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1 Abstract

Cancer is a disease driven by genetic as well as by epigenetic changes. Epigenetic changes include aberrant histone modifications, global DNA hypomethylation and hypermethylation at CpG islands in promoter sites or the deregulation and mutation of genes involved in epigenetic mechanisms. The aim of our study was to analyse epigenetic changes specific to either hormone sensitive or insensitive prostate cancers in order to distinguish prostate cancer stages based on epigenetic patterns. Moreover, we intended to define differences between prostate cancer characteristics to discover possible tumour associated biomarkers.

We applied several methods in the hormone sensitive LNCaP and the hormone insensitive LNCaP-abl, PC-3 and DU145 human prostate cancer cell lines as well as tissue samples of human prostate cancers with differences in hormone response and/or therapy. Western blot analyses revealed an increased level of EZH2 protein in LNCaP and LNCaP-abl cells compared to the androgen independent PC-3 and DU145 cells and also detected a reduced level of DNMT1.

Additionally, COBRA analysis displayed a higher methylation pattern of PC-3 cells compared to the LNCaP and LNCaP-abl cell lines. Further, DNA methylation analysis in patient samples exhibited elevated hypermethylation at EDNRß, GSTP1 and WNT2 promoter regions in hormone insensitive patient samples (HI) compared to cancer tissues of patients with or without hormone ablation therapy (naive and HAT patients). Finally, IHC staining showed elevated BMI-1, DNMT1 and DNMT3a levels in HI patients compared to naive and HAT patients. Our results indicate differences in epigenetic patterns between hormone sensitive and insensitive prostate tumours and it will be interesting to expand our analyses to genome-wide screens on defined patient cohorts.

2 Zusammenfassung

Krebs ist eine Erkrankung, die sowohl von genetischen als auch von epigenetischen Veränderungen vorangetrieben wird. Zu diesen epigenetischen Veränderungen zählen sowohl Abweichungen der Histonmodifikationen, Veränderungen der DNA Methylierung in so genannten „CpG islands" wie auch unterschiedliche Regulationen von Genen im epigenetischen Netzwerk der Zelle.

Ziel unserer Arbeit war es epigenetische Veränderungen zwischen Hormon-sensitiven und Hormon-insensitiven Prostatakrebsarten zu finden, welche es ermöglichen Unterschiede innerhalb verschiedener Prostatakrebsstadien aufzudecken und somit mögliche tumorassoziierte Biomarker zu definieren. Untersucht wurden die Hormon-abhängigen Zelllinien LNCaP und die Hormon-unabhängigen Zelllinien LNCaP-abl, PC-3 und DU145 sowie humanes Gewebe aus Hormon-sensitiven und -insensitiven Prostatakarzinomen und aus Prostatakarzinomen nach Hormonablationstherapie.

Die in dieser Studie durchgeführten Proteinanalysen konnten ein erhöhtes Level an EZH2 in den beiden verwandten Zelllinien LNCaP und LNCaP-abl nachweisen, als auch die verminderte Expression des Enzyms DNMT1 im Vergleich zu PC-3 und DU145 Zellen detektieren. Außerdem deuten die erhaltenen COBRA Analysedaten auf ein erhöhtes Methylierungsmuster in PC-3 Zellen im Gegensatz zu LNCaP und LNCaP-abl. In einer Mehrheit von Hormon-unabhängigen Patientenproben waren EDNRß, GSTP1 und WNT2 hypermethyliert. BMI-1, DNMT1 und DNMT3a wurden mittels immunohistologischem Nachweisverfahren verstärkt in Hormon-insensitven Patienten nachgewiesen.

Unsere Ergebnisse deuten darauf hin, dass epigenetische Unterschiede zwischen Hormon-abhängigen und unabhängigen Prostatakrebsstadien vorliegen und regen dazu an unsere Analysen auf genomweite Screens in definierten Patientenkohorten auszuweiten.

3 Introduction

3.1 Epigenetics

"Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo, Martienssen et al. 1996). The term "epigenetics" was introduced for the first time by Conrad Waddington, by combining the greek word epi- (above, over) and genetic, thereby illustrating the molecular mechanism which translates the genetic information into phenotypes. Epigenetic mechanisms are essential in many biological processes including gene and microRNA expression, DNA-protein interactions, suppression of transposable element mobility, cellular differentiation, embryogenesis, X-chromosome inactivation, genomic imprinting (Portela and Esteller 2010) and the protection against viral genomes (Vaissiere, Sawan et al. 2008).

Epigenetic regulation is accomplished by three main epigenetic mechanisms: DNA methylation, post-translational histone modifications and RNA-mediated processes (Egger, Liang et al. 2004). DNA methylation is catalysed by DNA methyl-transferases (DNMTs), which transfer methyl groups to cytosine residues at the C5 position. CpG islands, which are rich in CG content and located mainly in promoter regions, are normally kept free from methylation, but hypermethylation at these sides can lead to transcriptional gene silencing in cancer and to cancer progression (Bird 1986; Egger, Liang et al. 2004). Another major epigenetic mechanism relates to post-translational histone modifications. In the nucleus, 147 base pairs of DNA are wrapped around core histones, which build up nucleosomes. These molecular units contribute to the formation of highly structured chromatin. The level of chromatin compaction, which is partially regulated by histone modifications, determines the accessibility of DNA. The highly conserved amino-terminal tails of the histone proteins (mainly H3 and H4) are targets for post-translational modifications, such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADPribosylation. These modifications influence transcriptional activation or

inactivation of chromatin regions by guiding the emergence of euchromatin ("ON state") or heterochromatin ("OFF state") (Jenuwein and Allis 2001) and consequently set up an environment to facilitate chromatin dynamics in conjunction with other remodelling systems. These modifications are dynamic, enabling the cell to respond to different requirements during cell cycle and development such as DNA replication, repair, recombination and chromosome segregation (Russo, Martienssen et al. 1996).

The variety and the combination of covalent histone modifications on different residues of the amino-terminal tails were assumed to produce a so called "histone code" (Strahl and Allis 2000; Jenuwein and Allis 2001). Nevertheless, it is controversial, if the proposed histone code is a cause or the consequence of the chromatin state (Henikoff and Shilatifard 2011).

3.2 DNA methylation

Methylation of DNA is established by DNA methyltransferases (DNMTs), which catalyse the transfer of methyl groups from S-Adenosylmethionine (SAM) to DNA. DNMTs are divided into maintenance (DNMT1) and *de novo* (DNMT3a and DNMT3b) methyltransferases. During replication, DNMT1 copies the hemi-methylated DNA pattern from the parental DNA strand to a new daughter strand. In this way, the DNA methylation pattern is passed to next cell generations (Vaissiere, Sawan et al. 2008). On the other hand, the *de novo* methylation at unmethylated CpG sites is accomplished by DNMT3a and DNMT3b (Klose and Bird 2006), which are expressed at high levels in embryonic stem cells (Newell-Price, Clark et al. 2000), where *de novo* methylation occurs.

3.3 Histone modifications and Polycomb Group Proteins

Posttranslational histone acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP ribosylation are modifications, which are established by different enzymes. Histone acetyltransferases are enzymes

targeting lysine (K) residues by transferring acetyl groups and thereby opening chromatin and leading to transcriptional activation, whereas histone deacetylases (HDACs) are removing acetyl groups. Histone methyltransferases (HMTs) play an important role in epigenetic modifications by adding methyl groups on arginin (R) and lysine (K) residues. Mono-, di- or trimethylation on histone H3 and H4 can either lead to gene activation or repression (Lachner and Jenuwein 2002). Trimethylation of histone 3 at lysine 4 (H3K4me3) and acetylation at histone 3 at lysine 9 (H3K9ac) indicate active DNA (Liang, Lin et al. 2004), while in contrast H3K9me3 and H3K27me3 lead to transcriptional repression and represent an inactive or silent state of DNA (Fischle, Wang et al. 2003). Enhancer of Zeste homologue 2 (EZH2) is the catalytic subunit of the polycomb repressive complex 2 (PRC2) and is responsible for H3K27 trimethylation (Cao, Wang et al. 2002) due to its HMT-activity. The H3K27me3 serves as a hallmark for the recruitment of additional polycomb group (PcG) proteins, for example polycomb repressive complex 1 (PRC1) (Min, Zhang et al. 2003), thereby fostering and maintaining the heritable and repressive chromatin state and determining cellular memory (Ringrose and Paro 2004). Overexpression of or changes within this complex organisation has been observed during cancer formation and progression (Kondo, Shen et al. 2008; Yang, Karuturi et al. 2009).

3.4 Epigenetics and disease

Heritable epigenetic changes are detected early during tumour development (Nelson, De Marzo et al. 2009) (Baylin and Ohm 2006). Epigenetic changes can include the deregulation of HMTs and histone demethylases, the overexpression of HDACs and the global loss of histone acetylation, which has been observed in several types of cancers (Kelly, De Carvalho et al. 2010). Epimutations such as defects in DNA methylation and aberrant histone modifications dramatically change the cellular methylation profile and can contribute to carcinogenesis through several events, such as repression of tumour suppressor genes, activation of normally silent oncogenes, the

reactivation of embryonic genes, loss of imprinting, disordered microRNA expression, activation of retro-transposons (Nelson, De Marzo et al. 2009) and aberrant gene expression in foreign tissues. In general, the cancer genome can be characterised by a global loss of methylation (hypomethylation), whereas simultaneously a gain of methylation (hypermethylation) at promoter sites occurs (Herman and Baylin 2003; Feinberg and Tycko 2004). Hypermethylation of tumour suppressor genes is associated with cancer and is correlated to transcriptional silencing (Jones and Baylin 2002; Herman and Baylin 2003; Cooper and Foster 2008). A very common example for epigenetic gene silencing is the CDKN2A gene locus, which encodes the tumour suppressor proteins p14 and p16. It has been shown that the repression of the CDKN2A locus together with the PcG ring finger oncogene B lymphoma Mo-MLV insertion region 1 homolog (BMI-1) harbours oncogenic potential (Itahana, Zou et al. 2003) and that epigenetic silencing of CDNK2A occurs in many cancer types. The term cross-talk is used to link DNA methylation with chromatin modifications. Cross-talk implies that more than just one mechanism can contribute to gene silencing and lead to tumour development. For example, it was shown by Viré et al that EZH2 is needed for the CpG methylation of target promoters, through direct contact with DNMTs (Vire, Brenner et al. 2006). Another interesting study demonstrated the decrease of EZH2 and BMI-1 through inhibition of DNMT1 and DNMT3b. They showed that DNMTs regulate p16 expression levels by direct modification of DNA methylation and as well as indirect histone modification on the p16 promoter region (So, Jung et al. 2011).

3.5 Prostate cancer

Among males, prostate cancer (PC) is the second most frequently diagnosed cancer and the sixth leading cause of death in the western world (Jemal, Bray et al. 2011). Prostate specific antigen (PSA) screening of serum is used for early detection and determination of prostate cancer, although recent studies conclude that PSA screening is only useful to verify the presence of benign

prostatic hyperplasia, but is not specific for advanced stages of disease (Stamey, Caldwell et al. 2004). Observations indicate that this detection method is limited in specificity and sensitivity (Yegnasubramanian, Kowalski et al. 2004). Nevertheless, still one third of PC patients with elevated PSA levels undergo unneeded surgical castration, although they do not suffer from a malignant form of cancer (Sidransky 2002). Conventional therapies include irradiation and radical prostatectomy. As PC onset and progression are promoted by androgens, which operate through the androgen receptor (AR), applied treatments are frequently supplemented by androgen deprivation therapies in certain cases (Heinlein and Chang 2004). These therapeutic strategies can lead to the onset of androgen-independent cancer, that is almost exclusively untreatable and is termed as hormone refractory, androgen-independent or castration-resistant prostate cancer (Shen and Abate-Shen 2010).

Pathologically, PC can be classified as adenocarcinoma appearing mostly in the peripheral zone of the prostate. Tumours are further classified by Gleason scoring (Gleason 1-5) to histopathologically evaluate the grade of malignant tissue (Shen and Abate-Shen 2010).

3.6 Epigenetics and prostate cancer

Prostate carcinogenesis is a multi-step process driven by genetic and epigenetic changes (DeMarzo 2007). As PSA screening is very unspecific, the aim of many studies in prostate cancer is to find new biomarkers, for instance methylation markers (Laird 2003), which can discriminate between different stages of disease progression or allow for the detection of an existing disease (Laird 1997). One potential tumour marker for PC diagnosis is methylation of Glutathione S-transferase P (GSTP1), which is methylated in 90% and a potential biomarker in PC cases, but not in normal tissue (Lee, Morton et al. 1994; Jeronimo, Usadel et al. 2001; Nakayama, Bennett et al. 2003). The epigenetic silencing of GSTP1 may promote the vulnerability of prostate tissue to oxidative stress (Shen and Abate-Shen 2010), which results in DNA damage

and assists cancer initiation (Poulsen 1998). Besides GSTP1, other genes have been reported to be hypermethylated, such as adenomatous polyposis coli (APC) (Kang, Lee et al. 2004) and Ras association domain-containing protein 1 (RASSF1alpha) (Lui 2002). Co-occurrence of methylated GSTP1 together with APC facilitates a more sensitive detection (Jeronimo, Henrique et al. 2004). Also, endothelial receptor type B (EDNRß) methylation showed a strong correlation with disease progression in prostate cancer (Bastian, Palapattu et al. 2008) as well as down-regulation of estrogen receptor alpha (ESR-1), which is also due to promoter methylation and is linked to cancer metastasis (Li et al. 2004). Interestingly, ESR-1 promoter methylation is rather heterogeneous among PC, which may correlate with the tumour stage and/or disease progression (Yegnasubramanian, Kowalski et al. 2004; Kwabi-Addo, Chung et al. 2007; Vasiljevic, Wu et al. 2011). Aberrantly expressed mediators of the epigenetic machinery can also contribute to cancer formation. For example, increased expression of DNMTs and EZH2 was found in PC compared to normal tissue (Gravina, Marampon et al. 2011) (Varambally, Dhanasekaran et al. 2002). EZH2 can be seen as a potential oncogene and its over-expression or increase during disease progression is linked to elevated H3K27me2 and H3K27me3 marks as well as an increase in HDAC1 levels (Varambally, Dhanasekaran et al. 2002; Chen 2005; Saramaki, Tammela et al. 2006). Beke et al observed that the human prostate cancer cell line PC-3, which does not respond to androgens, and the AR positive cell line LNCaP significantly differ in H3K27me3 levels. This is correlated with either elevated or reduced PSP94 expression (Beke, Nuytten et al. 2007). PSP94 is a tumour suppressor protein (encoded by the *MSMB* gene) and its expression is progressively decreased during PC development from an early, androgen-dependent to a late androgen-independent state (Vanaja, Cheville et al. 2003; Stanbrough, Bubley et al. 2006). However, the Dnmt-1 increase is also an effect of increased proliferation (Szyf, Bozovic et al. 1991) as DNMT1 is cell cycle regulated, whereas EZH2 expression is cell cycle independent (Bracken, Pasini et al. 2003).

Recent studies suggest that the transition of prostate cancer from hormone dependent to hormone-resistant PC phenotypes is dependent on epigenetic

alterations (Gravina, Marampon et al. 2011). Aberrant DNMT1 expression is correlated with the occurrence of androgen independence in prostate cancer (Chen, Chen et al. 2010), as well as the up-regulation of DNMT3a and DNMT3b (Gravina, Marampon et al. 2011). WNT1 expression levels were shown to be higher in LNCaP cells compared to PC-3 cells (Thiele, Rauner et al. 2011), whereas the RNA expression pattern of patient tissues for WNT2, another gene of the WNT signalling pathway, was observed to be down-regulated in prostate tumours (Wissmann, Wild et al. 2003). However, it has to be taken into account that epigenetic changes are also an phenomenon of ageing, for example regarding the down-regulation of ESR-1 (Li, Shiina et al. 2004), which increases with patients age. The increase in PC incidences at an older age reflects this age-dependent process (Li, Shiina et al. 2004).

3.8 Project outline

In this study, we investigated whether hormone dependent and independent prostate carcinomas display an altered methylation profile at CpG islands in promoter regions of candidate tumour suppressor genes and if differences in histone modifications and expression of epigenetic enzymes can be seen between hormone dependent or independent prostate cancer. In order to answer these questions we performed combined bisulfite restriction analysis (COBRA), chromatin immunoprecipitation (ChIP), immunohistochemistry (IHC), MethylMiner and Western Blot analysis in selected prostate cancer cell lines and in patient samples of prostate carcinomas. We used formalin fixed paraffin embedded (FFPE) material of prostate cancer tissue to isolate DNA for methylation analysis (COBRA). Furthermore, we manufactured a tissue array containing the same prostate cancer samples to perform IHC analysis.

COBRA assay was performed on the three different human prostate cancer cell lines LNCaP, LNCaP-abl and PC-3, representing hormone dependent (LNCaP) or independent (LNCaP-abl and PC-3) cancer forms. We also analysed patient samples belonging to three cancer groups, which differed in therapy treatment and androgen response. Particularly, we used eight different patients that were

either treated with hormone ablation therapy or untreated (naïve) and seven hormone insensitive prostate cancer patients. We performed COBRA assays on CpG islands at the promoters of APC, death-associated protein kinase 1 (DAPK-1), EDNRB, ESR-1, GSTP1, p14, p16, RASSF1 and Wingless-type MMTV integration site family member 2 (WNT2).

Additionally, IHC staining was performed with the same patient samples as described above to determine epigenetic and cellular alterations between these different PC stages. Altogether, we analysed the status of ten proteins involved in epigenetic mechanisms. In this study, the expression status of BMI-1, CCCTC-binding factor (CTCF), DNMT1, DNMT3a, EZH2, HDAC1, HDAC2, HDAC3 as well as the level of H3K4 and H3K27me3 modifications were analysed. Next, we investigated histone modification patterns at the promoter site of the proto-oncogene WNT1, in the two prostate cancer cell lines LNCaP and LNCaP-abl by ChIP analysis in order to see if differences between these related cell lines with a different androgen response are reflected by histone modifications.

We applied MethylMiner assay on LNCaP cell lines as a first-line experiment in order to perform genome wide DNA methylation analyses in the future. Therefore, DAPK-1, SH2 domain-containing protein tyrosine phosphatase (SHP2), ubiquitin-conjugating enzyme E2B (UBE2B) and WNT2 were chosen as genes of interest. Finally, Western Blot analysis was performed on four human PC cell lines (LNCaP, LNCaP-abl, PC-3 and DU145) to evaluate the protein levels of EZH2 and DNMT1.

4 Material and Methods

4.1 Media, Solutions and Reagents

Hunt Buffer

20mM Tris pH 8

100mM NaCl

1mM EDTA

0.5% NP-40

Protease (Roche - 50xstock - complete)

Phosphatase Inhibitor (Halt Phosphatase Inhibitor Cocktail - Thermo Scientific)

in H_2O, filtered

RIPA Buffer for Western Blot

50mM Tris pH 8

150mM NaCl

1% NP-40

0.5% DOC

0.1% SDS

Protease and Phosphatase Inhibitor (Roche)

in dH_2O, filtered

SDS Loading Dye

100mM TrisHCl pH 6.8

200mM DTT

4% SDS

20% Glycerine

~20mg Bromophenolblue

Electrophoresis Buffer

14.4g Glycine

3g Tris

1g SDS
filled up to 1L with dH_2O.

Transfer Buffer

2.5g Tris
11.2g Glycerine
200ml Methanol
filled up to 1L with dH_2O.

Ponceau Red solution

0.5M TBS
150mM NaCl
50mM Tris Base)
in H_2O

Blocking Solution

1xTBS-T (1xTBS 0.5M; 0.1% Tween)
5% non-fat dry milk powder
0.02% Na-Azide

10x PBS

28.8g Sodiumhydrogenphosphate-dihydrate
5.2g Sodiumdihydrogenphosphate-monohydrate
90g NaCl adjust to pH 7.2-7.6
fill up with dH_2O to 1L.

10xTBS (0.5 M)

60.5g Tris-Base
90g NaCl Adjust with HCl to pH 7.5
(TBS-T: 0.1% Tween-20)

BSA/PBS

0.5% Bovine Serum Albumin

1xPBS

in H_2O

TE/EDTA Buffer

10mM TrisHCl pH 8.1

1mM Ethylene-diamine-tetraacetic-acid

in H_2O

RIPA Buffer for ChIP Analysis

50mM Hepes-KOH pH 7.6

1mM NP-40

0.7% Na-Desoxycholate

500mM LiCl

Protease Inhibitor (Roche)

in dH_2O, filtered

Cell lysis buffer (DNA extraction)

0.5M NaCl

0.2% SDS

0.1M Tris pH 8.3

5mM EDTA

in H_2O, filtered

ChIP Dilution Buffer

0.01% SDS

1.1% Triton X-100

1.2mM EDTA

16.7mM TrisHCl pH 8.1

167mM NaCl

Protease Inhibitor (Roche)

in H_2O, filtered

SDS-Lysis-Buffer (ChIP)

1% SDS

10mM EDTA

50mM Tris pH 8.1

Protease Inhibitor (Roche)

in H_2O, filtered

Cell Lysis Buffer 1 (ChIP)

50mM Hepes-KOH pH 7.5

140mM NaCl

1mM EDTA

10% glycerol

0.5% NP-40

0.25% Triton X-100

Protease Inhibitor (Roche)

in H_2O, filtered

Cell Lysis Buffer 2 (ChIP)

10mM TrisHCl pH 8

200mM NaCl

1mM EDTA

0.5mM EGTA

Protease Inhibitor (Roche)

in H_2O, filtered

Elution Buffer (ChIP)

1% SDS

0.1M $NaHCO_3$

prepared fresh on the same day

in dH_2O, filtered

12% Polyacrylamid Gel

12% PAA

1xTBE

1% APS

TEMED

in H_2O

4.2 Antibodies

4.2.1 Primary Antibodies for Western Blot Analysis

- ß-actin (Cell Signalling 4967S, rabbit) – 1:1000 dilution in blocking solution – 45kDa
- Dnmt1 (santa cruz sc-10222, goat polyclonal) – 1:1000 dilution in blocking solution – 183kDa
- EZH2 (Sigma E7031, rabbit polyclonal) - 1:1000 dilution in blocking solution –100kDa

4.2.2 Secondary Antibodies for Western Blot Analysis

- Polyclonal rabbit anti-mouse IgG (HRP) (DakoCytomation, R0270) – 1:10000 dilution in TBS-T
- Fox F(ab')2-fragment goat anti rabbit IgG (Invitrogen, L43000) – 1:10000 dilution in TBS-T

4.2.3 Antibodies for IHC staining

HDAC1 (Seiser laboratory - mouse)

HDAC2 (Seiser laboratory - mouse)

HDAC3 (Cell Signaling; #2632)

BMI-1 (Millipore; # 05-637)

CTCF (Cell Signaling; # 3418)

DNMT1 (abcam; # ab13537)

DNMT3a (santa cruz; # sc-20703)

EZH2 (sigma; # E6906)

H3K4me3 (abcam; # ab8580)
H3K27me3 (abcam; # ab6002)
Ki67 (Novocastra; # NCL-KI67-P)

4.3 Cell lines and cell culture

We used the human prostate cancer cell lines PC-3, LNCaP clone FGC (CRL-1740™) and the LNCaP subline LNCaP-abl. Cell lines were obtained from ATCC and were grown in RPMI 1640 medium (GIBCO) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO_2 and 95% room air. LNCaP and LNCaP abl cells were seeded and grown in cell culture dishes (greiner bio-one CELLSTAR; 145x20mm) until the plates were mostly overgrown and reached an adequate amount of cells.

4.3.1 Cell line description

In our study we used the prostate cancer cell lines PC-3, LNCaP and the LNCaP subline LNCaP-abl as well as DU145. PC-3 is a human prostate carcinoma cell line that was established from a bone marrow metastasis grade IV prostate cancer after androgen suppression therapy. Although PC-3 cells express the androgen receptor (AR), the cell line is androgen independent. LNCaP cells are derived from non-metastatic lymph nodes and are a slow growing, androgen dependent cell line, expressing the androgen receptor (AR). Their proliferation is regulated by androgens in a biphasic manner, which means that growth is enhanced by low concentrations of dihydrotestosterone (DHT – a metabolite of testosterone), whereas higher androgen levels reduce cell proliferation (Lee, Sutkowski et al. 1995). The new subline LNCaP-abl was established by Culig et al. after long-term androgen ablation of LNCaP cells (passages >75). LNCaP-abl cells are an androgen-independent (hormone insensitive - HI) cell line. Their proliferation is stimulated by bicalutamide, a non-steroidal anti-androgen, which leads to an increase in AR expression. This cell line has adapted to an androgen-depleted environment by generating a hyper-reactive AR (Culig, Hoffmann et al. 1999). In contrast, androgens have an inhibitory

effect on cell growth and proliferation in LNCaP-abl. For this reason, the LNCaP-abl cell line is a useful model system for studying therapy-resistant prostate cancer (androgen deprivation), whereas LNCaP represent an androgen dependent PC at an early tumour stage.

4.4 Tissue Array of prostate cancer patient samples

Three different cohorts of prostate cancer patients were analysed. The first cohort included patients undergoing radical prostatectomys followed by androgen deprivation therapy (n=8), the second cohort patients undergoing radical prostatectomys (n=8) and the third cohort patients include hormone insensitive tumours (n=7). As control, non-malignant tissue samples were used from patients undergoing cystectomy (n=1) serving as control. Formalin fixed paraffin embedded (FFPE) tumours from prostate cancer patients were provided by the Institute of Clinical Pathology at the Medical University of Vienna.

A tissue array was prepared by using a small paraffin block which served as an acceptor block. Patient tissues of interest were cut out from donor blocks and transferred to the acceptor block (Genxpress). The order of patient samples is shown in Supp.Figure3.

4.5 Immunohistochemistry – IHC

IHC is a method of detecting the presence of antigens in cells or tissue sections by utilization the specific binding of an antibody to its antigen. The antibody-antigen interaction is visualized by coupling the enzyme peroxidase to the antibody, which catalyse a colour-producing reaction.

4.5.1 IHC Protocol

Several sections of paraffin embedded tissues were sliced (about 4µm) using a microtome and mounted on slides. For deparaffinization, the slides were incubated over night (o/n) at 57°C, followed by washing and incubation steps with xylol, different dilutions (96%, 70% and 50%) of ethanol (EtOH) and

aqua dest (dAQ). For antigen retrieval citrate buffer (DAKO; pH6) was used followed by either microwave heating or heating by autoclave at 121°C. After heating, the slides had to cool down to room temperature and were washed with 1xPBS (pH 7.2 – pH 7.6). The tissue was blocked with H_2O_2 (GATT-KOLLER), avidin and biotin (Zamponi Diagnostik) for 10 min each at room temperature (RT) and in between washed with 1xPBS. Slides were blocked 7 min with Super Block (Zamponi Diagnostik), washed with 1xPBS, incubated with the diluted antibodies (diluted in 1% PBS/BSA) (see table) and incubated o/n at 4°C. After incubation the slides were washed with 1xPBS and incubated with the secondary antibody (anti-polyvalent biotinylated antibody) (Zamponi Diagnostik) for 10 min at RT and then washed three times with 1x PBS, followed by a final incubation with strepavidin horse radish peroxidase (HRP) for 10min and a final washing step with 1xPBS. AEC staining (3-amino-9-ethylcarbazole) (ID-Labs) was used for AB detection. Slides were counter stained with haemalaun solution (Merck) for 30 sec, washed with aqua dest. and mounted with aquatex (Merck) (Table 1).

Primary Antibody	Dilution	Buffer	Conditions
BMI-1 (Millipore)	1:100	Citrate	Autoclave
CTCF (Cell Signalling)	1:250	Citrate	Autoclave
DNMT1 (abcam)	1:100	Citrate	Autoclave
DNMT3a (santa cruz)	1:200	Citrate	Autoclave
EZH2 (active motif)	1:300	Citrate	Autoclave
HDAC1 (Seiser laboratory)	1:100	Citrate	Microwave
HDAC2 (Seiser laboratory)	1:100	Citrate	Microwave
HDAC3 (Cell Signalling)	1:100	Citrate	Autoclave
H3K27me3 (abcam)	1:400	Citrate	Autoclave
H3K4me3 (abcam)	1:100	Citrate	Autoclave
Ki67 (Novocastra, NCL-KI67P)	1:1000	Citrate	Autoclave

Table 1| Overview of antibodies and dilutions for IHC staining.

4.6 Combined Bisulfite Restriction Analysis – COBRA

For determination of the methylation status of prostate cancer patients and in human prostate cancer cell lines we performed COBRA assay. This method is

used to distinguish methylated CpG sites from unmethylated sites by treating genomic DNA with sodium bisulfite. Bisulite treatment converts unmethylated cytosines in the DNA sequence into uracils, whereas methylated cytosins remain unchanged. In the subsequent PCR the resulting uracils are amplified as thymines that allow the discrimination between originally methylated and unmethylated Cs by restriction enzymes, which have a CG in their recognition sequence and are thus cutting only initially methylated and unconverted CGs.

4.6.1 DNA Isolation of FFPE tissue samples

2-3 pieces of formalin fixed paraffin embedded (FFPE) patient tumour tissue samples were cut out with a tissue arrayer punch (Genxpress) and transferred into a 1.5mL Eppendorf tube. To remove the paraffin from tissues, 1000µL Xylol (Merck) were added. Compounds were mixed by inverting the tube. Centrifugation at full speed for 2 min was performed and the resulting supernatant was discarded. In addition, 800µL Xylol and 400µL EtOH (Merck) absolute were added and mixed by inverting the tube. Centrifugation at full speed for 2 min was performed and the resulting supernatant was discarded. Finally, the pellet was washed with 1000µL EtOH and dried at 55°C. 180µL ATL Puffer and 20µL Proteinase K (QIAamp DNA Mini Kit) were added to each pellet and incubated at 56°C on the thermomixer for 64 hours. Further steps for DNA purification were carried out according to QIAamp DNA Mini Kit protocol. In the final two steps of the QIAamp DNA Mini Kit the recommended amount of distilled water for elution was increased to 50µL. The DNA amount was measured with NanoDrop 2000 (Thermo Scientific).

4.6.2 DNA Isolation from cell lines

Cells were pelleted by centrifugation 5 min at 1500rpm and washed with 1x PBS. The cell pellet was then dissolved in 500-1000µL cell lysis buffer. 20µg/ml RNAseA were added and the cell lysate was incubated at 37°C for 30-60 min. 500µg/ml Proteinase K (10mg/mL) was added and incubated o/n at 55°C. After o/n incubation, a Phenol/Chloroform DNA extraction was performed using phase lock gel tubes (5prime). The DNA containing phase was taken off and

one volume of isopropanol (Merck) was added. The solution was mixed by inverting the tube and DNA was pelleted by centrifugation at full speed at 4°C for 10 min. Subsequently the DNA pellet was washed once with one volume 75% EtOH; dried at RT and dissolved in 100-500µL dH_2O at 37°C until well dissolved. DNA concentration was measured with the NanoDrop spectrophotometer (Thermo Scientific).

4.6.3 COBRA Protocol

After DNA Isolation, 1µg DNA of the patient samples was bisulfite converted according to EZ DNA Methylation™ Kit (Zymo Research). 16 cycles (95°C for 30 sec, 50°C for 60 min) were performed o/n in a thermocycler. The bisulite converted DNA was eluted in 30µL - 50µL dH_2O and stored at -20°C for further analysis. The bisulfite-converted DNA was amplified by PCR with methylation insensitive primers (Table 2). Primers for amplification of bisulfite converted DNA were designed using the SEQUENOM EpiDesigner program (www.epidesigner.com). DNA amplification was checked on a 1.5% agarose gel with 5µL of the PCR product. As positive control, DNA of peripheral blood mono-nuclear cells (PBMCs) was treated with SssI enzyme resulting in fully 100% methylated DNA (see below; SssI treatment). Untreated PBMC DNA served as unmethylated negative control. In the course of the experimental setup, two different PCR settings were performed, which differ in the application of used reagents. Either the JumpStart REDTaq DNA Polymerase Master Mix (Sigma-Aldrich) or AmpliTaq Gold 360 (Applied Biosynthesis) system was used.

Gene target	Primer Sequences	Fragment lenght basepairs	Annealing Temp. °C
APC fw	5′-GGTTGTGGGAAGTTAGTAATA-3′	232bp	57
APC re	5′-CAACTAAATACTTCACCTTCC-3′		
DAPK1 fw	5′-GAGGGTAGTTTAGTAATGTG-3′	323bp	57
DAPK1 re	5′-ACTATCCTCCTCACACTC-3′		
EDNRb fw	5′-GAGGGGTAAATAATATGTAGTG-3′	172bp	53
EDNRb re	5′-CTTAACCTCTAAATACCTTAATTC-3′		
ESR1 fw	5′-GTAAAGTTTAGGGAAGTTGT-3′	157bp	53
ESR1 re	5′-CCAACTTTAAATACTAATCTC-3′		
GSTP1 fw	5′-GGATTTGGGAAAGAGGGAAAG-3′	304bp	58
GSTP1 re	5′-CTAAAAACTCTAAACCCCATCC-3′		
p14 fw	5'-AAAAATGGGTTAGATATAAAGGATT-3'	248bp	53
p14 re	5'-CCTCTTCTAAATTTAAAAAACAAAC-3'		
p16 fw	5'-GGAGTTTTCGGTTGATTGGTTGGTT-3'	220bp	58
p16 re	5'-AACAACGCCCGCACCTCCTCTA-3'		
Rassf1/1 fw	5'-GAGTTTGGGTTAGTTTGGGTT-3'	413bp	57
Rassf1/1 re	5'-CTAAAACTTCCCTTCAATCTC-3'		
Wnt2 fw	5'-AGAGGGTAGTAGTTAGGGTT-3'	186bp	57
Wnt2 re	5'-CCTCTACTCTTAACCTAACTC-3'		

Table 2| COBRA PCR.

<u>PCR reaction setup 1:</u>

16.375µL dH_2O

5µL $MgCl_2$

2.5µL Buffer

2µL DNA sample

1µL Primer fw/rev (0.5µL each)

0.5µL dNTPs

0.125µL AmpliTaq Gold 360 (Applied Biosynthesis)

<u>PCR reaction setup 2:</u>

10µL of JumpStart REDTaq DNA Polymerase (Sigma-Aldrich)

7µL dH_2O

2µL DNA sample

1µL primer fw/rev (0.5µL each)

RestrictionEnzyme	BstUI (NEB)	HhaI (NEB)	HhaI (Fermentas)	TaqaI (NEB)
Sequence	5'...CG|CG...3'	5'...GCG|C...3'	5'...GCG|C...3'	5'...T|CGA...3'
	3'...GC|GC...5'	3'...C|GCG...5'	3'...C|GCG...5'	3'...AGC|T...5'
Buffer	10xBuffer	10xBuffer	10xTango Buffer	10xBuffer
amount	2µL	2µL	2µL	2µL
Temperature °C	60	37	37	65
Enzyme	0.25µL	0.25µL	0.5µL	0.25µL
Incubation time for digestion	3 hours	3 hours	3 hours	3 hours
dH_2O	2.75µL	2.75µL	2.5µL	2.75µL
PCR Product	15µL	15µL	15µL	15µL

Table 3| COBRA Restriction: Reaction-Setup for restriction digestion of PCR products.

The restriction of the PCR products was performed under appropriate conditions (Table 3) in a thermocycler. After restriction the sample was loaded onto a 12% polyacrylamid gel together with 1µL DNA-loading dye. Restriction fragments were separated and visualized by Midori Green (genxpress) staining.

4.6.4 SssI Treatment

For COBRA fully methylated DNA was generated by treating bisulfite converted PBMC DNA with the bacterial CpG Methyltransferase M.SssI. For this purpose, 30µg genomic DNA was used and 40µL 10xNEB2 buffer, 2µL S-Adenosyl methionine (SAM) and 5µL M.SssI (NEB - C_pG methylatransferase) was added and filled up to a total volume of 400µL with dH_2O. After 4 hours at 37°C the reaction was boosted by adding another 1µL SAM, 2µL M.SssI and 47µL dH_2O and incubated o/n under the same conditions. DNA was then purified either with Qiaquick Gel Extraction Kit (Gel Extraction Kit Protocol; Handbook 11/2006 page 25) or by DNA precipitation. For DNA precipitation, NaCl in a final concentration of 400mM, two volumes of 96% EtOH and 1µL glycogen were added to the M.SssI treated DNA sample and incubated one hour at minus 80°C. After centrifugation at full speed, 300µL of 70% EtOH was added to the sample and centrifuged again at full speed. The supernatant was discarded and the pellet was dissolved in 100µL TE buffer (10mM TrisHCl pH 8.1; 1mM EDTA). M.SssI treated DNA was stored at 4°C for further

experiments. Bisulfite converted PBMC DNA was used as unmethylated control, whereas SssI treated PBMC DNA served as methylated control.

4.7 Chromatin Immunoprecipitation – ChIP

Chromatin immunoprecipitation (ChIP) is used to investigate the interaction between proteins and DNA. It is also a major assay to analyse histone modifications, in which a histone modification specific antibody is used to precipitate cross-linked chromatin. Purified DNA can be analyzed afterwards by real time PCR.

4.7.1 ChIP Protocol

Using ChIP analysis, LnCaP and LnCaP-abl cell lines were cultured as described previously (2.3). The first step was to cross-link proteins to chromatin. Therefore, formaldehyde (Thermo Scientific ampoules) in an end-concentration of 1% per dish was added to the cultured cells in medium. The cells were then incubated shaking for 15 min at RT. To quench the reaction, 1/10 volume of 1.25M glycine (Bio-Rad) was added to cells and dishes were incubated shaking for 5 min at RT. Then the cells were washed with ice cold PBS. Adherent cells were scraped off, with a silicon cell scraper (Costar) together with ice cold PBS + phosphatase inhibitor (pH 7.2 – pH 7.6). The harvested cells were pooled in a 50mL tube (Greiner) and centrifuged at 2000 -2200rpm at 4°C for 5 min. Supernatant was discarded and the cell pellet was gently suspended in another 5mL ice cold PBS + PI and pelleted by centrifugation (2000rpm). The supernatant was discarded and the cell pellet was resuspended in 5mL of cell lysis buffer 1 + PI. The suspension was incubated on a rotating wheel at 4°C for 10 min, followed by centrifugation at 2000 – 2200rpm at 4°C for 5 min. The supernatant was taken off and in the next step 5mL of Lysis Buffer 2 + PI were added and incubated for 10 min at RT. The resulting cell pellet was resuspended in an adequate amount (depending on the cell pellet size) ranging between 200-1000µL in SDS-Lysis-Buffer + PI. The cell suspension was sonicated between 20-30 times (INVITROGEN Sonicator). Every 5 times the

water temperature was checked according to the heat development (temperature should not raise over 10°C). To determine the length of the chromatin, a 20µL aliquot was taken from the sonicated cells. 80µL dH_2O and 10µL 5M NaCl was added to the aliquote and boiled for 15 min at 95°C. Subsequently, 1µL RNAase (DNAse free) (Invitrogen) was added and incubated 30min at 37°C. Afterwards, 1µL Proteinase K (10mg/mL) (Invitrogen) was added and incubated another 30 min at 45°C. The DNA was purified with QIAquick PCR purification kit (QIAquick PCR Purification Microcentrifuge and Vacuum Protocol) and eluted in 30µL dH_2O. Finally, the DNA concentration was measured with NanoDrop 2000 (Thermo Scientific) and the remaining DNA was checked on a 2% agarose gel (500 and 1000 basepairs). For precipitation, 100µg chromatin, 10µg antibody (EZH2 active motif 39875/39876; H3K27 millipore cat.07-449) and 50-100µL Invitrogen Dynabeads Protein G (cat.100.03D; 100.04D) were used. Before incubation with the antibody, the chromatin was filled up with SDS Lysis Buffer (e.g. to 100µL) and was diluted to an end-concentration of 1x with ChIP Dilution Buffer + PI. As input control, 1% chromatin was taken away and stored at -20°C. Incubation of chromatin with the antibody was performed on a rotating wheel at 4°C for one hour. IgG beads were washed with 1mL BSA/PBS (0.5% BSA; 1xPBS) three times using a magnetic rack. After incubation of the antibody with the chromatin, the beads were added and incubated o/n at 4°C on a rotating wheel. As a control, chromatin of each cell line was also incubated with beads but without antibody. After incubation, the beads were washed 5 times with 1mL RIPA Buffer + PI using a magnetic rack and two times with TE Buffer. Chromatin DNA was eluted by incubating the beads two times with 200µL elution buffer for 15 min on a rotating wheel at RT. Chromatin input was filled up to a final volume of 400µL with elution buffer. For reverse cross-linking, 16µL 5M NaCl were added to the chromatin input and the precipitated chromatin was incubated 4 hours at 65°C. In addition 8µL 0.5M EDTA, 16µL 1M TrisHCl pH 6.5 and 2µL 10mg/mL proteinase K (Invitrogen) were added and incubated o/n at 55°C. For DNA isolation, a Phenol/Chloroform (25 Phenol: 24Chloroform: 1Isoamylalcohol) extraction was performed after incubation by the use of phase lock tubes

(5prime). Therefore, two volumes (800µL) EtOH, 80µL 3M NaOAc and 1µL glycogen were added to the DNA extract and incubated at -80°C for one hour. Then the sample was centrifugated at full speed at 4°C, the supernatant was taken off and the DNA pellet was washed with 70% EtOH once. The precipitated DNA was dried and dissolved in 100µL dH_2O for further analysis with real-time PCR using site specific primers. Quantitative real-time PCR was performed with FASTAKapa Mastermix (Peqlab Biotechnologies) on a Biorad cycler. For PCR reaction 10µL Mastermix, 7µL dH_2O, 2µl of cDNA and 1µL primer (fw + rev) were used in each run. Glyceraldehyde-3phosphate dehydrogenase (GAPDH) was used as control region.

Primer Sequences

WNT1_fw: 5'-CCA GGG TTG TTA AAG CCA GA-3' and rv: 5'-AGC AGC AGC GTA GAA AC-3'

MYT1pd_fw: 5'-AGG CAC CTT CTG TTG GCC GA-3' and rv: 5'-AGG CAG CTG CCT CCC GTA CA-3'

MYT1prox_fw: 5'-GGA CAC TGT GCG GAA GAG TT-3' and rv: 5'-TTG TCT GCT TTT CTG CAA TC-3'

GAPDH fw: 5’-GGT GGT CTC CTC TGA CTT CAA CA-3’ and rv: 5’-GTT GCT GTA GCC AAA TTC GTT GT-3’.

4.8 MethylMiner™ analysis of cell lines LnCaP versus LnCaP-abl

The MethylMiner™ Enrichment Kit was used for methylation analysis of LnCaP versus LnCaP-abl cell lines. This kit enables the enrichment and isolation of CpG methylated DNA only, by the use of the antibody against 5-methylcytosine (5mC). Afterwards specific gene targets of interest are analysed using RT-PCR method.

4.8.1 MethylMiner™ Protocol

The protocol was carried out as described in the handbook (Invitrogen life technologies handbook page 15-25). In our experimental setting 2µg DNA was

used for every sample. Accordingly, the protocol was performed in appropriate working steps based on the handbook. We analysed the cell lines LnCaP and LnCaP-abl. The methylated DNA (SssI treated) served as positive control.

4.9 Protein Isolation

For protein extraction from cells, 200µL HUNT extraction Buffer was used. The cell suspension was frozen in liquid nitrogen and thawed at 37°C, then again frozen and thawed on ice for 15 min. To remove the insoluble components, a centrifugation step was performed (4°C/13.200rpm). Supernatant was taken off and measured with NanoDrop 2000 (Thermo Scientific).

4.10 Protein Analysis - Western Blot

60µg of protein extract was loaded per lane on a SDS – Polyacrylamid Gel. 5% and 15% solution: 1.5M Tris pH 8.8; H_2O; 30% Polyacrylamid; Sodium dodecyl sulphate - SDS; Ammonium Persulfate; Tetramethylethyleneediamine-TEMED). Equal amounts of 2xSDS gel loading dye were added to 60µg isolated protein sample and incubated at 95°C for 5 min. Separation was performed on a 5-15% SDS PAGE at 120V for 2hours or 20V for 16hours. Colorplus Prestained Protein Ladder broad range 10-230 kDa (New England BioLabs) was used as marker. Blotting the separated proteins to a Nitrocellulose Membrane (Whatman) was performed with wet blotter system and further visualized with Ponceau Red solution to check for equal amounts of loaded samples. The blot was then blocked in Blocking Solution for one hour. The primary antibodies (see Table 2) were diluted in blocking solution and incubated o/n on a shaker. The blot was washed three times with TBS-T and incubated with secondary antibodies (see Table 2) 1:10000 diluted in TBS-T for one hour at RT. The blot was washed again three times with TBS-T. Protein bands were visualized by using GE* Healthcare Amersham* ECL Plus Western Blotting Detection Reagent and analysed with LUMI Analyst (Roche).

5 Results

5.1 Methylation analyses in cell lines and prostate carcinomas using COmbined Bisulphite Restriction Analysis – COBRA

COBRA was performed in human prostate cancer cell lines PC-3, LNCaP and LNCaP-abl as well as in three different prostate cancer patient groups to determine the methylation status of candidate tumour suppressor genes (**Table 4**). For our experiment, SssI DNA from peripheral blood mononuclear cells (PBMCs) served as a positive control for methylation. Results were categorised in either unmethylated (U) or hypermethylated (M).

Images of the restriction profiles for cell lines and patient samples are shown in **Suppl.Fig.1** and **2**.

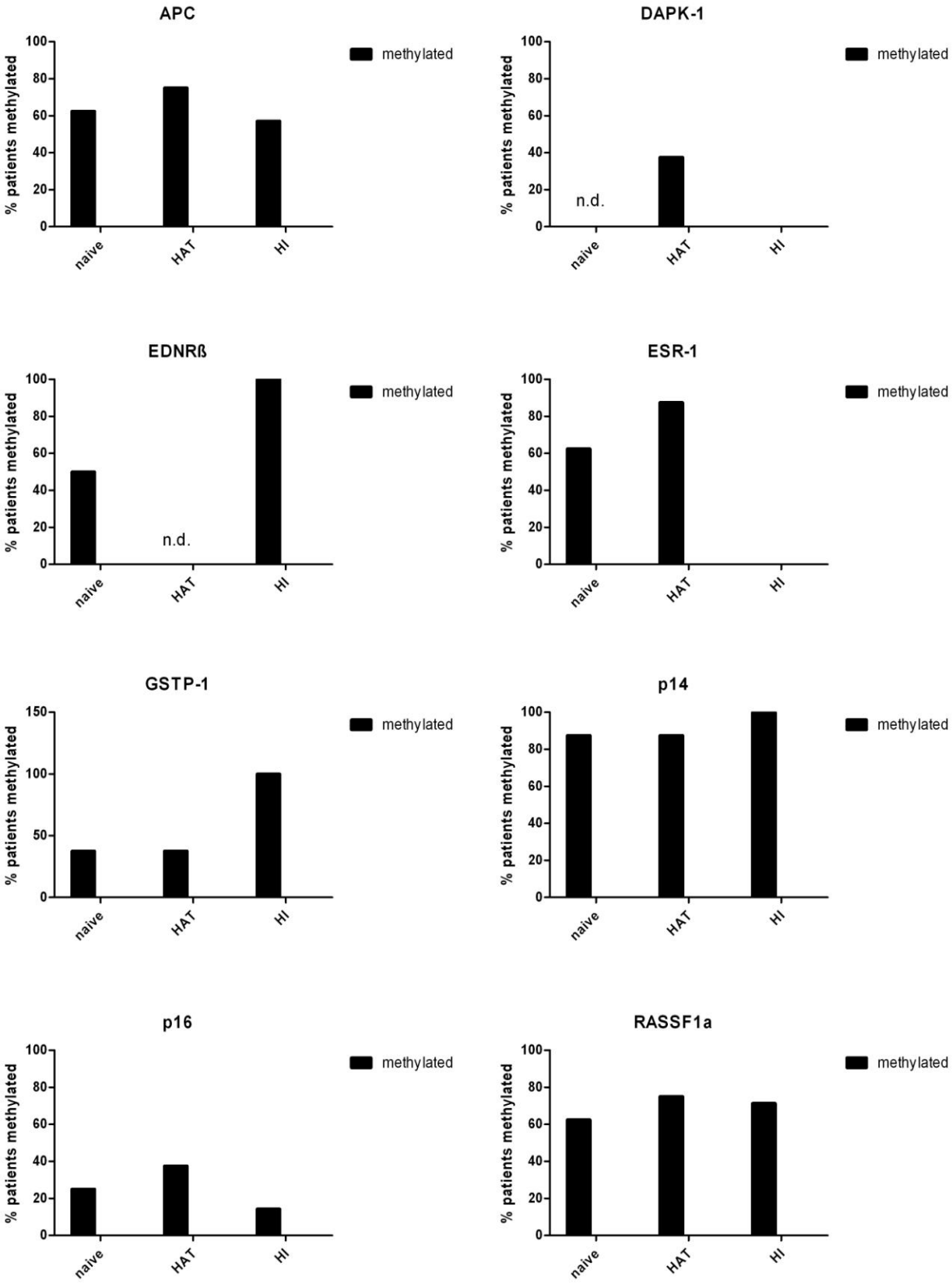
We chose a set of candidate genes known to be hypermethylated in different cancers for our analyses. APC and p14 were found unmethylated in all investigated cell lines. Interestingly, APC and p14 are the only targets found unmethylated in the hormone independent cell line PC-3, while the other seven remaining candidate gene loci DAPK-1, EDNRß, ESR-1, GSTP1, p16, RASSF1 and WNT2 were detected as hypermethylated. In regard to our data, the more aggressive hormone insensitive PC-3 cells exhibited a higher methylation level compared to hormone-dependent cell line LNCaP. Interestingly, the *in vitro* generated hormone insensitive LNCaP-abl subline shows slightly elevated methylation frequencies compared to their parental cell line, indicating that epigenetic changes are involved in the transgression from hormone sensitivity to hormone insensitivity in PC.

Patients	DAPK1	p16	ESR-1	WNT2	EDNRβ	RASSF1a	APC	p14	GSTP1
naive 1	n.d.	U	U	U	U	M	U	U	U
naive 2	n.d.	U	M	U	M	M	M	M	M
naive 3	n.d.	M	M	U	M	M	M	M	M
naive 4	n.d.	U	M	M	M	M	M	M	U
naive 5	n.d.	U	M	M	M	M	M	M	U
naive 6	n.d.	M	U	U	U	U	U	M	U
naive 7	n.d.	U	U	M	U	U	M	M	M
naive 8	n.d.	U	M	U	U	U	U	M	U
Methylated	**n.d.**	**2/8**	**5/8**	**3/8**	**4/8**	**5/8**	**5/8**	**7/8**	**3/8**
in %	**n.d.**	**25%**	**62.5%**	**37.5%**	**50%**	**62.5%**	**62.5%**	**87.5%**	**37.5%**
HAT 1	U	U	M	U	n.d.	M	M	M	M
HAT 2	M	M	M	U	n.d.	M	M	M	U
HAT 3	U	U	M	U	n.d.	M	M	M	U
HAT 4	U	U	M	U	n.d.	M	M	M	U
HAT 5	U	M	M	U	n.d.	M	M	M	M
HAT 6	U	U	U	U	n.d.	M	U	U	M
HAT 7	M	M	M	M	n.d.	U	U	M	U
HAT 8	M	U	M	M	n.d.	U	M	M	U
Methylated	**3/8**	**3/8**	**7/8**	**2/8**	**n.d.**	**6/8**	**6/8**	**7/8**	**3/8**
in %	**37.5%**	**37.5%**	**87.5%**	**25%**	**n.d.**	**75%**	**75%**	**87.5%**	**37.5%**
HI 1	U	U	U	M	M	M	M	M	M
HI 2	U	U	U	U	M	M	U	M	M
HI 3	U	M	U	M	M	M	M	M	M
HI 4	U	U	U	M	M	M	U	M	M
HI 5	U	U	U	M	M	U	U	M	M
HI 6	U	U	U	M	M	U	M	M	M
HI 7	U	U	U	M	M	M	M	M	M
Methylated	**0/7**	**1/7**	**0/7**	**6/7**	**7/7**	**5/7**	**4/7**	**7/7**	**7/7**
in %	**0%**	**14.3%**	**0%**	**85.7%**	**100%**	**71.4%**	**57.1%**	**100%**	**100.0%**
PBMC	U	U	U	U	U	M	U	U	M
Cystectomy	U	U	M	M	U	M	U	M	U
PC-3	M	M	M	M	M	M	U	U	M
LNCaP	U	U	U	M	M	U	U	U	M
LNCaP-abl	U	U	U	M	M	M	U	U	M

Table 4| Summary of COBRA results of prostate cancer cell lines and patients.

Next, we examined samples from patients, whose tumours were hormone-dependent (n=8) (naive), samples from patients who had received hormone ablation therapy (n=8) (HAT) followed by prostatectomy and from patients with hormone-insensitive prostate cancer (n=7) (HI). The cystectomy DNA sample was used as control for healthy tissue.

Our data show that HI patients display a higher methylation frequency for 4 of the 9 genes tested as compared to naive and HAT patients (**Tab.4** and **Fig.4**).


APC
% patients methylated
100
80
60
40
20
0
naive
HAT
HI
methylated
DAPK-1
% patients methylated
n.d.
naive
HAT
HI
methylated
EDNRß
% patients methylated
n.d.
naive
HAT
HI
methylated
ESR-1
% patients methylated
naive
HAT
HI
methylated
GSTP-1
% patients methylated
150
100
50
0
naive
HAT
HI
methylated
p14
% patients methylated
naive
HAT
HI
methylated
p16
% patients methylated
naive
HAT
HI
methylated
RASSF1a
% patients methylated
naive
HAT
HI
methylated

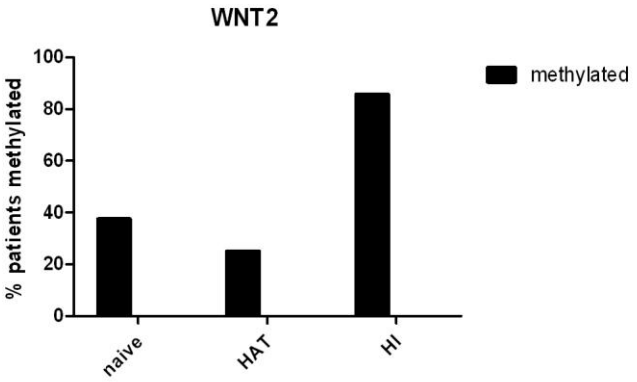


Figure 1| COBRA results of patient samples – all targets: methylated gene targets. naive patients n=8; HAT patients n=8; HI patients n=7.

Figure 1 shows a summary of the COBRA analyses comprising the naïve patient samples, those after hormone ablation therapy and those of hormone-insensitive patients. Most of the genes tested showed similar methylation levels in the three groups. Elevated methylation levels were detected in EDNRß, GSTP1, p14 and WNT2 in HI patients compared to naive and HAT patients, while ESR-1 methylation seems to be more distinct in hormone-dependent cancer stages. DAPK-1 and p16 showed low frequency of methylation in all patient groups. Because we obtained variable restriction patterns for the gene targets of DAPK-1 in naive and EDNRß in HAT patients, we could not interpret their methylation profiles. For this reason these data were designated as not determined (n.d.).

Together, our data demonstrate a trend towards higher methylation in hormone insensitive cell lines and tumours as compared to hormone dependent samples.

5.2 Chromatin Immunoprecipitation (ChIP) - H3K27me3 and recruitment of EZH2 at the WNT1 promoter in prostate cancer cell lines LNCaP and LNCaP-abl

In our study, we used chromatin immunoprecipitation to analyse the presence of the histone modification H3K27me3 at the WNT1 promoter encoding a proto-oncogene in two related prostate cancer cell lines. In particular, ChIP and

subsequently quantitative PCR (qPCR) were performed in the LNCaP cell line and its subline LNCaP-abl. It was reported previously that WNT1 expression levels are higher in LNCaP cells compared to PC-3 cells (Thiele, Rauner et al. 2011). The aim of this experiment was to find cell line specific histone modification patterns at the WNT1 locus due to the histone methyltransferase activity of EZH2. For this purpose, ChIP was performed with antibodies against the histone modification H3K27me3 as well as for the PRC2 subunit EZH2 (**Fig.2**).

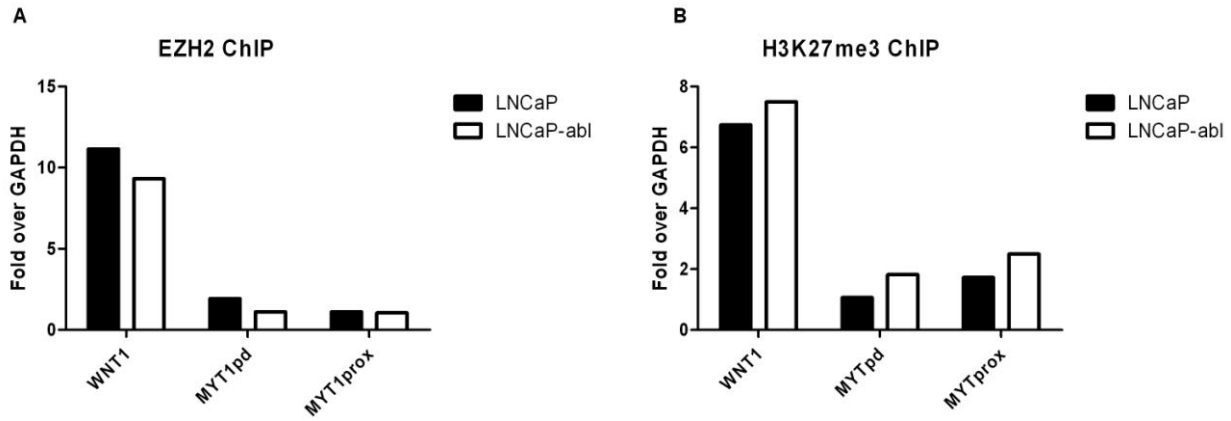


Figure 2| EZH2 (A) and H3K27me3 (B) ChIP: (A) EZH2 is recruited to the WNT1 gene locus; (B) The H3K27me3 mark is present at the WNT1 promoter in LNCaP and LNCaP-abl cells: Chromatin Immunoprecipitation with LNCaP and LNCaP-abl cells was performed with 100µg chromatin and 10µg antibody against EZH2 and H3K27me3. The ChIP protocol was performed as described in materials and methods above. Results are displayed as fold enrichment compared to GAPDH.

EZH2 is the major histone methyltransferase for the H3K27me3 modification. The performed EZH2 ChIP confirmed the recruitment of EZH2 to the WNT1 gene locus in both cell lines (**Fig.2A**). Furthermore, a strong enrichment of trimethylation of H3K27 was detected in both LNCaP and LNCaP-abl cells (**Fig.2B**). These findings suggest the recruitment of EZH2 to the WNT1 locus and consequently imply epigenetic silencing due to the repressive H3K27me3 mark. However, these results are in contrast to data which have shown that LNCaP cells express higher levels of WNT1 compared to PC-3 cells (Thiele, Rauner et al. 2011).

Summarizing our data, we could show EZH2-mediated trimethylation of H3K27

in the human prostate cancer cell lines LNCaP and LNCaP-abl. These histone modifications might indicate epigenetic silencing or down-regulation of WNT1. Our findings showed no cell line specific difference in the recruitment of EZH2 and the trimethylation of H3K27 at the WNT1 promoter. We suggest that this is due to the close relationship of LNCaP and LNCaP-abl cells.

5.3 CpG island methylation analysis in LNCaP and LNCaP-abl cell lines with MethylMiner™

The MethylMiner™ enrichment kit was used for LNCaP versus LNCaP-abl cell lines for methylation analysis. This kit enables the enrichment and isolation of methylated DNA by the use of the 5-methylcytosine binding protein MBD2. By the use of specific primers we analysed several genes including HIST, UBE2B, H19, SHP2, WNT2 and DAPK-1. The aim of this analysis was to find specific methylation changes or differences within these two cell lines in prospect to carry out genome-wide methylation analysis. The UBE2B gene is encoding the human ubiquitin-conjugating enzyme E2B. The modification of proteins with ubiquitin is an important cellular mechanism for targeting abnormal or short-lived proteins for degradation. SHP2 is a protein tyrosine phosphatase that regulates cellular processes including oncogenic transformations. DAPK-1 and WNT2 showed slightly enriched methylation in both cell lines compared to the positive control as determined by COBRA analysis.

As expected, we were able to precipitate known methylated regions within the WNT2, SHP2 and DAPK1 promoter regions, whereas no precipitation was obtained for HIST and UBE2B, two non-methylated control regions within the histone H3 and UBE2B promoter, respectively (**Fig.3**). The imprinted gene H19, which was expected to display 50% methylation, was also not precipitated. However, genome-wide methylation analyses in LNCaP cells available from the ENCODE consortium (ENCODE 2011) confirmed our results and did not show methylation of this region either (data were produced by the Dr. Richard Myers Lab and the Dr. Devin Absher lab at the HudsonAlpha Institute for Biotechnology).

Thus, we conclude, that these assays would be suitable for genome-wide analyses of hypermethylated DNA regions.

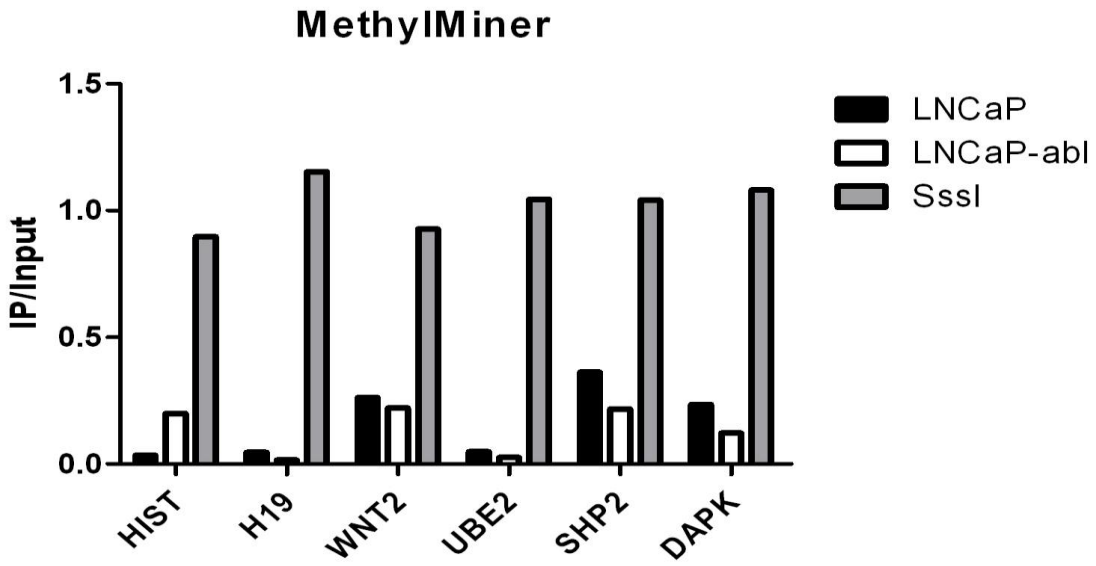


Figure 3| MethylMiner assay: Promoter methylation at promoter sites of WNT2, SHP2 and DAPK in LNCaP and LNCaP-abl cells. MeMiner assay performed on LNCaP and LNCaP-abl cells displays enrichment of methylated gene regions compared to control regions. SssI treated DNA was used as positive control.

5.4 Expression of enzymes involved in epigenetic processes

Protein analysis was performed in human prostate cancer cell lines LNCaP, LNCaP-abl, PC-3 and DU145 to compare expression levels in hormone-dependent and hormone-independent cells.

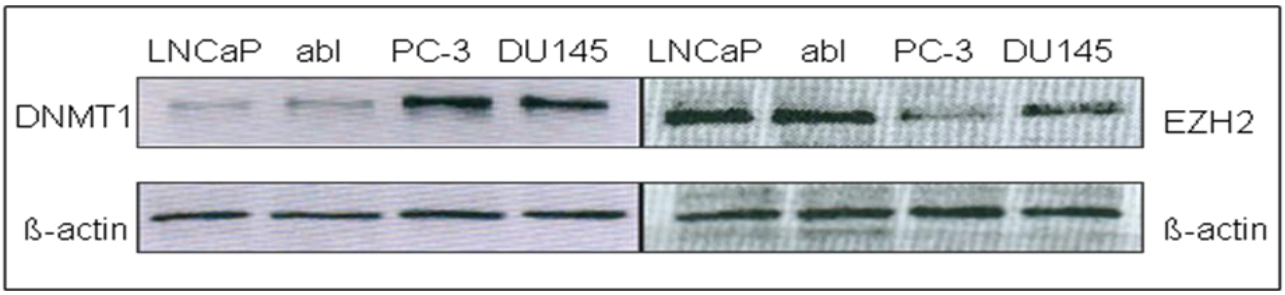


Figure 4| Expression of enzymes involved in epigenetic processes in human prostate cell lines. LNCaP, LNCaP-abl (abl), PC-3 and DU145 protein extracts were probed with antibodies against DNMT1 (left) and EZH2 (right). LNCaP and abl cells displayed reduced DNMT1 protein levels, whereas PC-3 and DU145 cell lines showed increased levels. Furthermore, the EZH2 level was distinctively lower in PC-3 cells and elevated in LNCaP cells. Per lane 60µg protein extract were loaded onto a 5-15% SDS-Gel. ß-actin was used as loading control.

Interestingly, PC-3 and DU145 exhibited elevated protein levels of DNMT1 compared to the LNCaP cell lines. Vice versa, EZH2 was increased in LNCaP and LNCaP-abl cells compared to PC-3 cells. DU145 cells were found slightly decreased in EZH2 expression at protein levels compared to LNCaP and LNCaP-abl (**Fig.4**).

These findings contribute to our hypothesis of an existing difference between hormone-dependent and hormone-independent cancer types based on epigenetic alterations, but do not correlate to previous findings where elevated EZH2 levels are linked to disease progression (Varambally, Dhanasekaran et al. 2002).

5.5 Immunohistochemical (IHC) staining of a tissue array from patient samples with different prostate cancer stages

Immunohistochemical staining was performed to determine putative differences in histone modifications or levels of epigenetic enzymes between

prostatectomy tissue samples of three different prostate cancer groups. These tissues were compared to normal tissue obtained from a cystectomy. We expected to detect specific modification changes during cancer progression. For this purpose, we stratified staining into three categories: positive (++), moderate (+) or negative staining (-) (see also **Suppl.Fig.3**).

As mentioned before, aberrant DNMT1 expression has been correlated to androgen independence in prostate cancer (Chen, Chen et al. 2010), as well as the increase of DNMT3a (Gravina, Marampon et al. 2011). In our current study, we observed the most prominent nuclear DNMT1 staining in HI patients (**Fig.6**, **Fig.9** and **Tab.5**) however, unspecific background staining might interfere with this observation. We also detected stronger staining for DNMT3a in HI cancer samples compared to moderate levels in naive patients (**Fig.6**, **Fig.** and **Tab.5**). DNMT3a was not detected in the cystectomy tissue and the majority of HAT patients (**Fig.6**, **Fig.** and **Tab.5**). BMI-1 was found positively expressed in HI and naive cancerous tissues (**Fig.6**, **Fig.9** and **Tab.5**). CTCF (**Fig.8**), H3K27me3 and H3K4me3 (**Fig.5** and **Tab.5**) (**Fig.7** and **Tab.5**) were detected in all patients at comparable levels. Concerning HDAC protein levels, HDAC1 expression was detected in all samples analysed with the most prominent staining in the cystectomy (**Fig.7** and **Tab.5**). Further, HDAC2 expression was found in all three cancer groups, whereas HI tissue samples showed the highest staining results compared to naive and HAT patients (**Fig.7**, **Fig.9** and **Tab.5**). A moderate HDAC3 staining was detected in naive, HI patients and the cystectomy sample, but no staining was found in HAT patients (**Fig.7** and **Tab.5**). However, the cystectomy sample showed strong expression levels of HDACs compared to probed tumour samples.

Although differences in protein expression levels were detected, it has to be pointed out that for statistically significant results a larger sample number of each patient cohort has to be analysed. For a summary of all patients see **Table 5**.

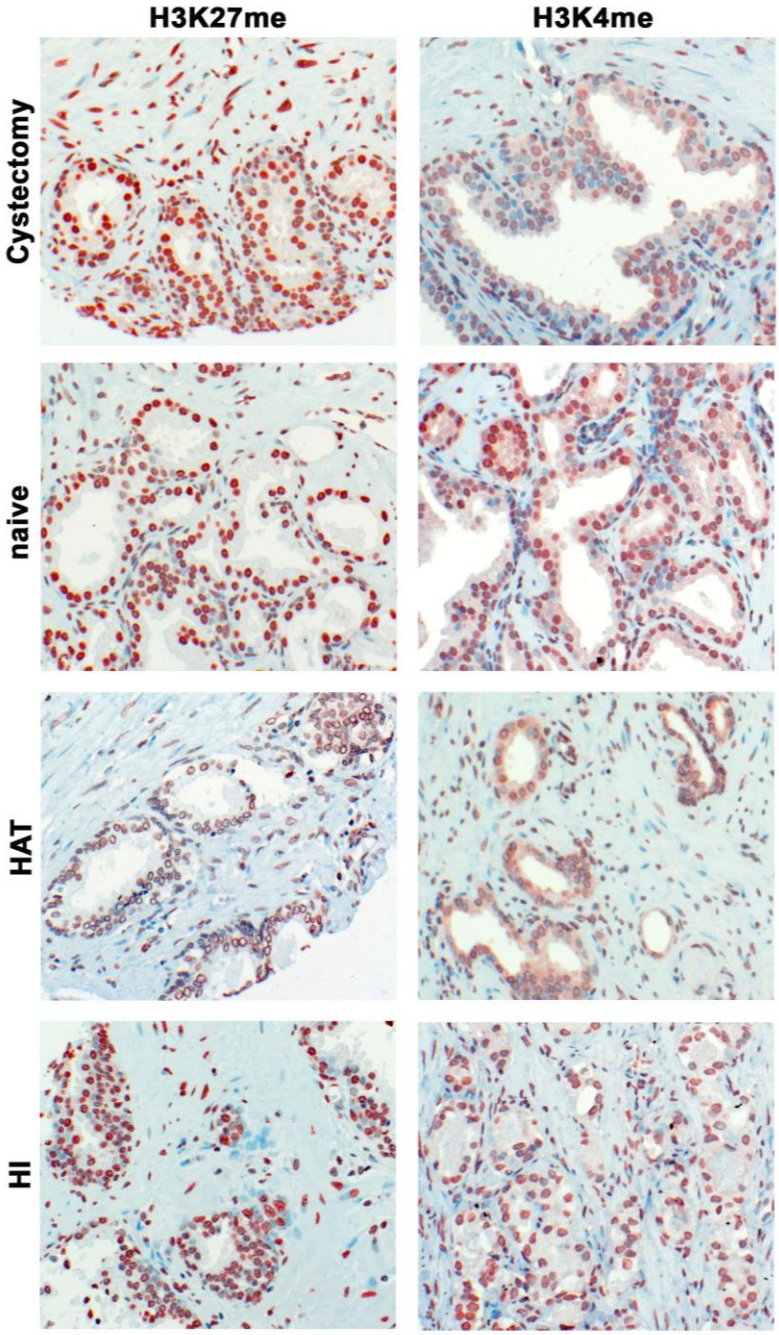


Figure 5| Tissue Array: IHC staining with ABs against H3K27me3 and H3K4. Cystectomy; naive tumours; HAT = tumours after hormone ablation therapy; HI = Hormone Insensitive tumours.

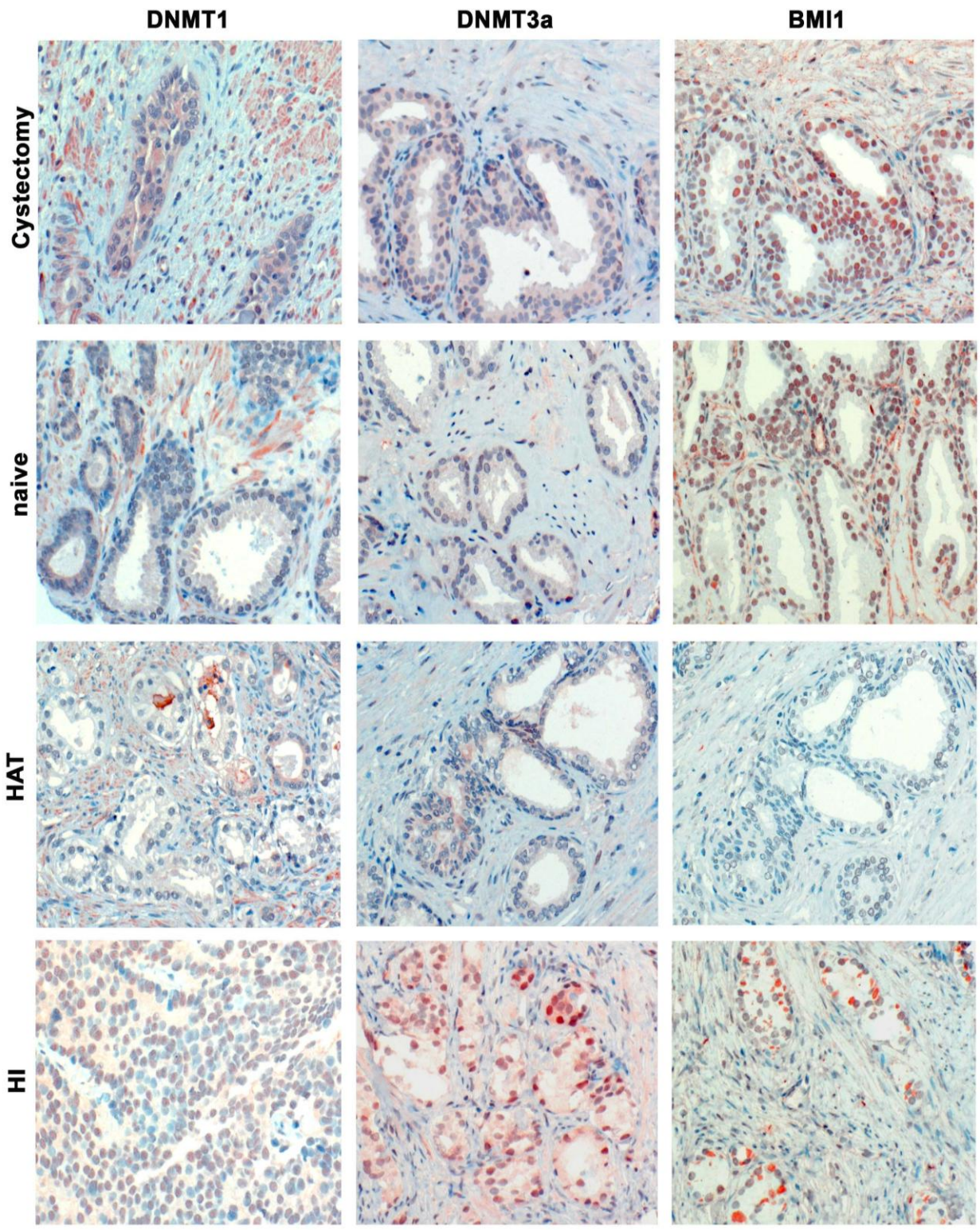


Figure 6| Tissue Array: IHC staining with ABs against DNMT1, DNMTt3a and BMI-1. Cystectomy; naive tumours; HAT = tumours after hormone ablation therapy; HI = Hormone Insensitive tumours.

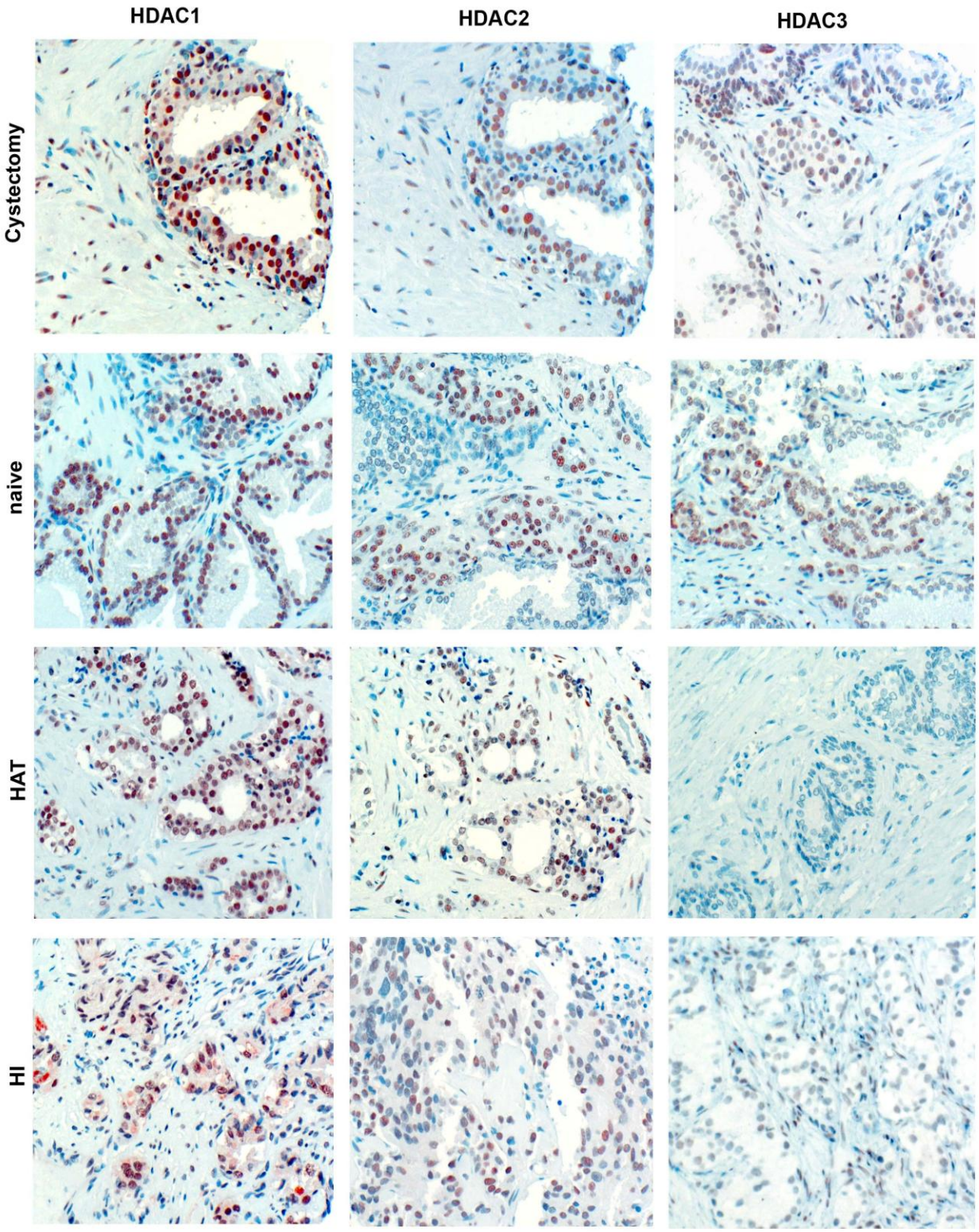


Figure 7| Tissue Array: IHC staining with ABs against HDAC1/2/3.
Cystectomy; naive tumours; HAT = tumours after hormone ablation therapy; HI = Hormone Insensitive tumours.

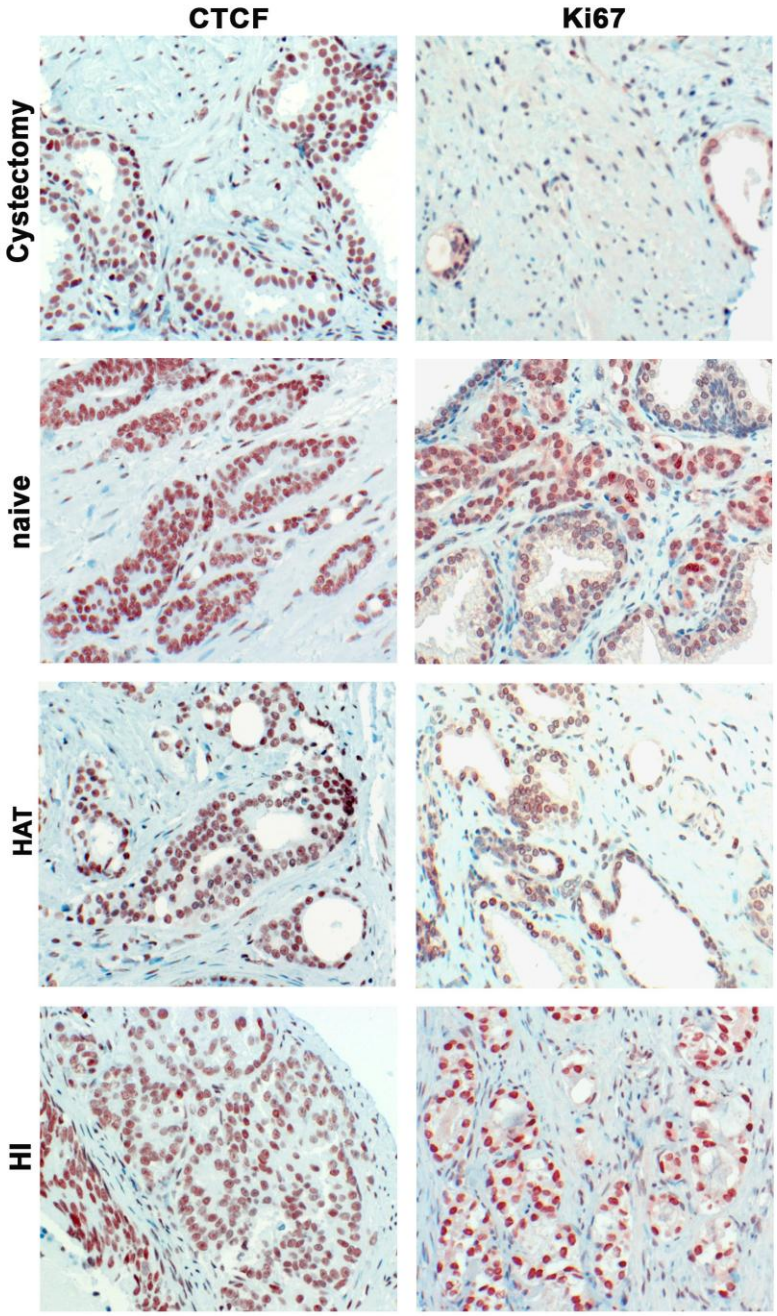


Figure 8| Tissue Array: IHC staining with ABs against CTCF and Ki67.
Cystectomy; naive tumours; HAT = tumours after hormone ablation therapy; HI = Hormone Insensitive tumours.

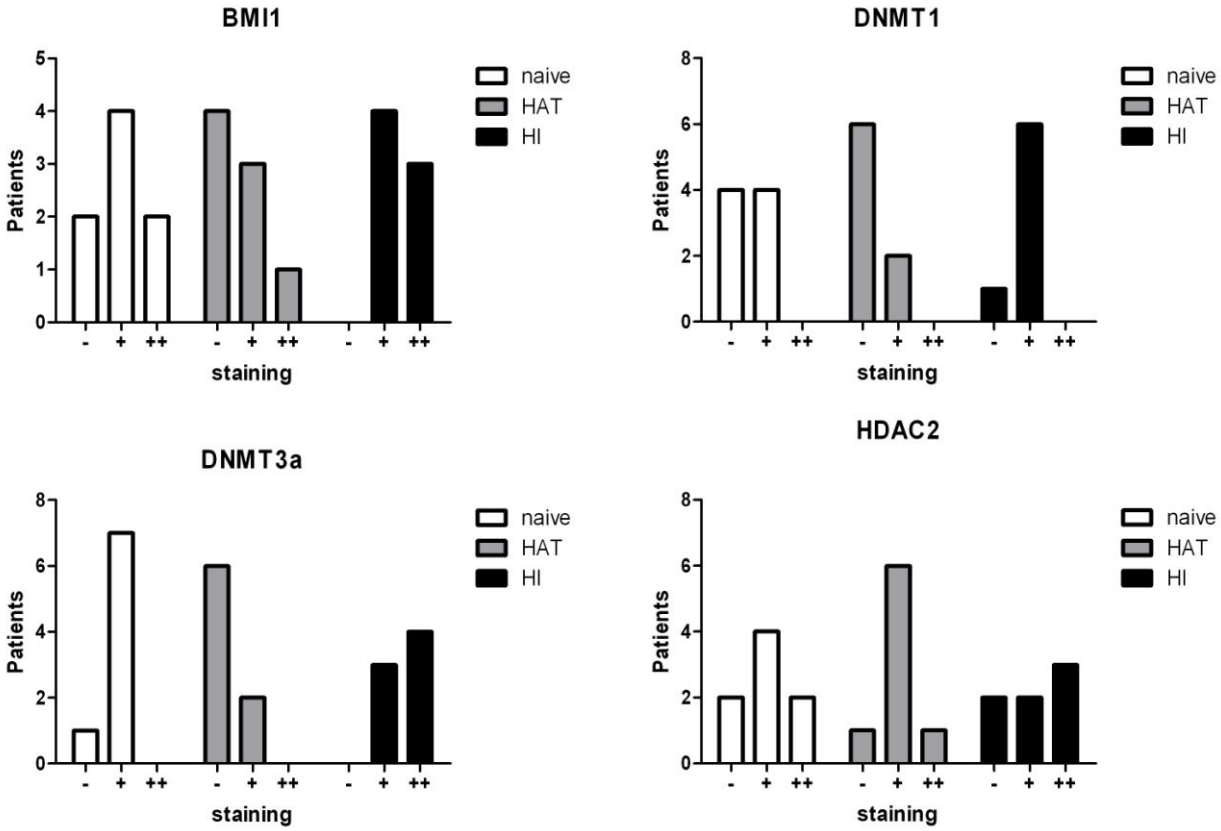


Figure 9| BMI-1, DNMT1 and DNMT3a IHC staining ratio among patient groups.

	BMI1	CTCF	DNMT1	DNMT3a	HDAC1	HADC2	HDAC3	H3K4me3	H3K27me3	Ki67
naive Patient	+	++	+	+	++	++	++	++	++	++
naive Patient	++	++	+	+	++	+	+	++	++	++
naive Patient	+	++	-	+	++	++	-	+	+	++
naive Patient	-	+	-	-	-	-	-	+	+	+
naive Patient	+	++	-	+	++	-	-	++	++	++
naive Patient	+	++	+	+	+	+	-	++	+	++
naive Patient	++	++	+	+	++	+	+	++	++	++
naive Patient	-	++	-	+	+	+	-	++	+	++
HAT Patients	-	+	-	-	+	+	-	+	++	+
HAT Patients	-	+	+	+	+	-	-	+	++	++
HAT Patients	-	++	-	-	+	+	-	+	++	+
HAT Patients	+	++	-	-	-	+	-	+	+	+
HAT Patients	+	++	-	-	++	+	-	++	++	+
HAT Patients	+	++	+	-	++	+	-	++	++	+
HAT Patients	++	++	-	+	++	++	++	++	++	++
HAT Patients	-	++	-	-	+	+	-	+	++	++
HI Patient 1	+	++	+	++	+	+	+	++	++	++
HI Patient 2	+	++	+	++	+	++	-	+	++	++
HI Patient 3	+	++	-	+	++	++	+	++	++	++
HI Patient 4	++	++	+	++	++	++	-	+	++	++
HI Patient 5	++	++	+	++	+	+	+	+	++	++
HI Patient 6	++	++	+	+	++	-	-	++	++	++
HI Patient 7	+	++	+	+	+	-	-	++	++	++

Table 5| IHC overview and evaluation of naive, HAT and HI patient samples.

Table 5 gives an overview of all evaluated patient samples. Tissue samples were classified according to moderate (+), positive (++) and negative (-) staining.

In conclusion, elevated expression of DNMT1, DNMT3a and HDAC2 in HI cancer samples compared to naïve and HAT patients implies a tendency towards different regulations of certain epigenetic enzymes between hormone-sensitive and insensitive cancer stages. Especially the observations of increased DNMT1 and DNMT3a levels are supported by previous studies for androgen independency in prostate cancer (Chen, Chen et al. 2010; Gravina, Marampon et al. 2011). Nevertheless, based on the small sample number of our study, a distinct conclusion is difficult to make.

6 Discussion

Our focus in this study was to find differences between hormone-dependent and independent cancer types, which are based on epigenetic alterations. This was tested in hormone-sensitive and insensitive patient samples and cell lines. One aim was to isolate a group of genes, which are specifically hypermethylated in certain stages of prostate cancer to identify differences between hormone sensitive and insensitive cancer groups.

In this study, we detected WNT2, EDNRß, RASSF1a, p14 and GSTP1 hypermethylated in hormone-insensitive cancer tissues (**Tab.4** and **Fig.1**). GSTP1, which is known to be methylated in 90% of PC cases and a potential biomarker in PC diagnosis, was also found to be hypermethylated in all cell lines in our study (Lee, Morton et al. 1994; Jeronimo, Usadel et al. 2001; Nakayama, Bennett et al. 2003).

Also, EDNRß methylation has been shown to correlate with disease progression before (Bastian, Palapattu et al. 2008). Interestingly, hypermethylation was exclusively found for the ESR-1 loci in hormone-sensitive cancer tissues. Previously studies showed that down-regulation of ESR-1 was linked to cancer metastasis and its methylation is age-dependent (Li, Shiina et al. 2004). However, ESR-1 methylation was observed to be differentially methylated in PCs (Yegnasubramanian, Kowalski et al. 2004; Kwabi-Addo, Chung et al. 2007; Vasiljevic, Wu et al. 2011). Interestingly, we found no methylation in HI patient samples (**Tab.4** and **Fig.1**). Normally, prostate epithelial cells are free of methylation in the ESR-1 promoter region. In cancer tissue, estrogens can affect prostatic cancerogenesis and neoplastic progression through an estrogen receptor-mediated process in human prostate tissue (Bonkhoff, Fixemer et al. 1999). Reduced expression of the ESR-1 gene has been shown in PC, particularly in hormone refractory tumours (Latil, Bieche et al. 2001).

Furthermore, WNT2 expression has been reported to be reduced in prostate cancer (Wissmann, Wild et al. 2003), which is in line with our analysis revealing a variable methylation pattern at the WNT2 promoter region (**Tab.4**, **Fig.1** and **Fig.3**). APC hypermethylation has been reported in the context of prostate cancer (Kang, Lee et al. 2004). We confirmed APC to be

hypermethylated in patient samples, although in HI patients a majority was not methylated (**Tab.4** and **Fig.1**). Jeronimo et al. pointed out that the occurrence of methylated GSTP1 together with the APC gene facilitates a more sensitive detection of prostate cancer (Jeronimo, Henrique et al. 2004). Oncogenic potential was reported by repression of the tumour suppressor genes p14 and p16 (Lowe and Sherr 2003). Interestingly, the majority of patient cancer tissue in our study showed hypermethylation at the p14 gene locus and weak to no methylation at the p16 promoter (**Tab.4** and **Fig.1**). No methylation was detected for p14 in the three cell lines tested and only PC-3 cells showed p16 specific methylation (**Tab.4** and **Fig.1**), indicating that the CDKN2A locus might not be a common target of DNA hypermethylation in prostate cancer.

Unexpectedly, ESR-1, p14, RASSF1a and WNT2 were methylated in the normal control sample (**Tab.4**). This sample was isolated from a patient with bladder cancer, who had undergone cystectomy. Frequently, such patients also display early stages of prostate cancer, which might explain our findings. Consequently, for further investigations a suitable negative control should be used.

Notably, in the course of our studies, we observed different restriction patterns in COBRA analysis for patients and cell lines in contrast to the positive SssI control sample. For example, a distinct methylation-restriction profile was seen at DAPK-1 and RASSF1a especially in PC-3 (**Suppl.Fig.1**). We assume that there must be a difference regarding the degree of DNA methylation, which is indicated by either strong and weak restriction bands or the loss of specific bands when compared to the positive control.

Another interesting finding in this study was the up-regulation of DNMT3a in HI patients (**Fig.6**, **Fig.9** and **Tab.5**). DNMT1 showed moderate nuclear staining in a majority of hormone insensitive patients (**Fig.6**, **Fig.9** and **Tab.5**), which is in line with previous studies (Gravina, Marampon et al. 2011). It was reported that during hormone ablation therapy DNMT activity is increased through epigenetic mechanisms, which favors the development of a hormone-resistant phenotype (Gravina, Marampon et al. 2011). It was also observed in

previous studies that increased EZH2 levels are correlated with enhanced H3K27 trimethylation (Cao, Wang et al. 2002). Unfortunately, we were not able to detect EZH2 by IHC-staining on our samples, which might be due to the lack of a specific antibody, however we could confirm higher EZH2 expression levels in cell lines using Western blot analysis (**Fig.4**).

We confirmed the proto-oncogene WNT1 as a target gene for H3K27 trimethylation by EZH2 in prostate cancer cell lines LNCaP and LNCaP-abl, using ChIP analysis (**Fig.2**). The down-regulation and inhibition of WNT1 was reported previously to induce apoptosis in colorectal cancer cells (He, Reguart et al. 2005) and is likely to have a function as a tumour suppressor.

Unfortunately, our aim to find differences between these related cell lines could was not met. Nevertheless, it would be interesting to include an additional hormone-insensitive cell line such as PC-3 for future studies. The LNCaP-abl cells have been generated *in vitro* and might not completely reflect the *in vivo* phenotype of a hormone insensitive prostate cancer cell line.

MethylMiner assays in LNCaP and LNCaP-abl cell lines showed methylation at the WNT2, SHP2 and DAPK-1 gene loci in both cell lines (**Fig.3**).These findings corresponded with methylation data provided by the ENCODE consortium. SHP2 was identified earlier to favour the risk of gastric cancer and its down-regulation has been shown to be due to DNA methylation (Katoh 2007). Additionally, we found elevated methylation at the DAPK-1 gene locus (**Fig.3)**, which did not correspond to our COBRA data (**Tab.4**). However, available ENCODE data confirmed our results, both for the COBRA and the MethylMiner assays, since we did examine different loci within the DAPK-1 gene, which displayed different methylation levels.

In conclusion, our results indicate that there are specific epigenetic alterations between hormone-sensitive and insensitive prostate carcinomas and provide the grounds for further genome-wide experiments. We expect that such analyses will help to classify prostate cancers by epigenetic molecular biomarkers and might be useful tools for diagnosis or prognosis of prostate cancer in clinical route in the future.

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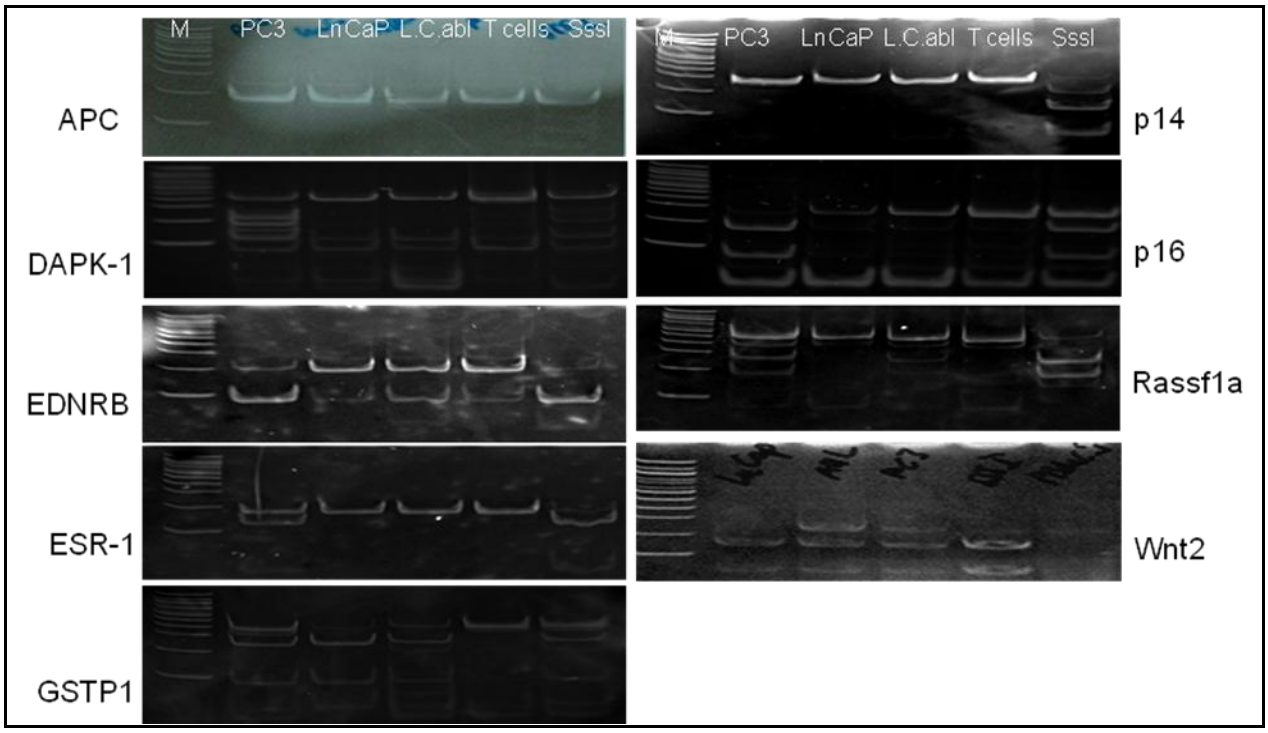
8 Abbreviations

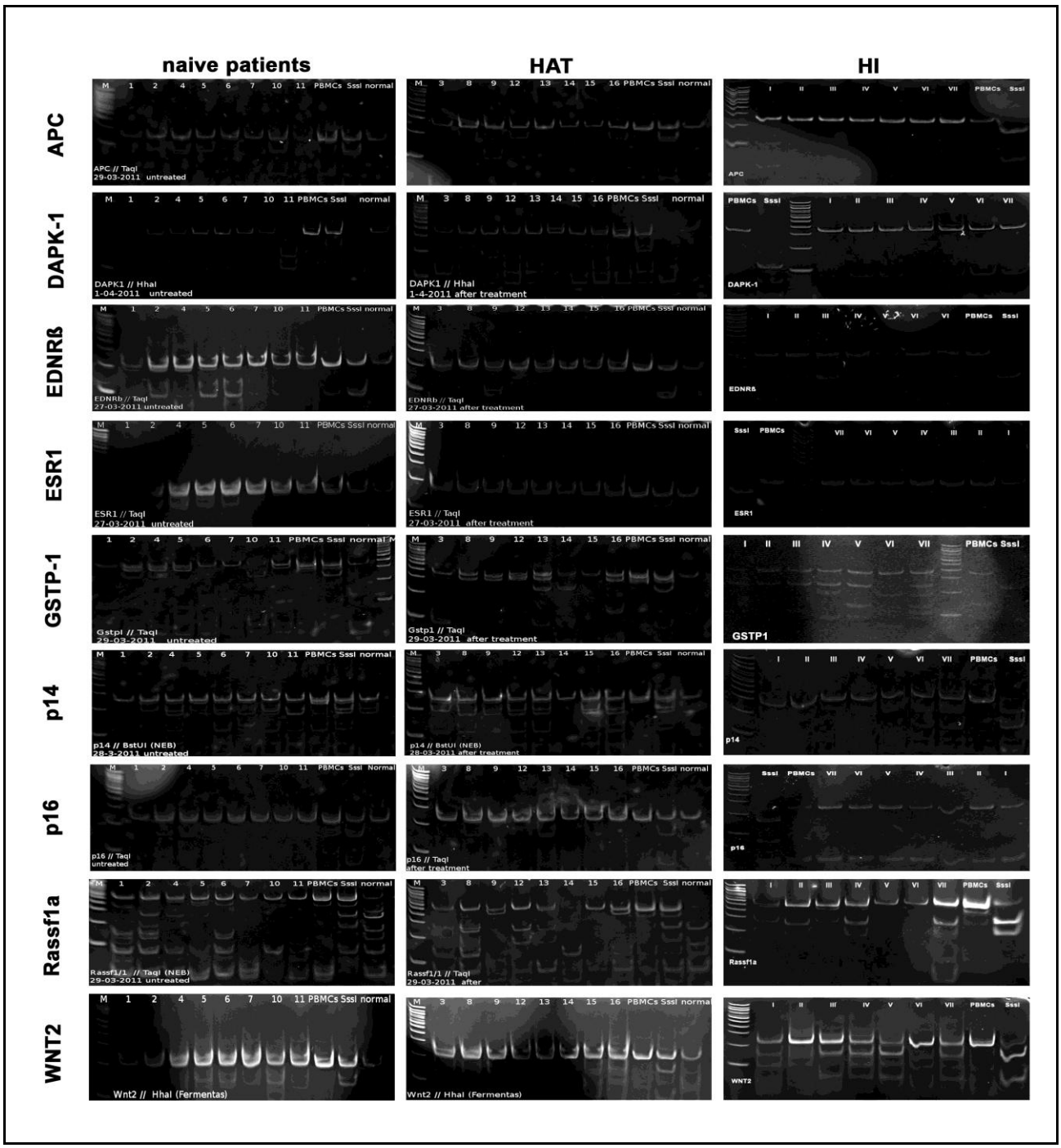
ABs	Antibodies
AP-1	Activator Protein 1
APC	Adenomatous polyposis coli
BMI-1	B lymphoma Mo-MLV insertion region 1 homologue
cJun	Jun proto-oncogene; AP-1 transcription factor family
CTCF	CCCTC-binding factor
DAPK-1	Death Associated Protein Kinase 1
DNMT	DNA methyltransferase
DNMT1	DNA (cytosine-5)-methyltransferase 1
DNMT3a/3b	DNA (cytosine-5)-methyltransferase 3 alpha/beta
EDNRB	Endothelin receptor type B
ESR-1	Estrogen Receptor alpha
EZH2	Enhancer of Zeste homologue 2
FFPE	Formalin fixed paraffin embedded
GSTP1	Glutathione S-transferase P 1
HAT	Hormone ablation therapy
HDAC	Histone deacetylase
HI	Hormone-insensitive
HMT	Histone methyltransferase
H3K27me3	trimethylation of Histone 3 at Lysine 27
H3K4	Histone 3 Lysine 4
JunB	Jun proto-oncogene; AP-1 transcription factor family
Ki-67	Antigen Ki-67
MYT1pd	Myelin-transcription-factor 1
MYT1prox	Myelin-transcription-factor 1 proximal
PBMC	peripheral blood mononuclear cells
PC	Prostate Cancer
PcG	Polycomb Group Protein
p14	Cyclin-dependent kinase inhibitor 2A - alternative reading frame of CDK2NA

p16	Cyclin-dependent kinase inhibitor 2A - CDK2NA
PRC2	Polycomb Repressive Complex 2
PSA	Prostate Specific Antigen
RASSF1	Ras association domain-containing protein 1
SAM	S-Adenosylmethionine
SHP2 (PTPN11)	SH2 domain-containing protein tyrosine phosphatase
SNPs	Single nucleotide polymorphism
TMA	Tissue Micro-array
TF	Transcription Factor
UBE2B	Ubiquitin-conjugating enzyme (E2B)
WNT1	Wingless-type MMTV integration site family member 1
WNT2	Wingless-type MMTV integration site family member 2

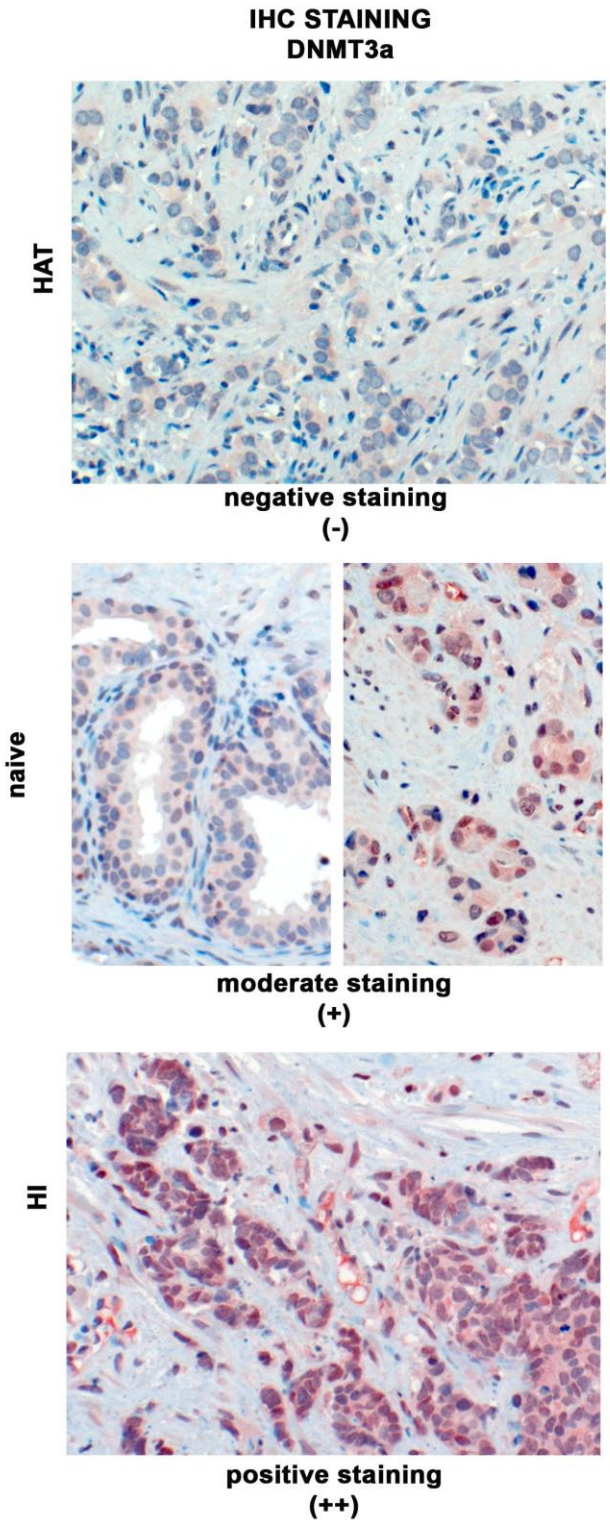
9 Supplementary Figures



Supp.Figures 1| COBRA restriction: Cell lines



Supp.Figures 2| COBRA restiction: naive ; HAT ; HI patients.



Supp.Figures 3| IHC staining intensity evaluation: Example of DNMT3a.

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Curriculum Vitae

Personal details

Name	Claudia Haider
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Education

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