

hCAP-D3 Expression Marks a Prostate Cancer Subtype With Favorable Clinical Behavior and Androgen Signaling Signature

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Abstract: Growing evidence suggests that only a fraction of prostate cancers detected clinically are potentially lethal. An important clinical issue is identifying men with indolent cancer who might be spared aggressive therapies with associated morbidities. Previously, using microarray analysis we defined 3 molecular subtypes of prostate cancer with different gene-expression patterns. One, subtype-1, displayed features consistent with more indolent behavior, where an immunohistochemical marker (AZGP1) for subtype-1 predicted favorable outcome after radical prostatectomy. Here we characterize a second candidate tissue biomarker, *hCAP-D3*, expressed in subtype-1 prostate tumors. *hCAP-D3* expression, assayed by RNA in situ hybridization on a tissue microarray comprising 225 cases, was associated with decreased tumor recurrence after radical prostatectomy ($P = 0.004$), independent of pathologic tumor stage, Gleason grade, and preoperative prostate-specific antigen levels. Simultaneous assessment of *hCAP-D3* and AZGP1 expression in this tumor set improved outcome prediction. We have previously demonstrated that *hCAP-D3* is induced by androgen in prostate cells. Extending this finding, Gene Set Enrichment Analysis revealed enrichment of androgen-responsive genes in subtype-1 tumors ($P = 0.019$). Our findings identify *hCAP-D3* as a new biomarker for subtype-1 tumors that improves prognostication, and reveal androgen signaling as an important biologic feature of this potentially clinically favorable molecular subtype.

Key Words: prostate cancer, molecular subtypes, prognostic markers, expression profiling, androgen signature

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Prostate cancer is the most frequently diagnosed cancer among men in the United States, with 1 in 6 men being diagnosed in their lifetime.⁶ Nevertheless, most men with prostate cancer will not die from their disease, suggesting that a large fraction of prostate cancers are clinically indolent. Although therapies have been documented to improve prostate cancer survival, there is a growing consensus that many men can be managed with active surveillance.^{9,17} Unfortunately, available clinical markers of tumor aggressiveness—preoperative serum prostate-specific antigen (PSA) levels, tumor stage, and biopsy Gleason grade—have significant shortcomings in accurately prognosticating individual patients, and, by default, most men opt for aggressive local therapies such as surgery or radiation therapy. Additional markers are needed to determine which men might benefit from more aggressive therapies and which might be spared unnecessary and potentially harmful interventions. Potential markers under evaluation include measures of tumor proliferation, DNA ploidy, and selected cancer genes.¹

In previous studies, we characterized the gene-expression patterns of prostate tumors using DNA microarrays.¹⁰ Unsupervised hierarchical cluster analysis identified 3 subtypes of prostate cancer, not previously recognized or distinguishable histologically, with different patterns of gene expression (illustrated in Fig. 1A). Subsequent tumor recurrences were observed only among patients from subtypes 2 and 3, suggesting that subtype-1 represented a relatively clinically favorable subclass of prostate cancer. Indeed, surrogate markers for the subtypes, assayed by immunohistochemistry (IHC) in tissue microarray (TMA) format on an independent cohort of patients, confirmed that subtype-1 cases [defined as AZGP1 (zinc-alpha-2-glycoprotein)+ and MUC1 (mucin 1)–] were associated with significantly lower recurrence rates compared with combined subtype 2 and 3 cases (AZGP1–, MUC1+), independent of pathologic stage, tumor Gleason grade, and preoperative serum PSA levels.^{5,10} Here we identify *hCAP-D3* as a second tissue biomarker for favorable prognosis subtype-1 prostate tumors, and characterize features of its expression in clinical prostate cancer specimens.

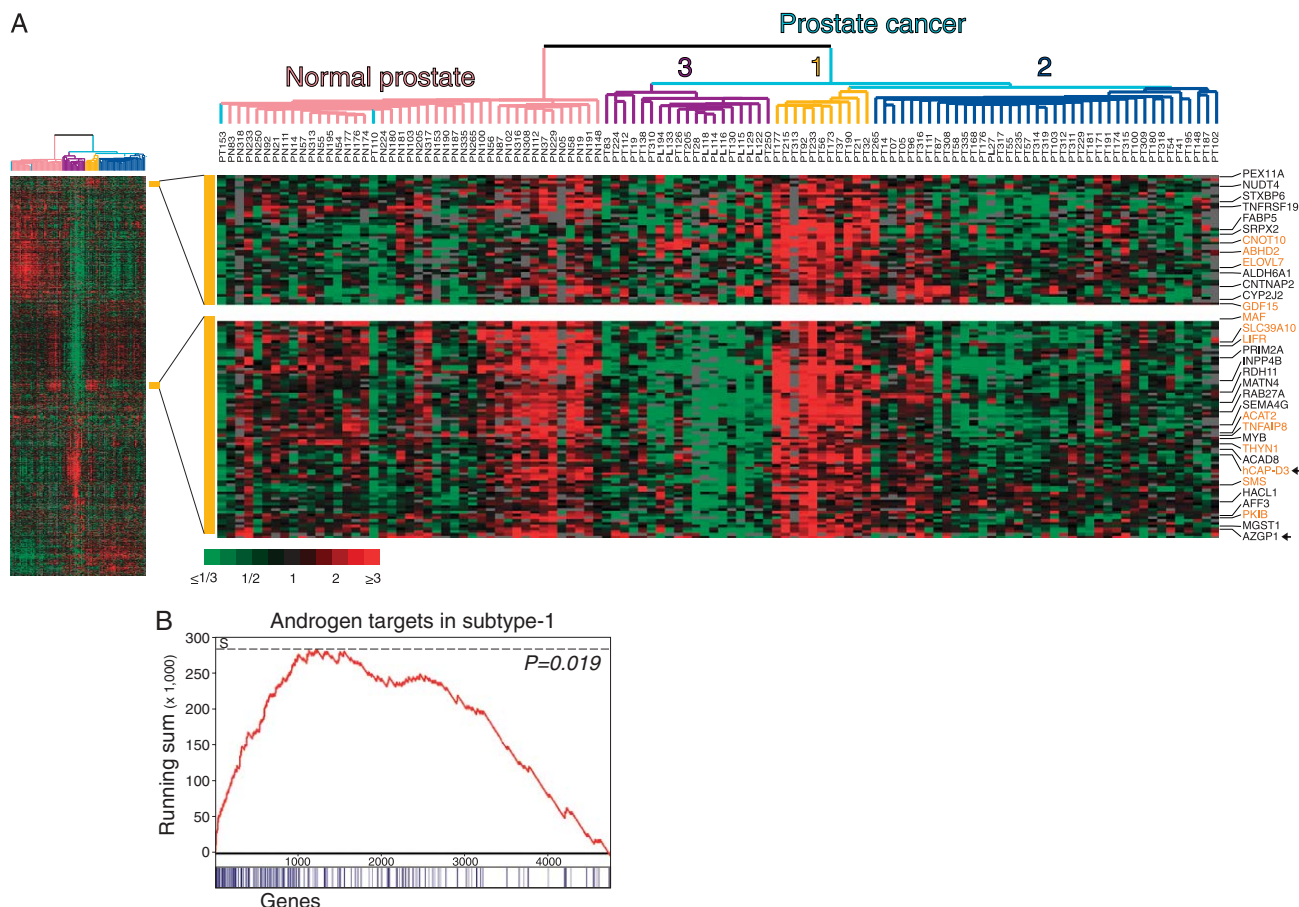


FIGURE 1. Expression patterns of subtype-1 prostate tumors. A, Cluster analysis of variable expressed genes, modified from *Proc Natl Acad Sci U S A.* 2004;101:811–816,¹⁰ distinguishes normal prostate (pink branches) and tumors, the latter further stratified into 3 different subclasses on the basis of patterns of gene expression (only a subset of patterns shown). *MUC1* (not shown) is among genes more highly expressed in tumor subtypes 2 and 3, whereas *AZGP1* and *hCAP-D3* (arrows) are more highly expressed in subtype-1 tumors. Androgen-induced genes are indicated by orange text. Note, although subtype-1 tumors comprise a minority of cases here, this likely reflects a bias in profiling larger tumors, less apparent in IHC studies¹⁰ or the RISH analysis, below. B, GSEA identifies enrichment of androgen-regulated genes in subtype-1 versus subtypes 2 and 3. Enrichment is evidenced by the early positive deflection of the Kolmogorov-Smirnov running sum (red line). The significance of the maximum running sum (*S*) is evaluated by comparison to 500 trials with randomly permuted subtype class labels; the *P* value is the frequency that *S* in the actual data is equaled or exceeded in the permuted data.

MATERIALS AND METHODS

TMA

The TMA comprised a set of 225 formalin-fixed, paraffin-embedded primary prostate tumor cases selected from diagnostic radical prostatectomy specimens collected at Stanford University, with Institutional Review Board approval, as previously described.¹⁰ Duplicate 0.6-mm tumor cores represented each case, and the series was associated with a minimum clinical follow-up of 5 years and a median follow-up of 8 years.

RNA In Situ Hybridization

RNA in situ hybridization (RISH) was performed as previously described.¹⁸ Briefly, 4-μm TMA sections

were deparaffinized, digested for 30 minutes with proteinase K, and hybridized overnight with an in vitro transcribed 445-nt antisense (gene-specific primer sequences: F-AGAAAATTGAAGGCCGGAGT, R-GGC TTGACACTTTCAAATGGA) or control sense digoxigenin-labeled riboprobe to *hCAP-D3*. Hybridized probe was then detected with a horseradish peroxidase-conjugated rabbit antidigoxigenin antibody using GenPoint tyramide signal amplification system (Dako). RISH scoring was based on recognizing a characteristic strong dotlike staining pattern associated with tumor cells.¹⁸ Kaplan-Meier survival analysis was performed using WinSTAT (R. Finch software), and multivariate proportional hazards analysis using the R software package.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA),¹⁵ performed as described,⁷ was applied to a set of 4741 variably expressed named genes from our previously published prostate tumor expression-profiling data set.¹⁰ The androgen responsive gene set comprised 330 named genes previously identified by microarray analysis to be induced in cultured prostate cells after exposure to androgen.²

RESULTS

We had previously defined 3 clinically relevant subtypes of prostate tumors on the basis of distinct patterns of gene-expression (Fig. 1A), or surrogate IHC markers.¹⁰ Because our IHC-based subtype assignments had relied on only 2 markers, we sought additional confirmation of the clinical relevance of our newly defined classes, focusing on markers that define subtype-1 cases because of potential application in identifying individuals

who could be spared aggressive primary therapies. We were unable to find antibodies that performed well for paraffin-IHC for nearly all candidate biomarkers for subtype-1 cancers; therefore, we used RISH to evaluate marker expression in an independent set of 225 primary prostate tumors represented in a TMA. One candidate biomarker, *hCAP-D3* (formerly *KIAA0056*; see Ref. 11), showed a pattern of expression that was closely correlated with *AZGP1* in a gene-cluster defining subtype-1 (Fig. 1A, arrow), and exhibited high microarray fluorescence intensities that indicated high absolute RNA levels in the subtype-1 cancers (not shown). *hCAP-D3* encodes a subunit of condensin II complex, a multiprotein complex involved in mitotic chromosome assembly (condensation),¹² and plays a critical role in cell division on the basis of functional siRNA screens.⁸

By RISH analysis, positive staining of *hCAP-D3* antisense probe (detecting sense mRNA) was associated with a statistically significantly decreased tumor-recurrence rate (Fig. 2A). Further, tumors scored positive for

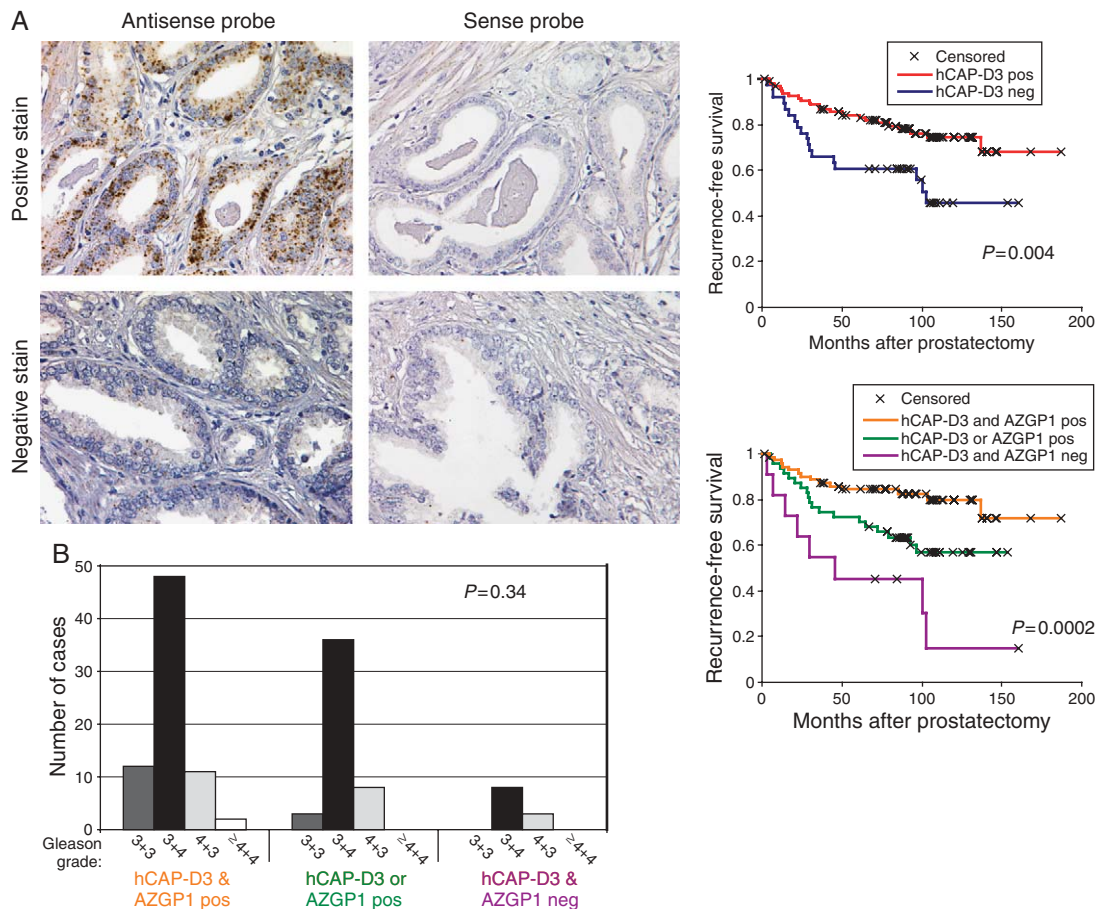


FIGURE 2. *hCAP-D3* expression associates with favorable prognosis. A, RISH staining of *hCAP-D3*, a surrogate marker for subtype-1 tumors, is associated with favorable PSA recurrence-free survival by Kaplan-Meier analysis (132 cases scorable). Representative positive and negative staining specimens, assayed in TMA format, are shown (400 × magnification). Right lower graph: combining *AZGP1* (previously scored¹⁰) and *hCAP-D3* markers improves risk stratification. B, Lack of association between subtype-1 markers [groups color-coded as per (A), bottom panel] and Gleason grade; χ^2 P value shown.

TABLE 1. Multivariate Proportional Hazards Analysis

| Variable | Hazard Ratio (95% Confidence Interval) | P* |
|---|---|--------|
| Gleason grade ($\geq 4+3$ vs. $\leq 3+4$) | 2.67 (1.37-5.20) | 0.004 |
| pT category ($\geq T3$ vs. $\leq T2$) | 3.55 (1.72-7.36) | 0.0006 |
| Preoperative serum PSA (per ng/mL)† | 1.03 (1.01-1.05) | 0.0005 |
| <i>hCAP-D3</i> staining (positive vs. negative) | 0.45 (0.24-0.85) | 0.014 |

*Wald test.
†Used as a continuous variable.

both *hCAP-D3* expression by RISH and AZGP1 expression by IHC were associated with an even lower recurrence rate compared with positive staining of either marker alone (Fig. 2A, right lower graph). Notably, *hCAP-D3* and AZGP1 markers were not merely surrogates for tumor Gleason grade, a strong predictor of tumor recurrence, as there was no significant association between marker staining and Gleason grade of the radical prostatectomy specimen (Fig. 2B). Indeed, like AZGP1,¹⁰ higher *hCAP-D3* expression by RISH was associated with significantly decreased recurrence after surgery independent of pathologic stage and Gleason grade, as well as preoperative serum PSA, in multivariate analysis (Table 1).

The subtype-1 gene cluster containing *hCAP-D3* and *AZGP1* was also expressed in a subset of normal prostate tissues (though not necessarily from the same patients) (Fig. 1A), suggesting it might reflect an aspect of normal prostate biology. Furthermore, we had previously identified *hCAP-D3* among the genes induced in cultured prostate cells exposed to androgen.² We, therefore, hypothesized that subtype-1 expression patterns might reflect in part androgen receptor signaling. We performed GSEA¹⁵ to assess whether the genes that distinguish between subtype 1 versus 2 and 3 were significantly enriched for the set of androgen-induced genes identified by microarray.² Consistent with our hypothesis, GSEA analysis identified an androgen signaling signature in subtype-1 cases ($P = 0.019$) (Fig. 1B; androgen-induced genes also highlighted in orange text in Fig. 1A).

DISCUSSION

Our findings are consistent with the existence of distinct molecular subtypes of prostate cancer that are distinguished by their unique gene expression signatures, biologic features, and clinical behavior. Prostate cancers that we have termed subtype-1 display gene expression features that overlap with normal prostate tissues and that distinguish them from other prostate cancers. We had shown that expression of AZGP1 protein, a gene more highly expressed in subtype-1 cancers, predicts favorable outcome after prostatectomy,¹⁰ a finding recently validated independently.^{3,5} We now demonstrate that increased expression of *hCAP-D3*, a new marker of

subtype-1 cancers, also predicts a favorable prognosis in an independent tumor set. Not only did *hCAP-D3* predict decreased recurrence after surgery independent of pathologic stage, grade, and preoperative serum PSA levels, when combined with AZGP1 expression levels it improved prognostication. Our findings provide further evidence that subtype-1 cancers represent a distinct molecular genetic entity with favorable clinical features and raise the possibility that they might represent clinically indolent tumors.

Our finding of increased expression of *hCAP-D3* in subtype-1 cancers also provides insights into the biologic features associated with this subtype. Consistent with our prior finding that *hCAP-D3* is androgen responsive, subtype-1 cancers show enrichment for androgen-induced genes by GSEA. Recently, other microarray studies have associated androgen signatures with low tumor grade,^{4,16} and low-grade tumors are known to have a favorable prognosis. It should be noted, however, that the subtype-1 tumors we have defined are not merely low-grade tumors, as there is no significant relationship between this subtype and Gleason grade. Rather, we speculate that the androgen signature, a distinguishing attribute of subtype-1, might reflect a state of molecular differentiation not appreciable histologically, but nonetheless potentially useful for tumor classification and prognostication. Whether a specific subset of androgen-regulated genes contribute to the pathogenesis of subtype-1 tumors or simply reflect tumor differentiation or activation of androgen signaling pathways in these tumors remains to be investigated.

In recent studies using array-based comparative genomic hybridization to profile genomic DNA copy number alterations in prostate cancer, we have discovered that subtype-1 tumors harbor characteristic DNA deletions on chromosomes 5 and 6 (manuscript submitted for publication). This finding implicates novel tumor suppressor genes at these loci in the pathogenesis of subtype-1 tumors. A potential link, if any, between these deletion loci/genes and the associated gene expression features of this cancer subtype such as AZGP1 and *hCAP-D3* expression and the androgen signature remains to be investigated. Given an apparent critical role of *hCAP-D3* in cell-cycle progression, it is somewhat surprising that expression of this transcript would be associated with a more favorable prognosis; alternatively, decreased

expression levels might be linked to defective mitotic chromosome assembly in the clinically less favorable subtypes 2 and 3. Whether *hCAP-D3* protein is functionally altered in a subset of prostate cancers is unknown and merits further investigation.

Our discovery of a molecular subtype of prostate cancer associated with favorable outcome and an androgen signature is reminiscent of recent findings in breast cancer. Gene expression profiling studies of breast cancer have defined distinct molecular subtypes, and tumors designated “Luminal-A” show an estrogen-receptor-associated gene-expression signature and favorable disease-free and overall survival.^{13,14} Although ours has been the first study to find similar “molecular subtypes” in prostate cancer, allelotyping studies have long identified heterogeneous changes in clinical prostate cancer samples. Furthermore, the recent association of androgen activation in low-grade tumors agrees with our findings. If validated, our findings indicate that, like breast cancer and other cancers, prostate cancer can be classified into distinct molecular subtypes, and molecular genetic features of these subtypes could serve as prognostic markers to aid in patient management.

Widespread application of prostate cancer screening, particularly with serum PSA measurements, has led to dramatic shifts in clinical prostate cancer. Given the dramatic rise in incidence and significant stage migration of prostate cancer toward smaller, lower grade tumors at the time of diagnosis, new prognostic biomarkers are desperately needed to identify tumors that do not require treatment, so that men might be spared unnecessary treatment and the resultant associated morbidities. The molecular subtypes of prostate cancer we have defined by gene-expression profiling represent a potential first step and have given rise to markers of poor and favorable prognosis, including *hCAP-D3*. Future work will entail defining a set of optimal markers of prognosis and developing models that can be validated in independent clinical samples. Ultimately, studies of biopsy material from patients followed but not treated (ie, “watchful waiting” cohorts) will be necessary to determine whether molecular markers of prognosis might effectively predict which patients progress to clinically significant disease requiring treatment. Discriminatory subtype markers might include gene-expression signatures, or selected surrogate biomarkers for tumor subtypes, like *AZGP1* or *hCAP-D3*. Although additional investigations are needed, our findings identify *hCAP-D3* as a biomarker for indolent subtype-1 prostate cancer with potential utility in improved prognostication, and identify androgen receptor signaling as an important biologic feature of this molecular subtype.

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