SUMMARY REPORT

NKFIH – PD-115616 grant

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Short summary (500-1500 character)

Our investigations revealed high preoperative serum MMP7, SDC1 and CGA levels as independent prognostic factors in clinically localized prostate cancer. Therefore, these serum proteins may help to distinguish between clinically indolent and potentially aggressive prostate cancer. Furthermore, MMP7 SDC1 and YKL40 proved to be able to predict resistance against docetaxel chemotherapy in castration-resistant prostate cancer. Accordingly, high levels of these proteins were associated with shorter patients' survival in patients who were treated with docetaxel chemotherapy. We performed functional analyses in docetaxel-resistant and parental (docetaxel sensitive) prostate cancer cell lines by downregulation (using the siRNA technique) and upregulation (by stable plasmid transfection) of MMP7 as well as extrinsic addition (rhMMP7) and pharmaceutical blocking of MMP7 (by Batimastat). These manipulations did not significantly affected docetaxel sensitivity of tumor cells. Only knock-down of MMP7 at high DOC concentrations resulted in a significant decrease of chemoresistance.

Rövid összefoglaló (500-1500 karakter)

Vizsgálataink a prosztatarák korai stádiumú eseteiben az MMP7, a SDC1 és a CGA független prognosztikai szerepét igazolták. Ezek a szérumfehérjék tehát alkalmasak lehetnek a klinikailag veszélytelen prosztatarákok potenciálisan magas kockázatú tumoroktól történő elkülönítésére. Továbbá az előrehaladott, kasztráció-rezisztens stádiumban a docetaxel kemoterápia előtti szérum MMP7, SDC1 és YKL-40 koncentrációk bizonyultak a túlélés független prediktív faktorainak. Funkcionális vizsgálatainkban prosztatarák sejtekben csendesítettük (siRNS technikával) illetve termeltettük (stabil MMP7 expressziós plazmid segítségével) az MMP7 molekulát. Ezen túlmenően rekombináns MMP7-tel kezeltük valamint farmakológiailag is blokkoltuk tumoros sejtjeinkben az MMP7-tet. Mindezen MMP7 manipulációs vizsgálatok nem gyakoroltak szignifikáns hatást a prosztatarák sejtek docetaxel iránti rezisztenciájára. Egyedül magas dózisú docetaxel adása mellett az MMP7 csendesített sejtekben találtunk csökkent doecetaxel-rezisztenciát.

Detailed summary

1. Sample collection

The serum collection from prostate cancer (PC) patients treated either with radical surgery or chemotherapy was continuous. The sample collection from surgically treated patients reached 295 samples from more than 101 PC patients. Further 373 serum samples from 72 docetaxel-treated patients and 26 samples from 8 Xofigo treated patients were also collected. Because of stringent health insurance regulations Xofigo therapy is available for strongly selected group of patients. This is why our Xofigo-cohort remained smaller as expected.

2. Evaluation of the prognostic effect of selected markers in early-stage PC

As the first step, the retrospective collection of clinical and follow-up data from patients with early-stage PC, who were treated with radical surgery, has been finished. On these samples, the following analyses have been performed:

2.1.1. Syndecan-1 (SDC1) ELISA in early-stage PC

SDC1 serum concentrations have been measured in samples of 99 PC patients. Of these 99 patients 81 were treated by radical prostatectomy (RPE). SDC1 levels did not correlate with patients' age, histological stage and serum PSA level. We found significant higher SDC1 levels in patients with lymph node metastases ($p=0.002$) and higher Gleason-score ($p=0.043$). In accordance, SDC1 serum concentrations were higher in patients with progressed disease (n=18) who could only treated with palliative transurethral resection ($p=0.002$). Most important, high SDC1 serum levels were independently associated with poor disease-specific survival (DSS).

2.1.2. SDC1 IHC in early-stage PC

In 101 of 103 FFPE PC samples also benign prostatic glands were available. In benign prostatic glands a strong and preferentially membranous SDC1-staining in the basal cell layer was observed. Both cytoplasmic and membranous SDC1-reactivities were significantly higher in benign prostate glands than in PC ($p=0.023$ and $p<0.001$). We did not detect correlations between SDC1-staining and patients' age, pathological tumor stage, PSA-level, presence of PC metastasis or palliative TURP versus RPE with curative intent. Cytoplasmic but not membranous SDC1-reactivity was stronger in patients with Gleason score higher than 6 ($p=0.016$).

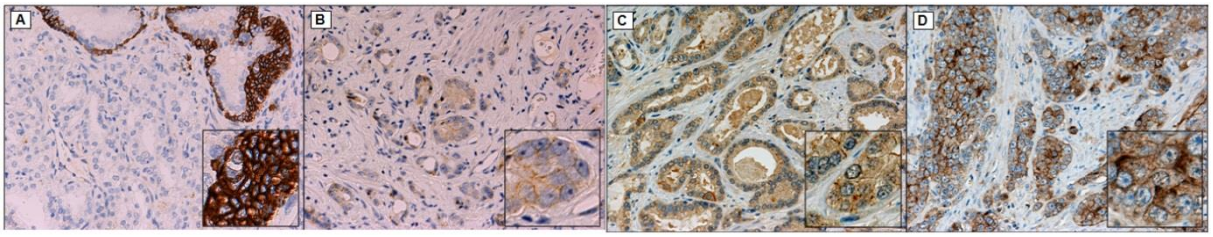

Fig 1 SDC1 expression in prostate cancer and benign prostate glands. A) Negative SDC1-immunoreactivity in PC with strong membranous staining of the basal cells of non-neoplastic (benign) prostatic glands (see inlay) and only minimal reactivity of the secretory epithelia. B) weak, C) moderate and D) strong SDC1-immunoreactivity in PC. Note the decrease of membranous and increase of cytoplasmic staining intensity. All images were taken at 200x magnification.

In conclusion, the increase of serum sSDC1 concentration in advanced PC suggests the involvement of SDC1 shedding in PC progression. In addition, high pre-operative sSDC1 serum levels may help to identify patients with lymph node or distant metastasis. The significant correlation between the serum levels of sSDC1 and MMP7 suggests that this protease is directly involved in SDC1 ectodomain shedding. Furthermore, analyzing a large cohort of PC patients with a long follow-up period and with available PC-specific survival data, we found no prognostic value for SDC1 IHC. In contrast, analyzing serum sSDC1 levels for the first time in PC, we found an independent prognostic value for sSDC1 in the pre-operative setting, suggesting its potential in therapeutic decision making.

Accepted publication:

These results have been published in „The Prostate" (IF=3.8).

Furthermore, these results have been presented in San Diego at the Annual Congress of the American Urological Association (2016).

2.2. MMP7 immunoassay in early-stage PC

Circulating MMP7 levels were determined in 106 controls and 240 PC patients divided in two independent cohorts with 130 serum and 110 plasma samples. For both cohorts, we used two immunoassay methods; ELISA (RnD Systems) and KRYPTOR assay (Thermo Fisher). The KRYPTOR method applies a fully automated measurement platform which has been designed for the needs of the daily clinical routine. MMP7 concentrations showed a high correlation (R Spearman: 0.97, $p<0.001$) between the two methods. Reagents for the KRYPTOR method have been sponsored by Thermo Fisher.

We found no diagnostic value for circulating MMP7 levels. MMP7 concentrations displayed no association with clinical or pathological tumor stage, grade and preoperative or postoperative Gleason score. In contrast, MMP7 serum and plasma levels were significantly elevated in metastatic compared to non-metastatic PC.

If considering all patients high MMP7 serum levels showed a clear association with shorter DSS. From clinical point of view a marker able to identify the small number of high-risk PCs in the group of early-stage tumors would have of major relevance. Therefore, metastatic PCs were excluded from further univariate and multivariate analyses. In this non-metastatic subgroup, high MMP7 levels proved to be a borderline significant risk-factor. Most important, if MMP7 serum levels were combined with those of PSA, risk-stratification could be largely increased ($p<0.001$).

MMP7 serum concentrations proved to be independent predictors of OS and DSS in the preoperative models when included as a continuous variable independently of being measured by the KRYPTOR or ELISA method.

2.3. Chromogranin A (CGA) immunoassay in early-stage PC

CGA is a glycoprotein commonly expressed in neuroendocrine cells. When a tumor develops in a neuroendocrine tissue, it becomes the main source of circulating CGA. Neuroendocrine differentiation in PC has been received attention in the last years because its potential implication as a prognostic and/or diagnostic factor. Therefore, we performed CGA analysis in our surgically treated (early-stage) PC cohort.

A significant correlation between CGA levels and tumor stage/grade, preoperative PSA level or lymph node status could not be observed. The most significant correlation was found between CGA levels and the presence of bone metastases. Furthermore, serum/plasma CGA analysis showed diagnostic relevance in PC. CGA levels in serum and plasma were elevated in metastatic compared to non-metastatic PC.

If considering all patients high CGA serum levels showed a clear association with reduced DSS ($p=0.008$). In the non-metastatic subgroup, high CGA levels proved to be a significant risk-factor.

CGA serum concentrations (>44.4 ng/ml) proved to be an independent predictor of OS and DSS in the preoperative models but not in the postoperative model.

In the subgroup of patients treated with RPE, CGA serum level proved not to be a significant prognosticator. As in the literature CGA are often discussed as a PSA-independent prognostic factor we also combined CGA and PSA levels in univariate and multivariate analyses. The subgroup of both low CGA and PSA value showed an excellent prognosis, suggesting that non-neuroendocrine differentiated PCs with low PSA may represent a clinically indolent subgroup.

Accepted publication:

These results have been published in „Pathology & Oncology Research" (IF=1.736).

2.4. Receptor activator of nuclear factor kappa-B ligand (RANKL) immunoassay

RANKL serum concentrations have been measured in samples of 68 PC and 13 BPH patients. 55 of the 68 PC patients were treated by radical prostatectomy (RPE) while 13 patients were treated by palliative transurethral resection. RANKL serum levels were significantly higher in BPH compared to PC patients (p=0.012). Serum RANKL levels did not correlate with patients' age, histological stage, PSA level, Gleason-score the presence of lymph node or distant metastasis. Also RANKL levels were not associated with overall or disease-specific survival. RANKL serum concentrations were similar between patients who were treated with RPE with curative intent (n=55) and those with progressed disease (n=13) who could only be treated with palliative transurethral resection.

3. Assessing the therapy predictive value of analyzed molecules in CRPC

3.1. MMP7, Fas and FasL immunoassay in DOC-treated CRPC patients

Serum levels of MMP7, Fas and FasL were determined in 987 serum samples of 96 CRPC patients. Men who completed the planned DOC treatment course with a good PSA response (PSA response was defined, according to the Prostate Cancer Clinical Trials Working Group Criteria [PCWG] I) and without experiencing radiographic progression were considered as DOC sensitive. These DOC sensitive patients underwent a retreatment with DOC. After the first retreatment (2nd series), further retreatments were offered based on the same response criteria. Based on these criteria 21 patients proved to be DOC-resistant and received one single series while 75 patients were considered DOC-sensitive and received repeated series of DOC.

3.1.1. Associations between patients' survival and serum levels of PSA, MMP-7, sFas and FasL at baseline

Pretreatment MMP7, sFas and FasL serum levels did not correlate with patients' age, ECOG performance status or the presence of lymph node metastases. In cases, present with bone metastases mildly elevated MMP7 levels have been observed (p=0.065), while in visceral metastatic cases MMP7 did not differ from those of non-metastatic cases. From the evaluated parameters, ECOG performance status (PS), bone or visceral metastases, elevated serum PSA levels were significantly associated with shorter DSS. Patients' age, ECOG PS, presence of lymph node or visceral metastasis and previously performed RPE had no significant impact on PSA, MMP7, sFas or FasL levels. Pretreatment with other first-line therapy were correlated with decreased MMP7 serum levels. PSA levels were 4-fold higher in bone metastatic castration-resistant PC. Most important, MMP7, sFas and PSA levels were significantly higher in DOC-resistant (single treatment) patients compared to those who were initially DOC sensitive (retreatment patients) (p=0.007, p=0.010 and p<0.001, respectively).

Higher pretreatment serum MMP7, sFas and PSA levels were significantly associated with both DOC-resistance (p=0.007, p=0.001 and p<0.001, respectively) and shorter cancer-specific survival (p<0.001, p=0.041, p<0.001, respectively). High MMP7 remained an independent predictor of poor cancer-specific survival (HR=2.11, 95%CI 1.36–3.30, p=0.001).

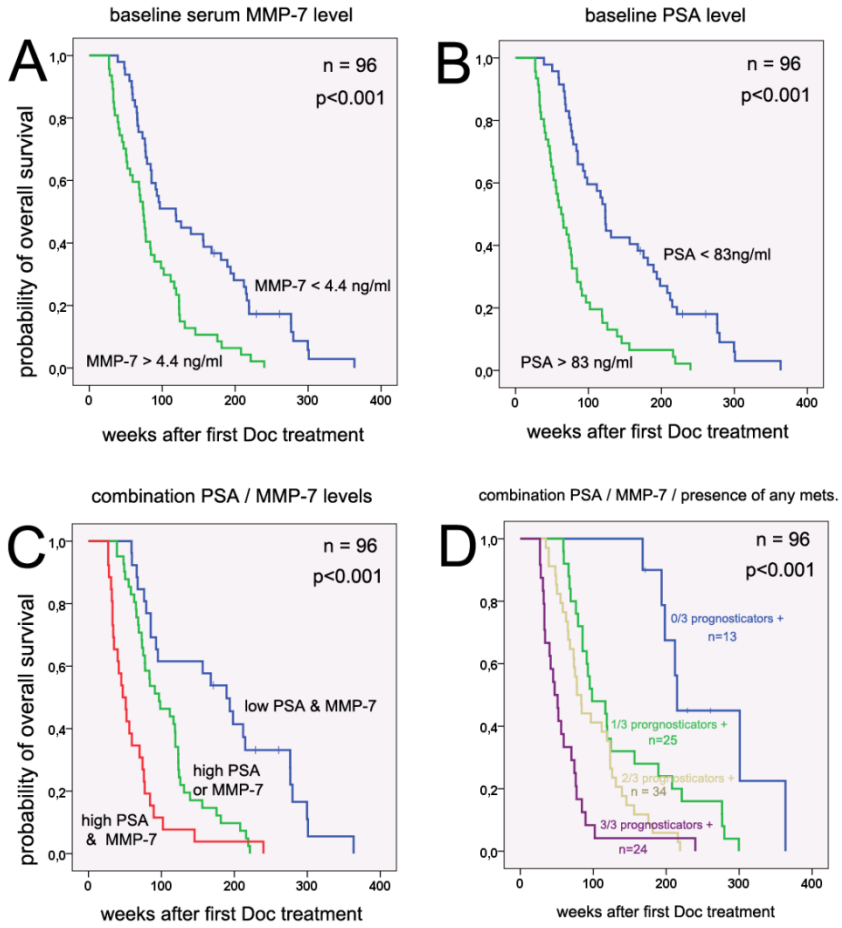

Fig. 2 Kaplan-Meier curves of overall survival according to (A) MMP-7 and (B) PSA levels. The risk-stratification improves when MMP-7 and PSA are combined (C). The combination of three prognostic factors (MMP-7, PSA and the presence of any metastasis) results the most accurate risk assessment (D).

3.1.2. Changes in MMP-7, sFas and FasL levels in response to DOC treatment

PSA levels consequently decreased during treatment series and increased in treatment holidays. MMP-7 levels showed no significant changes during DOC treatment if all patients were considered (figure 2). The subgroup analysis including only patients with high MMP-7 levels (>8 ng/ml) at baseline, however, revealed decreasing MMP-7 levels during treatments and increasing MMP-7 levels in treatment holidays (figure 2). In contrast, sFas and FasL levels exhibited no significant variations between different stages of DOC treatments.

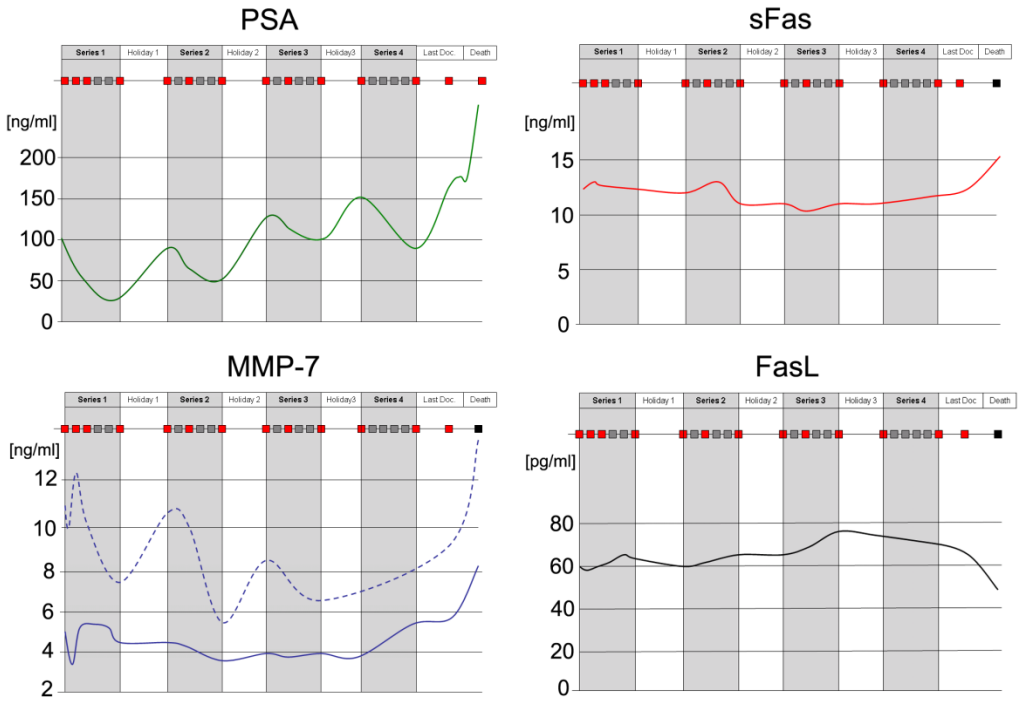

Fig. 3 Changes in PSA, MMP-7, sFas, and FasL serum levels during DOC treatment and treatment holidays. Red squares represent treatment days when serum samples were analyzed. Curves were drawn by using the median values of marker concentrations. In case of MMP-7 a separate curve was drawn including only those patients with high MMP-7 serum levels (>8 ng/ml) at baseline (dashed line).

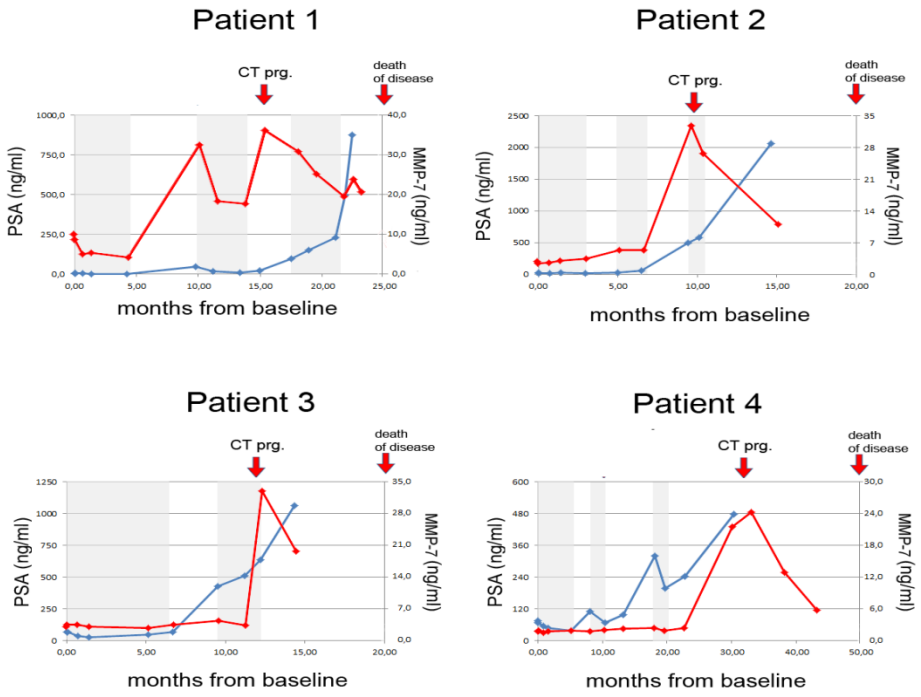

Fig. 4 Changes of PSA (dashed lines) and MMP-7 levels (black lines) in representative cases of CRPC. Grey area represents treatment periods (series). Note that PSA and MMP-7 levels strongly elevate prior or at the time of metastatic progression.

3.1.3. Conclusion

Better understanding of molecular mechanisms responsible for individual differences in treatment sensitivities is necessary for the careful selection of CRPC patients for different therapies. Our data reveals elevated MMP7 and sFas serum levels as markers of a DOC-resistant PCa phenotype. In addition,

pretreatment MMP7 levels were independently associated with poor patients' survival, making MMP7 a promising therapy-predicting marker in CRPC. Finally, not only the baseline levels but also the changes in PSA and MMP7 levels in treatment holidays were predictive for survival, suggesting these serum proteins as therapy monitoring markers. These results have to be validated in a larger prospective study before being proposed for everyday clinical practice.

Submitted manuscript:
These results have been submitted to The Journal of Urology.

3.2. SDC1 immunoassay in DOC-treated CRPC patients

Pretreatment SDC1 levels were determined in serum samples of 75 CRPC patients. Men who completed the planned DOC treatment course with a good PSA response (based on the PCWG criteria I) and without experiencing radiographic progression were considered as DOC sensitive. These DOC sensitive patients underwent a retreatment with DOC. After the first retreatment (2nd series), further retreatments were offered based on the same response criteria. Based on these criteria 49 patients proved to be DOC-resistant and received one single series while 26 patients were considered DOC-sensitive and received repeated series of DOC.

Patients' age, ECOG PS, presence of lymph node, bone or visceral metastasis had no significant impact on SDC1 levels. SDC1 levels were not significantly different between single treatment and retreatment patients. Similarly, pretreatment with other first-line therapy or with EMP did not correlate with SDC1 levels. In contrast, PSA was higher in patients with bone metastases (p=0.029).

Univariable Cox analysis found presence of any metastasis, bone metastasis or visceral metastasis as well as SDC1 levels (above the median) to be significantly associated with poor overall (OS) and disease-specific survival (DSS). Pretreatment PSA levels were associated with shorter disease-specific survival (p=0.041) and showed a trend to be associated with shorter overall survival (p=0.075).

Multivariable analyses revealed ECOG PS >0, presence of any metastases and high SDC1 serum levels as independent and unfavorable prognostic factors for both OS and DSS.

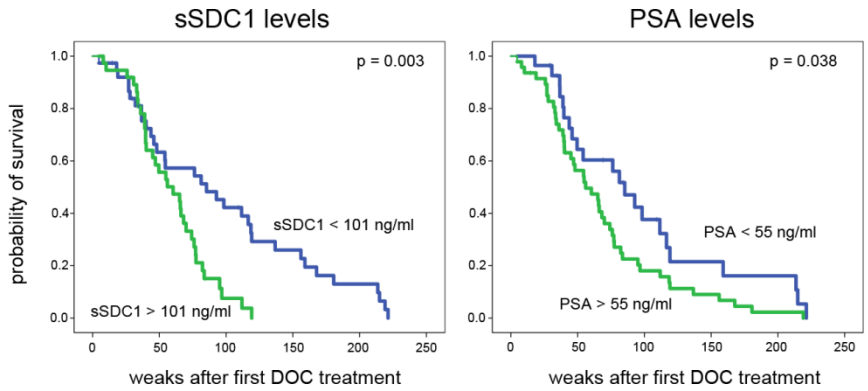

Fig. 5 Kaplan-Meier curves of cancer-specific survival according to sSDC1 and PSA pre-treatment (baseline) levels.

Submitted manuscript:
These results have been submitted to Urologic Oncology.

3.3. RANKL immunoassay in in DOC-treated CRPC patients

Pretreatment RANKL levels were determined in serum samples of 75 CRPC patients (see details on patient characteristics at 3.2. SDC1). RANKL levels were significantly higher in patients younger than 71 years (p=0.008). Higher RANKL levels were detected in lymph node negative compared to lymph node positive

patients (p=0.006). In contrast, RANKL levels tended to be higher in patients with compared to those without bone metastasis patients (p=0.066). Patients' ECOG PS, presence of visceral metastasis had no significant impact on RANKL levels. RANKL levels were not significantly different between single treatment and retreatment patients.

Univariable Cox analysis found presence of any metastasis, bone metastasis or visceral metastasis as well as PSA levels (above the median) to be significantly associated with poor overall (OS) and disease-specific survival (DSS). Pretreatment RANKL levels were not associated with OS or DSS.

3.4. YKL40 immunoassay in in DOC-treated CRPC patients

YKL-40, also known as chitalse 3-like 1 (CHI3L1) is a secretory glycoprotein and member of the '18 chitolectins family'. Under physiological conditions, it is produced in human embryonic stem cells, inflammatory cells and in tissues with high cellular activity. Elevated YKL-40 serum levels were reported in autoimmune and chronic inflammatory disorders as well as in a variety of solid tumors such as breast, colon, prostate, ovary and kidney tumors and glioblastoma. Further evidence suggests that elevated serum levels of YKL-40 are associated with drug resistance of cancer cells in glioblastoma. Based on these findings, we hypothesized that YKL-40 serum levels may predict response to DOC therapy in CRPC.

Pretreatment SDC1 levels were determined in serum samples of 109 CRPC patients. Men who completed the planned DOC treatment course with a good PSA response (based on the PCWG criteria I) and without experiencing radiographic progression were considered as DOC (DOC) sensitive. These DOC sensitive patients underwent a retreatment with DOC. After the first retreatment (2nd series), further retreatments were offered based on the same response criteria. Based on these criteria 56 patients proved to be DOC-resistant and received one single series while 53 patients were considered DOC-sensitive and received repeated series of DOC.

Patients' age, ECOG PS, pre-treatment with other first-line therapy or DOC in combination with EMP and the presence of visceral metastases and positive lymph node status had no significant impact on YKL-40 and PSA levels. In contrast, YKL-40 and PSA levels were significantly elevated in patients with bone metastases (YKL-40: p=0.032; PSA: p=0.010). Furthermore, YKL-40 levels were significantly higher in DOC-resistant patients who were treated with a single series of DOC than DOC sensitive patients who received repeated DOC treatments series (p=0.035). In contrast, PSA levels were not significantly different between DOC-resistant and DOC-sensitive patients. Patients with previous RPE had significantly lower YKL-40 and PSA levels (p=0.011 and p=0.008, respectively).

Univariable Cox analysis identified presence of any metastasis, bone metastasis or visceral metastasis as well as PSA levels (above the median) to be significantly associated with poor OS and DSS. Pre-treatment YKL-40 levels showed a trend to be associated with poor DSS (p=0.065). In patients who received DOC as first-line treatment, high YKL-40 levels were associated with significantly shorter OS (p=0.037) and DSS (p=0.017). In addition, ECOG PS >0 proved to be a significant prognostic factor for OS (p=0.002) and DSS (p=0.006). Patients who were pre-treated with RPE had better OS and DSS (p=0.036 and p=0.050). Multivariable analyses revealed ECOG PS>0, presence of any metastases and high PSA serum levels as independent and unfavorable prognostic factors for both OS and DSS.

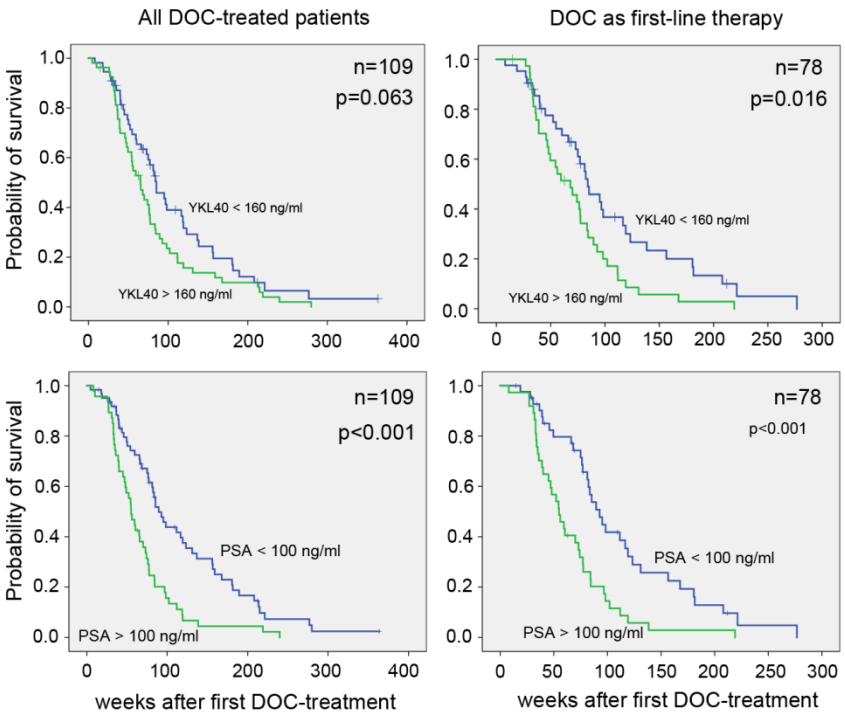

Fig. 6 – Kaplan-Meier curves of DSS stratified by pre-treatment YKL-40 and PSA

Submitted manuscript:
These results have been submitted to "The Prostate"

3.5. MMP7, Fas and SDC1 immunoassay by Xofigo-treated patients

Serum MMP7, Fas and SDC1 levels have been measured in 35 samples of 8 Xofigo-treated patients. SDC1 The median baseline serum SDC1, MMP7 and Fas levels were 21.1 (range 11.3 - 100.6 ng/ml), 2.89 (range 0.2 – 5.9) and 3934 ng/ml (range 3476 – 6856). We found no correlation between the serum concentrations of the assessed proteins. For further statistical evaluation of the prognostic/predictive value of these proteins for Xofigo treatment a longer follow-up time and larger patient numbers are needed.

4. Immunohistochemical analysis of PC bone metastasis

18 bone FFPE PC bone metastasis samples were available for MMP7, SDC1 and RANKL IHC analysis. MMP7 and SDC1 immunostaining showed a cytoplasmic positivity of the tumor cells. In 7 of 18 cases tumor cells showed an accentuated (strong or moderate) membranous SDC1 staining while 5 of the 18 of cases showed a strong/moderate MMP7 staining. No focal MMP7 or SDC1 immunorectivity has been observed at the tumor-bone interface. However, these observations have to be handled with careful caution as the decalcification of these samples may substantially influence their immunoreactivity.

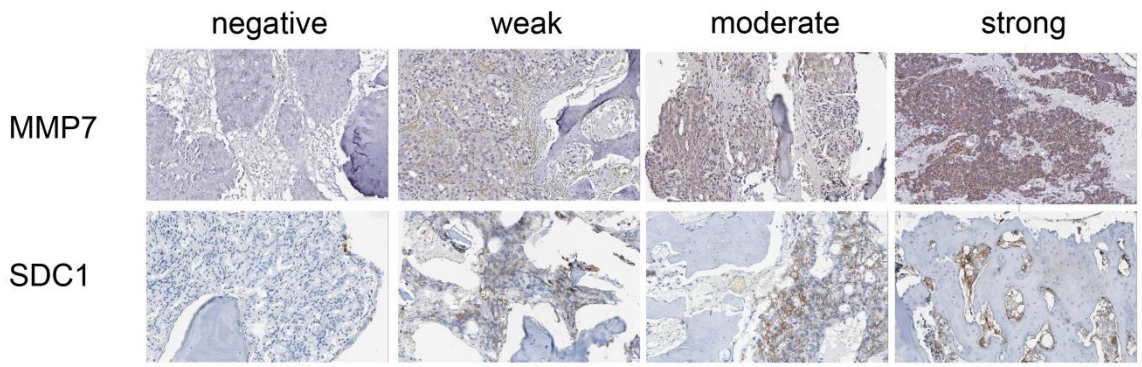

Fig. 7 – Representative MMP7 and SDC1 immunostaings of prostate cancer bone metastases.

5. Functional cell culture analyses of selected molecules

We investigated the DOC-sensitive vs. their DOC-resistant subclones of two widely used PC cell lines (PC3 /-DR, Du145 /-DR).

5.1. Comparison of baseline characteristics between docetaxel sensitive vs. resistant human prostate cancer (PC) cell lines (PC3 vs. PC3-DR / Du145 vs Du145-DR)

Aims: To describe differences in DOC-sensitivity as well as to assess possible expression-differences in target molecule's gene and protein expressions between DOC-resistant and their parental (DOC-sensitive) cell clones.

5.1.1. Comparison of characteristics between PC3 and Du145 cells (based on published literature)

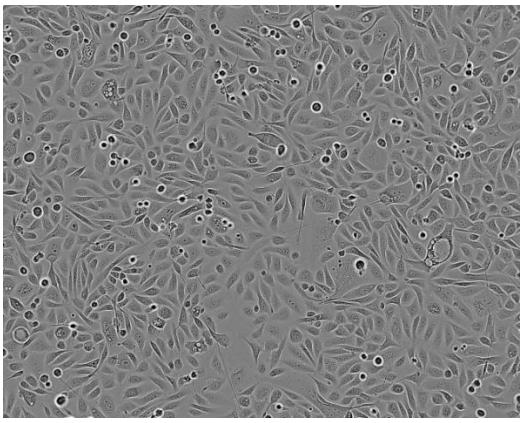

FIG 8A PC3 CELLS (4X MAGN.)

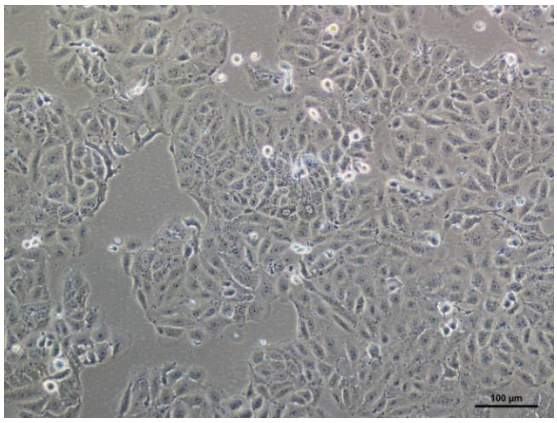

FIG 8B DU145 CELLS (4X MAGN.)

- High metastatic potential
- Derived from: Bone metastasis of grade IV of PC
- Not hormonsensitive
- No PSA expression
- Does not respond to glucocorticoids or fibroblast growth factors but influenced by epidermal growth factors

- Moderate metastatic potential
- Derived from: central nervous system metastasis of PC
- Not hormonsensitive
- No PSA expression
- It was demonstrated, that administration of RANKL (=NFkappaB ligand) promoted Du145 cell invasion in bone which leads osteolytic lesions
- DU145 cells can produce soluble factors that increase RANKL expression -> facilitate PC metastasis in bone

5.1.2. *Cell viability analysis by MTT-assay / dose response curve for DOC*

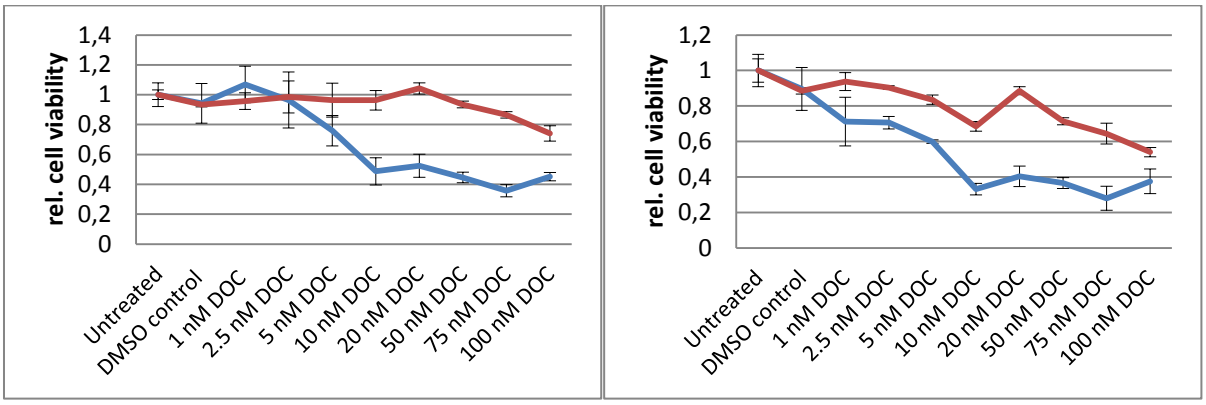

Fig 9 – PC3 (blue line) and PC3-DR (red) Docetaxel – Dose – response curve at 24 hours (left) and 48h (right). MTT-assays were performed on 96-well plates. 2000 cells were seeded in 100 ul RPMI 1640 Medium (10% FCS, 1% PS) per well. Quadruplicates were measured per condition.

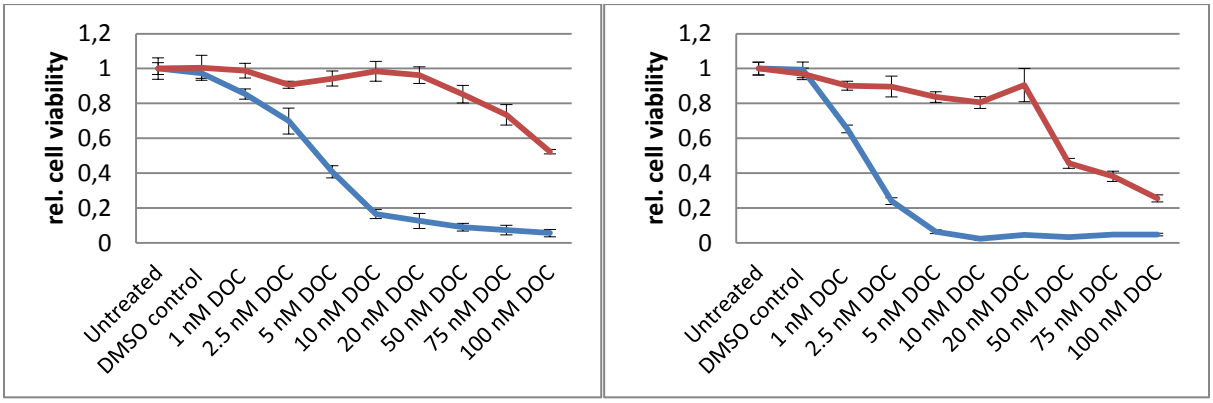

Fig. 10 – DU145 (blue line) and DU145-DR (red) Docetaxel – Dose – response curve at 24 hours (left) and 48h (right). MTT-assay execution: see the description of Fig 9.

5.1.3. *Cell cycle analysis by FACS*

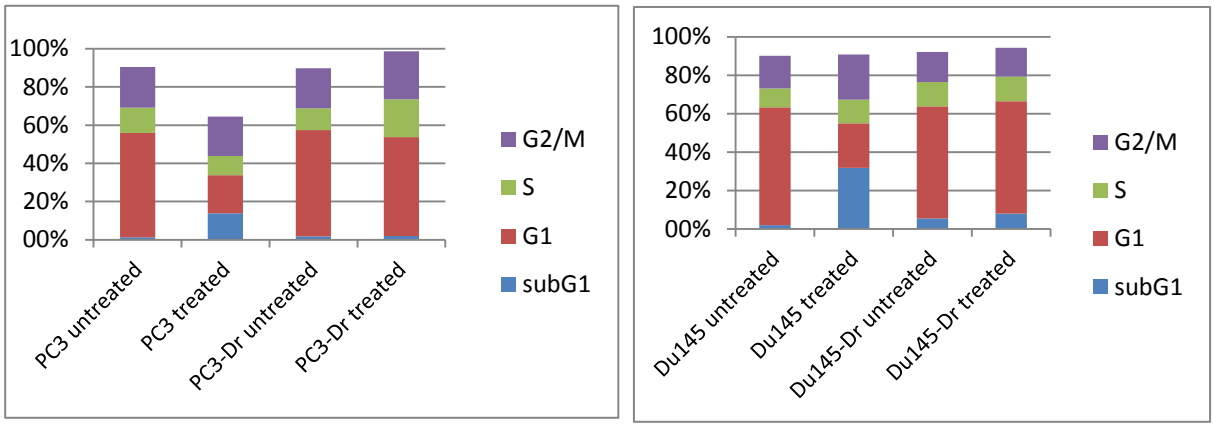

Fig. 11 Cells were stained with propidium-iodide and were investigated with and without DOC treatment (maintenance dose: 12.5 nM). Propidium iodide molecule binds to DNA. With FACS analysis the DNA content of the cells can be detected. The various quantity of DNA in the cells enables us to distinguish their current cell cycles phases with flow cytometry.

5.1.3. Basal gene expression of the molecules of interest assessed by RT-qPCR technique

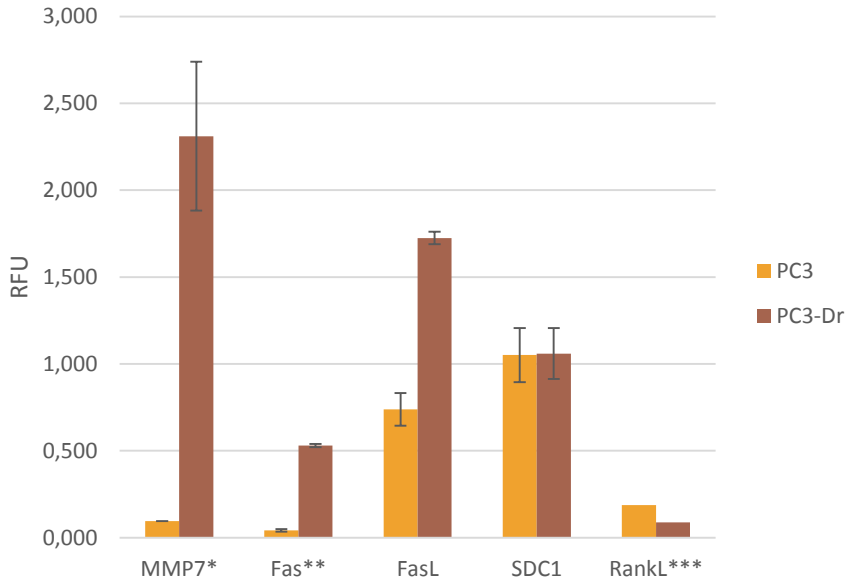

Fig. 12 – Gene expression analysis of target molecules in PC3 / -DR cells by RT-qPCR. TaqMan Gene Expression Assays were used for quantifying the expression of the genes of interest. The represented values are relative values normalized to the expression of HPRT housekeeping gene.

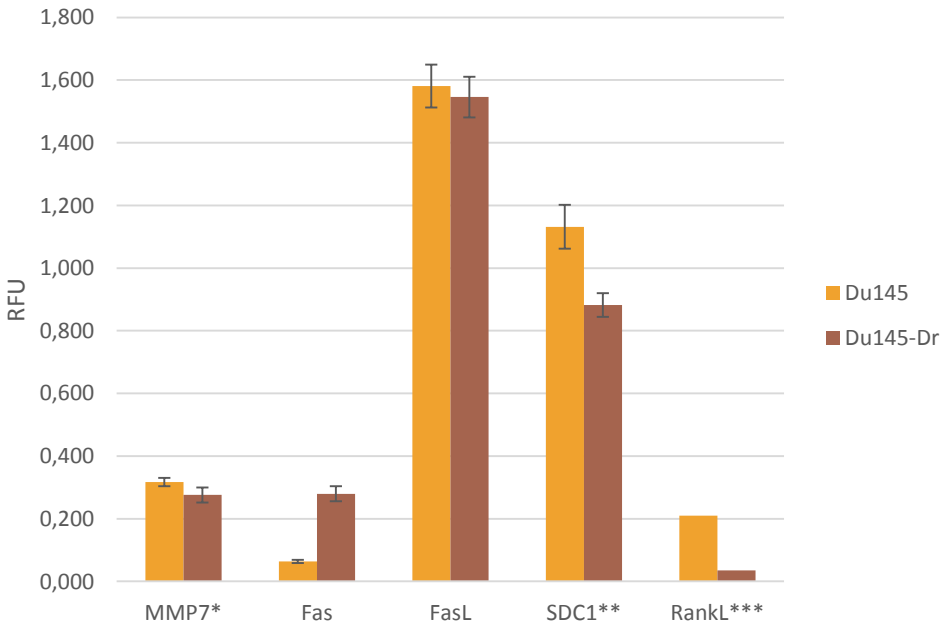

Fig. 13 – Gene expression analysis of target molecules in DU145 /-DR cells by RT-qPCR.

5.1.4. Protein expression of the molecules of interest (by Western blot analysis)

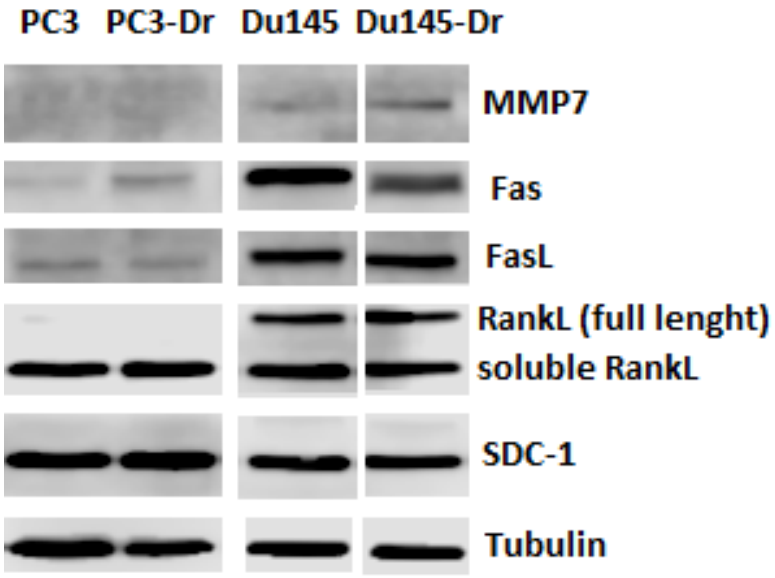

Fig. 14– Protein expression analysis of target molecules in PC3 / -DR and DU145 /-DR cells by Western blot analysis. 20 ug protein sample were loaded into each well. The anti-MMP7, -Fas, -FasL, -SDC-1, -Tubulin antibodies were blocked on the blot by using milk (5%), while anti-RankL antibody were blocked by BSA (5%). Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used for the detection of the target proteins.

5.1.5. Protein levels of the molecules of interest in conditioned medium (by ELISA analysis)

MMP7, SDC1 and Fas were undetectable in conditioned mediums of the assessed cell pairs.

Conclusions:

1) Cell viability (MTT) assay and FACS (Sub G1) analyses confirmed that PC3-DR and Du145-DR cells show significantly higher resistance against DOC.
2) MMP7 gene and protein levels are generally weak in all the four assessed cell lines as well as in conditioned medium. Overall the gene and protein expression levels are not concordant. PC3 and PC3-DR cells show very low protein levels of MMP7, Fas and FasL. MMP7 protein levels are higher in Du145-DR compared to Du145 cells.

5.2. *MMP7 knock-down by siRNA in DOC-resistant human prostate cancer (PC) cell lines (PC3 vs. PC3-DR / Du145 vs Du145-DR)*

Aims: To assess the effect of MMP7 knock down to DOC-sensitivity and expression of target genes in DOC-resistant cells

5.2.1. Cell viability analysis (by MTT-assay)

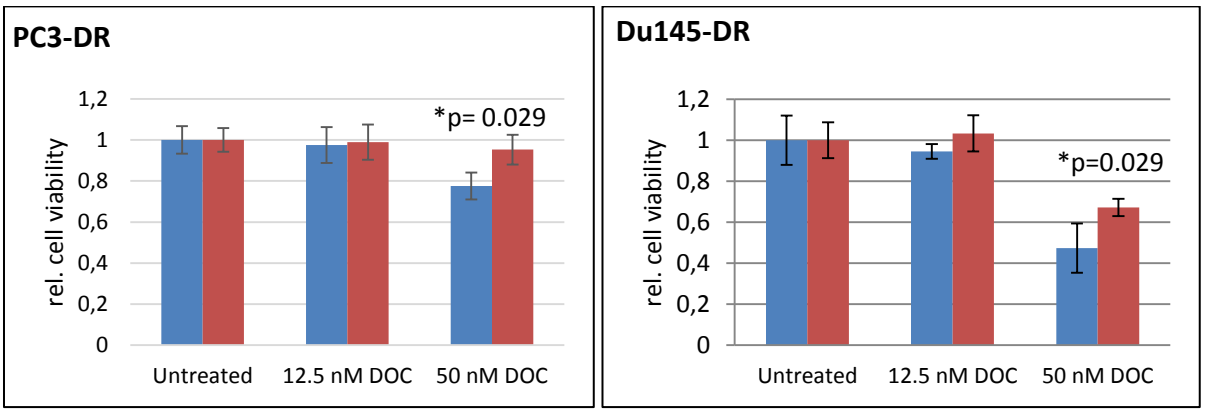

Fig. 15 – Effect of MMP7 knockdown to cell viability - MMP7 siRNA transfected - red bars, control DR cells blue bars. PC3-Dr and DU145-Dr cells were seeded on 6-well plates in RPMI 1640 Medium (10% FCS, without PS). The cells were transfected with control siRNA and siMMP7 by lipofection (transfection reagent: Lipofectamine 2000, Invitrogen). 24 hours after the transfection the cells were seeded on 96-well plate for performing MTT-assay.

5.2.2. Gene expression analysis by RT-qPCR

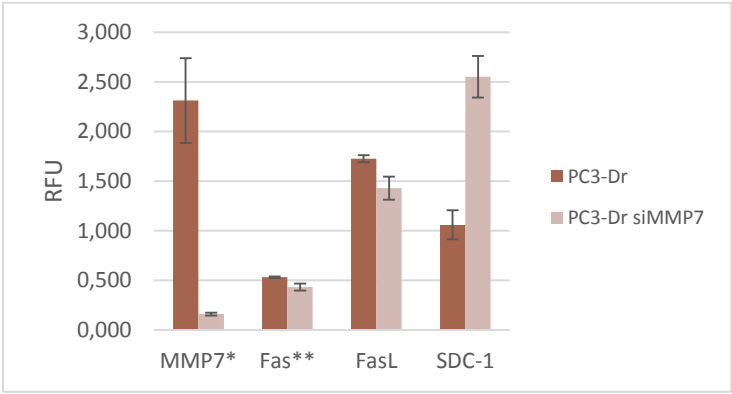

Fig. 16 – Gene expression analysis analysis of target molecules in PC3 / -DR cells by RT-qPCR. Details of the experiment: see the description of Fig 10. MMP7*: The relative MMP7/HPRT values were multiplied by 200. Fas**: The relative Fas/HPRT values were multiplied by 10 to facilitate data presentation.

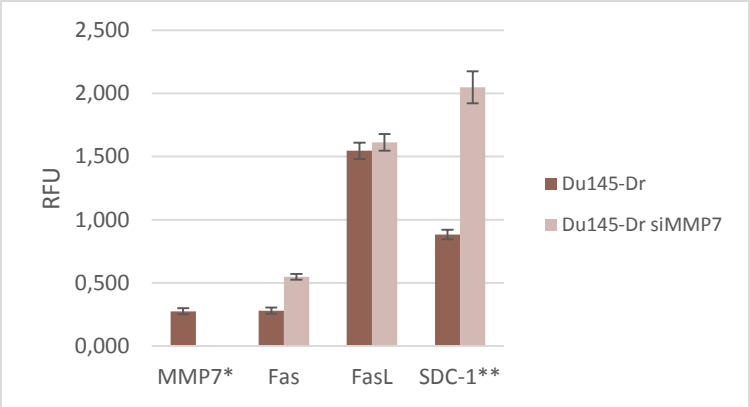

Fig. 17 – Gene expression analysis analysis of target molecules in DU145 /-DR cells by RT-qPCR. MMP7*: The relative MMP7/HPRT values were multiplied by 50. SDC-1**: The relative SDC-1/HPRT values were divided by 2.

5.2.3. Protein expression of the molecules of interest by Western blot analysis

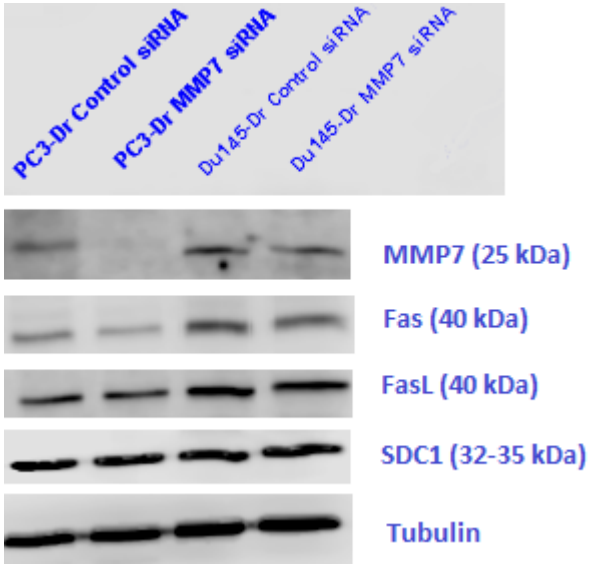

Fig 18 – Protein expression analysis of target molecules in PC3 / -DR and DU145 /-DR cells by Western blot analysis.

5.2.4. Protein levels of the molecules of interest in conditioned medium (by ELISA analysis)

MMP7, SDC1 and Fas were undetectable in conditioned mediums of the assessed cell pairs.

Conclusions:

1) Viability (MTT-assay) and FACS (Sub G1) analyses confirmed that PC-DR and Du145-DR cells show significantly higher resistance against DOC.
2) MMP7 knockdown was successful in both PC3-DR and DU145-DR cell lines as proven at both gene expression and protein levels.
3) MMP7 knockdown significantly increased chemosensitivity of both PC3-DR and Du145-DR cells, however this effect was only present at higher (50 nM) DOC concentrations.
4) MMP7 silencing didn't changed the protein expression of the assessed target molecules

5.3. *MMP7 inhibition by broad-spectrum MMP-inhibitor Batimastat (BB-94) in DOC-resistant human prostate cancer (PC) cell lines (PC3 vs. PC3-DR / Du145 vs Du145-DR)*

Aims: To assess the possible effect of pharmaceutical MMP inhibition on DOC-resistance of PC cells. As, to date, no MMP7-specific inhibitor is available, we used Batimastat which is a broad spectrum MMP inhibitor. Batimastat blocks MMPs – including MMP7 - by binding the zinc ion in the active site.

5.3.1. *Cell viability analysis (by MTT-assay)*

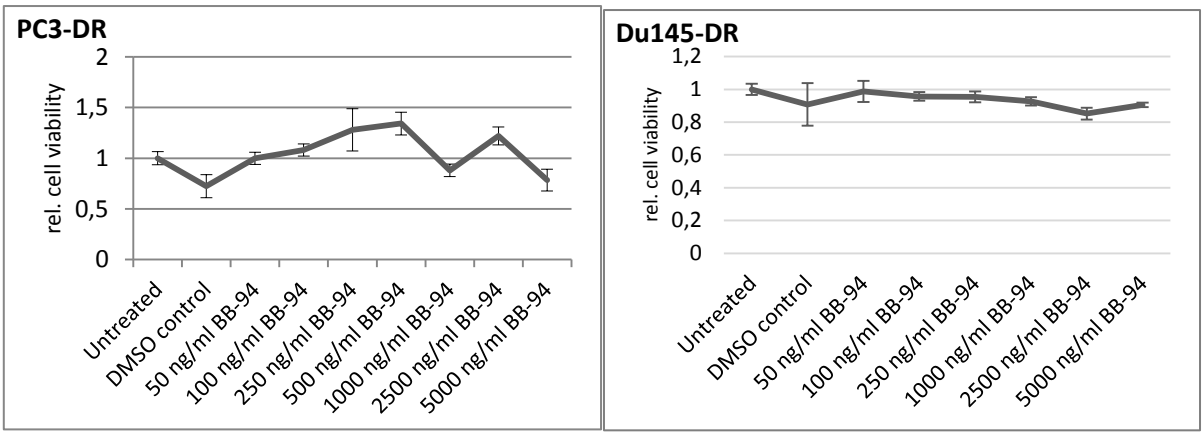

Fig. 19 A) Impact of BB-94 treatment on cell viability (at 48h) in PC3 / -DR (left) and DU145 /-DR (right) cells by MTT-assay

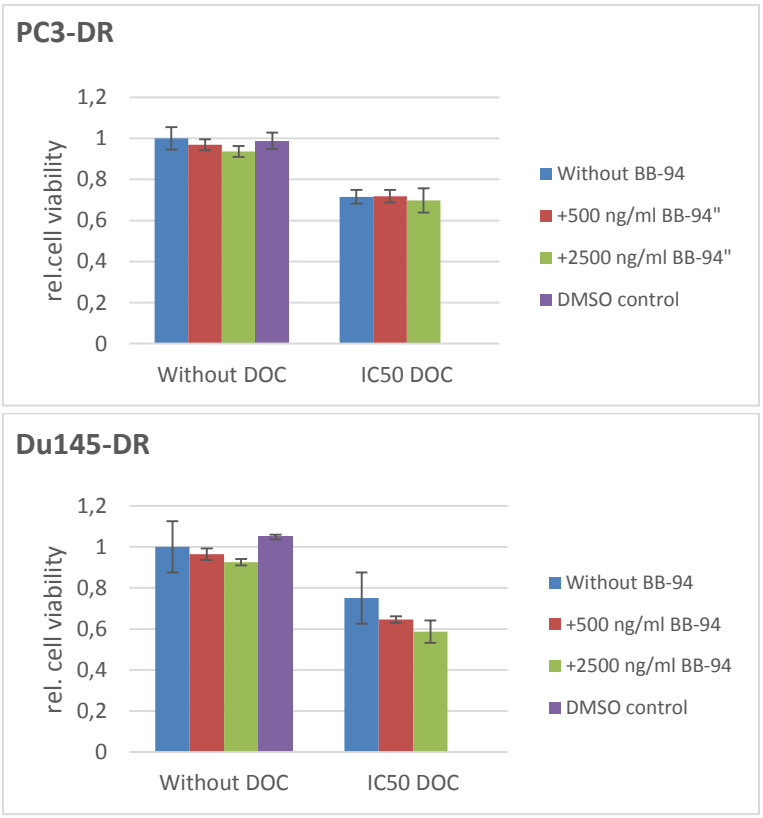

Fig. 20 – A) Impact of the simultaneous treatment with DOC and BB-94 treatment on cell viability (at 48h) in PC3 / -DR (and DU145 /-DR assessed by cell viability (MTT) assay

5.3.2. Protein expression of the molecules of interest by Western blot analysis

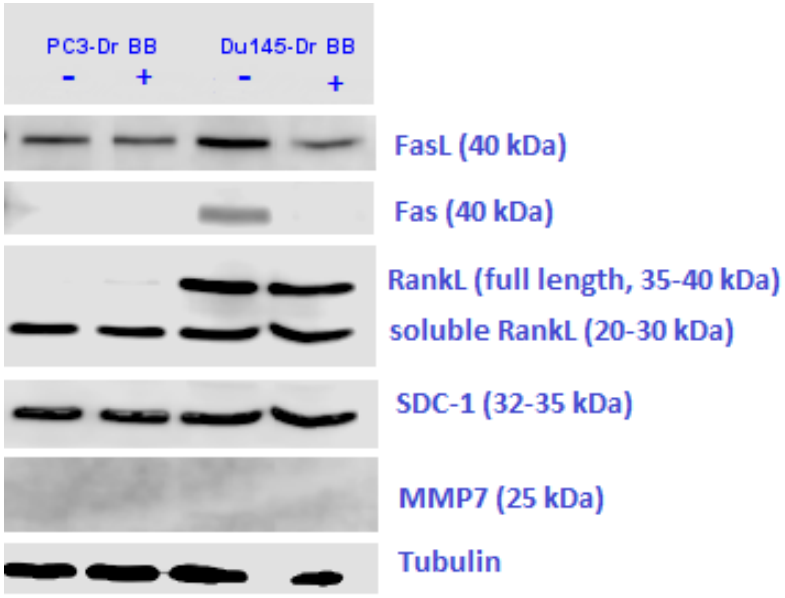

Fig. 21 – Protein expression analysis of target molecules in PC3 / -DR and DU145 /-DR cells by Western blot analysis. Western blot conditions were the same as prviously described.

5.3.3. Protein levels of the molecules of interest in conditioned medium (by ELISA analysis)

MMP7, SDC1 and Fas were undetectable in conditioned mediums of the assessed cell pairs.

Conclusions:

1) Viability (MTT-assay) analysis revealed no effect for Batimastat treatment on DOC resistance in PC3-DR cells.
2) Batimastat treatment did not significantly changed the expression of assessed genes

5.4. *Treatment of prostate cancer (PC) cell lines (PC3 vs. PC3-DR / Du145 vs Du145-DR)with recombinant human MMP7 (rhMMP7)*

Aims: To assess the possible external addition of MMP7 on DOC-resistance of PC cells.

5.4.1. *Cell viability analysis (by MTT-assay)*

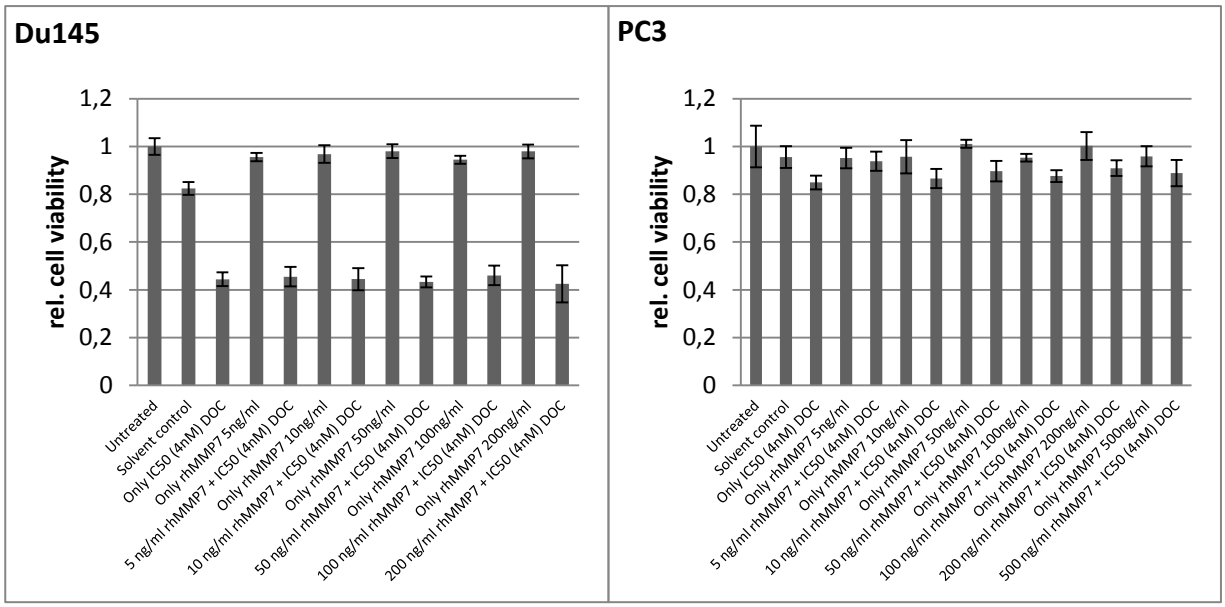

Fig. 22. – PC3 and Du145 parental cells were seeded on 96-well plates (2000 cells/well). 24 hours after they were pretreated with different concentrations of rhMMP7. Another 24 hours later the cells were treated with docetaxel and the maintenance dose of Dr cells). 48 hours after the docetaxel treatment the MTT-assay measurements were performed. These assays were performed twice (independently)

5.4.2. *Protein expression of the molecules of interest by Western blot analysis*

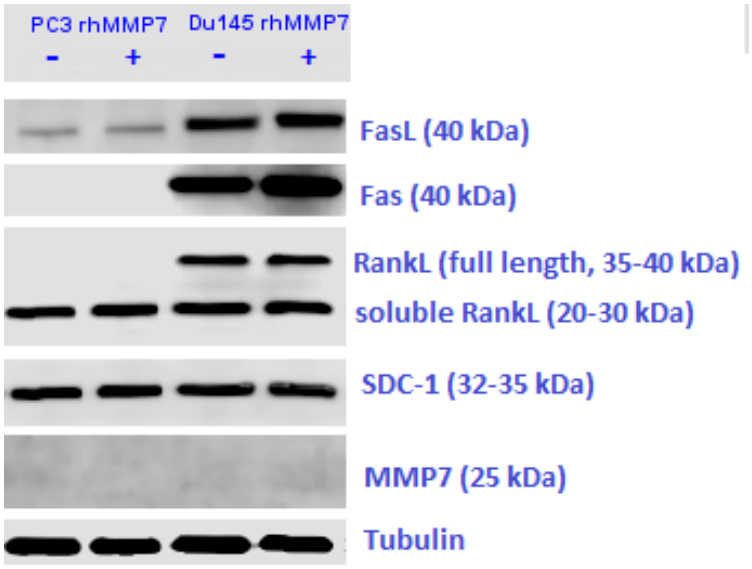

Fig. 23 – Protein expression analysis of target molecules in PC3 and DU145 cells by Western blot analysis.

5.4.3. Protein levels of the molecules of interest in conditioned medium (by ELISA analysis)

MMP7, SDC1 and Fas were undetectable in conditioned mediums of the assessed cell pairs.

Conclusions:

1) Viability (MTT-assay) analysis revealed no effect for human recombinant MMP7 treatment on DOC resistance of PC3 and Du145 cells.

5.5. Upregulation of MMP7 expression in (PC3 vs PC3-DR / Du14 vs, Du145-DR) with GFP-MMP7 plasmid transfection

Aims: To assess the effect of MMP7 upregulation on DOC-resistance of PC cells by stable plasmid transfection.

This experiment was not included in the original research plan. However, as the MMP7 levels were very low in both PC cell line pairs, this experiment has been additionally performed.

5.5.1. Cell viability analysis by MTT-assay

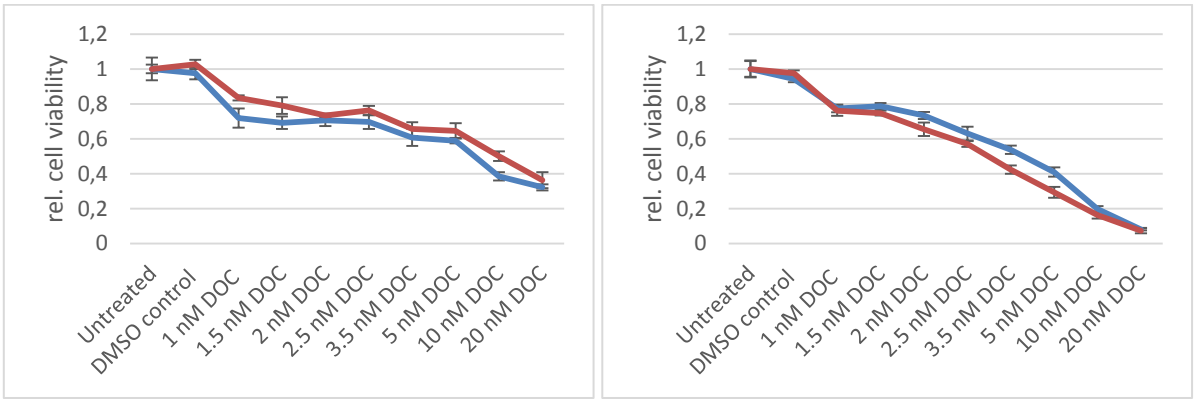

Fig. 24 –Docetaxel dose – response curves (48h) of PC3 (left) and Du145 (right) cells (blue lines) and stable MMP7 expressing cell lines (red lines).

5.5.1. Gene expression analysis by RT-qPCR

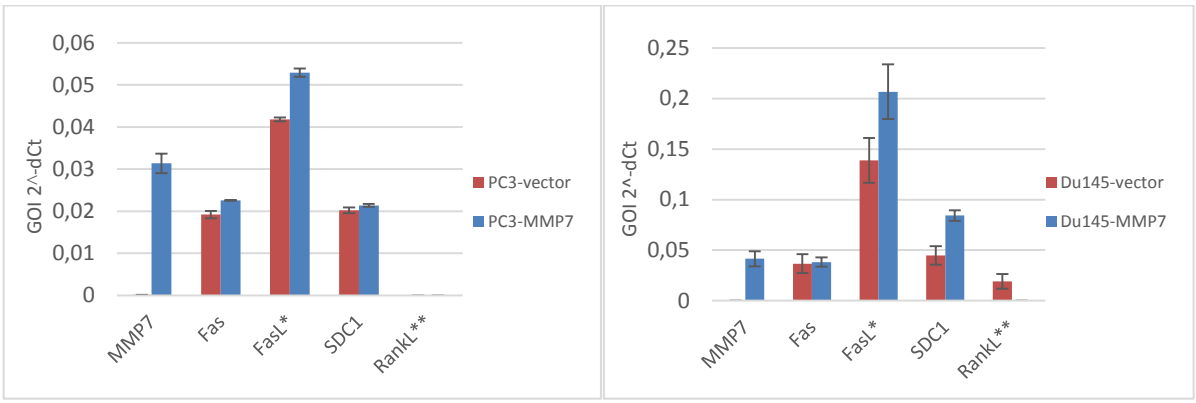

Fig. 25 Gene expression analysis analysis of target molecules in PC3-vector /-MMP7 (left) and in DU145-vector /-MMP7 cells by RT-qPCR. FasL*: The vales were multipied by 0.125. RanL values were multiplied by 100.

5.5.2. Protein expression of MMP7 by Western blot analysis

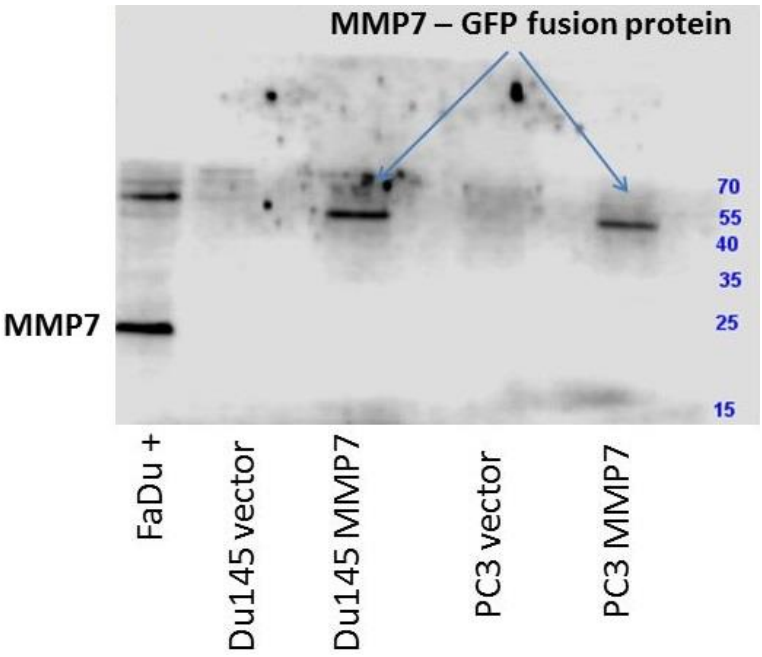

Fig. 26 – Protein expression analysis of MMP7 in PC3 and DU145 cells by Western blot analysis. FaDu cell protein lysate as positive control for MMP7 (about 25 kDa height). The delayed MMP7 bands to size of 50 kDa in DU145-MMP7 and PC3-MMP7 are due to the additional size of GFP fusion protein. Furher details of the Western blot conditions are he same as previously described.

5.5.3. Protein levels of the molecules of interest in conditioned medium (by ELISA analysis)

MMP7, SDC1 and Fas were undetectable in conditioned mediums of the assessed cell pairs.

Conclusions:

1) Gene expression and WB analysis confirmed that MMP7 plasmid transfection was successful
2) Stable transfection with MMP7 had no effect on the chemoresistance of PC3 or Du145 cells.

5.6. Prostate cancer – fibroblast co-culture

Aims: As in human tumor tissue samples MMP7 immunostaining often localize to the invasive front of the tumor, direct contact of tumor to non-malignant cells may be crucial for increased MMP7 production of tumor cells. Therefore, we aimed to assess, whether co-culturing tumor cells with non-tumorous stromal cells does increase the MMP7 production of PC cells.

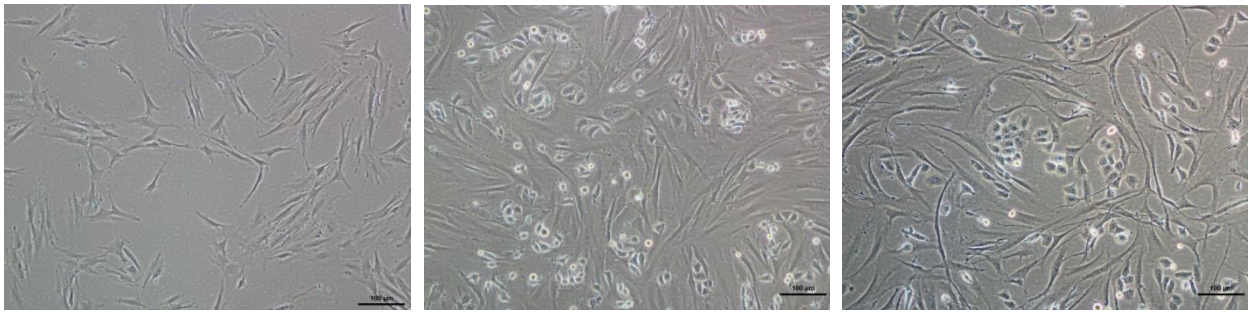

Fig. 27 Left: VHF fibroblast cells. Middle: VHF + PC3-Dr cells co-culture. Right: VHF + DU145-Dr. Co-culture method: The VHF cells were cultured for 48h in T25 flasks in RPMI 1640 Medium (10% FCS, 1% PS). Afterwards the Dr cells were seeded in these flasks with fresh medium. 72-120h later RNA and protein was extracted from the parallelly growing co-cultures.

5.6.1. Protein expression of MMP7 by Western blot analysis

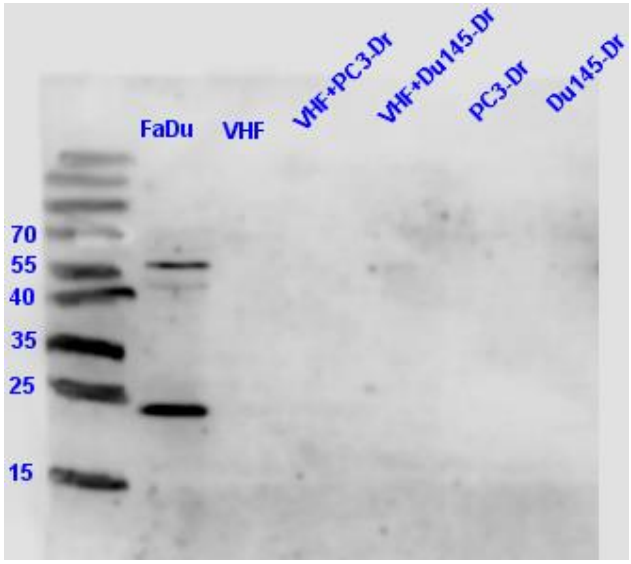

Fig. 28 – Protein expression analysis of MMP7 in PC3-DR and DU145-DR cells by Western blot analysis. FaDu cell protein lysate as positive control for MMP7.

Conclusions:

1) Co-culturing of PC3 and Du145 cells with normal fibroblasts did not influence the MMP7 expression of PC cells

Final conclusions

1) Our analysis on a large number of clinical samples reveled for the first time that pre-treatment serum MMP7 levels are independently associated with patients' survival under DOC chemotherapy. MMP7 may therefore help to identify DOC resistant patients. These patients may potentially benefit from alternative therapies.
2) Similarly SDC1 and YKL40 levels were associated with poor patients survival in CRPC patients who were underwent DOC chemotherapy.
3) Preoperative SDC1 and CGA levels add to the prognostic value of PSA in clinically localized PC and may therefore help to differentiate between clinically insignificant PC and those of aggressive and potentially lethal ones.
4) Functional analysis has been performed by downregulation (siRNA) and upregulation (by stable plasmid transfection) of MMP7 as well as extrinsic addition (rhMMP7) and pharmaceutical blocking of MMP7 (by Batimastat). These manipulations did not significantly affected chemosensitivity. Only knock-down of MMP7 at high DOC concentrations resulted in a significant decrease of chemoresistance.
5) Basal MMP7 expression was low in our cell culture model. Based on the observation, that MMP7 is ofthen focally expressed at the invasive tumor front, we established a co-culture with fibroblast cells. However, also in the PC-fibroblast co-culture MMP7 levels remained low.

Publication list

The grant support by NKFIH/OTKA has been acknowledged in all the below listed papers and manuscripts. A copy of the published manuscripts has been placed at the MTMT repository.

Published papers:

1. Szarvas T, Reis H, Vom Dorp F, Tschirdewahn S, Niedworok C, Nyirady P, Schmid KW, Rübben H, Kovalszky I. Soluble syndecan-1 (SDC1) serum level as an independent pre-operative predictor of cancer-specific survival in prostate cancer. Prostate. 2016 Aug;76(11):977-85. doi: 10.1002/pros.23186.

2. Niedworok C, Tschirdewahn S, Reis H, Lehmann N, Szücs M, Nyirády P, Romics I, Rübben H, Szarvas T. Serum Chromogranin A as a Complementary Marker for the Prediction of Prostate Cancer-Specific Survival. Pathol Oncol Res. 2017 Jul;23(3):643-650. doi: 10.1007/s12253-016-0171-5.

Submitted manuscripts (under review):

1. T Szarvas, Sevcenco S, Módos O, Keresztes D, Nyirády P, Ristl R, Puhr M, Hoffmann MJ, Niedworok C, Hadaschik B, Maj-Hes A, Shariat SF, Kramer G. MMP-7, sFas and FasL serum levels for predicting docetaxel resistance and survival in castration-resistant prostate cancer
   Under review at *The Journal of Urology*

2. Szarvas T, Sevcenco S, Módos O, Keresztes D, Nyirády P, Kubik A, Romics M, Kovalszky I, Reis H, Hadaschik B, Shariat SF, Kramer G. Syndecan-1 shedding is associated with chemotherapy-resistance in castration-resistant prostate cancer
   Under review at *Urologic Oncology*

3. Darr C, Krafft U, Hadaschik B, Sevcenco S, Csizmarik A, Nyirády P, Küronya Z, Reis H, Maj-Hes A, Shariat SF, Kramer G, Szarvas T. The role of YKL-40 in predicting resistance to docetaxel chemotherapy in prostate cancer
   Under review at *The Prostate*

Congress presentations:

1. Nyirády P, Sevcenco S, Módos O, Keresztes D, Kovalszky I, Reis H, Shariat SF, Kramer G Szarvas T
   Changes of tissue and serum levels of syndecan-1 during prostate cancer progression
   Annual Meeting of the American Association of Urology 2016, 06-10.05.2016 San Diego, USA

2. Szarvas T, Sevcenco S, Módos O, Keresztes D, Kovalszky I, Reis H, Shariat SF, Kramer G Nyirády P
   Prognosztikus és terápia-prediktív biomarkerek azonosítása prosztatarákban
   Magyar Uroonkológus Társaság (MUOT) Kongresszusa, 2017.05.19. Budapest, Magyarország