SHORT COMMUNICATION

Comparative proteome analysis identified CD44 as a possible serum marker for docetaxel resistance in castration-resistant prostate cancer

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Abstract
Baseline or acquired resistance to docetaxel (DOC) represents a significant risk for patients with metastatic prostate cancer (PC). In the last years, novel therapy regimens have been approved providing reasonable alternatives for DOC-resistant patients making prediction of DOC resistance of great clinical importance. We aimed to identify serum biomarkers, which are able to select patients who will not benefit from DOC treatment. DOC-resistant PC3-DR and DU145-DR sublines and their sensitive parental cell lines (DU145, PC3) were comparatively analyzed using liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). Results were filtered using bioinformatics approaches to identify promising serum biomarkers. Serum levels of five proteins were determined in serum samples of 66 DOC-treated metastatic castration-resistant PC patients (mCRPC) using ELISA. Results were correlated with clinicopathological and survival data. CD44 was subjected to further functional cell culture analyses. We found at least 177 two-fold significantly overexpressed proteins in the DOC-resistant sublines compared to the sensitive cell lines.
INTRODUCTION
Prostate cancer (PC) is the second most common solid cancer and the fifth leading cause of cancer mortality among men worldwide. Docetaxel (DOC) chemotherapy is one of the standard first-line therapy options for metastatic castration-resistant PC (mCRPC), but about half of the patients show initial resistance, and most of the patients will develop acquired resistance to DOC.

Despite significant progress in the understanding of the molecular background of DOC resistance, prediction of DOC treatment remains an unmet clinical need. Thus, our purpose was to gain insight into the molecular mechanisms of DOC resistance in PC and to identify novel therapy-predictive biomarkers and to find potential therapy targets. In order to identify differently expressed proteins between DOC-resistant vs. DOC-sensitive PC cell lines, we performed a comparative proteome analysis. Potentially secreted proteins were filtered by using bioinformatics approaches. Selected proteins were quantitatively analyzed in serum samples of mCRPC patients before DOC chemotherapy. Serum concentrations were correlated with clinical and follow-up data in order to determine their value for the prediction of response and survival of DOC-treated patients. Finally, we performed functional in vitro analyses by knocking down target proteins in PC cell lines and assessed its effect on DOC-sensitivity.

MATERIALS AND METHODS
2.1 Culturing and LC-MS/MS analysis of DOC-sensitive vs. -resistant PC cell lines
DU145 and PC3 human PC cell lines were purchased from ATCC (Rockville, MD), and their DOC-resistant sublines (DU145-DR, PC3-DR) were developed by adding increasing concentrations of DOC (Sigma-Aldrich, St. Louis, MO, USA) up to the maintenance concentration of 12.5 nmol/L as described earlier.

Proteome analyses were performed using the LC-MS/MS technique in order to identify differentially abundant proteins between the DOC-sensitive (PC3, DU145) versus DOC-resistant (PC3-DR, DU145-DR) cell lines (see Supplementary Methods).

2.2 Biomarker selection
Proteins quantified by LC-MS/MS were tested for differential abundance between parental and DOC-resistant PC cell lines. Proteins quantify with minimum two unique peptides, and those passing the applied significance thresholds (FDR-corrected p-value ≤0.05, fold change ≥2) were considered for further analysis. Bioinformatics methods were applied to identify potentially secreted proteins (see Supplementary Methods and Figure S1).

2.3 Patient cohort and samples
Serum samples were collected between 01/2014 and 03/2018 from 66 consenting mCRPC patients directly before treatment with first-line DOC.

PSA response was defined as at least 50% PSA decline from baseline during the first chemotherapy series. The Institutional Ethics Committee approved the study protocol (TUKEB: 55/2014). The main endpoint of the analysis was overall survival (OS).

2.4 Serum ELISA analyses
Serum concentrations of CD44, HGFR and IL13RA2 proteins were quantified using DuoSet ELISA kits (R&D Systems, MN, USA), while LNPEP and GSN levels were determined by using ELISA kits by
Nordic and LSBio (Nordic BioSite, Täby, Sweden; LSBio, WA, USA, respectively).

2.5 | Functional experiments

Based on the results of the ELISA analysis, we selected CD44 for further functional experiments. CD44 was knocked down in PC3-DR and DU145-DR cell lines by using the siRNA technique. Knock down was confirmed by Western blot and ELISA analyses, while cell cycle analysis was performed by flow cytometry. For RT-qPCR and Western blot analysis methods as well as for statistical methods please see Supplementary Methods.

3 | RESULTS

3.1 | Differentially expressed proteins between DOC sensitive vs. -resistant PC cells

Proteome analyses revealed 685 (DU145 vs. DU145-DR) and 248 (PC3 vs. PC3-DR) significantly differentially abundant (either increased or decreased) proteins of which 146 and 31 were at least 2-fold upregulated in DU145-DR and PC3-DR cell lines, respectively (see Figure S2).

We found ABCB1, SYPL1 and HSPB1 to be consequently highly abundant in both DOC-resistant sublines. The 10 most upregulated proteins in resistant cells are listed in Table S1.

Out of the 146 (DU145-DR) and 31 (PC3-DR) upregulated proteins, our prediction algorithm for secreted proteins identified five (CD44, GSN, CALU, COASY and HBS1L) and six (IL13RA2, COL6A1, MET, AUP1, ERAP1 and LNPEP) potentially secreted proteins, respectively. Five of these proteins (CD44, LNPEP, GSN, IL13RA2 and MET) were selected for ELISA analysis in clinical serum samples.

3.2 | Correlation of serum marker levels with clinicopathological parameters and survival

The main patients’ and follow-up characteristics are given in Table 1. Two proteins, GSN and IL13RA2, were undetectable in serum samples, while LNPEP showed detectable signals only in 15 of 53 samples; therefore, for LNPEP, results were dichotomized as positive vs. negative. We found no significant correlations between the assessed marker levels (MET, CD44, LNPEP and PSA) and clinicopathological parameters (see Table S2). Univariate Cox analysis showed ECOG performance status (>0) and PSA response to be significantly associated with shorter OS (p = 0.039, p = 0.001, respectively). In addition, CD44 levels greater than the median and upper 25% were correlated with poor OS (p = 0.014, p = 0.009, respectively) (see Table S3). Multivariate analysis revealed high CD44 serum level (upper 25%) to be independently associated with poor OS (p = 0.016) (see Figure 1A,B and Table S4).

3.3 | CD44 knockdown by siRNA and its effect on cell viability and cell cycle

CD44 mRNA and protein levels as determined by RT-qPCR and Western blot analysis correlated well with the protein levels determined by LC-MS/MS analyses (see Figure 1C,D). The siRNA transfection successfully silenced CD44 expression in DU145-DR cells and completely in PC3-DR cells according to Western blot and ELISA measurements (see Figure 1E,F).

Apoptosis analysis performed by flow cytometry using double staining (propidium iodide and Annexin V) revealed enhanced DOC-sensitivity in CD44 silenced DU145-DR cells compared to the controls under maintenance (12.5 nM) and also IC50 dose (100 nM) of DOC treatment as the rate of the living cells of the population decreased while the ratio of the apoptotic cells increased (see Figure 1G and Figure S3A,C).

### Table 1: Clinical characteristics of patients who underwent serum ELISA analyses for CD44, MET, IL13RA2, GSN and LNPEP

|                      | CD44, MET, IL13RA2 | LNPEP, GSNa |
|----------------------|--------------------|-------------|
| Total number of patients | 66                 | 53          |
| Age at baseline median (range) | 71 (44–86)       | 71 (44–86)  |
| ECOG PS at enrollment |                    |             |
| 0                    | 38                 | 30          |
| 1                    | 22                 | 19          |
| 2                    | 5                  | 3           |
| unknown              | 1                  | 1           |
| Bone metastasis      | 64                 | 51          |
| Lymph node metastasis (≥2 cm) | 29             | 21          |
| Soft tissue lesions (lung/ liver) | 14              | 10          |
| Previous prostatectomy | 12                | 10          |
| Previous radiation   | 7                  | 5           |
| PSA at baseline (ng/ml) | 87.8 (2.6–7312.0) | 88.9 (79–7312.0) |
| any PSA decline (yes / no / N.A.) | 46 / 11 / 9 | 36 / 10 / 7 |
| PSA decline 30%      | 36                 | 27          |
| PSA decline 50%      | 31                 | 23          |
| PSA decline 90%      | 16                 | 10          |
| PSA progression on DOC (yes/no) | 15 / 31         | 14 / 22     |
| Rad. progression on DOC (yes / no / N.A.) | 24 / 18 / 24 | 22 / 15 / 16 |
| Number of patients died / alive | 42 / 24 | 38 / 15 |
| Follow-up time in months median | 19              | 19          |

Abbreviation: ECOG PS, Eastern Cooperative Oncology Group Performance Status.

aFor the LNPEP and GSN analysis, because of limited sample volumes in some cases, only 53 samples were available.
Resistance of mCRPC to DOC remains incompletely understood. Currently known DOC resistance mechanisms include the overexpression of the drug transporter protein ABCB1 (also known as MDR1), inflammatory proteins (IL-6, YKL-40 and CCL2) and the transcription factor ERG, which is frequently (>50%) upregulated due to a chromosomal translocation resulting in TMPRSS-ERG gene fusion. Further studies pointed at the correlation between the presence of an androgen receptor splice variant (AR-V7) and taxane resistance. These results suggest that DOC resistance is molecularly divergent, and presumably more than one mechanism may contribute to therapy insensitivity.

The rapidly improving treatment landscape of mCRPC provides reasonable alternatives (cabazitaxel, abiraterone, enzalutamide, alapharadin, olaparib, rucaparib, pembrolizumab and 177lutetium-PSMA) for DOC-resistant patients. This development makes therapeutic decision-making increasingly complex. Lacking predictive and prognostic biomarkers, the optimal treatment sequence today does not account for molecular features of the tumour.

In the present study, using a hypothesis-free comparative proteomic analysis in DOC-resistant vs. sensitive parental PC cell lines, we identified a large number of proteins potentially involved in DOC resistance. These data provide a solid base for further research towards the elucidation of DOC resistance (see Table S5,S6). Five proteins (MET, CD44, LNPEP, GSN and ILL3RA2) were selected for quantitative analysis in serum samples of DOC-treated mCRPC patients. This analysis revealed high baseline serum CD44 levels as an independent predictor of shorter survival in DOC-treated patients. In accordance, our functional in vitro analysis showed that knockdown of CD44 re-sensitized resistant DU145-DR cells to DOC. These promising results warrant for further validation in an independent patient cohort. In this study, we focused on secreted proteins, but in the Supplementary Material, we provide a more detailed discussion on further promising marker candidates (see Supplementary Discussion).

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Dávid Keresztes: Conceptualization (supporting); Data curation (lead); Formal analysis (lead); Funding acquisition (supporting); Investigation (lead); Methodology (lead); Project administration (lead); Software (supporting); Visualization (lead); Writing – original draft (lead). Anita Csizmarik: Data curation (equal); Formal analysis (supporting); Project administration (lead); Writing – review & editing (supporting). Nikolett Nagy: Data curation (equal); Formal analysis (supporting); Visualization (supporting); Writing – review & editing (supporting). Orsolya Módos: Data curation (equal); Formal analysis...
DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are available in the Supplementary Material of this article.

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

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