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Award Number:
W81XWH-07-1-0149

TITLE:
Exploiting a Molecular Gleason Grade for Prostate Cancer Therapy

PRINCIPAL INVESTIGATOR:
Peter S. Nelson, MD

CONTRACTING ORGANIZATION:

Fred Hutchinson Cancer Research Center
Seattle, 98109

REPORT DATE:

March 2010

TYPE OF REPORT:

Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2010	Final	15 Feb 2007 - 14 Feb 2010

4. TITLE AND SUBTITLE
Exploiting a Molecular Gleason Grade for Prostate Cancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0149

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Peter S. Nelson, MD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fred Hutchinson Cancer Research Center

Seattle, WA 98109-1024

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
and Materiel Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

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. During this project period we have evaluated 37 prostate cancer antigens by Western blot and tissue-based assays and identified 15 with detectable levels in the plasma. Serum levels of osteopontin discriminated men with highly advanced cancer. Tissue levels of MAOA associated with relapse after prostatectomy. We determined that major challenge for the evaluation of all candidate outcome-associated proteins centers on the lack of antibodies with suitable performance characteristics of specificity and sensitivity. This limitation impacts both tissue (IHC) and ELISA-based studies of protein localization and quantitation. Newer technologies for protein quantitation such as SISCAPA and MRM may address these limitations, but they require additional optimization.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	31	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

This proposal was 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. We hypothesized that the specific molecular features that underlie prostate cancer grades define the capacity for tumor cell invasion and dissemination (progression) and may represent unique diagnostic markers and targets for therapeutic intervention.

The aims of the proposal remain unchanged: Aim #1: To compare the power of molecular versus histological Gleason categories for outcome predictions in the context of PSA relapse and prostate cancer-specific mortality. Aim #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: 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. In this context, the successful completion of this proposal has the potential to greatly improve clinical decision making by improving the accuracy of predicting which cancers may best be 'treated' by 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 (e.g. MAOA inhibitors).

BODY

The following summarizes the technical objectives for the proposal and the work accomplished during the 36-month interval between the initiation of the project (02/07) and the present completion of Year 3 Progress Report (02/2010).

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).

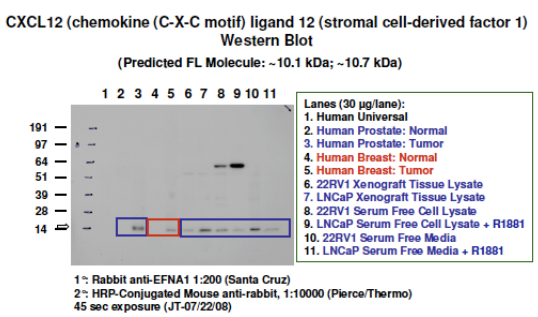
Objective 1a. *Antibody acquisition and evaluation.*

Task 1: purchase antibodies recognizing grade-determinant proteins (months 1-12). We purchased (or acquired) antibodies recognizing; TMPRSS2, MAOA, DAD1, ERG, Jagged, p63, AMACR, MUC1, FLNA, ALSCR2, CCNG2, FLH2, GSTMU1, PC4, RSK2, SMS. Postn, Arf1, Arf2, Cltc, Lamc2, Msn, Nrp1, Ppp1cc, Psma7, Tpm3, Tuba1a, Tuba1b, Tuba4a, AZGP1, ADAM7, ADAM9, ADAMTS5, ALCAM, CXCL12, EFNA1, HSPD1, JAG2, NOTCH3, STOML2 and PTPRF. (*Task Completed*)

Task 2: evaluate each antibody using a semi-quantitative immunoblot (months 1-12). We performed semi-quantitative immunoblots for the above antibodies with the specific intent of demonstrating a specific band. While we found that this approach is not definitive, and is influenced by profound heterogeneity in the tissue, and variable expression in cell lines, when moving forward to tissue staining, it is important to have an assessment of antibody specificity when in-

terpreting immunostains---e.g. avoid a false-positive signal. Very few of the commercial antibodies provided a single band of expected protein size and few discriminated between benign and neoplastic prostate tissues. However, a subset resulted in quite promising distinction between benign and cancerous prostate including MAOA, PTPRF, JAG2, and CXCL12 (CXCL12 protein expression shown in *Fig 1).* (*Task Completed*).

Figure 1. Elevated expression of CXCL12 protein in prostate cancer tissue. Note elevated expression in lane 3 (prostate cancer) relative to lane 2 (benign prostate. CXCL12 is also elevated in breast carcinoma and in prostate cancer cell lines



Task 3: optimize IHC staining using a fixation TMA, antibody dilutions and antibody retrieval methods (months 1-12). We optimized titering and conditions for antibodies against a panel of benign and neoplastic prostate cases: TMPRSS2, MAOA, DAD1, ERG, Jagged, p63, AMACR, MUC1, FLNA, ALSCR2, CCNG2, FLH2, GSTMU1, PC4, RSK2, and SMS, ABP280L, ITGAS, PSA, MIB1, Muc1, Jagged1, AMACR, CDK7, MTA1 and SKC4A1AP-----see Reportable Outcomes, *Datta et al (2007).* (*Task Completed*).

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 re-organized grade-defining genes present on our original microarray studies into a format for the direct comparison with protein expression levels determined by IHC. The majority of the expression levels between transcript and protein were not concordant. A component of this discordance is likely due to the protein localization in different cellular compartments (e.g. membrane vs nucleus). Another component of this discordance may lie in the lack of antibody specificity. A third component of this discordance may simply accurately reflect the physiological mechanisms of protein expression that includes transcriptional and post-transcriptional mechanisms. Overall, to date 10 genes gave clear concordant results between transcript and protein. These include MAOA, TMPRSS2, and DAD1 (see Reportable Outcomes *Lucas et al (2008)*). (*Task Completed*).

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). We completed 'outcomes' studies of the TMPRSS2 antibody on the Gelman TMA and found no correlation with relapse that was independent of Gleason Grade (see Reportable Outcomes *Lucas et al (2008)*). In the process of conducting these studies, we determined that this TMA is not optimal for completing all of the planned studies due to the limited numbers of these arrays that were actually be available. Thus, we initiated the construction additional TMAs with outcomes. We have developed a network of collaborating institutions (now 6 different sites) that will each construct outcomes TMAs suitable for cross-comparison of IHC results in the context of outcomes associations. We have identified 200 cases within our Center that will be used and construction of this TMA is in progress. We encountered delays in identifying, pulling, and marking the appropriate tissues due to manpower issues in the Department of Pathology. These are now resolved and construction is nearly complete. The planned IHC studies for Task 5 will commence at that

time. This effort was not under the direct control of the present proposal as the requirement of this resource for project completion was not anticipated and the development of this resource was not funded by this grant. Unfortunately, the full-completion of this objective must await the construction of the full TMA complement in order to have sufficient statistical power to determine protein expression, grade, and outcome associations. We are committed to this effort and plan on continuing this work beyond the project end-date. In the context of the work completed under the present proposal, we have acquired and evaluated more than 20 antibodies recognizing prostate cancer antigens, completed the protocols for using these antibodies for immunoblots and IHC, and evaluated these antibodies on test TMAs. Thus, we are poised to complete the outcome studies once the outcome TMAs are complete. (*Task 80% Completed*).

Task 6: Stain and read Stanford TMAs: 20 antibodies (months 16-22). The construction of the Stanford TMA was completed. However, the annotation of outcomes associated with these tissues is scheduled for updating by Dr. Stanford's group in March 2010. The most up-to-date outcomes data are required for accurately associating biomarker performance with clinical status. Thus, this task has not been completed to our satisfaction and, as with Task 6, we are committed to this effort and plan on continuing this work beyond the project end-date. (*Task 80% Completed*)

Task 7: Determine statistical associations with outcomes (months 22-24). We found no independent correlations with TMPRSS2 expression and clinical outcomes using the original Gelman TMA. We have completed a study of 4 grade/outcome-associated antibodies (ITGAS, PSA, MIB1, MTA1) and found no correlations with outcomes using the 50-case outcomes TMA. When the larger outcomes TMAs described above are completed and annotated, we plan to further evaluate outcome associations (Task 5 and 6). In studies of MAOA expression, we identified an association between altered MAOA expression and outcomes following primary treatment for localized prostate cancer (**Fig 2**) (See Reportable Outcomes: *Wu et al* (Submitted)). (*Task 80% Completed*).

A. Univariate model:

Risk Factors	Hazard Ratio	p-value	95% CI
Gleason Grade*	2.02	0.014	1.15-3.56
Age	1.65	0.464	0.43-6.27
Baseline PSA	1.01	0.671	0.97-1.05
MAOA Expression Change *†	1.66	0.027	1.06-2.59

B. Multivariate model

Risk Factors	Hazard Ratio	p-value	95% CI
Gleason Grade	1.85	0.038	1.03-3.32
MAOA Expression Change ‡	1.55	0.068	0.97-2.47

* p-value < 0.05
‡ 0.05 < p-value < 0.1
† MAOA expression in pre-treatment and post-treatment samples was measured by a quantitative realtime PCR. Expression changes were measured by cycle threshold difference (△CT) of MAOA between post-treatment and pre-treatment samples.

Figure 2. MAOA expression associates with Gleason Grade and with PSA relapse following primary therapy for prostate carcinoma.

Task 8: Refine antibody/protein list to minimal redundant set. (months 22-24). Currently, our minimal redundant set is comprised of MAOA and MUC1. Once outcome-association data are determined, additional antibodies/antigens will be added to this list. (*Task 80% Completed*)

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 acquired a panel of (anonymized) human serum protein samples that span a spectrum of a) absence of prostate can-

cer—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 Completed*).

Task 10: determine specificity of immunoreactivity and semi-quantitation (months 7-12). We completed Western analysis (blots) for 37 proteins/antibodies. Of these, 14 antibodies produced patterns indicating poor specificity, with multiple bands present. Of the remaining 23, 15 demonstrated good specificity. One protein, osteopontin, is capable of distinguishing metastatic cancer, but not early stage cancer, relative to individuals without cancer. Note, we have opted to use a stepwise approach for evaluating tumor-associated plasma proteins to save valuable human plasma. (The murine plasma samples were available from other ongoing studies and thus were not a cost to this project). Ten antibodies recognized an appropriate band in the plasma of mice bearing prostate tumors (see *Figure 3*). (*Task Complete*).

Objective 2b. *ELISA*

Task 11: Prepare assay plates (months 6-10). We prepared ELISA plates for eight antibody combinations to date. This effort exceeded the planned task objective of evaluating 6 targets (*Task Complete*).

Task 12: Run q/c with recombinant protein standards (months 10-11). This has proven to be a significant challenge and was not unexpected. The development of sensitive and specific ELISA systems is challenging, with major obstacles centered on specificity. Overall, four ELISAs have passed q/c metrics. For this reason, we also moved forward with SISCAPA analyses (see Task 16). (*Task Complete*).

Task 13: Analyze control and disease serum samples by ELISA (months 10-14). We have completed the analysis of serum samples representing low grade and high-grade cancer as well as benign and metastasis for the two antigens passing the ELISA q/c metrics. We identified a stage-association for serum osteopontin, but a grade-association was not identified for either protein (Figure 3). (*Task Completed*).

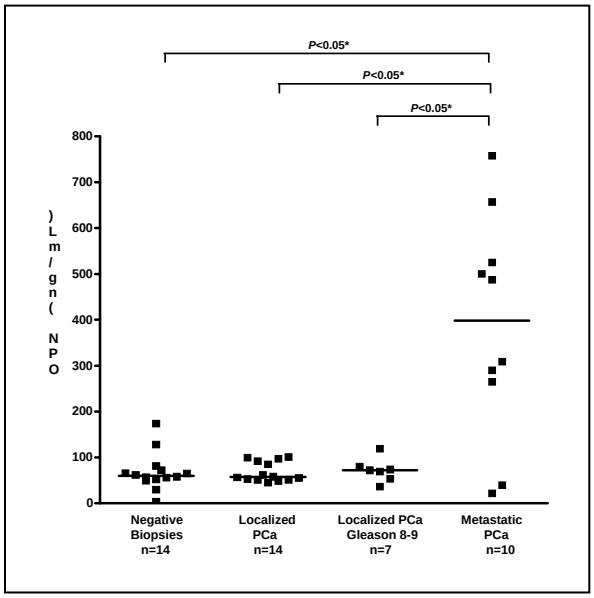
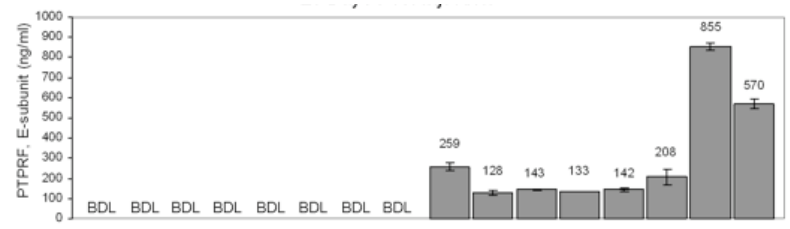


Figure 3. Scatter plots of osteopontin (OPN) in plasma from subjects with multiple negative biopsies, prostate cancer (PCa) patients with localized disease and Gleason scores of 7, PCa patients with localized disease and Gleason scores of 8 or 9, and PCa patients with bone metastases. Median values of 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.

Task 14: Repeat Tasks 10-13 for two additional proteins (months 14-24). We have completed the analysis of serum samples representing low grade and high-grade cancer as well as benign and metastasis for the two additional antigens passing the ELISA q/c metrics. We determined that PTPRF protein levels in the serum of tumor-bearing hosts significantly exceeded controls (Figure 4). To date, no grade association was identified (*Task Completed*).

Task 15: Repeat Tasks 10-13 for two additional proteins (months 24-34). Due to the failure of ELISA specificity, we evaluated the Luminex system for the utilization of single-

antibodies. Luminex assays were developed by Dr. Nicole Urban at our institution for 8 of the prostate cancer-associated proteins under analysis. None produced the requisite discriminatory power in a small test set of samples. These studies will be expanded to include a larger sample set in order to absolutely rule-out their utility. We have also begun studies using SISCAPA (see Objective 2c, below). (Task as specified, complete. Further studies are ongoing).



specific ELISA to measure concentrations of PTPRF in plasma. We found significantly higher levels of PTPRF in the plasma of tumor-bearing hosts (xenograft plasma) relative to control plasma in which levels of PTPFR were consistently below the limits of assay detection.

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). Due to the challenges we have observed in obtaining multiple high-quality (specific) antibody reagents, we evaluated SISCAPA and Multiple Reaction Monitoring (MRM) for protein quantitation. This involves the development of mass spectrometry peptide sprectra for the candidate proteins. We identified highly specific spectra for peptides corresponding to 18 candidate prostate cancer proteins. However, the assay detection limits have to date not been suitable for the accurate discrimination of control versus tumor-bearing hosts. Thus, our exploratory analysis indicates that while the overall technology is promising, further development will be required for low-abundance proteins or those with high normal variability. (*Task Complete*).

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

KEY RESEARCH ACCOMPLISHMENTS

- We completed the acquisition of 37 antibodies that recognize a subset of Gleason Grade-associated molecular changes found in human prostate cancer.
- We completed the analysis of 37 antibodies that recognize a subset of Gleason Grade-associated molecular changes found in human prostate cancer using IHC and plasma-based assays (Immunoblot).
- The levels of several proteins were capable of distinguishing benign from malignant prostate tissue (e.g. PTPRF, osteopontin, MAOA, CXCL12).
- MAOA expression levels associate with prostate cancer outcomes (relapse after radical prostatectomy) and may represent a assay of use in the clinical management of prostate cancers. Further, MAOA may also represent a target of prostate cancer therapies, further enhancing the utility of a tissue or blood-based assay of MAOA expression.
- We identified 2 proteins (osteopontin, PTPRF) that discriminated the presence of tumor from benign individuals using plasma-based assays. Additional studies will be required to determine if these are capable of discriminating cancer outcomes, and these studies are planned for the future.

REPORTABLE OUTCOMES

Year 1:

Datta MW *et al* (2007) The role of tissue microarrays in prostate cancer biomarker discovery. *Adv Anat Pathol*.Nov;14(6):408-18.

Lucas J et al (2008) The androgen-regulated type II serine protease TMPRSS2 is differentially expressed and mislocalized in prostate adenocarcinoma. *J Pathol*. 215:118-125.

Year 2:

Pritchard C *et al* (2009). Conserved gene expression programs integrate mammalian prostate development and tumorigenesis. Cancer Res. 2009 Mar 1;69(5):1739-47.

Year 3:

Wu M *et al* (Submitted) Chemotherapy-Induced Monoamine Oxidase Expression in Prostate Carcinoma Associates with Clinical Outcome and Functions as a Cytoprotective Resistance Enzyme. *Cancer Research*.

Whitmore, T et al (Submitted) Integrative analysis of N-linked human glycoproteomic datasets reveals PTPRF ectodomain as a novel plasma biomarker for prostate cancer. *Mol Cellular Proteomics*

*Whitmore, T et al (*In Preparation*)* Integrative analysis of transcript and protein profiling for the identification of serum prostate cancer biomarkers.

*Prueitt, R et al (*In Preparation*)* The value of plasma osteopontin in men with elevated PSA.

CONCLUSIONS

We have demonstrated that cancer and grade-associated mRNA abundance levels are associated with corresponding protein alterations for a subset (but not all) of candidates. We identified one very promising candidate protein, MAOA, that appears to have outcome discriminatory capability. While a component of the outcome prediction is linked to grade (we found that MAOA expression is associated with Gleason score), MAOA also exhibits features independent of grade, and can be induced by cytotoxic therapies. We developed an ELISA for quantitation of osteopontin and PTPRF and found that levels of these proteins in blood are associated with the presence of prostate cancer, though neither associates with Gleason grade. The major limitation to our studies at this juncture is the lack of availability of high quality antibody reagents that are essential for developing highly sensitive and specific protein-based assays. Although we evaluated SISCAPA and MRM-based methods, our conclusions to date is that these technologies are not yet superior to antibody-based assays, and thus, there is no simple route to assay development. Antibody-based assays remain the gold-standard, although the time and expense (and low success rates) substantially limits the rapid evaluation of protein-based biomarkers of disease presence and outcome.

REFERENCES

None

APPENDICES

Manuscript: Wu M *et al* (Submitted) Chemotherapy-Induced Monoamine Oxidase Expression in Prostate Carcinoma Associates with Clinical Outcome and Functions as a Cytoprotective Resistance Enzyme. *Cancer Research*.

Chemotherapy-Induced Monoamine Oxidase Expression in Prostate Carcinoma Associates with Clinical Outcome and Functions as a Cytoprotective Resistance Enzyme

Mengchu Wu[1*], Chung-Ying Huang[1*], William P. Harris[1], Hong Gee Sim[1], Jared Lucas, Celestia S. Higano[3,5], Lawrence D. True[4,5], Robert Vessella[5], Paul H. Lange[5], Mark Garzotto[6,7], Tomasz M. Beer[2] and Peter S. Nelson[1,3,4,5]

[1]*Divisions of Human Biology and Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA 98109-1024, Departments of* [3]*Medicine,* [4]*Pathology,and* [5Urology]*, University of Washington, Seattle, WA, and* [2]*Department of Medicine and Cancer Institute Oregon Health and Sciences University, Portland, OR.* [6]Division of Urology and Cancer Institute, *Oregon Health and Sciences University,* [7]Section of Urology, Portland VA Medical Center

*These authors contributed equally to these studies.

Running Title: MAOA influences chemotherapy resistance

Key words: prostate cancer, gene expression, monoamine oxidase, reactive oxygen, chemotherapy resistance

Address correspondence to:
Peter Nelson, M.D.
Division of Human Biology
Fred Hutchinson Cancer Research Center
Mailstop D4-100
1100 Fairview Avenue
Seattle, WA 98105-1024
Phone: (206)-667-3377
Fax: (206)-667-2917
Email: pnelson@fhcrc.org

ABSTRACT

Purpose: To identify molecular alterations in prostate cancers associating with relapse following neoadjuvant chemotherapy and radical prostatectomy.

Experimental Design: Patients with high-risk localized prostate cancer (TNM $\geq$ T2b or PSA $\geq$15 ng/ml or Gleason glade $\geq$ 4+3) were enrolled into a phase I-II clinical trial of neoadjuvant chemotherapy with docetaxel and mitoxantrone followed by prostatectomy. Pre-treatment prostate tissue was acquired by needle biopsy and post-treatment tissue was acquired by prostatectomy. Prostate epithelium was captured by microdissection, and transcript levels were quantitated by cDNA microarray hybridization. Gene expression changes associated biochemical relapse following chemotherapy and surgery were determined.

Results: Gene expression changes after chemotherapy were measured in 31 patients who completed 4 cycles of neoadjuvant chemotherapy. We identified 141 genes with significant transcript level alterations following chemotherapy that associated with subsequent biochemical relapse. This group included the transcript encoding monoamine oxidase A (MAOA). In vitro, cytotoxic chemotherapy induced the expression of MAOA and enhanced resistance to docetaxel. Elevated MAOA activity increased cellular reactive oxygen species and increased the expression and nuclear translocation of HIF1α. Inhibition of MAOA activity using the irreversible inhibitor clorgyline augmented the apoptotic responses induced by docetaxel.

Conclusions: The expression of MAOA is induced by exposure to cytotoxic chemotherapy, increases HIF1α, and contributes to docetaxel resistance. As MAOA inhibitors have been approved for human use, studies should be designed to exploit their potential for enhancing the clinical effectiveness of chemotherapy.

INTRODUCTION

Despite numerous clinical trials conducted over a span of more than four decades, only the administration of docetaxel has been shown to extend survival in patients with advanced castration resistant prostate cancer (CRPC) (1, 2). Unfortunately, the median life-span extension attributable to docetaxel therapy is only about 2 months, and few patients sustain durable complete remissions. For several types of solid tumors, notably neoplasms of the breast (3) and colon (4), cytotoxic drugs administered in conjunction with surgery or radiation have demonstrated survival benefits for those clinically-localized tumors with features indicating a predilection for early micrometastasis, or those with only documented local or regional spread. However, to date no studies have demonstrated a benefit for the addition of chemotherapy to primary surgical or radiotherapy approaches for prostate cancer as determined by outcomes of disease relapse or survival rates. Further, of four clinical studies evaluating neoadjuvant docetaxel alone or combined with other agents prior to prostatectomy, no complete pathological responses were identified in carefully studied resection samples (5-8). These results indicate that prostate cancers either exhibit a high degree of intrinsic resistance to taxanes and other chemotherapeutics, and/or rapidly acquire resistance phenotypes. Defining mechanisms underlying chemotherapy resistance is critical for selecting patients who may optimally benefit from specific regimens, and for designing new therapeutic strategies that either avoid---or specifically target resistance pathways.

To identify molecular changes associated with tumor cell exposure to chemotherapy agents commonly used in the treatment of prostate cancer, we conducted a prospective phase I-II clinical trial of neoadjuvant chemotherapy with docetaxel and mitoxantrone in patients with high-risk localized prostate adenocarcinoma (9). The neoadjuvant treatment sequence provides a unique setting for studying molecular characteristics of tumors before and after chemotherapy in which pre-treated and post-treated samples are readily available for analysis. There were no complete pathological responses identified at the time of prostatectomy, and thus viable cells evaluated following docetaxel and mitoxantrone treatment are presumably enriched for clones with molecular pathways contributing to therapy resistance. Although residual viable tumor cells were identified in each case, chemotherapy effects were evident. We previously reported the results of profiling gene expression changes in microdissected tumors acquired in the context of this study and found that post-therapy gene expression varied between individuals (10). However, the short follow-up post-therapy interval precluded analyses to determine if gene expression changes associated with tumor recurrence, a situation expected to occur through the survival

and subsequent proliferation of subclinical micrometastasis present at the time of initial treatment.

In the present study we sought to test the hypothesis that gene expression changes in prostate cancer cells following exposure to cytotoxic chemotherapy would associate with clinical outcomes. The median follow-up of the cohort of prostate cancer patients treated with neoadjuvant docetaxel and mitoxantrone was 40 months at the time of this analysis. Molecular changes associating with relapse included increases in transcripts encoding monoamine oxidase A (MAOA). MAOA is a key enzyme involved in the degradation of the biogenic and dietary monoamine neurotransmitters such as 5-hydroxytryptamine (5-HT, or serotonin) and norepinephrine. Amine metabolism is linked to essential cellular processes such as cell growth and differentiation, and the catalytic byproducts of MAOA, aminoaldyhyde and hydrogen peroxide, are known to cause oxidative damage that have potential implications for cancer, aging and neurodegenerative processes. We previously found that MAOA expression was upregulated in prostate cancers in association with higher Gleason grades (11), but effects on modulating cytotoxic drug effects are unknown. Two features of MAOA provided additional rationale for studies of this enzyme in the context of therapy resistance: approved inhibitors of MAOA are currently in clinical use for psychiatric illness, and the safety and toxicity profiles are established. Second, MAOA enzymatic properties have been exploited to develop positron-emission tomography (PET)-based imaging reagents for localizing MAOA activity in humans. These features provide a clear path for the rapid clinical evaluation of MAOA as a therapeutic target in the context of advanced prostate cancer once sufficient preclinical evidence of roles in therapy resistance are established.

MATERIALS AND METHODS

Patients and Study Description. Fifty-seven patients with high-risk localized prostate cancer (defined as TNM > cT2b or T3a or PSA $\geq$ 15 ng/ml or Gleason grade $\geq$ 4+3) were recruited between 2001 and 2004 for a phase I-II trial clinical trial of neoadjuvant chemotherapy. The design of this clinical trial has been previously described (9, 12). The protocol was approved by the Institutional Review Boards of the Oregon Health & Science University, Portland VA Medical Center, Kaiser Permanente Northwest Region, Legacy Health System, and the University of Washington. All patients signed informed consent.

Prostate Tissue Collection, Processing and Microarray Profiling of Gene Expression. Details of the tissue collection and gene expression profiling have been previously described in

detail (8). Briefly, transrectal ultrasound-guided needle biopsies were obtained from each patient at study entry and snap-frozen in liquid nitrogen prior to chemotherapy. At radical prostatectomy, cancer-containing tissue samples were snap frozen immediately after prostate removal. Benign and Neoplastic epithelial cells were separately acquired by laser-capture microdissection (LCM) using an Arcturus PixCell IIe microscope (Arcturus, Mountain View, CA). Total RNA was extracted from captured epithelium using a Picopure RNA isolation kit according to the manufacturer's instructions (Arcturus Inc., Mountain View, CA), and amplified using the messageAMP aRNA kit (Ambion, Austin, TX). Labeled cDNA probes were hybridized in a head-to-head fashion, pre-chemotherapy versus post-chemotherapy from the same individual, simultaneously to cDNA microarrays as we have previously described. Transcript abundance differences between patient-matched neoplastic epithelium before and after chemotherapy were compared using a random-variance t-test of log2 ratio measurements (13).

Quantitative reverse transcription PCR. cDNA was synthesized from 1µg amplified RNA (aRNA) from paired pre- vs. post-treated patient samples or 1µg RNA extracted from cultured cells, using 2µg random hexamers for priming reverse transcription by SuperScript II (200 U per reaction; Invitrogen). Quantitative reverse transcription real-time PCR (qRT-PCR) reactions were done in duplicate, using approximately 5ng of cDNA, 0.2uM of each primer, and SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in a 20 µl reaction volume and analyzed using an Applied Biosystems 7700 sequence detector. Samples were normalized to the cycle threshold value obtained during the exponential amplification of GAPDH. Control reactions with RNA or water as template did not produce significant amplification products. The sequences of primers used in this study were: GAPDH forward, 5'-CCTCAACGACCACTTTGTCA-3'; GAPDH reverse, 5'-TTACTCCTTGGAGGCCATGT-3'; MAOA forward, 5'-AAAGTGGAGCGGCTACATGG-3'; MAOA reverse, 5'-CAGAAACAGAGGGCAGGTTCC -3'; pleiotrophin (PTN) forward: 5'-GGGCAGCAATTTAAATGTTATGACTA-3'; PTN reverse: 5'-ACCCCCATTTTGCTGACTACATT-3'.

Cell Cultures and Treatments. The androgen responsive prostate cancer cell line, LNCaP and androgen insensitive prostate cancer cell line PC3 (both from the American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 100 IU/ml penicillin (Invitrogen Corp, Carlsbad, CA). The MAOA specific inhibitor, N-Methyl-N-propargyl-3-(2,4-dichlorophenoxy) propylamine hydrochloride (clorgyline), was purchased from Sigma-Aldrich (St Louis, MO). Docetaxel (provided by Sanofi-Aventis,

Bridgewater, NJ) was diluted in 70% ethanol and used at 1nM, 10nM, and 100nM concentrations for LNCaP cells and 50nM and 200nM for PC3 cells.

MAOA Expression. The full-length human MAOA cDNA was amplified from human placenta cDNA using the pair of primers, huMAOA_BstUI_170: GTCCGCGAAAGCATGGAG and huMAOA_EcoRI_1788: GCAGAGAGCATAAGAATTCAACTTCA. The amplification products were first cloned into pCR2.1 and then subcloned into the retro-viral vector pBABE-puro using BstU I and EcoR I sites. PBABE-puro-MAOA as well as the empty vector pBABE-puro were transfected into phoenix retro-viral packaging cells using Lipofectamine 2000 (Invitrogen Corp, Carlsbad, CA). The medium containing retrovirus expressing the vectors was collected and used to infect PC-3 cells followed by puromycin selection and confirmation of MAOA overexpression by both RT-PCR and western blot.

Cell proliferation and apoptosis assays. Cell proliferation was assessed using the MTS dye reduction assay (Promega Corp, Madison, WI). Briefly, 10 μl of MTS reagent was added to each well of the 96-well plate and incubated at 37°C for 60 min. The color change was assessed by measuring the absorbance value of each well at 450 nm with an ELx808 BioTek absorbance microplate reader (BioTek Instruments, Winooski, VT). The Apo-One Caspase-3/7 assay (Promega Corp, Madison, WI) was used to assess apoptosis as per the manufacturer's instructions.

MAO activity assay. MAO enzyme activity was assessed using the MAO-Glo assay (Promega Corp, Madison, WI). Cells were lysed using a passive lysis buffer (Promega Corp, Madison, WI) and a luminogenic MAOA substrate was added to the lysate to yield methyl ester luciferin. After incubating at room temperature for 1h, a detection reagent reconstituted from esterase and luciferase enzymes was added to each well and the luminescence measured using a Berthold L9505 BioLumat microplate luminometer (Berthold Technologies, Oak Ridge, TN).

Assay for Reactive Oxygen Species (ROS). ROS was detected by CM-H2DCFDA (Invitrogen Corp, Carlsbad, CA). Cells were trypisinized and washed with PBS, incubated either with 10μM of DCFDA in PBS or with PBS as a negative control at 37C for 30 min, washed with PBS and returned to media for a 30 min recovery period at 37C. Fluorescent cells in 1×10^5 cells were counted by flow cytometry. Mean fluorescence intensity was used as a measurement of ROS. The assay was done in a triplicate.

Western blot analysis. Whole cell lysates were prepared using RIPA buffer (25mM Tris pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with added protease inhibitors (Roche, Indianapolis, IN). Nuclear protein extracts were prepared using the Nuclear/Cytosol Fractionation Kit protocol (Biovision, Mountain View, CA). Thirty μg of protein were subjected to electrophoresis for 45 min at 200 V on a SDS polyacrylamide gel and transferred to a PVDF filter. The filters were blocked with 3% BSA for 1 and then incubated overnight with anti-MAOA polyclonal antibody, anti- NFkB (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HIF-1α (BD Biosciences, Franklin Lakes, NJ), or with anti-β-actin goat antibody (Promega Corp, Madison, WI). The filters were then incubated with species appropriate horseradish peroxidase labeled secondary antibodies (Thermo Scientific, Rockford, IL). Immuno blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

Statistical analysis. In order to identify genes whose expression was significantly related to biochemical (PSA) relapse free survival, gene expression profiles were analyzed for associations with biochemical relapse (http://linus.nci.nih.gov/BRB-ArrayTools.html). Briefly, we computed a statistical significance level for each gene related to biochemical relapse-free survival based on univariate proportional hazards models. Significant gene lists were determined by a threshold of p-value less than 0.01. The association of expression change of MAOA and time to PSA relapse was analyzed using a Cox proportional hazards model (Stata 8.0, Texus). Multivariate models included age, clinical stage, pathology Gleason grade, baseline PSA level, and post-chemotherapy MAOA expression change. The final model only included factors with a p-value less than 0.1. The statistical significance of assays of MAOA activity, cell proliferation, and apoptosis was evaluated using a Student's t test for 2-group comparisons. A value of $p<0.05$ was considered to be significant.

RESULTS

Clinical Trial Design and Patient Characteristics

Patients with high-risk localized prostate cancer (N=57) were enrolled in a neoadjuvant treatment protocol designed to administer four 28-day cycles of docetaxel 35 mg/m^2 and escalating mitoxantrone doses (Phase I) to a maximum of 4 mg/m^2 administered as 3 weekly doses followed by a 1-week off-treatment period (**Figure 1A**). We have previously reported details of this trial (8). Trans-rectal ultrasound-guided prostate biopsies were obtained prior to the first

course of treatment and within one month of completing chemotherapy, each patient underwent a radical prostatectomy. We quantitated gene expression changes in benign and neoplastic prostate epithelium from 31 patients who completed the full courses of chemotherapy with sufficient tumor in the pre- and post-therapy tissue samples to permit cell acquisition by microdissection. The attributes of the study participants are shown in **Supplemental Table 1** and in previous publications (8). The median Gleason score was 7 and approximately half of the participants (16/31) had a clinical stage equal to or exceeding T3. No patient had a complete pathological response. Median follow-up of the cohort is now 40 months, and to date, 11 patients (35%) have progressed to biochemical failure defined as a PSA ≥ 0.4 ng/ml or the initiation of therapy (14).

Alterations in MAOA Expression Associate with Biochemical Relapse Following Neoadjuvant Chemotherapy and Prostatectomy

We measured gene expression changes in prostate cancer cells exposed to chemotherapy *in vivo* with the hypothesis that these molecular alterations would comprise resistance mechanisms and pathways that could be exploited as therapeutic targets to improve treatment responses. As we previously reported, we used laser capture microdissection to acquire enriched populations of neoplastic epithelium from pre-treatment and post-treatment prostate tissue samples and measured gene expression changes by microarray analysis (10). Our initial studies performed at a median follow-up time of 19 months, did not have a sufficient post-treatment interval to assess for correlations of gene expression with subsequent clinical relapse. However, at a subsequent median follow-up time of 40 months, using an intermediate end-point of serum PSA of 0.4 ng/ml and rising as a surrogate indicator of ultimate progression to metastasis (14), 11 out of 31 patients were determined to have biochemical progression.

Of the chemotherapy-associated gene expression changes, 141 were significantly associated with PSA relapse-free survival (**Figure 1B** and **Supplemental Data File 3**). The gene encoding topoisomerase II alpha (TOP2A) was downregulated in patients with PSA relapse (**Figure 1B**), a finding consistent with a previous report demonstrated that chemoresistant testis cell lines had lower expression levels of TOP2A (15). Of those transcripts upregulated in neoplastic epithelial cells following chemotherapy (**Figure 1B**), we were particularly interested in monoamine oxidase A (MAOA), as we previously found MAOA to be upregulated in high-grade relative to low-grade prostate cancers (16). Of the 31 tumors evaluated, 23 (74%) expressed higher MAOA transcript levels in neoplastic relative to benign epithelium (**Supplemental Data File 2**).

We performed quantitative real-time PCR (qRT-PCR) to confirm MAOA expression levels and determined that 18/31 cases (58%) had increases in MAOA expression in prostate cancers following chemotherapy (**Figure 1C**). We next evaluated MAOA protein levels in two clinical cases where sufficient numbers of tumor cells could be assessed in needle biopsies before chemotherapy and in radical prostatectomy tissue post-chemotherapy from the same patient. In both cases, MAOA immunoreacitvity increased in neoplastic cells following chemotherapy (**Figure 1D**).

We incorporated the magnitude of MAOA mRNA alterations in a univariate Cox Proportional Hazard Model to estimate hazard ratios of several risk factors including age, baseline serum PSA before chemotherapy, pathologic stage, and histological Gleason grade, using time to PSA relapse as the clinical outcome. Of these variables, only greater MAOA transcript change and higher Gleason grade were significantly associated with biochemical failure after chemotherapy and prostatectomy (**Table 1**). In order to measure the net effect of MAOA expression change associated with time to PSA relapse, we further fit MAOA expression change and prostatectomy Gleason grade into a multivariate Cox Proportional Hazard Model. After adjusting for Gleason grade, the expression change of MAOA was marginally associated with time to PSA relapse (hazard ratio = 1.55, p-value= 0.068): the reduction in hazard ratio suggests that MAOA expression and status of tumor differentiation are associated.

MAOA Activity is Altered by Chemotherapy and Influences Cell Proliferation *In Vitro*

To confirm the effects of chemotherapy on MAOA activity, and evaluate the influence of MAOA on cellular phenotypes, we measured steady-state MAOA expression in several androgen-sensitive and androgen insensitive prostate epithelial cell lines. We found that androgen-responsive LNCaP cells expressed the greatest concentrations of MAOA protein (data not shown). VCaP cells also expressed relatively high amounts of MAOA compared to immortalized BPH-1 and 22RV-1 prostate epithelial cells while androgen-insensitive metastatic DU145 and PC-3 showed limited, but detectable MAOA protein expression (data not shown). We treated the LNCaP prostate cancer cell line with increasing concentrations of docetaxel ranging from 1 to 100nM. After 24 hours, MAOA activity increased significantly at each of the docetaxel concentrations, with higher levels affecting a greater magnitude of MAOA response (**Figure 2A**).

To determine if MAOA expression influences cell phenotypes, we expressed MAOA in PC3 cells, a line which exhibits very limited MAOA expression and activity at baseline. Relative to vector controls, PC3 cells expressing MAOA increased growth by 30% after 5 days in culture (**Supplemental Data File 2**). Inhibitors of MAOA enzymatic activity have been developed, in-

cluding several with approved clinical uses as anti-depressant agents. We determined that the MAOA inhibitor clorgyline could substantially reduce MAOA enzymatic activity in LNCaP cells at concentrations between 0.1-10μM (**Figure 2B**). The MAOA-induced elevated growth rate in PC3 cells was eliminated by treatment with the MAOA inhibitor clorgyline at doses that effectively suppressed MAOA enzyme activity (**Figure 2C**). Clorgyline treatment of LNCaP (**Figure 2D**) and VCaP (**Supplemental Data File 2**) cells that normally express high steady-state levels of MAOA also resulted in significant growth suppression.

Inhibition of MAOA Activity Modulates Docetaxel Effects on Prostate Cancer Cell Viability

In this clinical trial (**Figure 1A**), increased MAOA expression following chemotherapy associated with biochemical relapse, suggesting MAOA may confer resistance to chemotherapy-induced cell death. In order to determine if inhibition of MAOA modifies chemoresistance, we compared the effects of docetaxel toward wild-type PC3 cells that express very low levels of MAOA, and PC3 cells engineered to express high levels of active MAOA enzyme. The expression of MAOA significantly increased the number of viable cells after 48 and 72 hours of treatment with 200nM docetaxel (**Figure 3A**). The expression of MAOA reduced the cellular apoptotic response to docetaxel as determined by measurements of activated caspase 3 and 7 in the cells (**Figure 3B**). We next treated LNCAP and VCaP cells expressing high endogenous levels of MAOA with the MAOA inhibitor clorgyline followed one hour later by different concentrations of docetaxel. In LNCaP cells, clorgyline significantly augmented the growth suppression resulting from docetaxel administration, with the most pronounced additive effects seen at lower concentrations of docetaxel (**Figure 3C**). A similar effect was seen with the combination of clorgyline and docetaxel in VCaP cells (**Figure 3D**).

MAOA Expression Increases Reactive Oxygen Species and Activates Components of the HiF1α Program

We next sought to determine mechanisms by which MAOA could influence cellular resistance to chemotherapeutic agents. Monoamine oxidase enzymatic activity regulates oxidative deamination reactions of neurotransmitters such as serotonin, norepinephrine, and dopamine. Byproducts of this reaction include reactive oxygen species (ROS) such as H_20_2 and hydroxyl radicals (**Figure 4A**). Using a fluorescence-based assay we determined that PC3 cells engineered to express MAOA had significantly higher levels of ROS than control PC3 cells propagated in identical growth medium and assayed at the same cell density (**Figure 4B**). ROS have been shown to activate several signaling programs including NFκB, MAP-kinase, and HIF1α.

We determined that relative to control PC3 cells, PC3-MAOA cells expressed elevated levels of nuclear HIF1α (**Figure 4C**). Further, transcripts encoding the known HIF1α target gene vascular endothelial growth factor (VEGF), and the EMT-associated proteins vimentin (VIM), and pleiotropin (PTN) (17-20) were increased in PC3 cells expressing high MAOA levels (**Figure 4D**). To further evaluate the clinical relevance of MAOA and HIF1α, we evaluated whether elevated MAOA levels in prostate cancers treated with neoadjuvant chemotherapy associated with increased HIF1α. We found that changes in MAOA mRNA following chemotherapy were significantly correlated with changes in HIF1α levels, r=0.42 (p=0.02) (**Supplemental Data File 2, Panel E**).

DISCUSSION

For reasons that remain unclear, adenocarcinomas arising in the prostate appear to be particularly resistant to the cytotoxic effects of commonly-used anti-neoplastic drugs (1, 10). To identify resistance mechanisms, we designed a clinical trial to assess molecular features of tumor cells that associate with effects of chemotherapy exposure *in situ*. Among the gene expression changes we found to be altered by treatment, transcript levels of the gene encoding MAOA correlated with clinical relapse as defined by a rising PSA following radical prostatectomy. *In vitro* studies confirmed that docetaxel exposure increased the expression of MAOA in multiple prostate cancer cell lines, and inhibition of MAOA enzymatic activity using MAOA inhibitors enhanced the cytotoxicity of docetaxel.

Monoamine oxidase enzymatic activity functions to catalyze oxidative deamination reactions of neurotransmitters. Inhibition of MAO activity results in elevated levels of these amines in the central nervous system, a property responsible for antidepressant effects. Monoamine oxidase is encoded by two isozymes, MAOA and MAOB arranged in opposite orientation on the X chromosome, and expressed in the outer mitochondrial membrane in many diverse tissues throughout the body. MAOB preferentially metabolizes phenylethylamine and dopamine. MAOA metabolizes dopamine, serotonin and norepinephrine, though the dietary sympathomimetic tyramine is also a clinically-relevant substrate.

Studies of MAO expression or activity in the context of cell growth, stress responses, apoptosis and neoplasia have primarily focused on cells derived from the CNS. Most of the available data indicate that biogenic amines serve as antiapoptotic factors and protect mitochondria against pro-apoptotic events by permitting closure of the mitochondrial permeability transition pore and preventing the initiation or propagation of the pro-apoptotic cascade (21). In studies of neuronal cells, MAO inhibitors, leading to higher intracellular concentrations of monoamines and

reduced production of reaction products such as H202, function to protect cells from pro-apoptotic stimuli (22-24). A similar protective effect of MAO inhibitors toward melanoma cells has also been observed (21). Further, several studies of prostate cancer cells have demonstrated that serotonin, an MAO substrate, and serotonin receptor activity, associate with enhanced prostate cancer cell proliferation (25, 26).

In contrast to studies indicating that inhibiting MAO activity exerts effects that restrain tumor growth, several lines of evidence support a role for MAO in cancer promotion or progression. We previously found that MAOA expression correlated with prostate tumor cell differentiation status, with higher MAOA levels associated with higher Gleason patterns (27). Subsequent studies demonstrated that MAOA inhibits prostate cell differentiation (28), and the MAOA inhibitor clorgyline is capable of suppressing pro-oncogenic programs in prostate cancer cells (29). The expression of several other amine oxidases are increased in various cancer types such as those arising in the lung, breast, liver, and cervix (21). Of interest, the administration of L-deprenyl, an inhibitor of MAOB, resulted in significant reductions in tumor incidence in a rodent model of carcinogen-induced breast cancer (30). While a component of the anti-tumor effects were hypothesized to result from indirect activity toward prolactin production as well as neural-immune responses, direct effects of L-deprenyl on tumor cells were not ruled out.

An important area of inquiry centers on defining the mechanism(s) by which monoamine oxidases modulate tumor growth and chemotherapy resistance, and the corollary studies to determine how inhibitors of MAOA enhance chemotherapy sensitivity. The physiological functions of amine oxidases remain to be completely established, but known byproducts of amine metabolism include H_20_2 and hydroxyl radicals that have the ability to promote MAP-kinase signaling through redox-sensitive pathways (33). Our data indicate that one mechanism of therapy resistance links MAOA with the generation of ROS and activation of HIF1α. HIF1α expression---both protein stabilization and transcription, is promoted by elevated cellular ROS (31). Several studies have demonstrated that elevated HIF1α enhances cell survival in the context of cytotoxic chemotherapy and radiation (32-34). Thus, investigations into MAOA therapy resistance mechanisms should seek to determine if the primary effects of MAOA inhibition on prostate cancer treatment responses operate through HIF1α versus other ROS-dependent or ROS-independent pathways.

Two additional attributes of MAOA provide further impetus for studies involving therapy for prostate carcinoma. First, tracers for positron emission tomography (PET) imaging of MAOA activity have been developed. For example, [11C] clorgyline and L-[11C] deprenyl have been used to image MAOA and MAOB, respectively, in studies of CNS enzyme activity (35). The

MAOA ligand Harmine has also been labeled with [11C] and used to image a subset of neuroendocrine carcinoid and pancreatic tumors (36). These tracers offer the opportunity to quantitate on-target effectiveness of inhibitors, as well as overall tumor responses. A second notable feature of MAO involves a recent finding that MAO blockade appears to exhibit differential cytoprotective effects toward benign versus malignant tissues (37). Nontumorigenic and tumorigenic human cells were treated with MAOA and MAOB inhibitors, and exposed to gamma irradiation or cisplatin chemotherapy. The MAO inhibitors reduced radiation effects in the benign, but not the malignant cells, and MAO inhibition further suppressed the growth of malignant cells relative to those exposed only to radiation. MAO inhibitors also reduced cell death due to chemotherapy exposure in the benign but not malignant cells. As several MAOA inhibitors have already been approved for clinical use, exploiting their potential to enhance the effectiveness of therapies for prostate cancer should be straightforward.

ACKNOWLEDGEMENTS

We thank the patients who participated in this study for their altruism. We thank Alex Moreno for administrative assistance, David Gifford for assistance with tissue dissections, Roman Gulati, Ruth Etzioni and Sarah Hawley for statistical analyses, and Ilsa Coleman for figure preparation. We thank William Ellis and support staff in the Departments of Urology and Medical Oncology for contributions to patient care. MW was supported by a fellowship from the AUA Foundation. CYH was supported by a fellowship from the DOD CDMRP in Prostate Cancer (PC050489). This work was supported by grants R01 CA119125, PC060595, and the PNW Prostate SPORE (CA97186).

TABLES

Table 1. MAOA Expression and Post-Therapy Biochemical Relapse

A. Univariate model:

Risk Factors	Hazard Ratio	p-value	95% CI
Gleason Grade*	2.02	0.014	1.15-3.56
Age	1.65	0.464	0.43-6.27
Baseline PSA	1.01	0.671	0.97-1.05
MAOA Expression Change *†	1.66	0.027	1.06-2.59

B. Multivariate model

Risk Factors	Hazard Ratio	p-value	95% CI
Gleason Grade	1.85	0.038	1.03-3.32
MAOA Expression Change ‡	1.55	0.068	0.97-2.47

* p-value < 0.05
‡ 0.05 < p-value < 0.1
† MAOA expression in pre-treatment and post-treatment samples was measured by a quantitative realtime PCR. Expression changes were measured by cycle threshold difference (△CT) of MAOA between post-treatment and pre-treatment samples.

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FIGURE LEGENDS

Figure 1. MAOA expression is induced *in vivo* following exposure to docetaxel and mitoxanatrone and associates with biochemical relapse. **(A)** Schema of the neoadjuvant chemotherapy and prostatectomy trial. **(B)** Heat-map of gene expression changes in prostate carcinoma cells that associate with biochemical relapse following radical prostatectomy. Columns are 31 patients and rows are 141 genes. Red indicates increased expression following chemotherapy, green indicates decreased expression and black indicates no change. Grey indicates absent or poor quality data. R is relapse and NR is no relapse. **(C)** MAOA transcript alterations shown for 31 study participants. Ratios are intra-individual post-treatment versus pre-treatment MAOA transcript abundance measurements determined by qRT-PCR from microdissected neoplastic epithelium. **(D)** MAOA protein expression determined by immunohistochemistry. Representative images of neoplastic prostate epithelium acquired before and after chemotherapy exposure. Brown pigment indicates presence of MAOA protein.

Figure 2 MAOA expression is induced by chemotherapy *in vitro* and promotes cell proliferation. (A) Treatment of LNCaP prostate cancer cells with docetaxel increases MAOA enzyme activity. **(B)** The irreversible MAOA inhibitor clorgyline reduces MAOA activity in LNCaP cells (*$p<0.05$). **(C)** Expression of MAOA in the PC3 prostate cancer cell line (PC3-MAOA) increases cell growth which is inhibited by clorgyline (MAOI). **(D)** LNCaP cell growth is inhibited by the MAOI clorgyline (*$p<0.05$).

Figure 3. MAOA expression inhibits docetaxel cytotoxicity. (A) PC3 cells expressing MAOA exhibit enhanced cell survival following 48 and 72 hours of exposure to docetaxel (*$p<0.05$) **(B)** Elevated MAOA expression reduces cellular apoptosis following exposure to docetaxel. The addition of clorgyline enhances the cytotoxicity of docetaxel toward LNCaP **(C)** and VCaP **(D)** prostate cancer cells (*$p<0.05$)**.**

Figure 4. MAOA expression Increases ROS and the expression of HIF1α and HIF1α pathway genes. (A) Deamination reaction catalyzed by monoamine oxidase (MAO) enzymes produces H_20_2 as a reactive oxygen species (ROS) byproduct. **(B)** ROS levels are increased in PC3 cells expressing MAOA (*$p<0.05$). **(C)** Expression of MAOA in PC3 cells results in elevated nuclear HIF1α and NFκB protein. **(D)** Expression of MAOA in PC3 cells results in increased levels of transcripts encoding known HIF1α target genes.

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Figure 1

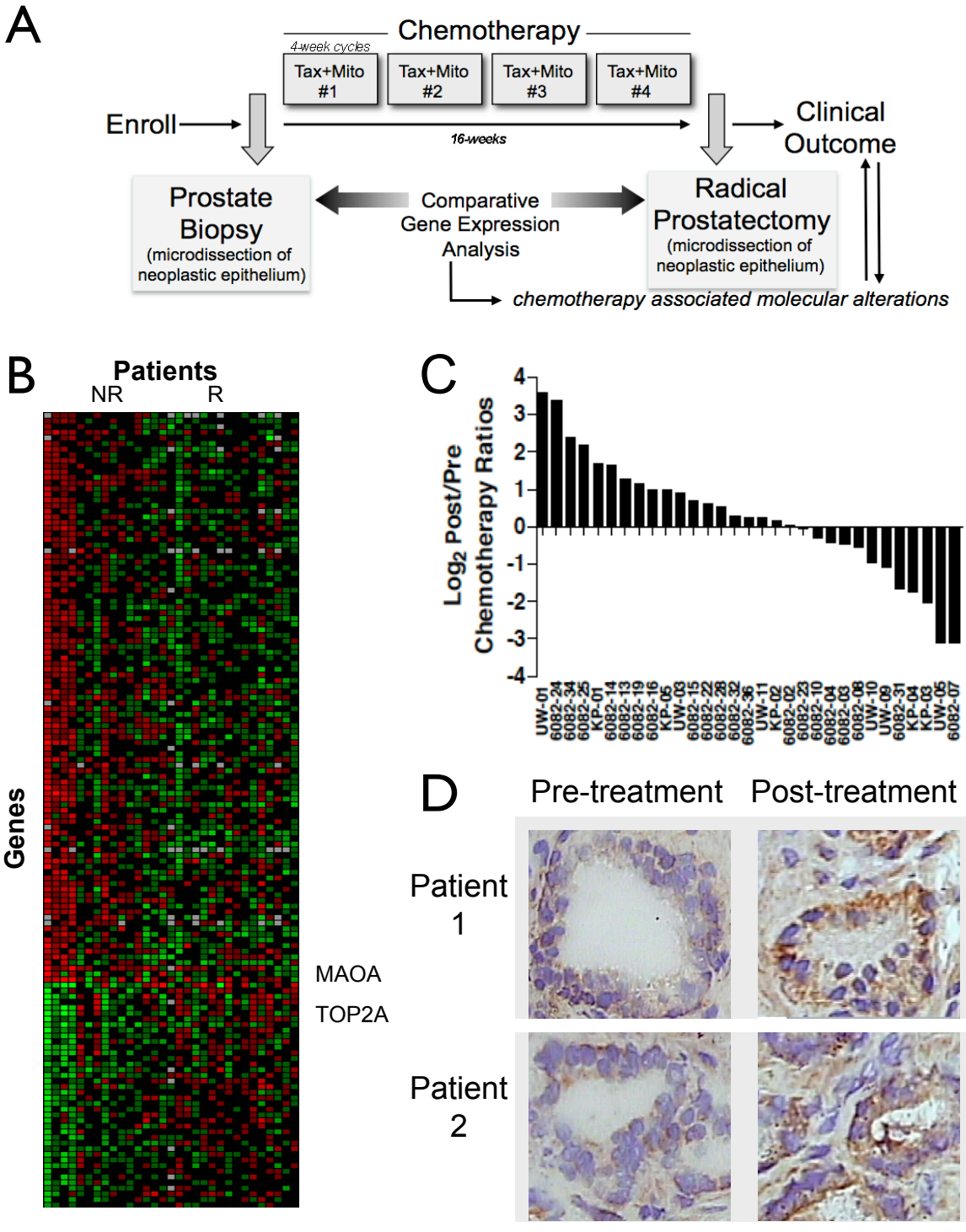

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Figure 2

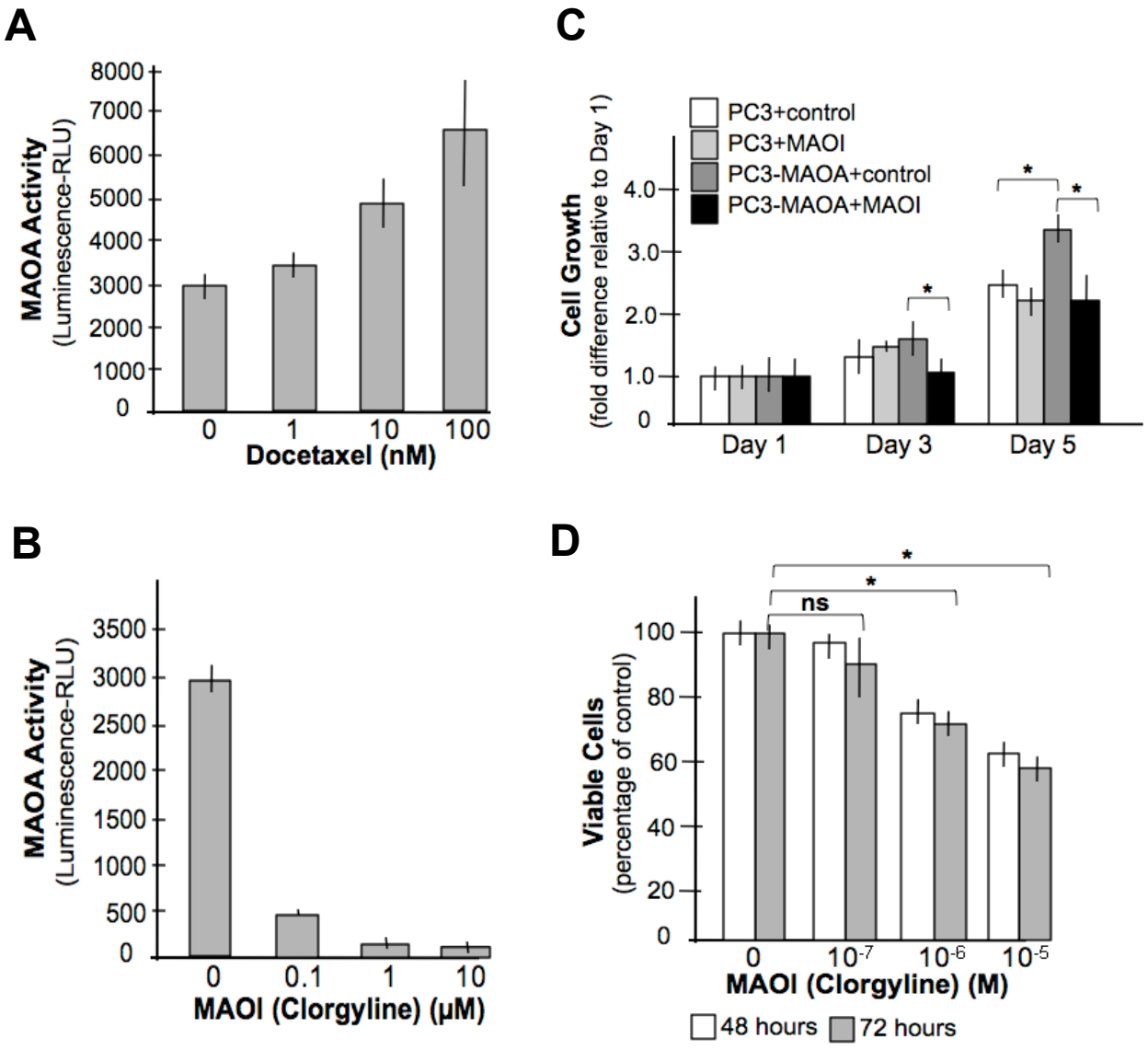

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Figure 3

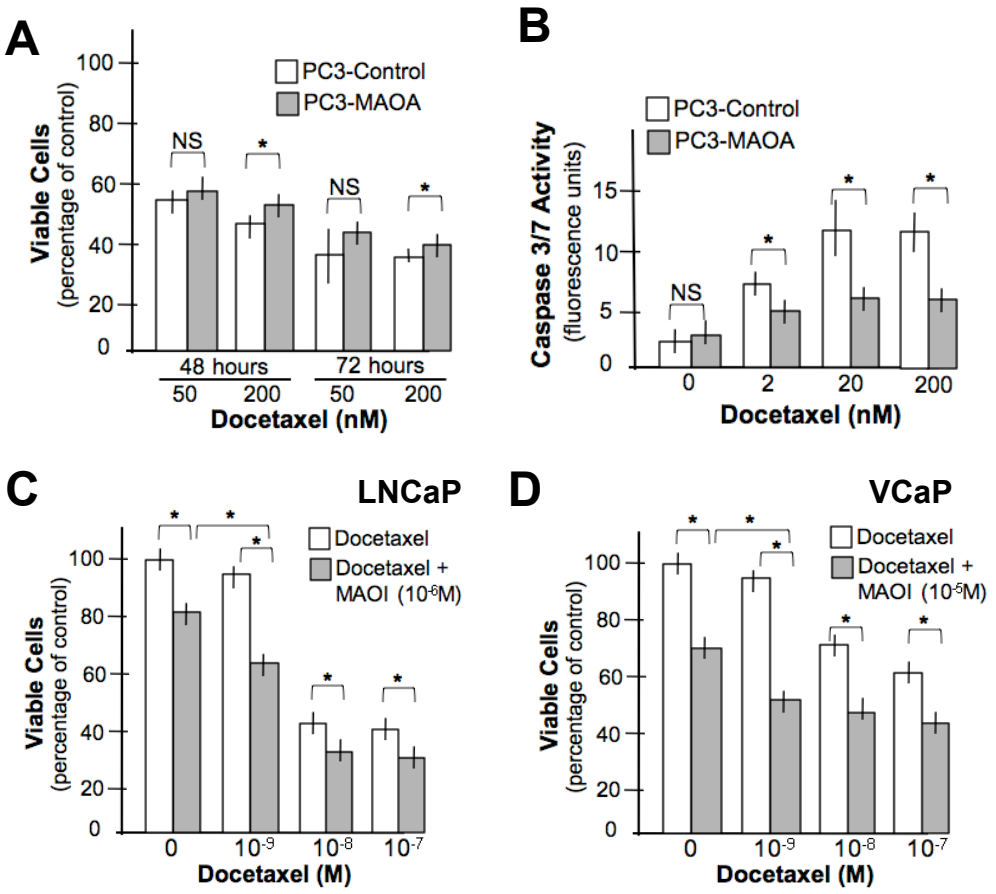

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Figure 4