Database-augmented Mass Spectrometry Analysis of Exosomes Identifies Claudin 3 as a Putative Prostate Cancer Biomarker*

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In prostate cancer and other malignancies sensitive and robust biomarkers are lacking or have relevant limitations. Prostate specific antigen (PSA), the only biomarker widely used in prostate cancer, is suffering from low specificity. Exosomes offer new perspectives in the discovery of blood-based biomarkers. Here we present a proof-of-principle study for a proteomics-based identification pipeline, implementing existing data sources, to exemplarily identify exosome-based biomarker candidates in prostate cancer.

Exosomes from malignant PC3 and benign PNT1A cells and from FBS-containing medium were isolated using sequential ultracentrifugation. Exosome and control samples were analyzed on an LTQ-Orbitrap XL mass spectrometer. Proteomic data is available via ProteomExchange with identifier PXD003651. We developed a scoring scheme to rank 64 proteins exclusively found in PC3 exosomes, integrating data from four public databases and published mass spectrometry data sets. Among the top candidates, we focused on the tight junction protein claudin 3. Retests under serum-free conditions using immunoblotting and immunogold labeling confirmed the presence of claudin 3 on PC3 exosomes. Claudin 3 levels were determined in the blood plasma of patients with localized (n = 58; 42 with Gleason score 6–7, 16 with Gleason score ≥8) and metastatic prostate cancer (n = 11) compared with patients with benign prostatic hyperplasia (n = 15) and healthy individuals (n = 15) using ELISA, without prior laborious exosome isolation. ANOVA showed different CLDN3 plasma levels in these groups (p = 0.004). CLDN3 levels were higher in patients with Gleason ≥8 tumors compared with patients with benign prostatic hyperplasia (p = 0.012) and Gleason 6–7 tumors (p = 0.029). In patients with localized tumors CLDN3 levels predicted a Gleason score ≥8 (AUC = 0.705; p = 0.016) and did not correlate with serum PSA.

By using the described workflow claudin 3 was identified and validated as a potential blood-based biomarker in prostate cancer. Furthermore this workflow could serve as a template to be used in other cancer entities. Molecular & Cellular Proteomics 16: 10.1074/mcp.M117.068577, 998–1008, 2017.

There is an urgent need for minimally invasively obtained biomarkers for multiple cancer entities. For each cancer entity, a biomarker must meet specific requirements. Prostate cancer (PCa) for instance is the most common cancer among men in industrialized countries (1) with most cases diagnosed in early stages. Many patients have insignificant carcinoma. These patients benefit from active surveillance, sparing them from overtreatment. On the other hand, in patients suffering from disease recurrence or incurable metastatic PCa, androgen deprivation therapy (ADT) typically fails after several years of therapy. Although an increasing number of therapeutic strategies has become available during the last decade (2, 3), more than 250,000 men die from aggressive late stage PCa per year worldwide (1). Therefore, an early discrimination between patients either suitable for surveillance strategies or in need of therapy is crucial to prevent overtreatment and to

* The abbreviations used are: PCa, prostate cancer; ADT, androgen deprivation therapy; BPH, benign prostatic hyperplasia; CLDN3, claudin 3; CM, conditioned medium; CPE, clostridium perfringens enterotoxin; EV, extracellular vesicle; NTA, nanoparticle tracking analysis; PSA, prostate specific antigen; SPECT, single photon emission computed tomography; TEM, transmission electron microscopy.
reduce PCa mortality. The value of prostate specific antigen (PSA), which is the most widely used screening marker, is still controversial (4) because it lacks specificity and only gives limited information about disease aggressiveness (5). More exact primary tumor histology needs harmful (6) and expensive transrectal multicore biopsies and still has a frequent rate of undergrading (7). Therefore markers, easily obtainable from blood or urine, that enable a precise risk prediction of PCa, are needed. Main efforts in PCa biomarker research have either focused on frequent genetic alterations like the TMPRSS2:ERG fusion gene, which has shown to be associated with worse outcome (8), or distinct biomarkers from blood (e.g. circulating tumor cells) or urine (like PCA3) (5, 9). Yet, none of these markers has made it into clinical routine.

Exosomes are small (40–100 nm in diameter) extracellular vesicles (EVs) which are shed by nearly all mammalian cells. They contain a varying mixture of different proteins and nucleic acid species (e.g. mRNA and miRNA) encapsulated in a lipid bilayer carrying transmembrane molecules which gives exosomes antigenic potency (10, 11). Recent findings suggest exosomes as important mediators in different physiological and pathophysiological processes (12). Especially long-range signaling via exosomes through the blood stream is associated with metastatic niche formation (13) and systemic inflammation (14). Because of their cancer-related function, exosomes themselves, or their components, could serve as valuable markers in different types of cancer. Recently, exosomal glypican 1 was reported as very promising early detection marker for pancreatic adenocarcinoma after identification of undergrading (7). Therefore markers, easily obtainable from blood or urine, that enable a precise risk prediction of PCa, are needed. Main efforts in PCa biomarker research have either focused on frequent genetic alterations like the TMPRSS2:ERG fusion gene, which has shown to be associated with worse outcome (8), or distinct biomarkers from blood (e.g. circulating tumor cells) or urine (like PCA3) (5, 9). Yet, none of these markers has made it into clinical routine.

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Until now, there is no established gold standard for the profiling and characterization of exosomal protein biomarkers in cancer. To integrate existing knowledge about potential protein biomarkers for PCa we combined an in vitro mass spectrometry (MS) approach with a subsequent extensive database search regarding biomarker-specific properties. The top candidate protein was validated immunologically in vitro and in clinical samples, using a facilitated approach without need for laborious exosome isolation.

**EXPERIMENTAL PROCEDURES**

**Experimental Design and Statistical Rationale**—Supernatant for exosome isolation for MS and quality control (immunoblotting, NTA, TEM) from PC3 and PNT1A cells and control medium was generated in two biological replicates each. Quality control experiments (Western blotting, NTA, TEM) under serum free conditions were performed in three biological replicates. The scoring system for candidate generation implemented user weighted factors for assumed specific biomarker features (Fig. 1). ELISA testing of patient samples was conducted in two technical replicates of each sample. ANOVA, post hoc Turkey test and Student’s t test were used to determine statistical significance in data with normal distribution.

**Cell Culture**—Human metastatic PC3 and benign PNT1A cells were expanded in a predefined FBS-containing modified DMEM medium (Quantum 286, GE Healthcare, Chalfont St. Giles, UK) under standard culture conditions. At a confluence of 70%, medium was discarded and cells were washed three times with sterile PBS (Life Technologies, Carlsbad, CA). Unlike other studies using serum-free or depleted medium, cells were then incubated for further 48 h with FBS-containing growth medium to generate conditioned medium (CM) for exosome isolation. This approach was chosen not to impair cellular growth conditions. Cells were detached with trypsin, harvested, washed with sterile PBS twice and stored as pellets at −80 °C. For retests PC3 and PNT1A cells were cultured and expanded under the same condition, but DMEM (Life Technologies) with no additives was used to generate CM for isolation of exosomes.

**Isolation of Exosomes**—PC3 and PNT1A exosomes both from FBS-containing and FBS-free CM were isolated as described by Théry et al. (21) with minor modifications. Additionally, two samples of FBS-containing medium, not in contact with prostate cells were subjected to the same isolation procedure as controls. In brief medium was centrifuged at 300 × g for 10 min, followed by 2000 × g for 10 min and 12,000 × g for 40 min. Pellets from each centrifugation step were discarded. Supernatant was then subjected to ultracentrifugation at 100,000 × g for 120 min. The resulting pellet was washed in sterile-filtered PBS and again centrifuged for 120 min at 100,000 × g. The final pellet was eluted in 50 µl of sterile-filtered PBS.

**Transmission Electron Microscopy (TEM)**—Five microliters of exosomes were placed on 100 Mesh formvar-coated copper grids (Plano, Wetzlar, Germany) for 5 min. Grids were then briefly rinsed three times with distilled water and negatively stained with 3% aqueous uranyl acetate for 3 min. Grids were subsequently air-dried and investigated with an EM900 or EM10 transmission electron microscope (Zeiss,
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Jena, Germany) equipped with a CCD camera at 30 k, 50 k, and 85 k magnification. For immunogold electron microscopy, the exosomes on grids were blocked for 5 min with 50 mM glycine/PBS and for 10 min with 50 mM glycine/PBS/0.8% BSA/0.1% fish skin gelatin, incubated for 20 min in the primary antibody solution (murine anti-CLDN3 (R&D Systems, Wiesbaden, Germany) 1:10 to 1:100, rinsed 2 × 5 min with PBS, incubated for 30 min in bridging antibody rabbit-anti-mouse (Dako, Agilent Pathology Solutions, Santa Clara, CA) 1:150 in blocking solution, incubated in Protein-A-Gold 10 nm (University Utrecht, Netherlands) 1:50 in blocking solution, rinsed 2 × 5 min in PBS, fixed in 1% glutaraldehyde in PBS, rinsed 5 × 5 min in PBS and 7 × 2 min in dH2O, contrasted on ice with 1.8% aqueous uranylacetate/0.8% methylcellulose, looped out and air dried for 5 min. Samples labeled with anti-CD63 (Active Bioscience, Hamburg, Germany, 1:50) were used as positive control, in negative controls the primary antibody was omitted.

**Nanoparticle Tracking Analysis (NTA)**—Two microliters of exosomes were diluted in sterile-filtered PBS and visualized using the LM10 NTA device (Malvern Instruments, Malvern, UK). Each sample was measured 6 times for 45 s (Screen Gain 1.0, camera level 15) with at least 200 valid tracks per video to obtain particle concentration and size distribution.

**Immunoblotting**—The protein content of exosomes and cell lysates was determined using BCA assay (Thermo Fisher Scientific, Waltham, MA). For immunoblotting sample preparation was performed both with reducing and nonreducing Laemmli buffer. Two micrograms of proteins from EVs or cell lysate were loaded on a 4–12% SDS gel for electrophoresis, followed by transfer to a PVDF membrane. Primary antibodies used were polyclonal rabbit anti-Calnexin, mouse monoclonal anti-HSC70 (clone W27), goat polyclonal anti-ALIX (all from Santa Cruz, CA), mouse monoclonal anti-CD9 (Immunotools, Friesoythe, Germany), rabbit polyclonal anti-CLDN3 and mouse monoclonal anti-beta Actin (both from Abcam, Cambridge, UK). HRP-conjugated goat anti-mouse, and goat anti-rabbit (both Jackson Immunoresearch, West Grove, PA) or donkey anti-goat (Santa Cruz) were used as secondary antibodies.

**Sample Preparation, Mass Spectrometry, and Interpretation of Mass Spectra**—Ten micrograms of protein from PC3 and PNT1A exosomes generated from FBS-containing CM or untreated FBS-containing medium were loaded on a 4–12% SDS gel. After 1D gel electrophoresis and Coomassie staining the stained area of each sample (2 cm) was cut into 3 individual pieces. In-gel digestion, peptide extraction and MS analysis were performed as described by Aretz et al. (22), with some modifications. In detail, gel pieces were chopped into small gel plugs and incubated with 150 μl water for 5 min at 37 °C. Proteins were reduced with 150 μl 10 mM DTT in 40 mM NH4HCO3 for 1 h at 56 °C, alkylated with 150 μl 55 mM iodoacetamide in 40 mM NH4HCO3 for 30 min at 25 °C in the dark, followed by three washing steps with 150 μl of water and water/acetonitrile at 37 °C.

Gel pieces were dehydrated with 150 μl neat acetonitrile for 1 min at room temperature, dried for 15 min and subsequently rehydrated with sequencing grade porcine trypsin (Promega, Madison, WI). After overnight digestion at 37 °C the supernatant was collected while gel pieces were subjected to four further extraction steps (acetonitrile/0.1% TFA 50:50 (v/v)). The combined solutions were evaporated to dryness and redissolved in 0.1% TFA/2.5% hexafluoroisopropanol.

Tryptic peptides mixtures were separated using a nanoAcquity UPLC system (Waters, Milford, MA). Peptides were trapped on a nanoAcquity C18 column (180 μm × 20 mm, particle size 5 μm) (Waters). The liquid chromatography separation was performed on a C18 column (BEH 130 C18 100 μm × 100 mm, particle size 1.7 μm, Waters) with a flow rate of 400 nl/min. The chromatography was carried out using a 3 h gradient of solvent A (98.9% water, 1% acetonitrile, 0.1% formic acid) and solvent B (99.5% acetonitrile and 0.1% μl formic acid). The nanoUPLC system was connected to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in the sensitive mode with the following parameters: capillary voltage 2400 V; capillary temperature 200 °C, normalized collision energy 35 V. Activation time 30000 ms. Data were acquired by scan cycles of one FTMS (resolution: 60000 at m/z 400; range: 370 to 2000 m/z) in parallel with six MS/MS scans in the ion trap of the most abundant precursor ions.

The peak list.mgf-files generated by Xcalibur software (version 2.1, Thermo Fisher Scientific) were used for database searches with the Mascot search engine (version 2.4.0, Matrix Science, Boston, MA) against the SwissProt database (version 2015_04, species: human, 20279 sequences). Trypsin was used as digestion enzyme (cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine) and the maximum of missed cleavages was set at 1. Fixed modification was set to carboxymethyl on cysteine residues and variable modifications were deamination of glutamine and asparagine and oxidation of methionine. Mass tolerance for precursor ions was 5 ppm and mass tolerance for fragments was 0.4 Da. No known contaminants were excluded during peptide matching. Using standard scoring, ion score cut-off was set at 20, the maximum significance threshold was set at 0.01 and the maximum number of hits was set at default. False discovery rate for matches above homology and identity threshold ranged between 1.49 and 4.45% per data set. Proteins identified were considered significant if at least two unique peptides had an individual ion score exceeding the Mascot identity threshold and a cumulative Mascot score >60 was reached. The mass spectrometry data have been deposited to the ProteomeXchange Consortium (23) after conversion into xml-files using PRIDE Converter 2 (24) with the data set identifier PXD003651 and 10.6019/PXD003651 (http://proteomcentral.proteomexchange.org/cgi/GetDataset?ID=PXD003651). A complete list of all identified peptides is given in supplemental Table S1.

In Silico Analyses and Scoring—For better compatibility, protein accessions from all data sets were transcribed to official gene symbols. Only proteins identified in both replicates of PC3 or PNT1A exosomes were counted as valid. Proteins identified in at least one sample of untreated medium were defined as background noise and subtracted from exosomal protein data sets. The remaining exosomal protein data sets of PC3 and PNT1A were compared and proteins only present in the PC3 data set were subjected to further analysis as potential biomarkers.

A custom-made scoring system incorporated information from four publicly available databases (Table I) and studies reporting proteomic mass spectrometry data sets of PCa tissue, identified from PubMed (date of search: December 12th 2016). CBioPortal is part of The Cancer Genome Atlas Project and harbors detailed clinical, mutational and transcriptional data from around hundred studies in different cancer entities (25). In two data sets (data set 1, Broad/Cornell, Nature Genetics 2012 (26), microarray, n = 31 and data set 2, TCGA provisional, RNA sequencing, n = 236) only data from primary tumors were available. In one data set (data set 3, MSKCC, Cancer Cell 2010 (27), microarray) both expression data from primary tumors (n = 131) and metastases (n = 19) were available. In data set 1 and 2 candidate gene expression was normalized to the expression in diploid tumors. In data set 3, gene expression in tumor samples was compared with benign controls. Expression data from primary tumors were pooled and resulted in a score ranging from −3 (largely underexpressed) to +3 (highly overexpressed). The same was done for metastasis data and both scores were added.

From the literature eight studies reporting proteomic mass spectrometry data of PCa tissue, either to identify proteins overexpressed in PCa compared with benign tissue or to identify proteins being associated with metastatic or high risk PCa, could be identified (28–

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TABLE I
Four publicly available databases were used for retrieval of biomarker relevant data of 64 proteins exclusively identified in PC3 exosomes and their corresponding genes

| Database               | Information                                | Contact address                   |
|------------------------|--------------------------------------------|-----------------------------------|
| CBioPortal             | Expression data from primary tumors and metastases | http://www.cbiportal.org/public-portal/ (date of access: January 26th 2017) |
| Peptide Atlas          | Expression in blood plasma                 | http://www.peptideatlas.org/ (date of access: February 8th 2017)            |
| PROTTER                | Protein conformation, association to membrane | http://wlab.ethz.ch/protter/ (date of access: February 7th 2017)             |
| Vesiclepedia           | Expression in exosomes                     | www.microvesicles.org (date of access: February 6th 2017)                    |

35). Protein candidates were compared with the protein data in each study and received a score of +1 per study and item (PCa versus benign, high risk versus low risk localized PCa, metastatic versus localized PCa) upon a positive match.

PeptideAtlas is a compendium of peptides identified in different organisms. As one of its subsets, it contains 91 data sets derived from blood plasma samples of healthy individuals, which can be used as a reference for the human blood plasma proteome (36). Proteins not described as normal plasma proteins received a score of +2, proteins only lowly abundant in blood plasma scored +1 (as measured by the frequency of identified peptides per 100,000 peptides). Proteins highly abundant in blood plasma did not score.

Those proteins having transmembrane or extracellular domains according to the PROTTER database (37), an open-source tool for visualization of protein structures, localizations, modifications, and potential interaction sites, received a score +1.

Using Vesiclepedia (38), a manually curated platform currently comprising more than 500 different studies of EVs with over 92,000 protein entries, proteins found on EVs of cancer cells or in plasma or urine samples of cancer patients scored positive (1 to 9 reports = +1, ≥10 reports = +2). The addition of the individual scores from CBioPortal, protein studies, PROTTER, PeptideAtlas, and Vesiclepedia resulted in a final score. For top candidates, a manual search regarding reports in PCa and exosomes using MEDLINE was conducted.

Patient Samples—Blood samples of patients with localized (n = 58, mean age 65.7 years (50–78 years), average PSA 9.96 ng/ml; 42 with a sum Gleason score of 6–7 and 16 with a sum Gleason score of 8–9, average PSA 8.11 ng/ml and 14.85 ng/ml, respectively) or metastatic PCa (n = 11, mean age 65.5 years (57–73 years)) or histologically proven benign prostate hyperplasia (BPH; n = 15, mean age 68.4 years (59–87 years)) without any known cancer in the past or present medical history were collected in the context of a local biobank study, following a standardized protocol using 9 ml K<sub>2</sub>EDTA-tubes (Sarstedt). After collection, tubes were placed for interim storage at 80 °C. The study is in accord with the institutional review board (ethics approvals 2012–358, 2013–359, 2014–360, 2015–361, 2016–362, 2017–363, 2018–364, 2019–365, 2020–366). Following an approach, exosomes from malignant PC3 and benign PNT1A cells were compared. Using sequential centrifugation and ultracentrifugation we obtained the exosomal fractions from the supernatant of both cell lines. Immunoblotting revealed a typical signature of proteins associated with EVs (ALIX<sup>+</sup>, TSG101<sup>+</sup>, HSC70<sup>+</sup>, Calnexin<sup>+</sup>); Fig. 2A). Visualized with TEM (Fig. 2B), samples contained cup-shaped particles in a size range typical for exosomes (39). Nanoparticle tracking analysis showed a homogenous size distribution around 110 nm of mean diameter (Fig. 2C).

RESULTS

Isolation of Exosomes from Prostate Cells—Following an in vitro approach, exosomes from malignant PC3 and benign PNT1A cells were compared. Using sequential centrifugation and ultracentrifugation we obtained the exosomal fractions from the supernatant of both cell lines. Immunoblotting revealed a typical signature of proteins associated with EVs (ALIX<sup>+</sup>, TSG101<sup>+</sup>, HSC70<sup>+</sup>, Calnexin<sup>+</sup>); Fig. 2A). Visualized with TEM (Fig. 2B), samples contained cup-shaped particles in a size range typical for exosomes (39). Nanoparticle tracking analysis showed a homogenous size distribution around 110 nm of mean diameter (Fig. 2C).

Identification of Differentially Expressed Exosomal Proteins—The isolated exosomes from PC3 and PNT1A supernatant and medium controls were subjected to MS in duplicates after 1D gel electrophoresis, trypsic digestion and peptide extraction to gain insight into their protein composition. Proteomic profiling identified 641 and 666 proteins in PC3 exosome samples and 809 and 843 in PNT1A exosome samples. Four hundred eighty-eight proteins were present in both PC3 replicates and 598 in both PNT1A replicates. Me-
dium controls contained between 441 and 462 proteins, with 382 proteins in both data sets. A full list of all identified proteins in each data set is given in supplemental Table S3. Following a restrictive protocol all 521 proteins identified in at least one of the control samples served as background data set, which was subtracted from the respective data sets of all proteins found in both replicates of PNT1A and PC3 exosomes. After subtraction of this background, 188 proteins remained in the PC3 data set and 227 in the PNT1A data set (supplemental Table S4). Sixty-four proteins were exclusively found in PC3 exosomes. The complete workflow to identify proteins exclusively detected in PC3 exosomes is described in Fig. 3.

**Scoring for Biomarker Features**—Sixty-four proteins found exclusively in PC3 exosomes were subjected to an extensive search in publicly available data sources. Using CBioPortal (25) RNA expression data from three different cohorts were analyzed. In data sets 1 (n = 31) and 2 (n = 236) only data from primary tumors were available. Data set 3 contained both expression data from primary tumors (n = 131) and metastases (n = 19). The average overexpression in primary tumors from all three data sets resulted in a score ranging from −3 to +3. The same was done for metastasis data.

The identified proteins were also compared with proteins that were reported to be associated with PCa tissue compared with healthy tissue or to be associated with metastatic or high risk PCa in mass spectrometry studies. Fatty acid-binding protein 5 (FABP5), inorganic pyrophosphatase (PPA1), junction plakoglobin (JUP), and tetraspanin 6 (TPSAN6) were reported in two data sets (+2). Eleven proteins were detected in one data set (+1).

Subsequently proteins were manually looked up in the PeptideAtlas (36) of normal human plasma proteins. Candidates not described as normal plasma proteins received a score of +2 (n = 44), proteins only slightly abundant in blood plasma (n = 15) scored +1. Proteins highly abundant in blood plasma did not score (n = 5). Transmembrane proteins and proteins with an extracellular domain according to the PROTTER database (37) received a score +1 (n = 38). Twenty-six proteins did not score. According to Vesiclepedia (38), most proteins had already been reported in one or several studies of EVs from cancer cells or on EVs from plasma or urine samples of cancer patients (0 reports 0, n = 3; 1 to 9 reports 1, n = 24; ≥ 10 reports +2, n = 37).

Addition of all scores allowed a ranking of the most potent biomarker candidates. All top candidates with a score of 8 or higher (n = 11) are displayed in Table II. Among these claudin 3 (CLDN3), scavenger receptor class B member 1 (SCARB1), gastrin-releasing peptide receptor (GRPR), MAGUK p55 subfamily member 6 (MPP6), FABP5, PPA1, and protein scribble homolog (SCRIB) achieved the highest score of +9. Supplemental Table S5 contains the complete list of all 64 proteins exclusively detected in PC3 exosomes including their results in each step of the scoring procedure.

In Vitro Validation of CLDN3 in PC3 Exosomes—Because of its high score and promising reports from the literature, suggesting claudins as potential exosome-based biomarker in
ovarian cancer (40), CLDN3 was chosen for further in vitro testing. CLDN3 is an epithelial tight junction protein mainly responsible to maintain obliteration of extracellular space in epithelial and endothelial cells (41). Until now, CLDN3 has not been described in context of exosomes in PCa and its function on exosomes is unknown.

To test whether CLDN3 was truly present on PCa exosomes, exosomes were generated from PC3 and PNT1A supernatants under serum free conditions. NTA (Fig. 4A) detected particles with a homogenous size distribution curve with its mean around 120 nm. In TEM (Fig. 4B) again cup-shaped vesicles in the same size range could be seen, show-

**Table II**

| Nr. | Symbol   | Protein                                      | Score |
|-----|----------|----------------------------------------------|-------|
| 1   | CLDN3    | Claudin-3                                    | 9     |
| 2   | SCARB1   | Scavenger receptor class B member 1           | 9     |
| 3   | GRPR     | Gastrin-releasing peptide receptor            | 9     |
| 4   | MPP6     | MAGUK p55 subfamily member 6                 | 9     |
| 5   | FABP5    | Fatty acid-binding protein, epidermal         | 9     |
| 6   | PPA1     | Inorganic pyrophosphatase                     | 9     |
| 7   | SCRIB    | Protein scribble homolog                      | 9     |
| 8   | ARRDCC1  | Arrestin domain-containing protein 1          | 8     |
| 9   | CLDN4    | Claudin-4                                    | 8     |
| 10  | ALCAM    | CD166 antigen                                 | 8     |
| 11  | SLC19A1  | Folate transporter 1                          | 8     |

Fig. 3. **Data set comparison.** A, Venn diagrams show a high overlap of identified proteins between replicates of PC3 and PNT1A exosomes. B, Comparison with medium control resulted in a reduction of data sets with 188 proteins remaining in the PC3 data set and 227 in the PNT1A data set. C, 64 proteins were exclusively found in PC3 exosomes, 103 in PNT1A exosomes.
ing a positive signal for the exosomal marker CD63 on the surface of the vesicles. Immunoblotting showed a typical signature of exosomal and cellular markers (CD9*, Calnexin*). CLDN3 was only present in PC3 cells and exosomes with a stronger signal in the exosomal fraction (Fig. 4C). Specific ultrastructural immune labeling with gold particles confirmed the presence of CLDN3 as a surface protein of PC3 exosomes (Fig. 4D).

Validation in Plasma Samples—To see whether CLDN3 is also detectable in clinical samples, we used an ELISA to determine the protein levels of circulating CLDN3 from plasma samples of patients with PCa or BPH and from healthy controls.

To be able to accurately detect CLDN3 with an antibody targeting the intravesicular C-terminal domain, thawed plasma samples were lysed prior to analysis. The lysis efficiency was validated by NTA of lysed and non-lysed PC3 exosomes and indicated a strong reduction of detectable particles (~61.5% for PC3 exosomes, data not shown).

All samples showed detectable CLDN3 plasma levels. The average CLDN3 plasma levels were: 4.06 ng/ml in healthy controls, 3.48 ng/ml in BPH patients, 3.84 ng/ml in patients with Gleason 6–7 tumors, 4.87 ng/ml in patients with Gleason 8–9 tumors and 4.77 ng/ml in metastatic tumor patients. ANOVA showed significantly different CLDN3 levels when comparing all groups ($p = 0.004$; Fig. 5A). Direct inter-group comparison proved a significantly higher expression in patients with Gleason score 8–9 tumors, compared with patients with BPH ($p = 0.012$) and Gleason score 6–7 tumors ($p = 0.029$). In metastatic PCa patients CLDN3 levels were not significantly elevated compared with BPH controls ($p = 0.053$) and patients with Gleason score 6–7 tumors ($p = 0.148$). There were no significant differences between healthy controls and the other groups.

Focusing on patients with localized tumors, no difference in CLDN3 levels were found for T-stage (T2 versus T3 or higher), N- or R-status.

Spearman correlation in localized tumors showed no correlation between CLDN3 levels and serum PSA levels (Spearman’s $\rho = 0.077$; $p = 0.568$). ROC analysis showed an AUC of 0.705 for CLDN3 to correctly predict a Gleason score of 8 or higher in 58 patients with localized tumors ($p = 0.016$; Fig. 5B).

DISCUSSION

The great clinical need for minimally invasive biomarkers is currently driving biomarker research. Because of their known cancer-related functions, exosomes and the proteins contained inside them bear great hope for cancer biomarker
research. Yet, a gold standard for profiling and characterization of exosomal protein biomarkers is lacking.

Here, we describe a novel workflow, completely covering the analysis of CLDN3 as exemplary exosome-based biomarker for PCa from \textit{in vitro} profiling of cancer exosomes over \textit{in silico} identification and \textit{in vitro} retesting to clinical validation. Using MS of the exosome fractions of cell culture supernatant to profile PCa exosomes, we could identify a higher number of proteins compared with other studies conducting exosomal proteomic profiling (19, 42, 43). Likely, this is because of our approach for exosome generation. Instead of FBS-free or exosome-depleted medium we used a complete growth medium. This approach has the advantage, that the cellular growth conditions are not impaired during the experiment, but comes along with the disadvantage of contaminating proteins originating from bovine exosomes present in the FBS. This bias was circumvented by analyzing medium without contact to cells as negative control in our MS experiment, allowing us to exclude medium-derived proteins from our exosome data sets. To validate our findings, subsequent analysis of exosomes generated under FBS-free conditions specifically confirmed the presence of our biomarker candidate CLDN3 on PC3 exosomes. For the purification of exosomes, we had chosen ultracentrifugation, based on a well-established protocol (21). Though other \textit{e.g.} filter- or immune-based protocols enable faster exosome recovery, ultracentrifugation is the far best described method, providing high sample purity and therefore still represents the gold standard for \textit{in vitro} exosome research (39). The results shown in our study are in accordance with current requirements formulated by the international society of extracellular vesicles (ISEV) for quality control of exosomes (39).

To date many studies have used MS for the proteomic profiling of exosomes for biomarker discovery, but for the downstream analysis of the generated data no standard procedure exists (44). Most reports simply focus on proteins already described as biomarkers. In contrast, we followed a structured, custom-made, database-augmented approach, utilizing existing knowledge to gain further insight into the biomarker capabilities of the identified proteins. Therefore, our approach on the one hand is a step toward a more structured screening of potential exosome-based biomarkers, but on the other hand lacks an accepted standard to be compared against.

The four used databases plus the identified MS data sets provide up-to-date information about gene and protein expression in cancer tissue, about protein abundance in blood plasma and on exosomes and about protein structure, all being critical parameters for a circulation-based biomarker. All data is publicly available and was used without need for further bioinformatic tools.

According to the screened data, our candidate CLDN3 is frequently overexpressed in localized and metastatic PCa, is annotated to the plasma membrane and exosomes, is of transmembranous structure with an extracellular domain, was repeatedly reported to be a part of EVs, and is typically not found in normal plasma. Multiple other proteins also ranked high in our scoring and seem to be valuable biomarker candidates to be followed up in further biomarker and functional studies.

Functionally, claudins are part of the multiprotein tight junction complexes and are mainly expressed in epithelial and endothelial cells (41). Both on the transcriptomic and the proteomic level, several members of the claudin family have been described to be differentially expressed in epithelial tumors. Hewitt \textit{et al.} reported CLDN3, CLDN4, and CLDN7 to be overexpressed in tumors originating from the pancreas, stomach, colon, bladder, ovary, breast, uterus, and prostate (45). Another study showed CLDN3 to be overexpressed in both primary and metastatic PCa tissue (46). Overexpression of CLDN3 was also found to be associated with perineural invasion of primary PCa (47).

Our own patient data show a significantly higher expression of CLDN3 in patients with Gleason 8 and higher tumors compared with patients with BPH or Gleason score 6–7 tumors, indicating an increase of CLDN3 plasma level with a higher
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grading of localized tumors. Correspondingly Sheehan et al. found a prognostic correlation for an elevated CLDN3 and CLDN4 tissue expression in localized PCa (48).

CLDN3 plasma levels in patients with metastatic disease were not significantly higher compared with BPH controls and patients with Gleason score 6–7 tumors. This fits to an integrated expression microarray study reported by Amaro et al. in which CLDN3 was found to be overexpressed mainly in primary tumor tissue, whereas expression in metastases was similar to those in primary tumors (49). A lower expression in metastatic compared with primary tissue might be associated to epithelial to mesenchymal transition (EMT).

CLDN3 has not been described as circulating biomarker, neither in association with exosomes nor as free circulating protein. Exosomal CLDN4 was identified as potential circulating biomarker in ovarian carcinoma (sensitivity 51%, specificity 98%) and initial results suggest a potential additional information in combination with established markers like CA125 (40). After immunogold labeling the same group could show CLDN4 to be located on the surface of exosomes of ovarian cancer patients. Using the same technique, we could demonstrate CLDN3 to be present on the membrane of PC3 exosomes.

In hepatocellular carcinoma CLDN3 is suggested to suppress metastasis formation by inactivating Wnt/β-catenin mediated EMT processes (50). In ovarian cancer xenografts, knockdown of CLDN3 and CLDN4 resulted in increased tumor growth and a decreased death rate of tumor cells (51). Controversially, another group found suppressed growth and metastasis formation of ovarian cancer xenografts upon gene silencing of CLDN3 (52).

Claudins have also raised interest as target structures for novel imaging approaches. An In(125)-labeled anti-CLDN4 antibody showed promising results for detection of pancreatic cancer in a xenograft mouse model using SPECT (53). Because several Claudins, especially CLDN3 and CLDN4, are coreceptors for the pore-forming Clostridium perfringens enterotoxin (CPE) (54), they also seem to bear some interesting aspects for cancer therapy. In vitro CPE-expression vector transfection caused lethal effects to tumor cell lines expressing either CLDN3 or CLDN4, whereas cell lines negative for these surface proteins did not show impaired viability (55). Romanov et al. reported a protoxin of Clostridium perfringens which is cleaved by the serine protease PSA and therefore leads to a selective lysis of PCa cells (56). Maeda et al. could show a reduction in growth of PCa xenograft tumors in mice after peritumoral injection of CPE (57). CLDN4 could also successfully be used as target structure for an In(111)-labeled c-terminal fragment of CPE as tracer for single photon emission computed tomography (SPECT) in a breast cancer mouse model (58). This points to a potential use of claudins as target structures for both diagnostic imaging and therapeutic strategies.

The present study is limited by its preliminary character and by the small number of patients in the clinical validation cohort. Especially in the small group of patients with metastatic PCa castration, current treatment regime and PSA levels varied a lot. Nevertheless, CLDN3 plasma levels were significantly higher in patients with high Gleason score, pointing to a potential predictive value of this marker. Yet, without appropriate follow-up data this cannot be validated.

Taken together CLDN3 was identified as a new circulating biomarker candidate in PCa by mass spectrometry profiling of cell culture exosomes, followed by in silico scoring in up to date high quality databases. Clinical validation was possible directly from human plasma using ELISA, a quantitative standard laboratory method, without the need for laborious isolation of exosomes.

Besides its potential relevance in PCa, the workflow presented here could function as a template for similar exosome-based biomarker studies in other cancer entities, implementing existing knowledge on features with relevance for biomarker discovery.

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DATA AVAILABILITY

The mass spectrometry data have been deposited to the ProteomeXchange Consortium (data set identifier PXD003651 and 10.6019/PXD003651) and is accessible under http://proteomcentral.proteomexchange.org/cgi/GetDataset?.

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