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Circulating Tumor Cell–Based Molecular Classifier for Predicting Resistance to Abiraterone and Enzalutamide in Metastatic Castration-Resistant Prostate Cancer

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Abstract

While circulating tumor cell (CTC)–based detection of *AR-V7* has been demonstrated to predict patient response to second-generation androgen receptor therapies, the rarity of *AR-V7* expression in metastatic castrate-resistant prostate cancer (mCRPC) suggests that other drivers of resistance exist. We sought to use a multiplex gene expression platform to interrogate CTCs and identify potential markers of resistance to abiraterone and enzalutamide. 37 patients with mCRPC initiating treatment with enzalutamide (n = 16) or abiraterone (n = 21) were prospectively enrolled for CTC collection and gene expression analysis using a panel of 89 prostate cancer–related genes. Gene expression from CTCs was correlated with PSA response and radioclinical progression-free survival (PFS) using Kaplan-Meier and Cox regression analyses. Twenty patients (54%) had detectable CTCs. At a median follow-up of 11.3 months, increased expression of the following genes was significantly associated with shorter PSA PFS and radioclinical PFS: *AR*, *AR-V7*, *PSA*, *PSCA*, *TSPAN8*, *NKX3.1*, and *WNT5B*. Additionally, high *SPINK1* expression was associated with increased PFS. A predictive model including all eight genes gave an area under the curve (AUC) of 0.84 for PSA PFS and 0.86 for radioclinical PFS. In comparison, the AR-V7 only model

Abbreviations: ARSI, androgen receptor signaling inhibitors; AUC, area under the curve; CTC, circulating tumor cell; mCRPC, metastatic castrate-resistant prostate cancer; EpCAM, epithelial cell adhesion molecule; EMT, epithelial mesenchymal transition; HR, hazard ratio; IQR, interquartile range; IRB, institutional review board; LHRH, luteinizing hormone-releasing hormone; PFS, progression free survival

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resulted in AUC values of 0.65 and 0.64.These data demonstrate that clinically relevant information regarding gene expression can be obtained from whole blood using a CTC-based approach. Multigene classifiers in this setting may allow for the development of noninvasive predictive biomarkers to guide clinical management.

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Introduction

Treatment options for patients with metastatic castration-resistant prostate cancer (mCRPC) include androgen receptor signaling inhibitors (ARSI), such as abiraterone or enzalutamide, and taxane-based chemotherapy [1–4]. However, optimal treatment algorithms remain controversial, and there is substantial heterogeneity in treatment response [5]. For example, 30%-40% of patients with mCRPC do not respond to ARSI treatment or develop resistance within a brief period of time [1,2,6]. Predictive biomarkers that can help align an individual's tumor biology with an appropriate treatment remain an area of great clinical need.

While there has been marked progress in the development of tissue-based approaches that integrate precision genomics into clinical workflows [7], it is not practical or feasible to perform repeat biopsies with each new treatment decision over time, limiting the clinical utility of these discoveries. As a result, the development of noninvasive liquid markers to recapitulate and/or augment tissue-based information remains a priority, and CTC-based *AR-V7* expression is the first such marker to accurately predict ARSI response in patients with mCRPC [6]. There is a critical need to identify additional markers, however, as *AR-V7*–positive patients account for only a small percentage of ARSI nonresponders and some patients expressing *AR-V7* do respond to ARSI treatment [8,9].

Here, we hypothesized that a CTC-based gene expression platform could be utilized to identify potential molecular markers of response and resistance to ARSI therapy. Through prospective enrollment of mCRPC patients undergoing treatment with abiraterone or enzalutamide, we sought to better understand the degree to which clinically relevant tumor profiles could be identified using blood-based CTC isolation strategies.

Patients and Methods

Patient Cohorts

Thirty-seven patients with mCRPC initiating treatment with abiraterone or enzalutamide and 27 normal controls were prospectively enrolled between January 2015 and September 2016. Genetic predictors of ARSI resistance were examined using a CTC-based mRNA expression assay. All patients consented to an IRB-approved protocol that permits blood sampling and tracking of clinical data.

CTC Isolation and Gene Expression

The CTC isolation and mRNA recovery methods are described in our previous study [10]. Approximately 5 ml of whole blood was drawn into 10-ml EDTA-containing Vacutainer tubes with a cell preservative. All blood samples were obtained before or within 4 weeks after initiation of ARSI therapy. CTCs were positively selected using anti-EpCAM antibody-conjugated Dynabeads (16,203; Thermo Fisher Scientific), which are antibody-coated magnetic beads that isolate cells that express epithelial cell adhesion molecule. Cells were washed and lysed to collect mRNA, which was captured using Oligo(dT)25 mRNA Dynabeads. Reverse transcription was performed to obtain cDNA, and multiplex qPCR was then performed to generate to target gene preamplified library. Real-time qPCR was then utilized to evaluate a panel of 89 genes, plus 3 internal controls (Supplementary Table 1).

CTC Characterization and Normalization of Gene Expression

To classify samples as positive or negative for CTCs, we previously developed an epithelial expression-based model using blood samples from 27 healthy controls with or without exogenous spike-ins of prostate cancer cell lines comprised of eight markers: CD326, CDH1, CDH2, DSG2, EGFR, KRT8, KRT18, and KRT19 (**Supplementary Methods**).

Gene expression was evaluated by qPCR in a 384-well format, and cycle threshold (Ct) values were normalized using the delta–delta Ct method ($2^{(\text{delta–delta Ct}+1)}$) [11]. A total of 92 genes were assessed in each sample of 89 prostate cancer–related genes along with 3 internal control genes (ACTB, TUBA1B, and HMBS). Genes were selected based on a combination of potential relevance as a clinical biomarker and analytic performance characteristics, as previously described [10]. With a limited sample of 20 CTC-positive patients relative to a large number of candidate genes, a preliminary screening step was used to discard genes that could not differentiate between CTC-positive samples and those from 27 healthy controls (**Supplementary Methods**). In a secondary processing step, genes were categorized as "high" or "low" expression relative to a quantile selected from three fixed candidates to maximize the association with the primary clinical outcomes.

Clinical Outcomes

The primary outcomes for this study were (1) prostate-specific antigen (PSA) progression-free survival (PFS) and radioclinical PFS. PSA progression was defined using the Prostate Cancer Working Group 3 definition as a ≥25% increase in PSA levels above the nadir (and by ≫2 ng/ml), with confirmation ≥4 weeks later [12]. Radioclinical progression was defined as a ≫20% increase in the sum of the soft tissue lesion diameters during computed tomography, ≫2 new bone lesions on nuclear medicine bone scan, or symptomatic progression (pain aggravation or cancer-related complications) [12,13]. Secondary outcomes included overall survival and best PSA response (maximum percentage decrease in PSA level from baseline).

Statistical Analysis

The association of each gene with PSA and radioclinical PFS was assessed using Kaplan-Meier analyses and the log-rank test, with *P* values adjusted using the Holm method to control the family-wise error rate across the three candidate quantiles for the gene-specific high/low cutoff values. These cutoff thresholds were selected for each gene to minimize the geometric mean of the *P* values from the PSA and radioclinical PFS analyses. Following selection of the split point,

within the analysis of each outcome, we computed the Holm-adjusted *P* values for the selected genes. Genes nominally significant at the 5% level following this adjustment were considered as candidates for multigene models. In secondary analyses, Cox proportional-hazards regression was used to further evaluate the association of each gene with PSA and radioclinical PFS. Best PSA response for each gene was compared using Fisher's exact test. The utility of single and polygene scores for predicting 90-day PFS was measured using area under the receiver operating characteristic estimated from survival data [14]. Polygene scores were computed as simple sums of the number of component genes scored as 1 or "high". The predictive performances of the polygene and single-gene models were compared. The association of the polygene score with PSA and radioclinical PFS was also assessed. All statistical analyses were performed using R software (version 3.3).

Results

Patient Characteristics

All patients enrolled continued to receive luteinizing hormone-releasing hormone (LHRH) agonist therapy while on secondary hormonal therapy, with 21 patients (56.8%) receiving abiraterone and 16 (43.2%) receiving enzalutamide. At the time of enrollment, the median baseline age was 72 years (interquartile range [IQR]: 67-79 years), and the median PSA level was 20.9 ng/ml (IQR: 11.6-96.8 ng/ml). Patients were followed for a median of 343 days (IQR: 142-640 days), and the majority of patients (54%) had received more than one previous treatment other than an LHRH agonist (Table 1). The baseline characteristics of the cohort are presented in Table 1.

CTC Isolation and Gene Analysis

Using a previously developed epithelial-based expression signature to assess for the presence of CTCs, 20 patients (54%) were classified as having detectable CTCs. A preliminary screening step was used to remove genes whose expression levels could not reliably distinguish between CTC-positive samples and those from healthy controls (Supplementary Figures S1 and S2). Based on this analysis, 41 genes (30 prostate cancer–related genes, 8 epithelial markers, and 3 internal controls) were excluded, and 51 genes were retained for survival analysis.

Compared to patients who were CTC-negative or ARSI responders, patients who were CTC-positive or ARSI nonresponders tended to express a number of prostate cancer–related genes (Figure 1, *A* and *B*). Continuous gene expression values were converted to categories of high and low expression for each gene based on association with survival in Kaplan-Meier analysis. As described in the methods, three potential thresholds were assessed for each gene, with cutoffs set at the 25th (A), 50th (B), and 75th (C) percentiles; i.e., for criteria A, the 15 samples with the lowest gene expression were

Table 1. The Baseline Demographic and Clinical Characteristics of Patients with Metastatic Castration-Resistant Prostate Cancer

Variable	Overall (n = 37)	CTC Positive (n = 20)	CTC Negative (n = 17)	*P* Value
Age, median, IQR, years	72(67-79)	73(64-78)	72(68-80)	.437
Race (%)				
Caucasian	35 (94.6)	18 (90.0)	17 (100.0)	.407
African American	1 (2.7)	1 (5.0)	0 (0.0)	
Hispanic	1 (2.7)	1 (5.0)	0 (0.0)	
PSA, median, IQR, ng/ml	20.9 (11.6-96.8)	65.8(17.4-359.3)	14.4 (7.8-25.8)	.011
No. of prior therapy (%)				
0	17 (45.9)	7 (35.0)	10 (58.8)	.259
1	15 (40.5)	9 (45.0)	6 (35.3)	
2	5 (13.5)	4 (20.0)	1 (5.9)	
Gleason score (%)				
≤7	16 (43.2)	8 (40)	8 (47.0)	.803
8	3 (8.1)	2 (10)	1 (5.9)	
≥9	17 (46.0)	10 (50)	7 (41.2)	
Unknown	1 (2.7)	0 (0.0)	1 (5.9)	
ECOG (%)				
0	21 (56.8)	8 (40.0)	13 (76.5)	.079
1	13 (35.1)	10 (50.0)	3 (17.6)	
2	3 (8.1)	2 (10)	1 (5.9)	
Opioid analgesic (%)				
Yes	26 (70.3)	10 (50)	16 (94.1)	.003
No	11 (29.7)	10 (50)	1 (5.9)	
Albumin, median, IQR, g/dl	4.1(3.9-4.3)	4.1(3.8-4.3)	4.2(4.0-4.4)	.502
Hemoglobin, median, IQR, g/dl	12.8(11.7-13.9)	12.6(10.6-13.2)	12.8(12.5-14.6)	.825
Alkaline phosphatase, Median, IQR, g/dl	102.0(80.5-170.5)	117.0(80.3-267.5)	102.0(82.5-123.5)	≪.001
CTC probability	0.95 (0.06-0.99)	0.99(0.98-0.99)	0.056(0.006-0.170)	≪.001
Extent of disease at baseline				
Bone metastasis				
Yes	33 (89.2)	19 (95.0)	14 (82.4)	.217
No	4 (10.8)	1 (5.0)	3 (17.6)	
Nodal metastasis				
Yes	24 (64.9)	16 (80.0)	8 (47.1)	.036
No	13 (35.1)	4 (20.0)	9 (52.9)	
Visceral metastasis (liver and/or lung)				
Yes	10 (27.0)	6 (30.0)	4 (23.5)	.659
No	27 (73.0)	14 (70.0)	13 (76.5)	

P value: CTC+ vs. CTC–. *ECOG*, Eastern Cooperative Group.

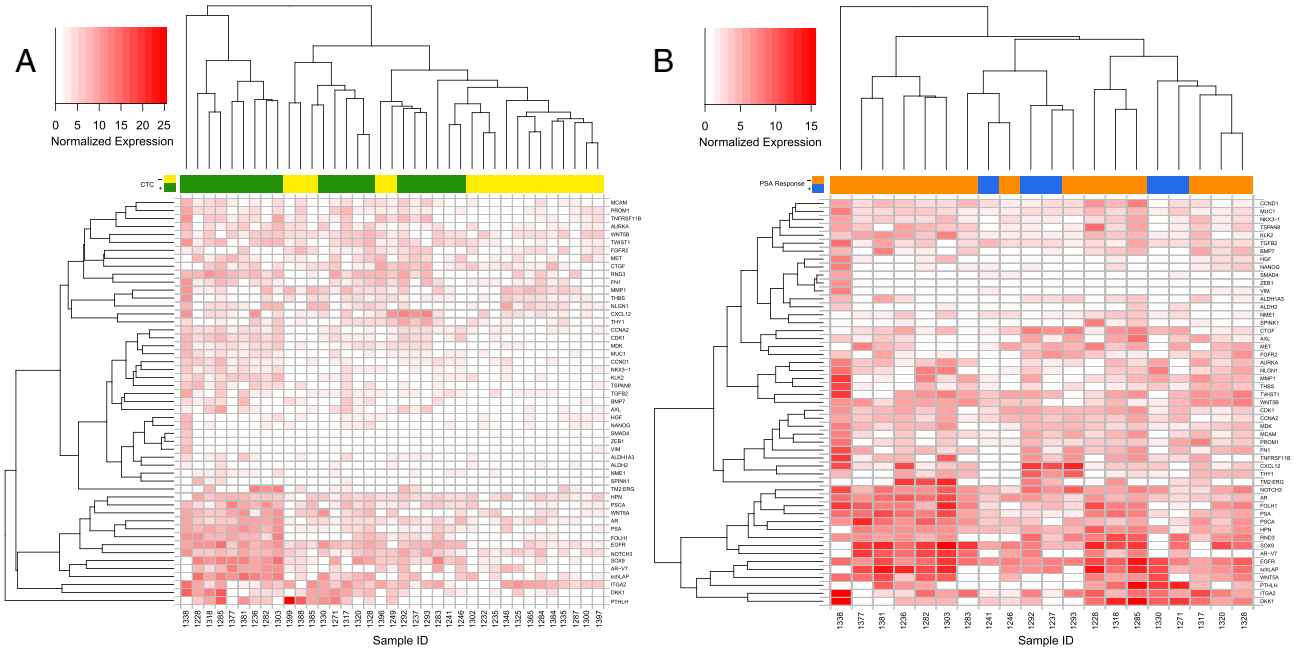

Figure 1. Integrative landscape analysis of gene signatures in metastatic castration-resistant prostate cancer with androgen receptor signaling inhibitor treatment. (A) A heat map representation of gene expression data from the CTC-positive samples (red) and the CTC-negative samples (blue). (B) Hierarchical clustering of gene expression in CTC-positive patients only. The 51 selected gene panels were enriched in the patients with a PSA response (≥50% decline in PSA level from baseline) (blue) compared to patients without a PSA response (red).

grouped as "low" and the remaining 5 grouped as "high." Threshold A was utilized for 18 genes, thrreshold B for 20 genes, and threshold C for 13 genes (Supplementary Tables S2 and S3).

Identification of Genes Associated with Oncologic Endpoints

Among CTC-positive patients, the median times to PSA progression and radioclinical progression were 91 days (IQR: 55-228 days) and 142 days (IQR: 42-492 days), respectively. Among the 51 candidate genes, increased expression of *AR*, *AR-V7*, *PSA*, *PSCA*, *TSPAN8*, *NKX3-1*, and *WNT5B* was significantly associated with decreased PSA PFS (Figure 2) and radioclinical PFS (Figure 3), while *SPINK1* was inversely associated with these outcomes. Univariate Cox regression also supported the association with both PSA and radioclinical PFS for each of these genes (Figure 4, *A* and *B*). Additionally, *BMP7*, *FOLH1*, *SOX9*, and *WNT5a* were nominally associated with PSA PFS, while *THY1*, *PTHLH*, *MDK*, and *HGF* were associated with radioclinical PFS (Supplementary Table S3). Waterfall plots displaying the maximum PSA response among CTC-positive patients and correlation with each of the candidate genes are shown in Supplementary Figure S3. The overall proportion of patients with a PSA response (≥50% decline from baseline) was 25% (5/20), and lack of response to ARSI treatment was significantly associated with high expression of *AR*, *TSPAN8*, *PSCA*, *WNT5B*, and *NKX3-1*. Finally, *AR*, *AR-V7*, *WNT5B*, and *SPINK1* were all associated with overall survival in the Kaplan-Meier analyses for this secondary endpoint (Supplementary Figure S4).

Pilot Development of Multigene Model

The multigene model comprised of all candidate genes (*AR*, *AR-V7*, *PSA*, *PSCA*, *TSPAN8*, *WNT5B*, *NKX3-1*, and *SPINK1*) was assessed as an exploratory analysis and compared to a single-gene model for *AR-V7* (Supplementary Figure S5). Receiver operating curves were constructed, and the AUCs for the multigene model showed increased accuracy compared to *AR-V7* alone for PSA PFS (0.84 vs. 0.65) and radioclinical PFS (0.86 vs. 0.64). In addition, the multigene model score was significantly associated with decreased PSA PFS and radioclinical PFS (Supplementary Figure S6).

Discussion

Given the variable patterns of response to ARSI therapy in mCRPC, there is a critical need for predictive markers to guide precision-based therapeutic strategies. While *AR-V7* provides an initial model for this approach, additional markers are needed [6,9]. The present study lends additional support to the utility of *AR-V7* in this setting, as all patients with high *AR-V7* expression were ARSI nonresponders. However, 10 of 15 patients (66.7%) with low *AR-V7* expression were also ARSI nonresponders, indicative of the variability of response and need to identify other detectable drivers of resistance. In this study, we demonstrate that multiple CTC-based biomarkers can be simultaneously evaluated and that there are several non–*AR-V7* biomarkers that may be predictive of ARSI response.

Many of the genes identified in this biomarker development study —specifically, *AR*, *AR-V7*, *NKX3-1,* and *PSA*—are consistent with the known importance of AR signaling–driven resistance to ARSI treatment. For example, numerous prior studies have confirmed that amplification of the *AR* gene predicts response to ARSI therapy [15,16]. Elevated *AR* transcription may also increase the generation of constitutively active truncated AR variants, as a CTC-based study revealed that *AR-V7* amplification was directly proportional to *AR* levels and truncated *AR* splice variants were associated with ARSI

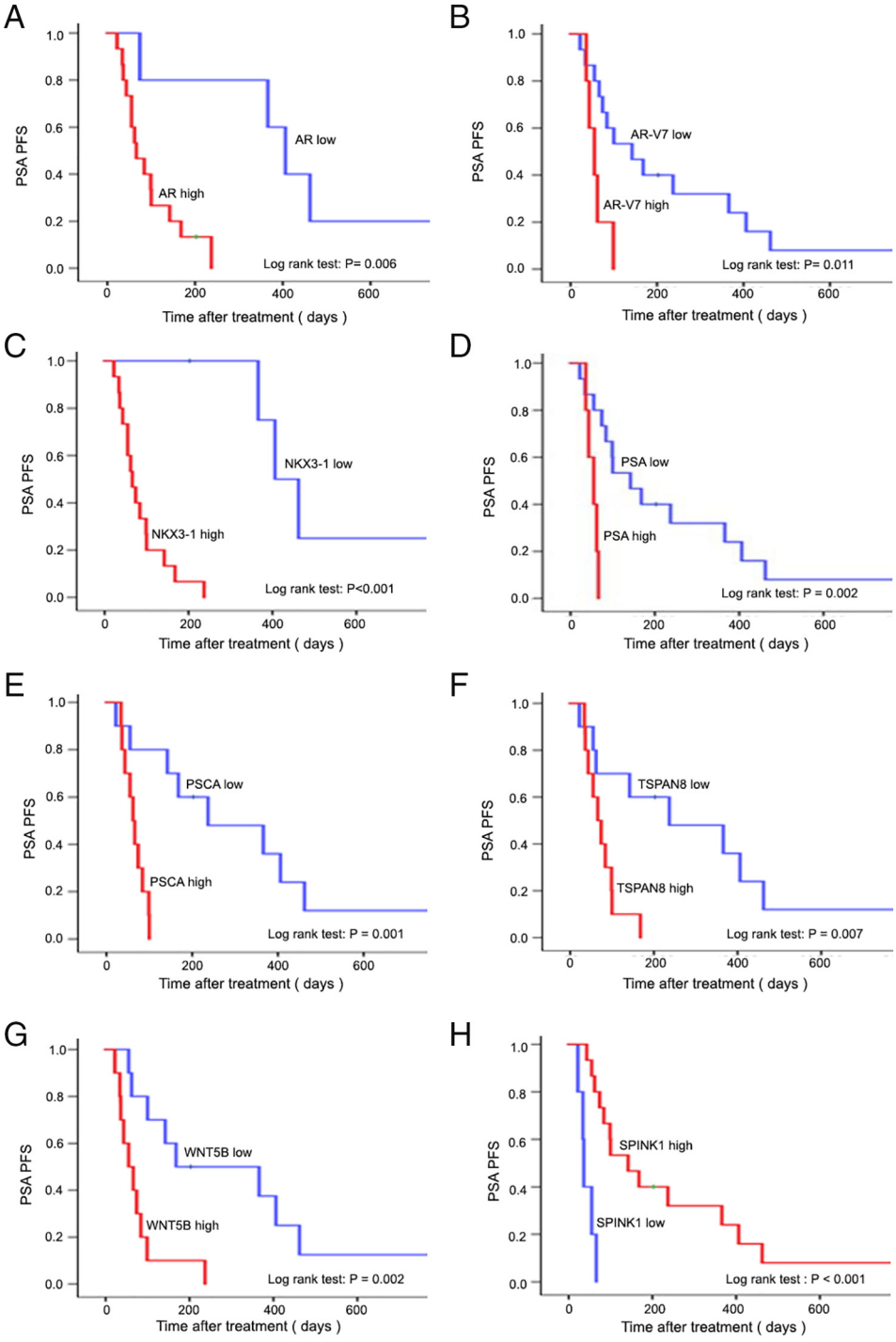

Figure 2. Kaplan-Meier plots for PSA PFS according to expression of eight genes (A: *AR*, B: *AR-V7*, C: *NKX3.1*, D: *PSA*, E: *PSCA*, F: *TSPAN8*, G: *WNT5B*, H: *SPINK1*). The *P* value is calculated using the log-rank test.

resistance [6,17]. *NKX3-1* is an AR-regulated homeobox gene and well-known marker of AR signaling [18]. *NKX3-1* has been shown to co-localize with AR and acts with other downstream pathways to promote cell survival in advanced prostate cancer [19]. Additionally, PSA transcripts, which are related to the AR signaling pathway, are known to be associated with time to ARSI treatment failure or death [20]. Fangfang et al. recently reported that detection of PSA transcripts in peripheral blood mononuclear cells could predict the time to ARSI treatment failure in patients with CRPC [21]. However, the present study is the first we are aware of to comprehensively and simultaneously assess AR signaling across multiple genes in this setting using a liquid-based approach.

In addition to the potential for AR signaling-based markers to serve as predictive markers, we identified markers of epithelial-mesenchymal transition (EMT) and "stemness" as having potential clinical relevance. Prostate stem cell antigen (*PSCA*) is a cell-surface 123–amino acid glycoprotein that is 30% identical to stem cell antigen type 2 (*SCA-2*) and was first identified in the LAPC-4 xenograft model of prostate cancer [22]. Elevated *PSCA* expression is correlated with higher tumor stage and progression to androgen

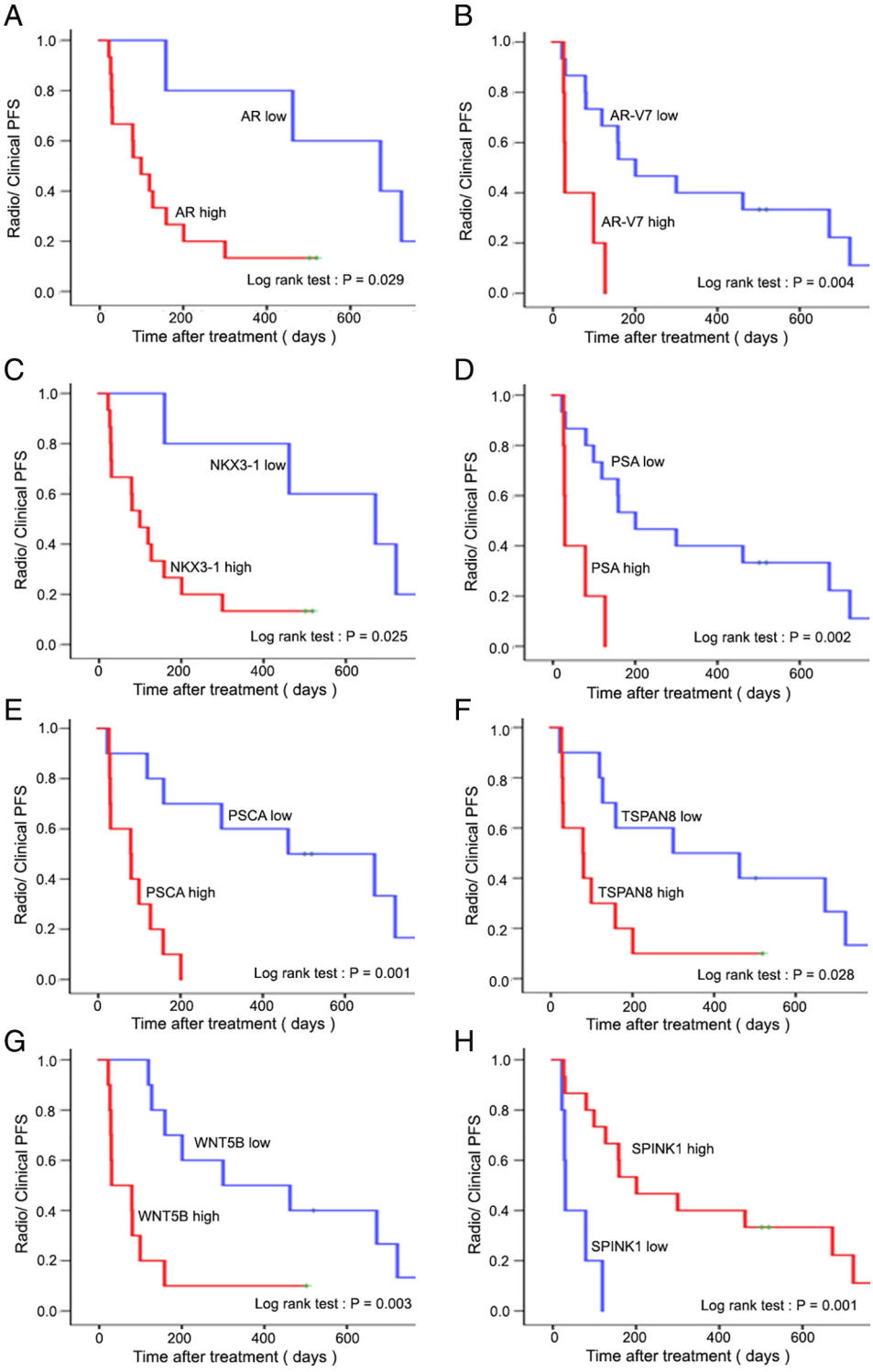

Figure 3. Kaplan-Meier plots for radiological and/or clinical PFS according to expression of eight genes (A: *AR*, B: *AR-V7*, C: *NKX3.1*, D: *PSA*, E: *PSCA*, F: *TSPAN8*, G: *WNT5B*, H: *SPINK1*). The *P* value is calculated using the log-rank test.

independence, and one previous study has indicated its potential prognostic value as a CTC-based marker [23,24]. The WNT pathway is also linked to treatment resistance in mCRPC [25], and *WNT5* signaling can induce epithelial-to-mesenchymal transition in cancer, with mesenchymal transitioned cancer cells instigating the invasion of neighboring epithelial cancer cells through *WNT5B* secretion [26]. While each of these markers—and other candidates, *TSPAN8* and *SPINK1*—needs validation, these data indicate that a liquid-based approach can provide clinically meaningful information surrounding the response to ARSI therapy.

The classification of patients as having high or low gene expressions is complicated, as the expression profiles vary for individual CTCs [25]. Technology for gene expression profiling based on CTCs is in its infancy, and at present, there are no robust reference sets against

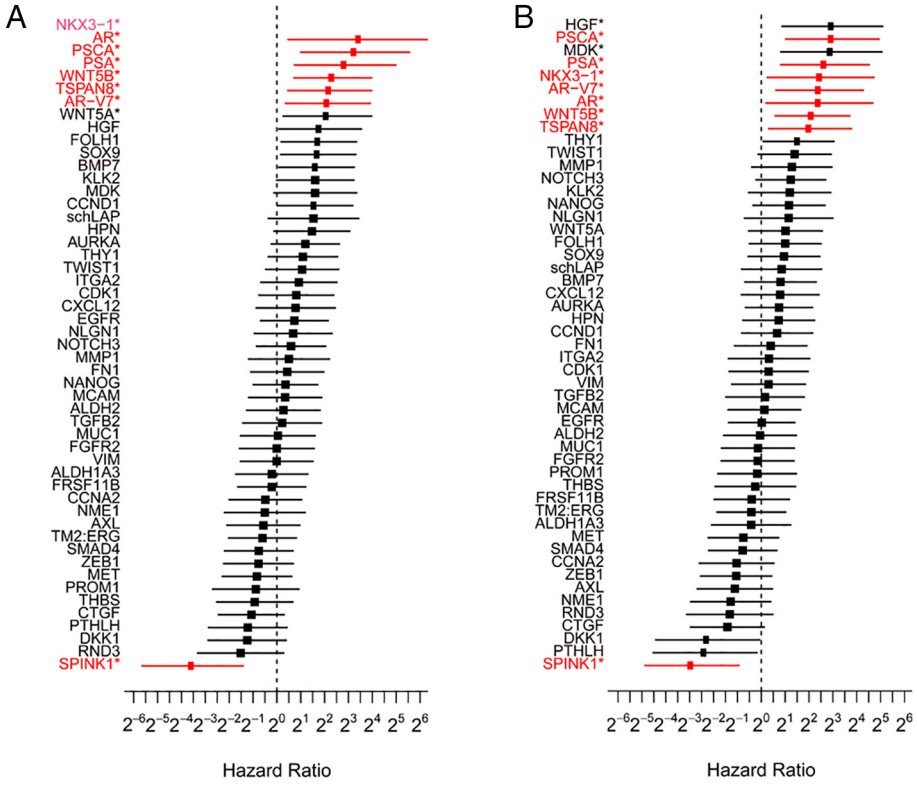

Figure 4. Cox proportional-hazard analyses of the associations between individual gene expression and PSA PFS (A) or radioclinical PFS (B). The estimate for the hazard ratio of NKX3-1 in the Cox model for time to PSA progression diverged to infinity and has consequently been omitted from the plot. Gene names shown in red were nominally significant for both clinical outcomes.

which to compare the relative expression of our samples. Instead, we used a data-driven approach to find relevant thresholds associated with PFS in order to demonstrate the potential utility of this technology. In doing so, we filtered out genes with expression patterns that could not distinguish between CTC-positive samples and normal controls, and optimized cutoff values for potentially useful genetic biomarkers. These analyses nominated a number of candidate genes consistent with potential ARSI resistance (*AR*, *PSA*, *PSCA*, *TSPAN8*, *SPINK1*, *NKX3.1*, *WNT5B*, and *AR-V7*), which may provide additional prognostic and predictive information beyond AR-V7 alone.

The present study has several additional limitations. First, the small sample size supports only a discovery-based approach, and prospective validation in larger cohorts is needed. Second, we relied on epithelial expression for enrichment and identification of CTCs, which likely misses some clinically relevant CTCs that have undergone EMT. However, the findings here suggest that this did not preclude identification of EMT/stem cell markers of progression. Third, drug resistance can occur through numerous mechanisms that may not be detected through RT-PCR, including translocation and mutation, and thus assessment of circulating cfDNA could augment the approach utilized here. Finally, we performed cell lysis immediately after cell enrichment, which precludes CTC enumeration and assessment of the level of CTC purity in the sample. However, the clinical utility of enumeration-based approaches remains unclear, and background leukocyte contamination is accounted for through normalization of gene expression to control samples [27].

Conclusion

We confirmed the potential for molecular analysis of enriched CTCs to help derive markers of treatment response and resistance in this prospective study of patients undergoing ASRI therapy. In addition to *AR-V7*, which is now a well-known marker in this setting, we identified multiple additional clinically relevant genes that can be detected through a simple blood draw. Although additional research is needed to validate these findings, CTC isolation and molecular characterization are an important and feasible avenue for biomarker discovery. Using methods such as the one described here, the ability to interrogate tumor expression via a simple blood draw offers substantial potential for enhancing precision-based treatment selection in mCRPC.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2019.06.002.

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Conflicts of Interest

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