Original Article

# Association of B7-H3 expression with racial ancestry, immune cell density, and androgen receptor activation in prostate cancer

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**BACKGROUND:** B7 homolog 3 (B7-H3) is an immunomodulatory molecule that is highly expressed in prostate cancer (PCa) and belongs to the B7 superfamily, which includes PD-L1. Immunotherapies (antibodies, antibody-drug conjugates, and chimeric antigen receptor T cells) targeting B7-H3 are currently in clinical trials; therefore, elucidating the molecular and immune microenvironment correlates of B7-H3 expression may help to guide trial design and interpretation. The authors tested the interconnected hypotheses that B7-H3 expression is associated with genetic racial ancestry, immune cell composition, and androgen receptor signaling in PCa. **METHODS:** An automated, clinical-grade immunohistochemistry assay was developed by to digitally quantify B7-H3 protein expression across 2 racially diverse cohorts of primary PCa (1 with previously reported transcriptomic data) and pretreatment and posttreatment PCa tissues from a trial of intensive neoadjuvant hormonal therapy. **RESULTS:** B7-H3 protein expression was significantly lower in self-identified Black patients and was inversely correlated with the percentage African ancestry. This association with race was independent of the significant association of B7-H3 protein expression with ERG/ETS and PTEN status. B7-H3 messenger RNA expression, but not B7-H3 protein expression, was significantly correlated with regulatory (FOXP3-positive) T-cell density. Finally, androgen receptor activity scores were significantly correlated with B7-H3 messenger RNA expression, and neoadjuvant intensive hormonal therapy was associated with a significant decrease in B7-H3 protein expression. **CONCLUSIONS:** The current data underscore the importance of studying racially and molecularly diverse PCa cohorts in the immunotherapy era. This study is among the first to use genetic ancestry markers to add to the emerging evidence that PCa in men of African ancestry may have a distinct biology associated with B7-H3 expression. ***Cancer* 2022;128: 2269-2280**. 

**LAY SUMMARY:**

- B7-H3 is an immunomodulatory molecule that is highly expressed in prostate cancer and is under investigation in clinical trials.
- The authors determined that B7-H3 protein expression is inversely correlated with an individual's proportion of African ancestry.
- The results demonstrate that B7-H3 messenger RNA expression is correlated with the density of tumor T-regulatory cells.
- Finally, in the first paired analysis of B7-H3 protein expression before and after neoadjuvant intensive hormone therapy, the authors determined that hormone therapy is associated with a decrease in B7-H3 protein levels, suggesting that androgen signaling may positively regulate B7-H3 expression.
- These results may help to guide the design of future clinical trials and to develop biomarkers of response in such trials.

**KEYWORDS:** African American, androgen receptor (AR), B7 homolog 3 (B7-H3), ERG, prostatic adenocarcinoma, PTEN, T cells, tumor-infiltrating lymphocytes.

## INTRODUCTION

B7 homolog 3 (B7-H3) (PD-L3, CD276) is a member of the B7 family of coregulatory molecules,[1] which also includes PD-L1 (B7-H1). In contrast to PD-L1, B7-H3 is widely expressed in prostate adenocarcinoma (PCa), and high

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expression has been associated with adverse pathologic features as well as a specific molecular phenotype.[2-6] Accordingly, several previous studies have demonstrated an association between B7-H3 expression and adverse oncologic outcomes in patients with surgically treated PCa, including early prostate-specific antigen (PSA) recurrence[3-5] as well as the development of castration-resistant PCa (CRPC) and cancer-specific death.[4] In light of this work, there is increasing interest in targeting B7-H3 in PCa. In recently reported phase 1 trial data, enoblituzumab—an Fc-optimized monoclonal antibody targeting B7-H3—has demonstrated clinical activity in advanced malignances, including PCa,[7] and several other trials are currently underway directed at B7-H3.

Despite this progress, several unexplored areas remain as trials targeting B7-H3 gain momentum. First, previous studies of B7-H3 expression in PCa were conducted predominantly in European ancestry cohorts.[2-6] Given recent evidence of differing immunobiology in prostate tumors arising in self-identified Black (BL) patients compared with self-identified White (WH) patients,[8-12] we hypothesized that B7-H3 expression may differ between races and could have a putative role in the differing immunobiology and immune therapy responses seen in these 2 groups of patients. In this framework, we considered the secondary hypotheses that B7-H3 expression is associated with the immune cell composition in PCa and that B7-H3 expression itself might be correlated with androgen receptor (AR) signaling through AR-binding site(s) upstream of the B7-H3 promoter. Herein, we conducted the largest study to date using a validated B7-H3 immunohistochemistry assay in ancestrally diverse cohorts of men with PCa (including 1 cohort that had previously published gene expression data) and quantitatively evaluated the association of B7-H3 expression with immune cell subpopulation densities and AR activity (AR-A). In addition, we investigated B7-H3 expression in pretreatment and posttreatment tumor tissues from a trial of intensive neoadjuvant hormonal therapy and characterized the AR-binding sites near B7-H3 from clinical tumors and patient-derived xenografts.

## MATERIALS AND METHODS

### Patients and Tissue Samples

With Johns Hopkins Institutional Review Board approval, this study included 1 test cohort tissue microarray (TMA), 2 previously described patient sets from Johns Hopkins Hospitals (a racial ancestry cohort and an intermediate-risk/high-risk cohort),[2,12-20] and a cohort from a previously described clinical trial of intensive neoadjuvant hormonal therapy[21] (see Supporting Methods). The intermediate-risk/high-risk cohort was included specifically because it provided an independent validation cohort for many of the findings in the racial ancestry cohort and also had previously published gene expression data available.[2,13]

### B7-H3 Staining

Immunohistochemistry (IHC) for B7-H3 (catalog no. 14058, RRID:AB_2750877; Cell Signaling Technology) was performed on the Ventana BenchMark system (Ventana/Roche) (see Supporting Methods). The assay was validated using a positive control cell line (JIMT-1; catalog no. ACC-589, RRID:CVCL_2077; DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) and a negative control cell line (NCI-H69/HTB-119; catalog no. HTB-119, RRID:CVCL_1579; American Type Culture Collection) based on immunoblotting data[22] (Fig. 1A,B).

### Digital B7-H3 IHC Scoring

We developed a digital quantitative scoring system to assess the intratumoral heterogeneity of B7-H3 protein expression (Fig. 1C-F) by combining the percentage of positive cells and the staining intensity using the H-score (range, 0-300), which was calculated using the Membrane version 1.7 algorithm in HALO (IndicaLabs) (Fig. 1C-F) (see Supporting Methods). For validation, we compared the digital score with a previously described visual scoring system[3] (see Supporting Methods).

### Ki-67 Staining and Interpretation

We performed IHC for Ki-67 in a Clinical Laboratory Improvement Amendments-accredited laboratory using methods similar to those described for B7-H3 (see Supporting Methods).

### Immune Cell Densities

T-cell densities (CD3, CD8, and FOXP3) were previously assessed and published for the racial ancestry cohort[18] and the intermediate-risk/high-risk cohort.[15] B-cell (CD79a) immunostaining for the racial ancestry cohort was also previously published,[12] and similar methods were used to assess CD79a-positive B-cell densities in the intermediate-risk/high-risk cohort and activated/anti-inflammatory (CD163-positive) macrophages in both cohorts in the current study (see Supporting Methods).

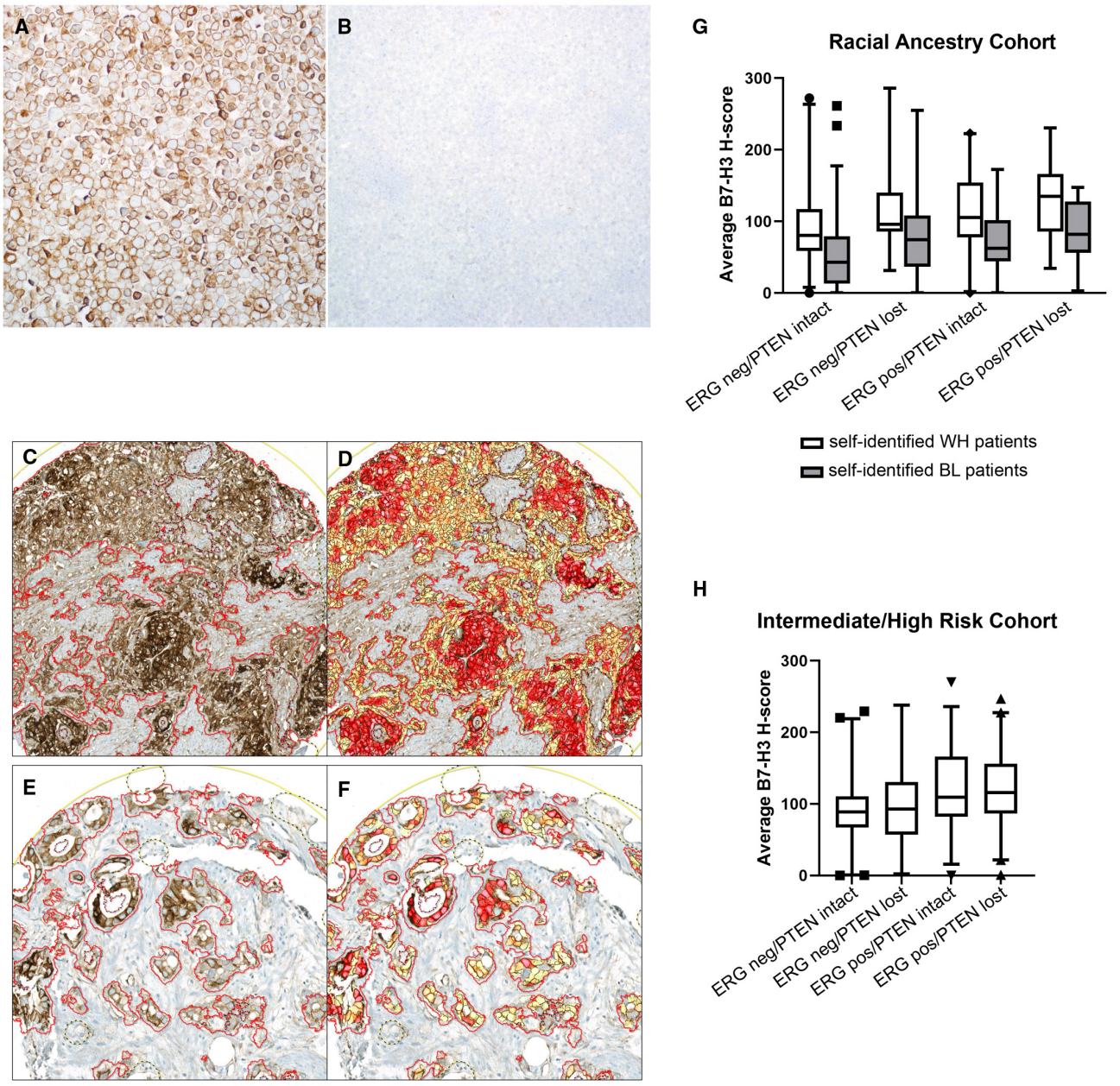


**FIGURE 1**. Automated, clinical-grade B7 homolog 3 (B7-H3) immunostaining with quantitative digital imaging analysis is illustrated. (A) B7-H3 immunostaining of JIMT-1 cells, a positive control cell line, demonstrates strong membranous staining. (B) B7-H3 immunostaining of NCI-H69/HTB-119 cells, a negative control cell line, is shown. (C) Representative B7-H3 immunostaining is shown in a primary prostate carcinoma. Tumor cells are delineated from stroma by the automated cell phenotyping algorithm (red lines). (D) Digital image analysis of tumor cells reveals cells with low-to-medium–intensity staining (yellow-orange areas) and high-intensity staining (red areas). (E) Representative B7-H3 immunostaining is shown in a primary prostate carcinoma. Tumor cells are delineated from stroma by the automated cell phenotyping algorithm (red lines). Areas that are manually excluded from scoring are delineated with yellow-black dotted lines. (F) Digital image analysis of tumor cells shows cells with low-to-medium–intensity staining (yellow-orange areas) and high-intensity staining (red areas; original magnification ×100 in A-F). (G) B7-H3 expression levels (average H-scores; range, 0-300) are illustrated according to ERG/PTEN status (negative [neg] vs positive [pos]) and self-identified African American (AA) versus White (WH) race in the racial ancestry cohort. (H) B7-H3 expression is stratified according to ERG/PTEN status in the intermediate-risk/high-risk cohort.

**TABLE 1**. Comparisons of B7-H3 and Clinicopathologic and Molecular Features in the Racial Ancestry Cohort

| | All Men | | | White Men | | | Black Men | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Average B7-H3 | | | Average B7-H3 | | | Average B7-H3 | |
| Variable | No. | Median H-Score | $P^a$ | No. | Median H-Score | $P^a$ | No. | Median H-Score | $P^a$ |
| Self-identified race | | | | | | | | | |
| White | 166 | 94.1 | <.001 | — | | | — | | |
| Black | 151 | 54.3 | | | | | | | |
| AJCC stage | | | | | | | | | |
| T2N0 | 141 | 75.4 | .7 | 74 | 88.6 | .5 | 67 | 56.6 | .9 |
| T3N0 | 140 | 79.2 | | 74 | 96.9 | | 66 | 51.8 | |
| N1 | 26 | 86.1 | | 15 | 97.9 | | 11 | 56.3 | |
| Gleason grade group | | | | | | | | | |
| <7 | 41 | 89 | .2 | 23 | 91.1 | .8 | 18 | 74.1 | .1 |
| 3+4 | 49 | 80.7 | | 26 | 88.6 | | 23 | 71.3 | |
| 4+3 | 142 | 76 | | 72 | 92.1 | | 70 | 44.2 | |
| 8 | 48 | 90.2 | | 24 | 111.4 | | 24 | 76.7 | |
| 9 | 37 | 65.8 | | 21 | 99.6 | | 16 | 17 | |
| PTEN | | | | | | | | | |
| Intact/ambiguous | 246 | 71.9 | <.001 | 119 | 88.3 | .01 | 127 | 50.3 | .02 |
| Loss | 71 | 95.9 | | 47 | 103.2 | | 24 | 78 | |
| ERG | | | | | | | | | |
| Negative | 215 | 69.4 | <.001 | 91 | 85.7 | .002 | 124 | 49.3 | .02 |
| Positive | 102 | 97.9 | | 75 | 110 | | 27 | 65.4 | |
| p53 nuclear accumulation | | | | | | | | | |
| Absence | 299 | 76.7 | .02 | 153 | 92.2 | .4 | 146 | 51.8 | .1 |
| Presence | 18 | 110 | | 13 | 110 | | 5 | 107.4 | |
| AR-A class | | | | | | | | | |
| Normal | — | | | — | | | 62 | 57.2 | .2 |
| Low | | | | | | | 40 | 47.2 | |

Abbreviations: AJCC, American Joint Committee on Cancer; AR-A, androgen receptor activity; H-score, histoscore.
[a]*P* values were determined using the Kruskal-Wallis test.

### *Genetic Ancestry Data*

Tumor DNA was isolated from the racial ancestry cohort as previously described (for 19 patients, benign tissue was used),[23] and all samples were profiled on Infinium Global Screening Arrays (see Supporting Methods).

### *B7-H3 AR Chromatin Immunoprecipitation Sequencing Analysis*

Published AR chromatin immunoprecipitation sequencing (ChIP-Seq) reads from clinical and patient-derived xenograft samples (GSE56288 [n = 20] and GSE130408 [n = 39]) were mapped to hg19. Alignments with a mapping score <30 and duplicate reads were removed, and peaks were called using the Model-Based Analysis of ChIP-Seq (MACS) (https://doi.org/10.1186/gb-2008-9-9-r137, Accessed January 10, 2021) with a *Q*-value threshold of .01 and no input file (see Supporting Methods).

### *Statistical Analysis*

All analyses were conducted using GraphPad Prism version 9 and SAS version 9.0 (SAS Institute Inc). Statistical tests were 2-sided, and *P* values < .05 were considered statistically significant (see Supporting Methods).

## RESULTS

### *Validation of B7-H3 IHC*

For B7-H3 IHC validation, we used formalin-fixed, paraffin-embedded cell line controls (Fig. 1A,B). The digital scoring algorithm (Fig. 1C-F) was validated by comparison with visual scoring (correlation coefficient [*r*] = 0.83 [*P* < .0001] and *r* = 0.84 [*P* < .0001] in the test TMA and in the cohort subset, respectively) (see Supporting Methods). We observed that previously reported B7-H3 messenger RNA (mRNA) expression[2] and B7-H3 expression, determined by IHC, were significantly correlated in the intermediate-risk/high-risk cohort (*r* = 0.29; *P* < .0001) (see Supporting Fig. 1).

### *Race and B7-H3 Expression*

The clinicopathologic and molecular features of the racial ancestry cohort and their associations with average B7-H3 protein expression are summarized in Table 1, and similar data for the intermediate-risk/high-risk subcohort

**TABLE 2**. Comparisons of B7-H3 and Clinicopathologic and Molecular Features in the Intermediate-Risk/High-Risk Subcohort

| | Average B7-H3 | | | B7-H3 mRNA | | |
|---|---|---|---|---|---|---|
| Variable | No. | Median H-score | *P*[a] | No. | Median Expression | *P*[a] |
| Race | | | | | | |
| White | 231 | 98.6 | .002 | 232 | 0.3208 | .3 |
| Non-White[b] | 23 | 73 | | 24 | 0.2462 | |
| Stage | | | | | | |
| T2[c] | 75 | 89.4 | .003 | 74 | 0.3034 | .0003 |
| T3a | 125 | 95.3 | | 125 | 0.2952 | |
| T3b | 51 | 118.7 | | 54 | 0.3726 | |
| Gleason grade group | | | | | | |
| <7 | 1 | 103.1 | .9 | 1 | 0.2413 | .03 |
| 3+4 = 4 | 108 | 95.9 | | 107 | 0.3149 | |
| 4+3 = 7 | 53 | 96.8 | | 54 | 0.2809 | |
| 8 | 29 | 92.4 | | 29 | 0.3568 | |
| 9 | 60 | 102.4 | | 62 | 0.3675 | |
| PTEN | | | | | | |
| Ambiguous/intact | 169 | 92.4 | .1 | 172 | 0.3066 | .006 |
| Loss | 85 | 103.5 | | 84 | 0.3665 | |
| ERG | | | | | | |
| Negative | 151 | 90.6 | .0003 | 153 | 0.3149 | .1 |
| Positive | 101 | 113.8 | | 101 | 0.3432 | |
| *ETS* | | | | | | |
| Negative | 128 | 89.1 | <.001 | 130 | 0.2956 | .005 |
| Positive | 126 | 109.5 | | 126 | 0.3437 | |
| p53 nuclear accumulation | | | | | | |
| Absence | 238 | 95.5 | .2 | 240 | 0.3124 | .01 |
| Presence | 16 | 121.8 | | 16 | 0.409 | |
| AR-A class | | | | | | |
| Normal | 146 | 98.1 | .7 | 151 | 0.3534 | .2 |
| Low | 52 | 94.7 | | 57 | 0.3568 | |

Abbreviations: AR-A, androgen receptor activity; H-score, histoscore; mRNA, messenger RNA.
[a]*P* values were determined using the Kruskal-Wallis test or the Mann-Whitney test.
[b]These included Black, Hispanic, other, and unknown men.
[c]These included T2, T2a, T2b, T2c, and T2x tumors.

are provided in Table 2. Higher pathologic stage was significantly associated with higher average B7-H3 protein expression in the intermediate-risk/high-risk subcohort ($P = .003$) (Table 2), although other clinicopathologic features were not associated with average B7-H3 protein expression. Average B7-H3 protein expression was associated with self-identified race in both cohorts: BL patients (racial ancestry cohort; $P < .001$) and non-WH patients (intermediate-risk/high-risk subcohort; $P = .002$) had lower B7-H3 protein expression compared with self-identified WH patients (Tables 1 and 2). In the racial ancestry cohort, in which WH and BL patients were matched for Gleason grade group, there was a nearly 40% reduction in the average B7-H3 H-score for self-identified BL patients compared with WH patients (Table 1, Fig. 1G). Self-identified race was highly associated with the estimated percentage of Yoruba in Ibadan, Nigeria (YRI) ancestry based on ancestry-informative markers by single nucleotide variant microarray, with only 4 of 267 patients appearing to be outliers (see Supporting Fig. 2). These outliers were not excluded from subsequent analyses. Self-identified WH patients had a median of 0.001% YRI ancestry and 97.3% Northern and Western European (CEU) ancestry according to ancestry-informative markers compared with self-identified BL patients, who had a median of 78.8% YRI ancestry and 18.5% CEU ancestry ($P < .001$). Accordingly, there was a significant inverse correlation between B7-H3 protein expression and the percentage YRI ancestry, ($r = -0.42$; $P < .0001$) (Table 3). A positive correlation was observed with the percentage CEU ancestry and the B7-H3 H-score ($r = 0.44$; $P < .0001$).

Higher average B7-H3 protein expression was observed in men who had ERG overexpression in the racial ancestry cohort ($P < .001$) (Table 1, Fig. 1G). Because ERG expression is less common in prostate tumors arising in self-identified BL men compared with WH men,[19,24,25] next, we investigated whether the association observed between B7-H3 protein expression and self-reported race in the racial ancestry cohort might be largely driven by differing frequencies of *ERG* rearrangement in the 2 populations. By using general linear regression models for the

**TABLE 3**. Spearman Correlation Coefficients of B7-H3 in the Racial Ancestry Cohort and the Intermediate-Risk/High-Risk Subcohort

| | Racial Ancestry Cohort: Average B7-H3 Protein | | | | | | Intermediate-Risk/High-Risk Subcohort | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | All Men | | White Men | | Black Men | | Average B7-H3 Protein | | B7-H3 mRNA | |
| Variable | *r* | *P*[a] | *r* | *P*[a] | *r* | *P*[a] | *r* | *P*[a] | *r* | *P*[a] |
| Age | 0.09 | .1 | 0.03 | .7 | 0.03 | .7 | 0.08 | .2 | 0.03 | .67 |
| Preoperative PSA | −0.12 | .04 | −0.01 | .9 | 0.01 | .9 | 0.05 | .4 | — | — |
| Percentage YRI ancestry | −0.42 | <.0001 | | | | | — | — | — | — |
| AR-A | | | — | — | 0.01 | .9 | 0.07 | .3 | 0.23 | .0007 |
| CD3 | −0.02 | .8 | 0.08 | .3 | −0.06 | .5 | 0.06 | .4 | 0.11 | .09 |
| CD8 | 0.03 | .6 | −0.01 | .9 | 0.05 | .6 | 0.04 | .6 | 0.05 | .5 |
| FOXP3 | −0.02 | .7 | −0.04 | .7 | 0.03 | .7 | −0.02 | .8 | 0.20 | .002 |
| CD79a | −0.09 | .1 | 0.02 | .8 | −0.09 | .3 | −0.04 | .5 | −0.10 | .1 |
| CD163 | 0.03 | .6 | 0.07 | .4 | 0.03 | .7 | 0.1 | .1 | 0.12 | .06 |
| Ki67 | — | — | — | — | — | — | 0.03 | .6 | 0.27 | <.0001 |

Abbreviations: AR-A, androgen receptor activity; mRNA, messenger RNA; PSA, prostate-specific antigen; *r*, correlation coefficient; YRI, Yoruba in Ibadan, Nigeria.
[a]*P* values were determined by Spearman correlation.

dependent variable of B7-H3 expression, we found that the median B7-H3 protein expression remained independently significantly different between self-identified BL men and WH men even after adjustment for ERG status (Table 4, model 1). The results were similar after adjustment for ERG status and clinicopathologic factors (Table 4, model 2) and also after additional adjustment for PTEN status (Table 4, model 3). Notably, the interaction between race and ERG status for B7-H3 protein expression was not statistically significant in any of the models. Higher B7-H3 protein expression was also observed in patients with previously reported ETS family gene expression ($P < .001$) or ERG overexpression ($P = .0003$)[26] in the intermediate-risk/high-risk subcohort (Table 2). This association was similar, although stronger, than that previously reported for B7-H3 mRNA and ERG mRNA expression in this cohort.[2]

Average B7-H3 protein expression was significantly higher in tumors with PTEN loss in the racial ancestry cohort ($P < .001$) (Table 1, Fig. 1G), and the results were similar for tumors with previously reported p53 nuclear accumulation (a sensitive proxy of underlying *TP53* missense mutation[14]), with significantly higher B7-H3 expression in tumors with p53 accumulation in the racial ancestry cohort ($P = .01$) (Table 1). Both of these relations were maintained for B7-H3 mRNA levels in the intermediate-risk/high-risk subcohort (Table 2), with significantly higher B7-H3 mRNA expression in tumors that had PTEN loss ($P = .006$) or p53 nuclear accumulation ($P = .01$). Although there was no significant correlation between average B7-H3 protein expression and Ki-67 levels in the intermediate-risk/high-risk subcohort ($P = .6$), Ki-67 levels were significantly correlated with B7-H3 mRNA expression in the same cohort ($r = 0.27$; $P < .0001$) (Table 3).

### *Immune Cell Density and B7-H3 Expression*

We assessed the association of B7-H3 protein expression with lymphocyte and macrophage subset densities in both the racial ancestry cohort and the intermediate-risk/high-risk cohort (Table 3). There was no significant correlation between B7-H3 protein expression and previously reported T-cell (CD3), cytotoxic T-cell (CD8), regulatory T-cell (FOXP3),[15,18] B-cell (CD79a) density,[12] or activated/anti-inflammatory (CD163-positive) macrophage density in the racial ancestry cohort or the intermediate-risk/high-risk subcohort (Table 3). However, in the intermediate-risk/high-risk subcohort, there was a significant positive correlation between regulatory (FOXP3) T-cell density and B7-H3 mRNA expression ($r = 0.20$; $P = .002$) (Table 3).

### *AR-A and B7-H3 Expression*

Previous studies have reported higher levels of AR protein expression in patients with higher B7-H3 protein expression[5,27] and an enrichment of AR signaling-related gene expression signatures among those with higher B7-H3 mRNA expression.[2] Next, we investigated whether the AR-A score, a previously reported 9-gene signature of AR-A in the intermediate-risk/high-risk subcohort,[17] was correlated with B7-H3 protein or mRNA expression (Table 3). Although there was no significant correlation between the average B7-H3 H-score and the AR-A score ($r = 0.07$; $P = .3$), there was a moderate positive

**TABLE 4**. Interactions Between B7-H3 and ERG and Race in the Racial Ancestry Cohort

| Variable | Model 1[a] | | Model 2[b] | | Model 3[c] | |
|---|---|---|---|---|---|---|
| | Average B7-H3 | | Average B7-H3 | | Average B7-H3 | |
| | Median Difference in H-score (95% CI) | *P* | Median Difference in H-score (95% CI) | *P* | Median Difference in H-score (95% CI) | *P* |
| Race | | | | | | |
| White vs Black | 40.4 (25.59-55.30) | <.0001 | 32.6 (12.59-52.71) | .002 | 33.4 (13.62-53.10) | .001 |
| ERG | | | | | | |
| Positive vs negative | 19.7 (−3.11, 42.60) | .1 | 21.7 (−2.14, 45.56) | .1 | 17.4 (−6.25, 41.00) | .1 |
| *P* for interaction | | .8 | | .9 | | .9 |

Abbreviation: CI, confidence interval; H-score, histoscore.
[a]Model 1 was a generalized linear regression model with race, ERG status, and the interaction between race and ERG status.
[b]Model 2 included all variables in model 1 with the additional adjustment of age, stage, grade group, preoperative prostate-specific antigen level, and cohort.
[c]Model 3 included all variables in model 2 with the additional adjustment of PTEN status.

correlation between B7-H3 mRNA expression and the AR-A score ($r = 0.23$; $P = .0007$). To explore the potential mechanism, we analyzed publicly available AR ChIP-Seq data from clinical or patient-derived xenograft samples of benign prostate, primary PCa, or CRPC (n = 59). Several AR peaks were observed near the promoter of the B7-H3 gene (*CD276*), with marked differences between the samples. Specifically, there was a low frequency of called AR peaks near B7-H3 in benign prostate samples (range, 0%-8%) that markedly increased in both primary PCa and CRPC (range, 40%-60%). Supporting the peak calling, we observed a significant increase in the normalized AR peak height in primary PCa and CRPC samples compared with benign prostate samples (see Supporting Fig. 3).

To further address the role of AR signaling in B7-H3 expression, we examined a cohort of paired samples from a recently described clinical trial of neoadjuvant intensive hormonal therapy with either combined apalutamide, abiraterone acetate, prednisone, and leuprolide or combined abiraterone, prednisone, and leuprolide for 6 cycles followed by radical prostatectomy.[21] B7-H3 protein expression was significantly higher in biopsies (pretreatment samples) compared with paired prostatectomies (posttreatment samples; $P = .03$), suggesting that intense hormonal therapy may downregulate the expression of B7-H3 (Fig. 2A,B).

### *B7-H3 Protein Expression and Metastasis-Free Survival*

In the racial ancestry cohort, there was no significant association of average B7-H3 protein expression (assessed continuously) with outcomes in the overall cohort ($P = .7$), or in either self-identified racial group (see Supporting Table 1), or in either univariable or multivariable analyses. The results were similar when B7-H3 protein expression was analyzed by quartile (see Supporting Table 2) and in Kaplan-Meier analyses (see Supporting Fig. 4). In the intermediate-risk/high-risk cohort (predominantly self-identified WH patients), B7-H3 protein expression, assessed continuously by a 1-unit increase in the H-score (hazard ratio, 1.006; 95% CI, 1.002-1.011; $P = .004$) or by comparing the highest quartile with the lowest quartile of expression (hazard ratio, 2.793; 95% CI, 1.546-5.046; $P = .001$), was significantly associated with the risk of metastasis in univariable analysis, and similar results were observed on Kaplan-Meier analysis (see Supporting Fig. 4). However, this association fell short of significance on multivariable analysis after adjusting for age, race,

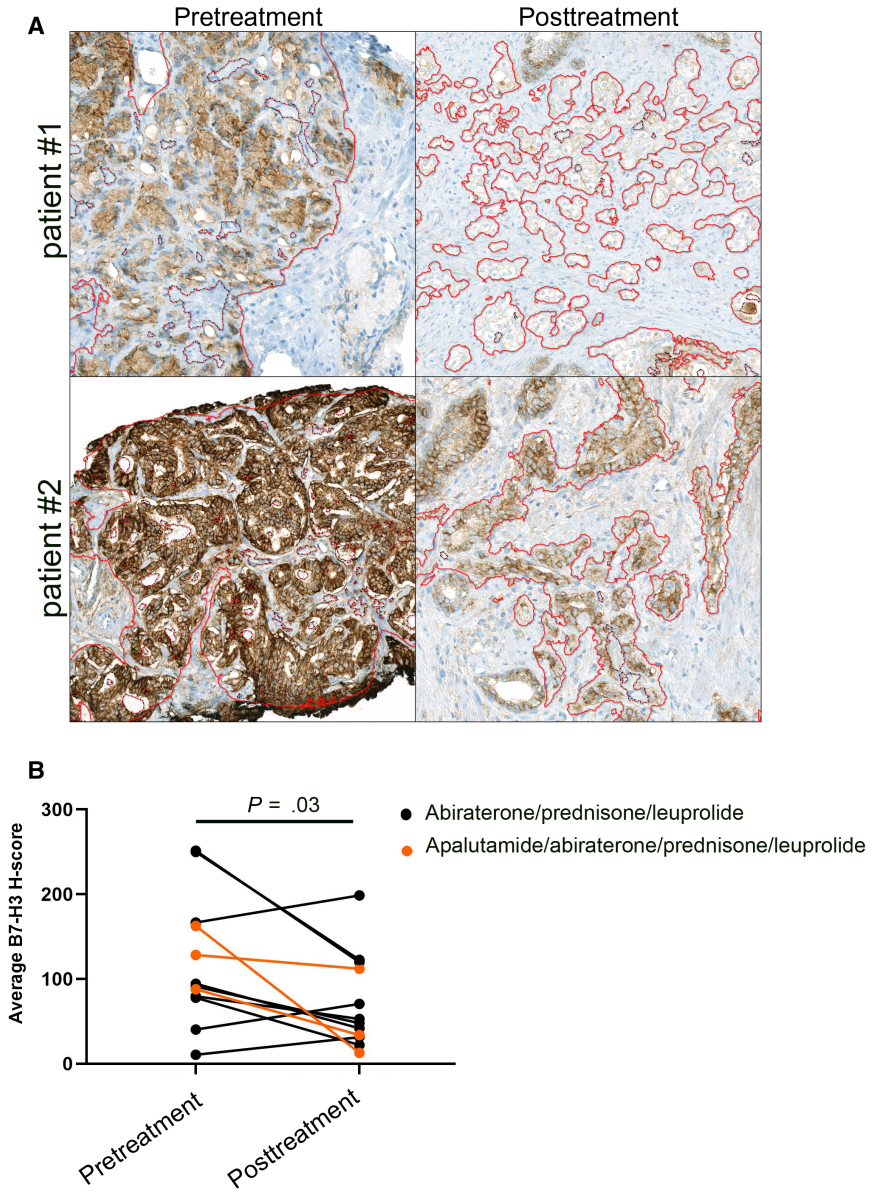


**FIGURE 2**. B7 homolog 3 (B7-H3) protein expression scores are illustrated in pretreatment and posttreatment samples from a clinical trial of intense neoadjuvant hormonal therapy. (A) Representative pretreatment and posttreatment samples of B7-H3 immunostaining are shown. Tumor cells are delineated from stroma by the automated cell phenotyping algorithm (red lines; original magnification ×100). (B) B7-H3 immunostaining quantification is illustrated in pretreatment and posttreatment samples.

Gleason grade group, preoperative PSA level, and body mass index (see Supporting Tables 1 and 2).

## DISCUSSION

The successful introduction of immunotherapy in cancer treatment has sparked intense interest in the B7 family of proteins. Among these, B7-H3 is unique because it is believed not only that it regulates antitumoral immunity (similar to the other B7 family members) but also that it has an important nonimmunologic role in tumorigenesis.[28,29] In addition, targeting B7-H3 using antibody-drug conjugates is of particular interest because of its limited protein expression in normal tissue, in stark contrast to its overexpression in many types of cancer,[28,30] potentially limiting toxicity while preserving treatment efficacy. In the current study, we developed and validated an automated, clinical-grade B7-H3 immunostaining protocol coupled with highly quantitative digital image analysis, improving upon previous

visual semiquantitative scoring systems, which are subject to interobserver and intraobserver variability. By using this assay, we tested the novel hypothesis that that B7-H3 expression differs by racial ancestry, reflecting the unique immunobiology of PCa arising in men of African descent, and we evaluated the interrelated hypotheses that B7-H3 expression may be associated with immune cell content and/or AR signaling. To our knowledge, this is the first study to examine B7-H3 expression in PCa from a racially diverse cohort with genetic ancestry annotations, the first to correlate B7-H3 expression with quantified immune cell densities, and the first to examine the expression of B7-H3 in paired samples before and after intensive hormonal therapy.

Numerous studies have documented a differing immune milieu in prostate tumors arising in BL men compared with WH men.[8-12,31] Intriguingly, a recent clinical trial of the immunotherapy sipuleucel-T in PCa produced a more pronounced response in self-identified BL men compared with WH men,[32] suggesting that these differences may be therapeutically significant. Notably, however, those prior studies used self-identified race rather than genetic ancestry to examine differences in PCa immunobiology. Here, we report that B7-H3 protein expression is significantly lower in PCa from self-identified BL men compared with grade-matched, self-identified WH men, and this association remains significant if we quantify genetic African or European ancestry. Importantly, B7-H3 remained significantly lower among BL men even after adjustment for ERG, PTEN, and clinicopathologic features, and there was no significant interaction between race and ERG or PTEN with respect to B7-H3 expression. Corroborating this finding in other tumor types, previous studies have suggested significantly lower B7-H3 expression in breast and colorectal cancers from self-identified BL patients compared with WH patients.[33-35] Whether these ancestry-related differences in B7-H3 expression are physiologically or clinically significant must be tested in future studies.

On the basis of its homology with PD-L1, it has been suggested that B7-H3 may have an important role in immune modulation, although detailed blocking studies have not been performed given current lack of knowledge about the B7-H3 receptor(s). To date, B7-H3 has been ascribed both immune-stimulatory and immune-inhibitory roles.[1,2,36-39] Only 1 previous study semiquantitatively evaluated the extent of immune infiltrate using hematoxylin and eosin sections of prostate tumors; and, although the results indicated that lymphocytic infiltrate was present at higher rates in tumors with marked B7-H3 intensity, the difference was not statistically significant.[3] In our current study of primary tumors, B7-H3 protein expression was not associated with changes in the density of infiltrating B cells, T cells, or macrophages. However, we observed that B7-H3 mRNA expression was positively correlated with regulatory T-cell density, which we estimated using IHC in the same tumor samples. Considering the overall high absolute level of B7-H3 expression in the setting of primary PCa, it is possible that small relative changes in B7-H3 expression are not physiologically significant. Alternatively, it is possible that we are unable to detect small changes in immune cell density, given that the heterogeneity of immune infiltrate across the tumor can be a limitation in TMA analysis. Additional spatial and functional studies in clinical trials will be required to elucidate the role of B7-H3 in immune regulation in PCa.

A positive correlation between B7-H3 and AR levels was previously identified for AR expression at the transcript[2] and protein[5] levels. This was proposed to occur through AR-binding sites upstream of B7-H3 that were observed in LNCaP cell lines. In the current study, we characterized AR ChIP-Seq data sets from clinical and patient-derived xenografts (n = 59) and identified several AR-binding sites upstream of the B7-H3 promoter that occurred at a marked greater frequency in primary PCa and CRPC compared with benign prostate samples. This is consistent with the increased B7-H3 protein expression we observed between benign prostate and prostate cancer. We did not observe a significant association between AR-A scores and B7-H3 protein expression; however, there was a significant correlation between B7-H3 mRNA expression and the AR-A score in the same cohort. In contrast, some in vitro data[2] and limited in vivo data[40] have been inconsistent with the hypothesis that AR signaling drives B7-H3 expression. Because those previous studies may have been confounded by clinicopathologic or molecular heterogeneity between treatment groups, we examined tumor samples before and after intensive hormonal therapy and found a significant decrease in B7-H3 expression in posttreatment samples compared with pretreatment samples in a paired analysis. The decrease in staining was observed in morphologically viable tumor cells that retained pan-keratin expression; however, we cannot entirely exclude the possibility that these changes were caused by nonspecific alterations in cellular viability. Future mechanistic work could validate this finding in additional model systems. To our knowledge, this study is the first to examine B7-H3 expression within the same prostate both before and after androgen ablation. We

are not able to ensure that the same tumor nodule was sampled pretherapy and posttherapy because most of the biopsies were not magnetic resonance imaging-guided, and this is an important limitation of most neoadjuvant studies published to date. Although our data support the possibility that AR signaling may drive, rather than suppress, B7-H3 protein expression, it is also notable that many samples retained significant B7-H3 expression after intensive hormonal therapy, suggesting that it remains an important therapeutic target even after hormonal suppression.

Multiple previous studies failed to demonstrate that B7-H3 expression is an independent prognostic factor in PCa in multivariable analyses, potentially because of its association with PCa stage and grade.[2,5,6] We observed that being in the highest quartile of B7-H3 protein expression was significantly associated with metastasis in univariate analyses among men in the predominantly WH intermediate-risk/high-risk cohort. However, B7-H3 expression was not significantly associated with metastasis among WH or BL men in the racial ancestry cohort, nor after multivariable adjustment in either cohort. Collectively, these analyses suggest the need for larger and diverse studies to further explore the association between B7-H3 and PCa outcomes, including by region-specific racial ancestry. However, regardless of the association with outcome, the high level of B7-H3 expression in PCa makes it an attractive potential therapeutic target.[7] For example, in the recently reported phase 1 cohort expansion for MGC018, an anti–B7-H3 antibody-drug conjugate, preliminary results showed that 5 of 9 patients with metastatic CRPC had a 50% reduction in PSA level,[41] and several other trials are underway.

The current study was limited by its assessment of only a single African ancestry cohort, and additional African, Asian, and European ancestry studies will be required to corroborate our findings. Furthermore, because mRNA levels may also be affected by poor fixation and because of the potential importance of posttranscriptional gene regulation by microRNA miR-29[42] or other mechanisms, it is unclear which metric (protein or mRNA) of B7-H3 is the most biologically representative. Clinical trials may be the best context in which to determine this. A strength of our study was our ability to assess B7-H3 with several patient and tumor characteristics. However, it should be noted that most (but not all) of our findings would remain statistically significant at the Bonferroni-adjusted *P* value of .006 for this study.

Despite these limitations, our study adds to the growing literature on B7-H3 expression in PCa. By using quantitative digital image analysis in high-risk and racially diverse cohorts, we confirmed the association of B7-H3 expression with pathologic stage, *ERG* rearrangement, and *PTEN* loss, and we made a novel observation that B7-H3 expression is inversely correlated with the percentage African ancestry, independent of ERG and PTEN status. This adds to emerging evidence that PCa in men of African ancestry may have a unique tumor biology, with potentially important clinical implications for immunotherapy in this population. Finally, in the first paired analysis reported to date, we found that intense neoadjuvant hormonal therapy was associated with a decrease in B7-H3 staining, suggesting that androgen signaling may positively regulate B7-H3 expression. Furthermore, from AR ChIP-Seq, we observed AR peaks near the promoter of B7-H3, indicating a marked increase in the frequency in PCa versus benign prostate. Taken together, these results may help to guide future clinical trial design around this promising target in PCa. This work provides a possible framework for determining B7-H3 treatment response cutoff levels as parameters for proper patient selection, for developing new agents targeting the AR–B7-H3 interaction, and for exploring B7-H3 as a predictive marker for targeted therapies, such as antibody-drug conjugates.

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## CONFLICT OF INTEREST DISCLOSURES

Ashley E. Ross reports personal fees and honoraria from Veracyte, Astellas, Bayer, Pfizer, Blue Earth, Myovant, and Janssen outside the submitted work. Mary-Ellen Taplin reports honoraria from AstraZeneca, AbbVie, Astellas, Bauer, Blue Earth, Constellation, Celegene, Epizyme, GlaxoSmithKline, Incyte, Janssen, Myovant, Roviant, UpToDate, the University of Pittsburgh, and the American Society for Clinical Oncology; and participation on a data safety monitoring board or advisory board for Clovis, Pfizer, and Myovant outside the submitted work. Nathan A. Lack reports personal fees from Nido Bioscience outside the submitted work. Angelo M. De Marzo reports research support from Janssen and Myriad during the conduct of the study and personal fees from Cepheid and Merck outside the submitted work. Emmanuel S. Antonarakis reports research funding to his institution from Janssen, Johnson & Johnson, Sanofi, Dendreon, Genentech, Novartis, Merck, Bristol Myers Squibb, AstraZeneca, and Constellation; grants or contracts from AstraZeneca, Celgene, Clovis, and Sanofi; personal fees from Aikido Pharma, Blue Earth Diagnostics, Dendreon, EcoR1, KeyQuest Health, Janssen, Pfizer,

and Projects in Knowledge; is a co-inventor of an AR-V7 biomarker technology that has been licensed to Qiagen; and reports participation on a data safety monitoring board or advisory board for Amgen, AstraZeneca, Bayer, Bristol Myers Squibb, Clovis, Constellation, Curium, Eli Lilly and Company, Exact Sciences, Foundation Medicine, Invitae, Ismar, Merck, Orion, Sanofi, and Tempus outside the submitted work. Corinne E. Joshu reports institutional grants or contracts from the American Cancer Society and the National Institutes of Health outside the submitted work. Eugene Shenderov reports institutional research funding from Macrogenics and personal fees from Biopharma, Inc, Firstthough.IO, and Guidepoint Global outside the submitted work. Tamara L. Lotan reports research support from Roche/Ventana, DeepBio, and Myriad Genetics and support for attending meetings and/or travel from the Prostate Cancer Foundation outside the submitted work. The remaining authors made no disclosures.

## AUTHOR CONTRIBUTIONS

**Adrianna A. Mendes:** Data acquisition and interpretation, revising for intellectual content, formal analysis, writing–original draft, and writing–review and editing. **Jiayun Lu:** Data acquisition, formal analysis, and writing–review and editing. **Harsimar B. Kaur:** Data acquisition and writing–review and editing. **Siqun L. Zheng:** Data acquisition and writing–review and editing. **Jianfeng Xu:** Data acquisition and writing–review and editing. **Jessica Hicks:** Data acquisition and writing–review and editing. **Adam B. Weiner:** Data acquisition and writing–review and editing. **Edward M. Schaeffer:** Data acquisition and writing–review and editing. **Ashley E. Ross:** Data acquisition and writing–review and editing. **Steven P. Balk:** Data acquisition and writing–review and editing. **Mary-Ellen Taplin:** Data acquisition and writing–review and editing. **Nathan A. Lack:** Data acquisition and writing–review and editing. **Emirhan Tekoglu:** Data acquisition and formal analysis. **Janielle P. Maynard:** Data acquisition and writing–review and editing. **Angelo M. De Marzo:** Data acquisition and writing–review and editing. **Emmanuel S. Antonarakis:** Data acquisition and writing–review and editing. **Karen S. Sfanos:** Data acquisition and writing–review and editing. **Corinne E. Joshu:** Formal analysis. **Eugene Shenderov:** Conceptualization, funding acquisition, data acquisition and interpretation, revising for intellectual content, writing–original draft, and writing–review and editing. **Tamara L. Lotan:** Conceptualization, funding acquisition, data acquisition and interpretation, revising for intellectual content, formal analysis, writing–original draft, and writing–review and editing.

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