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14. ABSTRACT The Purpose of this proposal is to exploit a molecular correlate of the Gleason grading system for prostate carcinoma in order to: a) develop improved outcome predictors; and b) identify therapeutic strategies. The Scope of the project involves a tissue and blood-based analysis of candidate cancer grade-discriminatory markers in terms of their sensitivity, specificity, and outcome associations. The Major Findings to date demonstrated that molecular alterations measured at the transcript level can be verified to occur at the protein level, and have the potential to be used as prognostic, treatment stratifying markers (e.g. TMPRSS2). We have also shown that grade associated proteins can be detected in the blood of men with prostate cancer. However, to date, the proteins we have evaluated (e.g. OPN) discriminate advanced, metastatic disease from localized disease, rather than high grade from low-grade cancers.					
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INTRODUCTION

This proposal is designed to exploit a molecular correlate of the Gleason grading system for prostate carcinoma in order to: *a)* develop improved outcome predictors; and *b)* identify therapeutic strategies targeted toward features unique to aggressive cancers. The hypothesis underlying this proposal is that the specific molecular features that underlie prostate cancer grades define the capacity for tumor cell invasion and dissemination (progression) and represent unique diagnostic markers, and targets for therapeutic intervention.

The aims of the proposal are unchanged. They are: #1: To compare the predictive power of molecular versus histological Gleason categories for outcome predictions in the context of PSA relapse and prostate cancer-specific mortality. #2: Determine if grade-associated differences in prostate cancer protein expression are reflected by levels of cognate serum proteins. #3 Write final report. (Note the original Aim 3 involving animal studies of altering prostate cancer grade-associated functions was deleted due to recommendations by the reviewers).

Disease relevance: The development and progression of human prostate cancer is driven by the accumulation of genetic changes and influenced by epigenetic events. Through comprehensive studies of genome and gene expression alterations, it is clear that prostate cancers are profoundly heterogeneous, both at the molecular and clinical level. This proposal has direct relevance to both basic studies of prostate carcinogenesis and clinical studies of therapeutic strategies. The successful completion of this proposal has the potential to greatly improve clinical decision making by improving the accuracy of predicting which cancers may potentially require observation rather than primary therapy, or defining those cancers that should have additional systemic therapy in addition to local therapy by virtue of their high malignant potential. Also in the context of clinical care, several of the key nodes distinguishing low grade from high-grade cancer feature metabolic components that currently can be targeted by FDA-approved drugs originally designed for the treatment of diseases other than neoplasia.

BODY

The following summarizes the technical objectives for the proposal and the work accomplished during the 12-month interval between the start of the project (02/15/07) and the preparation of this report (02/16/08).

D.1. Technical objective 1: To compare the predictive power of molecular versus histological Gleason categories for outcome predictions in the context of PSA relapse and prostate cancer-specific mortality. (Months 1-24).

Objective 1a. *Antibody acquisition and evaluation.*

Task 1: purchase antibodies recognizing grade-determinant proteins (months 1-12). To date, we have purchased (or acquired) antibodies recognizing; TMPRSS2, MAOA, DAD1, ERG, Jagged, p63, AMACR, MUC1, FLNA, ALSCR2, CCNG2, FLH2, GSTMU1, PC4, RSK2, and SMS. The majority of these have previously been used in immunohistochemical studies, though not in prostate cancer.

Task 2: evaluate each antibody using a semi-quantitative immunoblot (months 1-12). We performed semi-quantitative immunoblots for approximately 1/2 of these antibodies with the specific intent of demonstrating a specific band. However, we have found that this approach is not definitive, and does not provide additional data in our hands due to the profound heterogeneity in the tissue, and highly variable expression in cell lines.

Task 3: optimize IHC staining using a fixation TMA, antibody dilutions and antibody retrieval methods (months 1-12). We have optimized titering and conditions for the following antibodies against a small panel of benign and neoplastic prostate cases: TMPRSS2, MAOA, DAD1, ERG, Jagged, p63, AMACR, MUC1, FLNA, ALSCR2, CCNG2, FLH2, GSTMU1, PC4, RSK2, and SMS—see reportable outcomes, Datta et al (2007).

Objective 1b. IHC analysis/confirmation of protein expression and Gleason pattern

Task 4: compare protein expression patterns relative to transcript measures by microarray (months 12-16). We have initiated these studies. We have re-organized grade-defining genes present on our original microarray studies into a format that can be directly compared to protein expression levels determined by IHC. We are now in the process of quantitating the protein expression across Gleason-appropriate prostate cancer cases, and will import the data directly for comparison. We have completed a thorough analysis of the expression of TMPRSS2, a grade-associated protein. *Figure 1* demonstrates

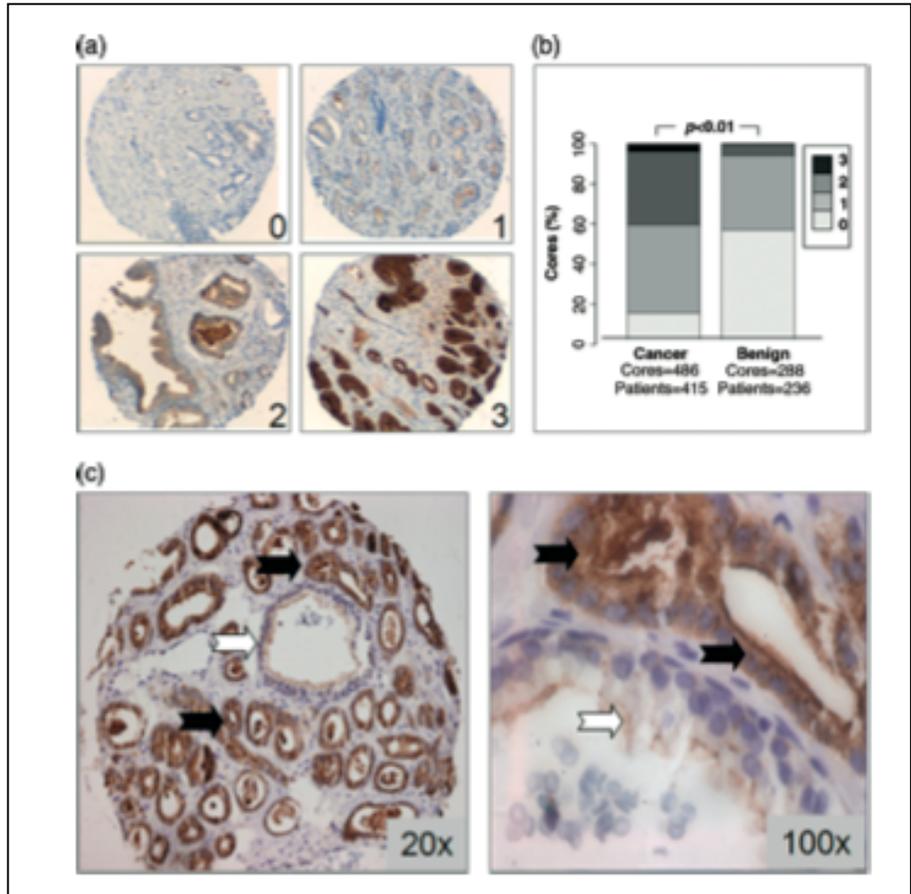


Figure 1. TMPRSS2 immunohistochemical staining of prostate tissue. (a) Examples of each scoring level of TMPRSS2 expression. Note TMPRSS2-stained material in some of the glandular lumina. (b) Summary of TMPRSS2 staining in benign and cancerous prostate epithelium. (c) Representative tissue microarray core showing intense TMPRSS2 staining in neoplastic epithelium (black arrows) relative to benign epithelium (white arrows) with loss of luminal polarity

TMPRSS2 expression in benign and cancer epithelium and shows mislocalization of TMPRSS2 in cancer cells. *Figure 2* demonstrates Gleason Grade-associated TMPRSS2 expression.

Objective 1c. IHC analysis of prostate cancer cohorts with outcomes reflected by PSA relapse and mortality.

Task 5: Stain and read Gelman TMAs: 20 antibodies (months 16-22)-pending

Task 6: Stain and read Stanford TMAs: 20 antibodies (months 16-22)-pending

Task 7: Determine statistical associations with outcomes (months 22-24)- pending

Task 8: Refine antibody/protein list to minimal redundant set. (months 22-24)- pending

D.2. Technical objective 2: *Determine if grade-associated differences in prostate cancer protein expression are reflected by levels of serum proteins (months 3-36).*

Objective 2a. Western Analysis for Antibody Q/C.

Task 9: prepare Western blots of serum proteins (months 3-6). We have acquired a panel of (anonymized) human serum protein samples that span a spectrum of a) absence of prostate cancer—biopsy proven; b) low grade prostate cancer; c) high grade prostate cancer; d) metastatic prostate cancer. The quality of the samples has been verified using Western analysis for abundant and low abundant proteins.

Task 10: determine specificity of immunoreactivity and semi-quantitation (months 7-12). We have completed Western analysis (blots) for 12 proteins/antibodies. Of these, four antibodies produced patterns indicating poor specificity, with multiple bands present. Of the remaining 8, three did not provide satisfactory detection, and these are being re-evaluated, or another antibody source is being evaluated. The remaining five demonstrating good specificity and we are scaling up a larger sample set to assess overall cancer/benign/grade evaluation. One protein, osteopontin, is capable of distinguishing metastatic cancer, but not early stage cancer, relative to individuals without cancer (*Figure 3*).

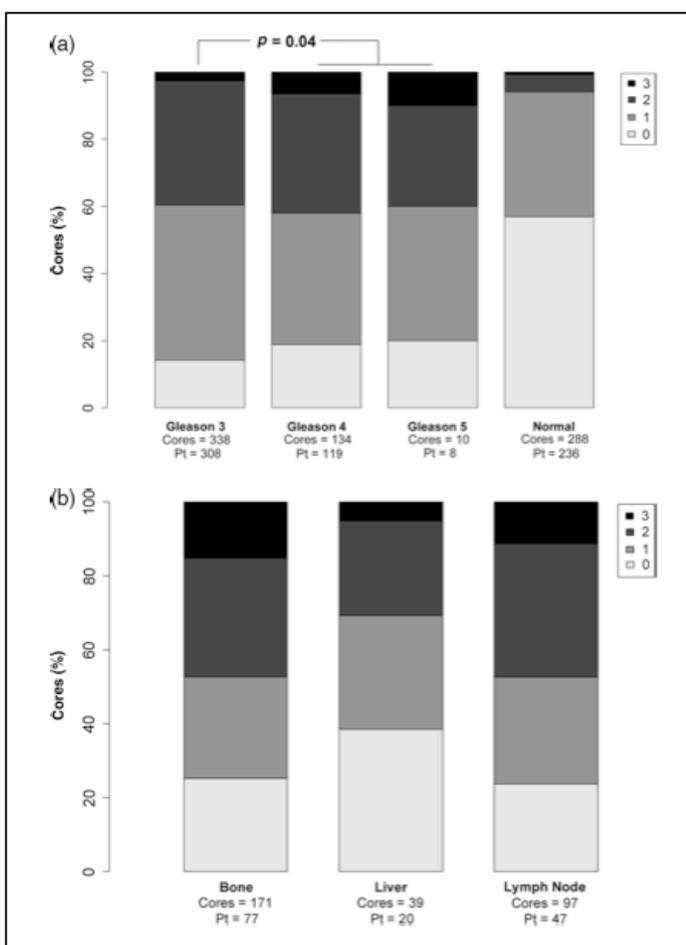


Figure 2. Tmprss2 expression and statistical analysis in primary and metastatic prostate cancer. (a) Percentage of Tmprss2-expressing cancer samples across Gleason grades 3, 4 and 5. The percentages of each scoring level are displayed. There is evidence that the intensity of staining increases in cancer ($p \leq 0.01$) and increases with Gleason grade ($p = 0.04$). (b) Percentage of Tmprss2-expressing prostate cancer metastases grouped by general tissue site. The percentages of each scoring level are displayed. There is no evidence that Tmprss2 is expressed differently at separate metastatic sites ($p = 0.88$)

Objective 2b. ELISA

Task 11: Prepare assay plates (months 6-10). We have prepared ELISA plates for three antibodies combinations to date. Data for our studies of osteopontin are shown in [Figure 1](#). We have established a system to avoid non-specific detection for these proteins. For the remainder of candidates, we are attempting to identify a second antibody as most ELISAs rely on two antibodies for specificity. If we are unable to identify a second antibody, we will proceed with a capture-ELISA or move toward other novel approaches such as SISCAPA (mass spectrometry-based assay—see task 16).

Task 12: Run q/c with recombinant protein standards. (months 10-11). For two proteins, we have run q/c for two proteins and these have passed our metrics.

Task 13: Analyze control and disease serum samples by ELISA (months 10-14). We have recently run serum samples representing low grade and high-grade cancer as well as benign and metastasis. Data analysis is in progress.

Task 14: Repeat Tasks 10-13 for two additional proteins (months 14-24)-pending

Task 15: Repeat Tasks 10-13 for two additional proteins (months 24-34)-pending

Objective 2c. SISCAPA

Task 16: Explore the utility of SISCAPA (Stable Isotope Capture by Anti-Peptide Antibodies) as an alternative (and improvement) to ELISA-based assays. (months 30-35)

D.3. Technical objective 3: Final Report: Complete data analyses, compile accomplishments and reportable outcomes and write final project report (Months 35-36)-pending.

KEY RESEARCH ACCOMPLISHMENTS

- We completed the acquisition and preliminary analysis of a panel of available antibodies that recognize a subset of Gleason Grade-associated molecular changes found in human prostate

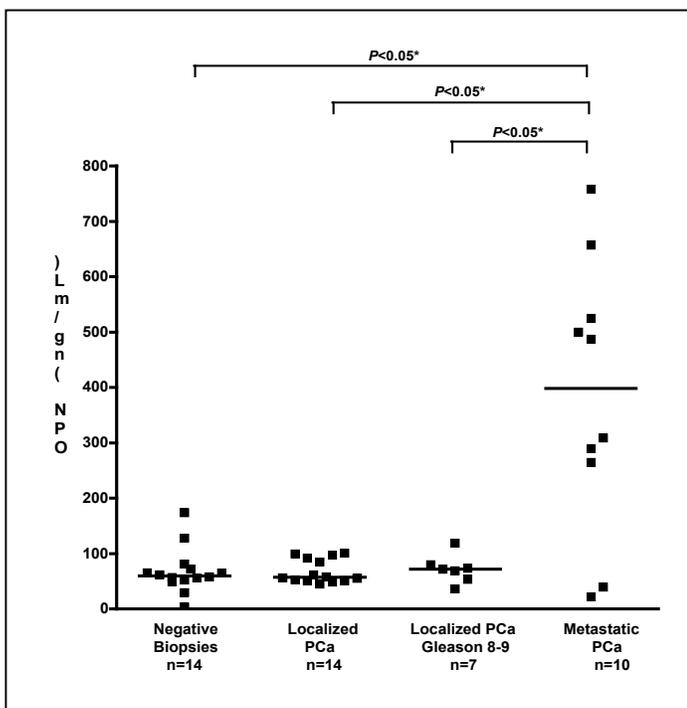


Figure 3 Scatter plots of osteopontin (OPN) in plasma from subjects with multiple negative biopsies, prostate cancer (PCa) patients with localized disease and Gleason scores of 7, PCa patients with localized disease and Gleason scores of 8 or 9, and PCa patients with bone metastases. Median values of each group are shown as horizontal lines. Significant differences across groups were tested with the Kruskal-Wallis nonparametric ANOVA test ($P=0.0156$) with Dunn's multiple comparison post-test. *Dunn's post-test P value for comparisons of metastatic PCa patients with the other subject groups.

cancer. The result of this study was published (see Datta et al), and a second manuscript is in preparation.

- We completed a comprehensive study of the Gleason Grade-associated protein TMPRSS2 and confirmed that TMPRSS2 protein (in addition to the transcript) corresponds to the degree of histological differentiation (grade). In addition, we found that TMPRSS2 is mislocalized in prostate cancer, and is expressed in metastasis. These results were published (see Lucas et al).
- Established ELISA methodologies in our laboratory, and determined that osteopontin protein is detectable in the blood of men with metastatic prostate cancer, but levels in localized prostate cancer are not sufficient to discriminate benign prostate from the presence of neoplastic disease (manuscript in preparation).

REPORTABLE OUTCOMES

Datta MW, True LD, Nelson PS, Amin MB. The role of tissue microarrays in prostate cancer biomarker discovery. (2007) *Adv Anat Pathol*. Nov;14(6):408-18.

Lucas J, True L, Hawley S, Matsumura M, Morrissey C, Vessella R, Nelson P. (2008) The androgen-regulated type II serine protease TMPRSS2 is differentially expressed and mislocalized in prostate adenocarcinoma. *J Pathol*. 215:118-125.

CONCLUSIONS

The research accomplished to date has demonstrated that molecular alterations measured at the transcript level can be verified to occur at the protein level, and have the potential to be used as prognostic, treatment stratifying markers (e.g. TMPRSS2). However, we have yet to identify a marker that can be used individually, with sufficient sensitivity/specificity to provide meaningful clinical outcomes across the population of men diagnosed with prostate cancer. Thus, proceeding with the aims of the proposal remain essential, as we anticipate that a panel of grade-associated proteins, rather than an individual marker, could achieve the requisite predictive values. We have also shown that grade associated proteins can be detected in the blood of men with prostate cancer. However, to date, the proteins we have evaluated discriminate advanced, metastatic disease from localized disease, rather than high grade from low-grade cancers. A key point in moving forward will include evaluating additional technologies that may enhance detection levels of tissue and blood proteins, ideally with a single rather than multiple antibodies.

REFERENCES

None

APPENDICES

None