Quantitative proteomics identifies brain acid soluble protein 1 (BASP1) as a prognostic biomarker candidate in pancreatic cancer tissue

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ABSTRACT

Background: Pancreatic cancer is a heterogenous disease with a poor prognosis. This study aimed to discover and validate prognostic tissue biomarkers in pancreatic cancer using a mass spectrometry (MS) based proteomics approach.

Methods: Global protein sequencing of fresh frozen pancreatic cancer and healthy pancreas tissue samples was conducted by MS to discover potential protein biomarkers. Selected candidate proteins were further verified by targeted proteomics using parallel reaction monitoring (PRM). The expression of biomarker candidates was validated by immunohistochemistry in a large tissue microarray (TMA) cohort of 141 patients with resectable pancreatic cancer. Kaplan-Meier and Cox proportional hazard modelling was used to investigate the prognostic utility of candidate protein markers.

Findings: In the initial MS-discovery phase, 165 proteins were identified as potential biomarkers. In the subsequent MS-verification phase, a panel of 45 candidate proteins was verified by the development of a PRM assay. Brain acid soluble protein 1 (BASP1) was identified as a new biomarker candidate for pancreatic cancer possessing largely unknown biological and clinical functions and was selected for further analysis. Importantly, bioinformatic analysis indicated that BASP1 interacts with Wilms tumour protein (WT1) in pancreatic cancer. TMA-based immunohistochemistry analysis showed that BASP1 was an independent predictor of prolonged survival (HR 0.468, 95% CI 0.257–0.852, $p = .013$) and predicted favourable response to adjuvant chemotherapy, whereas WT1 indicated a worsened survival (HR 1.636, 95% CI 1.083–2.473, $p = .019$) and resistance to chemotherapy. Interaction analysis showed that patients with negative BASP1 and high WT1 expression had the poorest outcome (HR 3.536, 95% CI 1.336–9.362, $p = .011$).

Interpretation: We here describe an MS-based proteomics platform for developing biomarkers for pancreatic cancer. Bioinformatic analysis and clinical data from our study suggest that BASP1 and its putative interaction partner WT1 can be used as biomarkers for predicting outcomes in pancreatic cancer patients.

Research in context

Evidence before this study

Pancreatic cancer is a heterogenous disease. There is a lack of molecular markers that can accurately predict the course of the disease and response to therapy. New prognostic and predictive biomarkers are urgently needed in order to characterise individual tumour biology and select optimal treatment.

Added value of this study

We conducted global and targeted mass spectrometry (MS)-based protein profiling of fresh frozen pancreatic cancer tissue specimens and healthy pancreas. Brain acid soluble protein 1 (BASP1) was found to be significantly upregulated in pancreatic cancer. External validation by tissue microarray (TMA) and immunohistochemistry in a large cohort showed that BASP1 overexpression significantly correlated to survival and response to chemotherapy in patients with pancreatic cancer. Pathway analysis linked to clinical data suggested that BASP1 interacts with Wilms tumour protein (WT1) in pancreatic cancer.

Implications of all the available evidence

Our study depicts how an MS-based proteomics platform can aid in biomarker development for pancreatic cancer. The results indicate that BASP1 and its putative interaction partner WT1 are useful biomarkers for predicting the outcomes of pancreatic cancer patients, although further validation in prospective clinical cohorts are necessary.

1. Background

Pancreatic cancer is an almost uniformly fatal disease. Tremendous efforts have been made to elucidate the mechanisms underlying pancreatic cancer in order to develop effective treatments. Although there have been significant scientific advancements, pancreatic cancer survival rates remain stagnant with a 5-year survival rate of 9%. In the United States, 56,770 patients are predicted to be diagnosed with pancreatic cancer and 45,750 individuals will die from the disease in 2019 [1]. Despite the continuous overall decline in the death rates from most cancer forms, both incidence and mortality rates for pancreatic cancer have increased during the past decade [2]. It is projected that pancreatic cancer will become the second leading cause of cancer-related death by the year 2030 [3].

Surgical resection is the only curative treatment option, yet only about 15–20% of patients are eligible for up-front radical surgery [4]. Furthermore, despite complete surgical resection and adjuvant chemotherapy, >60% of patients develop recurrences within 2 years post-operatively [5]. No molecular marker has yet been able to accurately predict the course of the disease or response to therapy [6]. Therefore, molecular markers are not used in routine clinical management of pancreatic cancer. To improve patient outcomes, novel prognostic and predictive biomarkers are needed in order to characterise individual tumour biology and select optimal treatment.

Proteomic profiling of biological samples has been shown to be a valuable approach for biomarker discovery in many cancers [7–10]. Analyses of patient serum samples and formalin-fixed paraffin-embedded (FFPE) tissue specimens using proteomic-based technologies have greatly increased the pool of potential biomarkers for pancreatic cancer detection and monitoring [11,12]. However, high abundance proteins in serum samples and chemical modifications acquired during the sample preparation of FFPE specimens hinder the accurate detection of low abundant and disease-specific proteins [13,14]. When examining disease-specific molecular information, including altered protein expression and post-translational modifications, fresh frozen tissues are considered superior for MS-based proteomics analysis [15].

In the present study, we utilised a quantitative proteomics approach using fresh frozen pancreatic cancer tissue specimens and healthy pancreas. Brain acid soluble protein 1 (BASP1) was found to be significantly upregulated in pancreatic cancer. Overexpression of BASP1 was closely correlated to survival and response to chemotherapy when examined in a large cohort by clinicopathological analysis. Based on further bioinformatic data mining coupled with clinical data analysis, we suggest that BASP1 interacts with Wilms tumour protein (WT1) in pancreatic cancer.

2. Materials and methods

2.1. Study design

The methodological workflow of the present study is illustrated in Additional file 1: Fig. S1. A Nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) platform was used for identification of candidate protein biomarkers for pancreatic cancer. Parallel Reaction Monitoring (PRM) was used for verification of protein biomarker candidates. Comprehensive bioinformatics analyses of candidate proteins and biological interaction partners were conducted to characterise functional relevance. Antibody-based validation was performed in a pancreatic cancer cell line and resected pancreatic cancer tissues from a larger cohort (Table 3). Protein expression levels were then integrated with clinicopathological information for survival analyses.

2.2. Patients and tissue samples

For MS analysis, fresh frozen pancreatic cancer tissue samples ($n = 10$ for MS discovery, $n = 8$ for targeted MS) were prospectively collected from patients undergoing pancreaticoduodenectomy due to tumours located in the head of the pancreas between July 2013 and April 2015 at the Department of Surgery, Skåne University Hospital, Lund, Sweden. Age and gender-matched fresh frozen normal pancreas ($n = 10$) from organ donors free of any pancreatic disease were obtained from Lund University Diabetes Center and used as healthy controls (HC). Written informed consent was obtained from participating patients. For tissue microarray (TMA) and immunohistochemistry (IHC) analysis, FFPE tissue samples ($n = 143$) were included from a retrospective cohort of pancreatic cancer patients who underwent surgery with curative intent from 1995 to 2017 at Skåne University Hospital in Lund and Malmö, Sweden. Following antibody optimisation and staining, biomarker expression could be evaluated in 141 of the 143 (98.6%) of tumour samples included in the TMA. All samples were re-evaluated by a pancreatic pathologist to confirm the diagnosis and uniformity of staging. The REMARK guidelines were followed where applicable [16].

2.3. MS studies

2.3.1. Tissue sample preparation

Individual fresh frozen tissue samples were pulverised in liquid N_2 and thoroughly homogenised in an extraction buffer consisting of 500 mM Tris-Cl [pH 8], 6 M guanidine-HCl in 50 mM ammonium bicarbonate (AMBIC) along with protease and phosphatase inhibitor cocktail. The obtained extracts were then subjected to 4 freeze-thaw cycles, followed by ultrasonic bath for 20 min at 0 °C. The soluble proteins were then reduced with 15 mM dithiothreitol (DTT) for 60 min at 60 °C, alkylated using 50 mM iodoacetamide (IAA) for 30 min at room temperature in the dark, precipitated with a sample to ethanol

(99.5%) ratio of 1:9 at −20 °C. The protein precipitates were dissolved in 50 mM AMBIC and digested at 37 °C overnight using Mass Spec Grade Trypsin/Lys-C Mix (Promega, Madison, WI, USA), with an enzyme to protein ratio of 1:100. The digested samples were dried and dissolved in 50 μl 0.1% Formic Acid (mobile phase A), and the concentration was specified using Pierce quantitative colorimetric peptide assay from Thermo Scientific (Rockford, IL, USA). Finally, to enable normalisation and as a control of the chromatographic performance, 25 fmol peptide retention time mixture (PRTC) (Thermo Fisher) consisting of 15 peptides was added to each sample.

2.3.2. LC-MS/MS analysis

The analytical platform, including a high-performance nanoflow liquid chromatography (HPLC) system (EASY-nLCTM™ 1000) and a Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Q Exactive™) equipped with a nanospray ion source (EASY-Spray™), were manufactured by Thermo Fisher Scientific (Bremen, Germany). Individual samples containing 1 μg of peptide mixture in mobile phase A were injected at a flow rate of 300 nl min^{-1}, separated by a 132 min gradient of 5–22% acetonitrile (ACN) in mobile phase A, followed by an 18 min gradient of 22–38% ACN in mobile phase A. Subsequent separation was conducted by a two-column system including the EASY-Spray analytical column (25 cm × 75 μm ID, particle size 2 μm, pore size 100 Å, PepMap C18) tandem with the Acclaim pre-column (2 cm × 75 μm ID, particle size 3 μm, pore size 100 Å, PepMap C18). The Orbitrap system was operated in the positive data-dependent acquisition (DDA) mode with an automatic switch between the full scan MS and MS/MS acquisition. On the precursors with the highest intensity, 15 data-dependent higher energy collision dissociation MS/MS scans were implemented. For the peptide detection, a full MS survey scan was performed in the Orbitrap detector. The MS scans with a resolution of 70,000 at 200 m/z, recording window between 400.0 and 1600.0 m/z, and automatic gain control (AGC) target value of $1 × 10^6$ with a maximum injection time of 100 ms. The resolution of the data dependent MS/MS scans was fixed of 17,500 at 200 m/z, values for the AGC target of $5 × 10^5$ and maximum injection time was 80 ms. The normalised collision energy was set on 27.0% for all scans.

2.3.3. Targeted proteomics analysis

PRM analysis was performed to verify differentially expressed proteins. One or 2 unique peptides of each targeted protein were selected from the discovery measurements, depending on detection frequencies >50%, missed cleavage = 0 and p-value <.05, along with peptide intensities and ranking of peptide spectrum matches. Finally, a spectral library of 81 selected proteins (from the 165 differentially expressed proteins as well as the proteins only detectable in one condition) including 150 peptides was created. Owing to inadequate tissue sample volume, we had to exclude 2 pancreatic cancer subjects from the PRM phase. The proteins extracted from 18 fresh frozen samples (8 pancreatic cancer samples vs. 10 healthy controls) were reduced, alkylated, and digested as described previously in sample preparation. One microgram of the sample was injected into the LC-MS/MS system, and the PRM assay was set in a time-scheduled acquisition mode with a retention time +/− 5 min and resolution at 35000 (AGC target to $5 × 10^5$, maximum injection time of 50 ms). The chromatographic peak width was 30s, normalised collision energy on 26.0%, and the isolation window of 2 m/z. Skyline software was used for relative quantification in the PRM study [17].

2.3.4. MS data analysis

Each sample was measured in duplicate by LC-MS/MS in a randomised order. The raw files generated from the duplicates were combined and evaluated using Proteome Discoverer software (Thermo Fisher) Version 1.4 focusing on high confidence peptides only. The spectra selection settings: minimum and maximum precursor mass at 350 Da and 5000 Da, respectively; signal-to-noise (s/n) threshold 1.5.

Parameters for SEQUEST HT [18] were set as follows: precursor mass tolerance of 10 ppm (p.p.m); fragment mass tolerance of 0.02 Da; trypsin as the enzyme; one missed cleavage site was accepted. Based on the UniProtKB human database [19], dynamic modifications were included, such as: methyl (+14.016 Da; K, R), dimethyl (+28.031 Da; K, R), acetyl (+42.011 Da; K), trimethyl (+42.047 Da; K, R), glygly (+114.043 Da; K), oxidation (+15.995 Da; M), and the fixed modification carbamidomethyl (+57.021 Da; C). The percolator was applied for the processing node, and the false discovery rate (FDR) value was set to 0.01. To quantify the peptides, the precursor ions area detector was used in the search engine (Proteome discoverer; Thermo Scientific), protein groups identified ≥2 peptides from all samples were considered for further analysis and only unique peptides were used for protein quantification.

2.4. Tissue microarray construction and immunohistochemistry

Archival FFPE pancreatic cancer specimens from the larger validation cohort were subjected to TMA. Employing an automated tissue array instrument (Minicore® 3, Alphelys, Plaisir, France), 4 cores of cancer tissue from each specimen (diameter at 2 mm, selected by a pathologist) were extracted and fixed into paraffin blocks. After quality control, the TMA blocks were sectioned into 3 μm thick slides for IHC analysis.

IHC was performed as described previously [20]. Briefly, after deparaffinisation, rehydration and antigen-retrieval, TMA-slides were incubated with primary antibodies (rabbit anti-human BASP1 (dilution 1: 100; Cat No. HPA045218, Atlas Antibodies); mouse anti-human WT1 (clone 6F-H2, Ready-to-Use, Cat No. IS05530-2, DAKO)) overnight at 4 °C. Next, slides were incubated with second antibody (for BASP1, biotinylated goat anti-rabbit (dilution 1:200; Cat No. BA-1000, Vector Laboratories, Burlingame, CA); for WT1, biotinylated horse anti-mouse (dilution 1:200, Vector Laboratories, Cat No. BA-2000)) followed by staining with avidin-biotin-peroxidase complex (Vectastain Elite ABC-HRP Kit, Cat No. PK-6100, Vector Laboratories, Burlingame, CA). The sections were then incubated with chromogen diaminobenzidine (DAB) (Cat No. SK-4100, Vector Laboratories, Burlingame, CA) and counter stained with haematoxylin and mounted with xylene based medium. The IHC scoring was performed by an experienced pancreas pathologist (A.S.) who was blinded to the clinical information. Scoring was based on the percentage of positive tumour cells and the staining intensity. IHC results were scored as follows: 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. For tumours that showed heterogeneous staining, the predominant pattern was taken into account for scoring.

2.5. Cell culture and immunofluorescence

The human pancreatic cancer cell line, PANC-1, was purchased from ATCC-LGC Standards (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, CA, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humified 5% CO_2 atmosphere at 37 °C.

For investigating intracellular localisation, PANC-1 cells were cultured ($8 × 10^3$ cells/well) in eight-well chamber slides (Lab-Tek II Chamber Slide System, Nunc). After 48 h, the cells were fixed with 4% formaldehyde, then permeabilised with 1% Triton X-100, blocked with 5% goat serum and incubated with mouse anti-human WT1 (clone 6F-H2, Ready-to-Use; Cat No. IS05530–2, DAKO) at room temperature for 2 h. After washing, cells were moved into dark environment, Goat-anti-Mouse Alexa Fluor 594 (dilution 1: 500; Cat No. A11032, Invitrogen) was added at room temperature for 1 h. Subsequently, the cells were blocked with 5% donkey serum and incubated with rabbit anti-human BASP1 (dilution 1: 50; Cat No. HPA045218, Atlas Antibodies) at room temperature for 2 h. Following washing, Donkey-anti-Rabbit Alexa Fluor 488 (dilution 1: 500; Cat No. A21206, Invitrogen)

was added at room temperature for 1 h. Finally, the cells were incubated with DAPI to stain the nuclei. Positive staining was visualised using a Nikon Eclipse 80i microscope with a Nikon DS-Qi1 camera and analysed using NIS-Elements software (Nikon Instruments Inc.; Melville, NY, USA).

2.6. Statistics and bioinformatics

Perseus software [21] version 1.6.0.7 was used for the statistical analysis of the MS results. The protein intensities were log2 transformed and normalised by subtracting the median intensity of all the proteins per sample. Replacing the missing values from a normal distribution was performed though data imputation by using the following settings: width 0.3 and downshift 0. A Two-Sample Student's t-test (two-tailed) followed by permutation-based FDR correction was performed to compare protein levels between the groups. The settings included S0 = 2, which is a parameter used to calculate the relative difference (ratio of change in protein expression to standard deviation) between group means. It defines the within groups variance, the relative importance of the resulted p-values, and the difference between means of log2 intensities [22]. Finally, the proteins with FDR adjusted p-value (or q-value) of 0.01 were considered as differentially expressed.

For bioinformatic analysis of networks involving the biological relationship between BASP1 and WT1, the Ingenuity Pathway Analysis software (IPA, Qiagen, Inc. Redwood City, CA, USA) was used. This toolset builds upon a literature-derived relationship knowledge base. A network involving all direct interactors of these proteins was built and analysed for pathway enrichment and functional annotations. Additionally, differentially expressed proteins between pancreatic cancer and healthy controls samples from MS discovery were mapped onto the BASP1/WT1 network. Subcellular localisation of significantly up- and down-regulated proteins in pancreatic cancer versus healthy control samples was manually assessed using UniProt [19] (https://www.uniprot.org/). PANTHER [23] (http://www.pantherdb.org/) was employed to identify gene ontology terms of the significantly differentially expressed proteins.

For IHC analysis, the correlation between the expression levels of protein biomarkers and clinicopathological parameters was estimated using the Mann-Whitney U test for continuous variables and Fisher's exact test or χ^2 for categorical variables. Kaplan–Meier analysis was used to calculate the cumulative probability of overall survival (OS), log-rank tests were used to evaluate the differences. Prognostic factors were calculated using univariable and multivariable analysis (Cox proportional hazards regression model). A value of $p < .05$ was considered statistically significant.

Statistical evaluation was conducted with Perseus software version 1.6.0.7, SPSS version 23.0 (SPSS Inc., Chicago, IL, USA), GraphPad Prism v.7 (La Jolla, CA, USA), and R [24] programming language version 3.5.1 (R Foundation for Statistical Computing, https://www.r-project.org/).
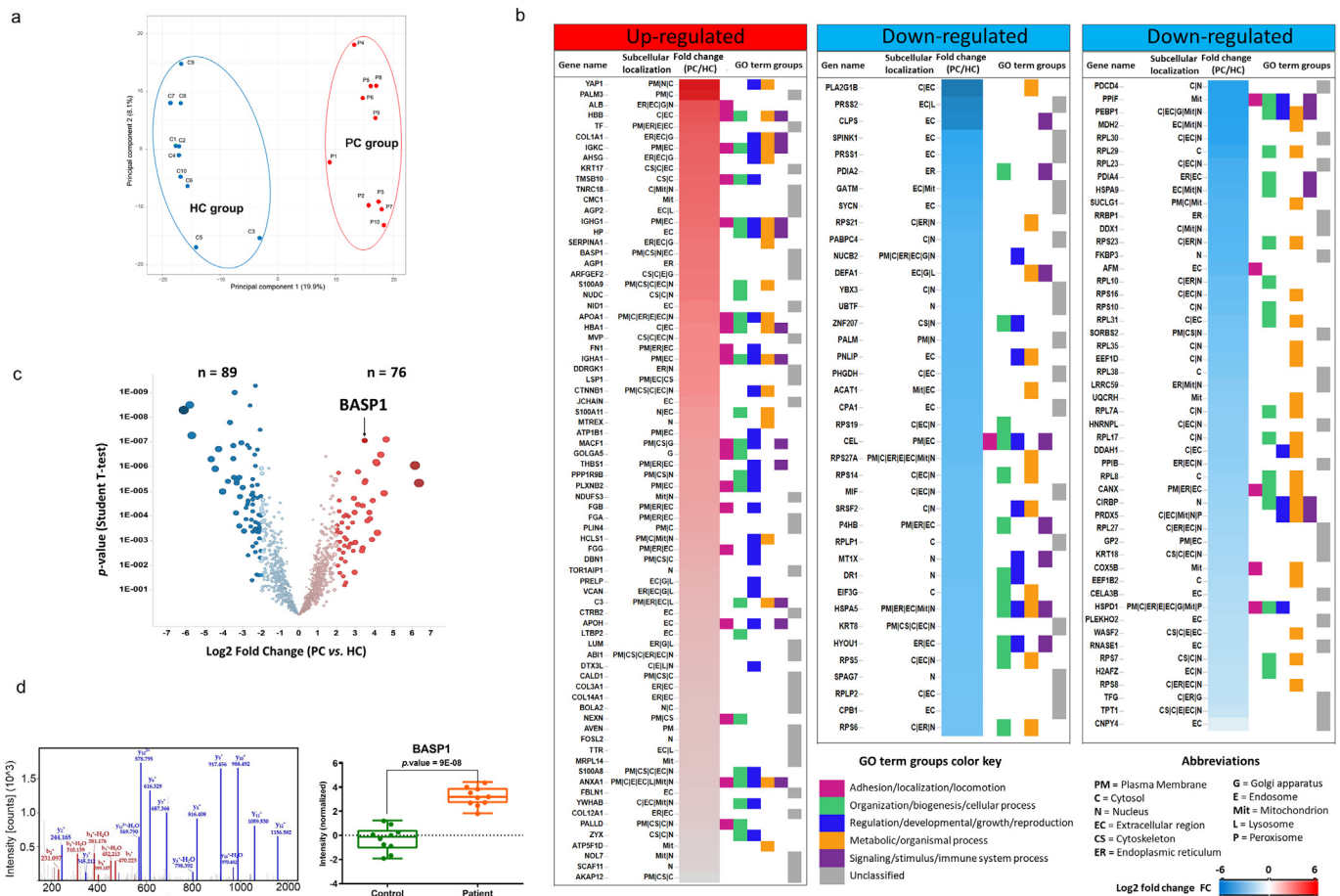

Fig. 1. Mass spectrometry (MS) discovery study. a. Principal Component Analysis of quantified proteins, a complete separation of pancreatic cancer (PC) and healthy control (HC) groups was observed. b. Heatmap of the 165 significantly altered proteins. The up-regulated and down-regulated proteins are ranked by log2 fold change and the subcellular location of each protein is also presented. PANTHER gene ontology (GO) analysis showed that GO terms of the 165 proteins related to biological processes such as localisation, biogenesis and signalling etc. c. Volcano plot of all proteins that were identified in this study, the dark red and dark blue dots denote the significantly up- and down-regulated proteins in PC compared to HC, respectively (q- value < .01 and log2 fold change over 2 in Student's t-test, the size of dots represent fold changes). BASP1 was one of the top-ranked candidate protein biomarkers. d. The label-free quantitative MS discovery, MS spectra of BASP1 (left), box-plot showing relative expression levels of BASP1 in PC and HC (right).

Table 1
PRM verified upregulated proteins in pancreatic cancer compared to healthy controls (ranked according to fold change).

Sr. no.	UniProt accession	Gene	Protein name	Unique peptide	P value	Fold change (PC/NC)
1	P02647	APOA1	Apolipoprotein A-I	K.LLDNWDSVTSTFSK.L	1.8E-08	39.12
2	B9A064	IGLL5	Immunoglobulin lambda-like polypeptide 5	K.VTVLGQPK.A	3.5E-09	35.02
3	P02765	AHSG	Alpha-2-HS-glycoprotein	K.FSVVYAK·C	2.1E-09	27.47
4	P0DOY2	IGLC2	Immunoglobulin lambda constant 2	K.AAPSVTLFPPSSEELQANK.A	1.6E-09	24.42
5	P02763	AGP1	Alpha-1-acid glycoprotein 1	R.YVGGQEHFAHLLILR.D	4.6E-06	24.25
6	P01857	IGHG1	Immunoglobulin heavy constant gamma 1	K.GPSVFPLAPSSK·S	2.2E-10	23.59
7	P01834	IGKC	Immunoglobulin kappa constant	K.VDNALQSGNSQESVTEQDSK.D	5.1E-11	22.78
8	P01876	IGHA1	Immunoglobulin heavy constant alpha 1	K.TPLTATLSK·S	1.3E-09	20.82
9	P02787	TF	Serotransferrin	K.EGYYGYTGAFR.C	1.6E-10	19.84
10	P02768	ALB	Serum albumin	K.DDNPNLPR.L	4.5E-09	19.70
11	P01009	SERPINA1	Alpha-1-antitrypsin	K.AVLTIDEK.G	6.2E-10	17.03
12	P80723	BASP1	Brain acid soluble protein 1	K.ETPAATEAPSSTPK.A	1.7E-05	12.91
13	P06703	S100A6	Protein S100-A6	K.LQDAEIAR.L	1.5E-05	12.13
14	Q05707	COL14A1	Collagen alpha-1(XIV) chain	R.YTAILNQIPSHSSSIR.T	6.5E-12	10.70
15	P16401	HIST1H1B	Histone H1.5	K.ATGPPVSELITK.A	1.6E-08	9.85
16	P23142	FBLN1	Fibulin-1	K.IIEVEEEQEDPYLNDR.C	4.8E-08	8.34
17	P52566	ARHGDIB	Rho GDP-dissociation inhibitor 2	K.TLLGDGPVVTDPK.A	1.9E-08	5.46

Abbreviations: NC, normal controls; PC, pancreatic cancer; PRM, parallel reaction monitoring.

3. Results

3.1. Identification of candidate biomarkers for pancreatic cancer

Representative fresh frozen pancreatic cancer ($n = 10$) and healthy control (n = 10) tissue samples were analysed using a LC-MS/MS platform. A total of 4138 proteins were identified (Additional file 2: Table S1) and 2950 proteins were quantified with one or more unique peptides (Additional file 3: Table S2). Among the quantified proteins, 2264 proteins were present in the pancreatic cancer group and 2354 proteins in the healthy control group, respectively. To demonstrate the general pattern of protein abundance variation within and between different groups, a two-dimensional Principal Component Analysis (PCA) was performed based on all quantified proteins by an online tool ClustVis [25]. Using the log2-ratio of each sample over the mean of all samples, a complete separation of the pancreatic cancer and healthy control groups was observed (Fig. 1a).

By employing the criteria of FDR adjusted p-value (or q-value) of 0.01, S0 = 2, the number of peptides >1 and the fold change >2 as a cut-off, a total of 165 proteins with two or more unique peptides were significantly differentially expressed between the two experimental groups (Fig. 1b). A volcano plot of significantly upregulated and down-regulated proteins is presented in Fig. 1c.

3.2. Development of targeted protein assays using PRM

To verify the differential expression changes of potential protein biomarkers from MS discovery, PRM was employed based on the same samples from the MS discovery phase ($n = 8$ in the pancreatic cancer group and $n = 10$ in the healthy control group). Eighty-one proteins with one or two unique peptides for each protein were selected and a panel of 45 proteins were successfully detected and quantified. Among these proteins, 17 proteins were significantly up-regulated ($p < .01$), while 28 proteins were down-regulated in pancreatic cancer

Table 2
PRM verified downregulated proteins in pancreatic cancer compared to healthy controls (ranked according to fold change).

Sr. no.	UniProt accession	Gene	Protein name	Unique peptide	P value	Fold change (NC/PC)
1	P04054	PLA2G1B	Phospholipase A2	R.AVWQFR.K	1.1E-06	56.89
2	P16233	PNLIP	Pancreatic triacylglycerol lipase	R.TGYTQASQNIR.I	8.7E-06	51.98
3	P09093	CELA3A	Chymotrypsin-like elastase family member 3A	R.WNWWGSTVK·K	1.7E-06	47.18
4	P04118	CLPS	Colipase	K.TLYGIYYK·C	1.2E-05	44.63
5	P19835	CEL	Bile salt-activated lipase	K.LGLLGDSVDIFK.G	4.5E-06	39.95
6	P07478	PRSS2	Trypsin-2	R.TLDNDILLIK.L	3.5E-06	36
7	P15085	CPA1	Carboxypeptidase A1	K.TEPVPDQDELDQLSK.A	2.4E-06	35.02
8	Q13087	PDIA2	Protein disulfide-isomerase A2	K.NFEQVAFDETK·N	3.4E-05	30.06
9	P07477	PRSS1	Trypsin-1	K.TLNNDIMLIK.L	3.3E-05	28.44
10	P09210	GSTA2	Glutathione S-transferase A2	K.LALIQEK.T	9.0E-05	10.78
11	O43175	PHGDH	D-3-phosphoglycerate dehydrogenase	K.TLGILGLGR.I	1.6E-06	10.27
12	Q13310	PABPC4	Polyadenylate-binding protein 4	K.SGVGNVFIK·N	8.4E-08	9
13	P07237	P4HB	Protein disulfide-isomerase	K.VDATEESDLAQQYGVR.G	1.1E-07	7.62
14	P43307	SSR1	Translocon-associated protein subunit alpha	K.GEDFPANNIVK·F	7.4E-07	7.21
15	P16989	YBX3	Y-box-binding protein 3	K.GAEAANVTGPDGVPVEGSR.Y	9.0E-07	6.36
16	Q9P2E9	RRBP1	Ribosome-binding protein 1	K.LLATEQEDAAVAK·S	1.8E-06	5.5
17	Q96AG4	LRRC59	Leucine-rich repeat-containing protein 59	K.LQQLPADFGR.L	3.9E-05	5.21
18	P13667	PDIA4	Protein disulfide-isomerase A4	K.VEGFPTIYFAPSGDK·K	1.9E-06	4.82
19	P11021	HSPA5	78 kDa glucose-regulated protein	K.NQLTSNPENTVFDAK.R	5.6E-07	4.5
20	Q9Y4L1	HYOU1	Hypoxia up-regulated protein 1	K.AANSLEAFIFETQDK.L	9.3E-05	4.23
21	O94760	DDAH1	N(G), N(G)-dimethylarginine dimethylaminohydrolase 1	R.ALPESLGQHALR.S	4.4E-07	3.66
22	P24534	EEF1B2	Elongation factor 1-beta	K.YGPADVEDTTGSGATDSK.D	2.0E-04	3.41
23	P30086	PEBP1	Phosphatidylethanolamine-binding protein 1	K.LYEQLSGK.-	1.4E-05	3.36
24	P63220	RPS21	40S ribosomal protein S21	K.DHASIQMNVAEVDK·V	4.7E-05	3.34
25	P61247	RPS3A	40S ribosomal protein S3a	K.TTDGYLLR.L	9.9E-06	3.34
26	P62263	RPS14	40S ribosomal protein S14	K.TPGPGAQSALR.A	2.9E-06	3.12
27	P30050	RPL12	60S ribosomal protein L12	K.IGPLGLSPK·K	1.8E-04	2.97
28	Q92734	TFG	Protein TFG	K.LLSNDEVTIK·Y	1.9E-05	2.43

Abbreviations: NC, normal controls; PC, pancreatic cancer; PRM, parallel reaction monitoring.

a

b

BASP1

p.value = 2E-05

c

d
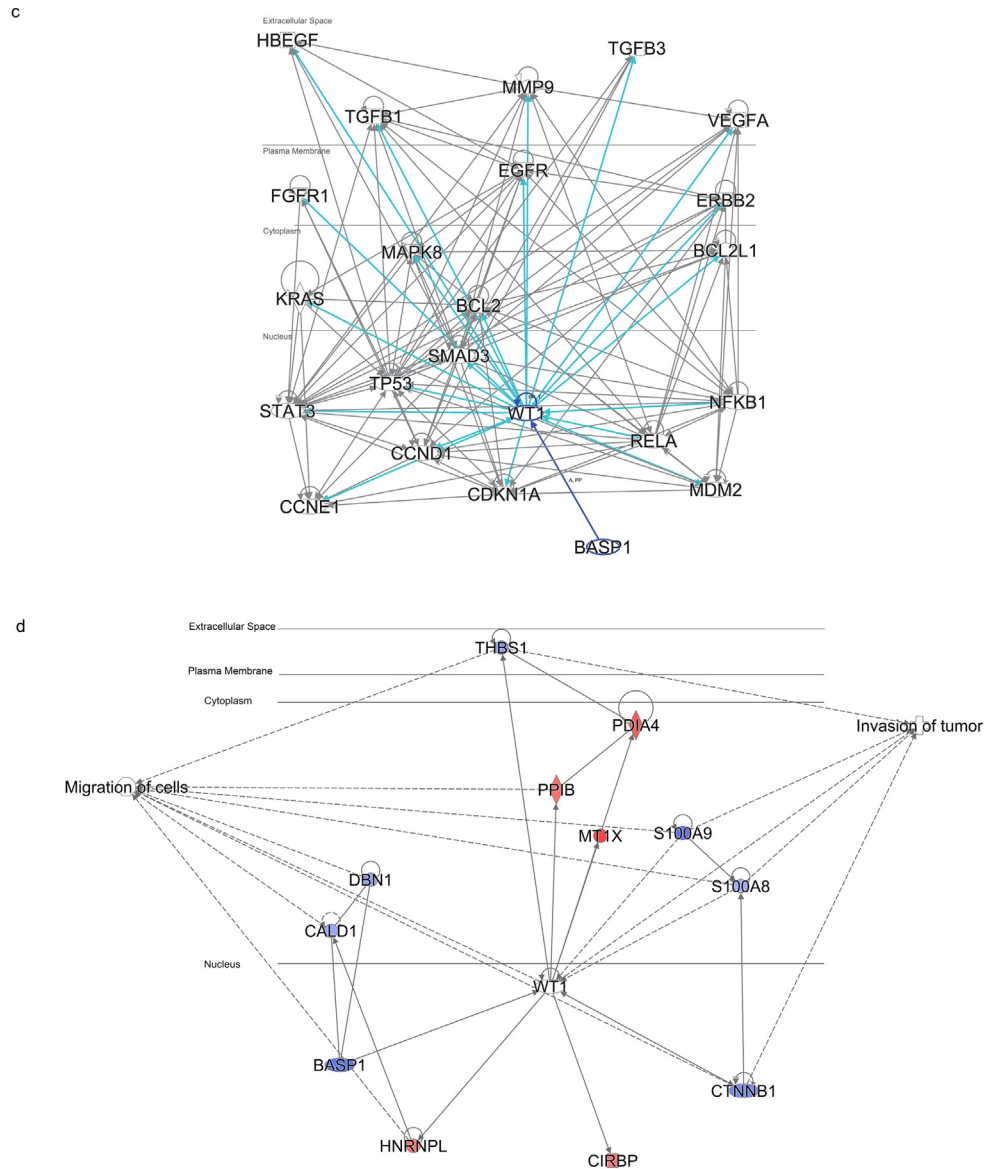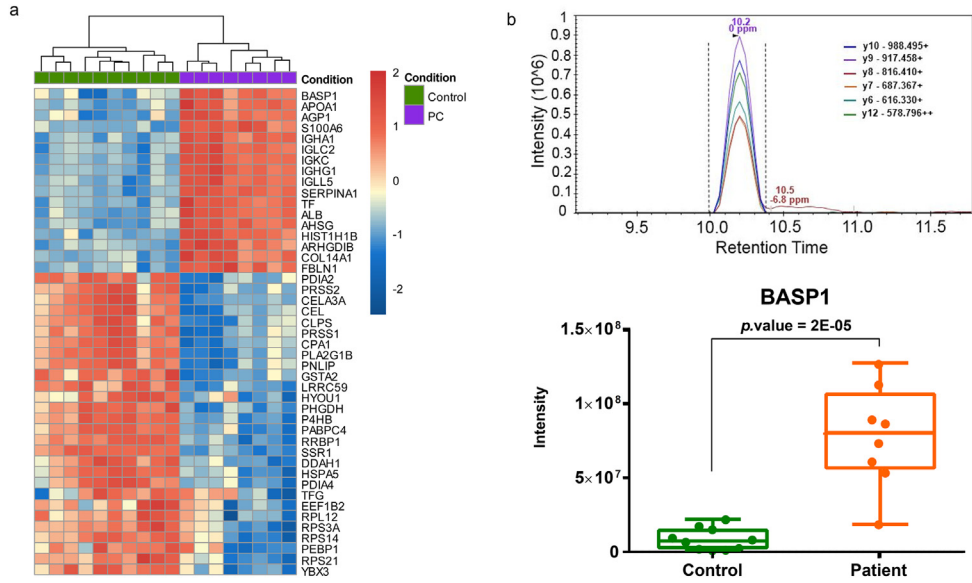
versus healthy controls, respectively (Tables 1 and 2). From the panel of 45 verified candidates, 16 extracellular proteins emerged that could theoretically be detected in serum and potentially be applied in non-invasive diagnosis and/or prognosis prediction, including S100A6, TF, FBLN1, HYOU1, PNLIP, P4HB, AHSG, PLA2G1B, AGP1, PRSS1, PRSS2, APOA1, ALB, SERPINA1, CLPS, and COL14A1 as previously reported by our group [26]. Subsequently, a consensus clustering heat map was created based on the 45 verified proteins and a clear discrimination between pancreatic cancer and healthy controls was observed (Fig. 2a).

3.3. Selection of BASP1 for further validation

BASP1 is a neuron enriched Ca(2+)-dependent calmodulin-binding protein with unknown function in pancreatic cancer. BASP1 was established as a top-ranked protein, being significantly up-regulated in the pancreatic cancer group by a fold change of 11.24, $p = 9E-08$ (Fig. 1d). Notably, based on quantification of the following unique peptides: SDGAPASDSKPGSSEAAPSSK and ETPAATEAPSSTPK, BASP1 presented as one of the most reproducible candidates, being significantly up-regulated in the pancreatic cancer group with a fold change of 12.91 and $p = 2E-05$ (Fig. 2b). As a potential novel biomarker, BASP1 was selected for further validation by bioinformatic and clinical association studies.

3.4. BASP1 is functionally related to WT1

In order to obtain an unbiased overview of the BASP1 functional relationships in a biological context, Ingenuity Pathway Analysis (IPA) was used to create a network involving all proteins with direct relationships (e.g. physical interaction or direct activation) to BASP1. This analysis, building upon a literature-derived relationship knowledge base, yielded a network including 412 proteins that were significantly enriched and involved in several canonical pathways (e.g. pancreatic adenocarcinoma signalling, regulation of the epithelial-mesenchymal transition pathway, ILK signalling, Additional file 4: Table S3) as well as tumorigenic conditions (e.g. apoptosis, cell migration, angiogenesis). Furthermore, among the top upstream regulators automatically identified by the IPA algorithm for the BASP1 interactor set, several well-known tumour-related signalling proteins emerged (e.g. TP53, TNF, TGFB1, EGF, HRAS).

Interestingly, the pathway analysis suggests that the link between BASP1 and pancreatic cancer is via WT1, and there are 21 proteins from the pancreatic adenocarcinoma signalling pathway that interact with WT1 (enrichment p-value 3E-16, Fig. 2c). Among these, extracellular signalling molecules TGFB1, TGFB3, VEGFA, HBEGF, receptor tyrosine kinases EGFR1, ERBB2 and FGFR1, apoptosis regulators BCL2, BCL2L1 and the recognised pancreatic cancer-related transcription regulator TP53, KRAS, and MAPK8 were annotated. Mapping of the differentially expressed proteins into the BASP1/WT1 network provided 11 hits out of 165 (Fig. 2d). Markedly, according to IPA analysis, most of these proteins are involved in cellular migration and tumour invasion processes.

3.5. BASP1 and WT1 expression in tumour samples and cancer cell line

The expression levels of BASP1 and WT1 were assessed in a larger cohort of pancreatic cancer patients by TMA-IHC. The clinical characteristics of the pancreatic cancer patients are shown in Table 3. Based on the validation cohort, 141 patients were successfully scored for BASP1 and 139 patients for WT1, respectively. Both markers were evaluable

in 137 patients. In the BASP1 cohort ($n = 141$), 15 (10.6%) tissue samples from pancreatic cancer patients showed negative staining (Score 0) and 126 (89.4%) samples displayed positive staining, where 25 (17.7%) samples were scored as weak (Score 1), 66 (46.8%) as moderate (Score 2), and 35 (24.8%) as strong (Score 3). The majority of the staining was observed accentuated in the cytoplasm/plasma membrane (PM), accompanied by weak nuclear staining (Fig. 3a). Interestingly, 135 (97.1%) pancreatic cancer tissue samples had positive staining of WT1 protein in the WT1 cohort ($n = 139$), and only 4 (2.9%) were observed as loss of positivity (Score 0). Moreover, the WT1 staining was predominantly presented in the cytoplasm of pancreatic tumour cells, while nuclear immunostaining was weak. Furthermore, the positively stained tissue samples were subdivided into weak 22 (15.8%, Score 1), moderate 51 (36.7%, Score 2), and strong 62 (44.6%, Score 3) staining (Fig. 3b).

In order to study the dual expression patterns of BASP1 and WT1 in human pancreatic cancer cell line, we performed immunofluorescence staining of BASP1 and WT1 in PANC-1 cell line. In accordance with our IHC results, BASP1 was mostly expressed in cytoplasm and PM, while WT1 was detected in the cytoplasm and mostly with perinuclear localisation (Fig. 3c).

3.6. BASP1 expression is an independent predictor of favourable survival

Kaplan-Meier analysis showed that pancreatic cancer patients with positive BASP1 expression had significantly prolonged overall survival (OS) compared to patients with negative BASP1 expression (median survival, 27.7 vs. 13.3 months, respectively, $p = .022$, Fig. 4a). The univariable Cox regression analysis indicated that apart from BASP1 positive expression ($p = .025$), three other variables, including smoking history ($p = .015$), presenting symptoms at diagnosis ($p = .044$), and histological grade ($p = .041$), were correlated with OS. In multivariable Cox regression analysis, positive BASP1 expression remained an independent prognostic factor with a hazard ratio (HR) of 0.468, 95% confidence interval (CI) 0.257–0.852, and $p = .013$ (Table 4).

3.7. High BASP1 expression predicts beneficial response to adjuvant chemotherapy

In the BASP1 cohort, patients with high expression of BASP1 (Score 3) exhibited significantly improved OS when they received adjuvant chemotherapy compared to those without adjuvant chemotherapy (median survival, 40.5 vs. 7.2 months, respectively, $p = .020$, Fig. 4b). No correlation to adjuvant chemotherapy ($p = .603$) was observed in patients with low expression of BASP1 (score 0, 1, and 2, Fig. 4c). These results suggest that BASP1 may function both as a marker for favourable prognosis and as a predictive biomarker for positive adjuvant chemotherapy response.

3.8. WT1 expression is correlated to poor survival and chemoresistance

Kaplan-Meier analysis revealed that patients in the high WT1 expression (Score 3) group had significantly shorter OS compared to those in the low WT1 expression (Score 0, 1, and 2) group (median survival, 22.2 vs. 25.7 months, respectively, $p = .028$, Fig. 4d). Further univariable Cox regression analysis demonstrated that besides high WT1 expression ($p = .029$), other factors such as smoking history ($p = .012$), presenting symptoms at diagnosis ($p = .049$), and high pathological grades ($p = .035$) were also associated with OS. In

Fig. 2. Targeted proteomics study and bioinformatic analysis of candidate protein biomarkers. **a.** Heat map of 45 Parallel Reaction Monitoring (PRM) verified biomarkers. An apparent discrimination between PC and HC can be observed, the verified protein biomarkers are listed to the right side. **b.** PRM verification, PRM transitions used for targeted verification of BASP1 (upper), box-plot showing relative expression levels of BASP1 in PC patients and matched healthy controls (under). **c.** Ingenuity Pathway Analysis (IPA) of all proteins from the pancreatic adenocarcinoma signalling pathway that have direct with biological relationships with BASP1 or WT1. **d.** All proteins with biological relationships with WT1 or BASP1 that are differentially expressed in pancreatic cancer vs healthy controls (Blue: proteins up-regulated in pancreatic cancer. Red: proteins down-regulated in pancreatic cancer).

Table 3
Clinicopathological variables stratified by BASP1 and WT1 expression.

Factors	BASP1 cohort				WT1 cohort			
	Total	Negative	Positive	p value	Total	Low	High	p value
	N = 141	N = 15	N = 126		N = 139	N = 77	N = 62	
Age (> 65 years)	93 (66)	9 (60)	84 (66.7)	0.58	91 (65.5)	54 (70.1)	37 (59.7)	0.214
Female gender	67 (47.5)	7 (46.7)	60 (47.6)	1	68 (48.9)	35 (45.5)	33 (53.2)	0.397
BMI (>25 kg/m^2)	57 (42.9)	9 (60)	48 (40.7)	0.175	56 (42.7)	30 (41.7)	26 (44.1)	0.86
Smoking history	66 (47.1)	6 (40)	60 (48)	0.596	65 (47.1)	38 (50)	27 (43.5)	0.495
Diabetes mellitus	33 (23.6)	1 (7.1)	32 (25.4)	0.188	33 (23.9)	20 (26)	13 (21.3)	0.553
Symptoms at diagnosis	132 (96.4)	14 (100)	118 (95.9)	1	130 (96.3)	73 (96.1)	57 (96.6)	1
Tumour location (head)	117 (83)	15 (100)	102 (81)	0.074	116 (83.5)	63 (81.8)	53 (85.5)	0.649
Tumour size (>2 cm)	118 (84.3)	13 (86.7)	105 (84)	1	115 (83.3)	64 (83.1)	51 (83.6)	1
T-stage (≥T2)	122 (87.1)	14 (93.3)	108 (86.4)	0.693	119 (86.2)	66 (85.7)	53 (86.9)	1
N-stage (≥N1)	106 (76.3)	10 (66.7)	96 (77.4)	0.349	103 (75.2)	62 (81.6)	41 (67.2)	0.073
AJCC 8th edition (≥IIA)	114 (82)	12 (80)	102 (82.3)	0.734	111 (81)	66 (86.8)	45 (73.8)	0.078
Histological grade (≥3)	82 (59)	10 (66.7)	72 (58.1)	0.589	83 (60.6)	47 (61.8)	36 (59)	0.86
Resection margin (≥R1)	55 (39.3)	6 (40)	49 (39.2)	1	53 (38.4)	28 (36.4)	25 (41)	0.601
Adjuvant chemotherapy	115 (84.6)	12 (80)	103 (85.1)	0.703	112 (83.6)	61 (83.6)	51 (83.6)	1
Recurrence of disease	102 (79.7)	12 (85.7)	90 (78.9)	0.734	102 (81)	55 (78.6)	47 (83.9)	0.5

Data were incomplete for some variables. Abbreviations: AJCC, American joint committee on cancer; BMI, body mass index; N-stage, nodal stage; T-stage, tumour stage.

multivariable Cox regression analysis, high WT1 expression was identified as an independent factor associated with OS (HR 1.636, 95% CI 1.083–2.473, $p = .019$, Table 4).

Interestingly, pancreatic cancer patients with strong expression of WT1, adjuvant chemotherapy displayed no significant impact on OS ($p = .335$, Fig. 4e). Of note, pancreatic cancer patients with weak-to-moderate WT1 expression, who received adjuvant chemotherapy presented significantly extended OS compared to patients that did not receive chemotherapy (median survival, 24.5 vs. 16.9 months, respectively, $p = .006$, Fig. 4f). These findings indicate that WT1 expression is correlated with chemoresistance in pancreatic cancer.

3.9. Patients with negative BASP1 and high WT1 expression have the poorest outcome

To examine the potential biological cross-talk between BASP1 and WT1 in terms of patient survival, we performed subgroup functionality analysis of these prognostic markers. For patients with negative expression of BASP1, the high WT1 expression group had significantly reduced OS compared to the low WT1 expression group (median survival, 9.4 vs. 20.4 months, respectively, $p = .022$, Fig. 5a). Interestingly, no significant difference in OS between high and low WT1 groups was observed in patients with positive expression of BASP1 ($p = .065$, Fig. 5b). These data suggested that BASP1 can potentially relieve the oncogenic effect of WT1 in pancreatic cancer patients.

Moreover, for patients with high WT1 expression, the positive BASP1 expression group presented significantly prolonged OS compared to the BASP1 negative group (median survival, 25.8 vs. 9.4 months, respectively, $p = .00012$, Fig. 5c). In addition, patients with high WT1 and positive BASP1 expression presented a similar survival pattern as the group of patients with low WT1 and negative BASP1 expression ($p = .822$, Fig. 5d). The results confirm the possibility that the protective role of BASP1 can impede the tumour promoting function of WT1. Finally, the best prognosis was seen in patients with positive
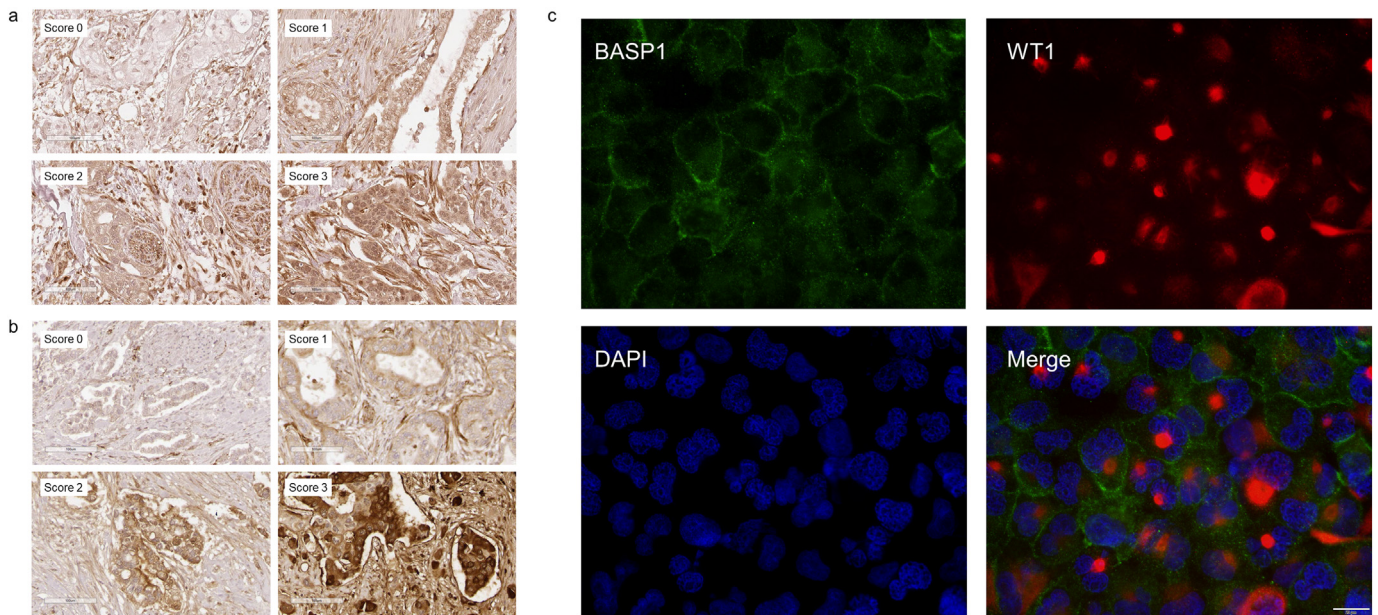

Fig. 3. Immunohistochemistry (IHC) and Immunofluorescence (IF) analysis of BASP1/WT1. **a.** Representative photomicrograph showing different levels of IHC staining of BASP1 expression in pancreatic cancer tissue samples. **b.** Representative photomicrograph showing different levels of IHC staining of WT1 expression in pancreatic cancer tissue samples. **c.** PANC1 cancer cells were labeled with antibodies for BASP1, WT1, and DAPI; green represents BASP1 (mostly expressed in cytoplasm and plasma membrane), red represents WT1 (detected in cytoplasm and mainly perinuclearly localised), and blue represents nuclear DNA staining by DAPI.

expression of BASP1 and low expression of WT1, whereas patients with negative BASP1 expression and high WT1 expression had the poorest outcome (median survival, 25.7 vs. 9.4 months, $p = .0001$, Fig. 5e). The multivariable Cox regression analysis, highlighted negative BASP1 expression and high WT1 expression as an independent factor associated with significantly shortened OS (HR 3.536, 95% CI 1.336–9.362, $p = .011$). These data suggest that BASP1 may act as a tumour suppressor rescuing the oncogenic effect of overexpressed WT1.

4. Discussion

In this study, we have employed nanoflow LC-MS/MS analysis to explore global protein expression patterns of fresh frozen pancreatic cancer tissues and healthy pancreas controls, and successfully identified a novel panel of potential protein biomarkers. This is the first report, presenting the functional role of BASP1 as a protein marker for prognosis and response to chemotherapy in pancreatic cancer. Importantly, we demonstrated that BASP1 could interact with WT1, providing valuable information for future research and clinical practice.

Currently, the gold standard for predicting outcomes in pancreatic cancer is the TNM classification system [27]. However, the TNM staging system is relatively non-discriminatory and tumours of the same stage may have different clinical behaviour in terms of prognosis and treatment response [28], which may lead to under- or overtreatment. Thus, tremendous efforts have been put into finding novel, reliable biomarkers for predicting clinical outcomes for pancreatic cancer patients [29–31]. Many interesting biomarkers have been proposed, however, few of those markers have been introduced into clinical practice, mainly due to the lack of sufficient validation [32]. To overcome this limitation, we constructed a TMA comprising large, clinically well-characterised pancreatic cancer cohort, and performed IHC analysis for assessing the validity of BASP1 as a targeted prognostic biomarker candidate.

BASP1 (also known as CAP-23 or NAP22) was originally identified as a cytoplasmic and plasma membrane-bound protein from brain extracts. It is known to be involved in axon regeneration and neuronal plasticity [33,34]. Recently, BASP1 was found to be a potential tumour suppressor and implicated in many cancers [35,36]. For instance, in hepatocellular carcinoma (HCC), aberrant promoter methylation of the
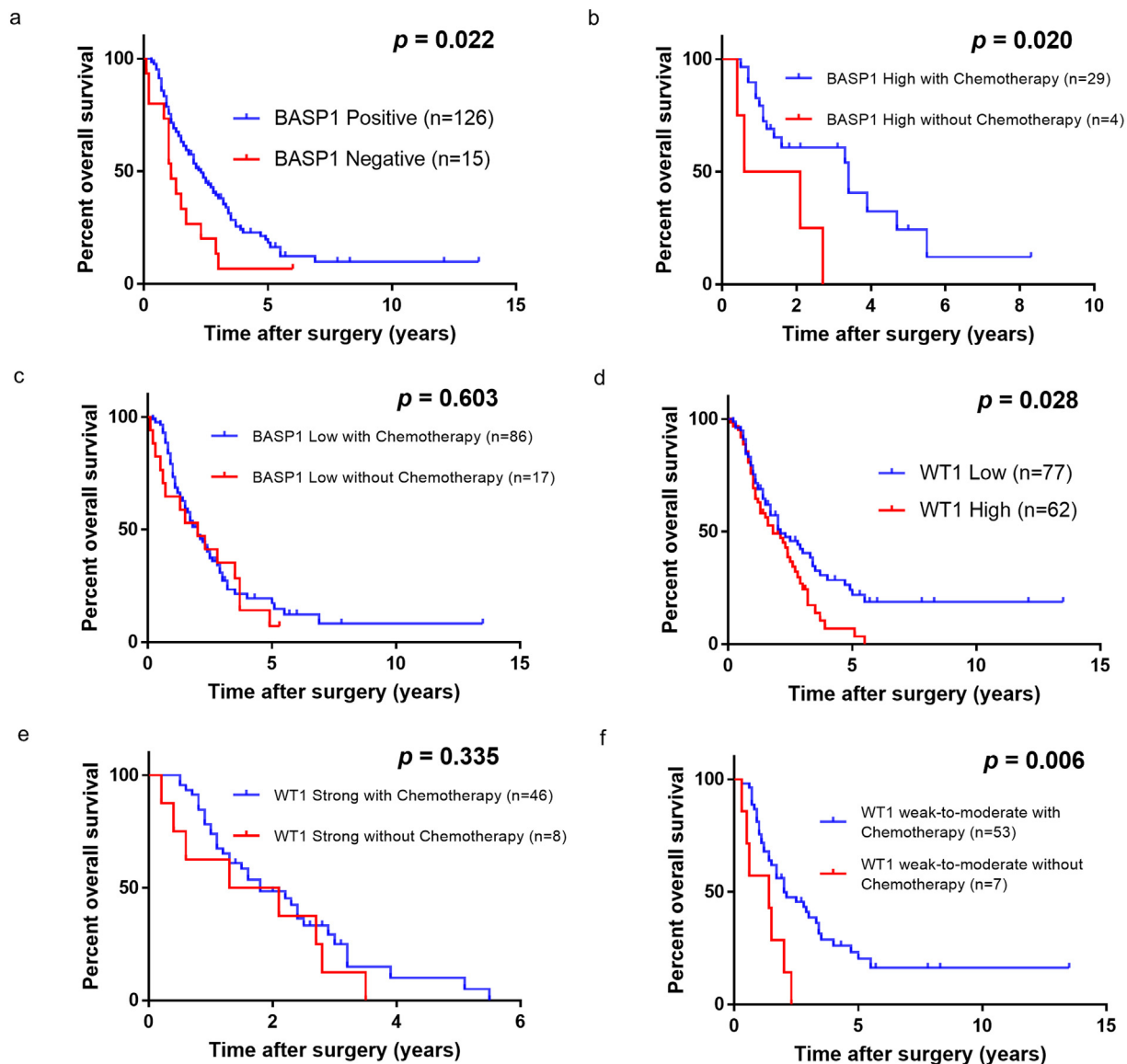

Fig. 4. Survival analysis of BASP1/WT1 in tissue microarray samples. **a.** Kaplan-Meier analyses for BASP1 (log-rank tests). **b.** When BASP1 exhibited high expression, adjuvant chemotherapy could significantly improve OS. **c.** Patients with low expression of BASP adjuvant chemotherapy showed no significance in improving OS. **d.** Kaplan-Meier analyses for WT1 (log-rank tests). **e.** When WT1 was strongly expressed, adjuvant chemotherapy displayed no significant impact on OS. **f.** When WT1 was weakly-to-moderately expressed, adjuvant chemotherapy significantly prolonged the OS.

BASP1 gene resulted in down-regulation of BASP1 protein expression, considered as useful finding for early detection of HCC [37]. In breast cancer, BASP1 interacts with the estrogen receptor α and enhances the anti-cancer effects of tamoxifen treatment. Additionally, high expression of BASP1 in breast cancer tissue is associated with better patient survival [36].

In the present study, the MS discovery and verification phases showed that the BASP1 protein was significantly overexpressed in pancreatic cancer tissues compared to healthy pancreas. These results are in accordance with previous findings from breast cancer studies, after IHC analysis, which indicated BASP1 as significantly up-regulated in malignant tissue compared to normal tissue [36]. However, the reason for this up-regulation remains unknown. In agreement with previous BASP1 studies, our current study highlights the tumour suppressor function of BASP1, supported by the link to favourable prognosis for pancreatic cancer patients after surgery. Additionally, pancreatic cancer patients with high BASP1 expression levels in tumour tissue showed a significantly enhanced benefit from adjuvant chemotherapy. This finding may aid clinicians to individualise chemotherapeutic treatment, hopefully improving patient outcomes and their respective survival patterns.

Interestingly, our bioinformatic analyses demonstrated that BASP1 shares a close interaction network with WT1 in pancreatic cancer. WT1 is a zinc finger transcription factor, which is a confirmed oncogenic factor. Overexpression of WT1 is associated with worse prognosis in patients with hematologic malignancies and various solid tumours [38], such as acute myeloid leukemia [39], breast cancer [40], and hepatocellular carcinoma [41]. Notably, WT1 was suggested as the most promising tumour-associated antigen for cancer immunotherapy by the National Cancer Institute [42], and many preclinical studies and clinical trials have demonstrated that WT1-targeted cancer vaccines have the potential to treat patients with pancreatic cancer [43–45]. Few studies have studied expression patterns of WT1 in pancreatic cancer [46,47]. However, only one recent study evaluated the prognostic value of WT1 in pancreatic ductal adenocarcinoma, indicating that cytoplasmic overexpression of WT1 correlated with unfavourable prognosis for the patient [48]. Notwithstanding, these findings were based on a small cohort of only 50 patients and additional studies would be necessary to confirm these results.

Consistent with other studies, we propose that WT1 may play an oncogenic role in pancreatic cancer, promoting tumour progression and being correlated to short-term relapse and poor survival. Furthermore, WT1 may be associated with chemotherapy resistance, which has not been reported previously.

There is evidence suggesting that BASP1 regulates and silences WT1 transcriptional activation [49]. Additional genome wide analysis indicated that the expression of BASP1 in leukemia cells leads to the transcriptional repression of >90% of the WT1 target genes [50]. Moreover, BASP1 and WT1 were found together in large complexes from cell lines and showed transcriptional repression activities [51,52]. It may be speculated that modulation of BASP1 in pancreatic cancer cells may facilitate WT1 targeted immunotherapy to achieve improved response rates.

A particular strength of our study was that healthy pancreas biopsies were used as control systems in the biomarker discovery phase. These unique and rare specimens were acquired from organ donors. Previous proteomic studies commonly use histologically normal tissue adjacent to the tumour as a control [53,54]. However, the regions adjacent to tumours have been found to have many aberrant morphologic and phenotypic alterations as predicted by the "field cancerisation theory" by Slaughter et al. [55,56]. The choice of healthy tissue as a comparative material for identification and further development of a discriminative biomarker is therefore preferable. Another important feature of the present study was that we used Trypsin/Lys-C Mix for protein digestion. >20% of cleavage sites may be missed by regular Trypsin [57]. The Trypsin/Lys-C Mix misses fewer lysine cleavage sites and enhances overall proteolytic efficiency as compared to Trypsin alone [58].

There are some potential limitations in our study that must be acknowledged. The fresh frozen samples used in the discovery phase were limited in number. The tissue microarray samples were accrued over a long time period with potential changes in histopathological characterisation, treatment and follow-up. However, all tissue specimens were re-evaluated by a dedicated pancreas pathologist to confirm diagnosis and uniformity of histopathological evaluation. Chemotherapy regimens varied during the study period, but most patients received gemcitabine-based chemotherapy.

5. Conclusion

We have demonstrated the feasibility of MS-based proteomic profiling of patient derived tissue specimens for biomarker development in pancreatic cancer. The proteomic strategy identified BASP1 as a promising biomarker candidate. The independent prognostic importance of BASP1 was validated in a large series of pancreatic cancer patients, together with its interaction partner WT1. We believe that our findings support that BASP1 and its putative interaction partner WT1 can be used as biomarkers for predicting the outcomes of pancreatic cancer patients, why further studies examining the function of BASP1 are warranted.

Table 4
Univariable and multivariable Cox regression analyses of overall survival.

Variables	BASP1 cohort				WT1 cohort			
	Univariable HR (95% CI)	p value	Multivariable HR (95% CI)	p value	Univariable HR (95% CI)	p value	Multivariable HR (95% CI)	p value
Age (>65 years)	1.028 (0.682–1.547)	0.896			1.035 (0.685–1.565)	0.871		
Female gender	0.785 (0.530–1.162)	0.227			0.769 (0.519–1.139)	0.19		
BMI (>25 kg/m^2)	1.346 (0.897–2.020)	0.151			1.273 (0.846–1.914)	0.247		
Smoking history	1.633 (1.100–2.422)	0.015*	1.626 (1.071–2.471)	0.023*	1.664 (1.116–2.480)	0.012*	1.727 (1.128–2.644)	0.012*
Diabetes mellitus	0.851 (0.526–1.377)	0.511			0.854 (0.522–1.395)	0.527		
Symptoms at diagnosis	0.353 (0.128–0.973)	0.044*	0.404 (0.143–1.142)	0.087	0.361 (0.131–0.995)	0.049*	0.459 (0.163–1.294)	0.141
Tumour location (head)	0.667 (0.394–1.128)	0.131			0.657 (0.389–1.110)	0.117		
Tumour size (>2 cm)	1.107 (0.663–1.849)	0.697			1.082 (0.654–1.788)	0.759		
T-stage (≥T2)	1.173 (0.684–2.011)	0.562			1.139 (0.673–1.927)	0.629		
N-stage (≥N1)	1.472 (0.916–2.366)	0.11			1.454 (0.911–2.321)	0.117		
AJCC 8th edition (≥IIA)	1.443 (0.855–2.436)	0.17			1.421 (0.852–2.370)	0.179		
Histological grade (≥3)	1.536 (1.018–2.317)	0.041*	1.647 (1.077–2.518)	0.021*	1.564 (1.033–2.368)	0.035*	1.696 (1.105–2.601)	0.016*
Resection margin (≥R1)	1.479 (0.986–2.219)	0.058			1.506 (0.996–2.277)	0.052		
Adjuvant chemotherapy	0.713 (0.431–1.180)	0.188			0.712 (0.435–1.166)	0.177		
BASP1 (positive)	0.523 (0.297–0.921)	0.025*	0.468 (0.257–0.852)	0.013*				
WT1 (high)					1.561 (1.047–2.328)	0.029*	1.636 (1.083–2.473)	0.019*

Abbreviations: AJCC, American joint committee on cancer; BMI, body mass index; CI, confidence interval; HR, Hazard ratio; N-stage, nodal stage; T-stage, tumour stage. Variables with p < .05 are marked with asterisk (*), variables with p < .05 in univariable analysis were included in multivariable analysis.
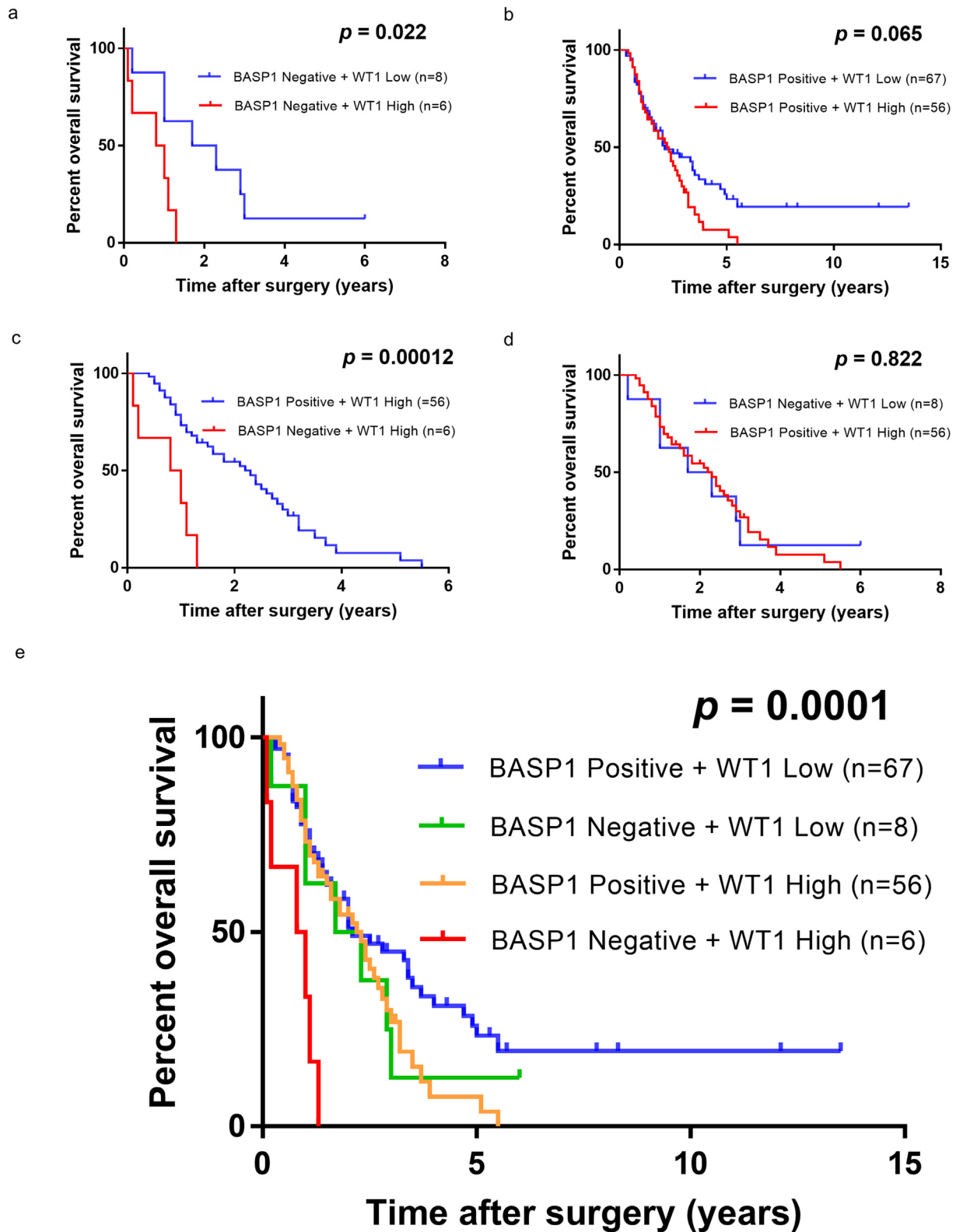
Fig. 5. Subgroup analyses of BASP1/WT1 expression and their correlation with overall survival (OS) in pancreatic cancer patients. **a.** When BASP1 was negative, high WT1 expression was associated with significantly reduced OS. **b.** When BASP1 was positive, high WT1 had no significant impact on OS. **c.** When WT1 was highly expressed, positive BASP1 correlated with significantly prolonged OS. **d.** When WT1 was highly expressed and BASP1 was positive, OS was similar to that of WT1 low expression level patients. **e.** Patients with positive BASP1 and low WT1 expression showed the best prognosis, whereas patients with negative BASP1 and high WT1 expression presented the poorest OS.

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Declaration of interests

RA, GMV and DA have filed a patent related to the findings presented in this manuscript. They are board members of Reccan Diagnostics. The other authors declare that they have no conflicts of interests.

Author contributions

QZ, TK, and DA conceived the original idea and designed the study with MZ, FL, GMV, and RA. QZ, DH, MB, TK, AS, KSH, and DA collected the data for the study, which were analysed by QZ, KP, and IPP. The data interpretation and manuscript drafting were performed by QZ and DA. The manuscript was revised by MB, KP, MZ, FL, and RA. All authors reviewed the manuscript and gave the final approval for submission.

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