

Normal and Prostate Cancer Cells Display Distinct Molecular Profiles of α -Tubulin Posttranslational Modifications

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BACKGROUND. Multiple diverse posttranslational modifications of α -tubulin such as detyrosination, further cleavage of the penultimate glutamate residue ($\Delta 2$ -tubulin), acetylation, and polyglutamylation increase the structural and functional diversity of microtubules.

METHODS. Herein, we characterized the molecular profile of α -tubulin posttranslational modifications in normal human prostate epithelial cells (PrEC), immortalized normal prostate epithelial cells (PZ-HPV-7), androgen-dependent prostate cancer cells (LNCaP), transitional androgen-independent prostate cancer cells (LNCaP-cds and CWR22Rv1), and androgen-independent prostate cancer cells (PC3).

RESULTS. Compared to PrEC and PZ-HPV-7 cells, all cancer cells exhibited elevated levels of detyrosinated and polyglutamylated α -tubulin, that was paralleled by decreased protein levels of tubulin tyrosine ligase (TTL). In contrast, PrEC and PZ-HPV-7 cells expressed markedly higher levels of $\Delta 2$ -tubulin. Whereas α -tubulin acetylation levels were generally equivalent in all the cell lines, PC3 cells did not display detectable levels of Ac-tubulin.

CONCLUSION. These data may reveal novel biomarkers of prostate cancer and new therapeutic targets. *Prostate* © 2006 Wiley-Liss, Inc.

KEY WORDS: prostate cancer; microtubules; detyrosination; acetylation; polyglutamylation; tubulin tyrosine ligase; HDAC6; SIRT2

INTRODUCTION

Prostate cancer is the leading cause of cancer-related illness and death among men in the Western world [1]. Intensive efforts to define early detection markers of the disease and to elucidate biomolecular mechanisms underlying prostate cancer initiation and progression have failed to solve these enigmatic problems to date. Therefore, approaches to further understand the biochemical/cellular phenotypes displayed during prostate carcinogenesis remain important endeavors, and will potentially lead to novel strategies for early diagnosis and treatment of prostate cancer.

Grant sponsor: California Cancer Research Program; Grant sponsor: DOD grant; Grant number: BC-024051; Grant sponsor: Paul F. Gulyassy Endowed Professorship; Grant sponsor: Phillip Morris External Research Program.

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Received 24 August 2005; Accepted 6 January 2006

DOI 10.1002/pros.20416

Published online in Wiley InterScience

(www.interscience.wiley.com).

The function of mammalian cells, including normal and tumor-associated prostate cells, is dependent upon the integrity, dynamics, and organization of the cytoskeleton. Among the many structural entities that make up the cytoskeleton, microtubules (MTs) are perhaps the most well characterized. MTs contribute, both directly and indirectly, to cellular processes such as growth, motility, secretion, intracellular organelle trafficking, and inter- and intra-cellular signaling [2]. Existing as large dynamic polymers, MTs are composed of heterodimers of α - and β -tubulin polypeptides translated from multiple genes [3]. The tissue distribution of the various α - and β -tubulin isoforms has not been clearly characterized, nor have specific functions been ascribed. However, expression of some β -tubulin isoforms are known to be associated with resistance to antimetabolic drugs [4,5], suggesting that each α - or β -tubulin isoform confers unique structural or functional differences. Moreover, and perhaps more importantly, the structural and functional diversity of MTs is further augmented by multiple posttranslational modifications on the α - and β -tubulin protein subunits (for review see [6,7]). While β -tubulin undergoes some posttranslational modifications, α -tubulin is modified to the largest and most diverse extent. Posttranslational modifications of α -tubulin occur at various positions within its primary structure, but the carboxyl terminus harbors the majority of these modifications except for acetylation (Fig. 1).

The most well studied posttranslational modification of α -tubulin is the cyclic removal and re-addition of the C-terminal tyrosine residue [8]. Removal of the gene-encoded C-terminal tyrosine (Tyr-tubulin), via tubulin-specific carboxypeptidase (TCP) activity, generates detyrosinated α -tubulin (Glu-tubulin). The reaction is reversible; that is, the enzyme, tubulin tyrosine ligase (TTL), is capable of catalyzing the addition of a tyrosine back onto the C-terminus of Glu-tubulin, in the absence of RNA or ribosome, thus regenerating Tyr-tubulin [9]. Previous studies have revealed that the

detyrosination/tyrosination cycle of α -tubulin is associated with progression through the cell cycle and cellular differentiation [10,11]. Most recently, an irreversible inhibitor of the α -tubulin detyrosination process (3-nitro-L-tyrosine; NO₂Tyr; [12]) has been utilized to demonstrate that strict regulation of tyrosination/detyrosination cycle is required for cellular differentiation [13], migration [14], and proliferation (Phung et al., submitted manuscript). Detyrosinated α -tubulin (Glu-tubulin) is elevated in tissue from breast cancer and neuroblastoma patients with poor prognosis [15,16], and this modification appears to parallel decreased expression of the TTL enzyme. Indeed, decreased TTL expression has been suggested to be a common feature of tumor cells [17]. Glu-tubulin is also subject to further proteolytic cleavage of the penultimate glutamate residue to form a "non-tyrosinatable" form of α -tubulin, termed Δ 2-tubulin, that is largely associated with neuronal cells, but is also elaborated in some non-neuronal cells [18].

Polyglutamylation and polyglycylation occur on the C-terminus of both α - and β -tubulins. Both modifications are common in neurons and ciliated cells, but the extent to which this occurs in non-neuronal cells remains poorly characterized. Polyglycylation has been shown to play important roles in motility and cytokinesis but is largely limited in its occurrence to *Protozoa*. Polyglutamylation, on the other hand, occurs in most organisms and appears to regulate interactions between MTs and microtubule-associated proteins (MAPs) [19]. Polyglutamylated tubulin (PGlu-tubulin) is detected in some proliferating mammalian cells and its level is increased during mitosis [20]. Although the precise role of tubulin polyglutamylation is not known in detail, studies have revealed that it is likely connected with centriole stability. Also, several studies have indicated the importance of this modification during cell differentiation and development where the stabilization of MTs is requisite [21].

In addition to posttranslational modifications occurring at the C-terminus of α -tubulin, acetylation of α -tubulin occurs on the ϵ -amino group of a highly conserved lysine residue located at position 40 of its primary sequence (the N-terminal domain). While the enzyme(s) responsible for α -tubulin acetylation have not been identified, two enzymes have been recently discovered that catalyze the deacetylation process, namely histone deacetylase-6 (HDAC6) [22] and the NAD⁺-dependent deacetylase sirtuin-2 (SIRT2) [23]. Modulation of α -tubulin deacetylation using pharmacological or genetic perturbation has revealed that the degree of α -tubulin acetylation modulates cell migration and motility [24,25].

Based upon these premises, it is evident that regulation and expression of these various α -tubulin

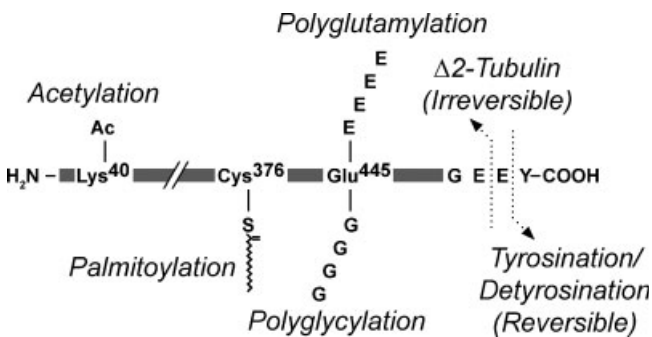


Fig. 1. Schematic illustrating the multiple posttranslational modifications of α -tubulin. All posttranslational modifications, with the exception of palmitoylation, were measured in this study.

posttranslational modifications are modulated during multiple biological processes. However, the extent to which α -tubulin undergoes posttranslational modification during the initiation and progression of prostate cancer remains unknown. We postulated that posttranslational modifications of α -tubulin are differentially expressed in normal versus prostate cancer cells and that comprehensive molecular profiling of these various posttranslational modifications may provide clues for gaining a better understanding of the molecular basis of prostate cancer. Furthermore, we hypothesized that establishing a focused proteomic approach to assess these posttranslational modifications would help to further define mechanisms underlying prostate tumor biology, and might also provide novel, targeted molecular therapeutic treatment strategies. Herein, we have characterized the molecular profile of α -tubulin posttranslational modifications in normal and tumor cells from human prostate epithelia, and present data suggesting that these molecular “signatures” might have potential diagnostic utility.

MATERIALS AND METHODS

Cell Culture

Primary cultures of normal human prostate epithelial cells (PrEC) were purchased from Cambrex Bio Science Walkersville (Cambrex Corporation; East Rutherford, NJ), cultivated in PrEBMTM (Prostate Epithelial Cell Basal Medium; Cambrex) and supplemented with PrEGM SingleQuots[®] (bovine pituitary extract, hydrocortisone, hEGF, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and gentamicin; Cambrex). Cells were maintained in accordance with manufacture instructions (CloneticsTM Prostate Epithelial Cell System). PZ-HPV-7, LNCaP, and PC-3 cells were obtained from the American Type Culture Collection (ATCC). PZ-HPV-7 cells were cultivated in Keratinocyte-Serum Free medium supplemented with human recombinant EGF and bovine pituitary extract (GibcoTM Invitrogen Corporation; Carlsbad, CA). LNCaP cells were cultivated in RPMI-1640 medium (GibcoTM Invitrogen Corporation) with 2 mM L-glutamine, streptomycin (0.1 mg/ml), penicillin (100 U/ml), and supplemented with 10% fetal bovine serum (Gemini Bio-Product; Woodland, CA). PC3 cells were cultivated in Ham's F12 medium (GibcoTM Invitrogen Corporation) with 2 mM L-glutamine, streptomycin (0.1 mg/ml), penicillin (100 U/ml), and supplemented with 10% fetal bovine serum (Gemini Bio-Product). Androgen-independent LNCaP cell lines (LNCaP cds1, cds2, and cds3) [26] were cultured in RPMI-1640 medium as noted above and supplemented with 10% charcoal/dextran-treated FBS (androgen-

depleted media; Hyclone, Logan, UT). The androgen-independent CWR22Rv1 cell line was previously established from a relapsed prostate cancer tumor (CWR22R-2152), which was derived from the parental androgen-dependent CWR22 tumor that had been serially transplanted after castration-induced regression and relapse [27]. The CWR22Rv1 cell line was cultivated in RPMI-1640 medium and supplemented with 10% FBS. All cell lines were cultivated in Nunc (Nalge Nunc International; Rochester, NY) and/or Corning (Corning Incorporated; Life Sciences, Acton, MA) cultivation dishes, flasks, and plates in a humidified incubator at 37°C in an atmosphere of 5% CO₂.

Electrophoresis and Western Blotting

Sub-confluent cells were washed twice in PBS and harvested in lysis buffer (50 mM TRIS-HCl pH 7.5, 20% glycerol, 1% SDS, and protease inhibitor cocktail; Sigma-Aldrich Corp.; St. Louis, MO). The protein concentration of the samples was determined using detergent-compatible BCA protein assay (Pierce Biotechnology; Rockford, IL). The samples were diluted to the same concentration and an equal volume of 2× SDS loading buffer (100 mM TRIS-HCl pH 6.8, 2% SDS, 0.02% bromophenol blue, 20% glycerol, 2% β -mercaptoethanol) was added. Equivalent quantities of protein (20 μ g) were separated by SDS-PAGE electrophoresis on 10% gels using established procedures. Proteins separated by one-dimensional electrophoresis were electro-transferred using a Mini Trans-Blot wet transfer system (Bio-Rad Laboratories; Hercules, CA) onto PVDF membranes (Millipore Corp.; Bedford, MA). Membranes were blocked in TBS (20 mM TRIS-HCl pH 7.6, 150 mM NaCl) containing 0.1% Tween 20 and 5% non-fat milk for 1 hr. The blots were then washed with TBS-Tween, and incubated with anti- α -tubulin clone DM1A (T9026, 1:5,000, Sigma-Aldrich Corp.), anti-polyglutamylated- α -tubulin clone B3 (T9822, 1:1,000, Sigma-Aldrich Corp.), anti-acetylated- α -tubulin clone 6-11B-1 (T6793, 1:2,000, Sigma-Aldrich Corp.), anti-tyrosine- α -tubulin clone TUB-1A2 (T9028, 1:3,000, Sigma-Aldrich Corp.), anti- Δ 2- α -tubulin (AB3203, 1:500, Chemicon International, Temecula, CA), anti-detyrosinated- α -tubulin (Gundersen et al. [28], 1:5,000), anti-polyglycylated tubulin (Kann et al. [29], 1:500), or anti- β -tubulin (T4026, 1:3,000, Sigma-Aldrich Corp.) primary antibodies overnight at 4°C. The membrane was extensively washed and then incubated with anti-mouse IgG (172-1011, 1:3,000, Bio-Rad Laboratories) or anti-rabbit IgG (172-1019, 1:3,000, Bio-Rad Laboratories) antibodies conjugated with horseradish peroxidase for 1 hr after washing with TBS-Tween. Detection of antibody reactivity was performed using SuperSignal

West Pico Chemiluminescent Substrate and visualized on CL X-posure films (both from Pierce Biotechnology). Equal sample loading was verified with immunodetection of β -actin (A5441, 1:10,000, Sigma-Aldrich Corp.) or staining of PVDF membrane with 0.1% amidoblack (in 1:3:6 volumes of acetic acid, methanol, and water) for non-specific visualization of proteins. Densitometric measurements were performed using a digital camera detection system (Kodak EDAS 290) and Kodak 1D Image Analysis software (both from Eastman Kodak; Rochester, NY) or ImageJ software (NIH, Bethesda, MD).

TubulinTyrosine Ligase (TTL) Antisera

An internal 15 amino acid sequence of the human TTL protein (amino acids 244–258; NH₂-CIQKEYS-KNYGKYEE-CO₂H) was used for synthesis of the peptide immunogen for preparing a rabbit polyclonal TTL antibody. This peptide sequence is identical in human, rat, and mouse TTL. The synthetic peptide was attached to keyhole limpet hemocyanin (KLH) via the thiol group of the N-terminal cysteine residue in the peptide sequence. Following pre-immune serum collection, New Zealand White rabbits were initially immunized with 200 μ g of the KLH-peptide conjugate in complete Freund's adjuvant. This was followed by five weekly immunizations with 100 μ g of the KLH-peptide conjugate in incomplete Freund's adjuvant. Two weeks following the final immunization, rabbits were exsanguinated and serum obtained. With the exception of peptide design, TTL antiserum production was performed by Sigma-Genosys (Sigma-Aldrich Corp.). Antiserum from the final bleed was diluted 1:1,000 for Western blot detection of TTL protein expression. Preimmune rabbit serum was not reactive against TTL or any other protein.

Immunofluorescence Microscopy

Cells were cultured on Lab-Tek II multi-chamber slides (Nalge Nunc International) coated with bovine skin gelatin until cells were just sub-confluent. Cells were fixed in methanol for 10 min at -20°C , rinsed with PBS, blocked in PBS containing 10% goat serum (1 hr at 25°C), and labeled with primary antibodies (1 hr at 25°C) including monoclonal anti- α -tubulin (1:2,000), polyclonal anti- $\Delta 2$ - α -tubulin (1:500), monoclonal anti-acetylated- α -tubulin (1:500) (same sources as those used for Western blot analyses); anti-detyrosinated- α -tubulin (1:500, AB3201, Chemicon International), polyclonal anti- α -tubulin, (1:50, sc-5546, Santa Cruz Biotech, CA). Secondary antibodies were Alexa488-conjugated goat anti-rabbit IgG and Alexa594-conjugated goat anti-mouse IgG (30 min at 25°C , 1:2,000, Molecular Probes, Eugene, OR). Nuclear counterstaining was

performed by using 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI; 0.1 μ g/ml, 5 min at 25°C). Samples were mounted in Mowiol 4–88/DABCO anti-photobleaching media. An LSM 5 Pascal Confocal Laser Scanning Microscope was used for imaging (Carl Zeiss; Thornwood, NY).

RESULTS

Western Blot Analysis of α -Tubulin Posttranslational Modifications in Cultured Human Prostate Cells

As shown in Figure 1, α -tubulin can undergo multiple posttranslational modifications that may be linked to altered cellular phenotypes related to growth, motility, signaling pathways, and metabolism. Given that normal and cancer epithelial cells of the prostate display distinct phenotypes, we hypothesized that characterizing the extent of α -tubulin posttranslational modifications will provide a molecular profile to distinguish normal PrEC from prostate cancer cells.

To test this hypothesis, we first performed a comprehensive analysis of α -tubulin posttranslational modifications in primary cultures of normal human PrEC, immortalized normal human prostate epithelial cells (PZ-HPV-7), androgen-dependent prostate cancer cells (LNCaP), and androgen-independent prostate cancer cells (PC3) using Western blotting with specific antibodies (Fig. 2). Compared to normal and immortalized human PrEC, both androgen-dependent and androgen-independent prostate cancer cell lines (LNCaP and PC3) displayed elevated levels of detyrosinated α -tubulin (Glu-tubulin), and accordingly moderately decreased levels of tyrosinated α -tubulin (Tyr-tubulin). Similarly, the level of α -tubulin polyglutamylation (PGlu-tubulin) was highly elevated in both of these prostate cancer cell lines, but undetectable in normal PrEC and immortalized PZ-HPV-7 cells. Therefore, enhanced α -tubulin detyrosination and polyglutamylation is a molecular signature of both androgen-dependent (LNCaP) and androgen-independent (PC3) human prostate cancer cell lines. In contrast, we have observed that the level of $\Delta 2$ -tubulin, the α -tubulin isoform missing both the C-terminal tyrosine and glutamate residues, is detectable in PrEC and PZ-HPV-7 cells, but undetectable in LNCaP and PC3 prostate cancer cell lines. It is noteworthy that the $\Delta 2$ -tubulin antibody recognized lower molecular weight proteins that may reflect further proteolytic modifications. Whereas PrEC, PZ-HPV-7, and LNCaP cells display comparable levels of acetylated α -tubulin (Ac-tubulin), PC3 cells do not display detectable levels of this posttranslationally modified form of α -tubulin. We found no evidence of polyglycylated α -tubulin in any of the prostate cell lines examined (data not shown). The expression of α -tubulin (as well as

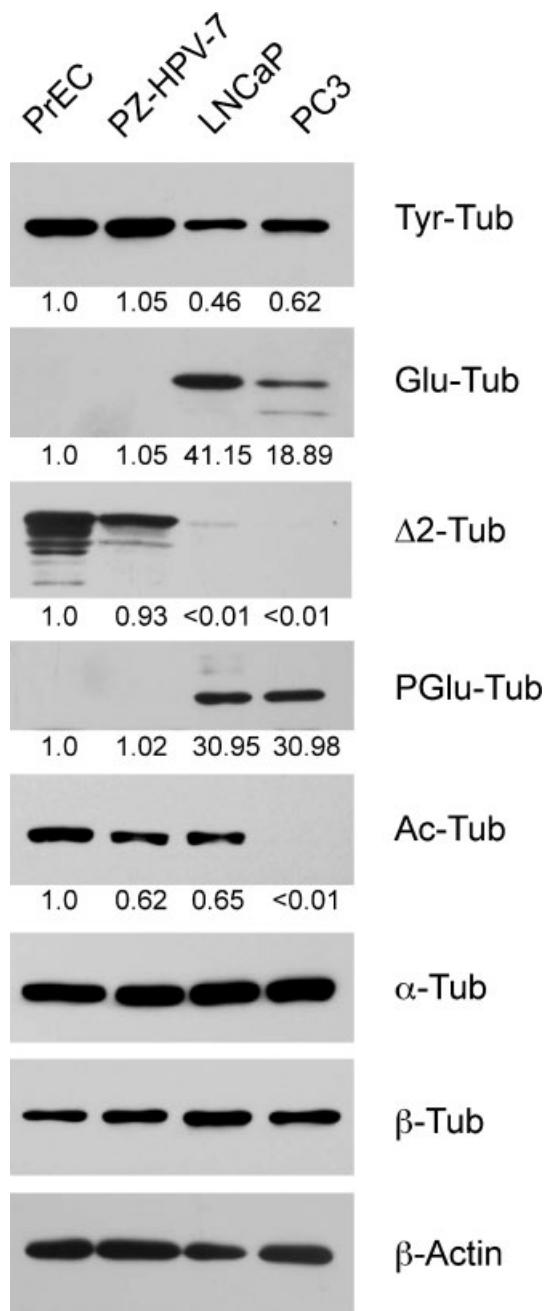


Fig. 2. Distinct profiles of α -tubulin posttranslational modifications are observed in normal human prostate epithelial cells and prostate cancer cells. The cell lines examined were normal human PrEC, immortalized normal human prostate epithelial cells (PZ-HPV-7), androgen-dependent prostate cancer cells (LNCaP) and androgen-independent prostate cancer cells (PC3). Cells were harvested just prior to confluence, proteins separated by one-dimensional SDS-PAGE, and the various posttranslational modifications (schematically illustrated in Figure 1) revealed by Western blotting using specific antibodies as described in the Materials and Methods section. Numbers below the blots indicate quantitative values relative to the normal human PrEC normalized to α -tubulin levels. Data are representative of the average of three separate experiments. Quantitative data are the average of at least three separate experiments.

β -tubulin and β -actin) were essentially indistinguishable amongst the four cell lines examined, revealing that the observed alterations in α -tubulin posttranslational modifications were not due to differential expression of total α -tubulin, but rather due to alterations in the expression and/or activity of the molecular machinery executing these posttranslational modifications.

Immunofluorescence Analysis of α -Tubulin Posttranslational Modifications in Cultured Human Prostate Cells

We next utilized indirect immunofluorescence and confocal microscopy to determine whether the results obtained from Western blotting recapitulated the distinct profile of posttranslational modifications of α -tubulin in the prostate cell lines. As shown in Figure 3 and consistent with Western blotting results, Glu-tubulin was observed in LNCaP and PC3 prostate cancer cell lines, but not in normal PrEC or immortalized normal PZ-HPV-7 cells. Similar observations were observed for polyglutamylated α -tubulin; that is, polyglutamylated α -tubulin was only detectable by immunofluorescence microscopy in prostate cancer cells, but not in normal or immortalized human PrEC (data not shown). Consistent with Western blotting data, Δ 2-tubulin was detectable only in normal and immortalized cells (PrEC and PZ-HPV-7) but not in the cancer cells (LNCaP and PC3). It is noteworthy that we observed that Δ 2-tubulin was localized primarily inside nuclei of normal and immortalized cells, but was not associated with microtubule polymers as illustrated in Figure 4. We also determined the presence and localization of Ac-tubulin in the PrEC using confocal microscopy. Our results confirmed observations from Western blotting; that is, Ac-tubulin was not detectable in PC3 cells (data not shown). Since Ac-tubulin and Glu-tubulin are both typically associated with “stable” microtubules [30], we next determined whether these two posttranslational modifications were colocalized in cells simultaneously expressing both modifications (i.e., LNCaP cells). Dual labeling strategies revealed a high degree of Glu-tubulin and Ac-tubulin colocalization in LNCaP cells (Fig. 5). Detailed profile analysis of signal distance versus intensity of a merged image showed the same trend in signal intensity of both red (Ac-tubulin) and green (Glu-tubulin) fluorescence. Therefore, Ac-tubulin and Glu-tubulin colocalize and consistent with previous notions likely reflect the existence of stable microtubules.

Expression of Tubulin Tyrosine Ligase (TTL) Protein in Human Prostate Cells

Since the diverse patterns of α -tubulin posttranslational modifications observed in normal and prostate

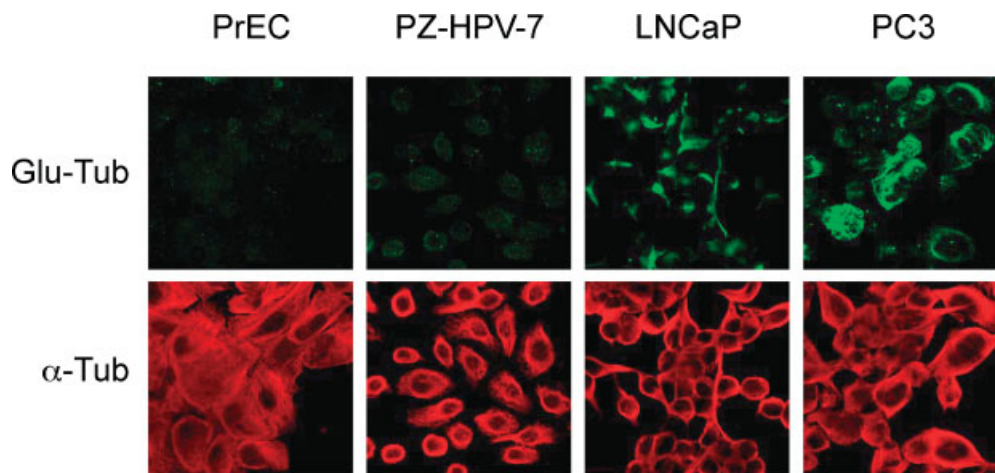


Fig. 3. Confocal immunofluorescence microscopy demonstrates that detyrosinated α -tubulin (Glu-tubulin) is elevated in prostate cancer cells. Immunofluorescence analysis of Glu-tubulin (green) and α -tubulin (red) was performed in PrEC, Pz-HPV-7, LNCaP, and PC3 cells. Cells were stained with both anti-Glu-tubulin and anti- α -tubulin antibodies. Detectable fluorescence staining for anti-Glu-tubulin antibody was observed only in LNCaP and PC-3 cells.

cancer cells could not be attributed to changes in total α -tubulin expression (Fig. 2), we reasoned that alterations in the expression and/or activity of the enzymes catalyzing these posttranslational modifications must

be altered. Since the tyrosination/detyrosination cycle is the most well understood posttranslational modification of α -tubulin, we next studied this pathway as “proof of concept” for our observations. The elevated

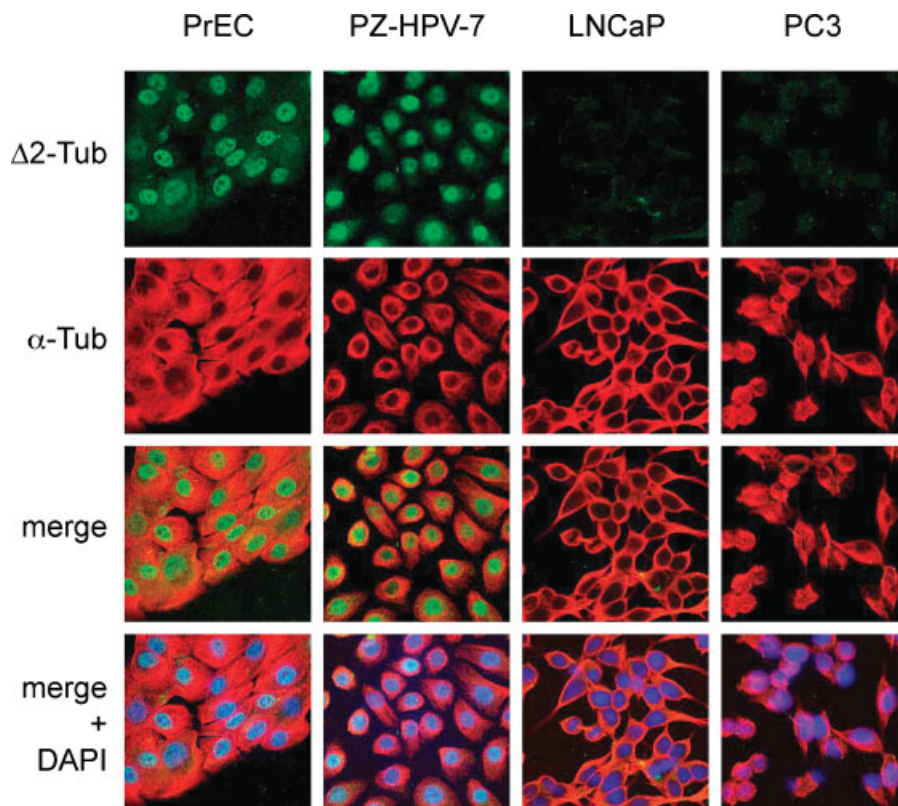


Fig. 4. $\Delta 2$ -tubulin is specifically localized in the nuclei of normal (PrEC) and immortalized (PZ-HPV-7) prostate epithelial cells. Immunofluorescence analysis of $\Delta 2$ -tubulin (green) and total α -tubulin (red) was performed in PrEC, Pz-HPV-7, LNCaP, and PC3 cells. Cells were fixed and stained with both anti- $\Delta 2$ -tubulin and anti- α -tubulin antibodies and counterstained with DAPI (nuclear stain). Signal of anti- $\Delta 2$ -tubulin antibody was detected in PrEC and Pz-HPV-7 cells only, and localized in the nucleus, rather than associated with cytoplasmic microtubules.

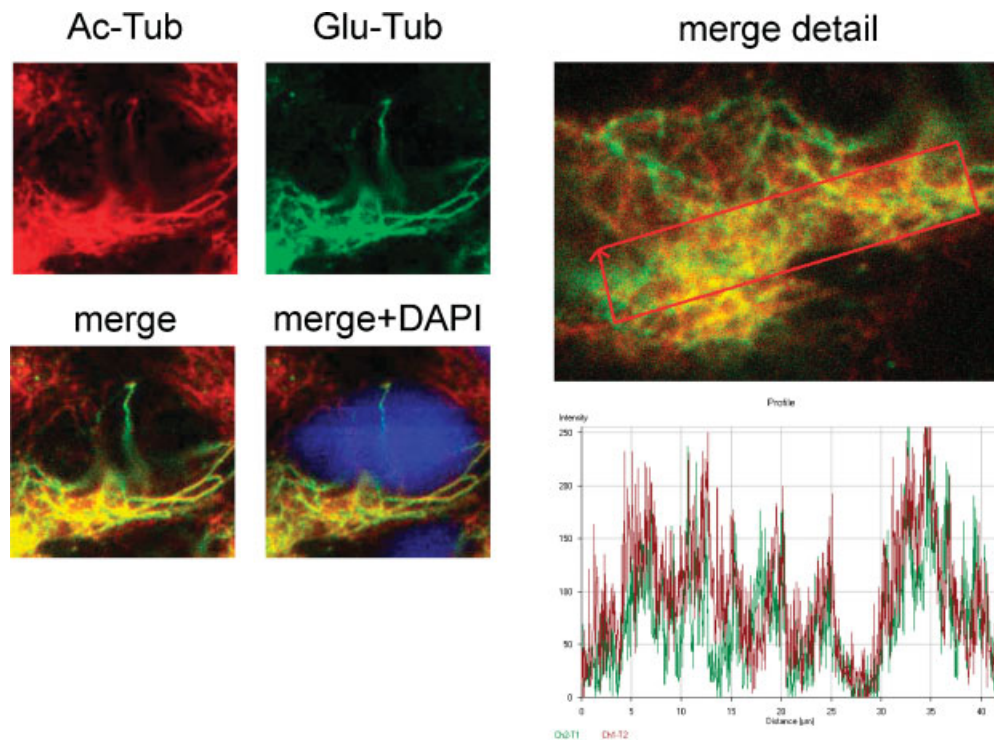


Fig. 5. Ac-tubulin colocalizes with Glu-tubulin in androgen-dependent LNCaP cells. Cells were double stained with both anti-Ac-tubulin (red) and anti-Glu-tubulin (green) antibodies and counterstained with DAPI. Colocalization was analyzed using profiling of intensity and distance of pixels for each color (Ac-tubulin—Ch1 ~ red, Glu-tubulin—Ch2 ~ green) from the outlined merged image.

levels of deetyrosinated α -tubulin (Glu-tubulin) in both LNCaP and PC3 cells could be the result of (1) decreased expression or activity of TTL, and/or (2) increased tubulin-specific carboxypeptidase (TCP) activity. To address this, we prepared a rabbit polyclonal antibody against TTL and probed the prostate cell lines for TTL protein by Western blot analysis. As shown in Figure 6A, both PrEC and PZ-HPV-7 cells expressed a protein with immunoreactivity to TTL, and consistent with the molecular weight (~43 kD) of recombinant TTL [31]. In LNCaP and PC3 cells, markedly lower levels of TTL protein were observed and quantification of the Western blot revealed approximately 90% and 50% reduction, respectively (Fig. 6B), consistent with the increased Glu-tubulin levels displayed by LNCaP and PC3 cells. These data demonstrate that decreased TTL expression in LNCaP and PC3 prostate cancer cells may prohibit the retyrosination of α -tubulin and explain the accumulation of deetyrosinated α -tubulin (Glu-tubulin). However, increased TCP activity and/or decreased TTL enzymatic activity may also equally contribute to the higher levels of Glu-tubulin expressed by prostate cancer cells, and remain issues that need further characterization.

α -Tubulin Detyrosination and Acetylation in “Transitional” Androgen-Independent AR-Positive Prostate Cancer Cell Lines

To further extend the validity and potential prognostic/mechanistic value of the α -tubulin posttranslational modifications we have observed, additional androgen-independent prostate cancer cell lines were examined. The androgen-independent cell line we have examined to this point, PC3, is androgen-independent and lacks expression of the androgen receptor (AR). Because of this, we have chosen to further study androgen-independent cell lines that express functional and mutant AR, since these cell lines are thought to represent “transitional” forms of refractory androgen-independent prostate cancer. The cell lines of this type that we have investigated are (1) three separate androgen-independent LNCaP cell lines (LNCaP cds1, cds2, and cds3) that were selected for androgen-independence by continuous passage in androgen-deficient conditions [26], and (2) the partially androgen-independent CWR22Rv1 cell line derived from a relapsed CWR22 tumor [27]. As shown in Figure 7, all of these cell lines displayed markedly increased (20–40-fold) levels of deetyrosinated α -tubulin (Glu-tubulin)

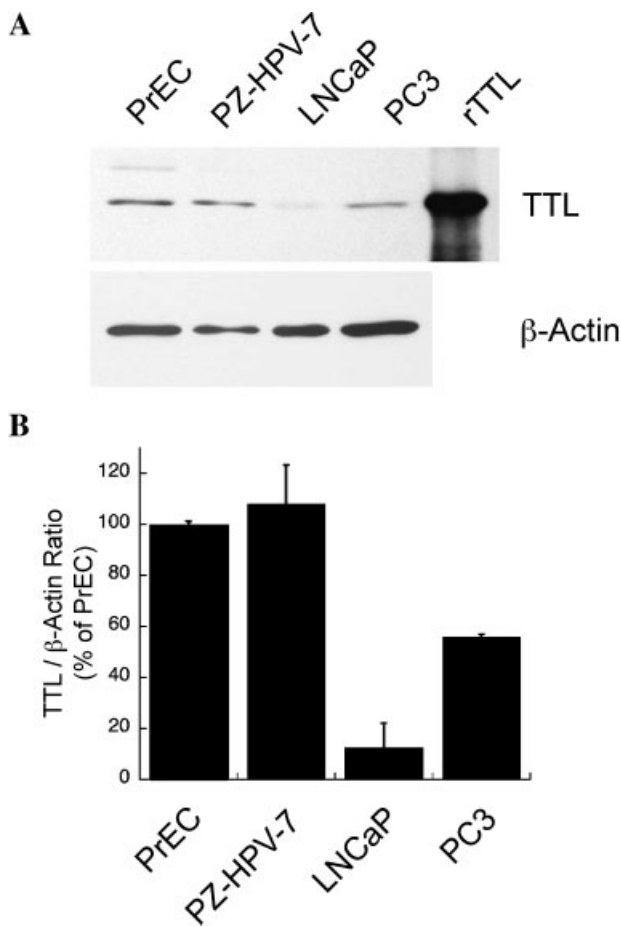


Fig. 6. Tubulin tyrosine ligase (TTL) protein level is decreased in prostate cancer cell lines. **A:** PrEC, PZ-HPV-7, LNCaP, and PC3 cell lysates were analyzed by Western blot for TTL protein expression. Recombinant TTL (rTTL) was used as a positive control of anti-TTL antibody specificity. Equal loading of cell lysates was verified using β -actin detection. **B:** Quantitation of TTL protein levels. Optical density per mm^2 was analyzed using ImageJ software and the calculated ratio to β -actin was related to expression in PrEC cells. Data are representative of at least three separate experiments.

compared to immortalized normal PZ-HPV-7 which like normal PrEC contain almost undetectable levels of Glu-tubulin (Fig. 2). Increased Glu-tubulin levels were paralleled by almost complete suppression of the TTL protein, consistent with our observations in the androgen-dependent parental LNCaP cell line and the androgen-independent AR-null PC3 cell line. Therefore, markedly increased levels of dephosphorylated α -tubulin (Glu-tubulin) and markedly decreased levels of TTL are common features of prostate cancer cell lines representing all the phases of prostate cancer. Having established that the androgen-independent AR-null cell line PC3 did not express acetylated α -tubulin (Ac-tubulin), we next examined the extent of α -tubulin acetylation in the androgen-independent LNCaP and

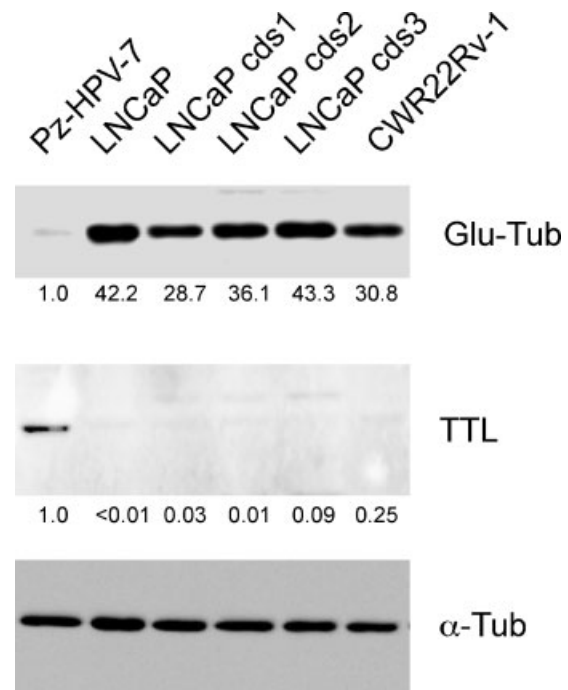


Fig. 7. Increased elaboration of dephosphorylated α -tubulin (Glu-tubulin) and decreased TTL protein expression is a common feature of “transitional” androgen-independent AR-positive prostate cancer cells. Cell lysates from androgen-independent LNCaP cell lines (cds1, cds2, and cds3) and cells derived from a relapsed tumor (CWR22Rv1) were probed by Western blotting for Glu-tubulin, TTL, and α -tubulin. Numbers below the blots indicate quantitative values relative to the immortalized normal human prostate epithelial cell PZ-HPV-7

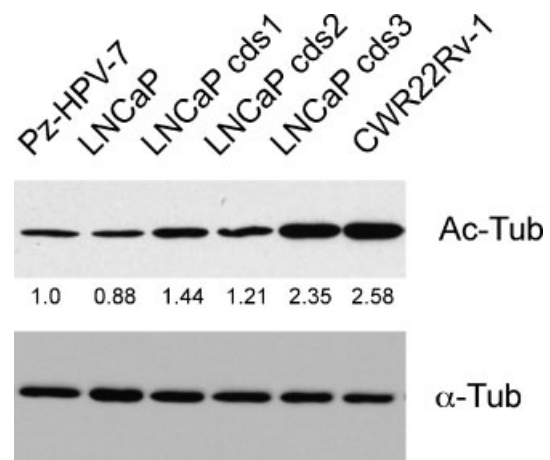


Fig. 8. Acetylated α -tubulin (Ac-tubulin) is marginally elevated in “transitional” androgen-independent AR-positive prostate cancer cells. Cell lysates from androgen-independent LNCaP cell lines (cds1, cds2, and cds3) and cells derived from a relapsed tumor (CWR22Rv1) were probed by Western blotting for Ac-tubulin and α -tubulin. Numbers below the blot indicates quantitative values relative to the immortalized normal human prostate epithelial cell PZ-HPV-7 normalized to α -tubulin levels. Data are representative of the average of three separate experiments.

CWR22Rv1 cell lines. In contrast to PC3 cells, both the LNCaP cds1-3 and CWR22Rv1 cell lines displayed detectable Ac-tubulin, and this was even marginally elevated in the LNCaP cds3 and CWR22Rv1 cells compared to immortalized normal PZ-HPV-7 (Fig. 8). Therefore, loss of α-tubulin acetylation appears to be confined to prostate cancer cells that are both androgen-independent and lack AR expression (PC3), suggesting that the function/expression of the molecular machinery regulating α-tubulin acetylation are dependent upon the AR in prostate cells.

DISCUSSION

Herein we have established a comprehensive molecular profile of α-tubulin posttranslational modifications in cultured PrEC representative of the phases of progression from a normal phenotype (PrEC and PZ-HPV-7), to androgen-dependent (LNCaP), to transitional AR-positive yet androgen-independent (LNCaP-cds and CWR22Rv1) and ultimately to androgen-receptor negative androgen-independent cells (PC3). We have utilized a combination of Western blotting and immunofluorescence microscopy with specific antibodies to establish these molecular signatures. Since cultured cells of human origin were examined, the characterization of α-tubulin posttranslational modifications (data summarized in Table I) may reveal important diagnostic “signatures” indicative of the initiation and progression through various stages of prostate cancer. We have established that normal human PrEC and immortalized human PZ-HPV-7 display qualitatively similar α-tubulin posttranslational modifications; that is, they express Tyr-tubulin, Δ2-tubulin, and Ac-tubulin, but little or no Glu-tubulin or polyglutamylated α-tubulin (PGlu-tubulin). A strikingly common feature of all prostate cancer cell lines examined (LNCaP, LNCaP cds1-3, CWR22Rv1, and

PC3) is the marked suppression of protein levels of TTL and concomitantly increased levels of detyrosinated α-tubulin (Glu-tubulin). Therefore, Glu-tubulin, Δ2-tubulin, and PGlu-tubulin are the posttranslational modifications of α-tubulin that most clearly distinguish normal PrEC from prostate cancer cells. Since androgen-independent, AR-null PC3 cells are the only prostate cell line examined that does not display Ac-tubulin, this could provide a molecular marker indicative of progression to complete androgen-independence and a marker and mediator of metastatic potential.

The distinctive profile of α-tubulin posttranslational modifications we observed illuminate potentially novel approaches and molecular strategies for diagnosing prostate cancer and evaluating its progression. Our findings also shed light on potential molecular mechanisms underlying prostate cancer and suggest novel molecular targets for treatment of this disease and other forms of cancer. In support of this notion, our observation that elevated levels of Glu-tubulin displayed by both androgen-dependent and androgen-independent prostate cancer cells (but not normal cells) is consistent with recent studies demonstrating that detyrosination of α-tubulin is associated with breast cancer and gliomas of poor prognosis [10,31]. Additionally, suppression of TTL protein expression paralleled by increased elaboration of Glu-tubulin has been proposed to be a common feature of several types of cancer cells [15–17]. Therefore, our findings strongly suggest that decreased expression of TTL protein and increased elaboration of Glu-tubulin is also a feature of prostate cancer. Despite these observations, the molecular mechanisms for suppression of TTL in prostate cancer and other cancers remains enigmatic and warrants further study. However, assessment of the promoter region of human TTL reveals the presence of two distinct CpG islands (unpublished observation)

TABLE I. Summary of the Levels of α-Tubulin Posttranslational Modifications in the Various Prostate Epithelial Cell Lines

Posttranslational modification	Androgen-dependent	Androgen-independent	Function
Detyrosination	↑↑↑	↑↑↑	Regulation of proliferation/differentiation switching
Δ2-Tubulin	↓↓↓	↓↓↓	Unknown; prevents re-tyrosination
Polyglutamylation	↑↑↑	↑↑↑	Interaction with MAPs/centriole stability
Acetylation	N.C.	↓↓↓ ^a /↑ ^b	Cell motility/migration
Polyglycylation	N.D.	N.D.	Cytokinesis (microtubule severing)

Changes in modifications are demonstrated by arrows with respect to normal and immortalized cells (PrEC, PZ-HPV-7), which were equivalent. ↑, increased level; ↓, decreased level; N.C., no change. The number of arrows indicate the degree of change.

^aPC3 cell line.

^bCWR22Rv1 and LNCaP cds1, LNCaP cds2, LNCaP cds3.

N.D., not detected.

that may be subject to hypermethylation and *TTL* gene silencing. Since hypermethylation of CpG islands is known to be important for silencing of critical genes in prostate cancer [32,33] and other cancers, this may provide a reasonable explanation for decreased *TTL* expression in cancer.

It was shown recently that tubulin polyglutamylase enzymes, responsible for polyglutamylation of α -tubulin, are members of the *TTL* domain protein family [34]. In our study we have detected high levels of polyglutamylated tubulin in androgen-dependent and androgen-independent cancer cell lines (LNCaP and PC3) and its expression correlates well with the presence of deetyrosinated α -tubulin. Therefore, future studies aimed at identifying the expression and functional activity of *TTL* and other enzymes of the *TTL* domain family (i.e., α -tubulin polyglutamylases) are warranted to clearly define their roles in prostate tumor progression and evaluate their utility as molecular targets for future therapeutic intervention.

We have demonstrated in cultured human PrEC that the level of $\Delta 2$ -tubulin, the form of α -tubulin lacking both C-terminal tyrosine and the penultimate glutamate residue is higher in normal and immortalized PrEC than in prostate cancer cell lines. This contrasts with previous studies of breast cancer tissues [16] and sarcomas produced by clonally selected 3T3 fibroblasts lacking *TTL* [17] and suggests that gender-specific tumor cells likely operate differently with regard to the expression of $\Delta 2$ -tubulin. It should also be noted that the proteolytic mechanism for generating $\Delta 2$ -tubulin is not currently known. In contrast to all other α -tubulin posttranslational modifications we examined in prostate cells, $\Delta 2$ -tubulin was found to be almost exclusively localized within the nucleus of PrEC and PZ-HPV-7 cells, as assessed by confocal microscopy. While the nuclear localization of tubulin is unexpected, it is not without precedent. Previous studies in various cancer cells have established that another member of the tubulin superfamily, β II-tubulin, is localized within the nucleus [35] where it has been reported to play a role in the regulation of cell signaling [36]. Although the functional significance of $\Delta 2$ -tubulin nuclear localization remains unknown (and has not been previously reported in the literature), our data suggest that $\Delta 2$ -tubulin may possess functions beyond those normally attributed to cytoplasmic microtubules, and perhaps related to cellular pathways controlling gene expression and cell-cycle progression [37,38].

Acetylation/deacetylation is typically perceived as a biomolecular pathway for regulation of gene expression by histone modification [32]. Histone acetyltransferases and histone deacetylases also regulate acetylation and deacetylation of non-histone targets [39]. Interestingly, the AR is a target of acetylation [40].

Acetylation can regulate AR function and, ultimately, proliferation of prostate cells. Deacetylation of other transcription factors such as Forkhead box protein O1A (FOXO-1) can play a role in androgen-independent reactivation of the AR in prostate cells, resulting in androgen-refractory cancer growth [41]. Another known non-histone target of protein deacetylases (HDAC6 and SIRT2) is α -tubulin. α -Tubulin acetylation occurs on the ϵ -amino group of a highly conserved lysine residue of α -tubulin. It has been shown that deacetylation of α -tubulin promotes chemotactic cell movement, supporting the notion that HDAC6-mediated deacetylation regulates microtubule-dependent cell motility [23,24]. Consistent with this notion, specific small molecule inhibitors of HDAC6 have been demonstrated to increase Ac-tubulin levels, which conferred decreased cellular motility [42]. Thus, our data demonstrating that α -tubulin acetylation occurs in all prostate cell types examined, with the exception of the androgen-independent and AR-null prostate cancer cell line PC3, is mechanistically intriguing and consistent with the known capacity of these types of prostate cancer cells to be highly motile and capable of aggressive metastatic potential [43]. It is tempting to speculate that the dramatic decrease in α -tubulin acetylation in the AR-null cells (PC3) may be a result of increased expression of the known α -tubulin deacetylases. It is noteworthy that both HDAC6 and SIRT2 contain CpG islands within their promoter regions [44,45], and that hypomethylation of these CpG islands may promote overexpression as has been previously demonstrated for urokinase-type plasminogen activator (uPA), cytochrome p450 1B1, and heparanase in prostate cancer [46–48]. Previous studies have demonstrated that HDAC6 mRNA expression in cultured PC3 cells is similar to that observed in LNCaP cells [49], so increases in HDAC6 expression are not likely responsible for the decreased α -tubulin acetylation we have observed in PC3 cells. However, the expression of SIRT2 has not been examined in prostate cancer cells, and this may provide important insights into the mechanisms underlying decreased α -tubulin acetylation in PC3 cells. The decrease in α -tubulin acetylation in PC3 cells may also result from a decrease in the expression and/or activity of α -tubulin acetyltransferase enzymes; however, at present the enzyme responsible for this process has not been identified. Moreover, the presence and co-localization of both indicators of stable microtubules, acetylated and deetyrosinated α -tubulin [30], were observed in androgen-dependent and androgen-independent LNCaP and CWR22Rv1 cells, but not in PC3 cells. This suggests that androgens (or a loss of the AR) may play a role in regulating α -tubulin acetylation/deacetylation, as well as microtubule stability, and that α -tubulin acetylation

and detyrosination may not always parallel each other as previously suggested.

CONCLUSIONS

Herein we report a comprehensive analysis of the α -tubulin posttranslational proteome in cultured human normal PrEC, immortalized normal PrEC, androgen-dependent prostate cancer cells and androgen-independent prostate cancer cells. Our studies revealed that these molecular signatures can distinguish normal cells from prostate cancer cells in culture, and that markedly increased polyglutamylation and detyrosination of α -tubulin that is paralleled by decreased expression of the TTL protein are common features of cultured prostate cancer cell lines. It is noteworthy also that decreased α -tubulin acetylation may provide a novel marker of the loss of AR expression. However, it remains to be demonstrated whether these paradigms prevail in human specimens. Despite this limitation, our observations illustrate a novel approach for assessing prostate cancer initiation and disease progression, and suggest a number of previously unrecognized molecular targets for therapeutic intervention in prostate cancer and perhaps other forms of cancer. The data presented also underscore the necessity to further characterize the identity and functions of the molecular machinery involved in catalyzing these diverse α -tubulin posttranslational modifications.

ACKNOWLEDGMENTS

The authors thank Dr. R. W. de Vere White for constructive comments on the manuscript and for providing androgen-independent LNCaP cell lines (LNCaP-cds), Dr. H.-J. Kung for providing the CWR22Rv1 cell line, Dr. M.-H. Bré for providing anti-polyglycylated tubulin antibodies, and Dr. J. Wehland for providing recombinant TTL. This work was supported by the California Cancer Research Program (J.P.E.), DOD grant #BC-024051 (J.C.B), the Paul F. Gulyassy Endowed Professorship (J.P.E.), and a post-doctoral fellowship from the Phillip Morris External Research Program (L.K.). We thank Dr. J.A. Last for helping to support A.K. through the UC Davis Fogarty International Training Program.

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