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Alterations of Histone H1 Phosphorylation During Bladder Carcinogenesis

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Abstract

There is a crucial need for development of prognostic and predictive biomarkers in human bladder carcinogenesis in order to personalize preventive and therapeutic strategies and improve outcomes. Epigenetic alterations, such as histone modifications, are implicated in the genetic dysregulation that is fundamental to carcinogenesis. Here we focus on profiling the histone modifications during the progression of bladder cancer. Histones were extracted from normal human bladder epithelial cells, an immortalized human bladder epithelial cell line (hTERT), and four human bladder cancer cell lines (RT4, J82, T24, and UMUC3) ranging from superficial low-grade to invasive high-grade cancers. Liquid Chromatography-Mass Spectrometry (LC-MS) profiling revealed a statistically significant increase in phosphorylation of H1 linker histones from normal human bladder epithelial cells to low-grade superficial to high-grade invasive bladder cancer cells. This finding was further validated by immunohistochemical staining of the normal epithelium and transitional cell cancer from human bladders. Cell cycle analysis of histone H1 phosphorylation by western blotting showed an increase of phosphorylation from G₀/G₁ phase to M phase, again supporting this as a proliferative marker. Changes in histone H1 phosphorylation status may further clarify epigenetic changes during bladder carcinogenesis and provide diagnostic and prognostic biomarkers or targets for future therapeutic interventions.

Keywords

Bladder Cancer; Histone H1; Phosphorylation; Mass Spectrometry

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INTRODUCTION

Bladder cancer is the fifth most common neoplasm in the United States and the second most prevalent cancer in men 60 years of age or older ^{1, 2}. Tobacco smoking is the most significant environmental risk factor for development of bladder cancer. For this reason, increased incidence of bladder cancer is a global concern due to worldwide expansion of tobacco use ^{2, 3}. Over 90% of bladder cancer cases are transitional cell carcinomas (urothelial carcinoma) with less than 10% being squamous cell carcinoma and adenocarcinoma ⁴. Of newly diagnosed urothelial carcinoma cases, approximately 75% are superficial non-invasive tumors and 25% are invasive tumors ⁵. These two clinical presentations of bladder urothelial cancers have two very distinct molecular pathways ⁵. The less aggressive, low-grade, non-invasive tumors often have alterations of the tumor suppressor p16 gene and Ras-MAPK signaling pathway, whereas the more aggressive, high-grade, invasive tumors exhibit alterations in the tumor suppressor p53 and retinoblastoma genes and their related signaling pathways ⁶. Non-invasive disease is usually successfully treated with transurethral resection of the tumor during cystoscopy, but has a 70% recurrence rate and a 10-20% chance of progression into a high-grade invasive disease. Invasive disease has a significantly worse prognosis with approximately a 50% risk of death regardless of treatment ⁷. Therefore, the proper management of bladder cancer mandates a very close surveillance of patients with frequent office visits, cystoscopies, urine cytology, urine biomarkers and radiographic imaging for identifying bladder cancer recurrence and/or progression in a timely manner. This lifelong monitoring results in a significant health care burden, adding billions to annual costs in the United States, which makes the care of bladder cancer the most expensive among all malignancies ^{2, 8}.

There are few commercially available urine marker assays that are used in conjunction with urine cytology and cystoscopy for bladder cancer surveillance, but there remains a clear need for more sensitive and specific biomarkers to improve the early detection of recurrent disease and identify those at risk of progression ^{4, 9}. Nucleosomal histones are basic proteins found in eukaryotic nuclei that package DNA into chromatin and have been reported as potential cancer biomarkers. Core histones (H2A, H2B, H3 and H4) form the histone octamer around which 147 base pairs of DNA are wrapped. The linker histone (H1) binds in the region where the DNA enters and exits the octamer. Core histone modifications are reported as having cancer biomarker potential in the bladder ^{10, 11} as well as prostate ¹², lung ¹³, kidney ¹⁴, breast ¹⁵ and pancreas ¹⁶. Linker histone phosphorylation has also been shown to be one of the early alterations in the progression of head and neck squamous cell carcinomas ¹⁷ and a potential cellular proliferation biomarker in head and neck ¹⁸⁻²⁰ and cervical ²¹ cancers.

The characterization of H1 modifications is complicated by the larger number of H1 variants and in some cases their high sequence homology. The human histone H1 variants H1.0, H1.1, H1.2, H1.3, H1.4, H1.5 and H1.X are expressed in somatic cells. Four additional histone H1 variants are expressed solely in germ line cells ²². H1.2, H1.3, H1.4 and H1.5 are expressed in all somatic cells. H1.1 is expressed in thymus, testis, spleen, lymphocytic, and neuronal cells. H1.0 is expressed in terminally differentiated and non-proliferating cells ²³. H1.X has been detected in a variety of human tissues. H1.X only has ~30% sequence similarity to the other H1 variants and is therefore distinct. H1.X has been reported as essential to mitotic progression ²⁴ and it appears that linker histones are essential for mammalian development ²⁵. It has been suggested that H1 variants have different functions yet these differences are still under investigation ²⁶.

Phosphorylation is the most predominant modification of H1²⁷ and can occur at the N- and C-terminus²⁸. H1 phosphorylation has been linked to important cellular processes such as DNA replication and cell cycling²², transcription by RNA polymerases I and II²⁹, and p53-dependent DNA damage response pathways³⁰. Paradoxically, H1 phosphorylation has been associated with both chromatin decondensation and chromatin condensation²².

Phosphorylation related to cell cycling occurs on cyclin dependent kinase (CDK) phosphorylation sites. Both Cdc2 and CDK2 have been linked to H1 phosphorylation. Techniques to specifically identify CDK-dependent linker histone phosphorylation have been reported and are suggested to have potential for cancer diagnosis and prognosis¹⁹. Protein phosphorylation is often aberrant in cancer states due to the dysregulation of cellular signaling pathways³¹, and its alterations can be used as a pharmacodynamic marker in patients treated with targeted kinase inhibitors³².

In this paper, the H1 phosphorylation status in bladder cancer and its potential as a biomarker of disease progression were investigated. Liquid Chromatography Mass Spectrometry (LC-MS) was used to profile histones in normal bladder urothelial cells and bladder cancer cell lines, representing the spectrum of disease, to determine the relationship between H1 phosphorylation and bladder cancer carcinogenesis. The experiments revealed that increased global phosphorylation of histone H1 is associated with progressive bladder carcinogenesis and its invasiveness. The H1 phosphorylation patterns were confirmed by western blotting with a phospho-H1 antibody (p-T146). Immunoblotting and immunohistochemistry with the same antibody showed that staining was highest in mitotic cells. Immunohistochemistry analysis of human bladder tissues revealed a significantly increased expression of histone H1 p-T146 in normal vs. cancer tissues, and this expression correlated with grade and invasiveness. These novel data demonstrate that histone H1 phosphorylation correlates with bladder cancer development and progression and thus this marker may have potential diagnostic, predictive and prognostic clinical implications, and a future role as a therapeutic target.

MATERIALS AND METHODS

Cell Growth

Human non-invasive low-grade (RT4), and human invasive high-grade (T24, J82, and UMUC3) bladder cancer cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Immortalized (hTERT) and normal human bladder epithelial cells were obtained from Dr. Margareta Knowles (St James's University Hospital, Leeds, UK) and Lonza Walkersville, Inc (Walkersville, MD), respectively. This collection of bladder cells represents the spectrum of bladder cancer carcinogenesis. RT4, T24, J82 and UMUC3 were maintained as monolayer cultures in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, and penicillin (10 U/ml) and streptomycin (10 mg/ml) (GIBCO, Grand Island, NY) at 37 °C in a 5% CO₂/95% air, humidified atmosphere. hTERT and normal human bladder epithelial cells were grown in Keratinocyte Serum Free Medium (KSFM; GIBCO, Grand Island, NY) with supplements of bovine pituitary extract and epidermal growth factor plus 30 ng/ml Cholera toxin (Sigma Chemical Company, St. Louis, MO). Seeding densities for different experiments were according to growth kinetics determined for each cell line in previous experiments (data not shown).

Histone Extraction

Cells were seeded, grown for 24-48 h (based on our previous experiences and the growth kinetics of each cell line), harvested by scraping and then snap frozen. A single sample of normal human bladder epithelium and at least three biological replicates of the four human

bladder cancer cell lines (RT4, J82, T24, and UMUC3) and the immortalized human bladder epithelial cell line (hTERT) were cultured for experiments. Due to limited availability of normal human bladder epithelial cells and in order to have similar amount of protein for each replicate, we had to use all the extracted histones from normal human bladder epithelial cells for one replicate. Histones were extracted as described previously³². Briefly, cell pellets were resuspended in 1 mL of NP-40 lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 0.15 mM spermine, 0.5 mM spermidine, 1 mM PMSF, protease inhibitor cocktail (1:1000)] and incubated on ice for 5 min. The nuclei were pelleted at $483 \times g$ for 15 min at 4 °C and the pellet washed with 1 mL TBS [10 mM Tris-HCl (pH 7.4), 150 mM NaCl]. Sulfuric acid (H₂SO₄) (0.2 M) was added to the washed pellet to extract the histones and it was vortexed and incubated on ice for 30 min. The solution was centrifuged at $12,045 \times g$ for 15 min at 4 °C to remove the cellular debris. Eighty percent acetone was added to the supernatant and precipitated at -20 °C overnight. The precipitated histones were centrifuged at $12,045 \times g$ for 15 min at 4 °C, allowed to air-dry for 10 min and resuspended in HPLC water.

LC-MS

Protein concentration was determined by Bradford analysis. Thirty micrograms of purified histones were characterized by LC-MS analysis. LC-MS analysis was performed with a Dionex U3000 HPLC (Dionex; Sunnyvale, CA) coupled to a MicroMass Q-ToF (MicroMass, Whythenshawe, UK). Reversed-phase separation was carried out on a Discovery Bio Wide Pore C18 column (1.0 mm $\times$ 150 mm, 5 μ m, 300 Å; Supelco, USA). Mobile phases A and B consisted of water and acetonitrile with 0.05% trifluoroacetic acid, respectively. The flow rate was 25 μ L/min and the gradient started at 20% B, increased linearly to 30% B in 2 min, to 35% B in 8 min, 50% B in 20 min, 60% B in 5 min and 95% B in 1 min. After washing at 95% B for 4 min, the column was equilibrated at 20% B for 30 min and a blank was run between each sample injection. The cone voltage on the Q-ToF was 25 V. LC-MS data was deconvoluted using MassLynx 4.1.

LC-MS Data Analysis

The raw LC-MS data was summed across each eluting peak in the chromatogram and the resulting summed mass spectrum was deconvoluted to produce a zero-charge mass spectrum. The LC-MS experiment was replicated a minimum of three times from different cell cultures. To determine the extent of phosphorylation, the relative abundance for all the phosphorylated isoforms were summed and divided by the relative abundance of the unphosphorylated isoform. Significance testing was performed using SigmaPlot 12 (Systat Software, Inc., San Jose, CA). A one way ANOVA was performed to determine differences in phosphorylation between the cell lines followed by the Holm-Sidak all pairwise multiple comparisons procedure ($\alpha=0.05$).

Western Blotting

Cells were seeded in culture dishes and grown for 24 h. Adherent and nonadherent cells were harvested by scraping and the total proteins were extracted in lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with 1mM PMSF and 1 $\times$ Halt protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL). Protein concentrations were determined by BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Protein samples were then electrophoresed, transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA), incubated with appropriate primary and secondary antibodies, and detected by chemi-luminescence (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibody against Histone H1 (phospho T146) was obtained from Abcam, Inc. (Cambridge, MA) and GAPDH antibody was obtained from Cell Signaling Technology Inc.

(Beverly, MA). Anti-rabbit secondary antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell Cycle Synchronization

At 25-30% confluency, UMUC3 bladder cancer cells were washed twice with 1× PBS and then incubated with RPMI 1640 + 2 mM thymidine (Sigma-Aldrich, St. Louis, MO) for 18 hours. Thymidine was then removed by thoroughly washing with 1× PBS and fresh RPMI 1640 media was added to allow cells to release for 9 hours. Subsequently, RPMI 1640 + 2 mM thymidine was added for a second thymidine block and kept for 17 hours. After the second block (synchronization in the G1 phase), thymidine was removed by thoroughly washing with 1× PBS and released by adding fresh RPMI 1640. Cells were collected by trypsinization at time zero and 2, 4, 7, 8, 9, 10, 12 and 24 h after thymidine removal to collect cells in each subsequent phase of the cell cycle. To obtain cells in the M-phase of the cell cycle, after the first thymidine block, thymidine was washed and cells allowed to release for 3 h. One hundred ng/mL nocodazole was then added in RPMI 1640 media for 12 hours and cells were washed in 1× PBS and collected by trypsinization. Samples were prepared in duplicate for paired cell cycle analysis by flow cytometry and western blot analysis.

Cell Cycle Analysis

After trypsinization, cells were washed in cold 1× PBS and fixed in ice cold 70% ethanol. Samples were stained with 50 µg/mL propidium iodide in PBS containing 0.1% Triton-X and 0.2 mg/mL RNase A for 30 minutes at room temperature. Flow cytometry was performed by a FACSCalibur (BD Biosciences, Franklin Lakes, NJ) instrument and cell cycle modeling analysis by ModFit (Verity Software House, Topsham, ME). Protein lysates were obtained from cells and western blots were run as described above, looking at Histone H1 (phospho T146) (Abcam, Cambridge, MA).

Immunohistochemistry

All human bladder tissue/tumor samples were selected after careful review by our expert genitourinary pathologist (IRB-approved protocol # 2010E0365; PI: Amir Mortazavi, MD). To represent the spectrum of bladder cancer carcinogenesis, non-invasive low-grade, non-invasive high-grade, and invasive high-grade bladder cancer tissues, as well as non-cancerous, normal appearing bladder urothelial tissues were selected (n = 8 cases for each group). Immunohistochemical analysis was conducted on formalin-fixed paraffin embedded tissue sections (4.5 micron). After rehydration, antigen retrieval was performed by boiling the slides in citrate buffer (Antigen Retrieval Citra Plus, BioGenex, San Ramon, CA) for 30-35 minutes. Endogenous peroxidase was inhibited by incubation in peroxide blocking solution (Dako Cytomation, Carpinteria, CA) for 15 min followed by 60 min incubation with either Histone H1 phospho (T146) antibody (0.1 µg/mL, rabbit polyclonal antibody, Abcam, Cambridge, MA) or Ki67 antibody (1:50, mouse monoclonal [MIB 1], Dako Cytomation, Carpinteria, CA). After three washes with wash buffer (Super Sensitive Wash Buffer, Biogenex, Fremont, CA), sections were incubated for 30 minutes with a matched labeled polymer-HRP (Dako Cytomation, Carpinteria, CA). Color was developed by 10 min incubation with DAB chromogen solution (Dako Cytomation, Carpinteria, CA). Slides were counterstained with Mayers hematoxylin (Dako Cytomation, Carpinteria, CA) for 2 min and mounted. Negative controls include omission of primary antibody. Images were captured from each tissue section at 200X or 400X magnification using bright field microscopy (Nikon ECLIPSE E 800, Tokyo, Japan) by a digital camera (Spot RT, Diagnostic Instrument, Inc., Sterling Heights, MI).

Immunohistochemistry Data Analysis

Images representative of non-invasive low-grade, non-invasive high-grade, and invasive high-grade bladder cancers; and non-cancerous, normal appearing bladder urothelium (n = 8 cases for each grade) were captured at 400X magnification from hematoxylin and eosin (H&E), H1 p-T146, and Ki67 stained tissue sections. The manual tag feature of the Image-Pro Plus 7.0 (Media Cybernetics, Inc., Bethesda, MD) image analysis software was used to quantify labeled (DAB; brown) nuclei and counterstained (hematoxylin; blue) nuclei among urothelial cells. The percentage of stained nuclei over total nuclei was calculated for one image per case: Percent positive (%) = $L/(L + C) \times 100$, where L = labeled nuclei and C = counterstained, unlabeled nuclei. Significance testing was performed using SigmaPlot 12 (Systat Software, Inc., San Jose, CA). A one way ANOVA was performed to determine differences in percentage of staining followed by the Holm-Sidak all pairwise multiple comparisons procedure ($\alpha=0.05$). Data are presented as the mean $\pm$ SEM. Cases with greater than 10% staining were categorized as positive. A Fisher Exact test was performed to characterize the proportion of positive staining between non-cancer and cancer and between high-grade cancer and non-high-grade (non-cancer plus low-grade cancer).

RESULTS

Phosphorylation of histone H1 changes through bladder cancer carcinogenesis

LC-MS profiling was performed to determine differences in post-translational modifications (PTMs) of histones in different cell lines, representing the spectrum of bladder urothelial carcinogenesis. These model cell lines included normal human urothelial cells, an immortalized human bladder cell line (hTERT), a non-invasive low-grade human bladder cancer cell line (RT4), and three invasive high-grade human bladder cancer cell lines (J82, T24 and UMUC3). The LC-MS profiles revealed distinct differences in the level of histone H1 phosphorylation between the normal, non-invasive and invasive cancer cells. We inspected the spectra of the core histones for differences that would indicate differential PTMs between the samples, but found none except for the J82 cell line. We did not look for differences in specific PTMs. Representative mass spectra of the core histones for one replicate can be found in Supplementary Figures 1-6. Figure 1 shows the LC-MS Total Ion Chromatograms (TICs) for the normal urothelium, immortalized and the bladder cancer cell lines. Histone H1 isoforms elute earlier than the core histones due to their lower hydrophobicity. Histone isoform H1.5 eluted first followed by isoforms H1.2, H1.3 and H1.4. Representative mass spectra from one replicate are shown in Figures 2-3 and all remaining replicates are provided in Supplementary Figures 7-15.

The deconvoluted mass spectra of histone H1 variant H1.5 is shown in Figure 2. The peak at 22,493 Da was identified as the N-terminally acetylated isoform of histone H1 variant H1.5. The peaks at 22,572, 22,652, and 22,731 Da are the N-terminally acetylated/mono-, di- and tri-phosphorylated isoforms of the histone H1 variant H1.5³³. The deconvoluted mass spectra of the histone H1 variants H1.2, H1.3 and H1.4 are shown in Figure 3. The peaks at 21,276, 21,777 and 22,262 Da were identified as the N-terminally acetylated isoforms of histone H1 variants H1.2, H1.4 and H1.3, respectively³³. The peak at 21,356 is the N-terminally acetylated/mono-phosphorylated isoform of the histone H1 variant H1.2. The peaks at 21,857, 21,936 and 22,015 are the N-terminally acetylated/mono-, di- and tri-phosphorylated isoforms of the histone H1 variant H1.4, respectively. The peak at 22,340 is the N-terminally acetylated/mono-phosphorylated isoform of histone H1 variant H1.3.

The mean ratio $\pm$ standard error for the summed peak heights of the phosphorylated isoforms relative to the unphosphorylated isoform for each cell line and H1 isoform was calculated (Figure 4). For histone variant H1.5, a statistically significant difference ($p<0.05$)

was observed between the invasive human bladder cancer cell lines and both the immortalized normal human bladder epithelium (hTERT) and the non-invasive human bladder cancer cell line (RT4). The J82 cell did not have a detectable amount of H1.5 and thus is not shown in Figure 4. A similar significant difference was observed for histone variants H1.2 and H1.4. In addition, for these variants, the invasive human bladder cancer cell lines T24 and UMUC3 demonstrated statistically significant differences ($p < 0.05$) relative to the invasive human bladder cancer cell line J82. Furthermore, J82 demonstrated a significant difference ($p < 0.05$) relative to the immortalized normal human bladder epithelium (hTERT) and the non-invasive human bladder cancer cell line (RT4) for the histone variant H1.4. For the histone variants H1.5, H1.2, and H1.4, there were no significant differences between the immortalized normal human bladder epithelium (hTERT) and the non-invasive human bladder cancer cell line (RT4) or between the invasive cell lines T24 and UMUC3. For histone variant H1.3, there were not significant differences observed between any of the cell lines.

The sites of phosphorylation of H1 histones in various human cell lines have been extensively characterized previously^{23, 29, 32, 34-36}. There is some variability in the level and sites of phosphorylation depending on the cell line, however, in all publications that looked at mitotic or asynchronous cells, mitotic-specific CDK-dependent phosphorylation on T146 (T147 for H1.3) was reported for histones H1.2-H1.4. We therefore used a commercially available immunohistochemistry-grade p-T146 antibody to confirm by western blotting the increased H1 phosphorylation observed by mass spectrometry. We have compared the non-invasive low grade bladder cancer cell line (RT4) with invasive high-grade bladder cancer cell lines (J82, T24, and UMUC3) and found an increase of H1 p-T146 from low-grade to high-grade (Figure 5, and Supplementary Figure 16). The relative level of H1 p-T146 in invasive high grade bladder cancer cells (J82, T24, and UMUC3) as compared to the RT4 non-invasive low-grade bladder cancer cell line was 50% higher.

Phosphorylation of p-T146 is cell cycle dependent

CDK-dependent phosphorylation has been reported as a possible marker for proliferation and neoplasia¹⁹. In addition, Sarg *et al.* reported that the p-T146 antibody stained HeLa cells undergoing mitosis³⁶. Therefore, the cell cycle dependence of T146 phosphorylation in bladder cancer was examined by western blotting synchronized UMUC3 cells against the p-T146 antibody (Figure 6). Cells were in G₀/G₁, early S, late S, early G₂/M, and late G₂/M phases at 0, 2, 4, 7 and 9 hours after release, respectively. Cells were blocked in mitosis with nocodazole treatment. Western blot analysis revealed that H1 phosphorylation increased with time as more of the cellular population progressed to M phase. Maximum phosphorylation was observed with the sample blocked during M, as expected for a CDK-dependent site²⁸. The staining in S phase is likely due to a small proportion of the cells already cycling to M. The cell cycle dependence of p-T146 can be seen by immunohistochemical staining of the formalin fixed, paraffin embedded cell block of unsynchronized UMUC3 cells (Supplementary Figure 17).

H1 p-T146 is a potential biomarker of human bladder cancer progression

As the high grade invasive bladder cell lines demonstrate increased phosphorylation compared to non-invasive low-grade bladder cancer and transformed normal bladder lines, immunohistochemical analysis for p-T146 and Ki67, a well characterized biomarker of proliferation³⁷, was conducted on human non-cancerous normal appearing bladder urothelium, non-invasive low-grade, non-invasive high-grade, and invasive high-grade bladder cancer tissues ($n = 8$ for each tissue type) (Figure 7). The percentage of positively stained nuclei was quantified in representative images for each case. ANOVA analysis demonstrated significant differences in percentage of p-T146 staining between grades

($p < 0.001$). Pairwise comparisons indicate that invasive high-grade (21.5% $\pm$ 2.9%) and non-invasive high grade (16.8% $\pm$ 2.3%) were significantly greater than non-cancer (1.2% $\pm$ 0.7%) ($p = 0.001$) and that invasive high-grade was significantly greater than non-invasive low-grade cancer (8.4% $\pm$ 2.9%) ($p = 0.002$). Although there was a trend in higher nuclear p-T146 staining for non-invasive high-grade as compared to non-invasive low-grade, this did not reach statistical significance ($p = 0.073$). The difference in percentage of positive nuclear staining with grade is strongly correlated with traditional markers of proliferation, including Ki67 ($p < 0.001$). Invasive high-grade (36.8% $\pm$ 6.6%) and non-invasive high-grade (48.2% $\pm$ 9.3%) was greater than non-cancer (7.9% $\pm$ 5.0%) ($p < 0.05$) and the non-invasive high-grade was greater than non-invasive low-grade (17.3% $\pm$ 5.0%) ($p = 0.01$).

Classification of cases as positive or negative based upon a 10% threshold of positive staining within the representative images as described above was used to investigate the potential use of p-T146 staining as a biomarker of aggressiveness. Of the cases with normal appearing epithelium of bladder, none demonstrated greater than 10% staining; whereas 18% of the non-invasive low-grade, 78% of the non-invasive high-grade and 82% of the invasive high-grade cases of bladder cancer could be classified as positive. The proportion of positive cases ($> 10\%$ positive staining) was significantly greater in those with cancer compared to non-cancer (58% vs. 0%; Fisher Exact Test; $p = 0.002$). More striking was the increased likelihood of positive staining in cases with high-grade cancers (non-invasive and invasive) compared to low-grade and non-cancerous tissue (80% vs. 10%; Fisher Exact Test; $p < 0.001$). The incidence of greater than 10% positive staining for Ki67 in the matched cases was assessed. Of the tissues with no cancer, 13% had greater than 10% positive staining. There was an increasing incidence of positive staining with aggressiveness of the cancer as 45% of the non-invasive low-grade, 100% of the non-invasive high-grade and 100% of the invasive high-grade cancers demonstrated greater than 10% staining. Similar to p-T146, there were significant differences between non-cancer and cancer (13% vs. 80%; Fisher Exact Test; $p < 0.001$) and between high-grade cancers and low-grade cancer and non-cancerous tissues (100% vs. 32%; Fisher Exact Test; $p < 0.001$). Similar trends and significant differences were observed when the threshold of stain was set for 5% positive (data not shown).

DISCUSSION

We have identified an increase in phosphorylation of histone H1 when analyzing global histone modifications by LC-MS in cultured human bladder cancer cells representing the spectrum of this disease, ranging from normal urothelial cells to low-grade non-invasive disease and then high-grade invasive bladder cancer. The above LC-MS data shows compelling differences in the H1 profiles that would otherwise not be observed using comparatively insensitive immunological reagents. Current H1 antibodies are known to cross-react with multiple H1 variants/isoforms or have broad specificity. The LC-MS analysis clearly demonstrates distinct differences in phosphorylation of the histone H1 variants H1.5, H1.2 and H1.4 between the normal bladder epithelium, low-grade non-invasive and high-grade invasive bladder cancer cell lines, while histone variant H1.3 displayed no differences.

Interestingly, the histone profiles of the immortalized normal human bladder epithelial cells (hTERT) were more similar to the RT4 bladder cancer cell line than normal human bladder epithelial cells, which correlates with the observed high proliferative rates for both lines in our other *in vitro* experiments.

The striking differences in H1 phosphorylation of variants H1.5, H1.2 and H1.4 between superficial (non-invasive) and invasive cell lines may be useful in bladder cancer screening, and/or predictive biomarkers of recurrence, invasiveness, progression and response to treatment. Of course, all these potential implications of these findings require future confirmatory large-scale studies.

During the cell cycle of invasive UMUC3 bladder cancer cells, H1 phosphorylation gradually increases from G₁ to M transition, with the most significant increase occurring during G₂/M stage and the maximum phosphorylation being observed during M²⁸. Initial H1 phosphorylation during S phase may weaken its binding to chromatin fibers because the negative charge decreases its interaction with the DNA²². This decreased interaction between H1 and DNA would result in chromatin decondensation and facilitate DNA synthesis. However, as H1 becomes hyperphosphorylated, a conformational change occurs, leading to aggregation of H1 globular domains. This aggregation is proposed to be linked to chromatin condensation and is important in mitosis^{22, 28}.

There is no current standard screening for bladder cancer in high risk populations, such as smokers. In addition, currently available predictive biomarkers of bladder cancer recurrence and progression lack adequate sensitivity or specificity for wide application, resulting in undiagnosed progressive disease, and thus surgical interventions (such as radical cystectomy), chemotherapy and a significantly worse overall survival^{4, 7}. There remains a significant need for the development of diagnostic, predictive and prognostic biomarkers for bladder cancer. Evidence supports the role of epigenetic modifications in bladder cancer carcinogenesis and monitoring these epigenetic changes (i.e. DNA methylation and histone modifications) as a plausible target for biomarker development^{38, 39}. Interestingly, Burstein, et al. demonstrated that the linker histone phosphorylation is one of the early alterations in the progression of head and neck squamous cell carcinomas¹⁷. While it is not known if histone phosphorylation drives malignant behavior of bladder cancer cells or if it is only a proliferation marker, it may serve as a clinically relevant biomarker for bladder cancer progression from low-grade to aggressive high-grade tumors. The differential expression of CDK-dependent H1 phosphorylation in invasive vs. *in situ* carcinomas of the head and neck was suggestive of the use of this marker for prognostication of this cancer¹⁹. These data support the hypothesis that cell-cycle related phosphorylation (CDK-dependent) has the potential to be a powerful marker for assessment of the degree of cellular proliferation. In our experiment, we showed that the percentage of positive H1 p-T146 staining in human bladder cancer cases correlates with the increasing histopathologic grade and invasiveness of bladder cancer as well as the proliferation rate, evident by Ki67 staining. Ki67 overexpression has previously been found to add prognostic information for prediction of clinical outcome after radical cystectomy in bladder cancer⁴⁰. These data support the assertion that H1 phosphorylation has the potential utility as a diagnostic, predictive and prognostic biomarker in bladder cancer patients and warrants further investigation and validation. Confirmation of the sites of H1 phosphorylation in these cell lines and development of IHC-grade immunological reagents targeting these sites also warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	analysis of variance
BCA	bicinchoninic acid
CDK	cyclin-dependent kinase
DAB	3,3' Diaminobenzidine
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
H&E	hematoxylin and eosin
KFSM	keratinocyte serum free medium
LC-MS	liquid chromatography-mass spectrometry
PTM	post-translational modification
Ras-MAPK	RAS-mitogen activated protein kinase
RNA	ribonucleic acid
TIC	total ion chromatogram
Tris	trisaminomethane

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Figure 1. LC-MS Total Ion Chromatograms (TIC) of histones from normal human bladder epithelial cells (Normal), immortalized human bladder epithelial cells (hTERT), non-invasive low-grade (RT4), and invasive high-grade human bladder cancer cells (J82, T24, and UMUC3).

Figure 2.

Deconvoluted mass spectra of histone H1 variant H1.5 from normal human bladder epithelial cells (Normal), immortalized human bladder epithelial cells (hTERT), non-invasive low-grade (RT4), and invasive high grade human bladder cancer cells (T24 and UMUC3). The peak at 22,493 Da is the N-terminally acetylated isoform of histone H1 variant H1.5. The peaks at 22,572, 22,652, and 22,731 Da are the N-terminally acetylated/mono-, di- and tri-phosphorylated isoforms of the histone H1 variant H1.5.

Figure 3.

Deconvoluted mass spectra of histone H1 variants H1.2, H1.3 and H1.4 from normal human bladder epithelial cells (Normal), immortalized human bladder epithelial cells (hTERT), non-invasive low-grade (RT4), and invasive high-grade human bladder cancer cells (J82, T24, and UMUC3). The peaks at 21,276, 21,777 and 22,262 Da are the N-terminally acetylated isoforms of histone H1 variants H1.2, H1.4 and H1.3, respectively. The peak at 21,356 is the N-terminally acetylated/mono-phosphorylated isoform of the histone H1 variant H1.2. The peaks at 21,857, 21,936 and 22,015 are the N-terminally acetylated/mono-, di- and tri-phosphorylated isoforms of the histone H1 variant H1.4, respectively. The peak at 22,340 is the N-terminally acetylated/mono-phosphorylated isoform of histone H1 variant H1.3.

Figure 4.

The ratio of the summed peak heights for the phosphorylated to the unphosphorylated isoforms for histone H1 variants H1.5, H1.2, H1.4 and H1.3 from normal human bladder epithelial cells (Normal), immortalized human bladder epithelial cells (hTERT), non-invasive low-grade (RT4), and invasive high-grade human bladder cancer cells (J82, T24, and UMUC3). A single replicate on the Normal cells was used due to limited availability of normal human bladder urothelial cells. All pairwise multiple comparisons (Holm-Sidak method) were performed to determine significant differences in phosphorylation between cell lines. Statistically significant differences are indicated (* = $p < 0.05$, compared to hTERT; # = $p < 0.05$, compared to RT4; \$ = $p < 0.05$, compared to J82). Error bars represent standard error ($N = 3$ for each cell line).

Figure 5. Western blot analysis of the expression of phospho-histone H1 (p-T146) in non-invasive low-grade (RT4) and invasive high-grade (J82, T24, and UMUC3) bladder cancer cell lines. This immunoblot is representative of three independent experiments.

Figure 6.

Cell cycle dependence of p-T146 in the invasive human bladder cancer cell line UMUC3. Cells were synchronized by double thymidine block and then released. For each time point, two plates of cells were grown. One plate was used for cell cycle analysis (A.) and the other for western blot analysis (B.). The grey lines represent asynchronous cells. The black lines represent synchronous cells. Cells were in G₀/G₁, early S, late S, early G₂/M, and late G₂/M phases at 0, 2, 4, 7 and 9 hours after release, respectively. Cells were blocked in metaphase with nocodazole.

Figure 7.

Tissues ranged from non-cancerous normal appearing bladder urothelium, to non-invasive low-grade, non-invasive high-grade, and invasive high-grade human bladder cancers were used. $N = 8$ for each tissue type. (A.) Hematoxylin and eosin staining (H&E) and immunohistochemical staining of human bladder tissues with H1 p-T146 and Ki67 antibodies. (B.) Quantification of the immunohistochemical staining of human tissues with H1 p-T146 and Ki67 antibodies. The percentage of nuclei staining positive. Error bars represent standard error. All pairwise multiple comparisons (Holm-Sidak method) were performed to determine significant differences in staining between tissue types. Statistically significant differences are indicated (* = $p < 0.05$, compared to non-cancerous bladder urothelium; # = $p < 0.05$, compared to non-invasive low-grade bladder cancer). (C.) The percentage of cases with >10% positive. Fisher Exact Tests were performed to determine significant differences in staining for cancer vs. non-cancer samples and non-cancer + low-grade cancer samples vs. high-grade cancer. Significant differences were found for both cases with both antibodies. For H1 p-T146, $p = 0.002$ for cancer vs. non-cancer and $p < 0.001$ for high-grade cancers (non-invasive and invasive) vs. low-grade and non-cancerous tissues. For Ki67, $p < 0.001$ for cancer vs. non-cancer; and $p < 0.001$ for high-grade cancers (non-invasive and invasive) vs. low-grade and non-cancerous tissues.