Biomarkers for Predicting Abiraterone Treatment Outcome and Selecting Alternative Therapies in Castration-Resistant Prostate Cancer

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Approximately one-third of patients with metastatic castration-resistant prostate cancer (CRPC) exhibited primary abiraterone resistance. To identify alternative treatment for abiraterone nonresponders, we performed drug discovery analyses using the L1000 database using differentially expressed genes identified in tumor biopsies and patient-derived xenograft (PDX) tumors between abiraterone responders and nonresponders enrolled in PROMOTE trial. This approach identified 3 drugs, including topoisomerase II (TOP2) inhibitor mitoxantrone, CDK4/6 inhibitor palbociclib, and pan-CDK inhibitor PHA-793887. These drugs significantly suppressed the growth of abiraterone-resistant cell lines and PDX models. Moreover, we identified 11 genes targeted by all 3 drugs that were associated with worse outcomes in both the PROMOTE and Stand Up To Cancer cohorts. This 11-gene panel might also function as biomarkers to select the 3 alternative therapies for this subgroup of patients with CRPC, warranting further clinical investigation.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Abiraterone is a major systemic treatment for metastatic castration-resistant prostate cancer, but the response rate is limited. Biomarkers to predict abiraterone prognosis and alternative therapies for patients who failed abiraterone treatment are not available.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ This study answered two questions, which patients are not likely to respond to abiraterone and what alternative therapies they may benefit from.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ We identified an 11-gene panel, the elevated expression of which not only are predictive of worse abiraterone response at baseline, but also serves as markers for alternative therapies that target a high proliferative phenotype, including a topoisomerase II inhibitor, a CDK4/6 inhibitor, and a pan-CDK inhibitor.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ Earlier clinical trials of mitoxantrone and palbociclib failed to find significant survival benefits, partially due to the lack of biomarkers to subgroup patients who may better benefit from these treatments. The gene panel identified in this study will provide new tools for individualize treatment for abiraterone-resistant patients.

Prostate cancer (PC) is the second most frequently diagnosed and the second most deadly cancer type for men in the United States.[1] Approximately 10–20% of patients will develop metastatic PC (mPC) and androgen deprivation therapies (ADTs) targeting androgen receptor are the first-line systemic treatment of mPC.[2] The majority of these patients eventually develop hormone-refractory

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PC, castration-resistant prostate cancer (CRPC),[2,3] when second-generation ADT drugs, such as abiraterone acetate, a cytochrome P450 17A1 (CYP17A1) inhibitor, have been shown to extend overall survival significantly.[4,5] However, at least 30% of patients do not respond to initial abiraterone treatment and nearly all patients will eventually develop acquired resistance.[6,7]

We have earlier reported pathways, including Wnt-signaling and cell cycle progression, as potential biomarkers of abiraterone response for patients with CRPC from the Mayo Clinic's PROMOTE study.[8,9] In this study, we aim to identify drugs that might overcome abiraterone resistance, and markers to select these drugs over abiraterone. We performed drug discovery analysis by applying a multivariate gene expression reversal approach using the Enrichr portal Chem Pert down/up database,[10,11] and identified mitoxantrone, a topoisomerase II inhibitor, palbociclib, a CDK4/6 inhibitor, and PHA-793887, a pan-CDK inhibitor as top candidate drugs. We have validated the efficacy of the three drugs in abiraterone-resistant prostate cancer cell lines, patient-derived xenograft (PDX)-derived organoid, and PDX mouse models. Moreover, we identified an 11-gene panel that was highly expressed in abiraterone acetate/prednisone (AA/P)-resistant patients and PDX models and can be suppressed by these 3 drugs. The gene panel was predictive of a worse prognosis in primary or metastatic patient cohorts, which might assist with the selection of alternative therapies in a subset of abiraterone-resistant patients.

METHODS

See **Supplementary Material** for methods.

RESULTS

Identification of candidate drugs to suppress abiraterone resistance expression phenotype

The drug identification workflow was illustrated in **Figure 1a**. To identify drugs that might be able to overcome abiraterone resistance, we performed gene enrichment analysis using LINCS L1000 Chem Pert down/up database with two gene sets: (i) differentially expressed genes (DEGs) between abiraterone responders and nonresponders, as defined by the 12-week composite progression end point from the bone metastatic samples obtained by the Mayo Clinic PROMOTE study,[9] and (ii) DEG between PDX models generated from abiraterone responders and nonresponders enrolled in the PROMOTE study.

We first searched the database using previously published DEGs in bone metastasis samples, which comprise 70% of the samples of PROMOTE cohort. Using 103 upregulated genes, we identified 689 drugs with at least one signature passing false discovery rate (FDR) 0.05 (**Figure 1b**, **Table S1**). Using 73 downregulated genes, we identified 141 drugs (**Figure 1c**, **Table S2**). We used the number of signatures and rank score, the weighted average rank of all signatures for a particular drug (see **Methods**) to select top candidate drugs. As shown in **Table S3**, out of all the top drugs, 3 were CDK inhibitors (palbociclib, PHA-793887, and CGP-60474), consistent with our previous findings.[9]

Four PDX models (MC-PRX-01, MC-PRX-03, MC-PRX-07, and MC-PRX-08) from AA/P nonresponders and one PDX model (MC-PRX-04) from AA/P responders were successfully established using the AA/P treatment naïve metastatic biopsies from the PROMOTE patients. Differential expression analysis of the RNAseq data from PDXs identified 377 upregulated and 763 downregulated genes in AA/P nonresponders' PDX tumors (FDR ≤ 0.05 and fold change ≥ 2; **Table S4**). The most significantly enriched pathways using the DEG were related to mitosis and G2/M checkpoints (**Table S5**). Using the upregulated genes, 117 drugs (**Table S6**) were identified. No drug was identified using the downregulated genes in PDX due to lack of significantly enriched pathways. Four drugs (palbociclib, mitoxantrone, PHA-793887, and PD-0325901) ranked top, either by total number of signatures or by rank score, were overlapped with the top drugs identified using the patients' DEG (**Figure 1d**, **Table S3**).

Efficacy of the identified drugs in inhibiting prostate cancer cell, organoids, and PDX mouse model

To examine whether the four identified drugs have therapeutic effect, especially in the abiraterone-resistant setting, we first used prostate cancer cell models 22Rv1 and LNCaP, both of which are widely applied for studying androgen receptor (AR) signaling.[12] They are at least partially androgen-dependent[13,14] and express the major target of abiraterone, CYP17A1.[15] We also developed abiraterone-resistant cell lines (22Rv1-AbiRes and LNCaP-AbiRes; **Figure 2e–l**). Compared with their parental cells, the expression of AR, AR variants V7 and del567es, and AR regulated hallmark genes, such as TMPRSS2, FKBP5, NKX3.1, and PSA were all increased in the resistant cells (**Figure S1a,b**). As shown in **Figure 2a–c**, mitoxantrone, palbociclib, and PHA-793887 inhibited cell growth in both cell lines regardless of the abiraterone-resistance status, whereas the effect was more significant for mitoxantrone and palbociclib in AbiRes cell lines. On the other hand, PD-0325901 (PD; **Figure 2d**) only exhibits inhibition effect in 22Rv1 but not LNCaP, probably because of the Q56P mutation in MEK, the target gene of PD-0325901 in that cell. MEK Q56P is a recurrent mutation in The Cancer Genome Atlas (TCGA) melanoma, lung, and gastric cohorts, and have been shown to cause resistance to several MEK inhibitor previously.[16] We then evaluated whether the identified drugs further sensitized the cells to abiraterone. As shown in **Figure 2e–g** and **Figure 2i–k**, despite that the AbiRes Cell lines are significantly resistant to abiraterone alone (**Figure 2a**), the combination treatment of mitoxantrone, palbociclib, or PHA-793887 almost completely diminished the resistance phenotype. To evaluate the potential synergistic effects between abiraterone and selected drug, we computed combination index (CI) using Chou-Talalay methods (**Figure S2a–c**).[17] Synergistic effects (CI < 1) were observed with mitoxantrone, palbociclib, and PHA-793887 at all concentrations tested in 22Rv1, and selected concentrations in LNCaP, whereas the effects for PD-0325901 was not consistent.

The potency of the identified drugs was further tested in organoids derived from the two PDX models, MC-PRX-01 and MC-PRX-05 (**Figure 3**), developed from AA/P nonresponder pretreatment tumors. As expected, the two organoids showed abiraterone resistant phenotype with less than 20% inhibition of organoid growth at the highest tested concentration of abiraterone (**Figure 3e–l**). On the other hand, although mitoxantrone,

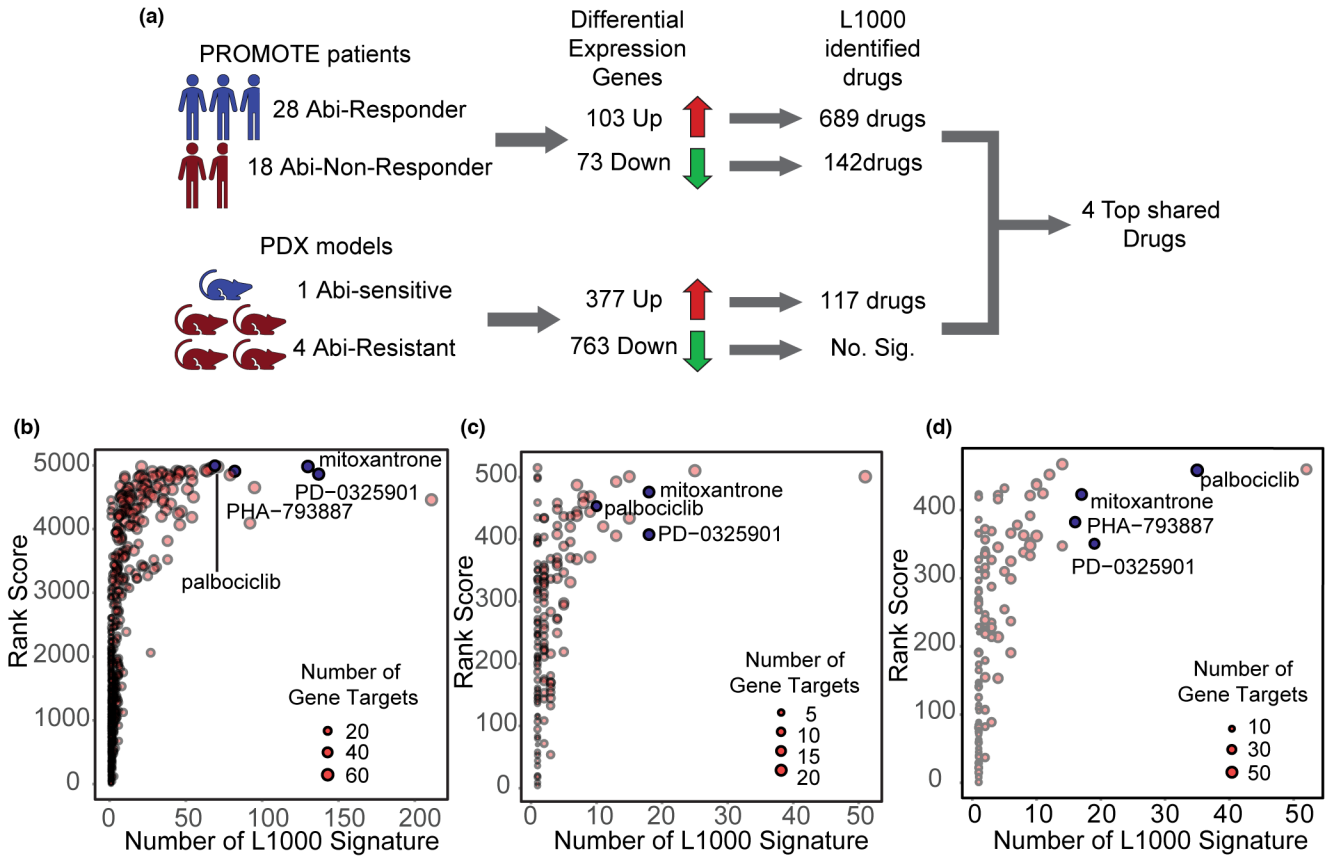

Figure 1 Drug discovery analysis based on patient tumor and xenograft genomic information. (**a**) The workflow for the drug discovery analysis. Bubble plots for Enrichr-LINCS L1000 Chemical Perturbation analysis using significantly, (**b**) upregulated in PROMOTE abiraterone nonresponders, (**c**) downregulated genes in PROMOTE abiraterone non-responders, and (**d**) upregulated genes in abiraterone resistant PROMOTE PDX model. Data was presented as rank score (see Methods section) vs. the number of signatures for each drug returned by L1000 database search with FDR ≤ 0.05. Size of the bubbles represents number of gene targets overlapped between the submitted list genes and gene signature. Abi, abiraterone; FDR, false discovery rate; PD, PD-0325901; PDX, patient-derived xenograft.

palbociclib, and PHA-alone exhibited weaker cytotoxic effects in organoids than in cell lines (**Figure 3a–d**), they still sensitized the organoids to abiraterone, as evidenced by decreased viability in an abiraterone dose-dependent fashion (**Figure 3e–g, i–k**). Synergistic effects (CI < 1) were also observed across almost all doses for mitoxantrone, palbociclib, and PHA-793887 (**Figure S2e–g**) in MC-PRX-05. Although CI cannot be evaluated in MC-PRX-01 due to the complete lack of efficacy of abiraterone in this model, it was nevertheless evidenced by the dose-response inhibition effect seen in the combination treatment (**Figure 3e–g**). PD-0325901 again exhibited inconsistent results between the two PDX-derived organoid models, with almost no inhibition in MC-PRX-05 (**Figure 3d**, **Figure S2h**). Because of the inconsistent response results of PD-0325901 in different models, we decided not to include this drug in our follow-up investigation.

Among the three drugs, mitoxantrone currently is the only US Food and Drug Administration (FDA)-approved drug for patients with CRPC. It was the most potent (nM scale) in our cytotoxicity experiments (**Figures 2** and **3**) and showed the most synergistic effects (**Figure S2**). Therefore, we first evaluated the efficacy of mitoxantrone, as well as doxorubicin, another topoisomerase II inhibitor commonly used in the treatment of breast cancer, in PDX mouse models. Consistent with organoids data, the PDX models responded minimally to abiraterone treatment alone (**Figure 4a–c**). However, mitoxantrone/doxorubicin treatment alone was able to decrease tumor growth by more than 50%. Abiraterone and TOP2 inhibitor combination treatments almost completely abolished tumor growth in 8 out of 10 mice in MC-PRX-01, although no statistical difference was observed between TOP2 inhibitors alone vs. combined with abiraterone (**Figure 4a–c**). We then evaluated the efficacy of two CDK inhibitors in the PDX models. Although both PHA-793887 and palbociclib inhibited tumor growth, palbociclib appeared to be effective as a single treatment, whereas PHA-alone failed to exhibit statistically significant inhibition at tested dosage but did appear to sensitize the tumor to abiraterone treatment (**Figure 4e–g**). Despite that the Chou-Talalay method indicated potential synergistic effects of the three drugs in *in vitro* experiments, our *in vivo* study indicated that mitoxantrone and palbociclib had the same potency as a single therapy compared with combination with Abi, whereas PHA-793887 might have synergistic effect with abiraterone. In all cases, no significant toxicities were observed as evidenced by mice body weight (**Figure 4d,h**).

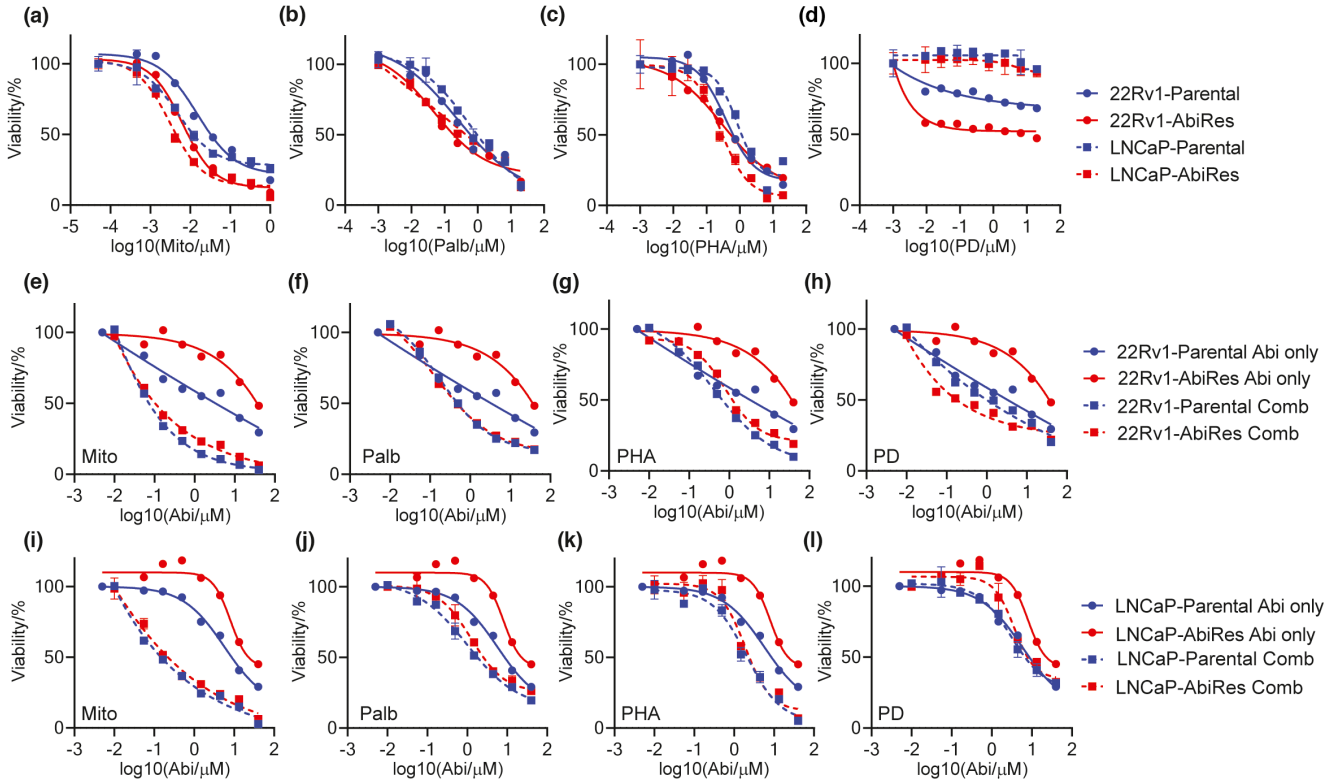

Figure 2 Single and combination drug treatments in abiraterone parental and resistant cell lines. Cytotoxicity assays in -v1 and LNCaP parental and AbiRes cell lines treated with (**a**) mitoxantrone, (**b**) Palbociclib, (**c**) PHA-793887, or (**d**) PD-0325901 alone at indicated concentrations. Cytotoxicity assays in 22Rv1 parental and AbiRes cell lines treated with combination of abiraterone and (**e**) mitoxantrone (1:200 mitoxantrone:abiraterone), (**f**) Palbociclib (1:12 Palb:abiraterone), (**g**) PHA-793887 (1:12 PHA-793882:abiraterone), or (**h**) PD-0325901 (1:12 PD-0325901:abiraterone). Cytotoxicity assays of LNCaP parental and AbiRes cell lines treated with combination of abiraterone and (**i**) mitoxantrone (1:200 mitoxantrone:abiraterone), (**j**) Palbociclib (1:12 Palb:abiraterone), (**k**) PHA-793887 (1:12 PHA-793882:abiraterone), or (**l**) PD-0325901 (1:12 PD-0325901:abiraterone). X-axis indicated concentrations of abiraterone treated alone or in combination. All data was presented as mean ± SD of at least two replicates, and normalized to vehicle treatment, as shown as the left most data points. AbiRes, abiraterone resistant; Comb, combination; Mito, mitoxantrone; PD, PD-0325901.

Identification of 11 genes modulated by mitoxantrone, palbociclib, and PHA-793887

In order to identify genes that may be targeted by the drugs and potentially can be used for selection biomarkers for these treatments, we then examined the gene targets potentially modulated by all 3 drugs as indicated from the L1000 signatures. Intriguingly, these drugs shared highly similar gene signatures. Using the top significant DEG from patients' tumors, 52 genes were shared among all drugs (**Figure S3a**). Whereas using the top significant DEG from PDX tumors, 24 genes were shared among all drugs (**Figure S3b**). Combining the overlapped genes from patients and PDX for each drug, we identified 11 genes (*CCNA2*, *CCNB1*, *CCNB2*, *PRC1*, *SMC2*, *DLGAP5*, *ECT2*, *FBXO5*, *CDK1*, *NCAPG*, and *KIF4A*) that were shared between the two datasets as commonly targeted genes by all 3 drugs (**Figure 5a**, **Figure S3c**). These genes were highly expressed in bone-metastatic samples from the AA/P nonresponders, as earlier reported.[9] We then examined the expression levels of these 11 genes in samples from all metastatic sites. All except 1 gene (PRC1) were significantly upregulated in AA/P nonresponders (Mann–Whitney's test; **Figure S3d**). All 11 genes were also significantly upregulated in PDX tumors derived from AA/P nonresponders (Mann–Whitney's test; **Figure S3e**) and in AbiRes cell lines (**Figure S1c,d**). The 11 genes are mostly enriched in the mitotic and G2/M checkpoint pathways (**Table S5**).

We then validated whether the drugs we identified were capable of suppressing the expression of these 11 genes in our abiraterone-resistant cell line, organoids, or PDX tumors by quantitative real-time polymerase chain reaction. As shown in **Figure 5**, the expression of the 11 genes was either not significantly affected or slightly elevated with abiraterone treatment, but was generally suppressed after treatment of the mitoxantrone, palbociclib, or PHA-793887 (**Figure 5b–g**), and the pattern of suppression did not appear to be different between single-drug treatment or combination treatment with abiraterone for mitoxantrone and palbociclib. Interestingly, the combination treatment of PHA-793887 and abiraterone appeared to have better gene suppression effect than PHA-793887 alone in most models with more significant observations in the PDX organoids and tumor (**Figure 5d,g**), which was consistent with our *in vivo* tumor treatment effect (**Figure 4e–g**). These results indicate that mitoxantrone, palbociclib, and PHA-793887 may inhibit tumor growth through the inhibition of these genes.

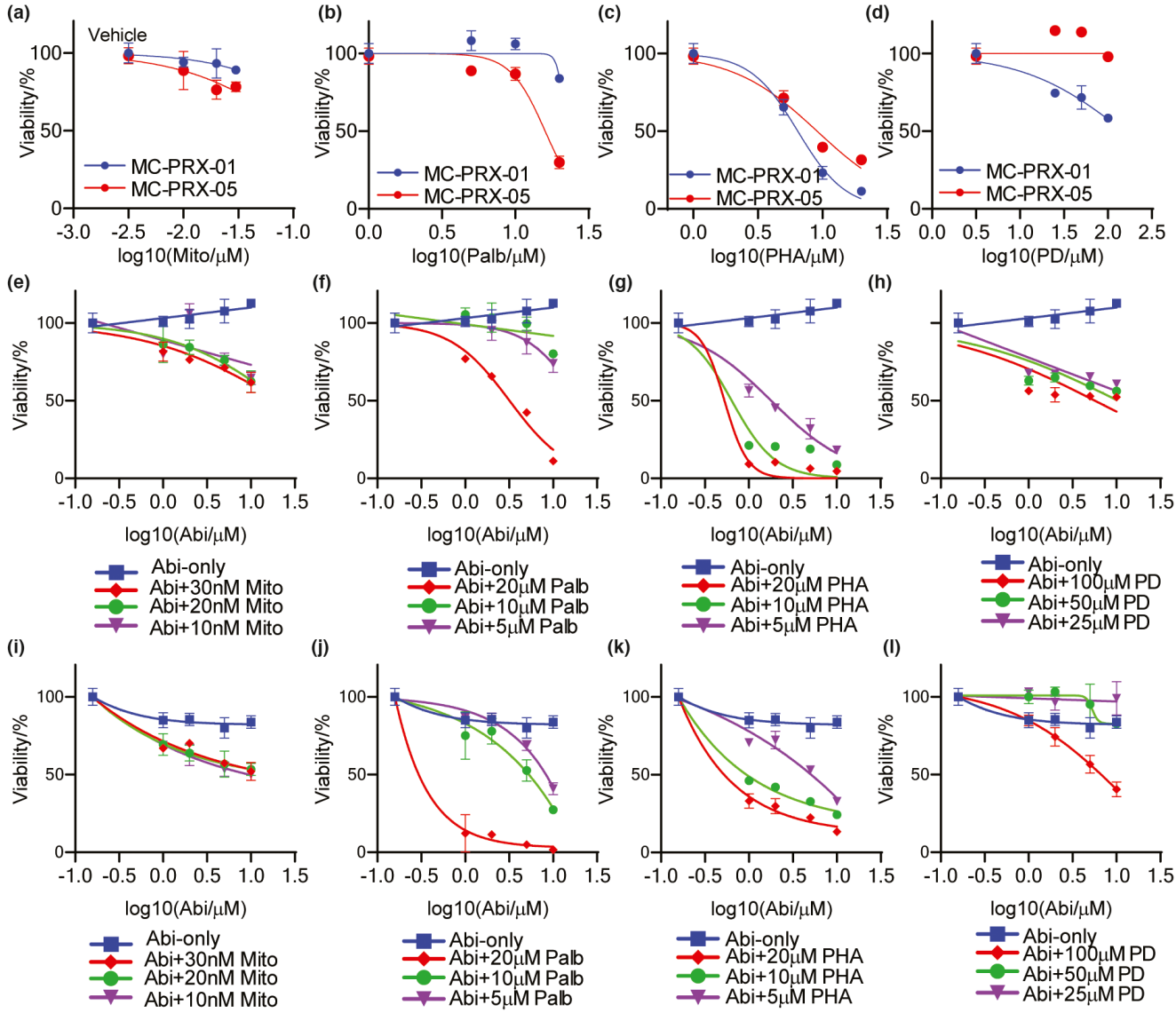

Figure 3 Single and combination drug treatments in PDX derived organoids. Cytotoxicity assays of MC-PRX-01 and MC-PRX-05 treated with (**a**) mitoxantrone, (**b**) Palbociclib, (**c**) PHA-793887, and (**d**) PD-0325901 at indicated concentrations. Cytotoxicity assays of MC-PRX-01 treated with abiraterone alone or in combined with (**e**) mitoxantrone, (**f**) palbociclib, (**g**) PHA-793882, or (**h**) PD-0325901 at indicated concentrations. Cytotoxicity assays of MC-PRX-05 treated with abiraterone alone or in combined with (**i**) mitoxantrone, (**j**) palbociclib, (**k**) PHA-793882, or (**l**) PD-0325901. All data was presented as mean ± SD of at least two replicates, and normalized to vehicle treatment, as shown as the left most data points. Abi, abiraterone; Mito, mitoxantrone; Palb, palbociclib; PD, PD-0325901; PRX, patient-derived xenograft.

The 11-gene panel is associated with treatment outcomes in CRPC

The 11 genes were upregulated in AA/P nonresponders' tumor samples and PDX models derived from these AA/P nonresponders (**Figure S3d,e**), but whether these genes can be used for prediction of long-term outcomes such as overall survival (OS) and time to treatment change (TTTC) in CRPC was not clear. In order to evaluate the prognostic value of the 11 genes for long-term outcome, we first performed unsupervised machine learning clustering analysis using the 11 genes from the 68 PROMOTE baseline patients' samples collected from all biopsy sites (**Figure 6a**). Based on the elbow method, the patient can be best clustered into three subgroups. One of the clusters, corresponding to ~25% of patients, exhibited apparently higher expression, based on the sum of z-scores of the 11 genes in that cluster, compared with the other two clusters. This cluster was designated as the "high-expression cluster." The other two clusters were collectively referred to as the "low-expression cluster." Moreover, the high-expression cluster identified by the gene panel was associated with significantly worse OS (P value = 0.00164; **Figure 6b**) and TTTC (P value = 0.017; **Figure 6c**). We performed similar clustering and Kaplan-Meier analysis on biopsy samples from bone metastasis only (**Figure S4a–c**). Although the trends were similar, the results were not statistically significant, which is likely due to the limited number of samples.

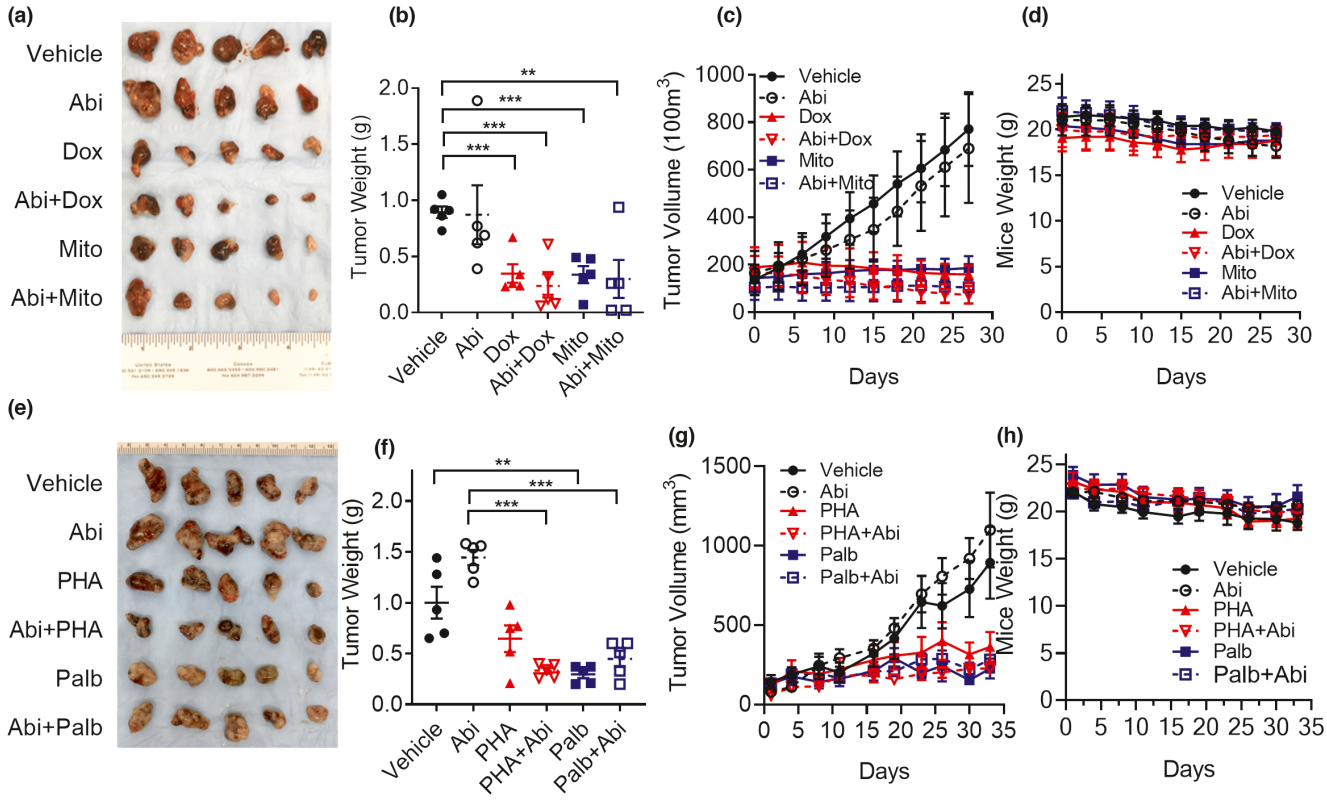

Figure 4 Topoisomerase II (TOP2) inhibitors and CDK inhibitors inhibit abiraterone resistant PDX tumor growth. MC-PRX-01 PDX model treated with TOP2 inhibitors mitoxantrone (Mito) and doxorubicin (Dox). (**a**) Tumors harvested after 28 days of treatments of abiraterone alone (Abi), TOP2 inhibitors (Mito and Dox) alone, or combination of the two. (**b**) Tumor weights at the time of harvest were quantified. Statistical significance indicated unpaired *t*-test: $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ ($n = 5$). (**c**) Tumor growth during the TOP2 inhibitors treatment period. (**d**) Mice body weight during the TOP2 inhibitors treatment period. MC-PRX-01 PDX model treated with CDK inhibitors Palbociclib (Palb) and PHA-793887 (PHA). (**e**) Tumors harvested after 35 days of treatments of abiraterone alone, CDK inhibitors (Palb and PHA) alone, or combination of the two. (**f**) Tumor weights at the time of harvest were quantified. Statistical significance indicated unpaired *t*-test: $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ ($n = 5$). (**g**) Tumor growth during the CDK inhibitors treatment period. (**h**) Mice body weight during the CDK inhibitors treatment period.

In order to further validate our findings, we analyzed a second independent metastatic prostate cancer cohort, the Stand Up To Cancer (SU2C).[18] To best resemble the PROMOTE cohort, we first analyzed data obtained in patients with available OS data enrolled only in the abiraterone treatment arm of this cohort to validate our finding (53 samples; **Table S6**). Using the 11-gene panel and clustering strategy that was applied to the PROMOTE dataset, the expression data of the SU2C cohort can also be clustered into high and low expression subgroups, and that patients in the high expression cluster also exhibited worse OS as compared with the low expression cluster (P value = 0.0208; **Figure 6d,e**). When including samples in the enzalutamide treatment arm (9 additional samples with available data), survival benefits retained the same (**Figure S4d,e**).

The 11 genes are associated with progression-free survival in the primary tumor

Relapse is the major reason for prostate cancer-related mortality, and ADT is often used as adjuvant therapy after surgical removal of the tumor. In order to examine whether our panel also has a prognostic value of disease relapse in the primary tumor, we further examined the TCGA primary prostate cancer cohort (**Figure 6f,g**) and German Cancer Research Center (DKFZ) early onset prostate cancer cohort (**Figure S4f,g**). We used progression-free survival (PFS) as the clinical outcome for the TCGA cohort and biochemical relapse (BCR) for the DKFZ cohort. Interestingly, the results also showed that the patients with high expression or in the high expression subgroup had a worse PFS outcome in the TCGA cohort (P value = 6.78×10^{-6}; **Figure 6f,g**) and a worse BCR outcome in the DKFZ cohort (P value = 6.55×10^{-5}; **Figure S4f,g**). These results also indicated the potential value of our gene panel in predicting the risk of disease progression in the primary tumor. Moreover, we evaluated our gene panel in other TCGA cancer types, including breast cancer, cervical cancer, and colorectal cancer. None of these cancer types we evaluated showed statistically significant associations between the clusters based on the 11 gene expression and the outcome (PFS), suggesting that this gene panel is more specifically related to prostate cancer prognosis or ADT prognosis (**Figure S5**).

Comparison of the 11-gene panel with other gene panel markers

We finally evaluated the effect of our gene panel in the context of other genomic alterations and clinical variables in the PROMOTE samples.[9] We first examined the Spearman correlation of the expression of the 11 genes with a number of clinical variables,

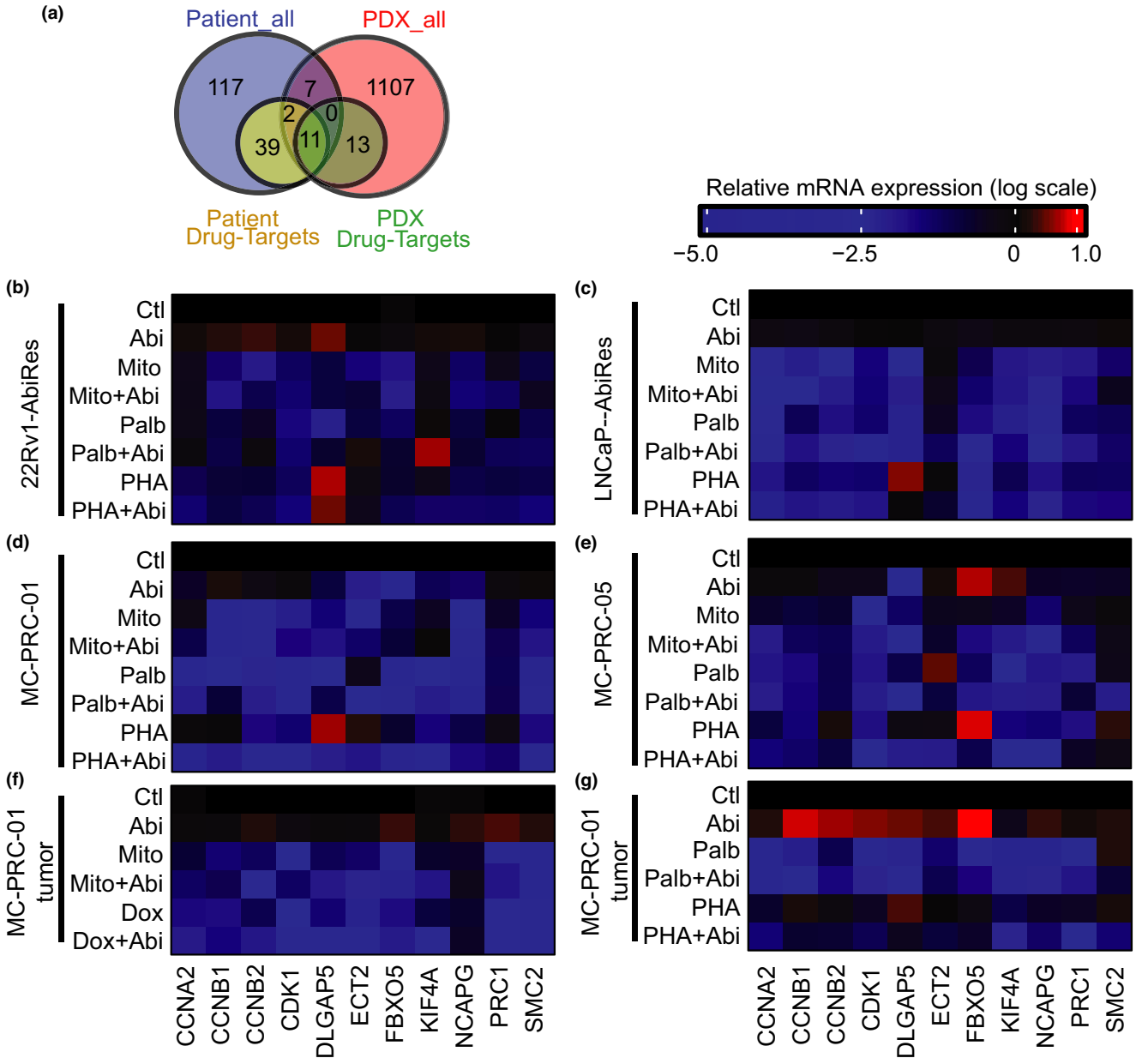

Figure 5 Expression modulation by the three drugs. (**a**) Venn diagram for all differentially expressed genes (DEG) in PROMOTE patients (Patient_all[9]), PDX models (PDX_all), and genes targeted by the top three candidate drugs that were also shared between patients (Patient Drug-Targets) and PDX models (PDX Drug-Targets). The 11 genes were shared among all comparisons. Expression modulation by qRT-PCR after abiraterone, mitoxantrone, Palb, PHA-793887 or combination treatment in (**b**) 22Rv1 AbiRes, (**c**) LNCaP AbiRes, (**d**) PDX organoids MC-PRX-01, (**e**) PDX organoids MC-PRX-05, (**f**) PDX tumor MC-PRX-01 treated with TOP2 inhibitors, and (**g**) PDX tumor MC-PRX-01 treated with CDK inhibitors. Expression was presented in presented as Log2-fold change after normalized to vehicle treatment in each cell line or PDX model after normalization to housekeeping gene, GAPDH. Abi, abiraterone; Ctl, control; Mito, mitoxantrone; Palb, Palbociclib; PDX, patient-derived xenograft; qRT-PCR, quantitative real-time polymerase chain reaction.

including PSA and testosterone levels, as well as three widely used gene panels or scores that are known to be associated with prostate cancer progression, including AR activity score (20 genes),[19] cell cycle progression score (CCP; 31 genes),[20] and neuroendocrine prostate cancer score (NEPC; 70 genes).[21] We found that the 11 genes were highly correlated with the CCP score (**Figure S6a,b**), despite that only three genes (CDK1, DLGAP5, and PRC1) were shared between the CCP gene panel and our 11-gene panel. The Mann–Whitney test of CCP scores between the high- and low-expression subgroups indicated a significant difference with P values as low as 9.5×10^{-15} (**Figure S6f**). Compared with the low-expression group, the high-expression subgroup appeared to be associated with a higher proportion of non-bone tissue biopsy origin, a higher proportion of erythroblast transformation specific (ETS) fusions, and a higher AR activity score, but not PSA, NEPC score, or mutation burden (**Figure S6c–i**). However, the high-expression subgroup carried a higher fraction of copy number variations (P value = 0.024), indicating increased genomic

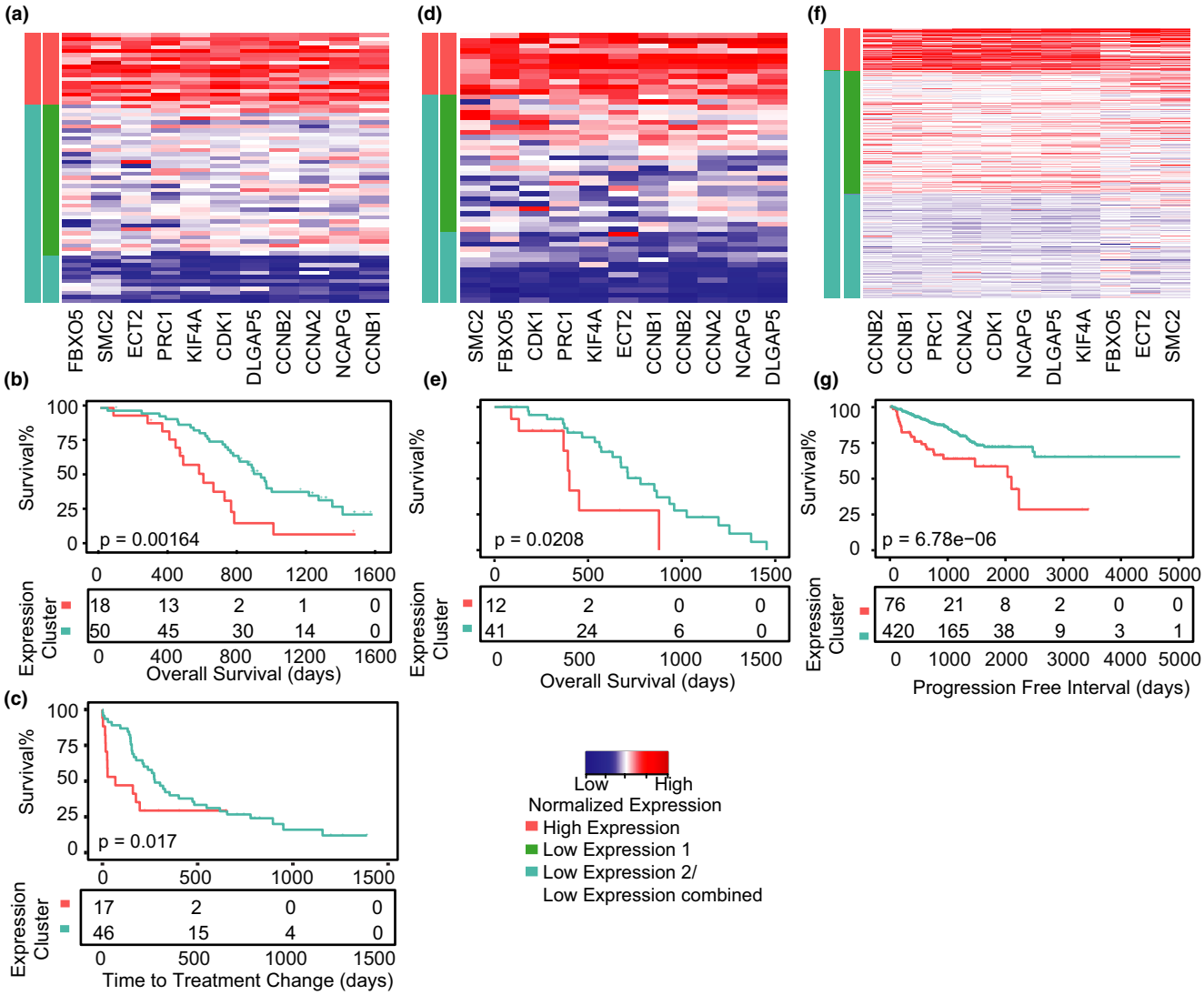

Figure 6 Clustering and survival analysis of the patients' profiles using the 11 gene panel. (**a**) Heatmap of expression of the 11 gene targets shared among the 4 candidate drugs. Patients were arranged after k-means clustering with the clusters arranged on the left indicated by colored bar ("high expression" cluster as red, and the two "low expression" clusters as green and cyan, respectively, or as cyan combined). Kaplan-Meier analysis of (**b**) overall survival (OS) and (**c**) time to treatment change based on high- and low expression clusters using the 11 gene panel. The *P* value of the Gehan-Breslow-Wilcoxon test is calculated and indicated in the figure and number of patients in each risk group is indicated below the figures. (**d**) Heatmap of 11-gene expression and (**e**) Kaplan-Meier analysis of OS in the SU2C cohort. (**f**) Heatmap of 11-gene expression and (**g**) Kaplan-Meier analysis of progression-free survival in the TCGA prostate cancer cohort. SU2C, Stand Up To Cancer; TCGA, The Cancer Genome Atlas.

instability that was likely responsible for the association with the worse outcome (**Figure S6j**). No mutations were identified in the 11 genes. However, gene gain and loss were observed in almost all 11 genes, with three genes (CCNA2, CDK1, and KIF4A) being most different between the high- and low-expression groups (**Figure S6k**).

We then evaluated the prognostic prediction values of clinical variables and the gene panels by COX proportional hazard models. Univariate analysis identified log-PSA as the only clinical variable associated with prognosis (**Figure 7a**). We then evaluated the prognostic values of the 11-gene panel and NEPC, CCP, and AR activity scores in two metastatic (PROMOTE **Figure 7b** and SU2C **Figure 7c**) and two primary (TCGA **Figure 7d** and DKFZ **Figure 7e**) cohorts. Compared with the other panels, our 11-gene panel could consistently predict clinical outcomes either as OS in the metastatic cohorts or as PFS in the primary cohorts. AR activity score was better indicative of prognosis in the primary cohorts, whereas NEPC, on the other hand, was only significant in the metastatic settings. The NEPC scores were near zero in most samples in the primary cohorts, indicating a lack of neuroendocrine phenotype in this setting, consistent with previous reports.[18,21] The CCP panel was consistent with our 11-gene panel, especially in the primary cohorts, as previously reported,[22,23] whereas less significant in the metastatic settings. Moreover, the hazard ratio of

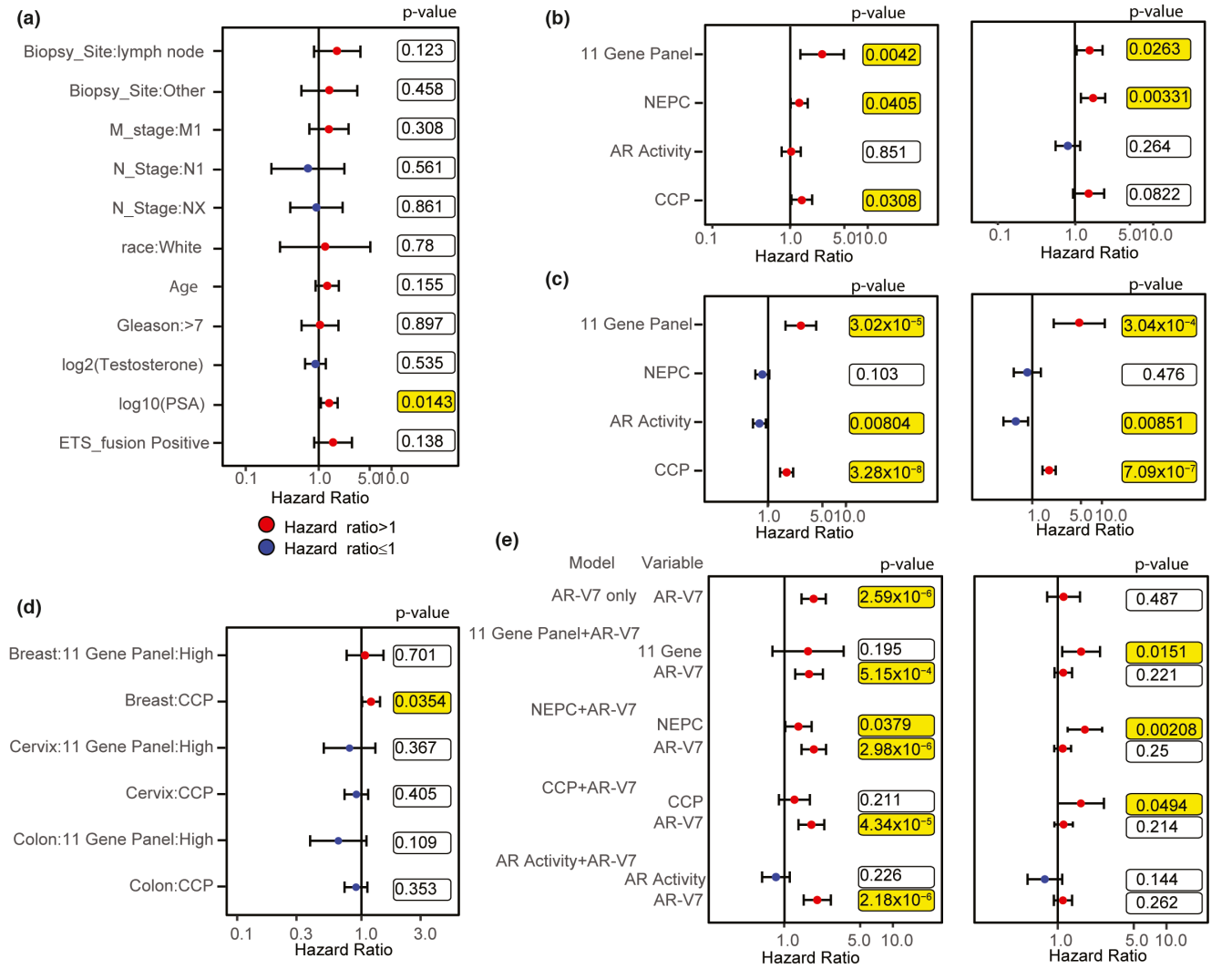

Figure 7 Gene panels serve as independent prognosis predictors using the COX proportional hazard model (**a**) Univariate analysis of overall survival (OS) against clinical variables in PROMOTE cohort. X-axis represents the hazard ratio, plotted in log scale, with error bars indicating 95% confidence interval. The *P* values are indicated on the right side of each variable with $P \leq 0.05$ highlighted in yellow. COX univariate model with the 11-gene panel, AR score, CCP score, or NEPC score as predicting variable, performed using (**b**) OS of metastatic cohorts (left) PROMOTE and (right) SU2C cohort, and (**c**) progression-free survival (PFS) of primary cohorts, (left) TCGA prostate cancer cohort, and (right) biochemical relapse interval for DKFZ cohort. (**d**) PFS for other TCGA cohorts. (**e**) COX model using AR-V7 (SRPM) or AR-V7 (SRPM) in combination with the 11-gene panel, AR score, CCP score, or NEPC score in the (left) PROMOTE and (right) SU2C cohorts. AR, androgen receptor; CCP, cell cycle progression; DKFZ, German Cancer Research Center; NEPC, neuroendocrine prostate cancer; SU2C, Stand Up To Cancer; TCGA, The Cancer Genome Atlas.

the 11-gene panel was consistently higher than that of CCP, suggesting that the high-expression subgroup identified by the 11 gene panel have worse clinical outcomes compared with those identified by CCP. Finally, we also examined the specificity of the 11-gene panel and CCP gene panel in other cancer types. Similar to a previous report,[24] CCP was also a prognostic marker in breast cancer. However, the 11-gene panel was not significantly associated with outcome in any other cancer types we tested (**Figure 7f**).

The expression of ligand-independent AR splice variants, especially AR-V7, has been shown to be associated with ADT failure in primary prostate cancer,[25] but its association with prognosis in metastatic setting was controversial.[26] We examined the expression of AR and various AR-splice variants in the PROMOTE cohort and found that the expression of AR variant, particularly ARV3 and V9, but not total AR or ARV7 was higher in ADT-resistant group as defined by 3 months comprehensive response (**Figure S7a,b**). However, this observation was not captured by the PDX model due to the limited number of PDX models we were able to develop (**Figure S7c-d**). Using univariate COX model, we found that AR-V7 expression was a significant predictor of OS in the PROMOTE cohort, but not in the SU2C cohort (**Figure 7e**). When included in the multivariate models with various gene panels that have shown association with prognosis or response, AR-V7 stayed significant in PROMOTE and insignificant in SU2C (**Figure 7e**). In fact, the 11-gene panel and CCP panel became insignificant in the multivariate model in PROMOTE because the 11-gene high-expression subgroup also expressed significantly higher AR-V7 than the low-expression subgroup (**Figure S7e**), thus coincidently predict the

same group of patients in PROMOTE. But this was not the case in SU2C (**Figure S7f**) in which the 11-gene panel predicted an independent subset of patients with worse outcome but not necessarily with high AR-V7 expression. Last, we examined whether the identified drugs inhibit cell growth via suppressing AR-V7 expression. Data implicated that PHA-793,882 reduced AR-V7 protein in both abiraterone-resistant cell lines, but not consistent for the other two drugs (**Figure S7g–h**). Therefore, suppression AR-V7 expression might contribute but was not essential to the proliferation-inhibition effects of all the drugs.

DISCUSSION

Despite the survival benefit of abiraterone to patients with mCRPC, the initial response rate is approximately half to two-thirds, and eventually almost all patients have progression of disease.[6,7] Alternative therapies, such as docetaxel or enzalutamide, only provide survival benefits in less than 50% of abiraterone-resistant patients.[27–30] Therefore, biomarkers to predict abiraterone response at the time of diagnosis, and, more importantly, to identify alternative therapies when abiraterone fails are of great interest. However, in our study, we found that clinical features, such as the Gleason Scores, has limited prediction values in CRPC metastatic settings (**Figure 7a**), whereas single gene markers, such as ETS transcription factor and expression of AR splice variants has been controversial in the metastatic setting,[31–33] and, as we found in this study, cohort dependent (**Figure 7e**). Various gene expression panels have been developed to either evaluate the aggressiveness or to molecularly classify prostate cancer. CCP score has been one of the most studied gene panels. It can identify aggressive primary prostate cancer with higher risk of recurrence after radical prostatectomy (**Figure 7b–e**).[20,22,23] The NEPC score was first developed in several cohorts, including SU2C, and has been primarily used to identify AR-independent, neuroendocrine subtype of prostate cancer, which has been associated with worse outcomes in the metastatic stage (**Figure 7b–e**).[21] However, the neuroendocrine phenotype is rare in the primary settings. AR activity score has been also used mostly to characterize androgen-dependent AR activity, and, in our study, AR activity score was mostly effective in predicting outcome in the early stage primary cohorts (**Figure 7b–e**).[19,34] In the current study, we found that a panel of 11 genes was able to identify a subgroup of ~25% of patients with the worst outcomes represented by TTTC or OS following AA/P treatment in the PROMOTE and SU2C mCRPC cohorts. This panel of 11 genes might also serve as a selection marker for alternative therapies, such as the three drugs identified here. Moreover, our 11-gene panel was also indicative of a higher risk of recurrence in ~ 15% of patients in the TCGA and DKFZ cohorts, two cohorts with primary disease (**Figure 6f,g**, **Figure S6f,g**). Our panel was also specific to prostate cancer (**Figure S5**), which was different from the CCP gene panel.[24]

Resistant mechanisms are heterogeneous, and our ability to identify the biomarkers was also limited by the number of patients and PDX models available in the PROMOTE study, which partially resulted in only genes upregulated in nonresponders rather than downregulated were included in our gene panel. This is at least partially due to the lack of abiraterone-sensitive PDX model. However, because tumors taken by mice are usually more proliferative, our approach by integrating data from both human tissues and corresponding PDX tumors might help identify one of the most common and aggressive mechanisms associated with poor outcomes (i.e., tumors with highly proliferative phenotype). Despite these limitations, the fact that our panel has been validated in four independent cohorts and the identified drugs have also been confirmed experimentally in different *in vitro* and *in vivo* systems provide additional confidence of this approach and the utility of the gene panel derived from this approach.

In this study, we identified three alternative drugs for a subset of ADT-resistant patients. The three drugs all target cell cycle progression but with different specificities. Palbociclib is a CDK4/6-specific inhibitor, PHA-793887 is a pan-CDK inhibitor, and mitoxantrone inhibits TOP2, which played critical roles in DNA replication, and chromosome condensation and segregation,[35] which is also required for cell cycle progression. It has been reported that cell cycle genes are often co-expressed or co-regulated.[20] Therefore, it is not surprising that all three drugs, despite differences in their mechanism of action, modulated the expression of the 11 genes that mostly function in G2/M checkpoint. In fact, in addition to palbociclib and PHA-793882, we also identified other CDK inhibitors, such as alvocidib, CGP-60474, and AZD-5438 (**Table S7**), all of which appear to suppress at least a subset of the 11-gene panel despite their different CDK specificity. Nevertheless, further mechanistic studies are needed to evaluate their efficacy.

Mitoxantrone has been approved for improving the quality of life for patients with mPC. There are a few phase Ib/II trials using a combination of mitoxantrone or other TOP2 inhibitor and hormonal therapies, such as NCT00004124, NCT00002855,[36] and NCT01240629[37]). So far, no significant clinical benefits of the combination therapy compared with the hormonal therapy alone has been reported. Similarly, three CDK4/6 inhibitors (palbociclib, abemaciclib, and ribociclib) have been tested in several early phase trials in combination with ADT for mPC (NCT02059213, NCT02555189, and NCT03706365) either failed to find survival benefits or are still ongoing. The lack of appropriate biomarkers for patient stratification may be one of the hurdles to show the clinical benefits of these drugs either alone or combined with standard care. Our 11-gene panel might help with patient stratification and individualize the therapy to benefit the patients who are at the highest risk of having the poorest outcome.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICT OF INTEREST

Drs. Wang and Weinshilboum are co-founders and stockholders of OneOme, LLC. All other authors declared no competing interests for this work.

AUTHORS' CONTRIBUTIONS

S.Q., H.G., L. Wei, W.T., A.B., R.W., and L. Wang wrote the manuscript. S.Q., H.G., R.W., and L. Wang designed the research. S.Q., H.G., W.K., H.Z., and Y.G. performed the research. S.Q., H.G., J.P.S., and K.K. analyzed the data. W.K., F.X, P.Y., K.K., J.A.S., J.Y., Y.Z., L. Wei, and B.Q. contributed new reagents/analytical tools.

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