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Transcription alterations of genes of the Ubiquitin-Proteasome Pathway in Prostate Carcinoma

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Mum

Dad

Uncle Marc

Abbreviations and Acronyms

AAA	ATPases associated with diverse cellular activities
ATP	Adenosine triphosphate
ß-catenin	Beta-catenin
Blk	B lymphoid tyrosine kinase
BPH	Benign prostate hyperplasia
cDNA	complementary Deoxyribonucleic acid
CML	Chronic myeloid leukaemia
CNrasGEF	Cyclic nucleotide ras GEF
Ct	Cycle - threshold
CSF1	Colony stimulating factor 1 (macrophage)
DCC	Deleted in colorectal cancer
DEPC water	Diethylpyrocarbonate water
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
Dvl1	Dishevelled-1
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
EGF-R	Epidermal growth factor receptor (EGFR)
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
Fig	Figure
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
HE	Haematoxylin eosin
HHR23A	UV excision repair protein Rad23 homolog A
HPRT	Hypoxanthine ribosyltransferase
HPV	Human papilloma virus

HRG	Heregulin-β1
IGF-IR	Insulin-like growth factor-I receptor
Jak-STAT	Janus kinases, Signal transducers and activators of transcription
LHRH	Leutinizing hormone releasing hormone
LNCaP	Lymph Node Carcinoma of Prostate
Mcm7	Multicopy maintenance protein 7
MHC	Major histocompatibility complex
OCT-compound	Optimal cutting temperature compound
p21	Cyclin-dependent kinase inhibitor 1 (p21; Cip1) [CDKN1A]
p53	Tumour supressor protein p53 [TP53]
PBS	Phosphate buffered saline
PC	Prostate carcinoma (prostate cancer)
PDGF-R	Platelet-derived growth factor receptor (PDGFR)
PIN	Prostatic intraepithelial neoplasma
PSA	Prostate specific antigen
qRT-PCR	quantitative Reverse Transcriptase – Polymerase Chain Reaction
RFDD-PCR	Restriction fragment differential display polymerase chain reaction
RNA	Ribonucleic acid
mRNA	messenger Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
RLT	Rolling liquid transporter
RNF11	RING finger protein 11
RQ	Relative quantitation ≡ Relative Expression
SGK	Serum/glucocorticoid regulated kinase
SMAD1	Mothers against decapentaplegic homolog 1
SMAD2	Mothers against decapentaplegic homolog 2
SMAD3	Mothers against decapentaplegic homolog 3

SMAD7	Mothers against decapentaplegic homolog 7
SMURF1	Smad specific E3 ubiquitin protein ligase 1 (Smurf1)
SOD1	Superoxide dismutase-1
SPSS	Superior Performing Software Systems
Tab	Table
TFRC	Transferin-receptor
TGF	Transforming growth factor
TGF-ß	Transforming growth factor-beta (TGF-beta; TGF-beta 1; TGF-ß 1)
TGF-ß receptor 1	TGF-beta receptor I (TGFBR1; TGF-ß type I receptor)
TH1	Trihydrophobin 1
TNM	Tumor, Nodes, Malignancy
TRAP-δ	Translocon-associated protein-delta
TTK	TTK protein kinase
TTYH2	Tweety homolog 2 (Drosophila)
TTYH3	Tweety homolog 3 (Drosophila)
UKE	Universitätsklinikum Hamburg-Eppendorf
UPP	Ubiquitin-Proteasome Pathway
UV	Ultra violet
Vav	Vav guanine nucleotide exchange factor

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1. Hypothesis and Principal task

1.1 Hypothesis

The pleiotropic pathways regulated by ubiquitin-proteasome-pathway-dependent protein degradation depict the fundamental importance of regulated proteolysis in retaining normal cellular homeostasis. It is postulated that expression aberrations of different proteins of this intricate enzymatic machinery play a role in the evolution of malignancies in the prostate and provide a potential molecular biological marker system for prostate carcinoma as well as further contribute to the development of novel prostate carcinoma therapy.

1.2 Principal task

UPP is a major player in regulatory processes in the cell. Alterations of this critical degradation machinery are involved in many human pathologies and this makes it a vital target for tumourigenesis and cancer progression. (Burger and Seth 2004; Mani and Gelmann 2005; Devoy et al. 2005) Its hierarchical nature further provides a platform for specific intervention of molecular targets that would allow a novel approach to innovative anti-cancer therapeutics. (Burger and Seth 2004) Investigating abnormal transcription patterns in the ubiquitin-proteasome system mark the principal goal of this study and would contribute to a better understanding of prostate cancer biology. Such findings could provide substantial information for the identification of highly sensitive and specific novel diagnostic markers and therapeutic strategies for prostate cancer via targeting the ubiquitin-proteasome cascade.

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2. Introduction

This chapter provides information on prostate carcinoma, the ubiquitin-proteasome pathway and the transcripts, whose expression patterns would be investigated.

2.1 Prostate carcinoma

This section briefly reviews the occurrence, pathophysiology, classification, diagnosis and therapy of prostate carcinoma.

2.1.1 Epidemiology

Cancer is a leading cause of death, accounting for 13% of all deaths in the world in 2005. Prostate cancer stands on position six of the leading cause of global deaths among men after lung, stomach, liver, colorectal and oesophagus cancer. In Germany it ranks third among the leading causes of cancer-related deaths after lung- and intestinal cancer. Prostate carcinoma is the second most common cancer in men and the most common urologic malignant tumour in males, especially at advanced age. Prevalence is identical in the different ethnic groups and demonstrates higher frequencies than incidence rates. Incidence and mortality rates on the other hand, vary across the globe with highest incidences in African-American men. In addition PC incidence increases remarkably with age, generally detected in men over the age of 50, with more than 50% in men above 70 years of age. The 5-year survival rate is approximately 82%. (WHO 2006; Nelen 2007; Hautmann and Huland 2006; Clements 2007; Bertz et al. 2006; Krebsinformationsdienst 2003; National Cancer Institute 2005)

2.1.2 Aetiology and Pathogenesis

The exact aetiology of prostate cancer is unknown. Postulations include genetics, hormones, ethnic disparity, age, diet and infectious diseases. Genetic predisposition is responsible for 10 -15% of all prostate cancers.

First grade (father, brother) or second grade (grandfather, uncle) relations account for a two to three time increase in PC risk and a 10 to 20 years earlier establishment of the disease. Genetic alterations on chromosome 1 and the X-chromosome have been found in these patients. A shared familiar risk for prostate and breast cancer has also been implicated. African-American men demonstrate higher incidence and aggressiveness of cancer as well as tumour diagnosis at an earlier age and higher age-specific mortality compared to Caucasian men, who in turn show higher incidences than Asian male. Eunuchs do not develop prostate carcinoma and PC progression is generally androgen dependent. Eating habits, especially animal protein and fat and environmental factors like exhaust fumes and air pollution influence prostate cancer growth. The direct link between the prostate gland and the urethra suggest that viral and venereal infections may induce prostate cancer genesis. Current evidence substantially holds up to the contention that PIN is a precursor for prostatic adenocarcinoma. (Hautmann and Huland 2006; Schwab 2001; Hanno et al. 2001; Rubin et al. 2005).

2.1.3 Anatomy and Pathophysiology

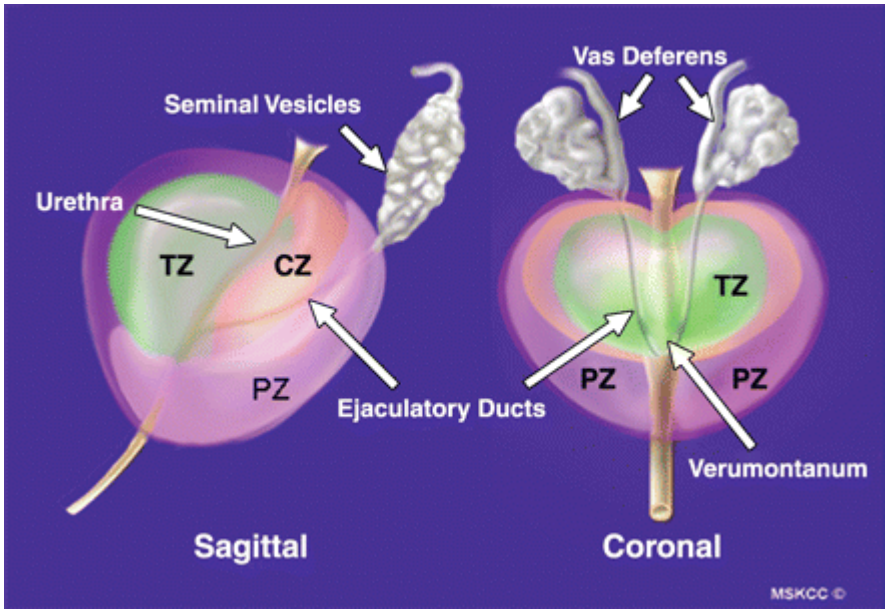


Fig 2.1.3: Zonal anatomy of prostate; PZ = peripheral zone, TZ = transition zone, CZ = central zone (Curran S et al., AJR Am J Roentgenol, 2007)

Prostate cancer is a malignant neoplasm of the prostate gland. The prostate, a genitourinary gland in male, contributes to normal sexual function and also plays a role in bladder control [Fig 2.1.3]. A trivial fraction of prostate cancers arise from the CZ. The TZ marks the area of origin of BPH and 10% of prostate cancers. 90% of prostate cancers originate in the PZ. (Schwab 2001; Clements 2007; Hautmann and Huland 2006)

Adenocarcinomas of the acini make up 95% of prostate cancers. Other histology variants are rare. Prostate carcinoma demonstrates slow growth rate, high grade heterogeneity and multifocality. (Clements 2007; Hautmann and Huland 2006; Mostofi et al. 2002; Schwab 2001) Malignancy is determined by deviations from normal gland architecture. These deviations demonstrate diverse variants of growth patterns from acinar over cribriform to anaplastic tumours. Capsular penetration and invasion of the seminal vesicles indicate tumour progression. Tumour invasion occurs primarily lymphogenous along perineural lymphatic channels and later haematogenous. Obturator lymph nodes mark the first site of metastatic spread and its invasion is applied for lymph node – staging. Osteoblastic metastasis marks the main location of haematogenous spreading, particularly in the vertebral column. Hepatic metastases are unusual. Metastases in order of decreasing frequency include lymph nodes, bones, lung, and liver. The major cause of death is an extensive dissemination of the tumour habitually with terminal pneumonia or sepsis (Rubin et al. 2005; Hautmann and Huland 2006; Clements 2007)

2.1.4 Staging and Grading

TNM staging and Gleason grading systems are the two most used classification systems for PC. The TNM staging system is the international standard for prostate carcinoma staging [Tab 2.1.4]. Gleason grading is based solely on the histological architecture of tumour gland formation and infiltration [Fig 2.1.4]. It recognises 5 patterns of decreasing differentiation. Pattern 1 being the most differentiated and pattern 5 the least. The sum of the primary and second most prevalent pattern, make the Gleason score,

thereby incorporating the heterogeneity of PC architecture: e.g. Gleason 3+4=7. The lower the score the better the prognostic outcome. Gleason score is a potential prognostic factor for natural history prediction and assessment of risk of tumour recurrence after total prostatectomy or radiotherapy. (Clements 2007; Egevad et al. 2004; Rubin et al. 2005; Schwab 2001)

2.1.5 Natural history of disease

Tumour volume correlates like in no other solid tumour with tumour aggressiveness. Micro tumours, less than 0.2cm³ and barely palpable via digital rectal examination, do not metastasise. Larger tumours, over 12cm³, almost always demonstrate metastasis. Advanced disease demonstrates 5-year mortality rates of 75% and a mortality rate of 90% at 10 years. Marked by its defined slow growth progression, only clinically apparent carcinomas have need of therapy. (Hautmann and Huland 2006; Hanno et al. 2001)

2.1.6 Clinical presentation

Clinical manifestation depends on tumour stage at time of presentation. Early stages generally stay asymptomatic while advanced tumour stages may demonstrate symptoms due to bladder outlet obstruction, like incontinence or haematuria, as seen by BPH. Bone pain, weight loss, lethargy or neurological compromise in the spine, result from metastases, are uncommon and represent very advanced disease. (Clements 2007; Schwab 2001; Hautmann and Huland 2006)

T – Primary Tumour

- TX Primary tumour cannot be assessed
- T0 No evidence of primary tumour
- T1 Clinically inapparent tumour not palpable or visible by imaging
  - T1a Tumour incidental histological finding in 5% or less of tissue resected
  - T1b Tumour incidental histological finding in more than 5% of tissue resected
  - T1c Tumour identified by needle biopsy (e.g., because of elevated PSA)
- T2 Tumour confined within prostate
  - T2a Tumour involves one half of one lobe or less
  - T2b Tumour involves more than half of one lobe, but not both lobes
  - T2c Tumour involves both lobes
- T3 Tumour extends through the prostatic capsule
  - T3a Extracapsular extension (unilateral or bilateral)
  - T3b Tumour involves seminal vesicle(s)
- T4 Tumour is fixed or invades adjacent structures other than seminal vesicles: bladder neck, external sphincter, rectum, levator muscle, or pelvic wall

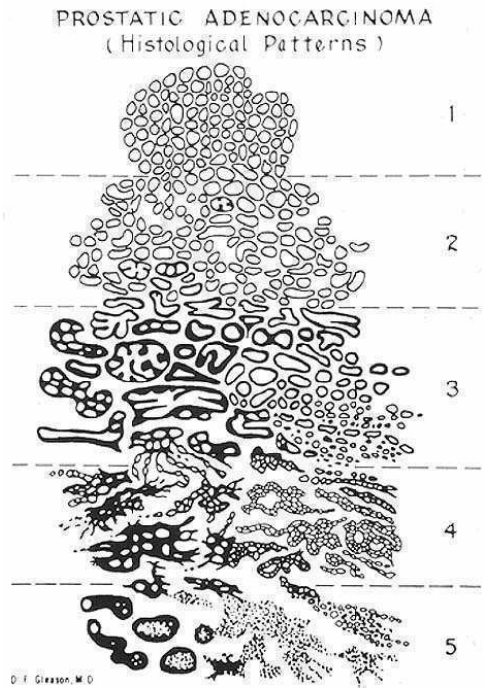
N – Regional Lymph Nodes

- NX Regional lymph nodes cannot be assessed
- N0 No regional lymph node metastasis
- N1 Regional lymph node metastasis

M – Distant Metastasis

- MX Distant metastasis cannot be assessed
- M0 No distant metastases
- M1 Distant metastasis
  - M1a Non-regional lymph node(s)
  - M1b Bone(s)
  - M1c Other site(s)

Tab 2.1.4: TNM Clinical Classification of prostate carcinoma (Sobin and Wittekind, UCIC, International Union Against Cancer, 2002)



Gleason pattern 1:

very well enclosed nodule of single, separate glands, closely packed, practically back-to-back, round or oval, fairly large in relation to most patter 3, approximately equal in size and shape and do not infiltrate adjacent benign tissue. Usually observed in transition zone carcinomas, very rare, and when present, habitually associated with pattern 2.

There are discussions on if pattern 1 be considered a tumour

Gleason pattern 2:

fairly well enclosed, nodule of single, separate glands, round or oval, with open lumens, smoothly rounded, generally larger than pattern 3 glands, more loosely arranged, not as uniform as pattern 1, with possible minimal invasion of non neoplastic tissue possible. Often observed in transition zone carcinomas

Gleason pattern 3:

glands vary in size and shape, usually small ("micro glands") in relation to pattern 1 and 2, some however moderate-sized to large, often elongated or angular, the small glands are in contrast to small poorly defined pattern 4 glands, distinct circumscribable units. Clearly infiltrative, expanding into non-neoplastic tissue. May also be cribriform glands, these are slightly larger than benign prostate glands with regular outer contours, reminiscent of intraductal mamma carcinoma and must be separated from cribriform pattern 4, intra-ductal cribriform proliferations and prostate duct adenocarcinoma. Small infiltrating pattern 3 glands are almost always associated with cribriform pattern 3.

Gleason pattern 4:

glands are either fused, poorly defined with only occasional lumen formation or cribriform. Fused glands are chains, nests or masses no longer separated by intervening stroma, may sometimes contain strands of residual stroma giving the appearance of partial separation and may have a scalloped appearance peripherally; the "hypernephromatoid" pattern is rare variant of fused glands resembling renal cell carcinoma. Poorly defined glands not originally described by Gleason are difficult to distinguish from small-acinar pattern 3 glands. Cribriform glands are either large with cribirform sheets or small with irregular infiltrating borders.

Gleason pattern 5:

virtually no glandular differentiation. This pattern is composed of solid sheets, solid cords, or single cells, as well as tumour nests with central comedonecrosis.

Fig 2.1.4: Gleason patterns of prostatic adenocarcinoma (Egevad, Allsbrook and Epstein, Johns Hopkins University, © 2004)

2.1.7 Screening

PSA is currently the single best test for PC screening and is widely used in PC diagnosis. PC-screening by means of serum PSA and DRE significantly improves early tumour discovery and yearly screening further improves PC detection. PC-screening is recommended in men above the age of 45 and for black Americans and individuals with familial disposition, screening is recommended above 40 years of age. Biopsies should be performed in men with abnormal DRE and PSA levels > 10ng/l. (Clements 2007; Hautmann and Huland 2006; Hanno et al. 2001)

2.1.8 Diagnostics and Diagnosis

Diagnosis is usually performed after further investigation via biopsy when abnormal DRE results, elevated PSA levels are detected or in tissue obtained during transurethral resection to treat BPH. Ultrasound-guided needle biopsy has become the principal method of diagnosis. Histological findings achieved from the biopsy cores together with serum PSA deliver the majority of staging information used for therapeutic decisions. (Clements 2007; Hautmann and Huland 2006; Rubin et al. 2005; Schwab 2001; Hanno et al. 2001)

Digital rectal examination:

DRE is a standard component of physical examination though not very sensitive nor specific. Palpated irregularities in case of tumour include dense rough nodules or disparity in tissue consistence and imply further analysis via TRB. (Hanno et al. 2001; Hautmann and Huland 2006)

Prostate specific antigen and other serum markers:

PSA is a glycoprotein with molecular weight of 30 000 Dalton, produced exclusively in prostate ductal epithelium and functions in the liquefaction of the seminal coagulum formed with ejaculation. Its serum level correlates with tumour volume. PSA is the most useful biochemical marker for PC detection and therapy monitoring. It demonstrates high sensitivity of almost

100%, but a rather lesser specificity. The reference serum cut-off level is 4ng/ml and biopsy is generally recommended by values >10ng/ml. Prostatitis, BPH and other processes also express increased levels of PSA. Tumours however present a 10 fold higher increase in PSA level than BPH. Serum PSA correlates directly with prostate volume and increases with age. Other classic markers like acid phosphatase, acid prostate phosphatase and alkaline phosphatase have lost their value in PC detection. (Hautmann and Huland 2006; Hanno et al. 2001)

Transrectal ultrasound (TRUS) and Transrectal needle biopsy (TRB):
The most important use of TRUS is to aid TRB. The classic approach of TRB is to gain six core biopsies, 3 from each lobe. Obtaining 10 or more systematic biopsies of the peripheral zone and transitional zone further increase the possibility of diagnosis. (Hanno et al. 2001; Hautmann and Huland 2006; Schwab 2001)

Bone scan:
Radionuclide imaging is the main method for assessing osseal metastases in disseminated disease and displays high sensitivity. (Hanno et al. 2001; Hautmann and Huland 2006)

Computer tomography (CT) and magnetic resonance imaging (MRI):
CT and MRI do not belong to standard procedures for PC diagnostics nor staging. The endorectal MRI display high performance of prostate anatomy and its surrounding tissue and may provide additional staging information to assess extracapsular disease in patients with intermediate PSAs and positive needle biopsies. (Hanno et al. 2001; Hautmann and Huland 2006)

2.1.9 Therapy

Various factors like age, co morbidities, tumour stage and Gleason score as well as therapy side effects are taken into consideration when deciding on the form of therapy. Radical prostatectomy and radiation therapy present

treatment options for localised (T1, T2, T3, N0, M0) organ –confined disease. Hormone therapy, androgen deprivation, marks treatment for advanced disease (metastases). Therapeutic strategies for refractory disease are generally palliative, present a mean survival of 11 months and include chemotherapy, immunotherapy, radiation and pain treatment. (Schwab 2001; Hanno et al. 2001; Hautmann and Huland 2006)

Watchful waiting:

This is an alternative in patients with early localised disease with good differentiated small tumours (T1, T2, N0, M0) and low life expectancy. (Sökeland et al. 2004)

Radical prostatectomy:

This treatment is applied for patients with a mean life expectancy > 10 years and generally below the age of 70 years. The entire prostate together with the seminal vesicles and the underlying fascia are taken out. (Hanno et al. 2001; Hautmann and Huland 2006)

Radiation therapy:

This is the treatment of choice for T3 disease. Interstitial brachytherapy is an alternative method to standard external beam radiation. External radiation is administered upon three-dimensional conformal as multi field radiation in divided doses. For transperineal brachytherapy radioactive iodide (^{125}I) and palladium (^{103}Pd) are implanted via ultrasound-guide in the prostate. After-loading therapy is a similar method to brachytherapy. In this case temporary radioactive iridium is brought to the prostate via ultrasound-guided perineal needles and additional percutaneous radiation is required to attain the necessary dose. These methods limit unwanted radiation to the bladder and rectum. Short term results show similar outcome to surgery or external beam radiation. (Schwab 2001; Hanno et al. 2001; Hautmann and Huland 2006)

Androgen ablation:

Radical or bilateral subcapsular orchiectomy (castration), LHRH agonists and steroid or non steroid antiandrogens (receptor blockers) present different methods for androgen deprivation. The prostate atrophies without testosterone. The main difference between these methods lies in the range of their individual side effects. Oestrogen therapy has been eliminated because of its cardiovascular side effects and mamma hyperplasia. The effects of inhibitors of androgen synthesis or complete androgen blockage are controversial. (Hanno et al. 2001; Hautmann and Huland 2006)

Hormone refractory disease:

Patients experiencing clinical or biochemical failure while on androgen ablation can experience regression on withdrawal, which may last for several months to a year. Chemotherapy with single or combined treatment regimen of cytostatic drugs delivers rather prolonged response. Immunotherapy can be administered only restrictedly and bone metastases can be eliminated by local radiation. (Hanno et al. 2001; Hautmann and Huland 2006)

2.1.10 Follow-up and Prognosis

PSA is the single most important and specific tumour marker for post operative and post radiation monitoring. Overall outcome depends on pre-treatment stage and post-therapy PSA level. PSA values after androgen ablation do not reflect therapy outcome. 10- to 15-year recurrence rate after surgery is ca. 20%. Highest success rates after radiation are reported when therapy is instituted before PSA > 1.0ng/ml. 10-year survival rate of patients with advanced disease is ca. 10%. (Schwab 2001; Hanno et al. 2001; Hautmann and Huland 2006)

2.2 The Ubiquitin-Proteasome Pathway

The Ubiquitin-proteasome pathway and Cancer:

The ubiquitin-proteasome pathway (UPP) is accountable for the ATP-dependent selective and timely removal of misfolded, damaged, unassembled polypeptide chains and regulatory proteins in the cell. (Schwab 2001; Zwickl and Baumeister 2002) The proteasome regulates in this manner numerous basic cellular processes: cell cycle progression, transcription, signal transduction, proliferation, apoptosis, cellular stress response, modulation of surface receptors, ion channels, metabolic regulation, antigene processing and presentation, regulation of tumour suppression proteins, combating cancer and viral infection. (Cooper 2000, Zwickl and Baumeister 2002; Mani and Gelmann 2005; Boston Biochem ©1998-2005; Bioinformatic Harvester) Its involvement in the pathogenesis of various cancers and its role in human cancer therapy have previously been reported and would be discussed later in this paper.

The Ubiquitin-proteasome machinery:

Cellular disposal of unwanted molecules is essential for normal cellular homeostasis. The UPP presents the major, oldest, energy dependent and most tightly regulated process for selective, rapid proteolysis in eukaryotes. Peroxisomes and lysosomes are other systems that mediate molecular breakdown processes. (Cooper 2000; Elliot and Elliot 2001; Zwickl and Baumeister 2002) Ubiquitin targets protein via conjugation of multiple ubiquitin moieties (ubiquitination) for ATP-dependent degradation by the proteasome [Fig 2.2.1]. The proteasome is further accountable for ubiqitin recycling. Ubiquitin is an abundant, highly-conserved, small 76 amino acid protein found only in eukaryotes, neither in archaea nor bacteria. Ubiquitination involves a cascade of three enzyme types, E1, E2 and E3. E1, encoded by a single gene, activates the C-terminus of the ubiquitin, ATP dependent, via a high-energy thiolester linkage to the E1. E2 then transfers ubiquitin to one of the multiple E3. E3 is responsible for the

specificity of protein recognition, thus ensuring the fidelity of selective proteolysis. E3 catalyse the covalent-attachment of the ubiquitin C-terminus to the ε-amino group of a lysine residue of the substrate (protein) via isopeptide linkage and subsequent formation of a long multiubiquitin chain, which serves as a signal for degradation by the proteasome. De-ubiquitination enzymes recover ubiquitin from ubiquitin-protein conjugates and breakdown abundant multiubiquitin chains. (Schwab 2001; Zwickl and Baumeister 2002) The proteasome is highly conserved in all three domains in life, archaea, bacteria and eukaryotes. The simplest form is found in bacteria and most complicated in eukaryotes. 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, each containing 7 subunits (28 non-identical subunits): two inner β-rings which contain the proteolytic active sites and two outer α-rings. The 19S cap is made of a base and a lid. The base contains three non-ATPase subunits and six ATPases that unfold the substrates and translocate them into the core (substrate specificity). The lid contains eight non-ATPase subunits; its role is still unclear. The proteasome is found free in the cytosol, attached to the ER and in the nucleus. (Schwab 2001; Zwickl and Baumeister 2002)

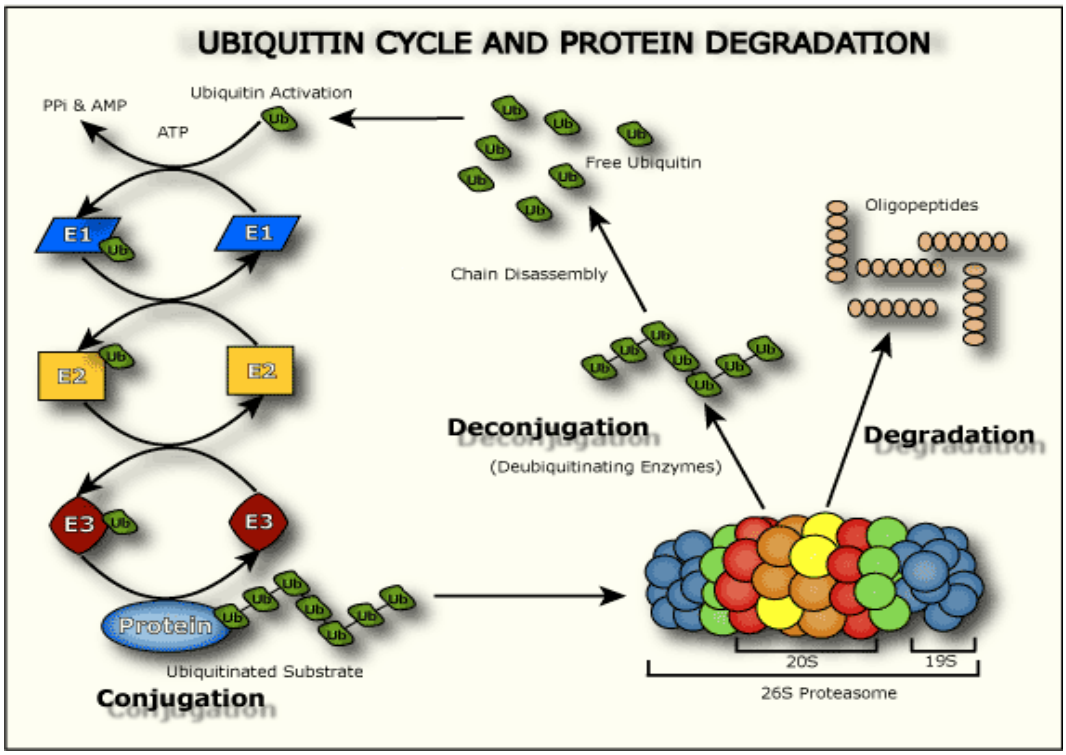


Fig 2.2.1: Ubiquitin-Proteasome degradation pathway (Boston Biochem © 1998-2005)

2.3 Transcripts

Expression patterns of ten transcripts from the two compartments of the ubiquitin-proteasome cascade, eight ubiquitin ligases and two proteasome subunits, were investigated in tumour and adjacent normal tissue of prostate carcinoma patients. Tab 2.3.1a-c display transcript information and their involvement in cancer. The transcripts have been shown to be dysregulated in various tumours or their functions could be relevant in cancerogenesis.

Symbol	Name	Aliases	Chromosome location	Function / Role in Cancer	Reference
UBE3A	Ubiquitin protein ligase E3A	E6-AP; Human papilloma virus E6-associated protein; Oncogenic protein-associated protein E6-AP;CTCL tumour antigen se37-2; AS; HPVE6A; EPVE6AP; ANCR;FLJ26981	15q11-q13	E3 ubiquitin-protein ligase. Targets p53, itself, Blk, TH1 for degradation. Mediates Mcm7, HHR23A ubiquitination. Over-expressed in breast cancer compared to adjacent normal tissue. Downregulated in invasive breast and prostate carcinomas compared to adjacent normal tissue.	Bioinfrmatic Harvester; Zwickl and Baumeister 2002; Huibregtse et al. 1993; Scheffner et al. 1993; Nuber et al. 1998; Mishra and Jana 2008, Crinelli et al. 2008; Oda et al. 1999; Yang et al. 2007; Kühne and Banks 1998; Kumar et al. 1999; Deng et al. 2007; Gao et al. 2005
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	E3 ubiquitin ligase SMURF2; hSMURF2; MGC138150; DKFZp686F0270	17q22-q23	E3 ubiquitin-protein ligase. Targets Smad1, Smad2, Smad3, Smad7, Smurf1 for degradation. Regulates TGF-ß signalling. Plays a role in breast cancer progression. Overexpressed in oesophageal squamous cell carcinoma.	Bioinfrmatic Harvester; Lin et al. 2000; Zhang et al. 2001; Fukunaga et al. 2008; Wiesner et al. 2007; Nakano et al. 2009; Jin et al. 2009; Fukuchi et al. 2002; Chen and Matesic 2007
NEDD4L	Neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4-2; hNedd4-2; KIAA0439; Ubiquitin-protein ligase NEDD4-like; Neural precursor cell expressed, developmentally down-regulated gene 4-like; Ubiquitin-protein ligase Rsp5; RSP5; FLJ33870	18q21	E3 ubiquitin-protein ligase. Targets ENaC for degradation. Mediates Smad2, TGF-ß type I receptor degradation. Mediates ubiquitination of itself, TTYH2, TTYH3. Decreased expression in prostate cancer compared to benign prostate tissue.	Bioinfrmatic Harvester; Harvey et al. 2001; Itani et al. 2005; Zhou et al. 2007; Kabra et al. 2008; Kuratomi et al. 2005; Bruce et al. 2008; He et al. 2008; Hu et al. 2009
HECW1	HECT, 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.	Bioinfrmatic Harvester; Miyazaki et al. 2004; Li et al. 2008

Tab 2.4.1a: Transcript information

Symbol	Name	Aliases	Chromosome location	Function / Role in Caner	Reference
CBL	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 tumourigenesis. Associated with gastric tumourigenesis and progression. May function as basal cell marker for prostate cancer.	Bioinfrmatic Harvester; Zwickl and Baumeister 2002; Yokouchi et al. 1999; Levkowitz et al. 1999; Ravid et al. 2004; Bonaccorsi et al. 2007; Pennock and Wang 2008; Umebayashi et al. 2008; Joazeiro et al. 1999; Ito et al. 2004; Reddi et al. 2007; Miura-Shimura et al. 2003; Sehat et al. 2008; Kamei et al. 2000; Knight et al. 2008
SIAH1	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, ß-catenin. Down-regulated in and associated with advanced stages of hepatocellular carcinoma. Associated wtih gastric cancerogenesis.	Bioinfrmatic Harvester; Zwickl and Baumeister 2002; Hu et al. 1997; Hu and Fearon 1999; Kim et al. 2004; Zhou et al. 2008; Wheeler et al. 2002; Santelli et al. 2005; Yoshibayashi et al. 2007; Matsuo et al. 2003; Kim et al. 2004
MDM2	Mdm2 p53 binding protein homolog (mouse)	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein; Ubiquitin-protein ligase E3 Mdm2; Mouse double minute 2 homolog; p53-binding protein MDM2; Double minute 2, human homolog of, p53-binding protein; HDM2; HDMX; 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.	Bioinfrmatic Harvester; Zwickl and Baumeister 2002; Honda et al. 1997; Fang et al. 2000; Honda and Yasuda 2000; Kawai et al. 2003; Sehat et al. 2008; Shiina et al. 1999; Leite et al. 2001; Zhang et al. 2003; Wang et al. 2003

Tab 2.4.1b: Transcript information

Symbol	Name	Aliases	Chromosome location	Function / Role in Caner	Reference
NEDD4	Neural precursor cell expressed, developmentally down-regulated 4	NEDD4-1; RPF1; Receptor-potentiating factor 1; KIAA0093; MGC176705	15q	E3 ubiquitin-protein ligase. Participates in ENaC ubiquitination. Mediates ubiquitination of PTEN, IGF-IR, CNrasGEF. Paricipates in Melan-A ubiquitination in melanoma cells. Associated with colorectal and gastric cancerogenesis. Up-regulated in prostate and bladder cancer.	Bioinfrmatic Harvester; Zwickl and Baumeister 2002; Staub et al. 1996; Abriel et al. 1999; Harvey et al. 2001; Itani et al. 2005; Wang et al. 2007; Kim et al. 2008; Sehat et al. 2008; Pham and Rotin 2001; Lévy et al. 2005; Chen and Matesic 2007
PSMC4	Proteasome (prosome, macropain) 26S subunit, ATPase, 4	Proteasome 26S ATPase subunit 4; Protease 26S subunit 6; S6; Tat-binding protein 7; TBP7; MIP224; MB67 interacting protein; 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.	Bioinfrmatic Harvester; Dubiel et al. 1994; Tanahashi et al. 1998
PSMB5	Proteasome (prosome, macropain) subunit, beta type, 5	Proteasome subunit, beta type, 5; Proteasome beta 5 subunit; MB1; Proteasome subunit X; X; Proteasome chain 6; Proteasome subunit MB1; Macropain epsilon chain; Proteasome epsilon chain; Proteasome catalytic subunit 3; MGC10214; Multicatalytic endopeptidase complex epsilon chain; PSX large multifunctional protease x; LMPX	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.	Bioinfrmatic Harvester; Deng et al. 2007; Lü et al. 2008; Oerlemanns et al. 2008

Tab 2.4.1c: Transcript information

3. Materials and Methods

3.1 Materials

All materials and solutions were either purchased in RNAse-free state or treated to deactivate RNAses. Dilutions were made with RNAse-free water.

3.1.1 Chemicals and solutions

DEPC water	Sigma-Aldrich, St. Louis, USA
RLT buffer	Quiagen, Hilden, Germany
RNA-later	Quiagen, Hilden, Germany
Sterile PBS-buffer	Dulbecco's, Invitrogen, Karlsruhe, Germany
OCT-compound	Tissue-Tek®, Torrance, USA
TaqMan® Universal Master Mix (2X)	Applied Biosystems, California, USA

3.1.2 Utensils

Eppendorf Tubes (0.5ml, 1.5ml, 2ml)	Eppendorf, Hamburg, Germany
Fast Optical 96-well reaction plates	MicroAmp™, Applied Biosystems, California, USA
Optical Adhesive Covers	Applied Biosystems, USA
P.A.L.M. Membrane Slides	(P.A.L.M. ® Microlaser Technologies AG, Bernried, Germany)
Pipettes	Eppendorf, Hamburg, Germany
Pipette Tipps with filter	Eppendorf, Hamburg, Germany
Powder-free Latex Gloves	HARTMANN, Heidenheim, Germany / Kimberly-Clarks, USA
Sterile Tubes (15ml, 50ml)	Greiner, Germany
Superfrost plus slides	Milian, Gahanna, USA
Surgical Disposable Scalpels	Braun, Germany

3.1.3 Machines

2100 Bioanalyzer	Agilent, Waldbronn, Germany
7900HT Fast Real-Time PCR System	Applied Biosystems, Darmstadt, Germany
Automatic ice machine	SCOTSMAN®, Germany
Biopsy Punch	Stiefel, Wächtersburg, Germany
Microscope	Zeiss, Germany
Microscope for microdissection	Zeiss, Germany
Microtom	2800 Frigocut E, Reichert-Jung, Germany
Oven	Heraeus, Germany
PTC-100™ Peltier-Effect Cycling	MJ Research, Inc., Massechusettes, USA
Centrifuge	Biofuge 15, Heraeus, Germany
Vortexer	CIRCOMIX, B. Braun, Melsungen AG, Germany

3.1.4 Kits

RNeasy Micro Kit (50)	Quiagen, Hilden, Germany
RNA 6000 Pico Reagents Part II	Agilent, Lithuania
High-Capacity cDNA Archive Kit	Applied Biosystems, California, USA

3.1.5 Assays

Assays on Demand (2X) Gene Expression Assay	Applied Biosystems, California, USA

Target Transcript Assays (Applied Biosystems, California, USA)

UBE3A	Hs00963673_ml
NEDD4L	Hs00969334_ml
NEDD4	Hs00406454_ml
HECW1	Hs01546585_ml
SMURF2	Hs00909283_ml
CBL	Hs01011446_ml
Mdm2	Hs00234753_ml
Siah1	Hs02339360_ml
PSMC4	Hs01035007_ml
PSMB5	Hs00605652_ml

Endogenous control Assays (Applied Biosystems, California, USA)

HPRT	Hs99999909_ml
GAPDH	Hs99999905_ml
TFRC	Hs99999911_ml

3.2 Methods

Changes in the ubiquitin-proteasome pathway were analyzed by investigating the expression level of eight ubiquitin E3 ligases and two proteasome subunits at the level of transcription, indirectly via running real time RT-PCR on cDNA from corresponding mRNA of the transcripts. The methods applied for specimen processing are standardized and routinely used in the laboratory of the Department of Urology of the University Hospital (UKE). A section of the methods had been described by T. Schlomm. (*Schlomm et al. 2005*)

3.2.1 Patient data

Patient data was achieved from a large data bank (Urodata) of the laboratory of the Department of Urology of the University Hospital. Urodata consists of patient information with prostate carcinoma at different stages including personal data, medical history, specimens acquired, test results, therapy/-ies obtained and the follow-up.

The patients selected [Tab 3.2.1] had tumours at stage pT2 or pT3 (TNM-System) and neither obtained radio-, chemo-, nor ablative hormonal therapy. The names of the patients were anonymised (e.g. patient number T3_003 for patient 003 with tumour stage T3). Each patient had a sample pair made of its tumour and adjacent normal tissue (e.g. patient T3_003 had a sample pair made of T3_003N for its normal and T3_003T for its tumour tissue).

3.2.2 Sample collection

Tissue specimens were obtained from prostate glands immediately after radical prostatectomy (2004-2005). After excision, the gland was macroscopically inspected. By invisible or impalpable tumours, a cut was made through the mid section of the lateral surface of the gland from the base to the apex. Samples were then taken with a 6mm punch biopsy instrument from foci with tumour (visible tumours) or areas suspected to be

cancerous (non visible/-palpable tumours) based on pre-operative diagnosis, systematic 10-location biopsies. The same procedure was used to obtain normal tissue samples from prospective tumour-free regions of the prostate. Each sample was immediately stored in a cryo-tube filled with 1.5ml RNA-later. This method guarantees an ischemia time of less than three minutes for each specimen. The samples were stored overnight at ambient temperature and then transferred to a -80°C freezer.

Patient-Nr	*Age	Tumour-Stage	Gleason-Score	Gleason-Sum	LN-Status	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 3.2.1: 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)*

3.2.3 Cryosections

The specimens in the cryo-tubes were thawed at room temperature. To elute most of the RNAlater form the tissue, two washings of 5minutes each were carried out in 10ml pre cooled (0°c) sterile PBS-buffer in an ice bath. These washings facilitate following procedures like, cryo-cutting and microdissection as well as microscopic visualisation, which are negatively influenced by RNA-later. The washed specimens were quickly refrozen in a drop of OCT-compound on the holder of the cryo-microtome at optimal cutting temperature of -25°C. After adequate freezing, slices of 5-7µm thickness were made and transferred to Super frost plus slides. The slides were then HE-stained and the histology subsequently evaluated.

Only specimens with adequate tumour and normal tissue were further processed and cryosections of 10 - 15µm thickness were prepared from these selected specimens on P.A.L.M. membrane slides (the slides were irradiated for 30minutes in a standard UV-Plate to increase adhesiveness and thereafter backed for 4hours at 180°C in an oven to eliminate RNase). The slides were air-dried on ice for 1minute and subsequently stained with cresyl violet acetate. This procedure was carried out for each patient collective, made up of tumour and normal tissue.

Cryosections were prepared at precooled temperatures to hinder RNAse activity. Working in cold temperatures ensures inactivation of tissue and environmental RNAse.

3.2.4 Staining

Stainings were performed according to standard laboratory staining protocols.

HE-Staining:

HE slides were prepared for histological evaluation via the microscope. The slides were successively incubated in the following staining solutions shown below [Tab 3.2.4_1].

Staining solution	Incubation time
Aceton	1 minute
Bidestilated water	30 seconds
Haematoxlin III (undiluted)	1 minute
Tap water	1 minute
Eosin (0.1% in bidestilated water)	1 minute
80% Ethanol	30 seconds
96% Ethanol	1 minute
100% Ethanol	1 minute
Xylol	2 minutes

Tab 3.2.4_1: Scheme for rapid HE-Staining

After staining, the slides were overlaid with acrylate and cover slips and air-dried. Tissue architecture (histology) was then evaluated under the microscope and the slides thereafter preserved in boxes at room temperature.

Cresyl violet-Staining:

P.A.L.M. slides were stained after microscopic evaluation as shown below [Tab 3.2.4_2].

Staining solution	Incubation time
70% Ethanol	2 minutes
Cresyl violet (1% dissolved in 100% Ethanol)	30 seconds
70% Ethanol	30 seconds
96% Ethanol	30 seconds

Tab 3.2.4_2: Scheme for Cresyl violet – Staining

After staining, the slides were air-dried for 5 minutes. All solutions were pre cooled at -20°C and the entire procedure was carried out on ice to ensure RNAse deactivation. The slides were either processed immediately or preserved in sterile plastic cases at -80°C for later procession.

3.2.5 Microscopic evaluation

The HE-slides were analysed under the microscope for tumour and normal prostate tissue architecture. Screening was self-based (sometimes controlled by the supervisor) and later sent to the pathologist for final confirmation. Tumour grading was carried out based on Gleason classification. Specimens with aberrant tumour or normal prostate tissue architecture were disqualified.

3.2.6 Microdissection

Tissue areas containing only tumour-/ normal prostate tissue were manually dissected from the prepared cresyl violet stained P.A.L.M. slides with a scalpel under a microdissection microscope. Areas of up to 40mm² were won. For RNA analysis the acquired tissue was collected in RNA-lysis buffer (10µl ß-mercapto-ethanol: 1ml RLT buffer) and stored at -20°C.

3.2.7 RNA-Isolation and quality control

RNA was isolated from the tissue samples using the RNeasy micro kit according to the manufacturers protocol *("Total RNA Isolation from Microdissected Cryosections"*). 12µl of purified RNA were eluated from each sample and stored at -80°C.

For RNA quality analysis 1µl of the RNA-eluate were applied. Processing was carried out with the Total-RNA Pico Kit as indicated by the manufacturer's protocol *("RNA 6000 Pico Assay Protocol"*) and run on the Agilent 2100 Bioanalyzer according to the program protocol *("Eukaryote Total RNA Pico").* The rRNA Ratio [28S / 18S] was used as a measuring stab for quality control. Only samples with a rRNA Ratio more than 1.5 and a total quantity of at least 60ng were further processed. 60ng of RNA were needed for later RT-PCR processing. Below is an example for the calculations of the RNA total quantity in the residual 11µl eluate [Tab 3.2.7].

Sample ID	Measured-concentration (pg/µl)	Dilution (1 :…)	Is-concentration (ng/µl)	Total RNA quantity in 11µl (ng)
T3_021T	7,088.8	20	141.776	1559.54

Tab 3.2.7: Calculated RNA-Concentration from obtained RNA 6000 pico Assay-Data

Samples with inadequate RNA quality were disqualified and for those with insufficient RNA (quantity), the frozen tissue was thawed and reprocessed as above to win more RNA.

3.2.8 cDNA synthesis

60ng of the RNA from each selected sample were reverse transcribed in a total volume of 20ng to cDNA using the High-Capacity cDNA Archive Kit in accordance with the manufacturer's protocol. The Master Mix was made up of the following components [Tab 3.2.8_1]:

Components	Master Mix (µl)
10x RT-Puffer	2.0
25x dNTP	0.8
10x Random Primers	2.0
MultiScribe RT Enzym (50U/µl)	2.0
RNase-free water	3.2
Total	10

Tab 3.2.8_1: Master Mix, cDNA-Synthesis (Applied Biosystems)

10µl Master Mix were added to 10µl of each RNA sample (= 60ng RNA) [Tab 3.2.8_2] in Eppendorf tubes (20µl total volume) and mixed by repeated pipetting. The tubes were then incubated in the Thermocycler at 25°c for 10 minutes, at 37°C for 2 hours and then cooled down to 4°C. The prepared cDNA samples were thereafter stored at -20°C.

Sample ID	Is-concentration (ng/µl)	Target-quantity (ng)	Volume to be withdrawn from eluted RNA (µl)	Target-volume (µl)	Volume difference (RNase-free water (µl))
T3_021T	141.776	60	0.42	10	9.58

Tab 3.2.8_2: Calculated RNA-Volume (equivalent to 60ng RNA) for cDNA synthesis

3.2.9 Real-Time qRT-PCR

4,5µl (equivalent to 0,5ng) of cDNA were applied for a single quantitative PCR using the TaqMan® PCR Master Mix from Applied Biosystems as recommended by the manufacturer. The fluorogenic TaqMan® probe (Taq polymerase) is heat-stable and detects specific PCR product generation synchronic (real time) to each amplification cycle (specific hybridization between probe and target is required to generate fluorescent signal).

Three housekeepers (endogenous controls), HPRT, TFRC and GAPDH, were applied. All transcripts were run in duplicates.

The reaction mix comprised of the following components [Tab 3.2.9]:

Reaction components	Volume / Well (µl)
TaqMan® Universal PCR Master Mix (2X)	5.0
Assays on Demand (2X) Gene Expression Assay	0.5
cDNA diluted in RNase-free water	4.5
Total	10

Tab 3.2.9: Reaction Mix, for qRT-PCR (Applied Biosystems)

The calibrator (healthy prostate tissue) was obtained from biopsies of a pool of 20 healthy probands. RNA-isolation/-quality control and cDNA-synthesis were performed on the calibrator as described above.

PCR was run in duplicates on a 96-well plate in the 7900HT PCR system for 40 cycles following the manufacturer's recommendation. The plate was heated for 15 seconds at 95°C for denaturation and cooled for 1 minute to 60°C for primer annealing and extension followed by fluorescence

measurement. The 7900HT runs the PCR in real time. In this case data is collected throughout the PCR, permitting detection in the period when increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated (exponential phase) rather than the amount of target accumulated at the end of the PCR (End-Point or Plateau) as in case of traditional PCR methods, which is not as precise. [Applied Biosystems]

Relative quantitation was performed using the comparative Ct method (delta-delta Ct method). This method eliminates the need for a standard curve and thus the adverse effect of any dilution errors made in creating the standard curve samples. [Applied Biosystems]

The validity of the comparative Ct method is only guaranteed when the amplification efficiency of the target and endogenous reference are approximately equal (Applied Biosystems guarantees this assumption for its assays). This method applies the arithmetic function $2^{-}\Delta\Delta Ct$ [RQ]. [Applied Biosystems]

$\Delta\Delta Ct = \Delta Ct$ sample - ΔCt calibrator

ΔCt sample = Ct target – Ct endogene control

ΔCt calibrator = Ct target – Ct endogene control

Ct target is the Ct value for the target gene of any sample or calibrator.

Ct endogene control is the Ct value of the endogene control for each sample or calibrator.

The Ct-value is read from each PCR curve at the point just as it begins to rise from the baseline, the point where it is closest to the function 2^n. This is done as early as possible as the PCR exhausts with time and the curve approaches the plateau phase.

RQ-values - representing the x-fold quantity of a given target mRNA in the sample compared to the calibrator (set automatically as 1) - were calculated from exported PCR-data (Ct-values) for all tumour (T) and normal (N) tissue samples using the formula $2^{\Delta\Delta Ct}$ - whereas the basis represents the ideal PCR-efficiency (two generated amplificates per sample-DNA per cycle). The PCR-derived RQ values of tumour (RQ(T)) and normal tissue

(RQ(N)) were then used to calculate RQ (T/N), representing the n-fold transcription of the target transcript in tumour in relation to adjacent normal tissue. Calculated RQ(T), RQ(N) and RQ(T/N) were later employed for statistical analysis.

3.2.10 Statistical analysis

PCR-derived RQ data were employed on Excel and SPSS for subsequent analysis.

The t-test was engaged mainly for verification. This study did not meet the assumption for applying the t-test; as not all investigated population demonstrated gaussian distribution (Kolmogorov-Smirnov tests revealed that not all derived RQ-values displayed normal distribution). Subsequently non-parametric tests were employed for statistical evaluation. Paired Wilcoxon tests were the primary tests applied to evaluate the change in level of transcript expression (RQ(T/N)) in tumour (RQ(T)) in relation to adjacent normal tissue(RQ(N)). Further non-parametrical tests employed were the unpaired Mann-Whitney-U-test and the Spearman-Rho-test. The Mann-Whitney-U-test was employed to analyse the difference in expression rate between two variables (tumour stage: pT2 and pT3; Gleason score: 0-6 and 7-10). Correlation of transcript expression between investigated target transcripts in tumour tissue was analysed via Spearman-Rho (bivariate test).

3.3 Software

Software applied in this study include Microsoft Word 2003, Microsoft Excel 2003, and SPSS 12.0.

4. Results

4.1 Sample collection, Cryosections and HE-staining

53 patients were randomly selected from the Urodata, 30 with tumour stage pT2 and 23 with pT3 [Fig 4.1.1]. 24 of these patients demonstrated a Gleason score ≤ 6 (G6) and the rest a Gleason score ≥ 7 (G7) [Fig 4.1.1]. Cryosections and HE-slides were prepared from corresponding tumour and normal tissue of all selected patient specimens.

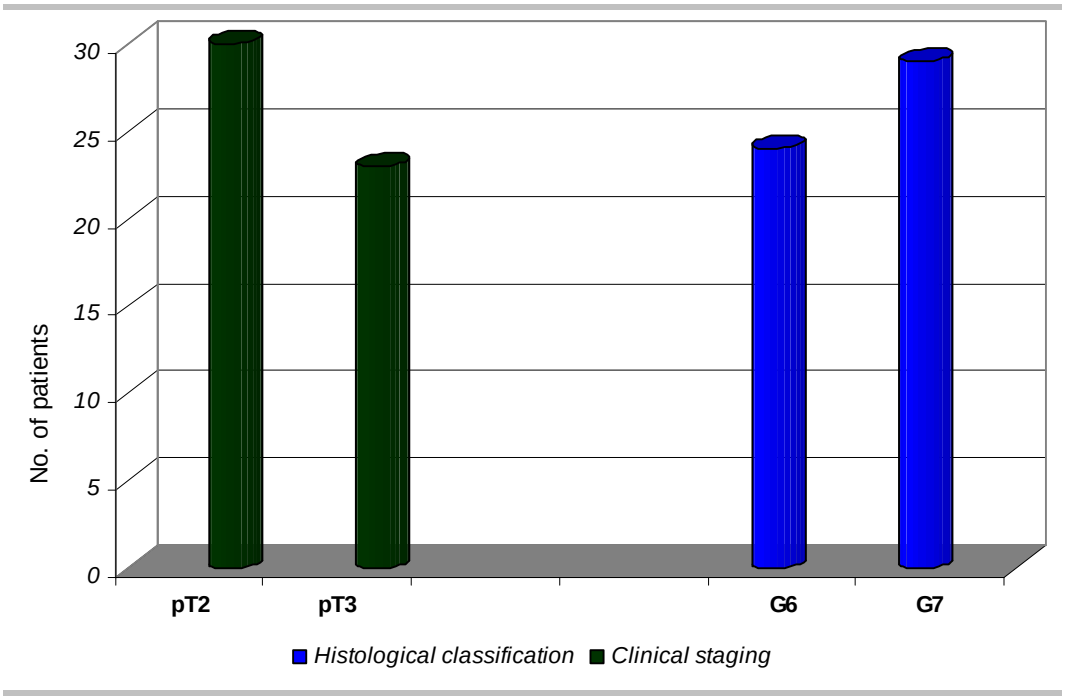


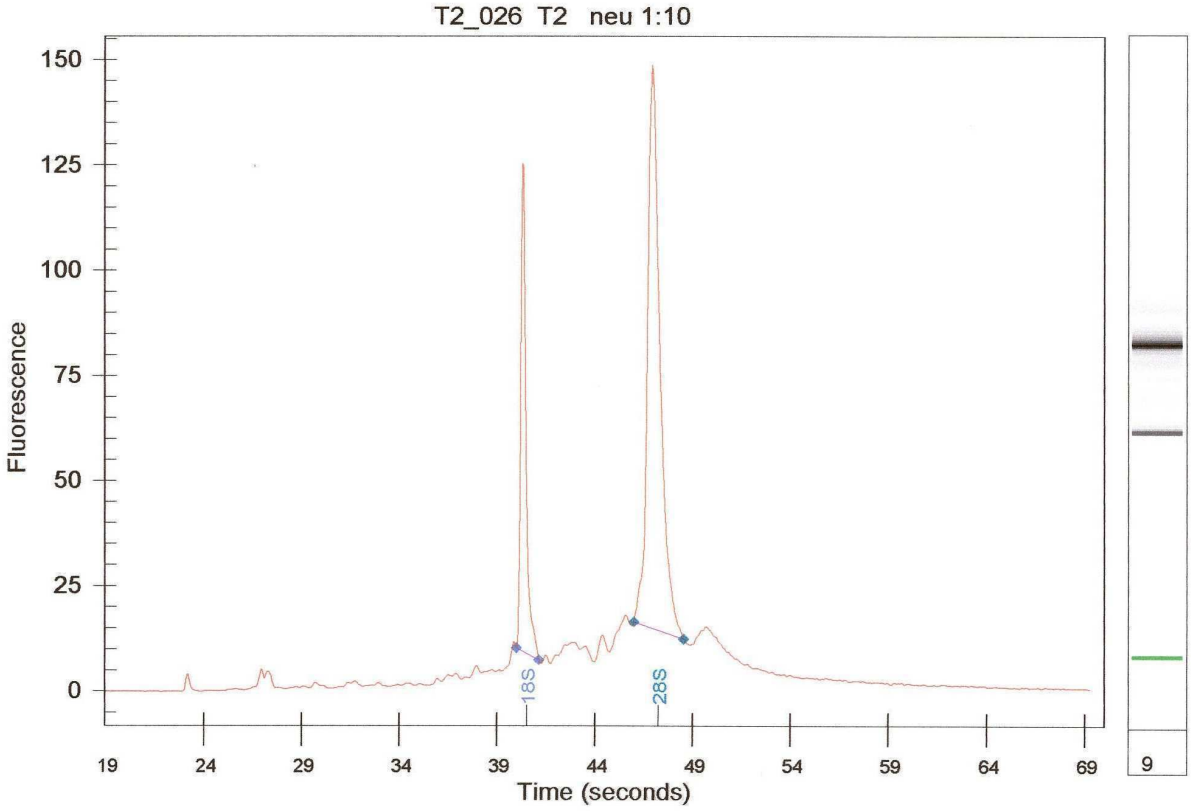
Fig 4.1.1: Selected patient samples – clinical staging and tumour histology (pT2: tumour stage pT2; pT3: tumour stage pT3; G6: Gleason score ≤6; G7: Gleason score ≥7)

4.2 Histological evaluation

29 of the 53 sample pairs, 16 with pT2 and 13 with pT3, displayed adequate tumour and normal prostate histology after microscopic evaluation. Palm slides were prepared from all 29 sample pairs. The disqualified specimens had either only tumour, only normal or mainly connective tissue.

4.3 RNA analysis and cDNA synthesis

RNA was isolated from tumour and normal tissue of the 29 samples after histological evaluation. Following RNA evaluation 22 of the 29 sample pairs, 11 with stage pT2 and 11 with pT3, revealed adequate RNA quality (rRNA Ratio > 1.5) and sufficient RNA material (RNA > 60ng) required for further investigation [Fig 4.3.1; Fig 4.3.2]. All 22 sample pairs were successfully run for cDNA synthesis and subsequent PCR-analysis.



Corrected RNA Area: 726.90 rRNA Ratio [28S / 18S]: 2.32
RNA Concentration: 6,360.03 pg/ul

#	Name	Start Time (s)	End Time (s)	Corr.Area	% of total Area
1	18S	39.99	41.15	91.22	12.55
2	28S	46.01	48.55	211.31	29.07

Fig 4.3.1: RNA analysis – an example on T2-026T (Agilent 2100 Bioanalyzer)

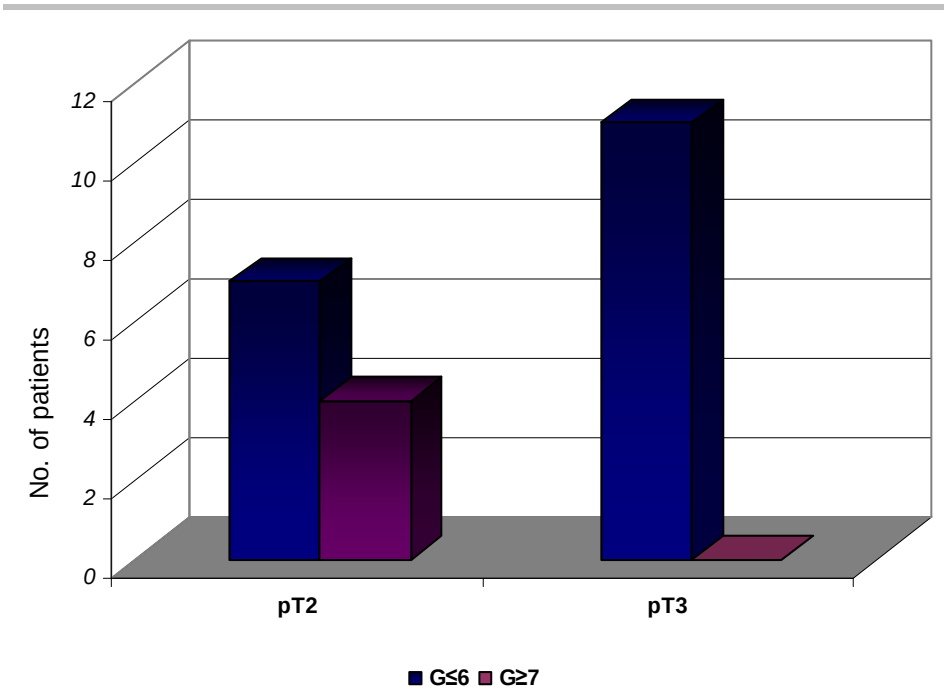


Fig 4.3.2: Samples applied for cDNA-synthesis and PCR-analysis histology (pT2: tumour stage pT2; pT3: tumour stage pT3; G6: Gleason score ≤6; G7: Gleason score ≥7)

4.4 qRT-PCR

The use of GAPDH as a housekeeping gene for differential expression studies has faced critic. Studies have shown that the expression of the GAPDH gene was increased in advanced prostate tumours (Rondinelli et al. 1997) and changed under chemotherapeutic drugs (Valenti et al. 2006). HPRT on the other hand has been shown to be sufficient as a single endogenous control for data normalisation in tumour research (deKok et al. 2005, Ohl et al. 2005). deKok et al. 2005 further showed that TFRC (TfR) presented a very high correlation coefficient for prostate tissue ($r^2 = 0.97$) in the study comparing different endogenous controls for gene normalisation measurements. Applying more than one reference gene encompasses the advantage of more accurate normalisation calculation than a single gene and thus improves the reliability of gene normalisation (deKok et al. 2005). Accordingly PCR data in this study was normalised on the endogenous

controls HPRT and TFRC. Cut-off niveau for significance was $p<0.05$. An example of calculated RQ(T/N) from PCR-derived RQ-values for PSMC4 is shown in Tab 4.4.1.

Sample	Tumour-stage	Gleason-score	LN-Status	RQ (N)	RQ (T)	RQ(T) / RQ(N)
T3-003	pT3b	4+4	N1	3.38459098	3.6332496	1.07346786
T3-004	pT3a	3+4	N1	4.17034431	2.0076308	0.48140649
T3-008	pT3b	5+4	N1	2.31272383	1.1023333	0.47663854
T3-012	pT3b	3+4	N0	2.81757497	3.1860955	1.1307935
T3-013	pT3b	3+4	N0	2.17324526	4.3147426	1.98539147
T3-016	pT3a	3+4	N0	5.7342627	3.9285352	0.68509858
T3-017	pT3a	4+4	N0	3.18414713	4.7238266	1.48354532
T3-019	pT3a	3+4	N0	3.60607774	5.4242832	1.50420584
T3-021	pT3a	4+5	N0	3.22258631	5.4823939	1.70124038
T3-022	pT3a	4+3	NX	1.91109501	4.2421055	2.21972507
T3-023	pT3a	4+3	N0	4.96169653	4.1645829	0.83934655
T2-007	pT2a	3+3	NX	3.42027541	5.7061731	1.6683373
T2-017	pT2c	3+3	NX	2.44519496	3.7471078	1.53243725
T2-022	pT2c	4+3	N0	5.46600394	2.7540409	0.50384906
T2-023	pT2c	4+3	N0	3.2271922	4.2275579	1.30998019
T2-024	pT2c	4+3	N0	2.88573636	4.176092	1.44714953
T2-025a	pT2c	4+3	N0	4.60904227	7.310829	1.58619265
T2-025b	pT2c	3+3	N0	3.25996393	6.6251334	2.03227199
T2-026	pT2c	3+3	NX	3.5140994	4.1655731	1.18538853
T2-027	pT2c	3+3	NX	6.15998569	5.6660075	0.91980854
T2-029	pT2c	3+3	NX	5.13495986	8.9361351	1.74025413
T2-030	pT2c	3+3	NX	2.68712698	11.659512	4.33902531
					RQ(T/N) = $\frac{\sum RQ(T)/RQ(N)}{22}$	
					= 1.44752519	

Tab 4.4.1: Calculated RQ(T/N) for PSMC4 normalised on HPRT (RQ(T/N): transcript expression in prostate tumour compared to adjacent normal tissue)

4.5 Transcript expression in Tumour vs Normal tissue

All analysed transcripts could be measured by real time RT-PCR in tumour and normal tissues of all samples [Tab 4.5.1]. The difference in transcript expression levels of the various transcripts in the analysed samples is displayed in Fig 4.5.1. The Wilcoxon test was applied to analyse the significance of the difference in transcript expression between tumor and normal prostate tissue [Tab 4.5.1]. Here, three transcripts, PSMC4, PSMB5 and NEDD4L, demonstrated significant up-regulation of expression in tumour compared to adjacent normal tissue [Tab 4.5.1; Fig 4.5.1].

Transcript	RQ(T/N)		RQ(T/N) - Average	p		P – average
	HPRT	TFRC		HPRT	TFRC	
PSMC4	1.44752519	1.69018234	1.56885376	0.028	0.026	0.027
PSMB5	1.93023847	2.22326931	2.07675389	0.001	0.001	0.001
CBL	0.9966721	1.07921078	1.03794144	0.249	0.858	0.554
HECW1	4.03811662	6.05025391	5.04418527	0.050	0.050	0.050
MDM2	1.88137563	2.08311161	1.98224362	0.355	0.077	0.216
NEDD4	1.11850741	1.22752684	1.17301712	0.338	0.408	0.373
NEDD4L	2.50125662	3.00693257	2.75409459	0.010	0.012	0.011
SIAH1	1.2819827	1.72419694	1.50308982	0.445	0.758	0.602
SMURF2	1.27116287	1.42283849	1.34700068	0.783	0.527	0.655
UBE3A	1.00688305	1.19421546	1.10054926	0.390	0.783	0.587

Tab 4.5.1: Increase in transcript expression [RQ(T/N)] in tumour (p: significance; RQ(T/N): transcript expression in prostate tumour compared to adjacent normal tissue)

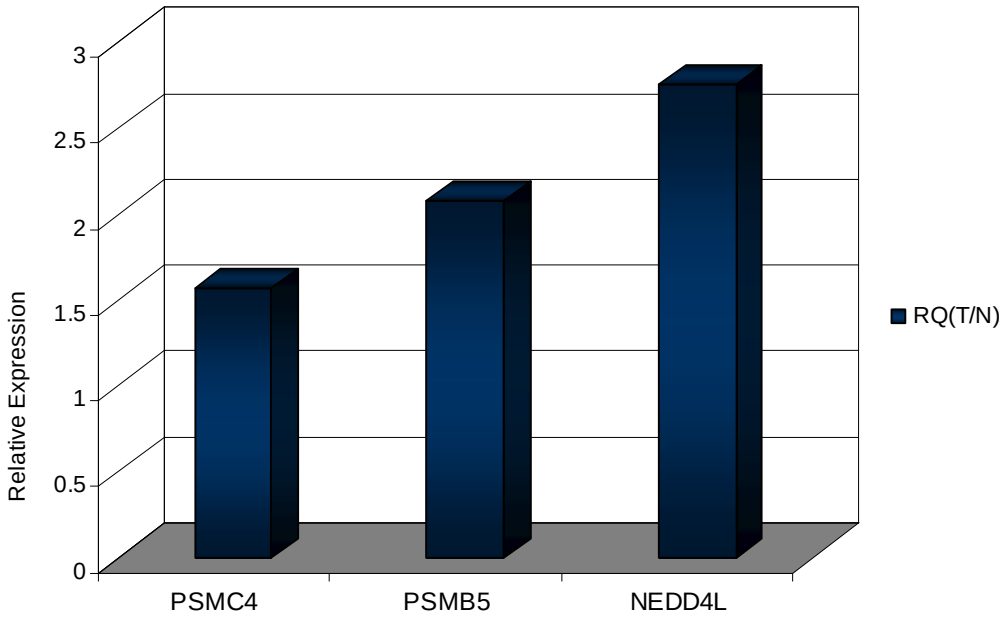


Fig 4.5.1: Avarage Transcript up-regulation in all tumours for significantly upregulated targets (RQ(T/N): transcript expression in prostate tumour compared to adjacent normal tissue)

PSMC4 was over-expressed in tumour tissue at 1.57-fold displaying a significance of 0.027 [Tab 4.5.1; Fig 4.5.2]. The over-expression of PSMB5 in tumour was > 2-fold with a significance of 0.001 [Tab 4.5.1; Fig 4.5.3].

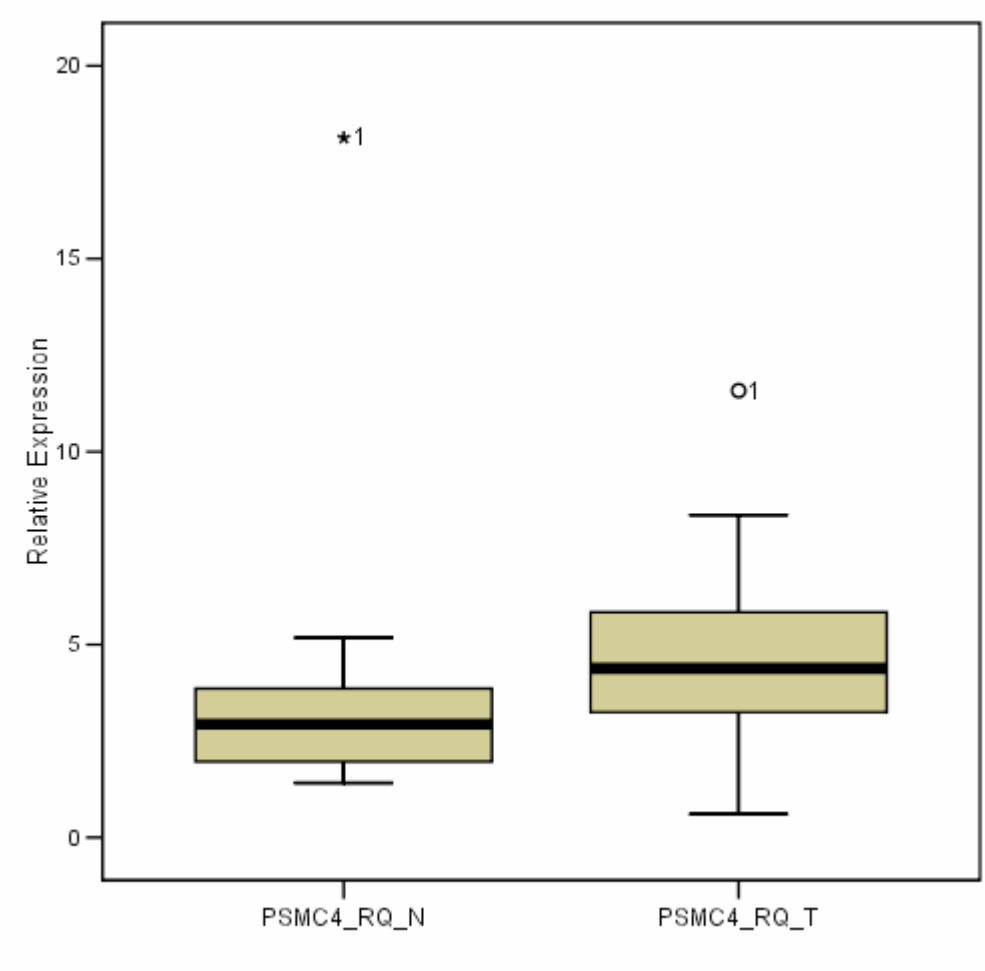


Fig 4.5.2: Up-regulation of PSMC4 in tumour (RQ_N: transcript expression in adjacent normal tissue; RQ_T: transcript expression in tumour tissue)

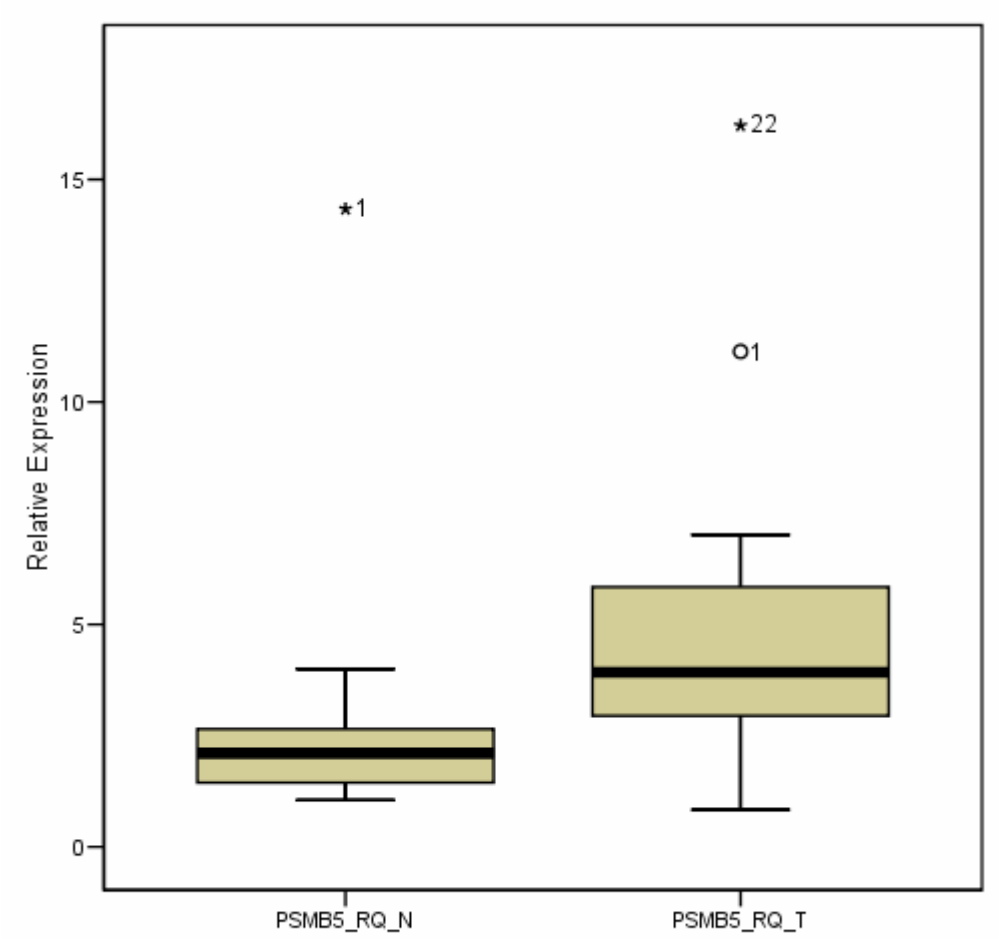


Fig 4.5.3: Up-regulation of PSMB5 in tumour (RQ_N: transcript expression in adjacent normal tissue; RQ_T: transcript expression in tumour tissue)

NEDD4L was the third of the three transcripts which displayed a significant up-regulation in tumour cells, demonstrating an over-expression of 2.75-fold with a significance of 0.011 [Tab 4.5.1; Fig 4.5.4].

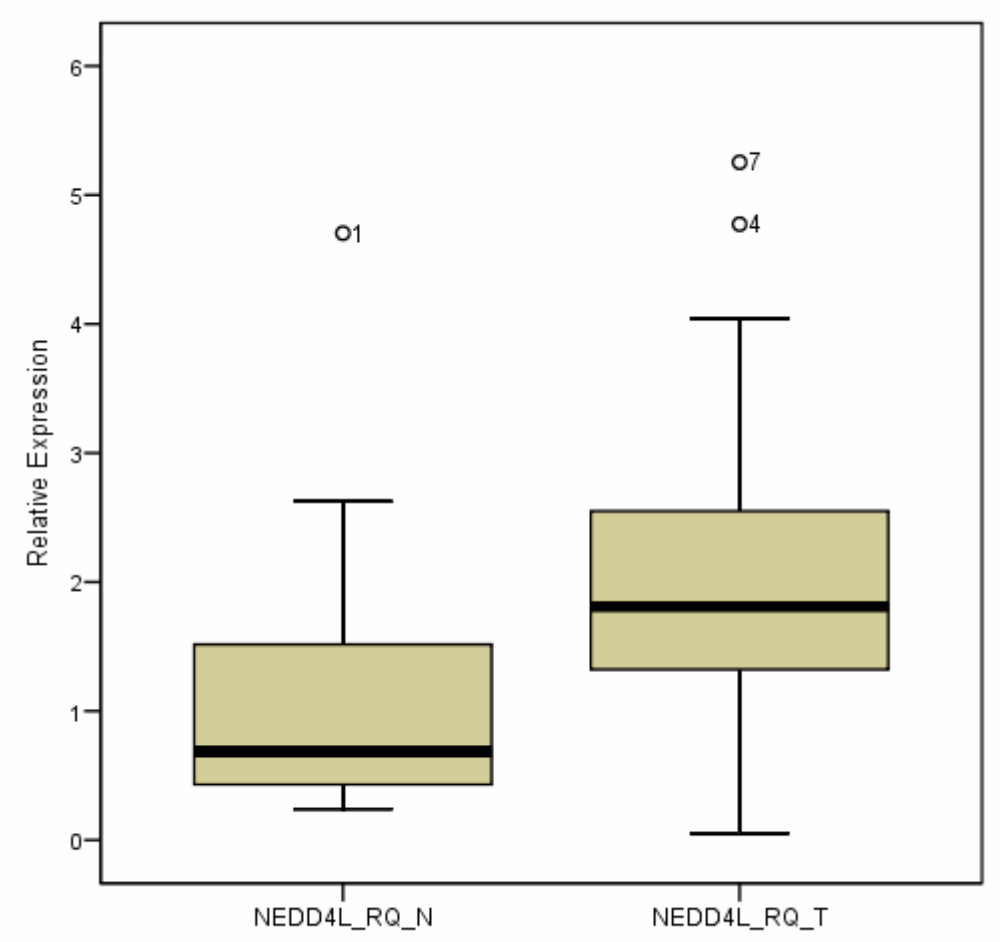


Fig 4.5.4: Up-regulation of NEDD4L in tumour (RQ_N: transcript expression in adjacent normal tissue; RQ_T: transcript expression in tumour tissue)

4.6 Transcript expression in relation to tumour stage and histology

The Mann-Whitney-U-test was applied to analyse the difference in level of transcription in tumour compared to normal tissue between tumours of stages pT2 and pT3 (pT_group 2, 3) as well as in relation to tumour histology, between Gleason scores from 4 to 6 (G6) and from 7 to 10 (G7). Only one transcript, SMURF2, demonstrated a higher level of expression in pT2 (pT_group2) over pT3 (pT_group3) tumours compared to normal tissue, displaying a significance of 0.028 [Fig 4.6.1]. The analysis in relation to tumour histology did not reveal any significant difference in transcript expression between the groups G6 and G7.

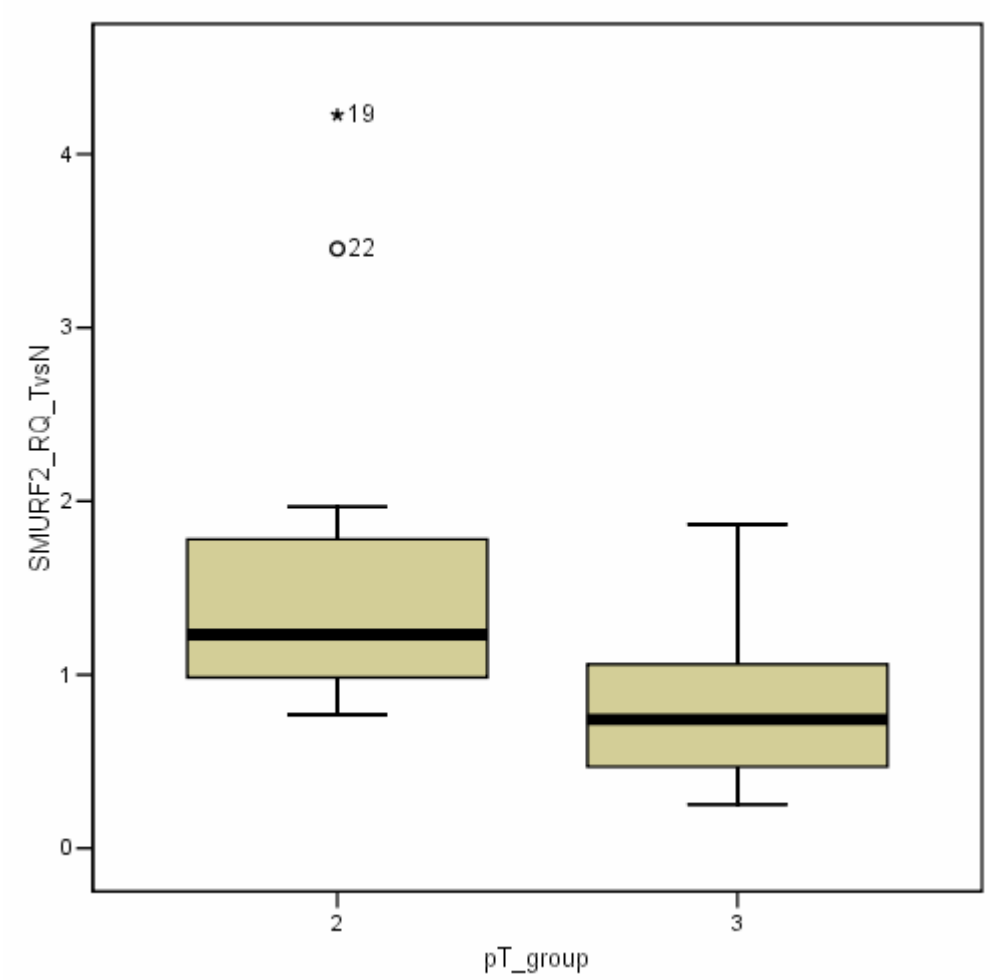


Fig 4.6.1: SMURF2 up-regulation higher in pT2-tumours than pT3-tumours when compared to normal tissue (pT_group2: tumour stage pT2; pT_group3: tumour stage pT3; RQ_TvsN: transcript expression in tumour compared to normal tissue)

4.7 Correlation between investigated transcripts

The Spearman-Rho test was employed to explore the correlation of expression between the transcripts in tumour cells. Correlations with coefficients, r, $r<0.3$ were considered weak, $0.3<r<0.7$ moderate and $r>0.7$ high. All significant correlations were positive [Tab 4.7.1; Tab 4.7.2].High positive correlations were found between UBE3A & PSMC4, UBE3A & PSMB5, UBE3A & CBL, UBE3A & SMURF2 and PSMC4 & PSMB5, all demonstrating very high level of significance ($p\leq0.001$).

The transcripts, PSMC4, PSMB5 and NEDD4L, which demonstrated significant up-regulation in tumour cells, showed significant positive correlation with each other [Fig 4.7.1].

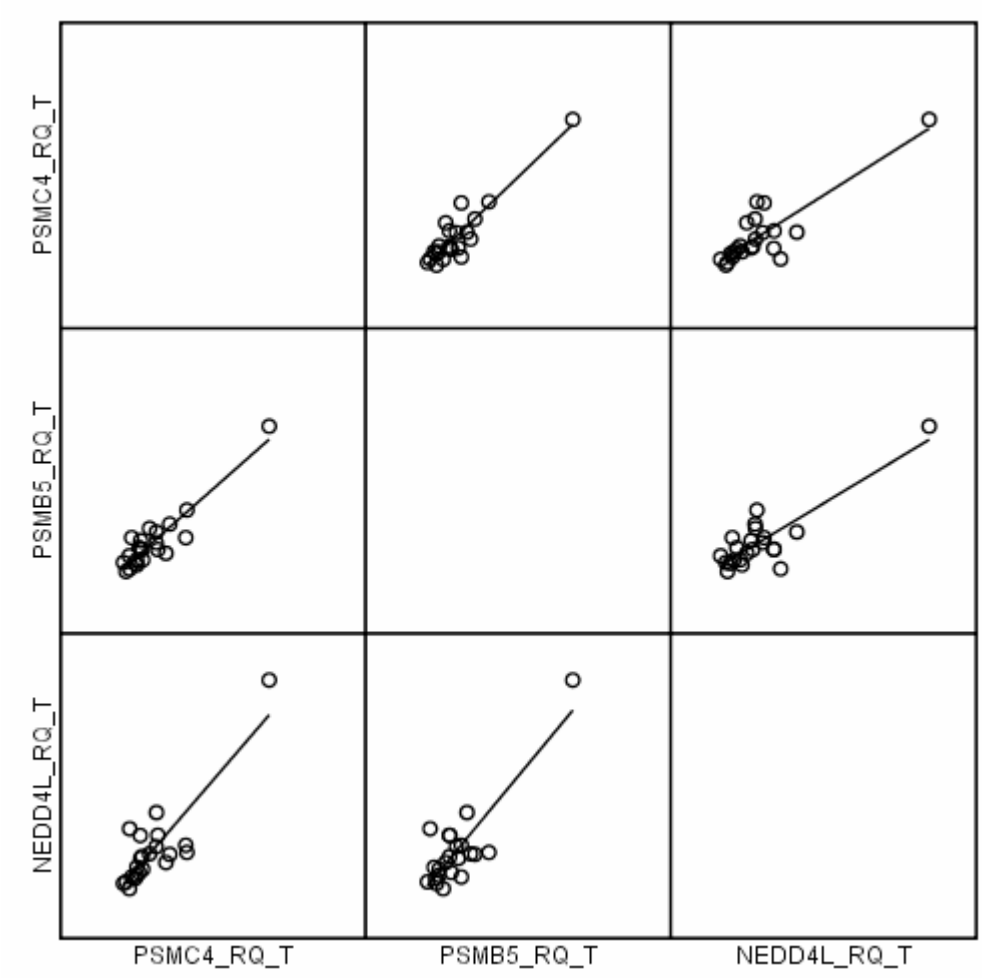


Fig 4.7.1: Positive correlation between PSMC4, PSMB5 & NEDD4L (RQ_T: transcript expression in tumour tissue)

	PSMC4	PSMB5	CBL	HECW1	MDM2	NEDD4	NEDD4L	SIAH1	SMURF2	UBE3A
PSMC4										
PSMB5	0.001									
CBL	0.014	0.006								
HECW1	0.025	0.538	0.005							
MDM2	0.046	0.005	0.004	0.045						
NEDD4	0.442	0.106	0.046	0.062	0.037					
NEDD4L	0.008	0.020	0.378	0.094	0.098	0.471				
SIAH1	0.026	0.064	0.421	0.792	0.170	0.668	0.359			
SMURF2	0.011	0.003	0.001	0.005	0.003	0.021	0.314	0.020		
UBE3A	0.000	0.001	0.000	0.007	0.001	0.136	0.176	0.035	0.000	

Tab 4.7.1: Significance of transcript correlation in tumour (value in box ≡ significance, p. Highlighted box ≡ significant correlation)

	PSMC4	PSMB5	CBL	HECW1	MDM2	NEDD4	NEDD4L	SIAH1	SMURF2	UBE3A
PSMC4										
PSMB5	0.734									
CBL	0.556	0.571								
HECW1	0.298	0.173	0.471							
MDM2	0.521	0.586	0.615	0.263						
NEDD4	0.360	0.579	0.452	0.132	0.483					
NEDD4L	0.496	0.412	0.156	0.256	0.270	0.117				
SIAH1	0.337	0.203	0.211	0.404	0.194	0.062	0.166			
SMURF2	0.614	0.673	0.641	0.361	0.608	0.633	0.159	0.340		
UBE3A	0.776	0.701	0.744	0.425	0.666	0.487	0.266	0.354	0.777	

Tab 4.7.2: Correlation coefficient of transcripts in tumour (value in box correlation coefficient, r: r>7 ≡ high correlation, 0.3>r<0.7 ≡ moderate correlation, r<0.3 ≡ weak correlation. Highlighted box ≡ significant correlation)

5. Discussion

Three of the investigated transcripts, PSMC4, PSMB5, NEDD4L, showed significant up-regulation in tumour compared to adjacent normal tissue of prostate cancer patients. Their impaired expression suggests their involvement in prostate cancer pathophysiology.

The critical role played by the ubiquitin-proteasome protein degradation system in cell cycle regulation, transcription, proliferation, apoptosis and regulation of tumour suppressor genes makes it a target for oncogenic mutation. Aberrations of this intricate regulatory cascade would induce oncogenic effects, inhibit tumour suppression activity and evade apoptosis, thereby enhancing malignancy. (Hanahan and Weinberg 2000; Devoy et al. 2005; Mani and Gelmann 2005)

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. PSMC4 and PSMB5 were over-expressed at 1.57-fold and 2.08-fold and further demonstrated significance of 0.027 and 0.001 respectively. Over-expression of these genes would enhance proteosomal degradation of some critical cellular regulatory proteins including tumour suppression genes and modulators of apoptosis, consequently inhibiting tumor suppressor activity and limiting apoptosis. PSMB5 has been shown to be up-regulated in breast cancer tissue compared to adjacent normal tissue and the inhibition of its activity was believed to be a useful method in human breast cancer treatment (Deng et al. 2007). Targeting the proteasome system for cancer therapy has already been demonstrated with bortezomib, (Velcade, PS-341), the first proteasome inhibitor to have undergone clinical trials, which confers anti-cancer activity in both solid and haematologic malignancies. It has been approved for the treatment of advanced multiple myeloma and is in ongoing

clinical trials for other haematological and solid tumors including prostate cancer. (Mitsiades et al. 2005; Roccaro et al. 2006) Proteasome inhibitors induce apoptosis and in vivo confer anti-tumour efficacy, as well as sensitise malignant cells and tumours to pro-apoptic effects of conventional chemotherapeutics and radiation therapy. (Voorhees et al. 2003; Burger and Seth 2004) Recent findings have further shown the role of oncogenic mutations in tumourigenesis. Mutation of PSMB5 and over-expression of mutant PSMB5 have been implicated with resistance towards bortezomib. (Lü et al. 2008; Oerlemanns et al. 2008) The findings above affirm the postulation of PSMB5 participation in prostate cancerogenesis. Selective intervention of PSMB5 activity could provide a useful approach for the treatment of human prostate carcinoma.

NEDD4L (Nedd4-2) was over-expressed in prostate tumour cells at 2.5-fold. It regulates diverse cellular processes like excretion, plasma membrane channel regulation, protein catabolism and TGF-beta signalling. NEDD4L targets ENaC for degradation, TTYH2, TTYH3 and itself for ubiquitination and negatively regulates TGF-ß signalling by targeting TGF-ß receptor type I and Smad2 for ubiquitination and degradation [Fig 5.1]. (Bioinformatic Harvester; Harvey et al. 2001; Itani et al. 2005; Kuratomi et al. 2005; Zhou et al. 2007; Kabra et al. 2008; He et al. 2008; Bruce et al. 2008) The up-regulation of NEDD4L would suggest higher tagging of its substrate proteins for ubiquitination and subsequent degradation, thus decreasing protein level of its substrates.

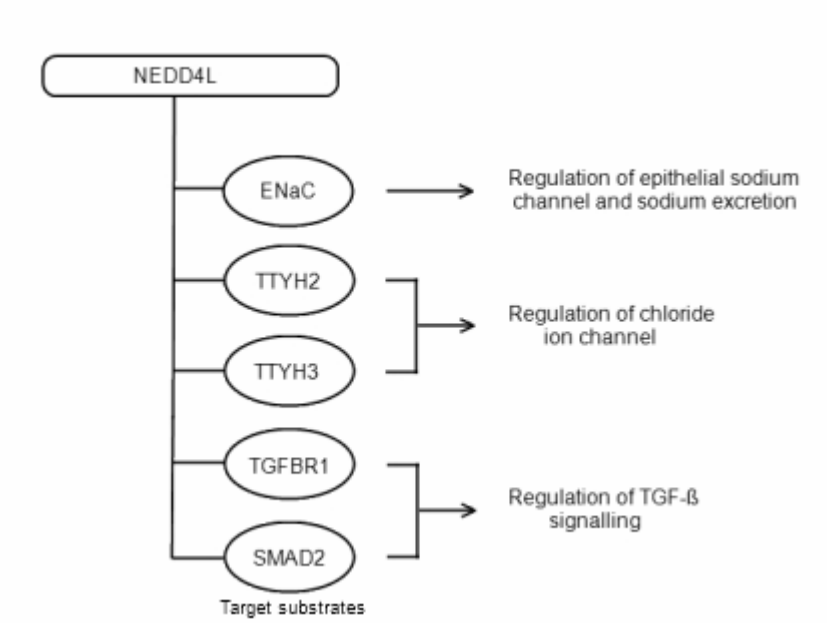


Fig 5.1: NEDD4L target substrates and function

ENaC is an amiloride-sensitive epithelial sodium channel that controls fluid and electrolyte homeostasis and its trafficking and activity is negatively regulated via NEDD4L. (Bioinformatic Harvester; Harvey et al. 2001; Itani et al. 2005; Zhou et al. 2007; Kabra et al. 2008) A higher expression of NEDD4L would induce a further reduction in ENaC-level and activity. ENaC action in blood pressure regulation and development of hypertension via ENaC-dependent sodium and fluid regulation has widely been investigated (Harvey et al. 2001; Dunn et al. 2002; Russo et al. 2005; Umemura et al. 2006). The role of NEDD4L-dependent ENaC degradation in prostate physiology has yet to be explored.

TTYH2 and TTYH3 belong to the family of tweety proteins which function as chloride ion channels. TTYH2 over-expression is associated with cancer progression and has been shown to be up-regulated in colon and renal cell carcinomas. (Bioinformatic Harvester; Rae et al. 2001; Toiyama et al. 2007; He et al. 2008) Their function in prostate physiology has yet to be investigated.

An interesting observation was made by Hu et al. (2009). They showed that NEDD4L-expression was decreased in prostate cancer compared to benign prostate hyperplasia and that the decreased expression of NEDD4L

correlated with higher Gleason score. Level of expression in this study was compared in prostate cancer cells of moderate Gleason score (3 out of 22 specimens with Gleason score 4+4 and 3 others with 4+4) against adjacent normal tissue. This may suggest that in the process of tumourigenesis the expression of NEDD4L is run lower compared to the benign prostate but that this expression is yet held higher in the cancerous cells compared to the "still" normal prostate cells (adjacent normal cells) in the prostate of carcinoma patients. Investigating the expression level of NEDD4L in prostate cancer cells against normal prostate cells (adjacent to cancer cells), PIN cells, BPH cells and healthy prostate cells would bring more light to this postulation and also provide better understanding in prostate cancer biology.

NEDD4L regulation of TGF-ß signalling presents as yet the role most implicated with tumourigenesis. TGF-ß (TGF-ß 1) is a pleiotropic growth factor that controls cell proliferation, differentiation and apoptosis [Fig 5.2]. It functions as a tumour suppressor of the prostate and maintains epithelial homeostasis in the prostate by inhibiting cell proliferation, stimulating cell differentiation and inducing apoptosis. (Bioinformatic Harvester; Cullig et al. 1996; Lee et al. 1999; Wikström et al. 2000; Park et al. 2003; Yang et al. 2009) At the same time, TGF-ß demonstrates oncogenic activity in malignant prostate. It is over-expressed in prostate cancer with even higher expression in metastatic carcinoma. In malignant prostate TGF-ß enhances cancerogenesis via tumour growth promotion, induction of angiogenesis, promotion of extracellular matrix modification and inhibition of host immunosuppression. (Lee et al. 1999; Wikström et al. 2000; Park et al. 2003; Kaminska et al. 2005; Perttu et al. 2006) TGF-ß receptor type I binds to TGF-ß to transduce TGF-ß signal from the cell surface to the cytoplasm. The binding of TGF-ß to its receptor induces complex formation of receptor-regulated Smads 2/3 with Smad 4, which is translocated into the nucleus to bind DNA and regulate transcription of specific genes. (Bioinformatic Harvester; Kaminska et al. 2005)

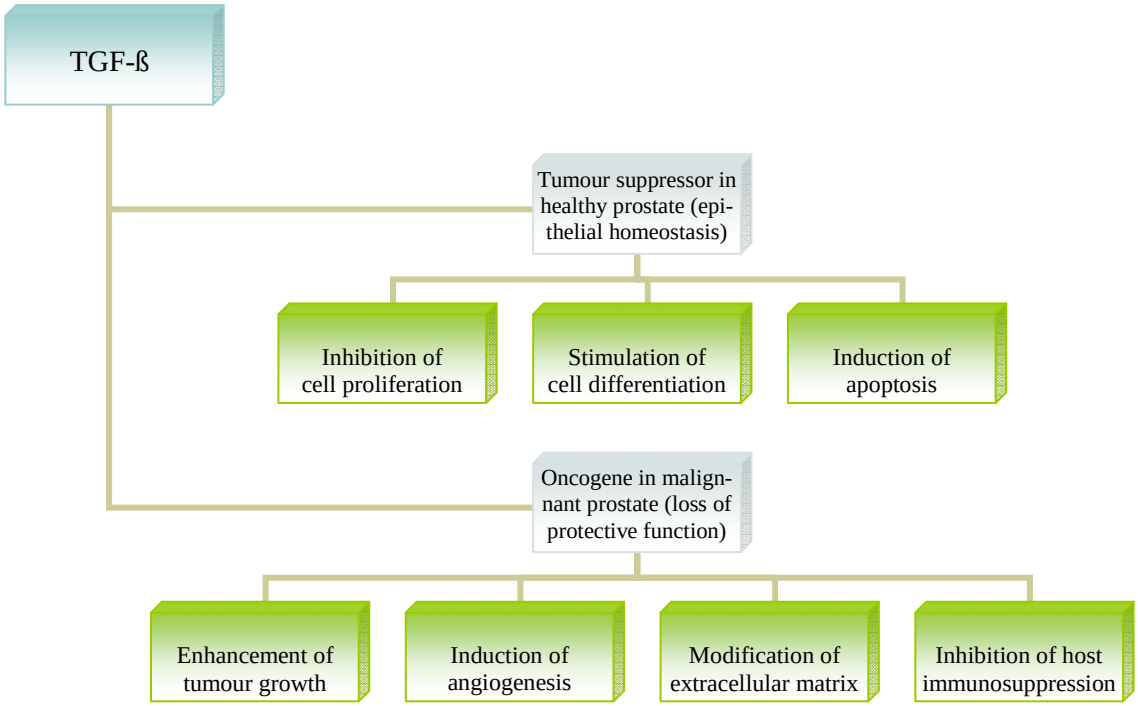


Fig 5.2: TGF-ß in healthy and malignant prostate

Smad2 belongs to the family of SMAD proteins that function as signal transducers and transcriptional modulators. Smad2 mediates signal transduction of TGF-ß and so regulates TGF-ß- induced apoptosis, gene expression, cell proliferation and differentiation. It is also phosphorylated by TGF-ß receptors upon response to TGF-ß signal. (Bioinformatic Harvester; Yang et al. 2009) Yang et al. (2009) suggest that Smad2 plays a role in tumour suppression in basal epithelium of the prostate. Consequently, Smad2 may play a role in prostate carcinogenesis via TGF-ß regulation. Phosphorylated Smad2 (p-Smad2) showed decreased expression in prostate cancer, primarily in malignant cells of Gleason grades 3 and 4, while cancer cells of Gleason grad 5 demonstrated more higher expression. (Perttu et al. 2006) A difference in NEDD4L expression in cancer cells of moderate Gleason score ($\leq$ 6) and higher Gleason score ($\geq$ 7) was not demonstrated in this study. A higher case number may reveal a change in expression level of NEDD4L in cancers of different Gleason score. The up-regulation of NEDD4L in malignant prostate cells could explain the decrease in Smad2 expression in prostate carcinoma [Fig 5.3]. TGF-ß receptor type I showed decreased expression in prostate cancer, which

provides tumour cells growth advantage over benign prostate cells. The down-regulation of TGF-ß receptors and over-expression of TGF-ß is associated with poorer prognosis. (Lee et al. 1999; Wiksröm et al. 2000; Park et al. 2003; Shariat et al. 2004) Shariat et al. (2004) further suggest loss of TGF-ß receptor I as a prognostic marker in patients with prostate cancer. Kaminska et al. (2005) reported that inhibition of TGF-ß action suppressed tumour viability, migration and metastases in mamma carcinoma, melanoma and prostate cancer model and further reported that TGF-ß inhibition could provide a novel approach for human cancer therapy. Earlier suggestions held that, manipulations in the TGF-ß signalling pathway could elucidate novel therapeutic strategies for prostate cancer. (Lee et al. 1999; Wikström et al. 2000) Kuratomi et al. (2005) showed that transcriptional activity of TGF-ß was inhibited through NEDD4L over-expression. Wikström et al. (2000) also reported that prostate cancer cells acquire resistance to anti-proliferative and pro-apoptotic effects of TGF-ß via down-regulation of TGF-ß receptors (type I, II). The up-regulation of NEDD4L in prostate carcinoma would induce a higher rate of TGF-ß receptor I targeting and subsequent degradation, which correlates with decreased quantities of TGF-ß receptor type I in prostate cancer cells and thus support the effect of higher negative regulation of TGF-ß signalling through NEDD4L up-regulation and subsequent loss of TGF-ß protective function [Fig 5.2; Fig 5.3]. The over-expression of TGF-ß in prostate cancer could be a response of malignant prostate cells to the down-regulation of TGF-ß receptor I. The effect of NEDD4L in TGF-ß signalling via Smad2 and TGF-ß receptor type I degradation depicts the role of NEDD4L in prostate cancerogenesis [Fig 5.2; Fig 5.3].

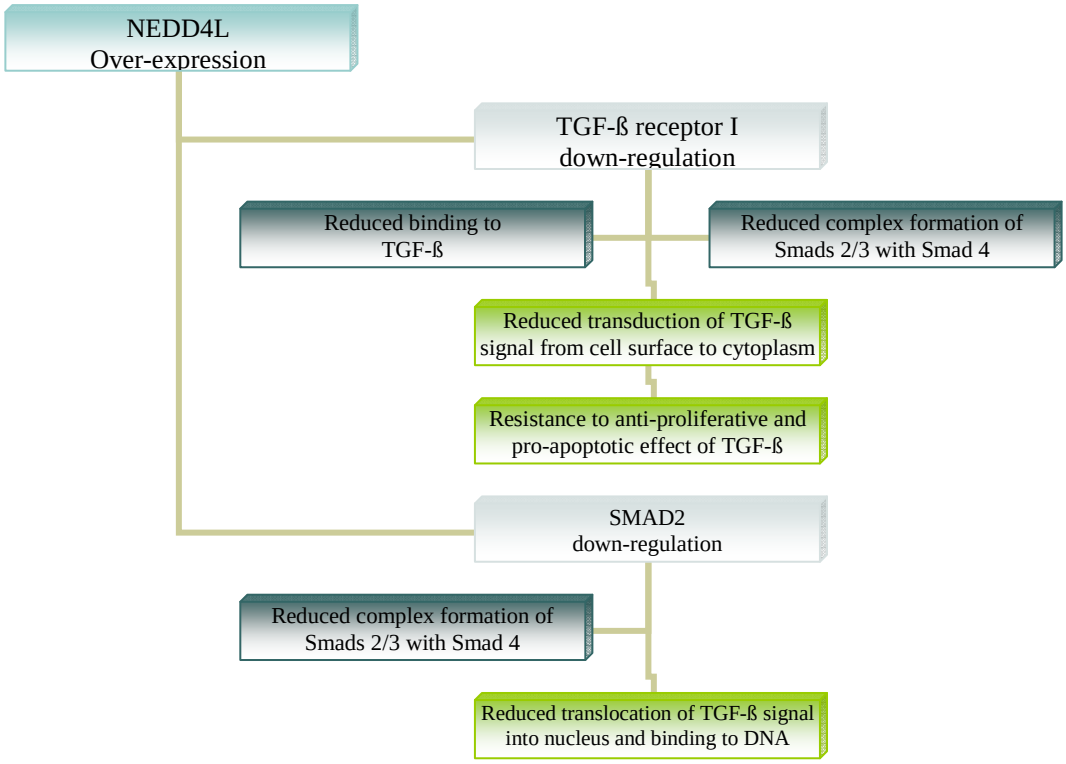


Fig 5.3: NEDD4L and TGF-ß signalling in malignant prostate

Androgen ablation is a well established and practiced form of (hormone) therapy for prostate carcinoma. (Hanno, Malkowicz and Wein 2005; Hautmann and Huland 2006) Qi et al. (2003) demonstrated an up-regulation of NEDD4L in prostate cancer cell lines (LNCaP) by androgen and suggested a potential role of NEDD4L in androgen action and prostate physiology. Increased expression of NEDD4L by androgen (Qi et al. 2003) would induce higher degradation of Smad2 and TGF-ß receptor I and subsequently lead to recession in TGF-ß activity, which would in turn lead to decrease in TGF-ß tumour suppressor activity (less apoptosis, less differentiation, less inhibition of proliferation) necessary for normal prostate growth and physiology. This effect of androgen on NEDD4L regulation via TGF-ß signalling provides opportunity for oncogenic mutation and may explain androgen action in prostate carcinoma. On this note, androgen ablation would imply down-regulation of NEDD4L and enhancement of TGF-ß tumour suppressor activity, which may explain tumour regression under androgen ablation in androgen-dependent prostate carcinoma. NEDD4L-dependent regulation of TGF-ß signalling thus may

disclose a mechanism of androgen action in prostate biology and therapeutic action of androgen ablation in androgen-dependent prostate carcinomas. These sum up to reinforce the role of NEDD4L in androgen action in prostate biology and the involvement of NEDD4L in prostate tumourigenesis. The important role of NEDD4L in TGF-ß signalling supports the postulation that manipulation of NEDD4L could elucidate a novel therapeutic strategy for prostate carcinoma treatment.

One transcript demonstrated significant difference in expression level at different tumour stages. The specimens examined were grouped in tumour stages pT2 and pT3 (pT_group 2, 3) and histological patterns with Gleason score ≤ 6 (G6) and Gleason score ≥ 7 (G7). SMURF2 displayed a higher rate of over-expression in pT2 tumour. This would suggest that different protein levels of SMURF2 are required at different stages of tumour progression. SMURF2 is also a negative regulator of TGF-ß signalling. It targets Smads 1, 2, 3 and 7 as well as Smurf1 for ubiquitination and degradation. Its up-regulation would imply increased negative regulation of TGF-ß signalling. The over-expression of Smurf2 in prostate cancer compared to adjacent normal tissue was however not significant.

The three transcripts which demonstrated significant up-regulation of expression displayed significant correlation of expression with each other in tumour tissue. Correlation between PSMC4 and PSMB5 was high. Correlation of NEDD4L with PSMC4 and PSMB5 was moderate. This suggests that increased ubiquitination of NEDD4L substrates also experience increased degradation. The effects of enhanced degradation have been discussed above. UBE3A also demonstrated high correlation of transcript expression in tumour cells with PSMC4, PSMB5, CBL, and SMURF2. The over-expression of UBE3A, CBL and SMURF2 in tumour tissue was however not significant. Up-regulation of HECW1, MDM2, SIAH1 and NEDD4 in tumour did not demonstrate significance as well.

NEDD4, CBL, UBE3A and MDM2 have demonstrated alterations in expression in prostate carcinoma as well as in other cancers. SMURF2 and SIAH1 also revealed change in expression in diverse human cancers. These transcripts all displayed up-regulation of expression in prostate tumour compared to adjacent normal tissue. Absence of significance of transcript expression in this study does not imply that the expression of these transcripts is not altered in prostate carcinoma. Examining a larger cohort may reveal changes in transcript expression and emerge as a more significant covariate.

Further examinations investigating expression alterations of NEDD4L and its target substrates in healthy, normal and malignant prostate tissue of increasing tumour stage and Gleason score would provide additional information on the role of NEDD4L in prostate cancer formation and progression as well as prostate biology. The effect of NEDD4L-dependent ENaC degradation as well as the outcome of NEDD4L on TTHY2 and TTHY3 degradation in the prostate (prostate biology, prostate cancerogenesis) could also be examined. Furthermore the possible correlation of NEDD4L expression with tumour aggressiveness via TGF-ß regulation could be elaborated and could provide a novel diagnostic factor for aggressive prostate carcinoma, which could possibly act as a prognostic marker. The effect of PSMB5, NEDD4L modification (PSMB5/NEDD4L inhibition) on tumour growth / size could provide useful information on possible therapy concepts for prostate cancer patients.

This study was carried out on a small tumour cohort and acts as a screening study, in which behavioural patterns of single transcripts were examined with the goal of finding out which transcripts reveal expression alterations and so provide basic knowledge of functional mechanisms of these transcripts in prostate carcinoma and prostate biology as well. Information from this study makes it then possible to run secondary investigations on those transcripts which could act as diagnostic markers as well as those

which could provide novel therapy approaches for prostate carcinoma. These additional examinations would however require a much larger cohort of tumours and healthy tissue to enable high statistical standards.

6. Summary

The profuse cellular processes controlled by the ubiquitin-proteasome degradation network make it a target for diverse human pathologies including cancerogenesis. (Devoy et al. 2005; Mani and Gelmann 2005)

Aberrations in the UPP were investigated by comparing transcript expression patterns of ten members of the system pathway in tumour and adjacent normal tissue of prostate carcinoma patients: eight of these transcripts are ubiquitin E3 ligases (UBE3A, NEDD4, HECW1, SMURF2, CBL, MDM2, SIAH1) and the other two, PSMC4 and PSMB5, belong to the two major components of the proteasome, the 19S regulator and 20S protease core respectively.

Three of the transcripts investigated, PSMC4, PSMB5 and NEDD4L, demonstrated significant up-regulation of expression in cancer cells compared to adjacent normal prostate tissue. Their dysregulation display the potential role of these transcripts in prostate tumourigenesis and progression. This study elaborated on the potential mechanism of NEDD4L action via regulation of TGF-ß signalling in prostate cancer pathogenesis and further discussed the role of NEDD4L in androgen action in the prostate. In addition, selective intervention in PSMB5 and NEDD4L action could provide novel therapeutic approaches for treatment of human prostate carcinoma.

7. References and Bibliography

Abriel H, Loffing J, Rebhun JF, Pratt JH, Schild L, Horisberger JD, Rotin D, Staub O (1999) Defective regulation of the epithelial Na+ channel by Nedd4 in Liddle's syndrome. J Clin Invest 103/5:667-73

Applied Biosystems (www.appliedbiosystems.com) Applications & Technologies. Real-Time PCR: Absolute vs Relative Quantitation

Applied Biosystems (www.appliedbiosystems.com) Applications & Technologies. Real-Time PCR: Real-Time PCR vs Traditional PCR

Applied Biosystems (PN 4303859) User Bulletin #2: Relative Quantitation of Gene Expression. Updated Oct 2001

Bertz J, Giersiepen K, Haberland J, Hentschel S, Kaatsch P, Katalinic A, Stabenow R, Stegmaier C und Ziegler H. (2006) Krebs in Deutschland, Häufigkeiten und Trends. Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. und RKI (online version), 5. überarb. u akt. Ausg., Saarbrücken

Bioinformatic Harvester EMBL Heidelberg (http://harvester.embl.de)
→ new site: Bioinformatic Harvester at Karlsruhe Institute of Technology (http://harvester.fzk.de/harvester/)

Bonaccorsi L, Nosi D, Muratori M, Formigli L, Forti G, Baldi E (2007) Altered endocytosis of epidermal growth factor receptor in androgen receptor positive prostate cancer cell lines. J Mol Endocrinol 38/1-2:51-66

Boston Biochem (© 1998-2005): Ubiquitin Proteasome Pathway (www.bostonbiochem.com/upp.php)

Burger AM, Seth AK (2004) The ubiquitin-mediated protein degradation pathway in cancer: therapeutic implications. Eur J Cancer. 40/15:2217-29

Canfield SE, Zhu K, Williams SA, McConkey DJ (2006) Bortezomib inhibits docetaxel-induced apoptosis via a p-21-dependent mechanism in human prostate cancer cells. Mol Cancer Ther 5/8:2043-50

Chen C, Matesic LE (2007) The Nedd4-like family of E3 ubiquitin ligases and cancer. Cancer Metastasis Rev 26/3-4:587-604

Clements R (2007) Prostate carcinoma. eMedicine, updated of Apr 18 2007 (http://www.emedicine.com/radio/topic574.htm)

Cooper GM (2000) Protein degradation. In: The Cell, A Molecular Approach. ASM Press Washington, D.C., Sinauer Associates Inc., Sunderland Massechusetts, 2nd edn, pp 305-309

Crinelli R, Bianchi M, Menotta M, Carloni E, Giacomini E, Pennati M, Magnani M (2008) Ubiquitin over-expression promotes E6AP autodegradation and reactivation of the p53/MDM2 pathway in HeLa cells. Mol Cell Biochem 318/1-2:129-45

Culig Z, Hobisch A, Cronauer MV, Radmayr C, Hittmair A, Zhang J, Thurnher M, Bartsch G, Klocker H (1996) Regulation of prostatic growth and function by peptide growth factors. Prostate 28/6:392-405

Curran S, Akin O, Agildere AM, Zhang J, Hricak H, Rademaker J (2007) Endorectal MRI of prostatic and periprostatic cystic lesions and their mimics. AJR Am J Roentgenol 188/5:1373-9

de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW, Span PN (2005) Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Lab Invest 85/1:154-9

Deng S, Zhou H, Xiong R, Lu Y, Yan D, Xing T, Dong L, Tang E, Yang H (2007) Over-expression of genes and proteins of ubiquitin specific peptidases (USPs) and proteasome subunits (PSs) in breast cancer tissue observed by the methods of RFDD-PCR and proteomics. Breast Cancer Res Treat 104/1:21-30

Devoy A, Soane T, Welchman R and Mayer RJ (2005) The ubiquitin-proteasome system and cancer. Essays Biochem 41:187-203

Dubiel W, Ferrell K, Rechsteiner M (1994) Tat-binding protein 7 is a subunit of the 26S protease. Biol Chem Hoppe Seyler 375/4:237-40

Dunn DM, Ishigami T, Pankow J, von Niederhausern A, Alder J, Hunt SC, Leppert MF, Lalouel JM, Weiss RB (2002) Common variant of human NEDD4L activates a cryptic splice site to form frameshifted transcript. J Hum Genet 47/12:665-76

Egevad L, Allsbrook WC and Epstein JI (© 2004) Gleason Grading of Prostatic Carcinoma. Johns Hopkins University (http://pathology2.jhu.edu/gleason/index.cfm)

Elliot WH and Elliot DC (2001) Cellular disposal of unwanted molecules. In: Biochemistry and Molocular Biology. Oxford, New York, 2nd edn, pp 267-273

Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J Biol Chem 275/12:8945-51

Fukunaga E, Inoue Y, Komiya S, Horiguchi K, Goto K, Saitoh M, Miyazawa K, Koinuma D, Hanyu A, Imamura T (2008) Smurf2 induces ubiquitin-dependent degradation of Smurf1 to prevent migration of breast cancer cells. J Biol Chem 283/51:35660-7

Fukuchi M, Fukai Y, Masuda N, Miyazaki T, Nakajima M, Sohda M, Manda R, Tsukada K, Kato H, Kuwano H (2002) High-level expression of the Smad ubiquitin ligase Smurf2 correlates with poor prognosis in patients with esophageal spuamous cell carcinoma. Cancer Res 62/24: 7162-5

Gao X, Mohsin SK, Gatalica Z, Fu G, Sharma P, Nawaz Z (2005) Decreased expression of e6-associated protein in breast and prostate carcinomas. Endocrinology 146/4:1707-12

Hanahan D and Weinberg RA (2000) The Hallmarks of Cancer. Cell 100:57-70

Hanno PM, Malkowicz SB, Wein AJ (eds) (2001) Adenocarcinoma of the prostate. In: Clinical Manual of Urology. McGraw-Hill Medical Publishing Division, New York Chicago San Francisco Lisbon London Madrid Mexico Milan New Dehli San Juan Seoul Singapore Sydney Toronto, 3rd edn, pp 520-536

Harvey KF, Dinudom A, Cook DI, Kumar S (2001) The Nedd4-like protein KIAA0439 is a potential regulator of epithelial sodium channel. J Biol Chem 276/11:8597-601

Hautmann R and Huland H (2006) Prostatakarzinom. In: Urologie. Springer, Heidelberg, 3., überarb Aufl, S 229-241

He Y, Hryciw DH, Carroll ML, Myers SA, Whitbread AK, Kumar S, Poronnik P, Hooper JD (2008) The ubiquitin-protein ligase Nedd4-2 differentially interacts with and regulates members of the Tweety family of chloride ion channels. J Biol Chem 283/35:24000-10

Honda R, Tanaka H, Yasuda H (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 420/1:25-7

Honda R, Yasuda H (2000) Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. Oncogene 19/11:1473-6

Hu G, Fearon ER (1999) Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. Mol Cell Biol 19/1:724-32

Hu G, Zhang S, Vidal M, Baer JL, Xu T, Fearon ER (1997) Mammalian homologs of seven in absentia regulate DCC via the ubiquitin-proteasome pathway. Genes Dev 11/20:2701-14

Hu XY, Xu YM, Fu Q, Yu JJ, Huang J (2009) Nedd4L expression is downregulated in prostate cancer compared to benign prostate hyperplasia. Eur J Surg Oncol 35/5:527-31

Huibregtse JM, Scheffner M, Howley PM (1993) Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol Cell Biol 13/2:775-84

Itani OA, Stokes JB, Thomas CP (2005) Nedd4-2 isoforms differentially associate with ENaC and regulate its activity. Am J Physiol Renal Physiol 289/2:F334-46

Ito R, Nakayama H, Yoshida K, Matsumura S, Oda N, Yasui W (2004) Expression of Cbl linking with the epidermal growth factor receptor system is associated with tumor progression and poor prognosis of human gastric cancer. Virchows Arch 444/4:324-31

Jin C, Yang YA, Anver MR, Morris N, Wang X, Zhang YE (2009) Smad ubiquitination regulatory factor 2 promotes metastasis of breast cancer cells by enhancing migration and invasiveness. Cancer Res 69/3:735-40

Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC (1999) The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science 286/5438:309-12

Kabra R, Knight KK, Zhou R, Snyder PM (2008) Nedd4-2 induces endocytosis and degradation of proteolytically cleaved epithelial Na+ channels. J Biol Chem 283/10:6033-9

Kamei T, Machida K, Nimura Y, Senga T, Yamada I, Yoshii S, Matsuda S, Hamaguchi M (2000) C-Cbl protein in human cancer tissues is frequently tyrosine phosphorylated in a tumor-specific manner. Int J Oncol 17/2:335-9

Kaminska B, Wesolowska A, Danilkiewicz M (2005) TGF beta signalling and its role in tumour pathogenesis. Acta Biochim Pol 52/2:329-37

Kawai H, Wiederschain D, Yuan ZM (2003) Critical contribution of the MDM2 acidic domain to p53 ubiquitination. Mol Cell Biol 23/14:4939-47

Kim CJ, Cho YG, Park CH, Jeong SW, Nam SW, Kim SY, Lee SH, Yoo NJ, Lee JY, Park WS (2004) Inactivating mutations of the Siah-1 gene in gastric cancer. Oncogene 23/53:8591-6

Kim H, Jeong W, Ahn K, Ahn C, Kang S (2004) Siah-1 interacts with the intracellular region of polycystin-1 and affects its stability via the ubiquitin-proteasome pathway. J Am Soc Nephrol 15/8:2042-9

Kim SS, Yoo NJ, Jeong EG, Kim MS, Lee SH (2008) Expression of NEDD4-1, a PTEN regulator, in gastric and colorectal carcinomas. APMIS 116/9:779-84

Knight JF, Shepherd CJ, Rizzo S, Brewer D, Jhavar S, Dodson AR, Cooper CS, Eeles R, Falconer A, Kovacs G, Garrett MD, Norman AR, Shipley J, Hudson DL (2008) TEAD1 and c-Cbl are novel prostate basal cell markers that correlate with poor clinical outcome in prostate cancer. Br J Cancer 99/11:1849-58

Krebsinformationsdienst (2003) Prostatakrebs. Deutsches Krebsforschungszentrum Heidelberg, Aktualisierung von Dezember 2003 (http://www.krebsinformationsdienst.de/Krebsarten/prostatakrebs.html)

Kühne C, Banks L (1998) E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. J Biol Chem 273/51:34302-9

Kumar S, Talis AL, Howley PM (1999) Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. J Biol Chem 274/26:18785-92

Kuratomi G, Komuro A, Goto K, Shinozaki M, Miyazawa K, Miyazono K, Imamura T (2005) NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. Biochem J 386/Pt 3:461-70

Lee C, Sintich SM, Mathews EP, Shah AH, Kundu SD, Perry KT, Cho JS, Ilio KY, Cronauer MV, Janulis L, Sensibar JA (1999) Transforming growth factor-beta in benign and malignant prostate. Prostate 39/4:285-90

Leite KR, Franco MF, Srougi M, Nesrallah LJ, Nesrallah A, Bevilacqua RG, Darini E, Carvalho CM, Meirelles MI, Santana I, Camara-Lopes LH (2001) Abnormal Expression of MDM2 in prostate carcinoma. Mod Pathol 14/5:428-36

Levkowitz G, Waterman H, Ettenberg SA, Katz M, Tsygankov AY, Alroy I, Lavi S, Iwai K, Reiss Y, Ciechanover A, Lipkowitz S, Yarden Y (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell 4/6:1029-40

Lévy F, Muehlethaler K, Salvi S, Peitrequin AL, Lindholm CK, Cerottini JC, Rimoldi D (2005) Ubiquitylation of a melanosomal protein by HECT-E3 ligases serves as sorting signal for lysosomal degradation. Mol Biol Cell 16/4:1777-87

Li Y, Ozaki T, Kikuchi H, Yamamoto H, Ohira M, Nakagawara A (2008) A novel HECT-type E3 ubiquitin protein ligase NEDL1 enhances the p53-mediated apoptotic cell death in its catalytic activity-independent manner. Oncogene 27/26:3700-9

Lin X, Liang M, Feng XH (2000) Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor-beta signaling. J Biol Chem 275/47:36818-22

Lü S, Yang J, Song X, Gong S, Zhou H, Guo L, Song N, Bao X, Chen P, Wang J (2008) Point mutation of the proteasome beta5 subunit gene is an important mechanism of bortezomib resistance in bortezomib-selected variants of Jurkat T cell lymphoblastic lymphoma/leukemia line. J Pharmacol Exp Ther 326/2:423-31

Mani A and Gelmann EP (2005) The ubiquitin-proteasome pathway and its role in cancer. J Clin Oncol 23/21:4776-89

Matsuo K, Satoh S, Okabe H, Nomura A, Maeda T, Yamaoka Y, Ikai I (2003) SIAH1 inactivation correlates with tumor progression in hepatocellular carcinomas. Genes Chromosomes Cancer 36/3:283-91

Mishra A, Jana NR (2008) Regulation of turnover of tumor suppressor p53 and cell growth by E6-AP, a ubiquitin protein ligase mutated in Angelman mental retardation syndrome. Cell Mol Life Sci 65/4:656-66

Mitsiades CS, Mitsiades N, Hideshima T, Richardson PG, Anderson KC (2005) Proteasome inhibitors as therapeutics. Essays Biochem 41:205-18

Miura-Shimura Y, Duan L, Rao NL, Reddi AL, Shimura H, Rottapel R, Druker BJ, Tsygankov A, Band V, Band H (2003) Cbl-mediated ubiquitinylation and negative regulation of Vav. J Biol Chem 278/40:38495-504

Miyazaki K, Fujita T, Ozaki T, Kato C, Kurose Y, Sakamoto M, Kato S, Goto T, Itoyama Y, Aoki M, Nakagawara A (2004) NEDL1, a novel ubiquitin-protein isopeptide ligase for dishevelled-1, targets mutant superoxide dismutase-1. J Biol Chem 279/12:11327-35

Mostofi FK, Sesterhenn IA, Davis CJ. In collab. With Sobin LH and participants from 10 countries (2002) Histological Typing of Prostate Tumours. World Health Organisation, International Histological Classification of Tumours, 2nd edn. Springer, Berlin Heidelberg New York

Nakano A, Koinuma D, Miyazawa K, Uchida T, Saitoh M, Kawabata M, Hanai J, Akiyama H, Abe M, Miyazono K, Matsumoto T, Imamura T (2009) Pin1 down-regulates transforming growth factor-beta (TGF-beta) signaling by inducing degradation of Smad proteins. J Biol Chem 284/10:6109-15

National Cancer Institute (2005) A Snapshot of Prostate Cancer. Updated August 2005 (http://planning.cancer.gov/disease/snapshots.shtml)

Nelen V (2007) Epidemiology of prostate cancer. Recent Results Cancer Res 175:1-8

Nuber U, Schwarz SE, Scheffner M (1998) The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate. Eur J Biochem 254/3:643-9

Oda H, Kumar S, Howley PM (1999) Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination. Proc Natl Acad Sci U S A 96/17:9557-62

Ohl F, Jung M, Xu C, Stephan C, Rabien A, Burkhardt M, Nitsche A, Kristiansen G, Loening SA, Radonić A, Jung K (2005) Gene expression studies in prostate cancer tissue: which reference gene should be selected for normalization? J Mol Med 83/12:1014-24

Oerlemans R, Franke NE, Assaraf YG, Cloos J, van Zantwijk I, Berkers CR, Scheffer GL, Debipersad K, Vojtekova K, Lemos C, van der Heijden JW, Ylstra B, Peters GJ, Kaspers GL, Dijkmans BA, Scheper RJ, Jansen G (2008) Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein. Blood 112/6:2489-99

Park JI, Lee MG, Cho K, Park BJ, Chae KS, Byun DS, Ryu BK, Park YK, Chi SG (2003) Transforming growth factor-beta1 activates interleukin-6 expression in prostate cancer cells through the synergistic collaboration of the Smad2, p38-NF-kappaB, JNK, and Ras signaling pathways. Oncogene 22/28:4314-32

Pennock S, Wang Z (2008) A tale of two Cbls: interplay of c-Cbl and Cbl-b in epidermal growth factor receptor downregulation. Mol Cell Biol 28/9:3020-37

Perttu MC, Martikainen PM, Huhtala HS, Bläuer M, Tammela TL, Tuohimaa PJ, Syvälä H (2006) Altered levels of Smad2 and Smad4 are associated with human prostate carcinogenesis. Prostate Cancer Prostatic Dis 9/2:185-9

Pham N, Rotin D (2001) Nedd4 regulates ubiquitination and stability of the guanine-nucleotide exchange factor CNrasGEF. J Biol Chem 276/50:46995-7003

Qi H, Grenier J, Fournier A, Labrie C (2003) Androgens differentially regulate the expression of NEDD4L transcripts in LNCaP human prostate cancer cells. Mol Cell Endocrinol 210/1-2:51-62

Rae FK, Hooper JD, Eyre HJ, Sutherland GR, Nicol DL, Clements JA (2001) TTYH2, a human homologue of the Drosophila melanogaster gene tweety, is located on 17q24 and upregulated in renal cell carcinoma Genomics 77/3:200-7

Ravid T, Heidinger JM, Gee P, Khan EM, Goldkorn T (2004) c-Cbl-mediated ubiquitinylation is required for epidermal growth factor receptor exit from the early endosomes. J Biol Chem 279/35:37153-62.

Reddi AL, Ying G, Duan L, Chen G, Dimri M, Douillard P, Druker BJ, Naramura M, Band V, Band H (2007) Binding of Cbl to a phospholipase Cgamma1-docking site on platelet-derived growth factor receptor beta provides a dual mechanism of negative regulation. J Biol Chem 282/40:29336-47

Roccaro AM, Vacca A and Ribatti D (2006) Bortezomib in the treatment of cancer. Recent Patents Anticancer Drug Discov 1/3:397-403

Rondinelli RH, Epner DE, Tricoli JV (1997) Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in late pathological stage human prostate cancer. Prostate Cancer Prostatic Dis 1/2:66-72

Rubin E, Gorstein F, Rubin R, Schwarting R, Strayer D (eds) (2005) Adenocarcinoma of the prostate. In: Rubin's Pathology: clinicopathologic foundations of medicine. Lippincott Williams & Wilkins, Philadelphia Baltimore New York London Buenos Aires Hong Kong Sydney Tokyo, 4th edn, pp 921-925

Russo CJ, Melista E, Cui J, DeStefano AL, Bakris GL, Manolis AJ, Gavras H, Baldwin CT (2005) Association of NEDD4L ubiquitin ligase with essential hypertension. Hypertension 46/3:488-91

Santelli E, Leone M, Li C, Fukushima T, Preece NE, Olson AJ, Ely KR, Reed JC, Pellecchia M, Liddington RC, Matsuzawa S (2005) Structural analysis of Siah1-Siah-interacting protein interactions and insights into the assembly of an E3 ligase multiprotein complex. J Biol Chem 280/40:34278-87

Scheffner M, Huibregtse JM, Vierstra RD, Howley PM (1993) The HPV-16 E6 and E6-AP complex functions a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75/3:495-505

Schlomm T, Luebke AM, Sültmann H, Hellwinkel OJ, Sauer U, Poustka A, David KA, Chun FK, Haese A, Graefen M, Erbersdobler A, Huland H (2005) Extraction and processing of high quality RNA from impalpable and macroscopically invisible prostate cancer for microarray gene expression analysis. Int J Oncol 27/3:713-20

Schwab M (ed) (2001) Prostate cancer. In: Encyclopedic Reference of Cancer. Springer, Berlin Heidelberg New York, pp 716-726

Schwab M (ed) (2001) Ubiquitin. In: Encyclopedic Reference of Cancer. Springer, Berlin Heidelberg New York, pp 937-943

Sehat B, Andersson S, Girnita L, Larsson O (2008) Identification of c-Cbl as a new ligase for insulin-like growth factor-l receptor with distinct roles from Mdm2 in receptor ubiquitination and endocytosis. Cancer Res 68/14:5669-77

Shariat SF, Menesses-Diaz A, Kim IY, Muramoto M, Wheeler TM, Slawin KM (2004) Tissue expression of transforming growth factor-beta1 and its receptors: correlation with pathologic features and biochemical progression in patients undergoing radical prostatectomy. Urology 63/6:1191-7

Shiina H, Igawa M, Shigeno K, Yamasaki Y, Urakami S, Yoneda T, Wada Y, Honda S, Nagasaki M (1999) Clinical significance of mdm2 and p53 expression in bladder cancer. A comparison with cell proliferation and apoptosis. Oncology 56/3:239-47

Sobin LH and Wittekind Ch (eds) (2002) Prostate. In: TNM Classification of Malignant Tumours. UICC, International Union Against Cancer. Wiley-Liss, New York, 6th edn, pp 184-187

Sökeland J, Schulze H, Rübben H (2004) Prostatakarzinom. In: Urologie: verstehen-lernen-anwenden. Georg Thieme Verlag, Stuttgart New York, 13. korr. und aktualisierte Aufl, pp 301-313

Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J, Rotin D (1996) WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. EMBO J 15/10:2371-80

Tanahashi N, Suzuki M, Fujiwara T, Takahashi E, Shimbara N, Chung CH, Tanaka K (1998) Chromosomal localization and immunological analysis of a family of human 26S proteasomal ATPases. Biochem Biophys Res Commun 243/1:229-32

Toiyama Y, Mizoguchi A, Kimura K, Hiro J, Inoue Y, Tutumi T, Miki C, Kusunoki M (2007) TTYH2, a human homologue of the Drosophila melanogaster gene tweety, is up-regulated in colon carcinoma and involved in cell proliferation and cell aggregation. World J Gastroenterol 13/19:2717-21

Umebayashi K, Stenmark H, Yoshimori T (2008) Ubc4/5 and c-Cbl continue to ubiquitinate EGF receptor after internalization to facilitate polyubiquitination and degradation. Mol Biol Cell 19/8:3454-62

Umemura M, Ishigami T, Tamura K, Sakai M, Miyagi Y, Nagahama K, Aoki I, Uchino K, Rohrwasser A, Lalouel JM, Umemura S (2006) Transcriptional diversity and expression of NEDD4L gene in distal nephron. Biochem Biophys Res Commun 339/4:1129-37

Valenti MT, Bertoldo F, Dalle Carbonare L, Azzarello G, Zenari S, Zanatta M, Balducci E, Vinante O, Lo Cascio V (2006) The effect of bisphosphonates on gene expression: GAPDH as a housekeeping or a new target gene? BMC Cancer 6:49

Voorhees PM, Dees EC, O'Neil B, Orlowski RZ (2003) The proteasome as a target for cancer therapy. Clin Cancer Res 9/17:6316-25

Wang H, Yu D, Agrawal S, Zhang R (2003) Experimental therapy of human prostate cancer by inhibiting MDM2 expression with novel mixed-backbone antisense oligonucleotides: in vitro and in vivo activities and mechanisms. Prostate 54/3:194-205

Wang X, Trotman LC, Koppie T, Alimonti A, Chen Z, Gao Z, Wang J, Erdjument-Bromage H, Tempst P, Cordon-Cardo C, Pandolfi PP, Jiang X (2007) NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. Cell 128/1:129-39

Wheeler TC, Chin LS, Li Y, Roudabush FL, Li L (2002) Regulation of synaptophysin degradation by mammalian homologues of seven in absentia. J Biol Chem 277/12:10273-82

WHO (2006) Cancer. Fact sheet N°297, February 2006 (http://www.who.int/mediacentre/factsheets/fs297/en/print.html)

Wiesner S, Ogunjimi AA, Wang HR, Rotin D, Sicheri F, Wrana JL, Forman-Kay JD (2007) Autoinhibition of the HECT-type ubiquitin ligase Smurf2 through its C2 domain. Cell 130/4:651-62

Wikström P, Bergh A, Damber JE (2000) Transforming growth factor-beta 1 and prostate cancer. Scand J Urol Nephrol 34/2:85-94

Yang J, Wahdan-Alaswad R, Danielpour D (2009) Critical role of Smad2 in tumor suppression and transforming growth factor-beta-induced apoptosis of prostate epithelial cells. Cancer Res 69/6:2185-90

Yang Y, Liu W, Zou W, Wang H, Zong H, Jiang J, Wang Y, Gu J (2007) Ubiquitin-dependent proteolysis of trihydrophobin 1 (TH1) by the human papilloma virus E6-associated protein (E6-AP). J Cell Biochem 101/1:167-80

Yokouchi M, Kondo T, Houghton A, Bartkiewicz M, Horne WC, Zhang H, Yoshimura A, Baron R (1999) Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. J Biol Chem 274/44:31707-12

Yoshibayashi H, Okabe H, Satoh S, Hida K, Kawashima K, Hamasu S, Nomura A, Hasegawa S, Ikai I, Sakai Y (2007) SIAH1 causes growth arrest and apoptosis in hepatoma cells through beta-catenin degradation-dependent and -independent mechanisms. Oncol Rep 17/3:549-56

Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Derynck R (2001) Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. Proc Natl Acad Sci U S A 98/3:974-9

Zhang Z, Li M, Wang H, Agrawal S, Zhang R (2003) Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. Proc Natl Acad Sci U S A 100/20:11636-41

Zhou R, Patel SV, Snyder PM (2007) Nedd4-2 catalyzes ubiquitination and degradation of cell surface ENaC. J Biol Chem 282/28:20207-12

Zhou Y, Li L, Liu Q, Xing G, Kuai X, Sun J, Yin X, Wang J, Zhang L, He F (2008) E3 ubiquitin ligase SIAH1 mediates ubiquitination and degradation of TRB3. Cell Signal 20/5:942-8

Zwickl P and Baumeister W (eds) (2002) The Proteasome-Ubiquitin Protein Degradation Pathway. Springer, Berlin Heidelberg New York

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10. STATUTORY DECLARATION

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Aussage (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

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