Quantitative Analysis of BTF3, HINT1, NDRG1 and ODC1 Protein Over-Expression in Human Prostate Cancer Tissue

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Abstract

Prostate carcinoma is the most common cancer in men with few, quantifiable, biomarkers. Prostate cancer biomarker discovery has been hampered due to subjective analysis of protein expression in tissue sections. An unbiased, quantitative immunohistochemical approach provided here, for the diagnosis and stratification of prostate cancer could overcome this problem. Antibodies against four proteins BTF3, HINT1, NDRG1 and ODC1 were used in a prostate tissue array (> 500 individual tissue cores from 82 patients, 41 case pairs matched with one patient in each pair had biochemical recurrence). Protein expression, quantified in an unbiased manner using an automated analysis protocol in ImageJ software, was increased in malignant vs non-malignant prostate (by 2-2.5 fold, p<0.0001). Operating characteristics indicate sensitivity in the range of 0.68 to 0.74; combination of markers in a logistic regression model demonstrates further improvement in diagnostic power. Triple-labeled immunofluorescence (BTF3, HINT1 and NDRG1) in tissue array showed a significant (p<0.02) change in co-localization coefficients for BTF3 and NDRG1 co-expression in biochemical relapse vs non-relapse cancer epithelium. BTF3, HINT1, NDRG1 and ODC1 could be developed as epithelial specific biomarkers for tissue based diagnosis and stratification of prostate cancer.


Introduction

Prostate carcinoma is a disease of the epithelium, the most common cancer in men and the cause of considerable morbidity and mortality [1]. Each year, over 30,000 men are diagnosed of prostate cancer and over 10,000 die of the disease in the UK. In 2010, 217,730 new cases of prostate cancer were diagnosed in the US, with 32,050 American males dying of the disease [2].

Diagnosis and prognosis of prostate cancer is based on tissue morphology from biopsies (~1 million procedures in USA and ~70,000 in the UK / year). First time biopsies identify cancer in 38% of cases whereas equivocal diagnosis or false negatives constitute ~25-30% of cases [3]. Descriptors of aggressiveness (e.g. Gleason grade) determine cancer management and therapy but have significant drawbacks and high variance, particularly for low grade cancers.

Immunohistochemistry (IHC) is used in resolving equivocal diagnosis, however, most IHC biomarkers have been identified using subjective (scoring) analysis introducing large variability [4]. For organ confined disease prostate biopsy remains a critical clinical tool, however, there is a need to resolve false negatives and refine diagnosis. By identifying cancers that have good prognosis over-treatment could be reduced but there are no quantitative protein markers to enhance the quality of diagnosis. A reproducible, quantitative approach could greatly facilitate this process.

Discovery of IHC markers, largely, and their analysis, is conducted by semi quantitative approaches (e.g. scoring of tissue sections stained with a chromo- or fluorophore) with large inter-observer errors [4,5]. Scoring of the intensity of a chromophore (or fluorophore) involves visual inspection followed by a score within a predetermined range by the experimenter. An approach based upon visual observation of
Biomarkers of Prostate Cancer

Results

Quantitative immunohistochemical analysis of proteins over-expressed in prostate cancer tissue

Expression of BTF3, HINT1, NDRG1 and ODC1 was largely epithelial and increased in malignant compared to benign or non-malignant prostate cores (Figure 1). We quantified the grayscale DAB-label (Figure 1 and Figure S1), in an unbiased manner, by using a reproducible, semi-automated particle analysis (Analyze Particles) protocol [8] using grayscale images of the stained tissue from over 450-500 individual prostate tissue cores for each biomarker (Figure 2). Calculations of total area and area fraction are given in Table 1. Integration of AUC revealed an increase in the expression of BTF3, HINT1, NDRG1 and ODC1 in malignant cores, diagnosed by histopathology, compared to non-malignant cores (p<0.0001, Mann Whitney U test). These results identify BTF3, HINT1, NDRG1 and ODC1 as proteins that are over-expressed in prostate cancer.

To investigate the utility of the proteins as putative biomarkers for prostate cancer diagnosis, we calculated the true positive rate (sensitivity) and false positive rate (1-specificity) by ROC curve (Figure 3). The area fraction data was transformed into probit and fitted to a Gaussian function. AUC values of 1.0 for an ROC curve represent high selectivity and sensitivity, whereas 0.5 suggests that the tested marker cannot distinguish between non-malignant and malignant categories. The dot plots of the transformed data are given in...
Table 1. Quantitation of protein expression in malignant and non-malignant human prostate tissue arrays using ImageJ software.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Condition (n)</th>
<th>Total Area</th>
<th>Area Fraction</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTF3</td>
<td>Benign (236)</td>
<td>22301 ± 1697</td>
<td>1.66 ± 0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malignant (228)</td>
<td>54750 ± 3588</td>
<td>4.09 ± 0.27</td>
<td>2.5</td>
</tr>
<tr>
<td>HINT1</td>
<td>Benign (214)</td>
<td>28564 ± 1882</td>
<td>2.13 ± 0.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malignant (225)</td>
<td>67173 ± 3977</td>
<td>4.93 ± 0.29</td>
<td>2.3</td>
</tr>
<tr>
<td>NDRG1</td>
<td>Benign (230)</td>
<td>27752 ± 1875</td>
<td>2.07 ± 0.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malignant (223)</td>
<td>72297 ± 4380</td>
<td>5.4 ± 0.33</td>
<td>2.6</td>
</tr>
<tr>
<td>ODC1</td>
<td>Benign (261)</td>
<td>38621 ± 4184</td>
<td>2.80 ± 0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malignant (243)</td>
<td>65428 ± 5389</td>
<td>4.88 ± 0.4</td>
<td>1.8</td>
</tr>
</tbody>
</table>

DAB label, representing protein expression for BTF3, HINT1, NDRG1 and ODC1 was quantified in an unbiased manner, by using a reproducible, semi-automated particle analysis (Analyze Particles) protocol with ImageJ software. Over 500 individual prostate tissue cores (see methods) RGB images were converted into 16 bit grayscale. The results are means ± SE for the calculated parameters of total area and area fraction. These data are standardized for amount of tissue on each core by using the inverse protocol on ImageJ (see methods). Fold increase in the expression of protein in malignant compared to non-malignant (benign) cores was confirmed by AUC calculations of data from Figure 2. (n)= number of usable cores included in the analysis. *Statistical analysis: expression, for all proteins, is significantly different in malignant compared to non-malignant prostate at P<0.0001 using Mann Whitney U test.

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Disease stratification using multiple markers and immunofluorescence staining

It is estimated that around 30% of patients undergoing radical prostatectomy for clinically localized disease will experience biochemical relapse (defined as detectable PSA ≥ 0.2 ng ml⁻¹ within 2 years of surgery). A cohort of patients used for the tissue array in this study, had undergone biochemical relapse [17]. We hypothesized that not only a change in expression but also co-localization of the identified biomarkers changes in prostate cancer epithelium. This was investigated by multi-labeled immunofluorescence staining using BTF3, HINT1 and NDRG1 antibodies (Figure 4). The 3 proteins were differentially expressed in non-malignant (Figure 4A) compared to malignant (Figure 4B) cores and found to be largely in the prostate epithelium, confirming the data from individual antibodies staining using 3,3-diaminobenzidine-horse radish peroxidase (DAB-HRP, Figure S1 and Figure 1). These results indicate not only that the expression of these protein biomarkers, individually, is increased in prostate cancer (Figure 2) but that the expression is largely epithelial and thus specific to the disease process (Figure 4).

The co-localization of BTF3, HINT1 and NDRG1 was next investigated in the multi-labeled immunofluorescence tissue cores from relapsed and non-relapsed patients from composite fluorescent images (Figure 5; BTF3, HINT1 and NDRG1, green, blue and red, respectively) in order determine the utility of this approach for prostate cancer stratification. There is an evident and quantifiable change in the pattern of staining of these protein biomarkers in biochemical relapse tissue cores:
BTF3 expression appears less in relapsed, compared to non-relapsed samples, whereas HINT1 and NDRG1 expression appears dominant in relapsed tissue samples (representative tissue cores from biochemical relapse and control patients are shown in Figure 5A and B); quantitative colocalization analysis of high magnification deconvoluted images (Figure 5C and D) showed that most of the apparent change in the pattern of expression, for multi-labeled tissue sections in non-relapsed vs relapsed samples, was due to the change in co-expression of BTF3 (fluorescein isothiocyanate, FITC, green) and HINT1 (cyanin 3, Cy3, blue). Calculated Pearson coefficients for colocalization for non-relapse vs relapse were 0.73 ± 0.02 vs 0.60 ± 0.07 (means ± SD, n=4, significance of difference p<0.02). Quantitative co-localization indicates that multi-labeling approach could be used for the stratification of prostate cancer in tissue cores.

Discussion

By using global gene expression analysis, tremendous progress has been made in the identification of dysregulated genes in prostate cancer over the past decade. What has been lacking is the translation of these genes into clinically useful biomarkers for the diagnosis and stratification of the disease. Human prostate was one of the first tissues to be investigated for global changes in gene-expression in disease almost a decade ago. Incidentally, the approval of new markers by Food and Drug Administration, USA, particularly protein markers, has declined over the past decade. This decline could be attributed to three major problems in the identification and development of tissue based protein biomarkers for the diagnosis and prognosis of prostate cancer: 1. Lack of specificity, i.e. not epithelium specific (the site of initiation of the disease). 2. Lack of an unbiased, automated, quantitative analysis of protein expression that could identify robust, reproducible data for putative biomarkers. 3. Lack of quantitative, proteomic approaches to for a molecular basis of prostate cancer prognosis.

Immunohistochemical markers could be extremely useful in primary or confirmatory diagnosis in support of histopathology, but due to the difficulties in identification or validation [18] only a handful of such markers exist. It also appears likely that histopathological analysis will remain a bulwark for prostate cancer diagnosis in the near to medium future. Thus in addition to continuing search for the identification of non-invasive biomarkers that could be detected in bodily fluids, it is important to keep refining and improving the immunohistochemical tissue biomarkers that could facilitate diagnosis, improve prognosis and provide useful predictive stratification of prostate cancer. Due to the increase in computing power over the last decade two technologies have matured that could help achieve these objectives. These are discussed below.

Morphological analysis is routinely used to diagnose prostate cancer, although IHC is used to assist morphological diagnosis, particularly in equivocal cases, e.g. in tissue samples from needle biopsies, transurethral resection specimens and metastatic tumor samples [19]. A complicating issue hindering discovery of new biomarkers for diagnosis and disease stratification of prostate (and other) cancer(s) from biopsy samples, has been a dearth of unbiased, quantitative methods to detect significant changes from IHC experiments for biomarker identification. There are, however, some exceptions to this approach. Rubin and colleagues [20] devised a
Multi-labeled immunofluorescence for BTF3, HINT1 and NDRG1. Co-expression of three proteins BTF3 (FITC-green), HINT1 (Cy3-blue) and NDRG1 (Cy5-red) in non-malignant (A) and malignant prostate tissue cores (B); images are representative tissue cores from the tissue array with over 200 samples. The three antibodies were incubated simultaneously and labeled with three Cy5–red different fluorophores using Bond automated staining system. Whole tissue cores were imaged using a Leica confocal system and 4x4 tiling at exactly the same settings for comparison. Images were zoomed 3x using digital zoom in ImageJ. Scale bar =100µm.
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Co-expression of three proteins BTF3 (FITC-green), HINT1 (Cy3-blue) and NDRG1 (Cy5-red) in malignant prostate tissue cores (A to D). Composite micrographs of immunofluorescently stained prostate tissue cores from patients with biochemical relapse (defined as PSA ≥ 0.2 ng/ml within 2 years of radical prostatectomy). Tissue cores from 4 patients (matched pairs for Gleason score and PSA), non-relapsed (A) and biochemical relapse (B) representative images used for quantitative co-localization analysis (see methods) are shown. Imaging was performed using a Leica confocal microscope; A and B are low magnification; C and D higher magnification; E is an example of the expression of the three markers in non-malignant tissue also at higher magnification. The expression of BTF3 (green), for example is evident in non-relapsed samples whereas it not very evident in cores from relapsed patients. Imaging was performed using an Olympus confocal microscope (40x tilled (A and B) or 40x with digital zoom 3.5-4 (C, D and E). High magnification images (C, D and E) were deconvolved using Huygens Profession software. 16bit tif files for each fluorophore (FITC, Cy3 and Cy5) were imported into ImageJ and pseudo colored (FITC, green), HINT1 (Cy3-blue) and NDRG1 (Cy5-red). Z-projections for up to 27 images are shown for each tissue core. Scale bar =100µm for A and B and 20µm for C and D. Quantitative co-localization analysis of images (e.g. Figure 5C and D) showed that most significant change in the pattern of expression, in non-relapsed vs relapsed samples, was due to the change in co-expression of BTF3 (FITC, green) and HINT1 (Cy3, blue) with Pearson coefficients for colocalization for non-relapse vs relapse = 0.73 ± 0.02 vs 0.60 ± 0.07 (means ± SD, n=4 , significance of difference p<0.02).

Figure 5. Differential pattern for BTF3, HINT1 and NDRG1 co-expression in malignant, biochemical relapse and non-relapse sample. Co-expression of three proteins BTF3 (FITC-green), HINT1 (Cy3-blue) and NDRG1 (Cy5-red) in malignant prostate tissue cores (A to D). Composite micrographs of immunofluorescently stained prostate tissue cores from patients with biochemical relapse (defined as PSA ≥ 0.2 ng/ml within 2 years of radical prostatectomy). Tissue cores from 4 patients (matched pairs for Gleason score and PSA), non-relapsed (A) and biochemical relapse (B) representative images used for quantitative co-localization analysis (see methods) are shown. Imaging was performed using a Leica confocal microscope; A and B are low magnification; C and D higher magnification; E is an example of the expression of the three markers in non-malignant tissue also at higher magnification. The expression of BTF3 (green), for example is evident in non-relapsed samples whereas it not very evident in cores from relapsed patients. Imaging was performed using an Olympus confocal microscope (40x tilled (A and B) or 40x with digital zoom 3.5-4 (C, D and E). High magnification images (C, D and E) were deconvolved using Huygens Profession software. 16bit tif files for each fluorophore (FITC, Cy3 and Cy5) were imported into ImageJ and pseudo colored (FITC, green), HINT1 (Cy3-blue) and NDRG1 (Cy5-red). Z-projections for up to 27 images are shown for each tissue core. Scale bar =100µm for A and B and 20µm for C and D. Quantitative co-localization analysis of images (e.g. Figure 5C and D) showed that most significant change in the pattern of expression, in non-relapsed vs relapsed samples, was due to the change in co-expression of BTF3 (FITC, green) and HINT1 (Cy3, blue) with Pearson coefficients for colocalization for non-relapse vs relapse = 0.73 ± 0.02 vs 0.60 ± 0.07 (means ± SD, n=4 , significance of difference p<0.02).
whether found to be fused to ERG or not, NDRG1 could be a useful protein biomarker for prostate cancer.

HINT1 belongs to the histidine triad (HIT) family and a protein kinase C interacting protein [30]. No information exists on HINT1 expression or function in prostate cancer although it may act as tumor suppressor in mice [31]. In a hepatoma cell line, HINT1 inhibits activity of Wnt/ß-catenin signaling and gene transcription via TCF4 [32]. We have shown, previously, that there is a suppression of ß-catenin/TCF4 mediated gene transcription of TCF4 transcription targets [8]. It is tempting to speculate that HINT1 may contribute towards the inhibition of Wnt/ß-catenin signaling in prostate cancer [8].

L-Ornithine decarboxylase 1 (ODC1) is an enzyme in the polyamine synthesis pathway [33] a key factor in normal cell proliferation and neoplastic growth. ODC1 has been identified as a genetic marker for colon cancer [34] and over-expression of ODC1 has been linked to high risk neuroblastomas [35] and colorectal and breast cancers [36]. These data correlate to in vivo experiments with ODC1 knock-out mice which developed fewer tumors in response to various carcinogenic promoters in line with the reduced gene copy number [37].

Our data suggests that using an unbiased, quantitative approach to identify epithelium specific biomarkers in combination with immunofluorescence could prove useful in diagnosis, stratification and prognosis of prostate carcinoma.

Materials and Methods

Prostate tissue array

Patient selection, disease state and construction details of tissue blocks are given elsewhere [8,17]. Briefly, tissue blocks were constructed using archival formalin-fixed, paraffin-embedded radical prostatectomy specimens from the 82 patients with pathological stage pT3a or b and pre-operative PSA stage of >3. 41 case pairs were matched for the following categories: pathological stage, Gleason grade and pre-operative prostate-specific antigen (PSA) concentration. One patient in each pair had biochemical recurrence (defined as PSA ≥ 0.2 ng mL-1 within 2 years of surgery) and the other remained biochemically free of disease (defined as undetectable PSA at least 3 years after surgery). The cores were diagnosed (as benign or non-malignant and malignant) and marked by a pathologist and 5-6µm sections were cut from the tissue arrays onto coated slides. To eliminate bias, the experimenters were blind to staining, imaging and analysis, with the latter being automated (see below). Clinical details of the tissue samples used are given in Table S2 and [17]; pathologists scored a majority of samples (>80%) as Gleason grade 4+3 and 3+4. A sample size calculation was made for differentiating between over-expression using DAB-IHC; the sample size for the protein expression was calculated prior to the construction of the tissue array (with an α and β of 0.05 and expected difference of 2.5 folds between the means of malignant and non-malignant samples to be 41 patients per group).

Ethics approval

Ethical approval was given by the Joint UCL/UCLH committees on the ethics of human research. The review board (RB) approved the use of human tissue for prostate cancer research, in compliance with the International Committee on Harmonization of Good Clinical Practice (ICH GCP). For tissue array construction the samples were anonymized during the construction of the tissue arrays [17] and used for various immunohistochemical studies in a project approved by the UCL/UCLH ethics committee. The RB waived the need for informed consent because the samples used for tissue array were archival pathological samples.

3,3-diaminobenzidine-horse radish peroxidase(DAB-HRP) staining

All staining was performed using Bond automated system according to manufacturer’s protocols. Details of the staining procedure for IHC using DAB and antibodies (BTF3, HINT1, NDRG1 and ODC1) used are given in Methods S1.

Immunofluorescence staining using 3 antibodies in prostate tissue arrays

The protocol for immunofluorescence staining was similar to that described for DAB staining, above, except that BTF3, HINT1 and NDRG1 antibodies incubated simultaneously and stained with FITC, Cy5 and Cy3 respectively, according to manufacturer’s protocol. Images were acquired using a Leica T710 confocal microscope with an EC Plan-Neofluar (40x) objective.

Image particle analysis using ImageJ software

An unbiased, reproducible, automated particle analysis method [8] was employed to quantify the DAB signal on non-malignant (benign) and malignant human prostate tissue cores using ImageJ software [21]. Macros were written to execute the following sequence of events for acquired jpeg images: 1. Open image 2. Convert to 16-bit image 3. Set threshold 4. Analyze particle (Size 0.5- Infinity, Circularity 0.00-1.00) 5. Save image 6. Save particle information (count, total area, average size and area fraction) into an excel spreadsheet (rsb.info.nih.gov/ij/docs/pdfs/examples.pdf). Units are default ImageJ setting (pixels). Standardization for the amount of tissue per core was also quantified by using the inverse function (EditInvert image) in ImageJ with the protocol described above. Quantified DAB signal is expressed as signal / amount of tissue in each tissue core. A flow chart for DAB signal quantitation is given in Figure S1. For all antibodies tested, expression was observed to be largely epithelial and analysis was also restricted to the epithelial expression; set threshold parameters were chosen after manual analysis of random cores for the subsequent quantitation of the signal. A contiguous spreadsheet for all the usable cores (between 211 and 260 cores) for non-malignant vs malignant comparison, for different antibodies was constructed. Tests for normal distribution (Kolmogorov-Smirnov test) of the acquired data and subsequent statistical tests for significance of difference between protein expression in malignant and non-malignant
cores was conducted using Mann Whitney U test. Mountain plots were constructed and analyzed for area under the curve (AUC) using Origin (Microcal). Receiver Operating Characteristics (ROC) curves were constructed after converting the data to normal distribution using probit using MedCalc software; likelihood ratios were also calculated from the ROC curves using MedCalc software.

For quantitative colocalization of BTF3, HINT1 and NDRG1 tissue cores were imaged using an Olympus IX81 confocal system using a dry 40x objective (3.5 to 4x digital zoom). The ‘oif’ files were imported into Huygens Professional software for image deconvolution. The deconvolved images were saved as 16bit TIFF files for each channel (excitation/emission (nm) FITC = 488 / 519, Cy3 = 559 / 567, Cy5 = 635 / 664). The 16bit TIFF files were imported into ImageJ and the colocalization plugin (Wright Cell Imaging Facility, University of Toronto) used to calculate Pearson co-efficient for colocalization.

Supporting Information

Figure S1. Schematic representation of the process to calculate DAB signal and amount of tissue per sample (core) using ImageJ particle analysis protocol. The DAB stained image (A) is converted to a grayscale image (B). For DAB signal calculation (C) a threshold is applied and the particles quantified. To calculate the amount of tissue, the grayscale image is inverted (D) and particles calculated.

Figure S2. Dot plots of area fraction values for BTF3, HINT1, NDRG1 and ODC1 used to obtain operating characteristics (Figure 4). N-M = non-malignant and M = malignant prostate tissue cores.

References


