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Original Article

Whole blood *GRHL2* expression as a prognostic biomarker in metastatic hormone-sensitive and castration-resistant prostate cancer

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Background: As potent systemic therapies transition earlier in the prostate cancer disease course, molecular biomarkers are needed to guide optimal treatment selection for metastatic hormone-sensitive prostate cancer (mHSPC). The value of whole blood RNA to detect candidate biomarkers in mHSPC remains largely undefined.

Methods: In this cohort study, we used a previously optimised whole blood reverse transcription polymerase chain reaction assay to assess the prognostic utility [measured by seven-month undetectable prostate-specific antigen (PSA) and time to castration-resistance (TTCR)] of eight prostate cancer-associated gene transcripts in 43 mHSPC patients. Transcripts with statistically significant associations ($P<0.05$) were further investigated in a metastatic castration-resistant prostate cancer (mCRPC) cohort (n=119) receiving contemporary systemic therapy, exploring associations with PSA >50% response (PSA_{50}), progression-free survival (PFS) and overall survival (OS). Clinical outcomes were prospectively collected in a protected digital database. Kaplan-Meier estimates and multivariable Cox proportional-hazards models assessed associations between gene transcripts and clinical outcomes (mHSPC covariates: disease volume, docetaxel use and haemoglobin level; mCRPC covariates: prior exposure to chemotherapy or ARPIs, haemoglobin, performance status and presence of visceral disease). Follow-up was performed monthly during ARPI treatment, three-weekly during taxane chemotherapy, and three-monthly during androgen deprivation therapy (ADT) monotherapy. Serial PSA measurements were performed before each follow-up visit and repeat imaging was at the discretion of the investigator.

Results: Detection of circulating Grainyhead-like 2 (*GRHL2*) transcript was associated with poor outcomes in mHSPC and mCRPC patients. Detectable *GRHL2* expression in mHSPC was associated with a lower rate of seven-month undetectable PSA levels (25% *vs.* 65%, P=0.059), and independently associated with shorter TTCR (HR 7.3, 95% CI: 1.5–36, P=0.01). In the mCRPC cohort, *GRHL2* expression predicted significantly lower PSA_{50} response rates (46% *vs.* 69%, P=0.01), and was independently associated with shorter PFS (HR 3.1, 95% CI: 1.8–5.2, P<0.001) and OS (HR 2.9, 95% CI: 1.6–5.1, P<0.001). Associations were most apparent in patients receiving ARPIs.

Conclusions: Detectable circulating *GRHL2* was a negative prognostic biomarker in our mHSPC and mCRPC cohorts. These data support further investigation of *GRHL2* as a candidate prognostic biomarker in metastatic prostate cancer, in addition to expanding efforts to better understand a putative role in therapeutic resistance to AR targeted therapies.

Keywords: Grainyhead-like 2 (*GRHL2*); biomarker; hormone-sensitive; castration-resistant; prostate cancer

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Introduction

In recent years, the management of metastatic hormone-sensitive prostate cancer (mHSPC) has undergone major change. Early treatment intensification with docetaxel chemotherapy or potent androgen receptor pathway inhibitors (ARPI) at the commencement of androgen deprivation therapy (ADT) has resulted in profound improvements in overall survival (OS) and quality of life, representing the new standard-of-care for this disease entity (1). As these potent systemic therapies move earlier into the prostate cancer disease course, and more treatment choices become available, it is imperative that prognostic and predictive biomarkers be identified to guide optimal treatment selection for both new and existing agents.

Biomarkers in metastatic prostate cancer have traditionally centred around clinicopathological factors to predict prostate tumour biology and response to systemic treatment. In mCRPC, these characteristics have included lactate dehydrogenase (LDH), prostate-specific antigen (PSA) level, alkaline phosphatase (ALP), albumin, Gleason score, Eastern Cooperative Oncology Group (ECOG) performance status, haemoglobin, presence of visceral disease and use of opioid analgesia (2-4). Similar clinical parameters have also been found to be prognostic in the mHSPC population (5,6). While these clinical parameters demonstrate reasonable concordance with clinical outcomes, they are insufficient to influence treatment selections. Furthermore, as sequencing of systemic therapy appears to influence the efficacy of subsequent lines of treatment (7), there is even greater imperative that methods beyond clinical variables be used to personalise metastatic prostate cancer treatment. These shortcoming of clinicopathological variables as decision aids have led many to propose molecular characterisation of prostate cancer as an alternative pathway towards identification of promising candidate biomarkers capable of being incorporated into clinical practice.

The conventional approach to molecular biomarker identification entails analysis of tumour tissue samples. However, the nature of metastatic prostate cancer is such that obtaining adequate tumour material for analysis remains technically challenging. Attention has instead shifted to the development of minimally invasive blood-based biomarkers. To date, the field has focused primarily on the analysis of circulating tumour cells (CTCs) (8-10) and circulating tumour DNA (11,12), supported by advances in high-throughput sequencing technologies and increasingly sophisticated bioinformatic analysis techniques. Characterisation of whole blood RNA expression is another approach for molecular profiling in prostate cancer patients (13-16). This technique offers the opportunity to investigate diverse sources of RNA within blood including extracellular vesicles, platelets and circulating cell-free RNA (17), with the added benefit of being amenable to

workflow automation, an important consideration that facilitates implementation in diagnostic laboratories outside of academic and research institutions (18,19).

We have previously developed a whole blood quantitative reverse transcription polymerase chain reaction (RT-PCR) assay to detect a panel of eight circulating prostate cancer-associated gene transcripts in a group of mCRPC patients (20). Building upon this work, we hypothesised that expression of specific transcripts may correlate with clinical outcomes related to tumour progression in mHSPC, and these same transcripts would have prognostic utility in our mCRPC cohort. We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/tau-20-1444).

Methods

Study population and sample collection

This cohort study included two distinct patient groups: mHSPC and mCRPC. Patients with mHSPC were prospectively enrolled from two separate Australian institutions: Monash Health (March 2018–June 2019) and St Vincent's Hospital (November 2016–October 2018). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the Human Research Ethics Committee at Monash Health (HREC 11571X) and St Vincent's Hospital (SVH 12/231). Written informed consent was obtained from all individual participants. Patients were eligible if initial blood draw occurred prior to or within six weeks of first administration of ADT (i.e., gonadotropin-releasing hormone analogues and/or first-generation antiandrogens). To optimise study recruitment, no other additional inclusion or exclusion criteria were mandated for this cohort.

The mCRPC cohort was prospectively enrolled from three Australian institutions (Monash Health, Chris O'Brien Lifehouse and Eastern Health) between September 2016 and September 2018, and has been previously described (20). Key eligibility criteria include patients with biochemically or radiographically progressive mCRPC planned for commencement of new systemic therapy, either ARPIs (e.g., abiraterone, enzalutamide) or taxane chemotherapy (e.g., docetaxel, cabazitaxel). Blood samples were taken immediately prior to the commencement of systemic therapy. Compared to prior reports (20), the present study represents an expanded patient sample with an extended follow-up period (up to September 30, 2019).

Gene expression assay

The RT-PCR assay was performed as previously described (20). Briefly, peripheral blood (2.5 mL) was collected in a PAXgene blood RNA tube (Qiagen, Hilden, Germany) and stored at –80 ℃ until batch sample processing. RNA was extracted from thawed whole blood, before undergoing qualitative and quantitative assessment. High-quality RNA was then reverse transcribed and pre-amplified. Gene expression was assessed using pre-designed, publicly available Taqman™ assays for the following target transcripts: *FOLH1*, *FOXA1*, *GRHL2*, *HOXB13*, *KLK2*, *KLK3*, *NPY* and *TMPRSS2*:*ERG*. Gene expression was defined as samples that reached cycle threshold within 35 cycles in at least two of three replicates per patient.

Data collection, outcome measures and follow-up

In both the mHSPC and mCRPC cohorts, the following data was collected at the time of PAXgene sample collection and stored in a protected clinical database: prior local and systemic treatment, racial background, Eastern Cooperative Oncology Group (ECOG) performance status, disease extent, complete blood count and serum biochemistry (including PSA level). In the mHSPC cohort, additional clinical characteristics related to disease volume and use of treatment intensification were also collected, both of which are known to have prognostic/predictive significance (21-23). Disease volume was categorised as per ECOG 3805 CHAARTED criteria (22), with high-volume disease defined by the presence of at least four bone metastases (with at least one beyond the axial skeleton), and/or the presence of visceral metastases. Treatment intensification was defined by concurrent administration of either docetaxel chemotherapy or AR targeted therapy with concurrent ADT therapy for mHSPC.

In the mHSPC cohort, the intermediate outcomes of seven-month undetectable PSA rate (defined as ≤0.2 ng/mL) and time to castration-resistance (TTCR) were selected based on their established prognostic utility (24,25). TTCR was defined by duration between commencement of ADT and development of PSA, symptomatic or radiographic

progression. In the mCRPC cohort, the outcome measures investigated were as follows: (I) PSA_{50} response (PSA decline from baseline of ≥50%, confirmed ≥3 weeks later), (II) progression-free survival (PFS) (time from treatment commencement to first of confirmed PSA progression, clinical or radiographic progression, or death from prostate cancer), and (III) OS (time from treatment commencement until death from any cause).

Follow-up interval was not strictly enforced for study patients, but left to the clinical discretion of the site investigator. In general, patients were reviewed monthly during ARPI therapy, three-weekly during taxane chemotherapy and three-monthly if receiving ADT monotherapy. Serial PSA measurements for disease monitoring were performed at the same laboratory prior to each follow-up visit, and repeat radiographic imaging was at the discretion of the investigator.

Statistical analysis

The associations between gene panel transcripts and categorical outcome measures (seven-month undetectable PSA in mHSPC cohort; PSA_{50} response in mCRPC cohort) were assessed using chi-square statistics. Univariable Cox proportional hazards models were used to assess the association between gene transcript expression and time-to-event outcomes (TTCR in mHSPC cohort; PFS and OS in mCRPC cohort), with P values adjusted for multiple testing using the Benjamini-Hochberg correction where appropriate (26). Exploratory subgroup analysis based on treatment administered (taxane chemotherapy and ARPI) was also performed in the mCRPC cohort. Gene transcripts reaching statistical significance (defined as $P<0.05$) in univariable analyses were incorporated into multivariable models with established clinical prognostic variables. Multivariable analyses in the mHSPC cohort (n=43) were restricted to three covariates to prevent overfitting: disease volume (high *vs.* low), use of treatment intensification, and haemoglobin [< lower limit of normal (LLN) *vs.* ≥ LLN]. Multivariable analyses in the mCRPC cohort (n=119) included prior chemotherapy exposure (yes *vs.* no), prior ARPI exposure (yes *vs.* no), haemoglobin (< LLN *vs.* ≥ LLN), Eastern Cooperative Oncology Group (ECOG) performance status (≥2 *vs.* 0–1), and presence of visceral metastases. All time-to-event outcomes were censored at date of last patient contact if the event had not occurred. All statistics and survival curves were generated using R Studio, version 1.1.463 (RStudio: Integrated Development Environment for R, Boston, Massachusetts). Given the exploratory nature of the study, no formal sample size calculations were undertaken. Instead, the study adopted a pragmatic design with few eligibility criteria, aimed at maximising patient recruitment.

Results

Study cohorts

Peripheral whole blood was obtained from 43 mHSPC patients. The clinical characteristics of this cohort are shown in *Table 1*. A total of 65% (28/43) of patients had *de novo* metastases at mHSPC diagnosis, including 40% (17/43) with high-volume disease. Upfront treatment intensification was administered in 49% (21/43) of patients. The relative detection frequency for each gene transcript is presented in Figure S1. At the data cut-off date of September 30, 2019, median follow-up time in the mHSPC cohort was 13.5 months [interquartile range (IQR), 7.8–20.5 months (mo)] for living patients. In the mCRPC cohort, pre-treatment whole blood samples were obtained from 119 patients immediately prior to commencing AR pathway inhibitors (n=83) or taxane chemotherapy (n=36). The clinical characteristics of this cohort are presented in *Table 2*. At the data cut-off date of September 30, 2019, median follow-up time in the mCRPC cohort was 22.8 mo (IQR, 12.0–30.6 mo) for living patients.

Gene transcript expression and outcomes in mHSPC cohort

Of the 43 patients, 39 (91%) had available data to assess seven-month undetectable PSA as an outcome measure. Table S1 summarises the undetectable PSA rates according to gene panel transcripts. Patients with detectable *GRHL2*, *HOXB13* or *KLK3* transcripts in blood were less likely to achieve an undetectable PSA, but these associations were not statistically significant (*GRHL2*: 25% *vs.* 65%, P=0.24; *HOXB13* 33% *vs.* 66%, P=0.24; *KLK3* 38% *vs.* 65%, P=0.27; adjusted for multiple testing). There was no significant difference in use of treatment intensification based on *GRHL2* status (*GRHL2*+ *vs. GRHL2*–: 50% *vs.* 49%, P=1.0), *HOXB13* status (*HOXB13*+ *vs. HOXB13*–:

Table 1 Patient characteristics of mHSPC cohort

Characteristic	Study cohort (n=43)
Treatment allocation, n [%]	
ADT alone	22 [51]
ADT plus docetaxel	20 [47]
ADT plus abiraterone acetate	1 [2]
Age	
Median age [range]	70 [45–87]
Race, n [%]	
White	39 [91]
Asian	4 [9]
ECOG PS, n [%]	
0–1	37 [86]
2	6 [14]
Extent of disease, n [%]	
Bone +/– lymph node	31 [72]
Lymph node only	11 [26]
Visceral	5 [12]
Gleason grade group, n [%]	
Grade Group 1–3 (Gleason ≤7)	6 [14]
Grade Group 4–5 Gleason ≥8)	26 [60]
No biopsy/unknown	11 [26]
Primary treatment, n [%]	
Surgery	9 [21]
Radiation +/– ADT	4 [9]
Metastatic disease at diagnosis	28 [65]
No treatment for localised disease	2 [5]
Baseline chemistry at study entry (median, range)	
PSA (ng/mL)	51 [1.3–2,910]
Haemoglobin (g/L)	140 [91–173]
ALP (U/L)	93 [31–1,457]
RNA concentration (ng/μL)	
Median (range)	88 [24–242]
Timing of sample collection, n [%]	
Sample collected before ADT initiation	35 [81]
Sample collected after ADT initiation [range in days]	8 [19] [1–35]

mHSPC, metastatic hormone-sensitive prostate cancer; ADT, androgen deprivation therapy; ALP, alkaline phosphatase; ECOG PS, Eastern Cooperative Oncology Group performance status; NLR, neutrophil-lymphocyte ratio; PSA, prostate-specific antigen.

Table 2 Patient characteristics of mCRPC cohort

Characteristic	Study cohort (n=119)
Treatment allocation, n [%]	
ARPI	
Enzalutamide	67 [57]
Abiraterone acetate	16 [14]
Chemotherapy	
Docetaxel	25 [21]
Cabazitaxel	11 [8]
Line of treatment	
First-line	59 [50]
Second-line	42 [35]
Third-line or beyond	18 [15]
Age	
Median age [range]	72 [46–91]
Race, n [%]	
White	109 [91]
Asian	5 [4]
African American/Black	3 [3]
Other	2 [2]
ECOG PS, n [%]	
0–1	111 [92]
2	8 [8]
Extent of disease, n [%]	
Bone +/– lymph node	111 [94]
Lymph node only	4 [4]
Visceral	12 [10]
Gleason grade group, n [%]	
Grade Group 1–3 (Gleason ≤7)	29 [24]
Grade Group 4–5 (Gleason ≥8)	61 [50]
No biopsy/unknown	29 [25]
Primary treatment, n [%]	
Surgery	23 [19]
Radiation +/– ADT	23 [19]
Metastatic disease at diagnosis	63 [53]
Primary ADT	6 [5]
No treatment for localised disease	4 [3]

Table 2 (*continued*)

Table 2 (*continued*)

Characteristic	Study cohort (n=119)
Prior use of chemotherapy, n [%]	
None	48 [47]
Any	71 [53]
Prior use of ARPI, n [%]	
None	89 [75]
Any	30 [25]
Baseline chemistry at study entry (median, range)	
PSA (ng/mL)	38.8 [0.51–2,719]
Haemoglobin (g/L)	126 [74–154]
ALP (U/L)	135 [45–5,918]
RNA concentration (ng/μL)	
Median (range)	91 [34–295]

mCRPC, metastatic castration-resistant prostate cancer; ADT, androgen deprivation therapy; ALP, alkaline phosphatase; ARPI, androgen receptor axis pathway inhibitor; ECOG PS, Eastern Cooperative Oncology Group performance status; PSA, prostate-specific antigen; ULN, upper limit of normal.

62% *vs.* 43%, P=0.3) or *KLK3* status (KLK3+ *vs.* KLK3–: 57% *vs.* 45%, P=0.6), suggesting that use of docetaxel chemotherapy or ARPI were unlikely to have impacted these findings.

The median TTCR for the mHSPC cohort was not reached. Cox regression analysis of the association between gene transcripts and TTCR are shown in *Table 3*. In univariable analysis, circulating *GRHL2* was strongly associated with shorter TTCR (median TTCR 9.7 mo *vs.* not reached; 12-month mCRPC progression rate 93% *vs.* 30%; P<0.001; *Figure 1A*). This remained significant in multivariable analysis when accounting for established clinicopathological prognostic markers of disease burden (Table S2) including disease volume, treatment intensification use and baseline haemoglobin (HR 7.3, 95% CI: 1.5–36, P=0.01). No other transcripts were significantly associated with TTCR.

GRHL2 expression and outcomes in mCRPC

Given the strong association of *GRHL2* expression and outcomes observed in the mHSPC cohort, we tested clinical outcomes with *GRHL2* expression in a separate cohort of mCRPC patients. In total, *GRHL2*-positive patients comprised 46% (55/119) of the cohort. Presence of the *GRHL2* transcript predicted for significantly lower PSA_{50} response rates [positive/negative: 25/55 (46%) *vs.* 44/64 (69%), P=0.01]. Analysis by treatment administered demonstrated that these results were driven primarily by ARPI-treated patients [positive/negative: 16/37 (43%) *vs.* 35/46 (76%), P=0.003], with no effect apparent in taxane chemotherapy-treated patients [positive/negative: 9/18 (50%) *vs.* 9/18 (50%), P=1.0]. Figure S2 shows the waterfall plots of best PSA response according to *GRHL2* status and treatment administered.

Median PFS and OS was 6.9 mo (IQR, 2.9–18.7 mo) and 20.6 mo (IQR, 9.8–34.5 mo), respectively. All but one patient in the mCRPC cohort died as a result of progressive prostate cancer. Patients with detectable circulating *GRHL2* transcript experienced significantly shorter PFS and OS (*Figure 1B,C*). These associations remained significant in multivariable analysis when adjusting for other baseline prognostic clinicopathological factors (Table S3) including prior chemotherapy and ARPI exposure, ECOG performance status, visceral disease and baseline haemoglobin (*Table 4*); similar to PSA response, these correlations were primarily observed in the ARPI-treated subgroup.

Discussion

In this prospective study, using a previously optimised whole blood RT-PCR assay, our aim was to identify a circulating biomarker(s) linked to treatment outcome in mHSPC patients commencing ADT and a separate cohort of mCRPC patients receiving contemporary systemic therapy. We found that detectable expression of the AR coregulator *GRHL2* in blood was a negative prognostic biomarker throughout the clinical spectrum of metastatic prostate cancer and was associated with rapid resistance to ADT, shorter response to systemic therapy and reduced OS. Notably, these associations were present even when accounting for baseline clinicopathological variables of prognostic significance.

The GRHL2 protein is part of the grainyhead-like transcription factor family and has been implicated in the

Table 3 Cox proportional hazards analysis of time to castration-resistance in mHSPC cohort based on gene transcripts

Gene transcript	n	Univariable[§]			Multivariable[†]		
		HR	95% CI	P	HR	95% CI	P
FOLH1[#]	35 [81]	5.0	0.63–651	0.24	–	–	–
FOXA1	22 [51]	1.4	0.37–5.1	0.64	–	–	–
GRHL2	8 [19]	7.8	2.0–30	0.02*	7.3	1.5–36	0.01*
HOXB13	13 [30]	4.9	1.3–19	0.08	–	–	–
KLK2	14 [33]	2.3	0.62–8.7	0.28	–	–	–
KLK3	14 [33]	2.6	0.71–9.8	0.24	–	–	–
NPY	34 [79]	3.3	0.40–27	0.31	–	–	–
TMPRSS2	3 [7]	4.4	0.88–22	0.19	–	–	–

All P values <0.05 are indicated with *. [§]Adjusted for multiple testing using the standard Benjamini-Hochberg correction. [†]Clinical variables incorporated into MVA: disease volume (high *vs.* low), docetaxel use in mHSPC (yes *vs.* no), haemoglobin (< LLN *vs.* ≥ LLN). [#]Cox regression fitted with Firth's penalized maximum likelihood bias reduction model. mHSPC, metastatic hormone-sensitive prostate cancer; CI, confidence interval; HR, hazard ratio; MVA, multivariable analysis.

pathogenesis of various cancers (27), though its functional relevance in prostate cancer has remained largely undefined until recently. Critical work by Paltoglou *et al.* supports dual oncogenic and protective roles of GRHL2, enhancing AR signalling through its function as an AR coregulator, whilst simultaneously downregulating intracellular signalling pathways responsible for epithelial-mesenchymal transition and cellular invasion (28). This delicate counterbalance between oncogenic and tumour suppressive phenotypes has also been observed in ovarian and breast cancer (29,30). The results here suggest that the balance in prostate cancer may be tipped towards GRHL2 as an oncogenic driver, though validation in larger independent cohorts is necessary before arriving at any definitive conclusions.

There is a relative paucity of data on the prognostic significance of circulating *GRHL2* in prostate cancer. Danila *et al.* selected *GRHL2* as a part of a five-gene whole blood RT-PCR assay, which also included the transcripts *KLK2*, *KLK3*, *HOXB13* and *FOXA1* (31). The authors found that presence of two or more transcripts was associated with shorter OS, and had superior prognostic value compared to CTC enumeration alone (31). However, the contribution of each individual transcript to the overall impact of this composite signature was not reported. Todenhofer *et al.* addressed the relative contribution of these transcripts in a cohort of mCRPC patients exclusively treated with abiraterone acetate (32). While there was a strong trend towards *GRHL2* expression and shorter OS, it did not correlate with other clinical endpoints, perhaps reflecting the relatively small sample size of 37 patients. Importantly, both these studies were exclusively in mCRPC population groups. To date, no data exists for GRHL2 in earlier disease states.

This study explored the relevance of *GRHL2* in an mHSPC cohort. Notably, *GRHL2*-positive patients experienced particularly poor outcomes, rapidly progressing from ADT commencement to mCRPC (median, 9.7 mo). We also observed poorer outcomes in mCRPC patients with detectable circulating *GRHL2* mRNA expression. These associations were most apparent in patients receiving ARPI therapy, consistent with preclinical evidence demonstrating GRHL2's role in enhancing transcriptional activity of both full length AR and constitutively active truncated AR variants (28), which are well-recognised resistance mechanisms to AR directed therapies (11,33).

Circulating *GRHL2* expression may reflect a tumour's increased reliance on the AR pathway as a primary method of growth signalling. Whilst theoretically this may render such tumours more susceptible to AR directed therapies, the presence of AR gain/amplification in plasma circulating

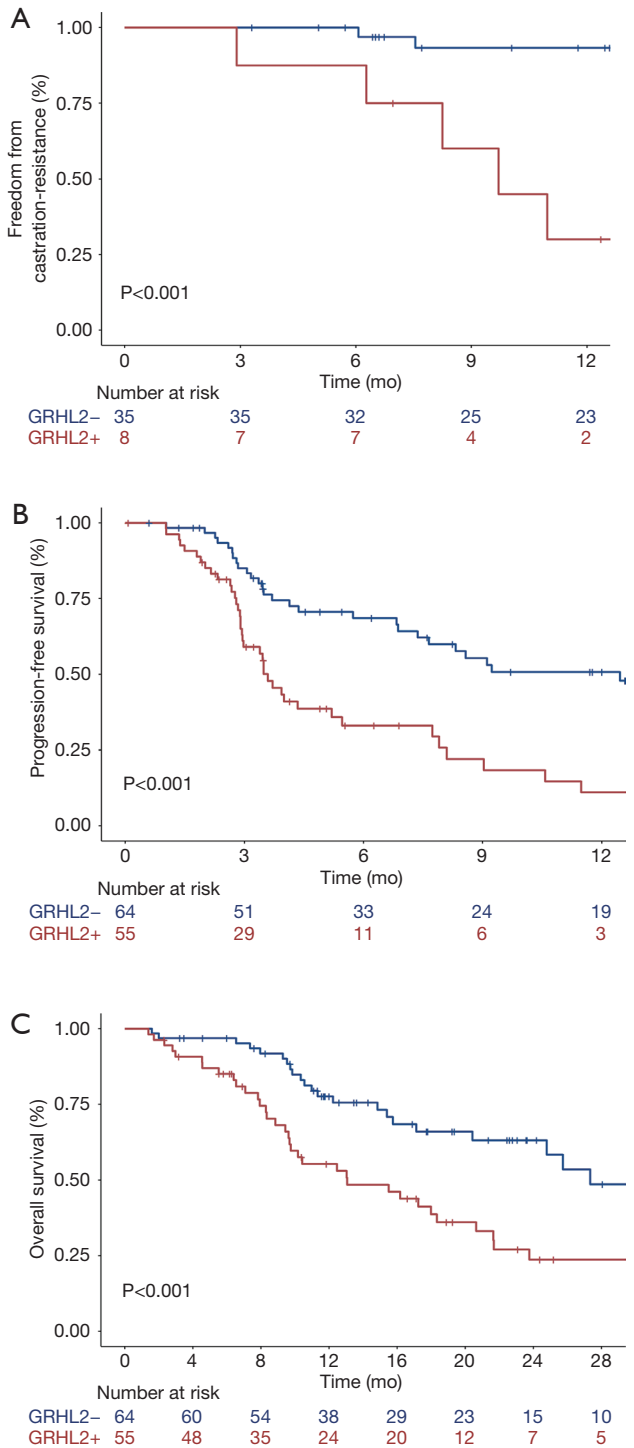

Figure 1 Kaplan-Meier analysis of clinical outcomes according to presence of *GRHL2* transcript in metastatic hormone-sensitive prostate cancer (mHSPC) and metastatic castration-resistant prostate cancer (mCRPC) cohorts. (A) Time to castration-resistance development (mHSPC cohort). (B) Progression-free survival (mCRPC cohort). (C) Overall survival (mCRPC cohort).

tumour DNA and circulating tumour RNA (ctDNA/ctRNA) next-generation sequencing assays has consistently been shown to be a negative predictive biomarker for response to both abiraterone acetate and enzalutamide (11,33-37). Similarly, detectable *GRHL2* mRNA in the mHSPC cohort could suggest greater dependency on AR-mediated proliferation that is incompletely suppressed with LHRH analogue monotherapy. Given the association between greater levels of testosterone suppression and improved clinical outcomes (38), it is feasible that *GRHL2*-positive mHSPC patients would benefit from more intense androgen suppression compared to *GRHL2*-negative patients. Multiple phase 3 trials have demonstrated that treatment intensification in the form of ADT combined with AR-directed therapies significantly improves OS in patients with mHSPC (39-44). Whether mHSPC patients with circulating *GRHL2* expression may preferentially benefit from these novel intensified AR inhibition approaches is an intriguing proposition worthy of greater exploration. Regardless, the established ability of GRHL2 to drive prostate cancer growth and disease progression in the castration-resistant environment, together with the data described here, justify further research into GRHL2 as a circulating biomarker candidate in metastatic prostate cancer.

Our study has several limitations. Small sample size especially in the mHSPC cohort, together with heterogeneity of treatment administered may impact the ability to detect meaningful associations and the overall generalisability of findings. Follow-up periods were relatively short, most notably in the mHSPC cohort, limiting the analysis of longer-term, more robust clinical outcomes such as OS. Future studies in independent cohorts are planned to ensure these results are reproducible and not just reflective of cohort-specific findings.

Conclusions

We demonstrated the utility of circulating *GRHL2* as a negative prognostic biomarker across the clinical spectrum of metastatic prostate cancer. *GRHL2* was associated with rapid resistance to ADT, shorter duration of response to novel AR directed therapies, more rapid tumour progression and poorer OS. These data support further investigation of *GRHL2* as a candidate prognostic biomarker in prostate cancer and expansion of efforts to better understand the role of *GRHL2* in mediating resistance to prostate cancer systemic therapy.

Table 4 Cox proportional hazards analysis of *GRHL2* expression with clinical outcomes in mCRPC cohort

Outcome	Univariable			Multivariable[†]		
	HR	95% CI	P	HR	95% CI	P
All patients (n=119)						
PFS	2.5	1.6–4.0	<0.001*	3.1	1.8–5.2	<0.001*
OS	2.5	1.5–4.2	<0.001*	2.9	1.6–5.1	<0.001*
ARPI-treated patients (n=83)						
PFS	3.1	1.8–5.3	<0.001*	4.8	2.6–8.8	<0.001*
OS	3.1	1.6–6.0	<0.001*	4.4	2.1–9.4	<0.001*
Taxane chemotherapy-treated (n=36)						
PFS	1.1	0.46–2.8	0.8	–	–	–
OS	1.5	0.60–3.9	0.4	–	–	–

All P values <0.05 in MVA are indicated with *. [†]Clinical variables incorporated into MVA: prior chemotherapy (yes *vs.* no), prior ARPI (yes *vs.* no), haemoglobin (< LLN *vs.* ≥ LLN), ECOG PS (≥2 *vs.* 0–1) and visceral disease (present *vs.* absent). mCRPC, metastatic castration-resistant prostate cancer; ARPI, androgen receptor pathway inhibitor; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group Performance Status; HR, hazard ratio; MVA, multivariable analysis; PFS, progression-free survival; OS, overall survival.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the Human Research Ethics Committee at Monash Health (HREC 11571X) and St Vincent's Hospital (SVH 12/231). Written informed consent was obtained from all individual participants.

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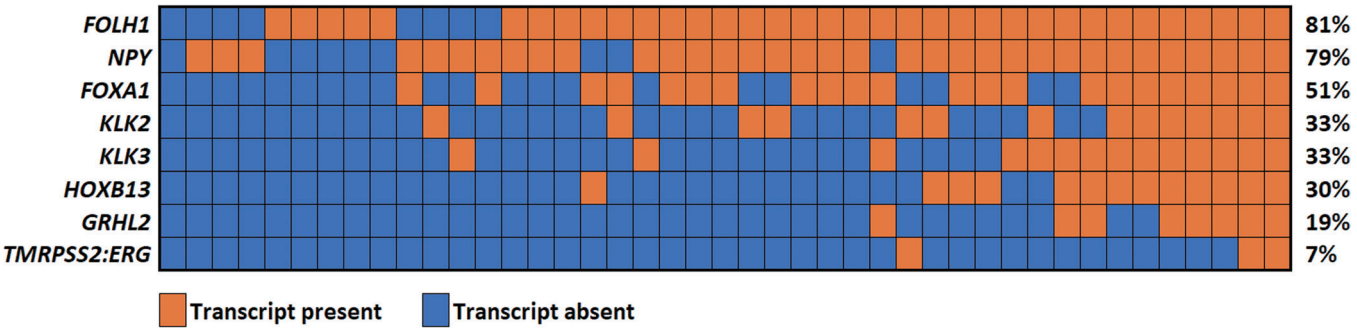

Figure S1 Distribution of circulating gene panel transcripts in the mHSPC cohort. Each column represents an individual patient sample. Percentage of patient samples positive for each transcript is shown on the right.

Table S1 Seven-month undetectable PSA rates according to presence of gene panel transcripts

	Undetectable PSA (%)[#]	P[†]
FOLH1 (+ *vs.* –)	18/32 (56) *vs.* 4/7 (57)	1.0
FOXA1 (+ *vs.* –)	10/19 (53) *vs.* 12/20 (60)	0.96
GRHL2 (+ *vs.* –)	2/8 (25) *vs.* 20/31 (65)	0.24
HOXB13 (+ *vs.* –)	4/12 (33) *vs.* 18/27 (66)	0.24
KLK2 (+ *vs.* –)	6/12 (50) *vs.* 16/27 (59)	0.96
KLK3 (+ *vs.* –)	5/13 (38) *vs.* 17/26 (65)	0.27
NPY (+ *vs.* –)	17/31 (55) *vs.* 5/8 (63)	1.0
TMPRSS2 (+ *vs.* –)	2/3 (66) *vs.* 20/36 (56)	1.0

[#]Based on 39 patients with available seven-month PSA data. [†]Calculated using Chi-square statistics (or Fisher's exact test if the expected frequency of the variable was less than 5) and adjusted for multiple testing using the standard Benjamini-Hochberg correction.

Table S2 Univariable Cox proportional hazard analysis of baseline clinicopathological factors associated with time to castration resistance

	HR	95% CI	P
Gleason score (≥8 *vs.* ≤7)	0.53	0.087–3.2	0.5
ECOG PS (2 *vs.* 0-1)	4.9	0.95–25	0.057
Visceral disease (yes *vs.* no)	2.3	0.47–11	0.3
Disease volume (high *vs.* low)	4.6	1.1–19	0.03
Treatment intensification# (yes *vs.* no)	0.62	0.16–2.3	0.47
Haemoglobin (< LLN *vs.* ≥ LLN)	0.94	0.90–0.98	0.03

All P values <0.05 are highlighted in bold. [#]Treatment intensification includes either docetaxel or AR pathway inhibitors as upfront therapy with ADT.

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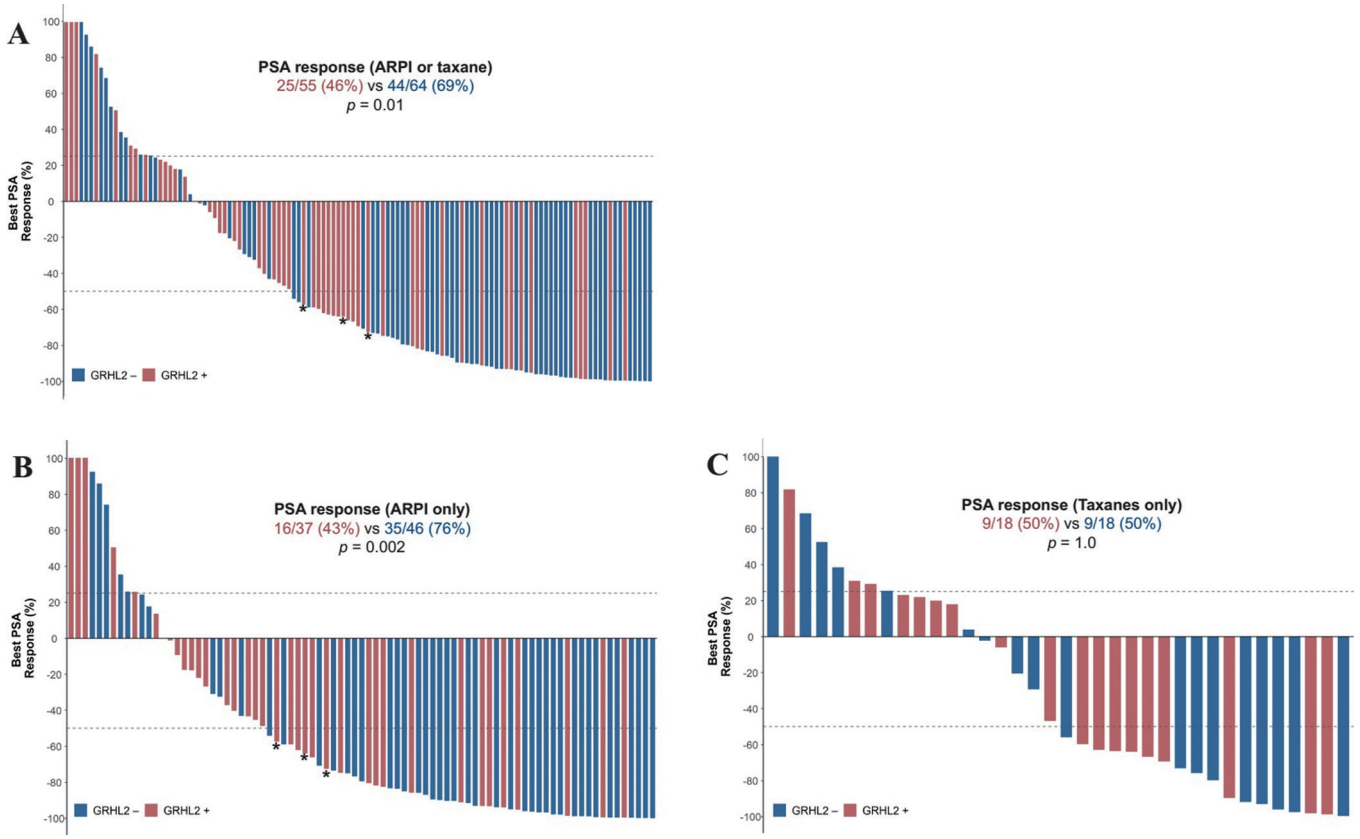

Figure S2 Waterfall plots of best PSA response and rates of confirmed PSA_{50} response according to *GRHL2* status and treatment received. Red bars represent *GRHL2*-positive patients and blue bars represent *GRHL2*-negative patients. Asterisk indicates non-confirmed PSA_{50} response. ARPI, androgen receptor pathway inhibitor (abiraterone acetate or enzalutamide).

Table S3 Univariable Cox proportional hazard analysis of clinical outcomes in the mCRPC cohort based on baseline clinicopathological factors

Variable	Progression-free survival			Overall survival		
	HR	95% CI	P	HR	95% CI	P
Gleason score (≥ 8 *vs.* ≤ 7)	1.3	0.72–2.2	0.4	1.1	0.54–2.1	0.9
Prior chemotherapy (yes *vs.* no)	1.4	0.88–2.2	0.2	1.6	0.97–2.7	0.07
Prior ARPI (yes *vs.* no)	2.8	1.6–4.6	<0.001	2.7	1.6–4.7	<0.001
ECOG PS (2 *vs.* 0-1)	1.5	0.66–3.2	0.4	2.5	1.1–5.5	0.03
Visceral disease (yes *vs.* no)	1.4	0.63–3.0	0.4	2.2	0.97–4.8	0.058
Haemoglobin (< LLN *vs.* ≥ LLN)	2.5	1.5–4.1	<0.001	3.0	1.6–5.4	<0.001

All P values <0.05 are highlighted in bold. ARPI, androgen receptor axis pathway inhibitor; ECOG PS, Eastern Cooperative Oncology Group performance status; ALP, alkaline phosphatase; NLR, neutrophil-lymphocyte ratio.

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