Predicting Prostate Cancer Aggressiveness through a Nanoparticle Test

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Prostate cancer (PCa) is the most common malignancy and the second leading cause of cancer death in American men. Due to the lack of accurate tests to distinguish aggressive cancer from indolent tumor, prostate cancer is often over-treated. Post-surgery pathology analysis revealed that 30% of tumors removed by radical prostatectomy are deemed clinically insignificant and would not have required such invasive treatment.[1] Over-diagnosis and treatment of low-risk prostate cancer has serious and long-lasting side effect: as high as 70% of the patients who receive radical prostatectomy treatment will suffer a loss of sexual potency that cannot be remedied by drugs such as sildenafil citrate.[2] We herein report a simple nanoparticle-serum protein adsorption test that not only can distinguish prostate cancer from normal and benign conditions, but also is capable of predicting the aggressiveness of prostate cancer quantitatively. This new test could potentially deliver the long-expected and very much needed solution for better individualization of prostate cancer treatment.

The nanoparticle test we report here is based on a new bioanalytical technique, nanoparticle-enabled dynamic light scattering assay (NanoDLSay™) that we developed earlier.[3-8] This technique detects protein analytes by monitoring the nanoparticle size change upon specific binding or non-specific adsorption of target protein analytes to the AuNPs. Serum proteins tend to adsorb to citrate-protected AuNPs through primarily electrostatic interactions, Au-N and Au-S bonding to form a so-called "protein corona".[9-11] Our initial thought of using serum-AuNP adsorption assay for cancer biomarker discovery was based on a simple hypothesis that there may be some differences in the proteins adsorbed to AuNPs between cancer and non-cancer samples. From our previous study,[8] we discovered that there is a significant difference in the serum-adsorbed AuNP size between mouse serum samples with and without prostate tumor. The average particle size of the assay solution is substantially smaller for mice carrying large tumor grown from orthotopically injected PC3 cells compared to healthy control mice and mice bearing smaller tumor grown from LnCaP cells.[8] However, we did not observe the same dramatic difference from human serum samples with and without prostate cancer. We also could not explain the mechanistic origin of the observed differences.

The biggest challenge for cancer biomarker discovery and early cancer detection is that at early stage, the amount of specific molecules that are released from the tumor to the blood is very small. In the mice model study we conducted previously,[8] the relative tumor mass versus body weight of the PC3 and LnCaP mice was approximately 5% and 0.3%, respectively. These ratios would correspond to a tumor mass of 2.5 Kg and 150 g in a human patient with a body weight of 50 Kg. Such tumor sizes are far exceeding the tumor size from human patients with early stage cancer. The volume of a high grand human cancer is about 3.5-4.0 cc.[12] The only

human setting with prostate cancer > 150 g would be the metastatic setting. It is not surprising that the difference found from mice models was not observed from human serum samples.

In order to determine if the same difference observed from the mice models can be observed from human serum samples, we attempted to increase the amount of cancer-specific components by spiking the serum samples with primary tumor tissue extracts prior to the AuNP adsorption test. We hypothesize that when tumor-associated molecules are released to the blood, this may cause certain molecular changes to occur in the serum and such molecular changes are reflected in the AuNP-serum protein adsorption assay. By spiking a tumor tissue lysate directly to the blood, the concentration of tumor-associated molecules in the blood is synthetically increased, and as a result, molecular change of the serum similar to what occurs in *in vivo* may be more easily and clearly observed.

We have now indeed observed the very same difference from the human serum samples as we observed previously from mice models: the average particle size of human serum samples spiked with prostate tumor tissue is significantly smaller than the serum samples spiked with normal prostate tissue lysates in the serum-AuNP adsorption test. In a first set of experiment, we tested 8 male serum samples (4 from normal donors and 4 from patients with benign prostate hypoplasia, BPH) spiked with 4 different prostate tissue lysates that were from normal healthy control, tissue with Grade 1, Grade 2, and Grade 3 prostate adenocarcinoma (Figure 1A and B, plot B is an expansion of plot A). All tissue lysates were prepared in the same buffer (a modified RIPA buffer) using exactly the same protocol and all tissue lysates have the same total protein concentration of 1 mg/mL. Among the 8 sets of serum samples, 7 sets exhibited a clear trend of decreased average particle size when the serum was spiked with prostate tumor tissue lysates. The average particle size is inversely related to the grade of the tumor. We tested additional sets

of normal and tumor tissue lysates-spiked serum samples (including data presented here, total approximately 100 samples made from the combination of 10 serum samples spiked with 10 different tissue lysates), and the tests all showed the same trend of nanoparticle size reduction. BPH21 is the only exception observed throughout the whole study so far. Linear regression analyses suggest that all but BPH21 sample mixes (R squared = 0.2061, p=0.1382) had significant linear inverse correlations between the average particle size seen in the nanoparticle assay and the increasing tumor grade/staging, with goodness of fit R squared values ranging from 0.7406, p=0.0003 for N17 to 0.9734, p<0.0001 for BPH 23 sample sets.

In a second set of experiment, we tested two sets of normal serum samples spiked with normal, BPH, and PCa tissue lysates, respectively. In the first set of samples, the two PCa tissue lysates are both from Grade 3 tumor: PCa1 has a Gleason Score of (4+5) and PCa2 has a Gleason Score of (5+4). Again, the spiking of PCa tissue lysates to the serum led to a much smaller average particle size of the assay solution. Between the two BPH tissue lysates, one behaved like the normal tissue, and another one caused the particle size decrease of the assay solution; however, the decrease is smaller than the PCa tissue lysates. There is also a difference between the two PCa tissue samples: PCa2 caused more substantial particle size decrease than PCa1, even though PCa1 has a Gleason score of (5+4) while PCa2 has a Gleason score of (4+5). In contrast to the pathological analysis, the AuNP adsorption assay we conducted here suggests that PCa2 is more aggressive than PCa1. We also observed a concentration-dependent effect: PCa2 tissue lysate was spiked into the same serum in 1:20 and 1:100 (lysate:serum, v/v) ratio, respectively. With an increased amount of tissue lysate spiked into the serum, the particle size decreasing effect caused by the tumor tissue lysate is more dramatic.

The second set of samples showed very similar results (Figure 1D): the BPH tissue lysate-spiked samples showed slight nanoparticle size reduction compare to the normal samples, while 4 out of 5 tumor tissue lysate-spiked samples showed substantial nanoparticle size reduction compared to the normal tissue lysates. The most aggressive tumor among the five samples, #15 from a donor of age 47 with a Gleason score of 8, showed the largest nanoparticle size reduction. On the other hand, sample #11, a tumor with a Gleason score of 7, exhibited a similar behavior as a normal tissue sample. Even with the small number of samples tested in this study, marked discrepancy can already be seen between the new test results and the pathology reports.

Based on ours and others' studies,[8,9] we suggested that the major components in the protein corona formed on the AuNPs are abundant serum proteins. Circulating immunoglobulin G (IgG) is one of the most abundant blood serum proteins, with a typical concentration in the range of 5-15 mg/mL. IgG is known to have strong affinity towards citrate-protected AuNPs, a property that has been used for decades as a general method to prepare AuNP immunoprobes through a simple adsorption process.[13] If IgG is indeed a major component in the protein corona adsorbed to the AuNPs, the observed difference between cancer and non-cancer samples could then have been due to the unique interactions between tumor-specific molecules and IgG. To test if this is the case, we conducted the same AuNP adsorption assay on pure human IgG solution spiked with 42 prostate tissue lysates. Remarkably, we observed the similar particle size differences between tumor, benign and normal tissue-spiked IgG solution (Figure 2). Furthermore, the average particle size of the assay solution is inversely related to the tumor grade. The normal and the most aggressive Grade 3 tumor can be clearly differentiated without any overlap. Most benign and Grade 1 tumor tissues gave similar results as normal tissues, but with two samples resembling a more aggressive tumor profile. The assay results of 11 Grade 2 tumor tissues

extend over a wide range, reflecting exactly the ambiguous aggressiveness of the Grade 2 tumor. If the 'normal range' threshold is set as 2SD below the mean of the normal control group (red dotted line), the Grade 2 and 3 tumors are detected with 100% sensitivity. However, most low-grade prostate tumors are slow-growing tumors. A more extensive clinical study on a larger data set needs to be conducted to establish the best cut-off value/range for treatment selection. According to the data obtained so far, it appears that this suitable cut-off value may appear somewhere in the average particle size range of 190-200 nm (blue dotted line).

Although we do not have the full landscaping of the interactions between tumor molecules and serum IgG, one possible model for the assay mechanism is illustrated in Figure 3 to explain the observed results. IgG, either as a monomer or oligomer, causes AuNP cluster formation when adsorbed to the AuNPs. When mixing IgG solution or serum with tumor tissue lysates, the specific binding of tumor-specific molecules with IgG inhibits the crosslinking of the AuNPs, leading to a decreased average nanoparticle size of the assay solution. This mechanism suggests that serum IgG, by interacting with tumor-associated, enhanced or altered molecules, may be providing a natural defense line against prostate cancer spreading in the blood. If this natural defense system is exhausted, cancer metastasis can begin. This model, very interestingly, echoes well with the findings on the association between cancer and the immune system/functions.[14-16]

The above model also implicates that the best serum biomarker for early detection of aggressive prostate cancer may be found from the complexes with serum IgG, not as individual molecules in the blood. If the tumor-specific molecules are interacting with circulating IgGs at a typical antibody-antigen binding constant of 10^{10} M^{-1}, a simple calculation (assuming the serum IgG concentration is at an average value of 10 mg/mL) reveals that the concentration of IgG-complexed tumor biomarkers would be 600,000 times of the free biomarker molecules in the

blood. This means at early stage of cancer development, almost all cancer biomarker molecules released from the tumor site to the blood are complexed to the serum IgGs. Current bioassays are set almost exclusively to detect target protein analytes in individual molecular forms, not in complexes. This is perhaps a key reason why no suitable serum biomarkers have been found for early detection of any type of cancer, despite the extensive efforts invested from the whole research community in the last few decades.

In summary, we reported here a simple nanoparticle assay for quantitative assessment of the prostate tumor aggressiveness. The significant inverse correlation of the average nanoparticle size of the assay solution with tumor histological diagnostic grading suggests that the nanoparticle assay could potentially provide a more accurate diagnostic tool to assess the tumor aggressiveness than the current diagnostic practices. A large scale clinical study to validate this new test is fully justified. The current assay still requires the use of biopsied tissue samples. Currently, we are conducting further studies to identify the specific tumor molecules that are complexed with serum IgG. We expect sensitive serum biomarkers for early detection of aggressive prostate cancer using only blood samples to be discovered from these studies.

METHODS

Materials: Citrate-protected gold nanoparticle (AuNP) (15708-9) was purchased from Ted Pella Inc. (Redding, CA). The average diameter of the citrate AuNP is 100 nm and the concentration of the nanoparticle is 10 pM. Pure human IgG (ab91102) was purchased from Abcam (www.abcam.com). All human serum samples were purchased from Asterand Solutions (www.asterand.com). Tissue lysate samples were purchased from Protein Biotechnologies (www.proteinbiotechnologies.com). The protocol used for preparing the tissue lysates can be

found from the website of Protein Biotechnologies and is also summarized in the Supplementary Information along with the clinical data of tissue samples. All human tissue and serum samples used in this study are de-identified, archived specimens. University of Central Florida IRB committee approved the use of these commercially acquired samples with IRB exemption.

Dynamic Light Scattering Analysis: Particle size analysis of the assay solutions was conducted using an automatic DLS instrument, *NDS1200*, from Nano Discovery Inc. (Orlando, Florida, www.nanodiscoveryinc.com). This system is equipped with a 12-sample holder carousel to allow automatic measurement of 12 samples within 5-6 minutes. The measurement error for the pure AuNP solution with an average diameter of 100 nm is ±2 nm.

Sample Preparation and Assay Methods: To prepare tissue lysate-spiked serum samples or pure IgG solutions, 1 µL lysate at a total protein concentration of 1 mg/mL was mixed with 20 µL serum or IgG solution (concentration of 1 mg/mL in phosphate buffer, pH 7.4). The mixed solution was set at 4°C overnight for tissue lysate-spiked serum samples, and 30 minutes at room temperature for tissue lysate-spiked IgG samples before nanoparticle assay was conducted. To conduct the AuNP adsorption assay, 2 µL sample solution was mixed with 40 µL AuNP solution. The serum-AuNP solution was incubated for 8 min, and the IgG-AuNP was incubated for 3 min at room temperature before particle size was measured. All assays were conducted in duplicate and the error bars in each plot represent the standard error of the assay.

Statistical Analysis Methods: Where possible, data were analyzed by one-way ANOVA for multiple variant analysis, and using Student t test or nonparametric Mann–Whitney U tests for pairwise analyses. The results of these analyses are listed with appropriate p values and statistical parameters (n, mean, SD and/or SEM) on the figures, in figure legends and/or in the text. The designation 'ns' is listed where statistical analysis was applied and no statistical significance

between the groups was obtained. Linear regression analysis for data presented in Figure 1 was done with significance determined by comparing slope deviation from zero and the R squared value for fit on each of the assay sets presented.

Note: Supplementary Information is available.

AUTHOR CONTIRBUTIONS

Q.H. developed the conceptual framework of the study, designed the experiments, conducted studies, analyzed data and prepared the paper. S.A.L. participated in the design of the experiment, explanation of the experimental data, conducted the statistical analysis of the data, and prepared the paper. S.S. and H.H. assisted Q.H to conduct the experiments. D.A.D. and I.R-R provided clinical insights to the study and data explanation, and participated in the preparation of the paper.

COMPETING FINANCIAL INTERESTS

QH is an owner and officer of Nano Discovery Inc., a company that has licensed the NanoDLSay™ technology from University of Central Florida for commercialization. Other authors declare no competing financial interest.

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<h1 style="text-align:center">Figure Legends</h1>

Figure 1. The AuNP adsorption assay results of serum spiked with different prostate tissue lysates. A and B: the assay results of 8 serum (normal healthy donor = 4; BPH = 4) spiked with 4 prostate tissue lysates from normal, tissue with Grade 1, Grade 2, and Grade 3 prostate adenocarcinoma. The Gleason scores of the three tumor tissues are: 4(2+2), 5(2+3), and 9(5+4), respectively. A is the scatter-plot of all 32 samples and B is an expansion of A with 6 samples that have relatively smaller average particle sizes. Linear regression analysis of each sample set suggests that all but BPH21 sample mixes (R squared= 0.2061, p=0.1382) had significant linear inverse correlations between the average particle size seen in the nanoparticle assay and the increasing tumor grade/staging, with goodness of fit R squared values ranging from 0.7406, p=0.0003 for N17 to 0.9734, p<0.0001 for BPH 23 sample sets. C and D: two sets of assay results of two different serum samples spiked with tissue lysates from normal healthy donors, BPH patients, and PCa donors. In the first set (C), PCa2 tissue lysate was spiked to the serum at two different ratios: 1:20 and 1:100 (tissue lysate:serum, v/v). Statistically significant differences were found in the assay results between the normal controls (mean 196.1± 11.3SD) and BPH samples (mean 183.8 ± 6.4SD; p=0.0078, Student t test), BPH and PCa samples (mean 158.4± 16.3SD; p=0.0051, Mann-Whitney U test), and normal and PCa samples (p<0.0001, Student t test).

Figure 2. The AuNP adsorption assay results of pure human IgG solution spiked with different prostate tissue lysates. The concentration of IgG solution was 1 mg/mL in phosphate buffer (10 mM, pH 7.4). The 'normal range' threshold (red dotted line) was set as 2SD below the mean of the normal control group analyses.

Figure 3. A mechanistic model to explain the observed difference between normal and prostate cancer with different tumor grades in the AuNP adsorption assay.
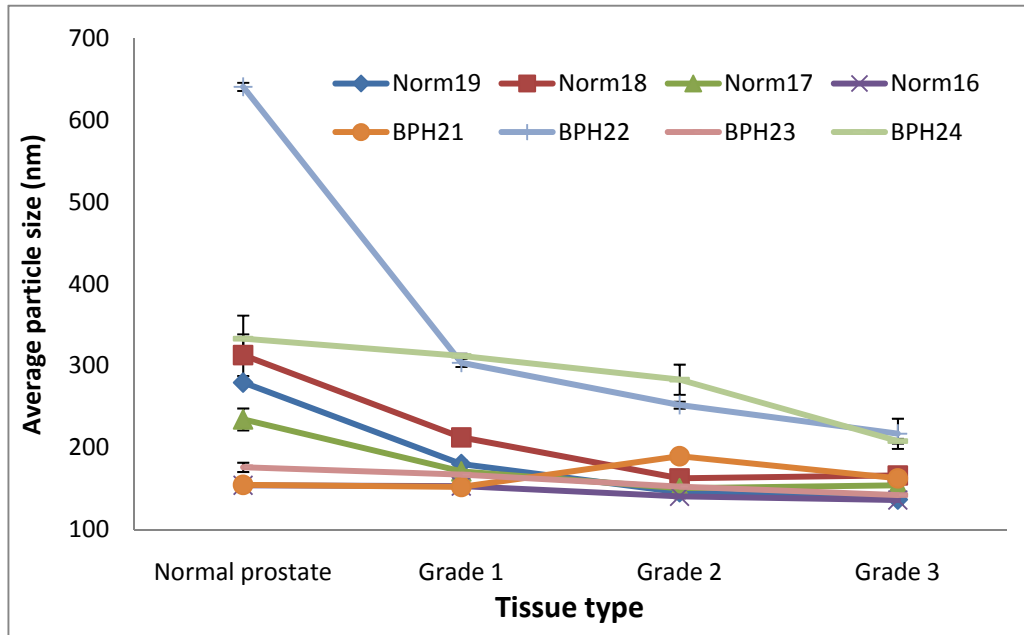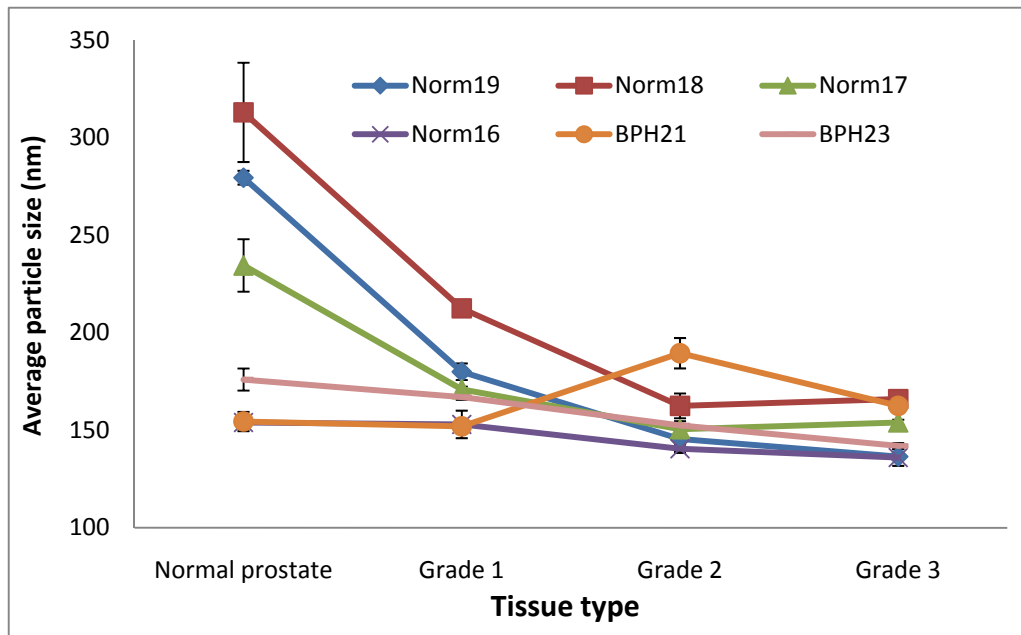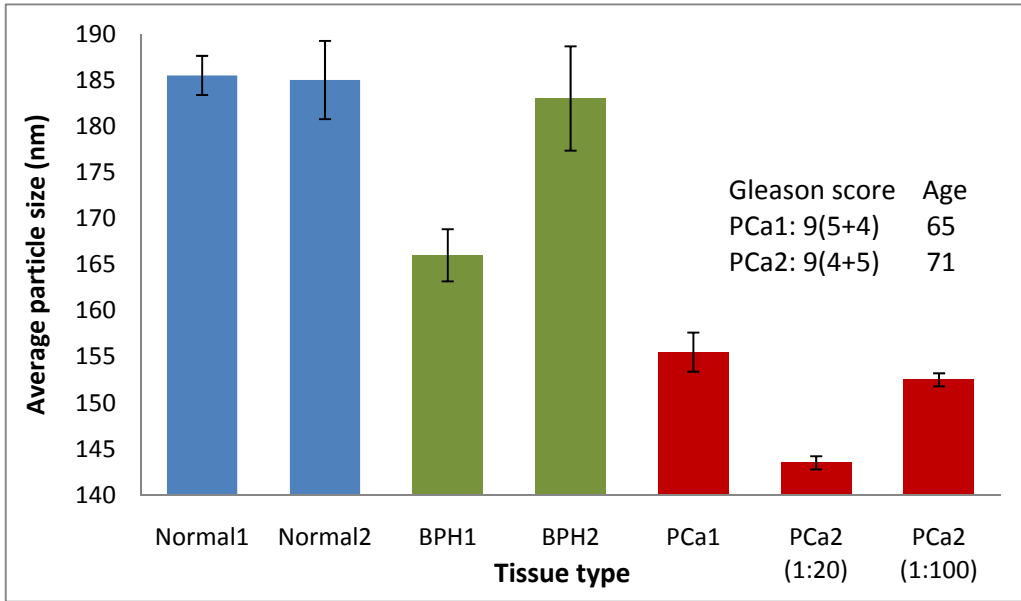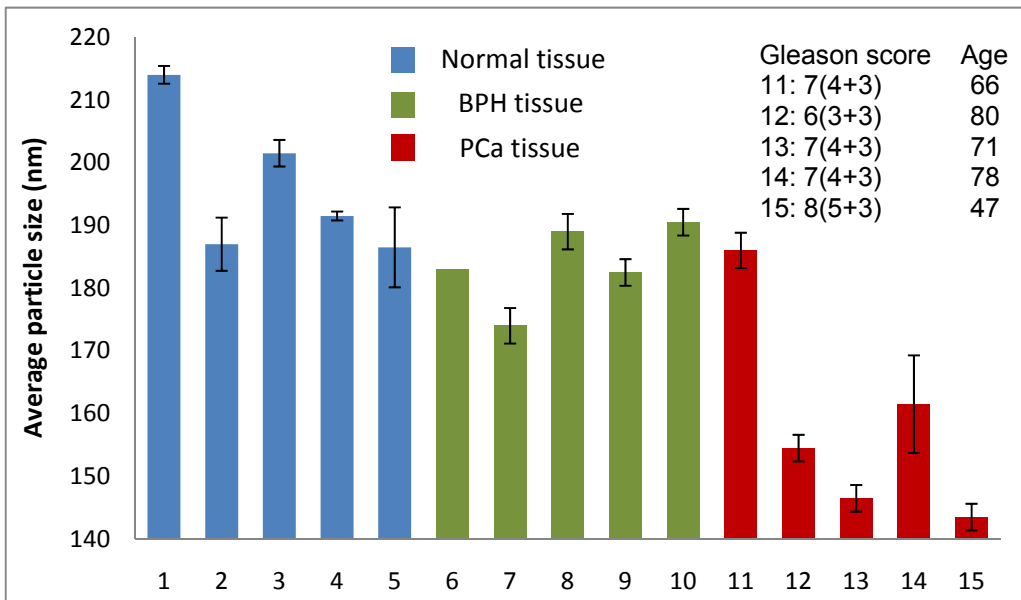
Figure 1A

Figure 1B

Figure 1C

Figure 1D

Figure 2

Figure 3

Without tissue lysate or with normal tissue lysate

AuNP
AuNP
AuNP

With tumor tissue lysate

AuNP

Y
IgG monomer, oligomer

Tumor molecule-complexed IgG