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Transcription alterations of members of the Ubiquitin-Proteasome-Network in Prostate Carcinoma

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Summary

Objective: Investigating the role of the ubiquitin-proteasome-network in prostate cancer (PCA) and eliciting potential markers for this disease. The ubiquitin-proteasome-network represents a key player in the maintenance of cellular homeostasis as a result of its fundamental function in the regulation of intracellular protein degradation. Members of this network play a role in the biology of haematological and solid tumours.

Subjects and Methods: Tumour-cells and normal epithelial-cells from 22 prostatectomy specimens were isolated by laser microdissection. Prostate biopsies from healthy individuals served for technical calibration and as controls. Transcript levels of eight selected genes with E3-ubiquitin-ligase activity (labelling target proteins for proteasome degradation) and two genes belonging to the proteasome-multienzyme-complex itself were analyzed by quantitative real-time RT-PCR.

Results: The proteasome-genes PSMC4 and PSMB5 and the E3-ubiquitin-ligase NEDD4L were significantly and coherently up-regulated in prostate cancer cells compared to the corresponding adjacent normal prostate tissue. Transcription of the E3-ubiquitin-ligase SMURF2 was significantly higher in organ confined tumours (pT2) compared to non organ confined cancers (pT3).

Conclusions: The results indicate a role of PSMC4 and PSMB5 and the E3-ubiquitin-ligase NEDD4L in prostate tumorigenesis, while SMURF2 downregulation could be associated with clinical progression. NEDD4L and SMURF2 both target TGF- β for degradation. This reflects the pleiotropic role of the TGF β -signalling pathway acting as a tumoursuppressor in normal and precancerous cells, but having oncogenic properties in progressing cancer. Further studies have to elucidate, if these alterations could represent clinically relevant PCA-diagnostic and progression markers.

Key-words: proteasome; E3 ubiquitin ligase; transcription; prostate carcinoma; tumour marker

Introduction

Knowledge about expression patterns of genes involved with the control of cellular homeostasis is important to understand malign transformation and cancer progression and could improve cancer diagnosis and staging. Cellular disposal of molecules is essential for normal cellular homeostasis. The ubiquitin-proteasome network (UPN) is accountable for the selective cellular removal of misfolded, damaged, and unassembled polypeptide chains and regulatory proteins (1-2). It regulates numerous basic cellular processes such as cell cycle progression, signal transduction, proliferation, apoptosis, modulation of surface receptors and regulation of tumour suppression proteins (2-4) (see figure 1). Proteins for proteasome degradation are labelled by conjugation of multiple ubiquitin moieties (ubiquitination). Ubiquitin, an abundant highly-conserved small 76 amino acid protein found only in eukaryotes, is transferred by a cascade of three enzyme types: protein E1 activates the C-terminus of the ubiquitin, while E2 transfers the activated ubiquitin to one of multiple E3 ubiquitin ligase proteins. E3-ubiquitin ligases are responsible for the specificity of protein recognition and ubiquitination, thus ensuring the fidelity of selective proteolysis. The proteasome is a 2.2MDa barrel-shaped multicatalytic proteinase complex, the 26S proteasome, composed of two complexes, a 20S core particle and a 19S regulator cap. The 20S core contains the protease subunits and is composed of four rings (two identical peripheral alpha-rings and two identical central beta-rings), each ring containing 7 non-identical subunits (resulting in 2 x 14 non-identical subunits). The 19S cap (alternative name PA700) is made of two sub-complexes, the lid and the base, and a total of 17 peptide molecules. It contains 11 non-ATPase subunits and six ATPases that unfold the substrates and translocate them into the core for proteolysis (substrate specificity; for further information see <http://www.atlasgeneticsoncology.org/Deep/UbiquitininCancerID20083.html>). The proteasome is found attached to the endoplasmatic reticulum (ER) and in the nucleus, but is also found free in the cytosol (1-2). Alterations of this critical degradation machinery play an important role in tumourigenesis and cancer progression (4-6). Its hierarchical character further provides a platform for a novel approach to innovative anticancer therapy (5).

The aim of this pilot study is a compact analysis of the transcription patterns of selected important “representatives” of those arms of the UPN, which control (prostate) cancer-relevant pathways (eight E3-ubiquitin ligases and two integral members of the proteasome, see table 1 and figure 1). Such findings could provide valuable information on prostate cancer biology and further indicate potential diagnostic and prognostic markers.

Subjects and Methods

Tissue Samples, cryo-section and laser microdissection

Informed consent from the patients involved in this project and the healthy volunteers (calibrator, see below) as well as the approval from the institutional review board (IRB) were obtained according to the directions of the German authorities.

Twenty-two PCA-patients undergoing radical prostatectomy were included in this study (Table 2; the identical collective has been investigated in an earlier study (see Hellwinkel et al. 2009 (7)). None of the patients had been treated with radio-, chemotherapy or ablative hormonal therapy. Tumour- and normal tissue samples were taken with a 6 mm punch biopsy instrument from tumourous and non-tumourous areas as described before (8). The specimens were incubated in RNAlater (Qiagen) at 4°C for two hours and then long-term stored at -80°C. To select epithelial (tumour and adjacent normal) cells for gene expression analyses, we performed laser micro-dissection as previously described (7-8). First, the specimens were briefly thawed on ice and then cryo-sections of up to 15 µm thickness were prepared and transferred to RNase-free Membrane Slides (P.A.L.M.® Microlaser Technologies AG, Bernried, Germany). The cryo-sections were air-dried and stained with cresyl violet following standard procedures. Additionally, representative sections were HE-stained for pathological examination and archival storing. To produce samples of highest uniformity, tissue areas containing either only tumour (always both Gleason components at proportions corresponding to the histopathological data given in table 2) or normal prostate duct cells (1000 – 3000 µm²) were micro-dissected and collected employing an UV-laser based laser microdissection system (P.A.L.M.® Microlaser Technologies AG, Bernried, Germany) according to manufacturer protocols. Micro-dissected samples were then collected in RNA-lysis buffer (RLT; Qiagen, Hilden, Germany). An existent set of histologically unsuspecting prostate biopsies from 24 healthy volunteers (for detailed information see supplemental table) served as an additional calibrator for RT-PCR analyzes.

RNA extraction and quality control

Collected tissue samples were processed applying the RNeasy Micro Kit (Qiagen) according to manufacturer protocols. From each sample, one micro-liter of the final eluted RNA volume (14 µl) was applied for analysis in an Agilent Bioanalyzer micro-capillary electrophoresis system (RNA 6000 Pico Kit, Agilent, Waldbronn, Germany). As initial quality check and for further comparisons with processed tissue, a complete fresh section of each biopsy was directly transferred to lysis buffer and processed as described above before starting the micro-dissections.

cDNA synthesis and real-time quantitative (RT-)PCR

From each selected sample RNA amounts of 20 ng (estimated on the basis of the Bioanalyzer analysis) were reverse transcribed to cDNA in a volume of 20 μ l applying random primers and the cDNA-archive kit (Applied-Biosystems, Darmstadt, Germany) according to manufacturer protocol.

Single transcript quantitative (q-) RT-PCRs were run in 20 μ l solutions (in duplicates) on the cDNA equivalent of each 1 ng RNA per sample using the PCR-master mix from Applied Biosystems as indicated by the producer. *Assays on demand* (Applied Biosystems; each 1 μ l per PCR-sample) were employed as pre-designed primer-pair and probe combinations to amplify specifically selected transcripts of the ubiquitin-proteasome network (UPN) as shown in table 1. Hypoxanthin-ribosyltransferase (HPRT) and Transferrin-receptor (TFRC) as house-keeping genes with low transcription rates served as independent internal controls (“house-keeping” genes; HKs). Real-time PCR-cycling was run using a Gene-analyser 7900 (Applied Biosystems) on 96-well fast-plates following manufacturer protocols. The relative quantification results (RQs) were calculated using the $\Delta\Delta$ Ct-method as specified by the producer; here, RNA from a pool of 25 healthy controls served as calibrator (set as 1; see above).

Statistic evaluation

Each experiment was run in duplicates. Descriptive and basic statistics involved calculation of means and/or medians and 95% confidence intervals. Relative quantification results (RQs; means of transcription levels normalized separately on 2 HKs and expressed as x-fold transcription compared to the [healthy control] calibrator set as 1) of all transcripts were first compared between the tumour- (RQ(T)) and the corresponding adjacent normal samples (RQ(N)). Non-parametric paired Wilcoxon-tests were applied to test differences between these groups for significance. Then, X-fold transcript quantities in tumours compared to the corresponding adjacent normal tissues were calculated by division of individual RQ(T)- by RQ(N)-values. The resulting RQ(T/N)-values were grouped depending on tumour stages (pT2 vs. pT3) or histopathological grades (Gleason-sum \leq 6 vs. Gleason-sum \geq 7). Non-parametric unpaired Mann-Whitney-U-tests were applied to test differences between tumour stages or histopathological grades.

Bivariate analyses were applied to test transcription rates of all genes for correlations (for this purpose, Spearman-Rho correlation coefficients were calculated).

Transcript level differences were considered to be significant when means of the double-HK normalized RQ sets displayed a p-value < 0.05 .

Statistic evaluation was performed using Microsoft-Excel and SPSS (SPSS-company).

Results

Transcription levels in Tumour vs. adjacent Normal tissue

All analysed transcripts could be analysed by real time RT-PCR in tumour and normal tissues of all samples (Tab 3). As demonstrated by paired Wilcoxon-tests, the proteasome-fractions PSMC4 (1.57-fold, figure 2a) and PSMB5 (2-fold, figure 2b) and the E3-ubiquitin-ligase NEDD4L (2.75-fold, figure 2c), were significantly up-regulated in tumour compared to the corresponding adjacent normal tissues. Another E3-ubiquitin-ligase, HECW1, was even 5-upregulated in tumour cells; however, this result did not meet our significance criteria.

Transcription levels in relation to tumour stage and histology

To evaluate transcription differences in tumours (normalized on adjacent normal tissue) of different clinical stages (pT2 vs. pT3) as well as in tumours of varying histopathological appearances (Gleason sum scores ≤ 6 vs. ≥ 7) Mann-Whitney-U tests were applied. Here, only one gene, SMURF2, demonstrated a significantly higher level of transcription in pT2- than in pT3-tumours (figure 3). However, the analysis on tumour histology did not reveal any significant difference in transcript levels among investigated transcripts between Gleason ≤ 6 and Gleason ≥ 7 tumours (not shown).

Correlation between investigated transcripts

To explore the coherence of transcription in tumour cells we employed multiple bivariate analyses of all examined transcripts and tested correlation coefficients by Spearman-Rho tests. All significant correlations were found to be positive indicating a coherent expression manner of the investigated UPN-genes: high positive correlations ($p \leq 0.001$) were found between the proteasomal units PSMC4, PSMB5 and the E3-ubiquitin ligase UBE3A and between the E3-ubiquitin ligases UBE3A and CBL or SMURF2 respectively (not shown). As displayed in figure 4, transcription rates of PSMC4, PSMB5 and NEDD4L which demonstrated significant up-regulation in tumour cells (figure 2a-c) also showed significant positive correlation with each other, signifying consistent and simultaneous expression patterns.

Discussion

The critical role played by the ubiquitin-proteasome protein degradation system in cell cycle regulation, proliferation, apoptosis and regulation of tumour suppressor genes makes it a target for oncogenic alterations (4, 6, 9). The UPN comprises of a large number of components; hundreds of E3-ubiquitin ligases have been described (10-11). To obtain compact information, we limited the number of investigated components to “representatives” of specific UPN-branches, which control important cancer relevant pathways. Our survey of course can not claim to be exhaustive, but it allows a quantitative assessment of the biological relevance and potential clinical applications of the UPN at the transcription level at a moderate effort.

Three of the investigated transcripts, PSMC4, PSMB5 and NEDD4L, showed moderate up-regulation in tumours compared to adjacent normal tissue of prostate cancer patients (figure 2a-c, table 3). The mean differences were relatively moderate (between approx. 1.6 and to 2.8-fold) but significant even with the limited number of samples of the investigated collective (22 tumour-normal tissue pairs). This annotation permits the suggestion of a possible role in prostate cancer biology.

PSMC4 and PSMB5 are members of the two complexes of the proteasome responsible for recognition and up-take of ubiquitin tagged substrates into the proteasome (19S regulatory complex) and their ATP-dependent proteolysis (20S core complex), respectively. Over-expression of these genes would enhance proteasomal degradation of some critical cellular regulatory proteins including tumour suppressors and modulators of apoptosis. Thus, the inhibition of the proteasome represents a promising strategy for cancer therapy. In recent studies, proteasome inhibitors display pro-apoptotic effects (5, 12-13). The first proteasome inhibitor (bortezomib, Velcade, PS-341) has now been approved for the treatment of advanced multiple myeloma (14-15). Additionally, trials for other haematological and solid tumours including PCA have been initiated (16-19).

NEDD4L acts as an E3-ubiquitin ligase and thereby regulates diverse cellular processes like excretion, plasma membrane channel regulation, protein catabolism and TGF-beta signalling. NEDD4L negatively regulates TGF- β signalling by ubiquitination of TGF- β receptor type I (TGF β -R1) and Smad2 (20). TGF- β (TGF- β 1) itself is a pleiotropic growth factor with tumour suppressor activity in normal prostate epithelia, which maintains epithelial homeostasis by inhibiting cell proliferation, stimulating cell differentiation and inducing apoptosis (21-24). The up-regulation of NEDD4L-transcription and subsequently increased protein-levels in prostate cancer cells found here would suggest higher tagging of its substrate TGF- β R1 for ubiquitination and subsequent proteasome degradation and thus decrease the protein-levels of its targets. This would result in downregulation of the TGF- β -cascade and its tumour suppressive functions. Furthermore, preclinical models suggest an

androgen responsiveness of NEDD4L (25). However, the effect of androgens on the ubiquitine network has not been validated in functional studies.

In an earlier study, Hu et al. (26) described downregulation of NEDD4L protein levels in prostate cancer. At the first instance, this observation appears to be contradictory to our results. However, we have to take into account, that Hu et al. compared their prostate cancer collective with cancer-adjacent and cancer-free benign prostate hyperplasia (BPH) samples. Now, prostate cancer represents a proliferative aberration dissimilar from benign hyperplasia; it does not develop from BPH. It is consequently quite problematic to state a downregulation of NEDD4L-expression during carcinogenesis based on a comparison of BPH and PCA only (as done by Hu et al.). In contrast, we compared prostate cancer and cancer-adjacent histologically *normal* prostate glands (not BPH!). This applies better to the hypothesis of an orthotopic and endogenous appearance of PCA-foci from histologically normal prostate gland tissues (eventually due to field cancerization (27)) and allows feasible conclusions on the natural history of gene transcription in PCA. Thus, a direct comparison of the study from Hu et al. and our own analysis is not viable.

PSMC4, PSMB5, and NEDD4L displayed a remarkable co-transcription (figure 4), indicating contiguously enhanced protein levels of these genes. This suggests an increased ubiquitination of substrate proteins of NEDD4L (and other E3 ubiquitin ligases), which is linked with a coherently augmented degradation capacity - demonstrating the coherence of expression rates of many genes belonging to the ubiquitin-proteasome network. A putative common regulation mechanism of the UPN could be a target for selective treatment strategies of PCA.

Only one transcript showed discriminated transcription levels at different clinical tumour stages: SMURF2 displayed moderate but significantly enhanced transcription level in pT2 tumours compared to the higher pT3-stage (figure 3). This would suggest that different protein levels of SMURF2 are required at different stages of tumour progression. SMURF2 targets Smads 1, 2, 3 and 7 as well as Smurf1 for ubiquitination and degradation and is (consequently) also a negative regulator of TGF- β signalling (28-33). At the first view, it appears illogical that an inhibitor of the TGF β -pathway – which is known to have tumoursuppressive properties in normal prostate (s. a.) – is seemingly fairly downregulated in progressing tumour. In this respect, it has to be reminded that TGF- β possesses pleiotropic properties. In malignant prostate cells, it promotes tumour growth, induces angiogenesis, supports extracellular matrix modification and inhibits host immunosuppression (21-23, 34-35). A downregulation of SMURF2 thus should support the oncogenic TGF β -action in malign cells.

From the clinical point of view, this work was carried out on a small but histologically well defined (laser microdissection) tumour cohort and acts as a pilot study. The results justify further exhaustive screening studies on all known members of the UPN. Independently, the next obligatory step is to

analyse the protein levels of those genes found here to be differentially transcribed. Immunohistochemistry (IHC) could provide such information in-situ - however (in contrast to RNA-studies) only semi-quantitatively and with a certain interpretative ambiguity. Thus, tissue micro arrays on large tumour cohorts along with comprehensive statistical analyses are mandatory to achieve a sufficient confidence of protein expression surveys via IHC. Such studies will clarify the diagnostic and therapeutic relevance of the provided results.

Conclusion

Parts of the ubiquitin-proteasome network (PSMC4, PSMB5 and NEDD4L) demonstrated significant and coherent gene-expression upregulation in prostate cancer cells. SMURF2 seems to be downregulated during clinical progression. Dysregulation of these genes display a potential role in prostate tumourigenesis and progression and could be further applied as diagnostic and therapeutic strategies.

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Tables and Illustrations

See attached Adobe pdf- and MS-Word files.

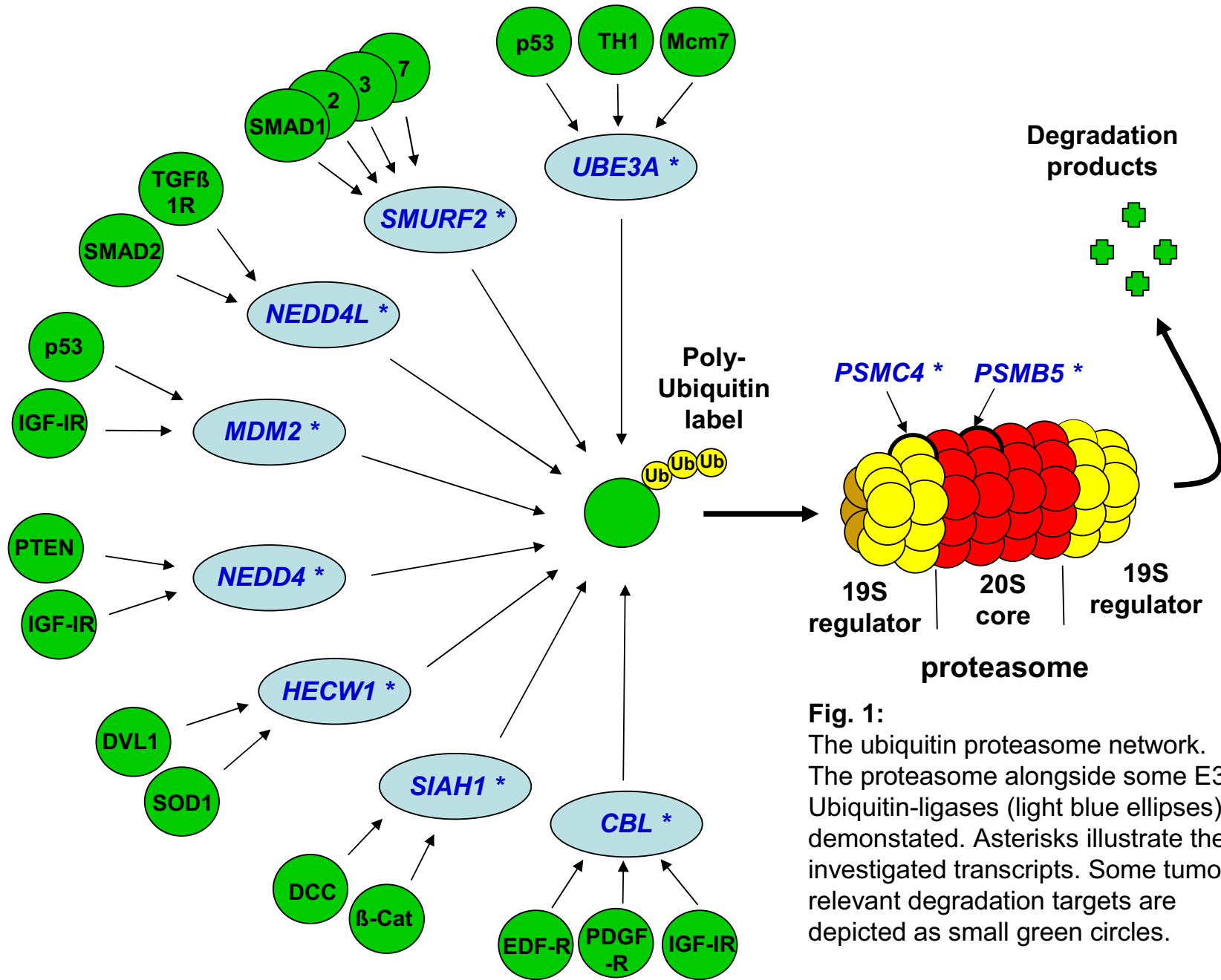


Fig. 1: The ubiquitin proteasome network. The proteasome alongside some E3-Ubiquitin-ligases (light blue ellipses) are demonstrated. Asterisks illustrate the investigated transcripts. Some tumor-relevant degradation targets are depicted as small green circles.

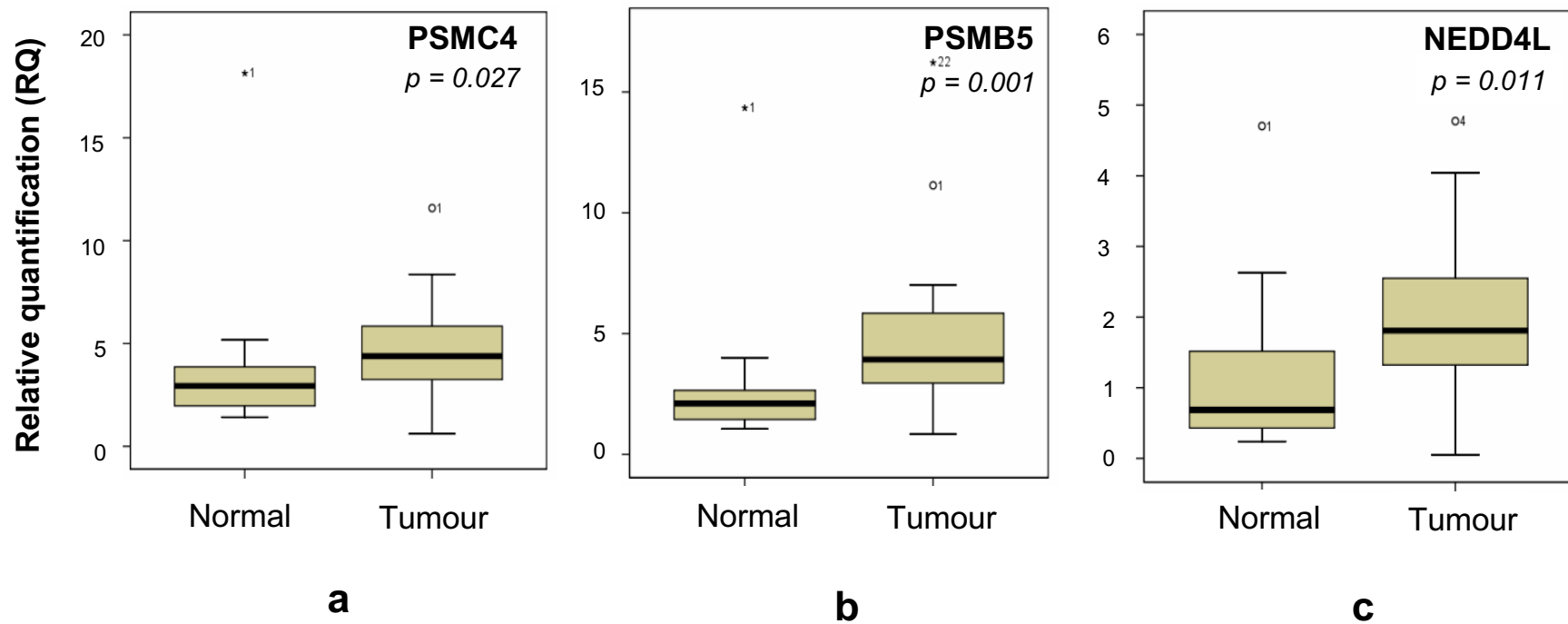


Fig. 2: PSMC4- (a), PSMB5- (b) and NEDD4L- (c) transcript expression levels in prostate tumours and adjacent normal tissues (RQ: relative quantity).

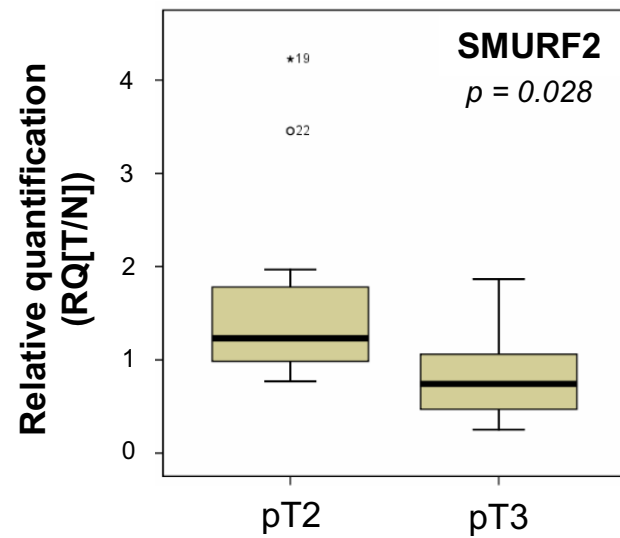


Fig. 3:

Levels of SMURF-transcript expression in pT2- and pT3- prostate tumours (RQ[T/N]: relative quantity in tumour compared to adjacent normal tissue).

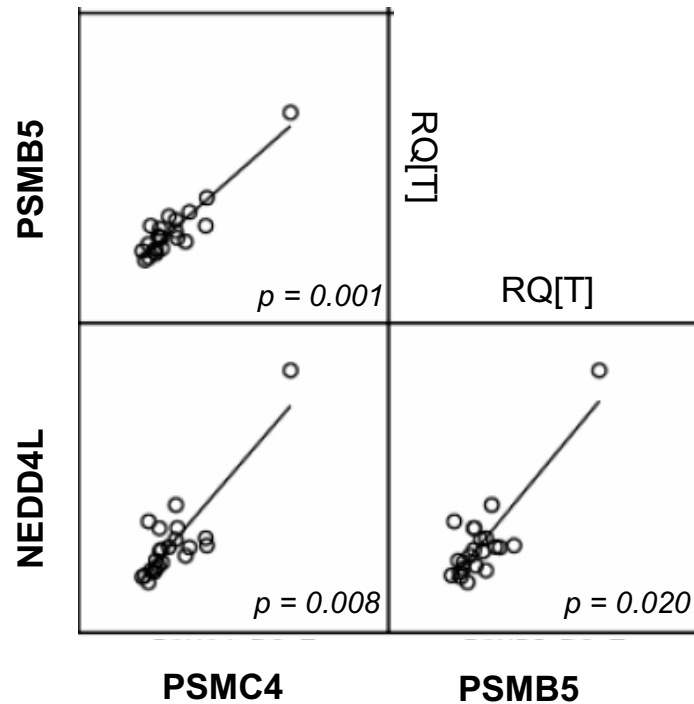


Fig. 4:

Correlations of PSMC4-, PSMB5- and NEDD4L-transcript expression in prostate tumours (RQ[T]: relative quantity in tumour). The axes are scaled linearly with RQ[T]-values ascending from left to right (x-axes) and from bottom to top (y-axes). Each circle depicts two-dimensionally the expression rates of two indicated transcripts in one tumor sample.

Gene-Symbol (TaqMan assay)	Name	Aliases	Chromosome location	Function / Role in Cancer
UBE3A (Hs00963673_ml)	Ubiquitin protein ligase E3A	E6-AP; Human papilloma virus E6-associated protein; FLJ26981	15q11-q13	E3 ubiquitin-protein ligase. Targets p53, itself, Blk, TH1 for degradation. Mediates Mdm2, HHR23A ubiquitination. Over-expressed in breast cancer compared to adjacent normal tissue. Downregulated in invasive breast and prostate carcinomas compared to adjacent normal tissue.
SMURF2 (Hs00909283_ml)	SMAD specific E3 ubiquitin protein ligase 2	E3 ubiquitin ligase SMURF2; DKFZp686F0270	17q22-q23	E3 ubiquitin-protein ligase. Targets Smad1, Smad2, Smad3, Smad7, Smurf1 for degradation. Regulates TGF- β signalling. Plays a role in breast cancer progression. Overexpressed in oesophageal squamous cell carcinoma.
NEDD4L (Hs00969334_ml)	Neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4-2; hNedd4-2; KIAA0439; Ubiquitin-protein ligase NEDD4-like; FLJ33870	18q21	E3 ubiquitin-protein ligase. Targets ENaC for degradation. Mediates Smad2, TGF- β type I receptor degradation. Mediates ubiquitination of itself, TTYH2, TTYH3. Decreased expression in prostate cancer compared to benign prostate tissue.
MDM2 (Hs00234753_ml)	Mdm2 p53 binding protein homolog (mouse)	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein; Ubiquitin-protein ligase E3 Mdm2; MGC71221	12q14.3-q15	E3 ubiquitin-protein ligase. Targets p53 for degradation. Targets itself for ubiquitination. Mediates IGF-IR ubiquitination. Mdm2 is associated with bladder cancer. Over-expressed in prostate carcinoma. Associated with prostate cancer growth and progression. Mdm2 inhibition could provide novel approach for anti-tumour therapy against human prostate cancer.
NEDD4 (Hs00406454_ml)	Neural precursor cell expressed, developmentally down-regulated 4	NEDD4-1; RPF1; Receptor-potentiating factor 1; MGC176705	15q	E3 ubiquitin-protein ligase. Participates in ENaC ubiquitination. Mediates ubiquitination of PTEN, IGF-IR, CNrasGEF. Participates in Melan-A ubiquitination in melanoma cells. Associated with colorectal and gastric cancerogenesis. Up-regulated in prostate and bladder cancer.
HECW1 (Hs01546585_ml)	C2 and WW domain containing E3 ubiquitin protein ligase 1	NEDL1; HECT type E3 ubiquitin ligase; NEDD4-like ubiquitin-protein ligase 1; KIAA0322	7p14.1-p13	E3 ubiquitin-protein ligase. Targets Dvl1 for degradation. Mediates ubiquitination of mutant SOD1. Enhances p53 apoptosis.
SIAH1 (Hs02339360_ml)	Seven in absentia homolog 1 (Drosophila)	Seven in absentia homolog; Siah-1; Siah-1a; hSIAH1; HUMSIAH; Sonic hedgehog homolog; FLJ08065	16q12	E3 ubiquitin-protein ligase. Targets DCC, itself, polycystin-1 for degradation. Mediates degradation of TRB3, synaptophysin, β -catenin. Down-regulated in and associated with advanced stages of hepatocellular carcinoma. Associated with gastric cancerogenesis.
CBL (Hs01011446_ml)	Cas-Br-M (murine) ecotropic retroviral transforming sequence	C-CBL; Oncogene CBL2; CBL2; RNF55	11q23.3	E3 ubiquitin-protein ligase. Targets EDF-R for degradation. Mediates ubiquitination of PDGF-R, Vav. Participates in IGF-IR ubiquitination. Plays a role in human tumorigenesis. Associated with gastric tumorigenesis and progression. May function as basal cell marker for prostate cancer.
PSMC4 (Hs01035007_ml)	Proteasome (prosome, macropain) 26S subunit, ATPase, 4	Proteasome 26S ATPase subunit 4; Protease 26S subunit 6; MGC8570; MGC13687; MGC23214	19q13.11-q13.13	ATPase subunit of the Base of 19S regulator cap complex of the 26S Proteasome that confers ATP dependency and substrate specificity.
PSMB5 (Hs00605652_ml)	Proteasome (prosome, macropain) subunit, beta type, 5	Proteasome subunit, beta type, 5; Proteasome beta 5 subunit; MB1; Proteasome subunit X; Proteasome chain 6; Proteasome subunit MB1; MGC10214	14q11.2	Beta subunit of the Inner ring of 20S core complex of the 26S Proteasome, accountable for proteolyses. Over-expressed in breast cancer compared to adjacent normal tissue. Mutation of PSMB5 associated with bortezomib resistance.

Tab 1: Information on the analysed transcripts (catalogue numbers of the used TaqMan real-time RT-PCR assays from Applied Biosystems are given between brackets). Detailed data and corresponding references can be obtained at Bioinformatic Harvester (<http://harvester.fzk.de/harvester/>)

Patient-Nr	*Age	pT	Gleason-Score	Gleason-Sum	pN	PSA (ng/ml)	free PSA (ng/ml)
T2_007	67	pT2a	3+3	6	NX	6.1	0.97
T2_017	65	pT2c	3+3	6	NX	4.01	0.88
T2_022	60	pT2c	4+3	7	N0	7.6	1.1
T2_023	66	pT2c	4+3	7	N0	5.38	0.53
T2_024	68	pT2c	4+3	7	N0	8.21	1.33
T2_025a	64	pT2c	4+4	8	N0	7.28	0.89
T2_025b	64	pT2c	3+3	6	N0	7.28	0.89
T2_026	63	pT2c	3+3	6	NX	3.5	0.61
T2_027	54	pT2c	3+3	6	NX	5.56	0.73
T2_029	63	pT2c	3+3	6	NX	9.33	0.82
T2_030	58	pT2c	3+3	6	NX	3.83	0.61
T3_003	59	pT3b	4+4	8	N1	7.14	0.63
T3_004	67	pT3a	3+4	7	N1	24.99	1.55
T3_008	41	pT3b	5+4	9	N1	3.03	0.39
T3_012	65	pT3b	3+4	7	N0	27.43	1.36
T3_013	59	pT3b	3+4	7	N0	3.44	1.16
T3_016	70	pT3a	4+4	8	N0	1.91	0.75
T3_017	61	pT3a	4+5	9	N0	13.13	1.67
T3_019	62	pT3a	3+4	7	N0	18.93	1.98
T3_021	54	pT3a	4+5	9	N0	9.64	0.8
T3_022	63	pT3a	4+3	7	NX	14.14	0.96
T3_023	62	pT3a	4+3	7	N0	4.12	0.08

Tab 2: Patient data: clinical history & tumour histology (Nr: Number; *: Age at surgery; LN: Lymph node; N0: no tumour in LN; N1: tumour in LN; NX: LN not analysed)

Transcript	RQ(T/N)		RQ(T/N) - Average	p
	HPRT	TFRC		
PSMC4	1.44752519	1.69018234	1.56885376	0.027
PSMB5	1.93023847	2.22326931	2.07675389	0.001
CBL	0.9966721	1.07921078	1.03794144	0.554
HECW1	4.03811662	6.05025391	5.04418527	0.050
MDM2	1.88137563	2.08311161	1.98224362	0.216
NEDD4	1.11850741	1.22752684	1.17301712	0.373
NEDD4L	2.50125662	3.00693257	2.75409459	0.011
SIAH1	1.2819827	1.72419694	1.50308982	0.602
SMURF2	1.27116287	1.42283849	1.34700068	0.655
UBE3A	1.00688305	1.19421546	1.10054926	0.587

Tab 3: Increase in transcript expression [RQ(T/N)] in tumour (p: significance; RQ(T/N): transcript expression in prostate tumour compared to adjacent normal tissue. Results at normalisation on the house-keeping genes HPRT (Hypoxanthinribosyl-transferase) and TFRC (Transferrin-receptor) and means are shown.

