported. Disappointingly, no accurate biomarkers for non-invasively detecting or staging bladder cancer have been uncovered. Nonetheless, the serum concentrations of five of the proteins investigated are statistically significantly associated with bladder cancer and two of the proteins show an association with reduced bladder cancer specific survival. Several of the UBC associated proteins are trans-membrane proteins which

are most likely present as soluble forms in the serum due to ectodomain shedding (20). The elevated serum concentrations may be due to increased expression within the tumour, increased sheddase activity in the tumour microenvironment, or a combination of both. Consistent with the current findings, we have previously reported that increased urinary concentrations of the shed ectodomains of EpCAM and EGFR indicate a poor prognosis (20,21)

The most significantly bladder cancer-associated protein was nectin-4, a calcium-independent transmembrane cell-adhesion molecule which has not previously been investigated in UBC, and is expressed both in normal urothelium and HG UBC at moderate to high-levels (The Human Protein Atlas). Nectin-4 did not discriminate well between NMIBC and MIBC or show significant prognostic potential but has previously been reported as a biomarker for breast, lung and ovarian cancers (22-24), and it is believed that the extracellular domain is shed into the circulation via ADAM17-mediated cleavage (25).

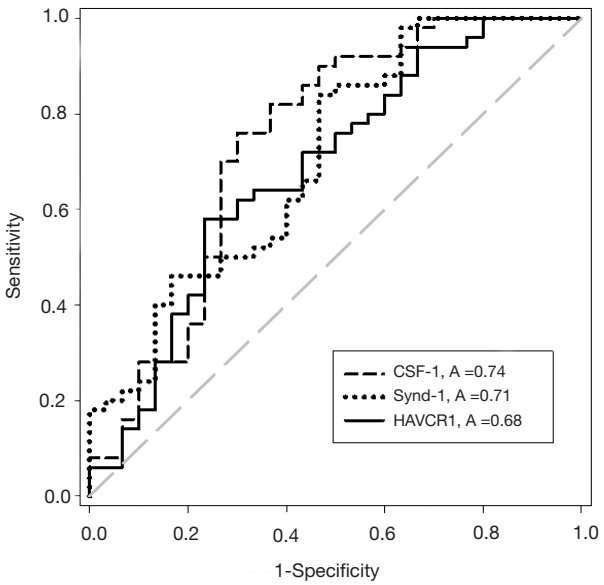

Figure 3 Receiver operator characteristic curves for discrimination between NMIBC and MIBC based on serum concentrations of CSF-1 (dashed line), syndecan-1 (dotted line) and HAVRC1 (solid line) (n=50 and 30 respectively). CSF-1, colony stimulating factor-1; NMIBC, non-muscle invasive disease; MIBC, muscle-invasive disease; HAVCR1, T-cell immunoglobulin mucin receptor 1.

Syndecan-1 was the second most cancer-associated protein in multivariate analysis, and also shows some discrimination between NMIBC and MIBC. Like nectin-4, syndecan-1 is a transmembrane protein most likely shed into the circulation by ADAM17-mediated cleavage (26). We find that elevated serum syndecan-1 is a very significant indicator of poor outcome in UBC patients. This is in agreement with the study by Szarvas *et al.* in which serum syndecan-1 was found to be an independent prognostic indicator in a cohort of 79 patients (27).

Our third UBC-associated molecule, T-cell immunoglobulin mucin receptor 1 (HAVCR1), is again a transmembrane protein whose extracellular domain can be released into the circulation by proteolytic cleavage (28). There are no publications relating to serum levels of this protein in UBC patients, but it is reportedly overexpressed in renal and ovarian cancers and an anti-HAVCR1 antibody-drug conjugate is currently being developed to treat renal, lung and ovarian cancers (29).

Macrophage colony stimulating factor 1 (CSF-1) is our fourth most cancer-associated protein and shows the best discrimination between NMIBC and MIBC of any of the proteins studied. It is a secreted cytokine (released by ectodomain shedding) which is released by several UBC cell lines (30); increased serum concentrations have been reported in several cancer types including breast, lung and ovarian (31-33) and are associated with a poor prognosis. Higher CSF-1 serum levels in some UBC cases may reflect high levels of tumour associated macrophages, which itself is associated with worse prognosis (34).

Matrix metalloprotease 7 (matrilysin or MMP7) is a

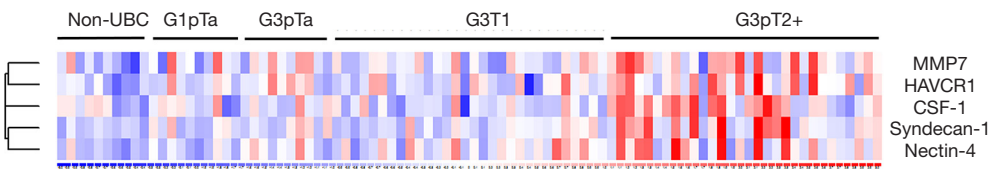

Figure 4 Heatmap of NPX values for the five UBC associated proteins across all patients. Samples are ordered left to right: non-UBC, G1pTa, G3pTa, G3pT1 and G3pT2+, whereas proteins are clustered revealing similarities in expression patterns. Expression is graded from low (blue) to high (red). UBC, urothelial bladder cancer; CSF-1, colony stimulating factor-1; NPX, normalised protein expression.

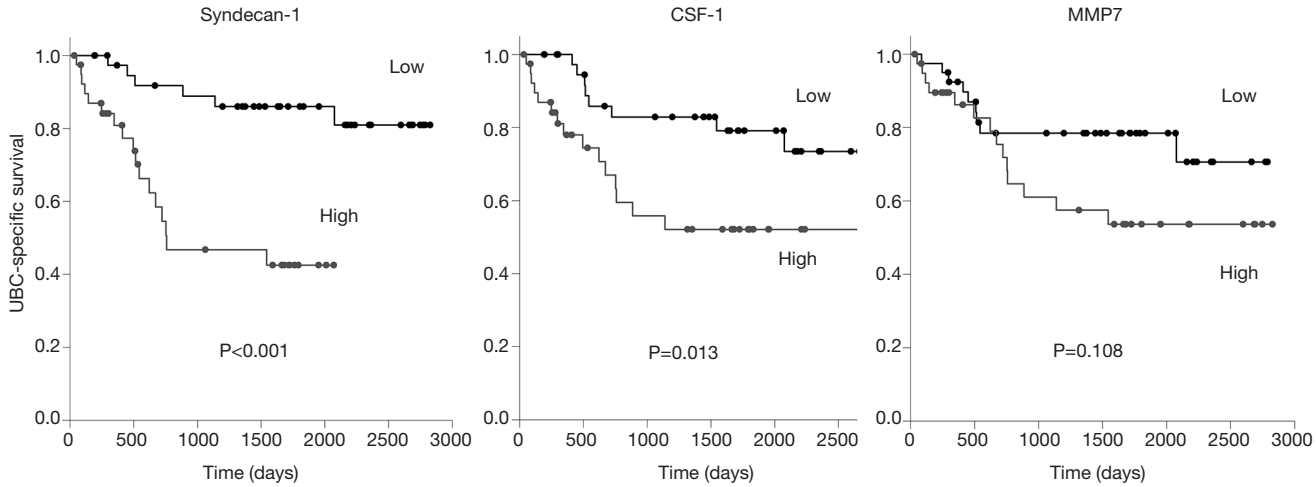

Figure 5 UBC-specific survival analysis. Patients were stratified according to whether their serum syndecan-1, CSF-1 or MMP7 levels were above or below the median value for the 80 UBC patients in the study and survival curves drawn for the high and low biomarker groups. P values were determined by log rank test. CSF-1, colony stimulating factor-1; UBC, urothelial bladder cancer.

secreted protease involved in tissue remodelling which has previously been detected at increased levels in advanced bladder cancer; it has been reported to be an independent prognostic indicator in a study of 79 UBC patients (16). Whilst our results corroborate the finding of elevated serum MMP7 in advanced UBC, serum MMP7 failed to reach statistical significance as a prognostic indicator in our study.

Conclusions

We have studied the relationship between UBC and the serum concentrations of 422 proteins. Those which reach statistical significance have been discussed and include both known biomarkers and new candidates that may warrant further investigation. Although UBC does cause many biologically plausible changes in the serum proteome, the disease is highly heterogeneous and none of the proteins studied appears to be suitable for non-invasive staging of bladder cancer. Our data suggest that the majority of the proteins studied here do not merit further investigation as UBC serum biomarkers, with the possible exceptions of syndecan-1 and CSF-1 which do appear to be highly prognostic. A major strength of our work is the use of the Proseek platform which has enabled, for the first time, measurement of hundreds of low abundance proteins in the sera of bladder cancer patients. The limitations of our study include the modest sample size and that the Proseek assays are a biomarker discovery tool that provides relative quantitation of a large number of proteins across a patient cohort rather than being an approved clinical test. To develop the latter would require either developing a custom "bladder cancer panel" multiplex assay (possibly using Proseek technology) or running one or more ELISAs. Validation in independent prospective studies is required to determine if these proteins are robust prognostic indicators and to determine whether they provide information over and above clinicopathological parameters.

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Footnote

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of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by NRES Committee East Midlands—Derby (UK ethics ref: 06/MRE04/65) and written informed consent was obtained from all patients.

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