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Multigene profiling of CTCs in mCRPC identifies a clinically relevant prognostic signature

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Abstract

The trend towards precision-based therapeutic approaches dictated by molecular alterations offers substantial promise for men with metastatic castration resistant prostate cancer (mCRPC).

However, current approaches for molecular characterization are primarily tissue based, necessitating serial biopsies to understand changes over time and are limited by the challenges

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inherent to extracting genomic material from predominantly bone metastases. Therefore, a circulating tumor cell (CTC)-based assay was developed to determine gene expression across a panel of clinically relevant and potentially actionable prostate cancer related genes. CTCs were isolated from the whole blood of mCRPC patients (n=41) and multiplex qPCR was performed to evaluate expression prostate cancer related target genes (n=78). A large fraction of patients (27/41, 66%) had detectable CTCs. Increased androgen receptor (*AR*) expression (70% of samples) and evidence of Wnt signaling (67% of samples) was observed. The *TMPRSS2:ERG* fusion was expressed in 41% of samples and the aggressive prostate cancer associated long non-coding RNA *SChLAPI* was upregulated in 70%. *WNT5a* (HR 3.62, 95% CI 1.63 – 8.05, p=0.002), *AURKA* (HR 5.56, 95% CI 1.79 – 17.20, p=0.003), and *BMP7* (HR 3.86, 95% CI 1.60 – 9.32, p=0.003) were independently predictive of overall survival (FDR<10%) after adjusting for a panel of previously established prognostic variables in mCRPC (Halabi nomogram). A model including Halabi, *WNT5a*, and *AURKA* expression, termed the miCTC score, outperformed the Halabi nomogram alone (AUC=0.89 vs. AUC=0.70). Understanding the molecular landscape of CTCs has utility in predicting clinical outcomes in patients with aggressive prostate cancer and provides an additional tool in the arsenal of precision-based therapeutic approaches in oncology.

Introduction

Prostate cancer is the most common cancer diagnosis and the second leading cause of cancer-related deaths among men in the United States [1]. While most patients harbor indolent versions of the disease, a large subset experience metastatic progression, recurrence, and resistance to therapy. Although the androgen signaling pathway is the most common driver of prostate cancer disease progression, a number of other genes and pathways have been recognized as potential prognostic markers and therapeutic targets, and after an initial response to androgen deprivation, metastatic prostate cancer will often progress to castrate resistant disease (mCRPC) [2–8].

Multiple adaptive mechanisms have been delineated that combat pharmacologic androgen starvation in men with mCRPC, including androgen receptor (*AR*) amplification, splicing, and mutation, or initiation of parallel androgen synthesis and proliferative pathways [9–11]. Wnt signaling is one such pathway that has been implicated as a downstream mediator of AR signaling in mCRPC and it has been suggested that AR-regulated noncanonical Wnt signals promote prostatic tumor growth, the development of osteoblastic metastasis, prostate cancer stem cell self-renewal and proliferation, and resistance to anti-androgen therapy [12–16].

Through extensive tissue-based sequencing, it is now clear that prostate cancers harbor diverse, clinically actionable drivers of disease progression that may inform both prognosis and potential response to therapy [4, 17–19]. While these findings highlight the promise of precision oncology testing in prostate cancer, the utility of these techniques may be limited both by the challenges of extracting viable tissue from bone metastases and the need for serial sampling as new resistance mechanisms arise [18, 20].

Emerging comprehension in the realm of circulating tumor cell (CTC) biology has unveiled the potential for non-invasive, continuous monitoring of cancer and previous studies have

established a correlation between CTC, primary tumor, and metastatic tissue gene expression profiles [21, 22]. Previous efforts to inform disease severity in prostate cancer have relied simply on the presence or absence of CTCs, but these studies have not yielded adequate clinical utility to guide therapeutic decision-making for individual patients [23–26]. Additionally, genomic profiling of cell-free DNA (cfDNA) represents another feasible and clinically informative liquid biopsy avenue in mCRPC [27, 28]. While newly developed approaches show promise in CTC detection, including CTC-iChip and the AdnaTest, the overall landscape of liquid approaches to assess changes in tumor behavior in men with mCRPC represent a major unmet clinical need as robust, readily reproducible methods for isolation and subsequent characterization of CTCs in prostate cancer remain elusive [9, 29, 30].

To facilitate non-invasive detection of genomic alterations and molecular expression in these patients, we developed and implemented a qPCR-based platform for analysis of CTCs from men with mCRPC. This approach was modeled after commercially available, tissue-based qPCR platforms for prostate cancer profiling, with the primary goal of eventual integration into clinical workflows [31, 32]. Given that CTCs likely represent cells from disparate metastatic sites, we hypothesized that understanding the expression of key genes of interest within CTCs could have unique utility in precision medicine approaches to predict clinical outcomes in patients with advanced prostate cancer [33].

Methods

CTC isolation, gene expression determination, and platform validation

Circulating tumor cells were isolated from 5 mL whole blood using anti-EpCAM antibody conjugated microbeads (Thermo Fisher Scientific). *DAPI*, *CD45*, *FITC*, and *PCa-CT-PE* (antibody cocktail: *PSMA*, *EGFR*, and pan-cytokeratin) were used to confirm the identity of CTCs. Following washing and cell lysis, mRNA from CTCs was captured using Oligo(dT)25 mRNA Dynabeads (Thermo Fisher Scientific). Reverse transcription was performed to obtain cDNA, followed by preamplification to generate target gene amplified libraries, and multiplex qPCR was then performed to evaluate a panel of 96 genes, including controls (Supplementary Table 1). Genes were selected based on relevance to prostate cancer progression and metastasis, as well as EMT and cancer stem cell biology. To account for leukocyte contamination, blood processed from 27 healthy controls was used as a baseline referent. To evaluate gene amplification efficiency for both pre-amplification and real-time PCR, prostate cancer cells (1.65×10^4 cells, including equal amount of PC3, VCaP and LNCaP) were lysed with 1 mL lysis buffer. Ten fold serial dilution was applied with lysis buffer to obtain samples equal to 3300, 330, 33, 3.3, and 0.33 cells per 200 μ L to evaluate the linear relationship between cell number and Ct value of individual genes (Fig. S1). To further validate the RT and amplification steps, we included Array control RNA Spikes (Thermo Fisher AM1780), using primers from the Fluidigm RNA Spikes Assay kit (100-5582) to validate the reverse transcription, preamplification, and qPCR steps. The kit contains RNA from *E.coli* with no known homology to mammalian sequences. Spike mix was prepared as described in the Fluidigm delta gene assay protocol, PN 100-4904 J1. Three of the RNA spikes were used, Spike-1 (750 nucleotides), Spike-4 (1000 nucleotides) and

Spike-7 (1474 nucleotides) and mixed. Ct value of each RNA spike was acquired with qPCR. The average value plus standard deviation of Ct value from different spikes was calculated and plotted against the copy number of different spikes, which showed a linear correlation between Ct value and copy number ($R^2 = 0.95$) (Fig. S1).

For platform validation, 180 GFP-labeled prostate cancer cells (PC3, LNCaP, VCaP) were sorted into 5 mL whole blood from a normal donor. Cells were captured using immunomagnetic bead enrichment and visualized by immunofluorescence. Cells were then counted, rather than lysed, to measure the recovery rate. Additionally, 300 PC3 & LNCaP cells were spiked into 5 mL of whole blood from healthy controls and captured by immunomagnetic bead enrichment, followed by staining with *DAPI*, *CD45*, and PCa-CT (antibody cocktail: *PSMA*, *EGFR*, and pan-cytokeratin) to visualize CTCs. To validate gene expression after CTC isolation, 10 prostate cancer cells (PC3, LNCaP, or VCaP) were spiked into 5 mL of whole blood from a normal control. CTCs were captured by immunomagnetic bead enrichment, lysed, and gene expression was then assessed by qPCR. qPCR gene expression was compared with known microarray profiles for each of these lines in order to evaluate whether the expression profiles matched that of what was expected for PC3, LNCaP, and VCaP cells.

Normalization of qPCR gene expression data

For each sample, gene expression of 96 genes was determined from qPCR in a 384-well format as described above. Cycle threshold (Ct) values were normalized using the delta-delta Ct method as previously described [34]. Briefly, genes within each sample were first normalized against endogenous controls by subtracting the sample-specific average Ct value for *ACTB*, *HMBS*, and *TUBA-1B*. For each gene, the resulting delta Ct values were corrected for background from contaminating blood cells by subtracting the average delta Ct value for that gene across the 27 healthy donor reference controls to obtain delta-delta Ct values. Undetermined Ct values were imputed as 40 (Supplementary Table 2). Finally, gene expression was quantified as $2^{-(\text{delta-delta Ct})}$ which we refer to as 'normalized expression.' All analyses were performed with $\log_2(\text{normalized expression} + 1)$, with the logarithm serving as a variance-stabilizing transformation and the addition of the constant serving to compress expression values comparable to background. In addition, we set gene expression values to zero when the underlying Ct value was greater than 35 as Ct values in this range are generally not quantifiable.

Classification of samples as CTC positive or negative

Given that this protocol involves cell lysis rather than visualization and enumeration, we established a model for identifying CTCs using the gene expression of eight epithelial markers: *CD326*, *CDH1*, *CDH2*, *DSG2*, *EGFR*, *KRT8*, *KRT18*, and *KRT19*. CTC-positive controls were created using serial dilutions of prostate cancer cell lines, PC3 and LNCaP, spiked into whole blood from healthy donors. Data from 27 healthy donor controls were used as negative controls. We first performed a principal components analysis to verify the accuracy of these genes for distinguishing positive from negative controls. The first principle component clearly separates positive from negative controls, with the second component primarily separating cell lines (Fig. S2). These two components account for 92% and 4% of

variance for these samples, respectively. The weights of each gene on this component are shown as a bar graph in Figure S2. We define an ‘epithelial score’ (ES) as the weighted sum of the eight epithelial marker genes using these weights. We then built a classification model for identifying new samples with CTCs using this epithelial score in a logistic regression. The fitted model is:

$$P(\text{CTCs present} \mid \text{gene expression}) = \exp(a + b * \text{ES}) / (1 + \exp(a + b * \text{ES})), a = -7.80, b = 0.94$$

Patient samples were considered CTC positive if they are above the conservative threshold of 75% on the estimated probability using the model above. In both the control and clinical setting, the majority of samples were towards the very high or very low end of the CTC probability model (Fig. S2).

Cox proportional-hazard survival models

The primary endpoint for this study was overall survival, and in order to identify CTC-based genes associated with mortality, we performed univariate Cox regression analysis in CTC positive patients for each of the measured genes [35]. The Halabi nomogram was utilized to account for baseline clinicopathologic variables, including ECOG performance status, disease site, opioid analgesic use, albumin, hemoglobin, alkaline phosphatase, and PSA in a multivariate Cox model. We obtained 18-month survival probabilities from <https://www.cancer.duke.edu/Nomogram/firstlinechemotherapy.html>. We subtract this probability from one to define a ‘Halabi’ score so that hazard ratios above one indicate increased risk of death. Cox bivariate regression analyses were then performed for each gene, adjusting for Halabi score, to identify genes associated with mortality independent of measureable clinical factors.

Cox regression was performed for 78 genes, after removing endogenous controls (*ACTB*, *HMBS*, *TUBA-1B*), epithelial markers (*CD326*, *CDH1*, *CDH2*, *DSG2*, *EGFR*, *KRT8*, *KRT18*, *KRT19*), blood-cell markers (*CD20*, *CD45*), artificial RNAs used in assay development (Spike1, Spike4, Spike7), alternate primers for *SChLAPI*, and *SPANXB2*. Log-transformed normalized expression values for each gene were rescaled by the interquartile range in order to make hazard ratios easier to interpret. Models were fit using the R function *coxph* in the *survival* package [36]. Genes were ranked using p-values from bivariate proportional hazards models with a single gene and the Halabi score as predictors. These p-values were transformed to FDR q-values using the Benjamini-Hochberg method [37]. The three genes significant at <10% FDR in the CTC positive cohort, *AURKA*, *BMP7*, and *WNT5A*, were selected for further analysis. Cox regression and Kaplan-Meier analysis was performed to assess whether CTC positive or negative status was associated with survival within the cohort. To aid in the interpretation of hazard ratios, we repeated the analysis using CTC probability based on our epithelial score as a continuous variable. This probability “*p*” was scaled using the transform: $2*(p-0.5)$, so that one unit change can be thought of as going from $p=0.25$ to $p=0.75$.

Determining prognostic potential of selected genes

We constructed waterfall plots based on expression of WNT5a, AURKA, and BMP7 for the 39 patients (26 CTC positive) with at least 6-month follow up with bars colored by vital status at 6 months. In these plots, patients in the CTC-positive subgroup were ordered from lowest to highest normalized expression and patients without CTCs ordered below the CTC-positive group. To assess significance in these plots, we used the hypergeometric distribution to test whether deaths were over-represented in the top 25% of patients.

We next examined composite scores using these three genes. First, in the CTC-positive group we assigned patients points for each gene, with normalized expression values in the 50–75th percentiles given one point, and those above the 75th percentile given two points. Then, we defined prognostic scores by adding point values across either all three genes or one of the three two-gene pairs. For these four scores, we constructed receiver operating characteristic (ROC) curves for predicting vital status at 6-months using the *survivalROC* function with smoothing parameter $\lambda=0.002$ from the *survivalROC* package in R [38, 39].

From the ROC analysis, we identified the best performing genes (*WNT5a* and *AURKA*) and added those to the Halabi score to generate a combined molecular and clinical signature. The resulting miCTC score ranges continuously from 0–5, with integer values of 0, 1, 2, 3 or 4 coming from AURKA and WNT5A gene-expression. We use 1 minus the Halabi 18-month survival probability as the ‘Halabi component’ of the score. As before, we use the *survivalROC* function in R to construct ROC curves for predicting survival at 6 months (180 days) using the miCTC and Halabi scores. Confidence intervals for the difference in AUCs between these two curves were computed using the appropriate quantiles from 10,000 bootstrap samples. Finally, we define a ‘high-risk’ subgroup as those patients having a miCTC score of two or more, with others forming a ‘low-risk’ subgroup. We compare these risk groups using Kaplan-Meier survival curves and assess significance using the log-rank test. Survival curves and log-rank p-values are computed using the *survfit* and *survdiff* functions from the *survival* package [40].

Survival analysis

Formalin-fixed paraffin embedded prostatectomy samples were from two published retrospective patient cohorts from the Mayo Clinic (n=780) [41–43]. As previously described, RNA extraction and hybridization to Affymetrix Human Exon 1.0 ST Arrays (Affymetrix, Santa Clara, CA) were performed in a CLIA-certified, clinical operations laboratory (GenomeDx Biosciences, Inc, San Diego, CA). Microarrays were normalized using Single Channel Array Normalization [44]. Microarray data are available with NCBI GEO accession numbers GSE46691 and GSE62116. The miCTC score was applied to the retrospective samples as described above. Kaplan-Meier curves were generated for the pooled Mayo Clinic cohorts and hazard ratio estimates were generated using Cox proportional hazards modeling.

All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cell lines were maintained using standard conditions. To ensure identity, all cell lines were

genotyped at the University of Michigan Sequencing Core using Profiler Plus (Applied Biosystems) and compared with profiles of respective cell lines available from ATCC. Cell authentication and mycoplasma testing was performed for all cell lines. This study was approved by the Institutional Review Board (IRB# HUM00052405), and written informed consent was obtained from all patients for collection and analysis of samples in accordance with recognized Declaration of Helsinki guidelines.

Results

Development of CTC isolation and gene expression analysis platform

CTC isolation and subsequent gene expression analysis was conducted using an anti-epithelial cell adhesion molecule (*EpCAM*) coated microbead protocol, followed by multiplex qPCR of 78 prostate cancer related genes plus internal controls (Fig. S3). Our panel contained genes previously shown to be involved in prostate cancer aggressiveness, metastasis, or progression, including *TMPRSS2:ERG*, *PSA*, *AR*, *WNT5a*, *NKX3.1*, *EGFR*, and *SChLAP1* [16, 45–49]. Recovered cells were identified by cell surface staining for *EpCAM* and absent staining for *CD45*, a common leukocyte marker [50]. In order to assess the performance of this platform, small numbers of cells (n=10) were spiked into 5 mL of whole blood. Gene expression analysis of these cells showed expression profiles that were congruent with previous microarray analyses of these cell lines, supporting the validity of our isolation and expression analytic platform (Fig. S4).

Isolation and analysis of CTCs from whole blood of patients with mCRPC

To characterize the CTC gene expression pattern in patients, we evaluated 41 patients with mCRPC (Table 1). The median age was 68 (IQR 61–75) and median PSA was 19.7 ng/mL (IQR 3.6–70.6 ng/mL). At the time of enrollment, all patients were on luteinizing hormone releasing hormone (LHRH) agonist therapy (leuprolide, goserelin), 5 patients were on additional treatment with abiraterone, 11 on enzalutamide, 8 on docetaxel, 4 on cabazitaxel, 1 on radium 223, and 1 on dexamethasone. Patients had received a median of two prior therapies (IQR 1–3). All patients had a minimum of 90 days follow up and there were 21 (51.2%) deaths. The median time to next therapy was 251 days (IQR 119–329) and patients received a median of one subsequent therapy (IQR 0–1).

CTC positivity was determined based on a model using the gene expression of eight epithelial markers (Fig. S2) and we identified 27 (66%) patient samples with detectable CTCs. In our protocol, CTC positive samples are identified after expression profiling, and each profile is potentially a mixture of CTC targets and contaminating blood cells, as this is an enrichment rather than pure isolation approach. For the healthy controls, due to the absence of epithelial CTCs, all RNA in the sequence library is from contaminating blood cells. As described in the Methods, gene expression in patient samples is normalized to both endogenous controls and then to expression in healthy controls to account for background contamination. In order to define potential thresholds regarding positive or negative expression of a given gene in CTCs, we used *k*-means clustering for each gene to find subgroups among 27 healthy controls and 27 CTC positive samples. When the composition of these two groups was such that one had significantly more CTC positive samples than

healthy controls (as determined by a Chi-squared test), we used the midpoint between the cluster means to define a threshold at which to call specific samples “positive” for a given gene. Using this approach, 19 samples (70%) showed increased expression of *AR* and 15 (55%) showed significant expression of *AR-V7*, with 14 (52%) shared between these two groups. Increased downstream *AR* signaling was seen in 14 (52%) samples based on joint expression of *KLK2 (PSA)*, and *NKX3-1*, and 18 (67%) samples showed significant upregulation of Wnt signaling based on expression of *WNT5a*, *WNT5b*, and *BMP7*. Among epithelial-mesenchymal transition (EMT) genes, 9 samples (33%) expressed *SOX2* and 21 (67%) had elevated levels of *TWIST1*, with 7 samples (26%) expressing both genes. Similarly, clustering jointly on *MDK* and *CCNA2*, 15 samples (56%) showed high levels of cell cycle activity. Among long non-coding RNAs (lncRNA) associated with prostate cancer, 19 samples (70%) showed elevated levels of the lncRNA *SChLAPI*, but only 8 (30%) showed increased *PCA3* levels. The *TMPRSS2:ERG* gene fusion was significantly expressed in 11 samples (41%) and based on concordant expression across two distinct amplicons, evidence for the *TM2:ETV1* fusion was seen in 4 samples (15%) (Fig. 1A).

To acquire a clinically relevant gene signature, we sought to identify genes expressed in CTCs independently associated with survival. To do this, we performed univariable and multivariable Cox regression analyses of individual genes and used a previously validated prognostic model to account for baseline clinicopathologic variables in mCRPC [51]. Increased expression of *WNT5a* (HR 3.62, 95% CI 1.63 – 8.05, p=0.002), *AURKA* (HR 5.56, 95% CI 1.79 – 17.20, p=0.003), and *BMP7* (HR 3.86, 95% CI 1.60 – 9.32, p=0.003) were strongly associated with earlier mortality (FDR<10%) after adjusting for the Halabi nomogram score. Other genes implicated in prostate cancer biology, including *WNT5b*, *KLK2*, and *PSA* were significantly associated with overall survival on univariate, but not multivariate, analysis (Fig. 1B). CTC positive or negative status alone was not significantly associated with survival in a Kaplan-Meier analysis (p=0.143) or univariate Cox model (HR 1.9, 95% CI 0.6 – 5.7, p=0.28) comparing the two groups. CTC status was also not significantly associated with survival in a bivariate model controlling for Halabi 18-month survival probability (HR 1.2, 95% CI 0.3 – 4.0, p=0.81). We repeated this analysis using the CTC epithelial score as a continuous variable, and it was marginally significant in the univariate analysis (HR 3.4, 95% CI 1.0 – 11.9, p=0.06) but not the bivariate model adjusted for Halabi 18-month survival probability (HR 2.1 95% CI 0.5 – 8.5, p=0.28) (Fig. S5).

Development of a CTC-based prognostic score

Given the ability of *WNT5a*, *AURKA*, and *BMP7* expression to independently predict survival in this cohort and their previously elucidated roles in metastatic, variant forms of prostate cancer and treatment resistance, we hypothesized that increased expression of these genes within CTCs would correlate with worse prognosis [16, 52–54]. Focusing on these three genes, we confirmed that early mortality (< 6-months) occurred more frequently in patients with higher expression of *WNT5a*, *AURKA*, and/or *BMP7* individually (Fig. 2A). We next developed prognostic models in the 27 CTC-positive patients using composite scores for all three genes and evaluated individual combinations of genes to optimize the model. Scores were determined by normalizing the expression values for each gene. One point was given for expression of a given gene in the 50–75th percentile of the CTC cohort

and two points for expression above the 75th percentile. While all three genes were individually prognostic, a composite score comprising *WNT5a* and *AURKA* expression outperformed all other combinations (AUC=0.91) (Fig. 2B).

This data was then used to derive a CTC-based prognostic score (hereafter referred to the Michigan CTC [miCTC] score). This score ranges from 0–5, with values of 0, 1, 2, 3 or 4 derived from *AURKA* and *WNT5A* gene expression (0–2 points for expression of each gene as described above). In order to account for baseline clinical variables, the Halabi nomogram score was incorporated as the fifth component into the overall score using the 18-month survival probabilities calculated from the Halabi nomogram (see Methods) (Fig. S6). For CTC-negative patients, this meant the miCTC score was based solely on the Halabi survival probability and the absence of *WNT5A* or *AURKA* gene expression. The miCTC score outperformed the Halabi nomogram alone (AUC=0.89 vs. AUC=0.70) across the entire 41 patient cohort (Fig. 2C). When categorizing the present cohort into high-risk (miCTC ≥ 2) vs. low-risk (miCTC <2) subgroups, Kaplan-Meier analysis demonstrated significant differences in overall survival over time (Fig. 2D).

External application of miCTC score using known sequencing data from prostate cancer tissue

To further assess the performance of the miCTC prognostic signature, we first evaluated the level of concordance of gene expression between our qPCR-based platform and tissue-based RNA-sequencing data for a subset of patients enrolled in an ongoing mCRPC profiling study [55]. In the 7 patients with both CTC-based qPCR and tissue-based RNA-seq data, a significant level of correlation existed among CTC-to-tissue gene expression (spearman correlation=0.38, p=0.0002), suggesting the genomic data obtained from CTCs accurately reflected tissue-based disease (Fig. 3A-B). We then queried the tissue-based expression of *WNT5a* and *AURKA* across a larger database containing samples from normal prostate tissue, localized prostate cancer, and metastatic samples to globally understand the landscape of *WNT5a* and *AURKA* expression [18, 56]. As expected, *WNT5a* and *AURKA* expression was found to be significantly upregulated in metastatic samples compared to normal prostate or localized prostate cancer tissue, further supporting the relevancy of these genes in mCRPC (Fig. 3C). Calculation of the miCTC score (using gene expression only) in this cohort revealed a significantly increased proportion of samples with high-risk scores (miCTC ≥ 2) in the metastatic group (92%) compared to the primary cancer group (52%) (p<0.001) (Fig. 3D).

miCTC score predicts long-term clinical outcomes in high-risk prostate cancer

Lastly, we applied the gene expression component of the miCTC score to a previously published radical prostatectomy cohort in order to assess its ability to predict long-term, objective outcomes in patients with high-risk, clinically localized prostate cancer [41–43]. This cohort included 780 patients with a median follow-up of 11.2 years after prostatectomy. A total of 513 patients (65.8%) suffered biochemical recurrence following prostatectomy, 288 (36.9%) developed metastatic disease, 166 (21.3%) died of their disease, and 336 (43.1%) died of any cause. *WNT5a* and *AURKA* expression were determined from the genomic data and used to calculate the miCTC score (on a scale of 0–4 without the

additional factor of the Halabi score). These scores were then matched with clinical outcomes. As shown in the Kaplan-Meier analyses, high-risk and increasing miCTC scores were associated with a greater risk of biochemical recurrence, metastasis, and death, and decreased prostate cancer-free survival when compared to those with lower-risk miCTC scores (Fig. 4A-D).

Discussion

Given the relative simplicity of obtaining single blood draws, CTCs obtained from whole blood may provide a compelling source of molecular information, and previous studies have shown that CTCs closely reflect the clinical and functional characteristics of solid tumors [57, 58]. While the clinical significance of the presence of CTCs has long been theorized, improvements in the downstream manipulation and comprehensive molecular characterization of liquid biopsy tools are more recent. For example, a plasma based real-time PCR test for the qualitative detection of defined mutations of the epidermal growth factor receptor (*EGFR*) gene was recently developed to aid in the selection of non-small cell lung carcinoma (NSCLC) patients for treatment with erlotinib [59]. The cobas *EGFR* Mutation Test, along with the CellSearch platform [50, 60, 61], currently represent the only FDA approved liquid biopsy tests that may help guide first-line treatment decisions in patients, further underscoring the significant impact prospective cfDNA and CTC-based diagnostic and prognostic tools may have.

While *AR-V7* expression in CTCs has received a great deal of attention, we sought to develop a reproducible platform allowing CTC-based expression analysis across a broader panel of prostate cancer related genes. This targeted approach was selected to improve the efficiency of the assay and potential clinical applicability, given the challenges inherent to CTC isolation and subsequent gene expression profiling. Although an unbiased approach would capture increased genetic heterogeneity and support the discovery of novel gene targets, it would be increasingly difficult to differentiate possible prognostic targets from genetic noise, especially using a qPCR based approach [62]. Additionally, the likelihood of type 1 errors due to multiple testing are substantially greater with a broad, unbiased approach. Our primary goal was to utilize this platform to identify clinically relevant genes able to predict key oncologic outcomes in mCRPC.

In this cohort of mCRPC patients on a variety of therapies, *AR-V7* expression was not significantly associated with survival. However, a number of findings appeared to support the clinical validity of the approach utilized here. First, the expression data demonstrated a high degree of AR signaling in a majority of samples, with over half of samples showing AR splice variants. Second, we detected a global increase in pro-metastatic factors within CTCs, including genes involved in EMT, cell cycle regulation, and lncRNA-mediated tumor suppression [49, 52, 53, 63]. Third, in patients with matched tissue-based gene expression available, there was a close correlation between the tissue and CTC-based results. Lastly, the tissue-based analysis in the localized disease setting provides evidence that the markers identified in our CTC-based approach also have applicability in earlier stage disease. While a “proof of concept” rather than a validation, the crude miCTC score based solely on

expression was applied to clinical outcomes in prostatectomy patients as a preliminary analysis to further corroborate the prognostic relevance of the genes identified here.

Strikingly, our results add further support to the importance of noncanonical Wnt signaling as an alternative resistance pathway contributing to prostate cancer progression and worse clinical outcomes. While Wnt signaling has been previously linked to prostate cancer aggressiveness through various cellular functions [13, 14, 18, 64, 65], our data strongly corroborates another recent study linking Wnt signaling to anti-androgen resistance in CTCs [16]. Additionally, Beltran and colleagues established the role of *AURKA* in prostate cancer proliferation and, in particular, in driving the development of neuroendocrine prostate cancer (NEPC). This has led to an ongoing phase II trial of Aurora Kinase A inhibition in NEPC (NCT01799278) [4, 53]. The miCTC score, driven by *WNT5a* and *AURKA* expression, reliably predicts overall survival in this development cohort, suggesting CTC genomics may mirror tissue-based drivers of variant or lethal prostate cancer.

The present study is limited by its relatively small sample size, and further prospective validation in larger cohorts will be required in order to confirm the clinical validity of these findings. Additionally, patients in this study were on a variety of therapies at the time of analysis. Evaluation of more homogenous cohorts is needed in order to ascertain the impact of specific therapeutic modalities on changes in gene expression over time and to develop predictive signatures of therapeutic response. However, the high event rate allowed us to identify several potential prognostic markers after adjusting for baseline clinical characteristics and determining false discovery rates.

While our CTC isolation protocol allows for simple, rapid processing of samples with high sensitivity, the approach itself has inherent limitations. Specifically, this protocol does not allow for CTC enumeration and no cell morphological assessment can be conducted. As a result, gene expression is not adjusted for number of CTCs and may be impacted by CTC quantity within a sample. However, the lack of correlation between epithelial score and survival indicates that CTC quantity itself is not a useful marker with this platform. Additionally, given that the platform is based on enrichment rather than isolation, gene expression analysis requires adjustment for background leukocyte contamination. Finally, as with other epithelial-based CTC detection approaches, the enrichment platform utilized here could miss CTCs that have undergone EMT and no longer express EpCaM on their cell surface [66].

These limitations notwithstanding, we developed a clinically relevant, multigene signature from CTCs using a scalable qPCR-based platform. Though external validation is needed, this molecular signature appears to strongly inform prognosis in patients with advanced prostate cancer. The strong prognostic performance of this signature in a tissue-based analysis of a large prostatectomy cohort further supports the potential clinical applicability of this data. We anticipate that this approach will facilitate serial patient assessments over time and identification of molecular changes as they occur in response to therapy. With continued improvement in the ability to determine key drivers of tumor progression using CTCs, we anticipate that this non-invasive, liquid-based approach will circumvent the need

for invasive extraction of genomic material from bone metastases and guide precision medicine therapeutics in prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of implication

Analysis of circulating tumor cell gene expression reveals a clinically prognostic “liquid biopsy” signature in patients with metastatic castrate-resistance prostate cancer.

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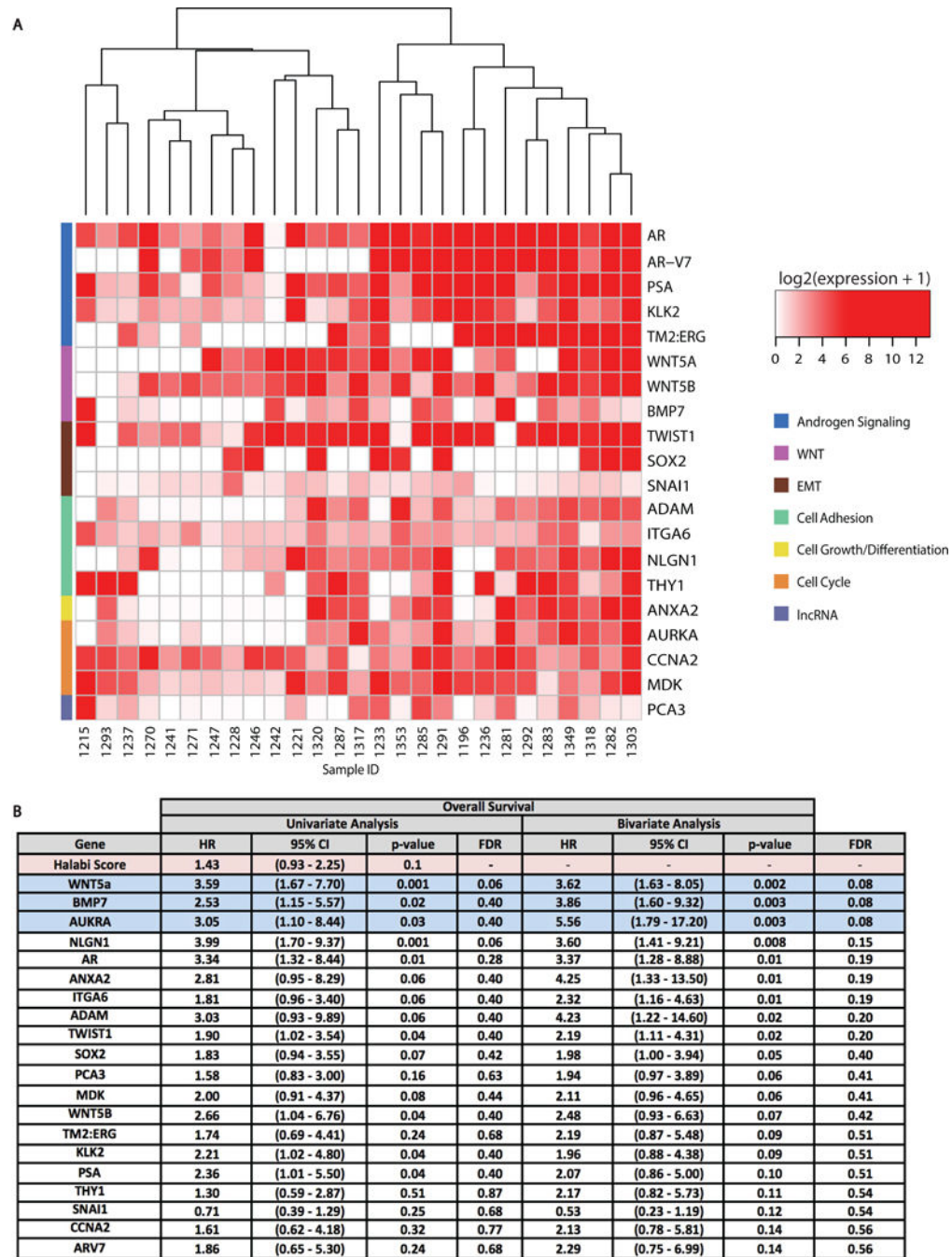


Fig. 1. CTC gene expression analysis reveals WNT5a, AURKA, and BMP7 are independently associated with overall survival in mCRPC

(A) A heatmap representation of the top 20 independently predictive genes as determined by Cox proportional hazards model. Each column represents a different sample and each row represents a separate gene. The heatmap color spectrum corresponds to lower relative expression (white) and higher relative expression (red). The left colored column represents the associated functional pathway for each separate gene (B) WNT5a, BMP7, and AURKA (highlighted in blue) are independently associated with overall survival with $p < 0.05$ and $FDR < 10\%$ when adjusting for Halabi variables.

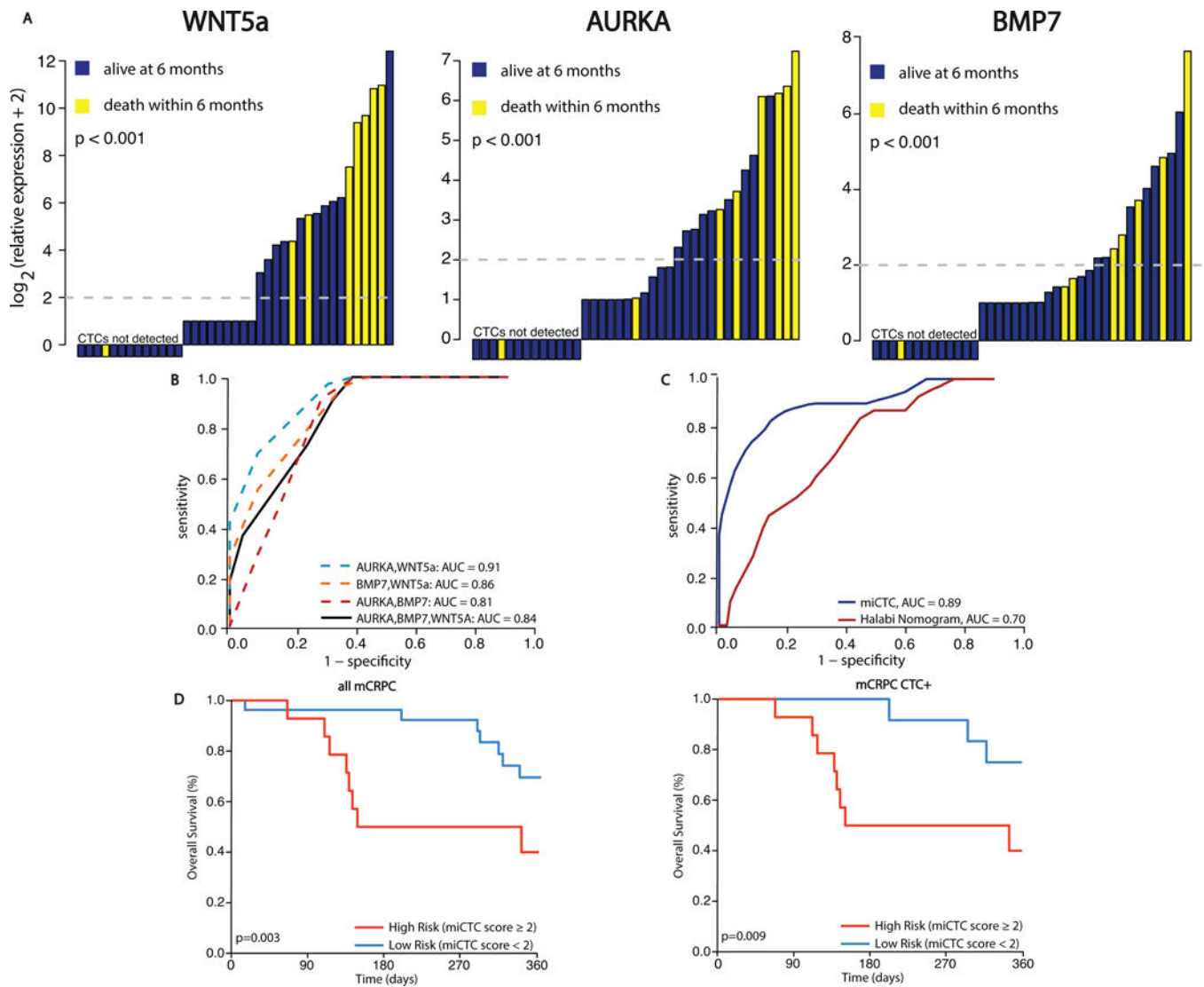


Fig. 2. A score combining Halabi, WNT5a and AURKA improves prediction of overall survival at 6 months over Halabi alone

(A) Survival status at 6 months based on normalized expression of WNT5a, AURKA, and BMP7 in 39 (26 CTC positive) patients with mCRPC. The hypergeometric distribution was used to test whether deaths were over-represented in the top 25% of patients. Deaths were significantly over-represented at the 1% level for all three genes ($p < 0.001$). (B) Receiver operating characteristic (ROC) curve compares accuracy of WNT5a, AURKA, and BMP7 expression for predicting overall survival at 6 months. (C) ROC curve compares accuracy of Halabi score alone (AUC=0.70) vs. miCTC score (AUC=0.89) combining Halabi score, WNT5a, and AURKA expression. (D) Kaplan- Meier curves depicting overall survival in patients with high vs. low miCTC score. Left curve represents entire mCRPC patient cohort (CTC+/-), right curve represents only CTC positive mCRPC patients.

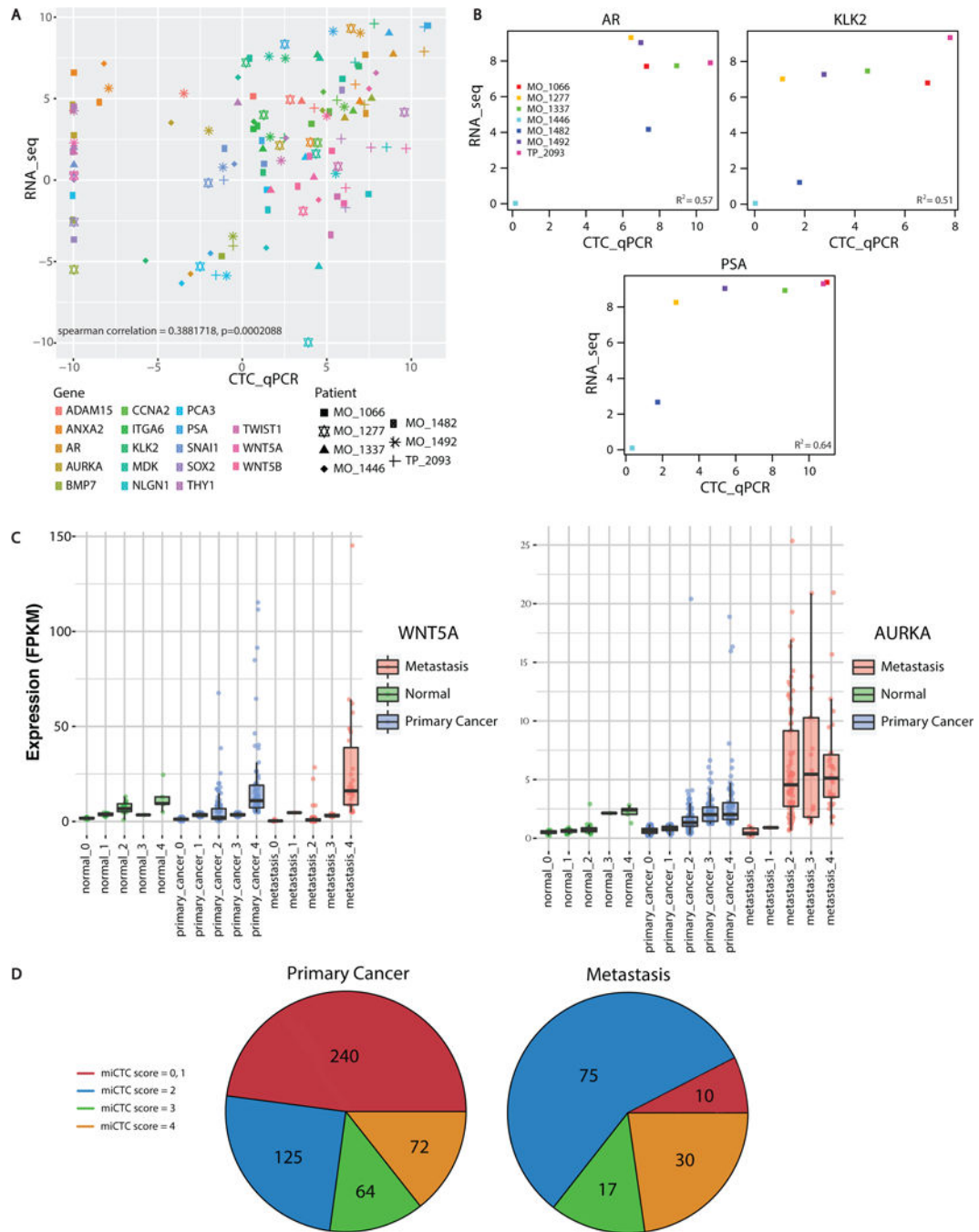


Fig. 3. Concordance of CTC gene expression by qPCR with known tissue-based RNA-seq expression

(A) Global analysis of expression for select genes in CTCs of 7 patients after isolation and qPCR via platform with known RNA-seq data from MI-ONCOSEQ (spearman correlation = 0.38, p=0.0002) (B) AR ($r^2=0.57$), KLK2 ($r^2=0.51$), and PSA ($r^2=0.64$) show concordance of expression between CTC qPCR and RNA-seq from MI-ONCOSEQ of 7 patients (C) Analysis of expression of AURKA and WNT5a by RNA-seq in an external cohort* (D) calculated miCTC score in external cohort of patients with primary and metastatic prostate

cancer* (numbers within each pie represent number of samples with that specific miCTC score), *primary tumor samples obtained from TCGA, metastatic samples from SU2C

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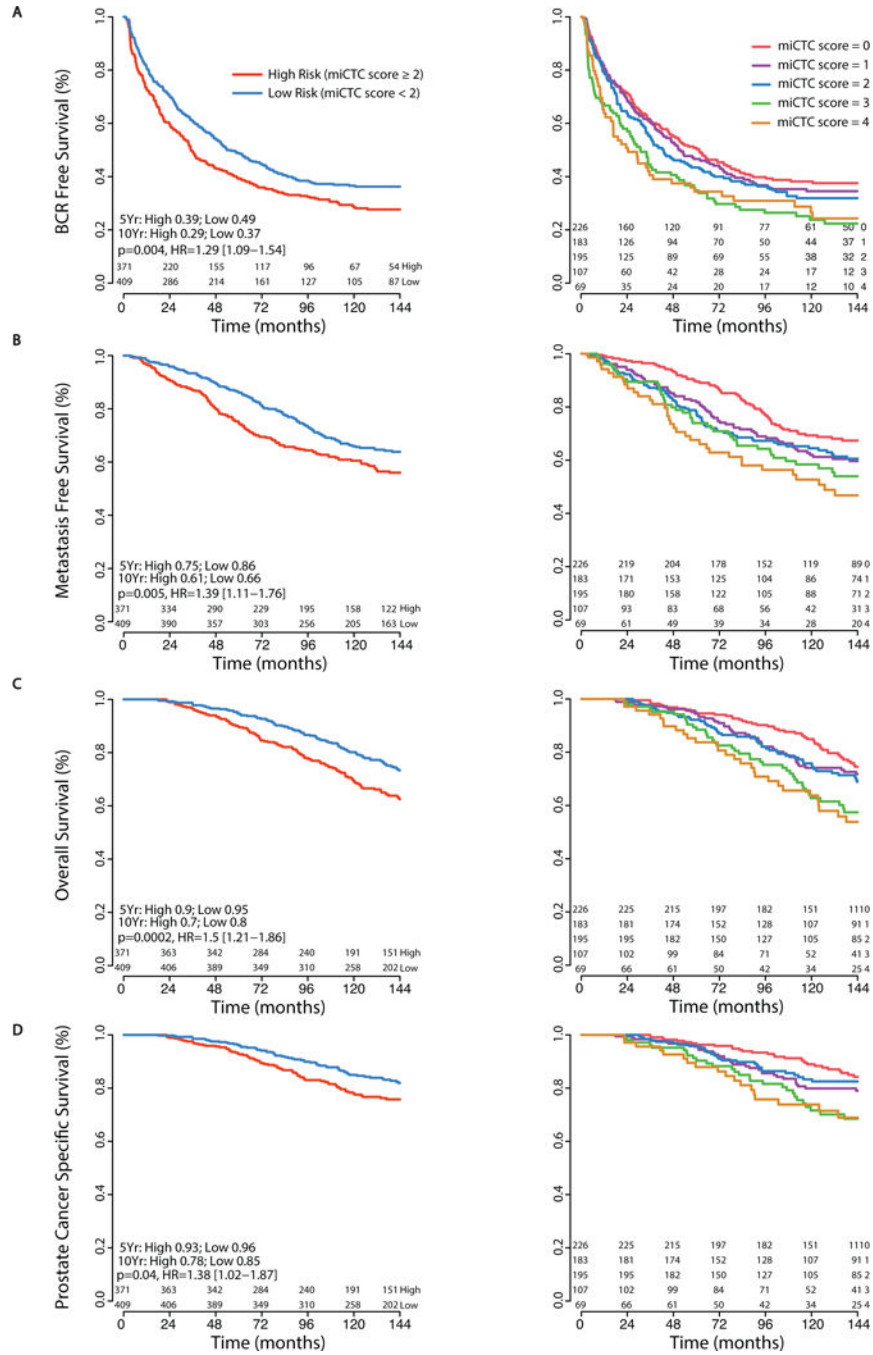


Fig. 4. miCTC score predicts long-term outcomes in patients with high-risk prostate cancer Kaplan-Meier curves showing high risk (miCTC ≥ 2) and increasing miCTC score is associated with decreased (A) biochemical recurrence free survival (p=0.004) (B) metastasis free survival (p=0.005) (C) overall survival (p=0.002) (D) and prostate cancer specific survival (p=0.04) in high-risk, clinically localized prostate cancer (Mayo Clinic)

Table 1

Demographics and baseline characteristics of mCRPC patient cohort

Variable	Overall n (%)	CTC positive n (%)	CTC negative n (%)	p-value
All	43	27 (63)	16 (37)	
Age⁺	68 (61–75)	64 (59–74)	71 (68–75)	0.943
Race				0.537
Caucasian	41 (96)	25 (92)	16 (100)	
African American	1 (2)	1 (4)	0 (0)	
Hispanic	1 (2)	1 (4)	0 (0)	
ECOG PS				0.235
0	18 (42)	9 (33)	9 (56)	
1	23 (53)	16 (59)	7 (44)	
2	2 (5)	2 (8)	0 (0)	
Metastatic Site				
Bone	29 (67)	18 (67)	11 (69)	
Visceral	9 (21)	6 (22)	3 (19)	
Regional LN	21 (49)	12 (44)	9 (56)	
Distant LN	4 (9)	3 (11)	1 (6)	
# of Metastatic sites				
1	15 (35)	9 (33)	6 (38)	
2	16 (37)	9 (33)	7 (44)	
3	11 (26)	8 (30)	3 (19)	
4+	1 (2)	1 (4)	0 (0)	
Prior Local Therapy				
Surgery	17 (40)	11 (26)	6 (38)	
Radiation	11 (26)	8 (30)	3 (19)	
Opioid Analgesic Use	18 (42)	16 (59)	2 (13)	0.001
Albumin⁺	4.1 (3.8–4.3)	4.1 (3.8–4.3)	4.1 (3.95–4.3)	0.769
Hemoglobin⁺	12.55 (11.1–13.0)	12.5 (10.4–13.2)	12.6 (11.95–13.25)	0.898
Alkaline Phosphatase⁺	103 (78.5–183)	153 (84–209)	85.5 (72.5–105)	0.008
PSA⁺	19.75 (3.55–70.6)	31.2 (4.7–100.5)	12.45 (12.1–32.1)	0.101

⁺ median value (IQR)