Original Article

Predictive value of pseudouridine in prostate cancer

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Abstract: Background: Recent studies have shown that certain small nucleolar RNAs (H/ACA snoRNAs) and the protein dyskerin (DKC1) are upregulated in prostate cancer and are thought to contribute to progression of disease. These components convert uridine to pseudouridine (abbreviated ψ), a type of post-transcriptional modification of RNA. Given the increased abundance of H/ACA snoRNAs and expression of DKC1 in prostate carcinomas, and because whole-body turnover of RNA increases in support of rapidly-growing cancer cells, we examined the value of pseudouridine as a biomarker for prostate cancer. Methods: Using a monoclonal antibody against pseudouridine, we tested its ability to distinguish between two 25-base RNA oligonucleotide sequences that differed by only one ψ-substitution, and subsequently measured ψ in RNA isolated from several prostate cancer cell lines representing different stages of disease using dot blot assays and pseudouridinylated RNA linked immunosorbent assay (PURLISA). We also performed immunohistochemistry on a tissue micro array (12 cases/24 cores) containing prostate adenocarcinomas and normal adjacent tissue (NAT). Results: High levels of pseudouridine were detected in androgen-independent cell lines (PC3 and Du145) compared to androgen-sensitive (LNCaP) and immortalized human prostate (RWPE) cells. Immunohistochemistry of a tissue micro array (TMA) containing normal adjacent and cancerous prostate tissue revealed a significant difference in immunoreactivity between normal and malignant tissue (P ≤ 0.0001). Conclusion: Our results provide new information on the relationship between pseudouridine expression and clinical progression of prostate cancer.

Keywords: Biomarker, epitranscriptomics, ELISA, H/ACA snoRNA, prostate tumors, tissue micro array

Introduction

Prostate cancer (PCa) is a leading public health concern in developed Western countries where an estimated 11.2% of men will be diagnosed with PCa in their lifetime [1]. Serum prostate-specific antigen (PSA) tests have been routinely used to screen for PCa, yet these measures fail to detect PCa in as many as 15% of cases [2]. Thus, there is a major clinically unmet need for accurate, novel biomarkers to detect PCa and prevent unnecessary treatment of indolent disease.

Recent next-generation sequencing (NGS) technologies have hastened the discovery of novel, RNA-based biomarkers that appear to hold promising diagnostic and/or prognostic properties. A study by Martens-Uzunova et al. [3] examined the small non-coding transcriptome of organ-confined and metastatic lymph node PCa and found that fragments of H/ACA box small nucleolar RNAs (snoRNAs) in metastatic tumors nearly doubled compared to those from organ-confined tumors. These H/ACA snoRNAs guide small nucleolar ribonucleoprotein (sno-RNP) complexes that convert specific uridine bases to the isomer pseudouridine (abbreviated ψ), a type of post-transcriptional modification [4].

The existence of post-transcriptional modifications of RNA has been known for decades but the NGS methods used to examine them at the transcriptome-wide level have only been recently developed [5, 6]. Nonetheless, elevated levels of modified nucleosides such as 1-methyladenosine, 1-methylguanosine and pseudouridine [7] have been found in the urine of patients with various malignancies [8] including cancers of the bladder [9], breast [10], esophagus [11], liver [12] and lung [13]. Since pseudouridine cannot be salvaged and has to be generated de novo, it presumably gets excreted in body fluids [14]. Thus, it has been hypothesized that high levels of excreted modified nucleosides such as pseudouridine could serve as biomarkers of tumor growth [7]. Among the approximately 20
different modified nucleosides that have been examined for use as diagnostic markers [8], pseudouridine is one of the most frequently evaluated in studies of this type, indicating that a deregulation of the pseudouridylation process could contribute to, or result from, the development of cancer [4].

Isomerization of uridine to Ψ in rRNA is accomplished by ribonucleoprotein (RNP) complexes consisting of a Ψ-synthase, 3 core proteins (NOP10, GAR1 and NHP2), and ncRNAs known as box H/ACA snoRNAs that guide the complex to the appropriate modification site [4]. The Ψ-synthase dyskerin (DKC1) is the catalytic portion of the snoRNP while the H/ACA snoRNAs function as guide RNAs that locate the appropriate RNA substrate via complementary base-pairing. Recently it has been shown that there is an increased expression of several of these snoRNAs in PCa [4], including SNORA74A, SNORA42, and SNORA64 [3]. Furthermore, prostate carcinomas were found to have a higher expression of the Ψ-synthase DKC1, and overexpression of the protein has been associated with the progression of the disease [15].

In this study we investigated the potential predictive value of pseudouridine in detecting the progression of prostate cancer to advanced disease, positing its use as a biomarker. We analyzed the expression of several H/ACA snoRNAs in primary and metastatic tumors and the expression of DKC1 and other snoRNP proteins using cBioPortal. Our results demonstrate that increased levels of pseudouridine are found in prostate cancer with potential to serve as a biomarker of prostate cancer progression.

Materials and methods

cBioPortal analysis of publically available datasets

To analyze the mRNA expression levels of the 14 upregulated H/ACA snoRNAs identified previously [3] we used cBioPortal [16, 17]. Specifically, we selected ‘Prostate Studies’ under the Query section, and then the ‘Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010)’ study. Next, we selected ‘mRNA Expression Z-Scores vs Normals’ under the ‘Genomic Profiles’ section and set the z-score threshold ± 2.0 (default). We then selected either the ‘Primary Tumors with mRNA (131)’ or ‘Mets with mRNA (19)’ option to examine mRNA expression in primary or metastatic tumors and submitted the 14 H/ACA snoRNAs (SNORA13, SNORA14B, SNORA15, SNORA42, SNORA46, SNORA50, SNORA58, SNORA6, SNORA60, SNORA62, SNORA64, SNORA65, SNORA74A, and SNORA7A) as query genes. The percentage of patients/cases with mRNA upregulation of the 14 individual genes in primary tumors was compared to those in metastatic tumors.

To generate the copy number alteration profile for the set of genes belonging to the DCK1 H/ACA snoRNP complex we again utilized www.cbioportal.org. Specifically, we selected ‘Prostate Studies’ under the Query section, and then the ‘Neuroendocrine Prostate Cancer (Trento/Cornell/Broad 2016)’ study. Next, we selected the ‘Putative copy-number alterations adjusted by ploidy and purity with CLONET’ option, and selected ‘CRPC-Adeno’ in the ‘Disease Code’ section and then queried the expression of the following genes: DKC1, NOP10, GAR1, and NHP2.

Design of RNA oligonucleotides, selection of antibody and dot blot analysis

Anti-pseudouridine mAB (D347-3) was purchased from Medical and Biological Laboratories (MBL, Nagoya, Japan). A 25-base 28S rRNA sequence that was shown to undergo pseudouridylation was used as a template and regular and Ψ versions were custom synthesized from Integrated DNA Technologies (IDT, Coralville, IA, USA) (5’-CGGCGGGAGUAAC [U or Ψ] AUGACUCUCUUA-3’).

For the dot blot assay, Biodyne B Pre-cut modified nylon membranes (0.45 µm) were purchased from Thermo Scientific (Waltham, MA, USA). Synthetic RNA oligonucleotides were diluted so that a 4.0 µL spot would yield 50, 100, 200, 400 or 800 ng of oligonucleotides, which were spotted on a nylon membrane. Water was used as the control (0 ng). Once spotted, the RNA was UV cross-linked to the membrane (1200 mJoules/cm²) and the membrane was then incubated for 1 hr in blocking buffer (3% bovine serum albumin [BSA] in 1× TBST) followed by incubation in primary antibody (anti-pseudouridine, 1:1000). After incubation the blot was washed 3×5 minutes each in 1× TBST, and incubated for 1 hr in secondary antibody (anti-mouse IgG, HRP-linked, #7076)
from Cell Signaling Technology [Danvers, MA, USA]) diluted 1:5000. After incubation and washing signal was developed using the WesternBright ECL kit from Advansta (San Jose, CA, USA). Images were developed using the ImageQuant LAS 4000 luminescent image analyzer (GE Healthcare Life Sciences, Chicago, IL, USA).

Cell lines and materials

Human prostate cancer cell lines (RWPE, LNCaP, 22Rv1, PC3 and Du145) were obtained from the American Type Culture Collection (Manassas, VA, USA). RWPE cells were cultured in Keratinocyte-SFM media (Gibco, Gaithersburg, MD, USA) supplemented with epidermal growth factor 1-53 (EGF 1-53) and bovine pituitary extract (BPE). LNCaP, 22Rv1, PC3 and Du145 cells were cultured in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin [100 U/mL] and streptomycin [100 μg/mL]). Trypsin (0.25% and 0.05%, with phenol red) was purchased from Gibco. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Dot blot analysis of prostate cancer cell lines

Total RNA was isolated from the different prostate cancer cell lines using the PureLink RNA Mini kit from Invitrogen (Carlsbad, CA, USA). The RNA from each cell line was diluted so that a 4.0 μL spot would yield 300 ng of RNA, and they were spotted on a nylon membrane. The 25-base [U or Ψ] 28S rRNA oligonucleotides were used as positive and negative controls. The membrane was incubated with primary and secondary antibody, and the signal developed as mentioned in the previous section. ImageJ was used to measure the intensity of each spot as compared to background intensity. Several equally-sized measurements were taken of the background to provide average intensity values, and one measurement made for each spot. The average background intensity was subtracted from each spot and plotted as folds over the intensity of the negative control.

Pseudouridinylated RNA-linked immunosorbent assay (PURLISA)

The PURLISA is designed for a 96-well plate format using 8-well strips that are added as necessary. Nunc CovaLink NH strips were purchased from Thermo Scientific. Nunc CovaLink NH strips contain polystyrene wells that are coated with molecules containing secondary amine (NH) groups. In the presence of a binding solution (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide [EDC, Sigma-Aldrich, St. Louis, MO, USA] and 1-methylimidazole [Sigma-Aldrich, pH 7.0] the Ψ’ phosphate group of RNA can bind to this solid phase via a covalent phosphoramidate bond. To each well, 80 μL of freshly made binding solution (0.2 M EDC dissolved in 10 mM 1-methylimidazole) was added, followed by the addition of one of the following: pseudouridine-containing synthetic oligonucleotides (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ng of 5’-GUAAACGGCGGGAGUAACΨAUGACUCUCUUAAGGUA-3’ oligos) to serve as the positive control, non-pseudouridylated oligos (100 ng of 5’-GUAAACGGCGGGAGUAACUAUGACUCUCUUAAGGUA-3’ oligos) to serve as the negative control, and 100 ng of RNA isolated from PCa cell lines (unknowns). For positive and negative oligos, 36-base sequences were used instead of 25-base sequences to enhance binding. After this the plate was covered with parafilm and incubated at 50°C for 5 hours. Next the wells were washed 3×5 minutes each with 200 μL of 1× wash buffer (.15M NaCl and 0.05% Tween20 dissolved in 1× PBS), after which 50 μL of anti-pseudouridine antibody (diluted 1:1000 in 3% BSA made with 1× wash buffer) was added to the wells. The plate was incubated at room temperature for 1 hr, washed, and 50 μL of biotin-conjugated anti-mouse antibody (Rockland, Limerick, PA, USA, diluted 1:2000) was added to the wells and the plate was then incubated for 30 mins. After incubation the wells were washed, and 50 μL of HRP-conjugated streptavidin (Thermo Scientific, diluted 1:5000) was added and the plate, and allowed to incubate for 30 minutes. The plate was then washed for a final time, and 100 μL of 3, 3’, 5’, 5’-tetramethylbenzidine (TMB, Thermo Scientific) was added to the wells for 5 minutes to allow the color to develop, upon which 100 μL of 1 M HCl was added to stop the reaction. The plate was then read at 450 nm in a spectrophotometer (SpectraMax M5, Molecular Devices, San Jose, CA, USA) and the amount of Ψ present in the wells is proportional to the measured absorbance. A standardized calibration curve is created using the mass of pseudouridine present per input of total Ψ-positive oligonucleotides and the line of
best fit equation is used to calculate the amount of Ψ in (in nanograms) in a given sample. All data were expressed as the mean ± SEM (standard error of the mean).

**TMA immunohistochemistry and analysis**

Human prostate cancer tissue microarray (TMA) PR242b was purchased from US Biomax, Inc. (Derwood, MD, USA). The TMA was deparaffinized and rehydrated with successive washes of xylene (3×10 minutes each) and ethanol (100% 3×5 minutes, 95% 1×5 minutes, 70% 1×5 minutes). The slide was then washed with double distilled (dd) H₂O for 5 minutes, followed by 1× PBS for 5 minutes, and then incubated in 3% H₂O₂ for 15 minutes. The TMA was then washed with ddH₂O for 5 minutes and 1× PBS for 5 minutes. Antigen retrieval was performed using citrate (pH of 6.0) with a pressure cooker and after removal the TMA was allowed to cool at room temperature for 15 minutes. Next the slide was washed with ddH₂O (3×1 minute) and 1× PBS (3×2 minutes) and incubated in blocking buffer (10% horse serum in BSA) for 1 hr. After blocking the slide was incubated with anti-pseudouridine antibody (1:1000) suspended in 10% horse serum in BSA for 1 hr at room temperature followed by washing with 1× PBS (3×5 minutes). Secondary anti-mouse HRP antibody was applied at 1:200 for 1 hr. After washing the signal was developed using 3, 3′-diaminobenzidine (DAB) and visualized under a microscope. When signal developed the slide was washed in running tap water for 5 minutes. The TMA was then counterstained with hematoxylin for 1 minute, washed for 5 minutes, and was then treated with Bluing Reagent (Thermo Fisher) for 30 seconds, and washed for 2 minutes. The slide was then dehydrated with 3×5 minute washes of 100% ethanol, and 3×5 minute washes of xylene. After dehydration a coverslip was then added. Immunohistochemical staining was done by the Biorepository and Pathology core at Mount Sinai.

Brightfield digital images of the TMA were captured at 40× using the Olympus Scanner at the Biorepository and Pathology core at Mount Sinai. Each individual core was evaluated for the presence of cancer by an in-house pathologist and given an H-score. The percentage of positively-stained epithelial cells and the relative intensity of the stain were measured. The final H-score is the summation of (1+i) pi where i is the intensity score [0 (lowest) 1+, 2+, 3+, 4+(highest)] and pi is the percent of the cells with that intensity. A final scatterplot showing the individual H-scores of normal adjacent tissues (NATs) vs. adenocarcinomas was made using Prism. The mean of each group ± SD (standard deviation) was also graphed. The p-value was calculated using a two-tailed unpaired t-test in Prism, where an alpha value of 0.05 was considered significant.

**Results**

**cBioPortal analysis**

A study by Martens-Uzunova et al. [3] was one of the first to examine the small non-coding transcriptome of different types of PCa in an effort to identify novel diagnostic and prognostic ncRNA expression profiles. Deep sequencing of organ-confined PCa and metastatic lymph node PCa (LN-PCa) revealed that the total amount of snoRNA fragments in metastatic tumors increased by >20% [3]. In addition several new, unique H/ACA snoRNAs were detected in metastatic PCa. To cross-validate the study’s findings we decided to compare the expression of the 14 non-putative LN-PCa associated H/ACA snoRNAs in primary and metastatic tumors sequenced from another study that examined the genomic profile of prostate cancer [18] (Table 1). We compared the mRNA expression levels of all 14 snoRNAs from primary (n=131) and metastatic (n=19) tumors and found that the percent of cases with upregulated mRNA levels increased in the metastatic tumors (10 out of the 14 snoRNAs), with 7 snoRNAs increasing 2-fold.

A recent study examining the clonal evolution of CRPC and castration-resistant neuroendocrine prostate cancer (CRPC-NE) [19] provided crucial whole exome sequencing data on these lethal subtypes of PCa. Interrogation of the data set revealed significant homozygous amplification of each gene comprising the pseudouridinylation-complex (Figure 1A).

**Selectivity of anti-pseudouridine antibody and detection of pseudouridine in pca cell lines**

To test the selectivity and specificity of the anti-pseudouridine antibody, we generated two 25-base 28S rRNA oligonucleotide sequences
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Table 1. Overexpression of H/ACA snoRNAs in PCa

<table>
<thead>
<tr>
<th>Name</th>
<th>Primary tumors (131 samples)</th>
<th>Metastatic Tumors (19 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNORA13</td>
<td>11%</td>
<td>16%</td>
</tr>
<tr>
<td>SNORA14B</td>
<td>8%</td>
<td>37%</td>
</tr>
<tr>
<td>SNORA15</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>SNORA42</td>
<td>14%</td>
<td>0%</td>
</tr>
<tr>
<td>SNORA46</td>
<td>8%</td>
<td>5%</td>
</tr>
<tr>
<td>SNORA50</td>
<td>5%</td>
<td>0%</td>
</tr>
<tr>
<td>SNORA58</td>
<td>13%</td>
<td>32%</td>
</tr>
<tr>
<td>SNORA6</td>
<td>24%</td>
<td>63%</td>
</tr>
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<td>SNORA60</td>
<td>4%</td>
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</tr>
<tr>
<td>SNORA62</td>
<td>22%</td>
<td>47%</td>
</tr>
<tr>
<td>SNORA64</td>
<td>30%</td>
<td>42%</td>
</tr>
<tr>
<td>SNORA65</td>
<td>28%</td>
<td>42%</td>
</tr>
<tr>
<td>SNORA74A</td>
<td>15%</td>
<td>37%</td>
</tr>
<tr>
<td>SNORA7A</td>
<td>17%</td>
<td>42%</td>
</tr>
</tbody>
</table>

Analysis of the mRNA levels of 14 H/ACA snoRNAs using the Cancer Cell, 2010 dataset available from the cBioPortal for Cancer Genomics (https://www.cbioportal.org/). The Cancer Cell, 2010 dataset was used to cross-validate the Oncogene, 2012 study's findings. The mRNA expression levels of the H/ACA snoRNAs of primary tumors were compared to levels found in metastatic tumors. The percent of patients/cases with upregulated mRNA levels increased in the metastatic group for 10 out of the 14 snoRNAs, with 7 snoRNAs increasing at least twice-fold.

(Figure 1B) that were that were shown to undergo pseudouridylation, identical except for a single Ψ substitution, and tested the performance of the antibody in a dot blot assay. The antibody was able to differentiate between the two sequences at a threshold of 50 ng, as shown in Figure 1C. Non-specific binding to the Ψ-negative sequence control was minimal, with signal intensity of the 800 ng spot comparable to or slightly lower than 50 ng of Ψ-positive RNA (a 16-fold difference in quantity).

We subsequently performed a similar dot blot analysis with RNA isolated from PCa cell lines and measured signal intensity of each spot relative to the non-pseudouridylated control with ImageJ [20]. Pseudouridine expression was measured in five different PCa cell lines that are representative models of different stages of disease (Figure 2A). RWPE cells are SV-40 immortalized prostate epithelial cells that are representative of 'normal' non-cancerous cells. LNCaP cells are AR-positive and respond to androgen stimulation. PC3 and Du145 cells are AR-negative models that are androgen-independent. 22Rv1 cells are AR-positive but express a splice variant and can model CRPC. Our results demonstrate that 22Rv1, PC3 and Du145 cells contain higher levels of Ψ-RNA compared to RWPE and LNCaP cells (Figure 2A).

Standardization of pseudouridinylated RNA linked immunosorbent assay (PURLISA) and quantification of pseudouridine in PCa cell lines

To measure Ψ in unknown RNA samples, a standardized calibration curve (Figure 2B) was created using Ψ-positive oligonucleotides (5'-GUAACGGCGGAGUAACΨ AUGACUCUUAA-GGUA-3') and the line of best fit equation was used to calculate the amount of Ψ (in nanograms) in a given sample. The R² value was 0.9236 (Figure 2B). Having standardized our assay, we next quantified Ψ in prostate cancer cell lines. PC3 and Du145 cells were found to have the highest quantities of pseudouridine (0.404 ng and 0.245 ng respectively, Figure 2C) as compared to LNCaP cells (0.033 ng), and RWPE cells (0.044 ng).

TMA immunohistochemistry and analysis

We performed immunohistochemistry (IHC) on a TMA (12 cases/24 cores, Supplementary Figure 1) containing prostate adenocarcinomas and adjacent normal tissue (NAT). We found strong immunoreactivity localized to the cytoplasm of glandular cells (Figure 3A), with negligible staining of the gland cells belonging to NATs. We then calculated the H-score (see Methods) which showed a significant difference between adenocarcinoma cores and NATs (P < 0.0001), though no relationship between Gleason sum and intensity of staining could be established (Figure 3B).

Discussion

There are alternatives to PSA testing [21], such as measuring levels of Prostate cancer antigen 3 (PCA3, gene that expresses a non-coding RNA in elevated levels in prostate tumor tissue) in urine and prostatic fluid, however these and other biomarker-based tests (e.g. the Prostate Health Index [PHI] and 4Kscore®) are not per-
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... with reported sensitivities ranging from 66% to 80% and specificities ranging from 45% to 76% [21]. It is therefore imperative to continue to search for alternative biomarkers to, or to be used in conjunction with, PSA.

Existing evidence supports that elevated levels of pseudouridine and other modified nucleosides are found in the urine of patients with various malignancies [8]. Exploitation of the urinary metabolome including pseudouridine continues to be investigated, as in the case of a recent study which found that the urine from men with prostate cancer have different metabolite profiles (e.g., branched-chain amino acids, glutamate, glycine, fumarate, etc.) compared to men with benign prostatic hyperplasia (BPH) and could be used to distinguish between malignant and benign disease [22]. Furthermore, significantly greater levels of urinary pseudouridine were also found in men with PCa [22]. While pseudouridine seems promising as a biomarker, the typical methods used to detect it are prohibitively expensive, laborious or time-consuming (e.g., chemical modifications with CMC [23, 24], mass spectrometry [25] and 1H nuclear magnetic resonance (1HNMR) [22]). We sought to characterize an anti-pseudouridine monoclonal antibody D347-3 (APU-6). This antibody was first prepared by Itoh et al. [26] and was used to measure the levels of pseudouridine from urine via inhibition ELISA. Upon characterization, they found that clone APU-6 was highly specific for pseudouridine with minimal cross-reactivity to related compounds (uridine, uracil, etc.) and performed nearly as well as HPLC [26]. Our study revealed that the antibody was able to differentiate between two 25-base 28S rRNA sequences known to be pseudouridinylated with a single Ψ substitution at the lowest threshold tested (50 ng), with minimal signal from non-specific binding even at the highest quantity tested (800 ng) and thus concluded that the antibody was sufficiently specific towards pseudouridine to be utilized further.

Analysis of pseudouridine in RNA from prostate cancer cell lines representing different stages of progression (i.e. normal prostate tissue, androgen-sensitive, castration-resistant, and androgen-insensitive disease), revealed that the androgen-independent cell lines (PC3 and Du145) exhibited the highest levels of pseudouridine, with strong signal also given by the CRPC 22Rv1 cells (serially propagated in mice after castration-induced regression and rela-
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This observation is in line with our predictions based on genomic analysis using cBioPortal, that advanced disease contains a wider variety and increased abundance of H/ACA snoRNAs, as well as the amplification of genes comprising the snoRNP complex (especially the catalytic component DKC1) and as such these cell lines would be expected to contain higher quantities of pseudouridine (Table 1 and Figure 1A).

We developed our PURLISA to be a facile and high-throughput method to detect and quantify pseudouridine from isolated total RNA, as the technical limitations of dot blot assays proved to be cumbersome (low-throughput and requirement of overnight incubation). Using the Nunc CovaLink NH strips we designed an assay that would bind the 5’ phosphate group of RNA to the solid phase for subsequent antigen detection. To validate our PURLISA, as well as our results obtained with the dot blot assay, we used PURLISA to quantify the amount of pseudouridine present in the same set of PCa cell lines. Similar to our previous results we found that PC3 and Du145 cells contained the greatest amounts of pseudouridine (0.404 ng and 0.245 ng, respectively). Thus, based on two different techniques, pseudouridine was found to be in significantly greater quantities in AR-negative, androgen-independent cell lines compared to AR-positive, androgen-sensitive cells (LNCaP) and non-cancerous cells (RWPE).

Immunohistochemical analysis of a prostate TMA (12 cases/24 cores) revealed that pseudouridine was highly expressed in glandular cells belonging to adenocarcinomas with very
little staining of glandular cells within normal adjacent tissues (NATs) (P ≤ 0.0001). While there was no relationship between H-score and Gleason sum, this may be due to the range of Gleason scores tested (Gleason scores 6-9) and a relationship may be established if lower Gleason scored tissue is also sampled (Gleason scores < 6). Clinical information regarding the PSA levels of the tissue donors would have been useful to consider but was not available. Besides an increased expression of DKC1 [15] and H/ACA snoRNAs [3] in prostate tumors an increase in ribosome biogenesis could also influence the levels of pseudouridine found in malignant tissue. A study by Arthurs et al. [28] which analyzed prostate tissue arrays stained for one of three ribosomal proteins (RPS19, RPS21 and RPS24) found that these proteins were highly expressed in malignant tissue compared to non-malignant tissue. Additionally RPS19 and RPS21 were also found to correlate with Gleason grade, with RPS19 expression significantly increasing between Gleason grades 5 and 8, and 6 and 8 and RPS21 expression significantly increasing between Gleason grades 5 and 8 [28]. In view of the relative abundance of pseudouridine in rRNA compared to most other types of RNA [14] it is reasonable to assume that higher levels of ribosomal proteins in malignant tissues contribute to the increased expression of pseudouridine seen in our analysis.

Figure 3. A. Representative images (10× with 40× insert) of normal adjacent tissues (NAT) and adenocarcinomas. A tissue micro array (TMA) purchased from US Biomax, Inc. (PR242b) was stained for pseudouridine and imaged at 10X and 40X. Cores with Gleason 3+3, 4+3 and 5+4 were used for representative images. Staining is primarily localized to the cytoplasm of glandular cells with negligible staining of the gland cells belonging to normal adjacent tissues (NATs). B. Plot of H-scores of normal adjacent tissues and adenocarcinomas. Each individual core was evaluated by an in-house pathologist for the presence of cancer, and given an H-score. The final H-score is the summation of (1+i)pi where i is the intensity score [0 (lowest)1+, 2+, 3+, 4+(highest)] and pi is the percent of the cells with that intensity. The p-value was calculated using a two-tailed unpaired t-test in Prism, where an alpha value of 0.05 was considered significant.
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Conclusions

Our findings present the first evidence that deregulation of the pseudouridinylation process is associated with the progression of prostate cancer. Whether elevated pseudouridine levels functionally contribute to prostate cancer initiation, and are a direct consequence of increased expression of H/ACA snoRNAs, DKC1, increased ribosome biogenesis or other pseudouridine synthases [29] remains to be elucidated. DKC1 is currently undergoing target validation, as a recently-discovered inhibitor of DKC1 (pyrazofurin), was found to reduce both in vitro pseudouridylation activity, as well as cell viability of a breast cancer cell line (MCF-7) [30]. Evaluating pseudouridine expression in the blood and/or serum of patients in the context of relevant clinical (e.g. Gleason grade, PSA levels, tumor volume, TNM stage, development of metastasis, etc.) and genetic (e.g. PTEN loss, genomic alterations in AR, PI3K/AKT and/or DNA repair pathways, presence of neuroendocrine disease, [31] etc.) factors would greatly aid in parsing pseudouridine’s utility as a diagnostic or prognostic tool for PCa.

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Disclosure of conflict of interest

None.

Abbreviations

Ψ, Pseudouridine; AKT, Protein kinase B; AR, Androgen receptor; BPE, Bovine pituitary extract; BPH, Benign prostatic hyperplasia; BSA, Bovine serum albumin; CMC, N-cyclohexyl-N'-β-(4-methylmorpholinum)ethylcarbodiimide; CNA, Copy number alteration; CRPC, Castration-resistant prostate cancer; CRPC-NE, Castration-resistant neuroendocrine prostate cancer; DAB, 3, 3'-diaminobenzidine; ddH₂O, Double-distilled H₂O; DKC1, Dyskerin; DNA, Deoxyribonucleic acid; EDC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide; EGF 1-53, Epidermal growth factor 1-53; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; GAR1, H/ACA ribonucleoprotein complex subunit 1; H/ACA snoRNA, H/ACA box small nucleolar ribonucleic acid; ³¹HNMR, Proton nuclear magnetic resonance spectroscopy; HPLC, High performance liquid chromatography; HRP, Horseradish peroxidase; LN-PCa, Lymph node prostate cancer; Mab, Monoclonal antibody; mRNA, Messenger ribonucleic acid; NAT, Normal adjacent tissue; ncRNA, Non-coding ribonucleic acid; NGS, Next-generation sequencing; NHP2, H/ACA ribonucleoprotein complex subunit 2; NOP10, H/ACA ribonucleoprotein complex subunit 3; PBS, Phosphate buffered saline; PCa, Prostate cancer; PCA3, Prostate cancer antigen 3; PHI, Prostate health index; PI3K, Phosphoinositide 3-kinase; PSA, Prostate specific antigen; PTEN, Phosphatase and tensin homolog; PURLISA, Pseudouridinylated RNA linked immunosorbent assay; RNA, Ribonucleic acid; RNP, Ribonucleoprotein; RPS19, Ribosomal protein S19; RPS21, Ribosomal protein S21; RPS24, Ribosomal protein S24; rRNA, Ribosomal ribonucleic acid; SEM, Standard error of the mean; SD, Standard deviation; SNORA13, Small nucleolar RNA, H/ACA box 13; SNORA14B, Small nucleolar RNA, H/ACA box 14B; SNORA15, Small nucleolar RNA, H/ACA box 15; SNORA42, Small nucleolar RNA, H/ACA box 42; SNORA46, Small nucleolar RNA, H/ACA box 46; SNORA50, Small nucleolar RNA, H/ACA box 50A; SNORA58, Small nucleolar RNA, H/ACA box 58; SNORA6, Small nucleolar RNA, H/ACA box 6; SNORA60, Small nucleolar RNA, H/ACA box 60; SNORA62, Small nucleolar RNA, H/ACA box 62; SNORA64, Small nucleolar RNA, H/ACA box 64; SNORA65, Small nucleolar RNA, H/ACA box 65; SNORA7A, Small nucleolar RNA, H/ACA box 7A; SNORA74A, Small nucleolar RNA, H/ACA box 74A; snRNP, Small nucleolar RNA-protein complex; SV-40, Simian virus 40; TMA, Tissue micro array; TMB, 3, 3', 5, 5'-tetramethylbenzidine; TBST, Tris-buffered saline, 0.1% Tween 20; U, Uridine.

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References


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Supplementary Figure 1. A. PR242b Key. Key for the PR242b human prostate tissue TMA indicating the placement of each core and Gleason score of each adenocarcinoma. B. PR242b Stained with Pseudouridine. PR242b human prostate tissue TMA stained with pseudouridine. The boxed region indicates the position of the normal adjacent tissues.