Integrative Analysis of Androgen Receptor Interactors Aberrations and Associated Prognostic Significance in Prostate Cancer

Zhu Wang, Ying Zhang, Qiong Deng, Jianwen Zhang, Xisheng Wang*, Hui Liang*

Purpose: Much progress has been made by directing against the adrogen receptor (AR) pathway in the treatment of prostate cancer in past decades. However, AR-interactors related metastatic castration resistant prostate cancer eventually developed. Here, we aimed to characterize the aberrations and therapeutic implication in advanced disease.

Materials and Methods: STRING database, UALCAN web portal and cBioPortal platform was used to analyze the AR interaction network, gene alterations, as well as the prognostic significance. GO and KEEG analysis was performed to characterize the functional enrichment of the identified AR-interactors.

Results: Ten first shell AR-interactors were identified, among of which FOXA1 and PELP1 was significantly up-regulated, while CCND1, CTNNB1, NCOA4 and HSP90AA1 exhibited a significantly decreased pattern. The median survival period of altered group (n = 227) was 70 months (95% CI, 60-105M), while that of non-altered group (n = 545) was 141 months (95% CI, 115.13-NA, $P < 0.001$). GO and KEGG enrichment showed that the identified AR-interactors were particularly enriched in prostate cancer and thyroid hormone signaling pathway, as well as endocrine resistance.

Conclusion: The AR-interactors might be useful markers for prostate cancer diagnosis and prognosis, and provide a new sight for revealing the molecular mechanism of CRPC progression.

Keywords: androgen receptor, prostate cancer, interactors, castration-resistance

INTRODUCTION

Prostate cancer is ranked the second most common male malignancies, and the fifth leading cause of cancer death among men worldwide[1]. To inhibit or block androgen receptor (AR) signaling pathway by androgen deprivation therapy (ADT) by chemical or surgical castration is the first-line treatment for advanced metastatic prostate cancer. However, with the progression of the disease, a variety of molecular mechanisms lead to the restoration of activity of AR signaling pathway, and then termed castration-resistant prostate cancer (CRPC) [2,3]. Accumulating evidence assigns a key role to the continuous activation of the androgenic receptor (AR) signaling pathway in CRPC progression, as well as alternative independent routes[2,4-6].

In the classical AR signaling pathway, AR translocates into the nucleus with the ligand-binding domain occupied by androgen, to govern the target gene expression via DNA-binding domain binds to androgen-responsive elements (ARE) and recruits additional coregulators and transcriptional modulators[7,8]. The transcriptional activity of AR signaling is greatly modulated by a number of coregulators (such as EP300 [E1A binding protein P300], SRC1 [Steroid Receptor Coactivator-1] and SRC3 [Steroid receptor coactivator 3])[9,10], and several key proteins including FOXA1 (Forkhead box protein A1), PTEN (Phosphatase and tensin homolog), ERBB2 (Erb-B2 receptor tyrosine kinase 2) and ERBB3 (Erb-B2 receptor tyrosine kinase 3)[11,12]. In addition, AR crosstalk pathways including MAPK, PTEN/PI3K/Akt/mTOR pathway, STAT3, Wnt/β-catenin and other signaling pathways play an important role in promoting the transformation of CRPC[13,14]. Overall, these key modulators and signaling molecules are just the tip of the iceberg of a coordinated and may redundant network that acts in concert with AR signaling pathway to promote tumor growth and development in prostate cancer.

At present, significant progress has been made in understanding the mechanism of CRPC, and several novel AR-directed therapies have been developed and clinically validated. Enzalutamide is the first second-generation nonsteroidal antagonist with a strong binding affinity to AR, could significantly prolong overall survival (OS) time for patients with lethal metastatic CRPC, which thus been approved by the US Food and Drug Administration (FDA) for the treatment of CRPC in 2012 [15-17]. Abiraterone acetate (AA), an inhibitor of the steroidal enzyme 17α-hydroxylase/C17-20 lyase (also known as CYP17A1 or P450c17) playing a central role in androgen biosynthesis from the adrenal glands [18,19], demonstrated to improve OS time in patients who had chemo-naive and docetaxel pretreated, becoming a therapeutic alternative to docetaxel and enzalutamide for metastatic castration-resistant prostate cancer (mCRPC)[18]. Darolutamide is another newly developed

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Table 1. List of first shell interactors of AR identified by the STRING database

Accession	Symbols	Description	Confidence Score
P10275	AR	Androgen receptor	-
P24385	CCND1	G1/S-specific cyclin-D1	0.998
P35222	CTNNB1	Catenin beta-1	0.999
P55317	FOXA1	Hepatocyte nuclear factor 3-alpha	0.999
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	0.999
Q00987	MDM2	E3 ubiquitin-protein ligase Mdm2	0.998
Q15788	NCOA1	Nuclear receptor coactivator 1	0.999
Q15596	NCOA2	Nuclear receptor coactivator 2	0.999
Q13772	NCOA4	Nuclear receptor coactivator 4	0.999
Q8IZL8	PELP1	Proline-, glutamic acid- and leucine-rich protein 1	0.999
P12931	SRC	Proto-oncogene tyrosine-protein kinase Src	0.999

non-steroidal androgen receptor antagonist and recently approved for the treatment of non-metastatic castration-resistant prostate cancer (nmCRPC)[17,20,21]. Several other targeting agents and new therapeutic modalities such as poly ADP ribose polymerase (PARP) inhibitors, histone deacetylase (HDAC) inhibitors and prostate-specific membrane antigen (PSMA)-ligand therapy are developed or being tested in clinical trials[22,23]. However, lots of patients will ultimately develop subsequent resistance to the individual agents via various complicated mechanisms, such as continuously active, truncated AR splice variant-7 (AR-V7)[24], bypass and alternative pathway of AR signaling. To identify the right agents or better the right combination and proper sequencing of treatments are becoming challenge in the near future.

Here, we comprehensively evaluated the expression and alteration of AR and its interactors from the existing database by bioinformatics method. So as to provide a new insight for the study of prostate cancer progression mechanism and prognosis evaluation.

MATERIALS AND METHODS

STRING analysis

The STRING protein network database (http://string-db.org/), a web resource for protein-protein physical and functional interactions[25], was used to compute the protein-protein interaction (PPI) network between AR and its related factors as previously reported[26]. We selected protein by name from the menu bar, entered the protein name AR, and selected the organism Homo sapiens. The network provides a summary of all the evidence channels, including based on a certified database,

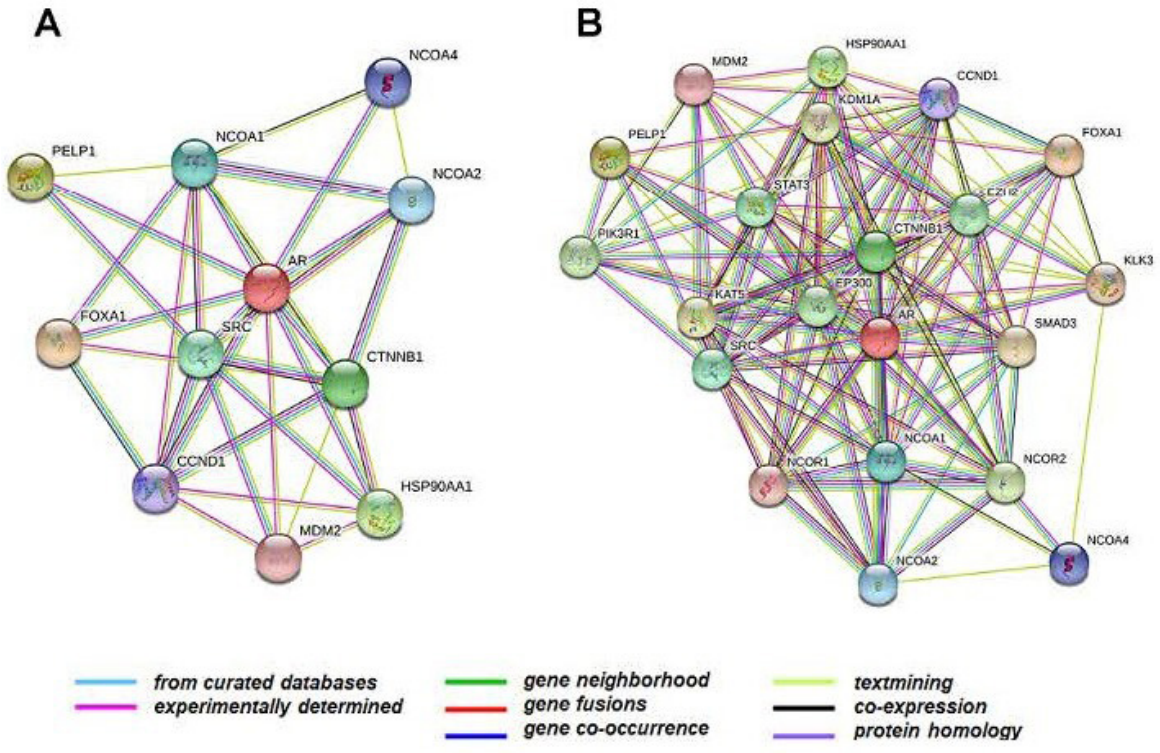


Figure 1. First shell AR-interactors identification based on the STRING database. **(A)** First shell AR-interactors limited to 10 with a medium confidence (score > 0.400). **(B)** AR-interactors limited to 20 with medium confidence (score > 0.400).

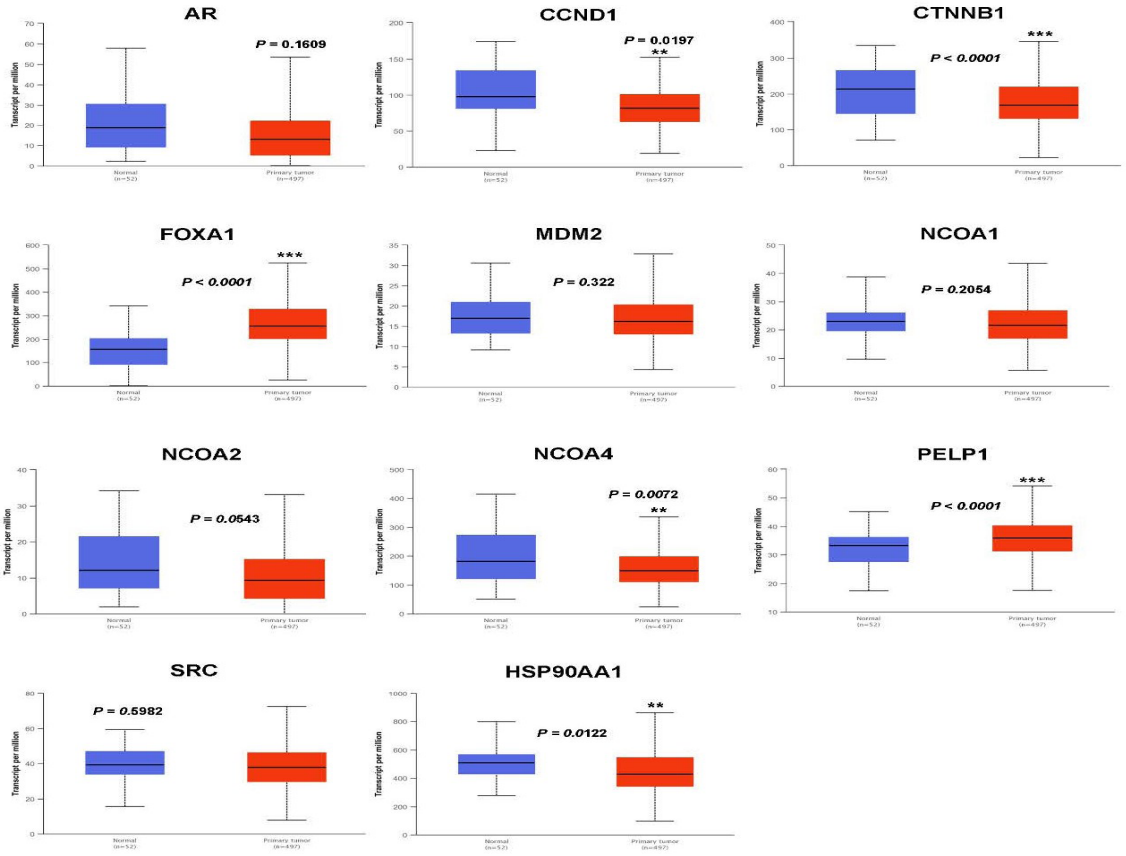


Figure 2. Expression profiles of AR-interactors in prostate cancer by UALACN. Graphs showing expression level of AR and 10 first shell AR-interactors using UALCAN web-portal in normal prostate and primary tumors. Red boxplot depicts the expression level of quired gene in primary tumors, while blue boxplot indicate expression in normal samples. **, $P < 0.05$; ***, $P < 0.0001$.

experimental verification, gene proximity, co-expression, homology speculation and text mining, to create the link between the protein nodes. A brief description of the colored lines and nodes is provided, to determine the relationship between AR and its related factors. The confidence score was set to medium (score > 0.400). A list with details of 10 proteins identified as first shell interactors of AR using the STRING database is shown in **Table 1**.

UALCAN analysis

UALCAN web-portal (http://ualcan.path.uab.edu), an integrated cancer data analysis platform based on the data of The Caner Genome Atlas (TCGA)[27], was used to evaluate the expression of AR and its interactors in prostate cancer according to the online instructions. The symbols of target genes were typed into the scan box and the TCGA dataset (Prostate adenocarcinoma) was chosen, and mRNA expression, survival (with tumors categorized into low and high expression groups), correlation, and the pan-cancer view was explored.

cBioPortal analysis

The cBioPortal (http://cbioportal.org) for Cancer Genomics is a publicly available platform for exploring, visualizing, and analyzing multidimensional tumor genomics and clinical data. Using the cBioPortal database, we created a virtual study using a combination of data provided by 16 prostate cancer studies (Supplementary Table S1), which included 6270 samples of 5981 patients (accessed on 14th March 2022). The genetic alterations of AR and identified AR-interactors were evaluated as described previously[28]. The overall survival (OS) and disease-free survival (DFS) differences between the altered group and the unaltered group of the query genes were simulated, and the results were displayed as Kaplan-Meier plots with *P* values from a logrank test[29].

GO enrichment and KEEG pathways analysis

Gene-ontology (GO) functional enrichment analysis was performed to investigate the biological significance of AR-interactors, which includes biological processes (BP), cellular components (CC) and molecular functions (MF). The enrichment factor is presented as log10(observed/expected). The Benjamini-Hochberg (BH) adjustment was applied for multiple associations, and $P < 0.05$ was recognized as significant. Kyoto Encyclopedia of Genes and Genomes (KEEG) enrichments were analyzed to determine the most enriched signalling pathway of AR-interactors involved in.

Statistical Analysis

In survival data, the median was estimated based on the Kaplan-Meier estimate of the survivor function. The statistical analysis was performed in the software of SPSS Statistics 25.0. $P < 0.05$ was recognized statistically significant.

RESULTS

First shell interactors of AR identified by the

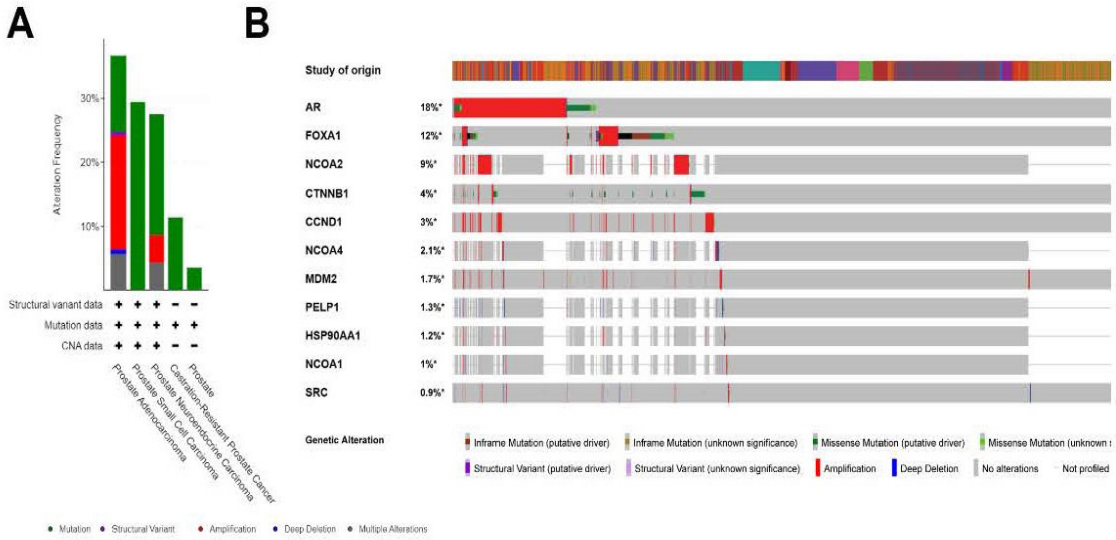


Figure 3. The integrated alteration distribution and The OncoPrint tabs of AR-interactors. **(A)** Alterations distribution was based on cancer types. Histogram indicates the alteration frequencies of the AR-interactors according to cancer types. **(B)** The OncoPrint tab summarizes genomic alterations of the quired genes across a set of prostatic samples. Each row represents a gene, and each column represents a tumor sample. Red bars indicate gene amplifications, blue bars are homozygous deletions, and green squares are nonsynonymous mutations.

STRING database

The STRING database (http://string-db.org/) was used to simulate the protein interaction network between AR and other interactors. Limiting the number of confirmed/predicted first shell interactors to a maximum of 10. Confidence interaction score was set to medium (score > 0.400). Results showed that an eleven proteins network includes AR, CCND1 (Cyclin D1), CTNNB1(Catenin Beta 1, also known as β-Catenin), FOXA1, HSP90AA1 (Heat shock protein 90 alpha family class a member 1), MDM2 (Mouse Double Minute 2, human homolog of p53-binding protein), NCOA1 (Nuclear Receptor Coactivator 1), NCOA2 (Nuclear Receptor Coactivator 2), NCOA4 (Nuclear Receptor Coactivator 4), PELP1 (Proline, glutamate and leucine rich protein 1) and SRC (Steroid receptor coactivator) was computed using the STRING interaction database (**Figure 1A**). The number of interaction network nodes is 11, the number of edges is 33, the average node degree is 6, the average local clustering coefficient is 0.815, the expected number of edges is 21, and the PPI enrichment *P* value is 0.00943. In addition, a PPI network with number of first shell interactors limited to 20 was simulated with medium confidence (score > 0.400) and shown in Figure 1B. Ten additional AR interactors were added to the network, including SMAD3 (SMAD family member 3), EP300, KLK3 (Kallikrein related peptidase 3), STAT3 (Signal transducer and activator of transcription 3), PIK3R1 (Phosphoinositide-3-kinase regulatory subunit 1), KAT5 (Lysine Acetyltransferase 5), NCOR1 (Nuclear Receptor Corepressor 1), NCOR2 (Nuclear Receptor Corepressor 2), KDM1A (Lysine Demethylase 1A) and EZH2 (Enhancer Of Zeste 2). (Supplementary Table S2). These data indicated that AR interaction network is complicated and exerted a complex with little is known in prostate cancer development.

Expression profile of AR and its interactors in prostate cancer

The mRNA expression levels of AR and its 10 first shell interactors were analyzed using UALCAN web-portal (http://ualcan.path.uab.edu) in prostate adenocarcinoma. Among these 11 proteins, we found that FOXA1 and PELP1 were significantly up-regulated in prostate cancers compared to normal prostate tissues ($P < 0.001$, < 0.001). On the contrary, CCND1, CTNNB1, NCOA4 and HSP90A were significantly down regulated in prostate cancer ($P = 0.0197$, < 0.001, 0.0072, 0.0122). The expression levels of AR, MDM2, NCOA1, NCOA2 and SRC exhibited no significant changes ($P = 0.322$, 0.2054, 0.0543, 0.5982). (**Figure 2 and Supplementary Table S3**).

Overview of AR-interactor alterations in prostate cancer

Sixteen prostate cancer datasets, which including 5981 patients / 6270 samples, from the cBioPortal database were involved. The parameters of genomic profiles, mutations and DNA copy number alterations (CNAs) were specified by default.

Our results showed a visual summary of alterations across 16 prostate cancer datasets based on the query of AR and its interactors (AR, CCND1, CTNNB1, FOXA1, HSP90AA1, MDM2, NCOA1, NCOA2, NCOA4, PELP1 and SRC). The required genes were altered in 34% (2139/6270) samples, including 17.86% amplification and 11.97% mutation in prostate carcinoma. Mutation is the main alteration type in prostate small cell carcinoma (29.41%, 5/17), neuroendocrine prostate carcinoma (NEPC, 18.84%, 13/69) and castration-resistant prostate cancer (CRPC, 11.43%, 8/70) as showed in Figure 3A. The details of the genomic alterations of AR and its interactors across the prostate cancer samples were summarized in the OncoPrint tab (Figure 3B). For AR, the frequency of alteration is 18%, mainly including gene amplification and mutation. The altera-

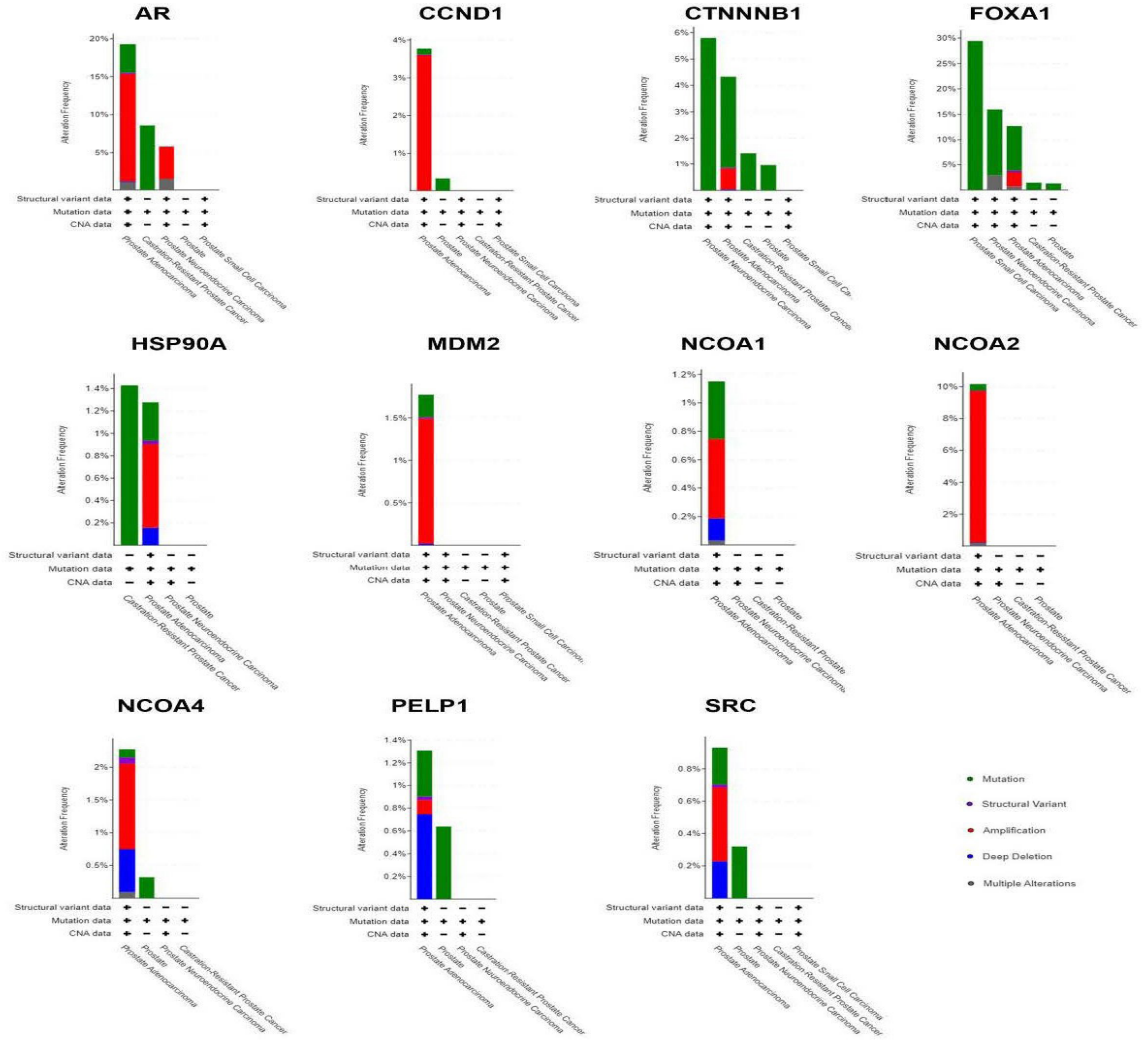


Figure 4. Alteration distribution of AR-interactors. Histogram indicates the alteration frequencies of the queried gene according to cancer types. Red bars indicate gene amplifications, blue bars are homozygous deletions, and green squares are nonsynonymous mutations.

tion rate of FOXA1 was 12%, and the main variation type was gene amplification. The alteration frequency of SRC was 0.9%, which relatively lower than other AR-interactors.

Alteration landscape of AR-interactors in prostate cancer

The alterations of all ten AR-interactors are shown in **Figure 4**. It's well known that AR acts as an important driver in castration resistance, and AR amplification and mutation are critical mechanisms that contribute to the progression[30,31]. Here, we found that the amplification of AR occurred in 14.22% prostate cancers, remarkably, 8.57% CRPC samples have AR mutation. The most common alteration type of CNND1, MDM2 and NCOA2 was amplification, accounting for 3.6%, 1.47% and 9.52% respectively. NCOA2, also known as SRC-2, was more frequently amplified or upregulated in patients with metastatic PCa, facilitating the development of CRPC[32]. Interestingly, the androgen deprivation treatment could also induce NCOA2 expression, which in turn activated PI3K signaling and promoted PCa metastasis and castration resistant progression[32]. These findings indicated that the amplification of AR-interactors including NCOA2, MDM2 and CNND1, might be highly correlated with tumor progression and act as critical regulators in CRPC. In the present study, we found that mutation was most frequently observed in CTNNB1, FOXA1 and HSP90A in all types of prostate cancer. Notably, the FOXA1 gene was mutated in 29.41% prostate small cell carcinomas. FOXA1, a known direct interacting AR cofactor, with high frequency mutations in coding and noncoding sequences leading to functional alterations, was recognized as drivers in prostate cancer progression[11,31]. CTNNB1 is an important co-activator downstream of the oncogenic Wnt signalling pathway. Therefore, mutations in the CTNNB1 gene have been implicated in oncogenesis of many cancers[33]. However, the high incidence mutations of CTNNB1 related functional alterations in PCa were not quite clear.

Prognostic impact of AR-interactor alterations in prostate cancer

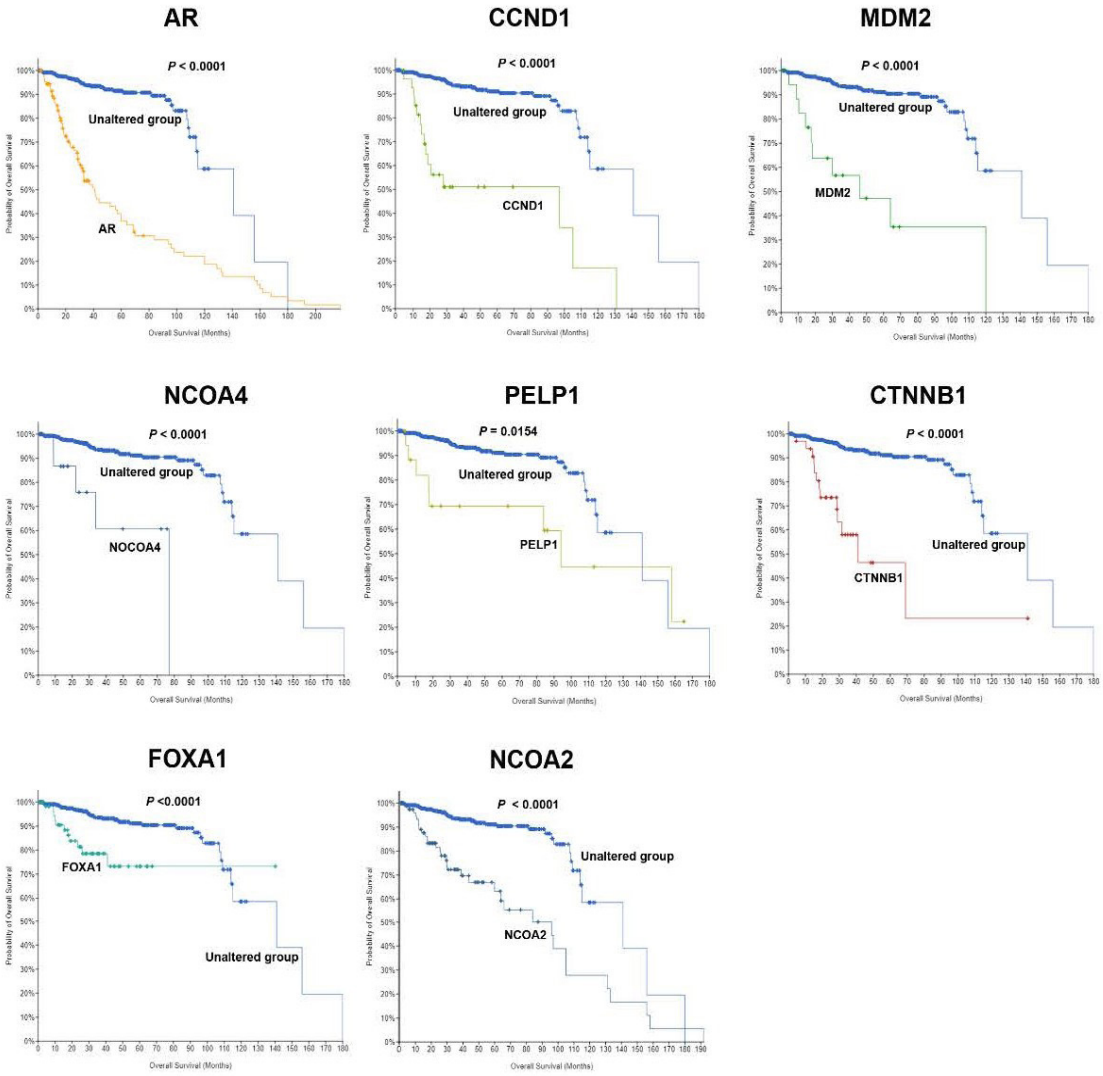


Figure 5. Prognostic impact of AR-interactor alterations in prostate cancer. The impacts of AR-interactors (HSP90AA1, NCOA1 and SRC) on OS with no-significance ($P > 0.05$) are not showed. 95% CI, 95% confidence interval.

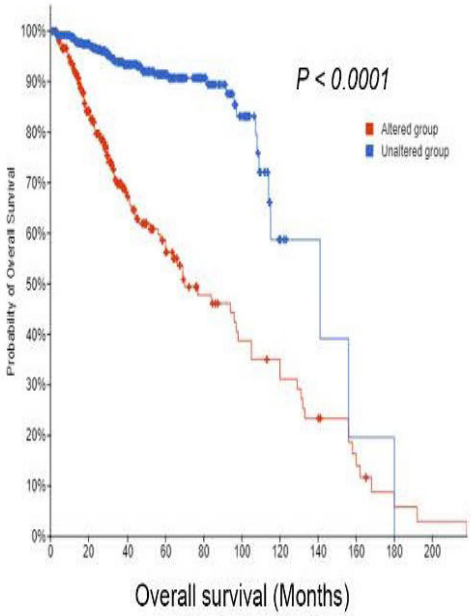
Prognostic impact of alterations of individual AR-interactor was investigated by cBioPortal platform. The results were displayed as Kaplan-Meier plots with P values from a logrank test. Results showed that among the 10 first shell AR interactors, 6 of them (CCND1, MDM2, NCOA4, NCOA2, PELP1, CTNNB1 and FOXA1) were significantly associated with inferior overall survival (OS) as shown in **Figure 5**.

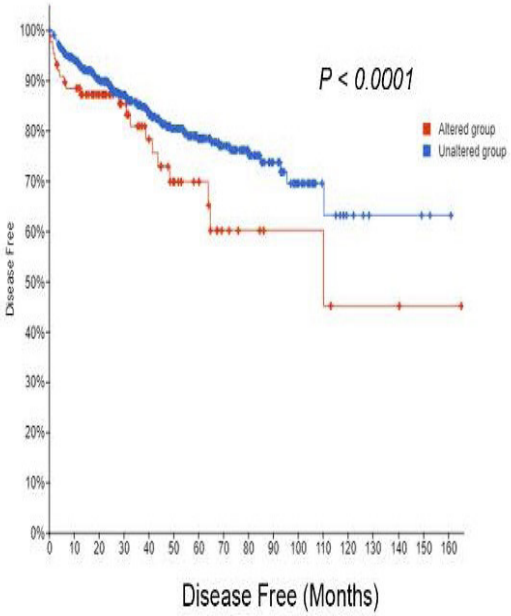
A previous study reported that NCOA2 was altered in 13% of the cohort, which is associated with poor outcomes in metastatic castration-resistant prostate cancer (mCRPC)[34]. In addition, the other four AR-interactors (HSP90AA1, NCOA1, and SRC) with alterations have no significant impacts on the prognosis of prostate cancer (data not shown). Importantly, we found that patients with dual AR and AR-interactors alterations had significantly shorter disease-free survival (DFS) and overall survival (OS) on univariable analyses. The median survival time of the altered group (n = 227) was 70 months (95% CI, 60-105M), while that of the unaltered group (n = 545) was 141 months (95% CI, 115.13 -not available (NA), $P < 0.001$). The DFS in the altered group (n = 82) was 110.16 months (95% CI, 64.66 - NA), however, the disease-free survival time in the unaltered group (n = 533) is NA, $P = 0.0552$ (**Figure 6, Supplementary Table S4 and S5**). These findings indicated that AR-interactors altered-grouping might be a stronger predictor of poor prognosis than AR-interactor aberrations alone.

GO enrichment analysis of AR-interactors

We analyzed the corresponding GO terms of the AR-interactors. A total of 168 GO terms were exported. From the overall GO terms distribution, the BP terms indeed are more informative and significant. In detail, 142 were BP GO terms, 5 were CC GO terms and 21 were MF GO terms (**Supplementary Table S6**).

Here, all 5 CC terms and the top 10 enriched BP terms and MF terms were selected and presented according to the strength of the terms (**Figure 7**). Among the 10 selected BP terms, “Cellular response to thyroglobulin triiodothyronine (GO:1904017)” and “Positive regulation of epithelial cell proliferation involved in prostate gland development (GO:0060769)” obtained the highest strength value **(Figure 7A)**.





	Number of Cases, Total	Number of Events	Median Months Overall (95% CI)
Altered group	227	95	70.00 (60.00 - 105.00)
Unaltered group	545	45	141.00 (115.13 - NA)

	Number of Cases, Total	Number of Events	Median Months Disease Free (95% CI)
Altered group	82	22	110.16 (64.66 - NA)
Unaltered group	533	91	NA

Figure 6. The Survival tab of integrated impacts of AR-interactor alterations on prognostics. **(A)** Overall survival. **(B)** Disease free survival.

This indicated that the AR-interactors were mainly involved in prostate cell proliferation, differentiation and prostate gland development. The enriched CC GO terms showed that all the AR-interactors were functional and located in the nucleus (**Figure 7B**). Apart from the BP and CC GO terms, the top 10 enriched MF GO terms mainly includes transcription factor/nuclear receptor binding and signalling receptor binding (Figure 7C), which have been confirmed to be essential for the initiation and development of prostate cancer.

Additionally, we performed KEEG pathway enrichment analysis to determine the predictive signaling pathways of identified AR-interactors. KEGG enrichment showed that the identified AR-interactors were particularly enriched in thyroid cancer, bladder cancer, prostate cancer and thyroid hormone signalling pathway, as well as endocrine resistance (**Figure 7D and Supplementary Table S7**). These results indicated that the first shell AR-interactors were located in the nucleus and widely involved in prostate cancer development through transcriptional regulation.

DISCUSSION

AR signalling pathway is recognized as a driver of prostate cancer initiation, progression and recurrence, hence, androgen-deprivation therapy directed toward a reduction in serum androgens and the inhibition of AR is generally the first-line therapy adopted[5]. However, multi-patients will experience cancer progression to CRPC, which is currently incurable and with a worse outcome[35]. Although several therapeutic agents have been approved and applied in clinical treatment for CRPC, an urgent need for rational biomarkers and treatment strategies to improve survival is indeed.

Mounts of studies have focused on and evidenced that continuously active of AR signaling pathway through mutations, splice variants, and aberrant coregulation critically contributes to tumor progression[36,37]. Few studies attended the aberrations of AR-interactor gene group and its prognostic significance in the PCa initiation and progression. Here, we comprehensively evaluated the aberrations of AR-interactors from the existing database by bioinformatics method, and provided new insights for the study of prostate cancer progression

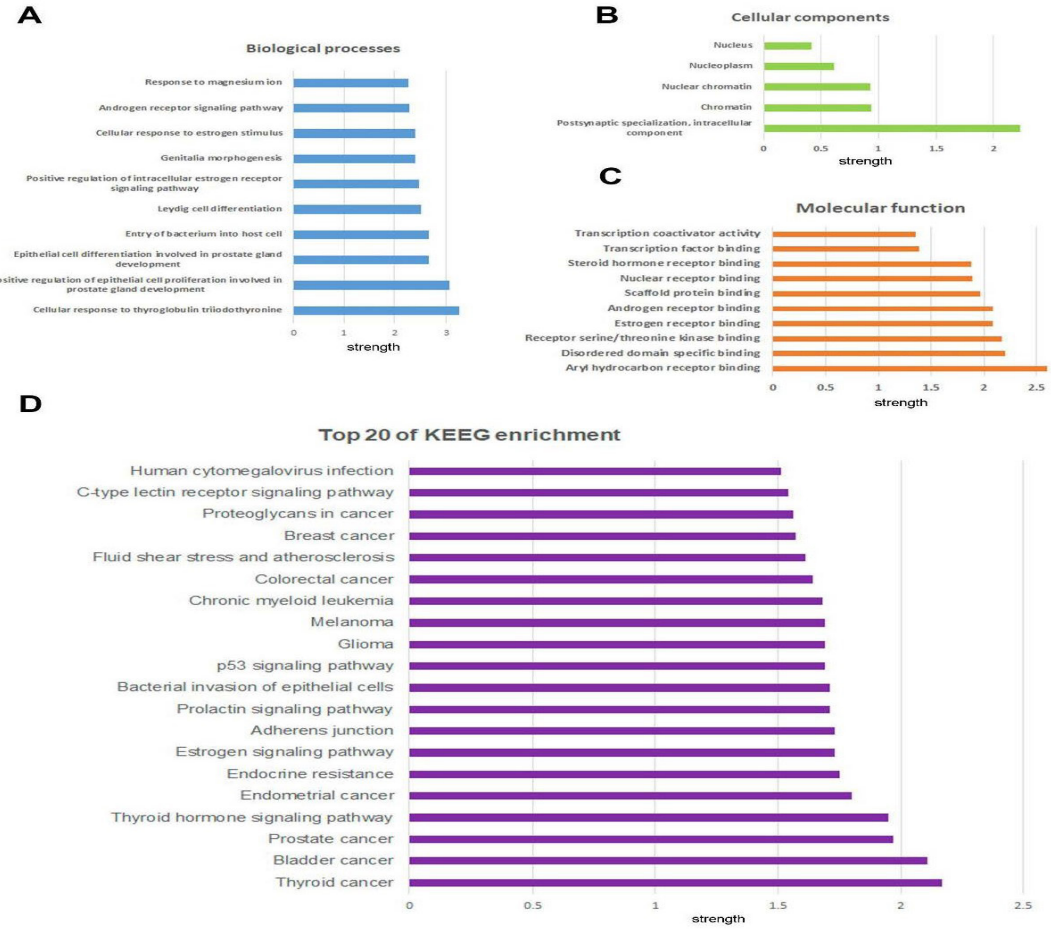


Figure 7. Functional enrichment analysis of AR-interactors. **(A)** BP GO terms. **(B)** CC GO terms. **(C)** MF GO terms. **(D)** Top 20 of KEEG pathways enrichment analysis.

mechanism and prognosis evaluation.

A 11-protein interaction network was computed, which included AR, CCND1, CTNNB1, FOXA1, HSP90AA1, MDM2, NCOA1, NCOA2, NCOA4, PELP1 and SRC. Of which FOXA1 and PELP1 were significantly up-regulated in prostate cancers. On the contrary, CCND1, CTNNB1, NCOA4 and HSP90AA1 were significantly down-regulated in cancer tissues as compared to normal control. FOXA1 (Forkhead box A1) also known as hepatocyte nuclear factor 3-alpha, is an important pioneer factor that directly interacts with AR to drive the growth and survival of prostatic cells[11]. The increased expression of FOXA1 could suppress STAT2 DNA-binding activity and interferon (IFN) signaling gene expression, promote cancer immune- and chemotherapy resistance in prostate cancer[38]. PELP1 is a scaffolding protein that acts as a coregulator of several nuclear hormone receptors and sequence-specific transcription factors, which is up-regulated in several cancers and plays essential roles in hormonal signalling, cell cycle progression and therapy resistance [39]. Particularly, PELP1 could facilitate 17-estradiol (E2)-induced activation of AR signalling by forming a protein complex with AR, providing cancer cells with a distinct growth and survival advantage in the absence of androgen[39,40]. These findings indicated that the co-expression of AR, FOXA1 and PELP1 might have critical significance in prostate cancer initiation and development.

The CCND1 gene could directly bind to the N-terminus of AR and function as a co-suppressor to inhibit ligand-dependent AR activation[41]. However, contradictory data have been reported that CCND1 could enhance DHT-induced prostate cancer cellular proliferation and facilitate the resistance of prostate cancer cells to DNA damage therapies[42,43]. Here, we demonstrated that CCND1 was significantly down-regulated in primary prostate cancers, and the patients with alteration of CCND1 have poor overall survival. Further investigations of the mechanism of CCND1 in prostate cancer development is needed. CTNNB1(β-catenin) is an intracellular scaffold protein that interacts with adhesion molecules, transmembrane-type mucins, signaling regulators and epigenetic or transcriptional regulators and plays a crucial role in cell adhesion, proliferation and multiple cellular signalling pathways[44]. Increasing evidence indicated that WNT/CTNNB1 signalling is an important AR-independent pathway in contributing to prostate cancer progression and acquisition of resistance[45-47]. Here, we found that CTNNB1 was significantly down-regulated in primary prostate cancers, and the main alteration type was a mutation, which might play a critical role in prostate cancer progression. However, the mechanism remains unclear. NCOA4 (nuclear receptor coactivator 4), was also known as androgen receptor-associated protein 70 (ARA70), which could enhance the androgen receptor transcriptional activity by binding to the N-terminus of AR in prostate cancer

cells[48]. HSP90AA1 (HSP90α) interacts and supports numerous oncoproteins that promote oncogenesis, including AR functional maturation and stability. Co-targeting HSP90 and AR strategy can achieve a better blockade of androgen signalling than targeting AR or HSP90 alone to enhance prostate cancer cell death[49]. However, in this study, we found that NCOA4 and HSP90AA1 were down-regulated in prostate cancers, with the mechanism unknown.

This study systematically analyzed the expression, alteration and interaction of AR and its first shell interactors in prostate cancer, as well as the effects on the prognosis. Among the 10 first shell AR interactors, 6 of them (CCND1, MDM2, NCOA4, PELP1, CTNNB1 and FOXA1) were negatively associated with the overall survival time. Remarkably, there was a significant difference of the overall survival and disease-free survival between the altered group and unaltered group, which suggested that the AR-interactor group might be a useful prognostic indicator for prostate cancer.

We found that the AR-interactors were related to 168 GO terms, most of which were associated with prostate cell proliferation, differentiation and prostate gland development, as well as transcriptional binding process, providing new sights for further study of the molecular mechanism of CRPC progression.

CONCLUSIONS

Taking together, the present study comprehensively analyzed the AR and its first shell interactors expression and alterations in prostate cancer. The AR-interactor gene set could provide new sights for prostate cancer treatment and might be a useful marker for the prognosis of prostate cancer. Further investigations are needed to better understand the significance of AR-interactors in prostate cancer initiation, progression and therapeutic resistance. However, despite the comprehensive analyses of AR and AR interactors, we acknowledge several limitations of our study. The expression and alteration data were obtained and analyzed from different database portals, which may limit the confidence and generalizability of our findings.

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CONFLICT OF INTEREST

The authors declare seriously that external funding sources and interest conflicts did not exist in the study.

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