

RESEARCH ARTICLE

Nanocytological Field Carcinogenesis Detection to Mitigate Overdiagnosis of Prostate Cancer: A Proof of Concept Study

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Abstract

Purpose

To determine whether nano-architectural interrogation of prostate field carcinogenesis can be used to predict prognosis in patients with early stage (Gleason 6) prostate cancer (PCa), which is mostly indolent but frequently unnecessarily treated.

Materials and Methods

We previously developed partial wave spectroscopic microscopy (PWS) that enables quantification of the nanoscale intracellular architecture (20–200nm length scale) with remarkable accuracy. We adapted this technique to assess prostate needle core biopsies in a case control study from men with Gleason 6 disease who either progressed (n = 20) or remained indolent (n = 18) over a ~3 year follow up. We measured the parameter disorder strength (Ld) characterizing the spatial heterogeneity of the nanoscale cellular structure and nuclear morphology from the microscopically normal mucosa ~150 histologically normal epithelial cells.

Results

There was a profound increase in nano-architectural disorder between progressors and non-progressors. Indeed, the Ld from future progressors was dramatically increased when compared to future non-progressors (1±0.065 versus 1.30±0.0614, respectively p = 0.002). The area under the receiver operator characteristic curve (AUC) was 0.79, yielding a sensitivity of 88% and specificity of 72% for discriminating between progressors and

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non-progressors. This was not confounded by demographic factors (age, smoking status, race, obesity), thus supporting the robustness of the approach.

Conclusions

We demonstrate, for the first time, that nano-architectural alterations occur in prostate cancer field carcinogenesis and can be exploited to predict prognosis of early stage PCa. This approach has promise in addressing the clinically vexing dilemma of management of Gleason 6 PCa and may provide a paradigm for dealing with the larger issue of cancer overdiagnosis.

Introduction

Overdiagnosis is an emerging deterrent towards implementation of cancer population screening[1]. The detection of indolent/clinically insignificant disease frequently triggers a myriad of harms from unnecessary diagnostic/therapeutic interventions including costs, discomfort and complications[2]. Prostate cancer (PCa) epitomizes this conundrum in that it results in ~30,000 deaths in Americans, but ~60% of all men >80 years old incidentally harbor this malignancy[3]. The widespread use of serum prostate specific antigen (PSA) for PCa screening has accentuated detection of clinically-indolent cancers. Since the harms of treating early-stage (T1c/T2a), low-grade (Gleason 6) disease (the most common PCa presentation) generally outweigh the benefits, many clinical societies now recommend delaying surgery in favor of active surveillance, which involves close monitoring with serial prostate biopsies[4]. Unfortunately, given the vagaries of predicting behavior of an individual's PCa (with the potential progression to fatal disease), only ~10% of eligible men actually undergo active surveillance[5]. Therefore, better prognostication tools are urgently needed to make this strategy more acceptable to both patients and their physicians[6].

One emerging approach for risk assessment is through exploiting field carcinogenesis. Field carcinogenesis (a.k.a. field cancerization, etiological field effect, field of injury etc.) is a well-established phenomenon in cancer biology that a focal neoplastic lesion develops in a permissive mutational environment from the interactions between genetic substrate and exogenous factors (diet, obesity, smoking, diabetes etc.) [7–9]. These changes may occur in the epithelial and/or stroma [10]. From a clinical perspective, field cancerization with the associated synchronous/metachronous lesions is widely used for management of neoplasia in the colon, lung, head and neck etc. While less well explored in prostate, there is compelling evidence of field carcinogenesis such as multi-focality and abnormalities in molecular markers (e.g. gene expression, methylation, microRNA, FISH, mitochondrial alterations) seen in the microscopically normal epithelium in patients who harbor this malignancy. [11–15]

Our group has developed a novel optics technology, partial wave spectroscopic microscopy (PWS), or nanocytology, which allows quantification of cellular structure at the nanometer length scales.[16] We have utilized PWS nanocytology for detection of the nano-architectural consequences of the diffuse genetic/epigenetic alterations that are the hallmark of field carcinogenesis. We have previously demonstrated that PWS interrogation of normal epithelium accurately predicted risk of colon[17] lung [18], esophagus[19], pancreas [20]and ovarian cancers [21]. To adapt this biophotonic breakthrough to PCa, we wanted to target the most vexing issue, management of early stage (Gleason 6 disease). Since this is typically diagnosed by needle biopsy, we hypothesized that nanocytological interrogation of the microscopically normal

prostatic epithelium would predict prognosis in men with early stage (Gleason 6) disease undergoing active surveillance.

Materials and Methods

Patients

This study was approved by the Institutional Review Board at NorthShore University Health-System. Samples were obtained from the NorthShore University active surveillance trial initiated in November 2008. Informed written consent was obtained from the participants. The inclusion criteria for the cohort of patients included: 1) age ≥ 60 ; 2) clinical stage $\leq T2a$; 3) Gleason score (grade) < 7 ; 4) $\leq 3/12$ positive cores on initial biopsy; and 5) $< 50\%$ linear involvement of any single core. Patients underwent their first surveillance biopsy 6–12 months after enrollment. Progression was defined as any change in criteria 3, 4 or 5. Thirty-eight patients were randomly chosen from the database of patients adjudicated as progressors and non-progressors by the chief study urologist (CBB).

Sample Procurement and Preparation

Transrectal biopsies were obtained under three dimensional (3-D) ultrasound guidance. Four of these cores were designated as research based and were fixed in an alcohol-based fixative and paraffin-embedded. Four micrometer sections were cut onto charged glass slides and deparaffinized for PWS. Hematoxylin and eosin (H&E) sections were reviewed by the study pathologist (MP) to direct the PWS analysis towards non-malignant tissue.

Overview of PWS

Briefly, as previously described, PWS marker disorder strength (L_d) is proportional to the mean and standard deviation of the spatial variations of the macromolecular density of the fundamental cellular building blocks (proteins, nucleic acids, lipids). Thus, L_d , colloquially, can be described as measuring the “clumpiness” of nanoscale structure. We have demonstrated that PWS is sensitive to structures from 20–200 nm through the spectral analysis of the interference spectra of light reflected from intracellular refractive index variations within microscopic spatio-temporal coherence volume, as opposed to typical light microscopy whose resolution is restricted 200–500 nm, the diffraction limit of light. Thus, histology is insensitive to many structures known to be involved in early carcinogenesis (macromolecular complexes, ribosomes, mitochondria, high-order chromatin etc.) that may be PWS detectable. Previously, we have shown that PWS was able to predict risk of colon, lung, esophagus, pancreas and ovarian cancers[22].

PWS Data Acquisition and Analysis

PWS analysis was performed as previously described. Regions of interest were identified (histologically normal glands) by the pathologist and calculated over ~ 30 regions per patient slide examined ($\sim 50K$ - $75K$ pixels or ~ 150 cells). The operator did not have clinical information for any of the 38 samples. However, the clinical information was available to technological investigators in the first 18 (“unblinded”) but not the last 20 (“blinded”) samples was performed in the first 18 patients but not the last 20.

Statistical Methods

All statistical analyses were performed using SAS 9.3 (SAS Inc., Cary, NC). Patient demographics were compared between progressors and non-progressors using t-test (for continuous

| | Non-Progressors (n=18) | Progressors (n=20) | p value |
|-----------------------------------|----------------------------|----------------------------|---------|
| Age (mean± standard deviation) | 67.1±5.9 years | 65.9±5.4 years | 0.52 |
| BMI (mean± standard deviation) | 27.1±2.4 kg/M ² | 29.0±4.9 kg/M ² | 0.14 |
| Race (% Caucasian) | 83.3% | 65.0% | 0.20 |
| Smokers (% current/former) | 44.4% | 60.0% | 0.35 |

Fig 1. Relevant demographic characteristics at time of first surveillance biopsy for patients who were later determined to be progressors versus non-progressors.

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variables) and Chi-square test. A standard two tailed t-test (assuming unequal variances) and analysis of covariance (ANCOVA) were performed to compare the Ld between progressors and non-progressors with a p value ≤0.05 considered statistically significant. The effect size was calculated using Cohen’s *d*: $Effect\ size = \frac{\mu_1\mu_2}{\sqrt{\sigma_1^2 + \sigma_2^2}}$ where μ_1 and μ_2 are the means for progressors and non-progressors and σ_1 and σ_2 are the corresponding standard deviations, respectively. The study statistician (CHW) also performed receiver operator characteristic curve (ROC) to assess the sensitivity and specificity of PWS in differentiating progressors and non-progressors for PCa in active surveillance.

Results

Cohort

We enrolled two sets of patients, an initial cohort of 18 patients (8 non-progressors and 10 progressors) where the PWS operator was blinded to clinical status but, followed by a distinct group of 20 patients (10 progressor and 10 non-progressors) in which our PWS analysis was performed by investigators blinded to group. The demographic factors are listed in Fig. 1. Median length of follow up was 34.2 months, mean age 66.5 ±5.6 years, and mean BMI 28.1±4.0. There were no significant differences in baseline patient characteristics between progressors and non-progressors.

PWS Analysis of Prostate Tissue Sections

PWS was performed in histologically normal glands identified by our pathologist. As can be noted from bright field imaging, we primarily targeted epithelium adjacent to glands for the defined region of interest. While there was no apparent difference on the bright field, there was a profound increase in Ld (more red in pseudocolor map) in progressors versus non-progressors (Fig. 2).

Mean per patient Ld in Progressors versus NonProgressors

We calculated the mean Ld from the region of interest per patient. Elevated Ld has been a hallmark of neoplastic transformation in a number of organs[20]. The Cohen d effect size of mean cellular Ld in this case-control study was 110% (Fig. 3) with a significant difference between

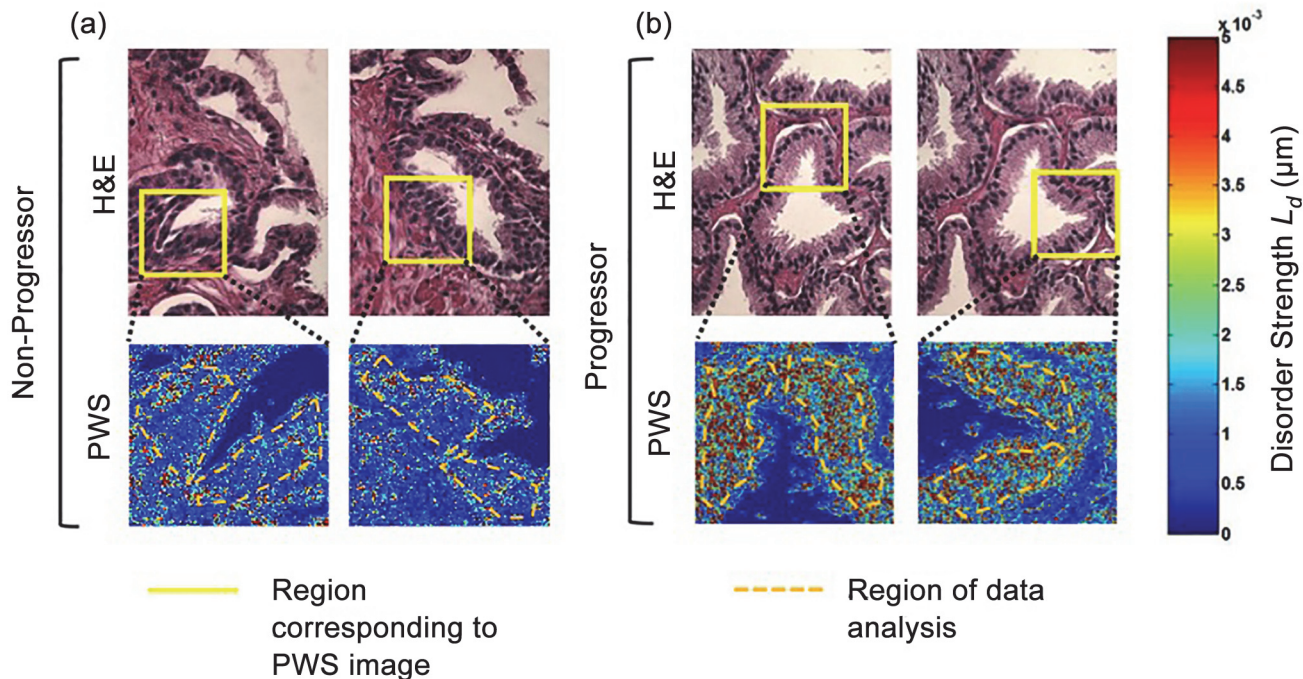


Fig 2. Representative images of prostate sections with bright field microscopy and Ld pseudocolor image maps. The bright field images from non-progressors (a) and progressors (b) are indistinguishable on the initial surveillance biopsies. However, the PWS images in the progressors show markedly higher Ld (e.g. red areas) when compared to the non-progressors, indicating profound nano-architectural abnormalities in the pathologically normal prostate epithelium. Median length of follow up was 34.2 months.

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progressors and non-progressors ($p = 0.002$). We analyzed half the dataset in a blinded fashion and noted that the AUC was equivalent to unblinded samples (0.81 versus 0.75, respectively), supporting the robustness of methodology. The combined dataset yielded a sensitivity of 88% and specificity of 72%, (Fig. 4) while the blinded dataset alone ($n = 20$) ad a sensitivity of 100% and a specificity of 70%. These strong diagnostics represent the minimal performance characteristics, with future refinements (e.g. assaying separately cytoplasmic versus nuclear Ld and epithelial versus stromal Ld) promising to improve discriminatory ability.

Potential Confounders

Since clinical factors (e.g. age, race[23,24], BMI[25], and smoking[26]) may modulate PCa aggressiveness, we performed analyses of covariance (ANCOVA) comparing Ld between progressors and non-progressors while controlling for these potential confounders. Somewhat surprisingly (based on previous literature) [27], serum prostate specific antigen was noted to be more elevated in progressors than nonprogressors, although statistical analysis showed that this did not impact upon Ld's diagnostic ability.

Discussion

The clinical need to differentiate aggressive from indolent PCa is clear since it is estimated that 233,000 American men are diagnosed annually with PCa, but only 29,480 are projected to die (still the second leading cause of male cancer deaths)[28]. While PSA screening has been widely practiced, the harms associated with the diagnosis and treatment of clinically indolent disease have led the US Preventive Services Task force to recently recommend against PSA screening [29].

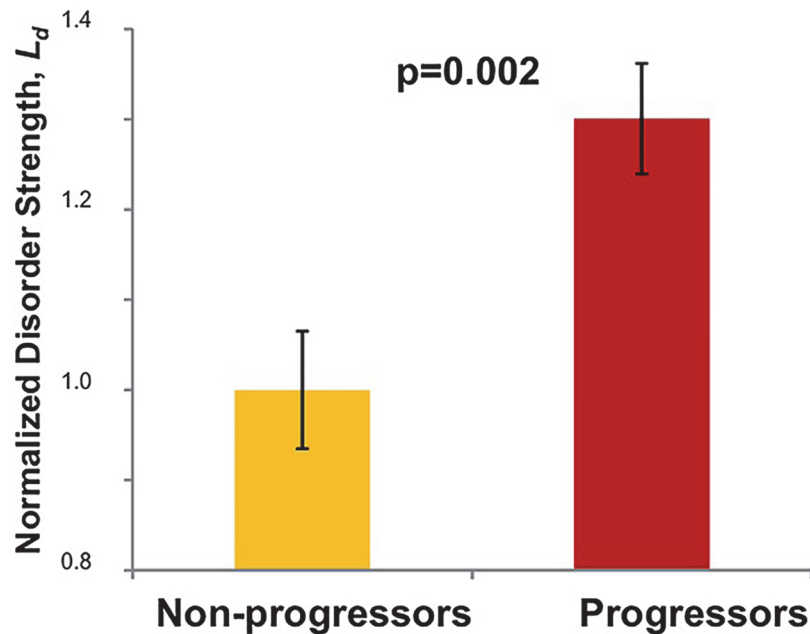


Fig 3. (a) Mean disorder strength (L_d) in non-progressors ($n = 18$) and progressors ($n = 20$) show a statistically significant difference between the two patient groups ($p = 0.002$) with an effect size of diagnosis = 110%. The median follow up time was 34.2 months.

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The issue of overdiagnosis now presents a major impediment to cancer screening in other cancers as well, including mammography for breast cancer and low-dose computerized tomography (LDCT) scanning for lung cancer[1]. Indeed, while LDCT resulted in a 20% reduction in lung cancer deaths in a high risk population, >95% of positives were false positive[30].

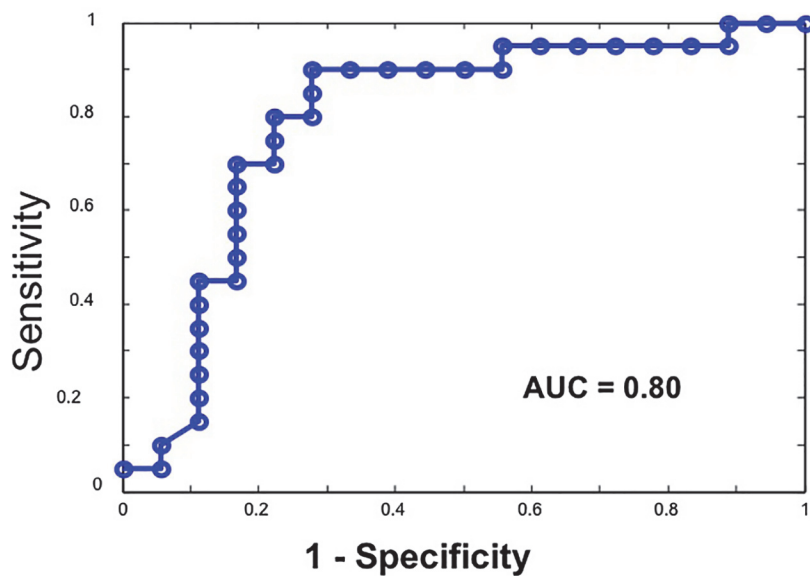


Fig 4. The ROC curve obtained from the composite dataset ($n = 38$) demonstrates an area under curve (AUC) of 0.80. This translated into a sensitivity = 88% and specificity = 72% for discriminating between progressors and non-progressors.

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Importantly, nanocytological field carcinogenesis detection has been demonstrated to be useful in screening for a variety of cancers that manifest field carcinogenesis (colon, lung, esophagus, pancreas, ovarian etc)[19–22,31]. One of the major novel aspects of this study is that it is the first to not simply assess presence/absence of tumor (screening), but rather prognosis. This has been fostered by our ability to overcome technical challenges and actually perform PWS nanocytology on intact tissue sections (the previously reported studies were all from cytological brushings). Thus, PWS nanocytology represents a platform technology that is applicable to screening/prognostication of a large number of malignancies.

Our approach of exploiting field carcinogenesis is particularly apropos for PCa given its multifocal nature. Indeed, the Gleason score is calculated by assessing the grade of the most common and next most common (or most aggressive) clone. While assessing individual tumor clones (e.g. 3-gene immunohistochemical signature) may correlate with the natural history of some PCa[32], given the clonal heterogeneity of most PCa and the semi-quantitative nature of these tests, they may have limited clinical utility. Intuitively, assaying field carcinogenesis within prostate specimens with or without PCa is potentially more attractive than focusing on genetic signatures obtained from specimens containing only PCa, as currently offered by tests such as Oncotype Dx, Prolaris, Decipher etc. [33]. There have been numerous other field effect markers (methylation, microRNA, gene expression) with putative prognostic ability, albeit performance to date has been modest[13,33,34]. Assessing nano-architecture is particularly powerful since it reflects the final common denominator beyond converging disparate molecular pathways. While other modalities (transmission electron microscopy[35] or karyometry[36] have noted abnormalities, their utilization as clinical tools are not feasible. The ability to distill PWS information into single biomarker (Ld) has considerable appeal from both a practicality and robustness perspective. Moreover, PWS quantifies the nano-architecture of specific compartments as well as throughout the cell.

Our previous work on nanocytological field carcinogenesis has been to assess presence of neoplasia; thus, this report represents a major new application for this approach, i.e. prognostication. Intuitively, it does seem logical to believe that field carcinogenesis profiling may be more promising than focusing on the tumor alone because of the multi-clonality that is the hallmark of prostate cancer. Indeed, the Gleason score is based on the grade of the most common and then either the next most common or most aggressive clone. Thus, it would be unclear which clone to analyze or how to synthesize gene expression data from a variety of clones. Our work parallels some of the data with liver cancer (another frequently multifocal disease) that profiling the uninvolved hepatocytes predicted recurrence in the liver after resection. [37]

Biologically, increased cellular nano-architectural disorder is intimately related to molecular events. From a physical perspective, an increased Ld implies “clumpiness” at the 20–200 nm length scales. These length scales encompass structures ranging from macromolecular complexes to small organelles. In the nucleus, disorder strength (Ld) is a measure of high order chromatin organization, which impacts upon multiple processes controlling gene expression. In keeping with this, we have demonstrated that nuclear Ld correlates with transcriptional activity[38]. In field carcinogenesis it is well established that gene expression is altered[37,39]. Furthermore, various gene alterations have been shown to be altered in prostate field carcinogenesis. However, given the genetic heterogeneity in tumors, finding the precise gene array is difficult. Since it appears that nano-architecture may be a “final common denominator” to the myriad of genetic/epigenetic alterations in early carcinogenesis, it should be particularly powerful. [20] In the cytoplasm, the determinants are more varied but an overarching theme is cytoskeletal organization[40]. Numerous cytoskeletal proteins (e.g. smooth muscle gamma actin) have been shown to be dysregulated in prostatic epithelium, further supporting the relevance to PCa[41].

There are many strengths of this study including its innovative nature and the well-characterized, prospectively followed cohort of men undergoing active surveillance. The clinical novelty includes use of field carcinogenesis to predict natural history of early stage PCa. From a biological perspective, this provides important insights into nano-architectural abnormalities in early carcinogenesis. Technologically, this study not only used PWS to assess prognosis, rather than just the presence of tumor, but also overcame the challenge of performing nanocytology on whole tissue sections.

Weaknesses that need to be acknowledged include modest cohort size and follow-up. On the other hand, this study represents the gold standard with the samples collected in a PRoBe-like fashion (samples prospectively collected and last half of dataset blinded; given that even in the unblinded dataset the investigator responsible for data acquisition was unaware of the clinical status, there is no possibility of bias). Another potential weakness of this study was the lack of clinically unequivocal endpoints, e.g. metastasis or death from PCa. Both of these endpoints, however, would require at least 10 years of follow up and would therefore be impractical. Furthermore, our definition of progression (increased Gleason score or increased volume of cancer) is well validated and widely used in clinical practice.

In conclusion, we demonstrate there are profound nano-architectural alterations in prostate cancer field carcinogenesis. Assessment of this through PWS nanocytology may represent a powerful biomarker to predict progression for men with early stage PCa. This approach may make PCa population screening more viable by mitigating the harms associated with identification of indolent disease. Moreover, this proof of principle study suggests that PWS nanocytology may potentially have a role in the clinical armamentarium against the screening overdiagnosis conundrum for a number of malignancies.

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Author Contributions

Conceived and designed the experiments: HR CB HS KK BH VB. Performed the experiments: HS DZ CM JC LB KK BH MQ JP MP. Analyzed the data: HS DZ CM JC LB CW. Contributed reagents/materials/analysis tools: HS DZ CM JC LB CW KK. Wrote the paper: HR CB HS VB.

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