Asian Journal of Andrology (2021) 23, 109–115
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ORIGINAL ARTICLE

Prostate Cancer

Clinical significance of EPHX2 deregulation in prostate cancer

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The arachidonic acid (AA) metabolic pathway participates in various physiological processes as well as in the development of malignancies. We analyzed genomic alterations in AA metabolic enzymes in the Cancer Genome Atlas (TCGA) prostate cancer (PCa) dataset and found that the gene encoding soluble epoxide hydrolase (*EPHX2*) is frequently deleted in PCa. *EPHX2* mRNA and protein expression in PCa was examined in multiple datasets by differential gene expression analysis and in a tissue microarray by immunohistochemistry. The expression data were analyzed in conjunction with clinicopathological variables. Both the mRNA and protein expression levels of *EPHX2* were significantly decreased in tumors compared with normal prostate tissues and were inversely correlated with the Gleason grade and disease-free survival time. Furthermore, *EPHX2* mRNA expression was significantly decreased in metastatic and recurrent PCa compared with localized and primary PCa, respectively. In addition, EPHX2 protein expression correlated negatively with Ki67 expression. In conclusion, *EPHX2* deregulation is significantly correlated with the clinical characteristics of PCa progression and may serve as a prognostic marker for PCa.

Asian Journal of Andrology (2021) **23**, 109–115; doi: 10.4103/aja.aja_34_20; published online: 17 July 2020

Keywords: arachidonic acid metabolism; metastasis; prognosis; prostate cancer; soluble epoxide hydrolase

INTRODUCTION

Prostate cancer (PCa) is one of the most frequently diagnosed non-cutaneous cancers among men and the fifth leading cause of cancer-related mortality among men worldwide.[1] The survival rate is higher when the cancer is diagnosed at a localized stage but decreases substantially when diagnosed at a metastatic stage. Although the prostate-specific antigen (PSA) testing has been used widely in the clinic and has led to early diagnosis of PCa,[2] its utility in reducing PCa mortality remains controversial.[3,4] In addition, PSA screening often leads to overdiagnosis due to the lack of a clear cutoff point for high sensitivity and specificity.[5] PCa growth is initially dependent on circulating androgens, and hormonal therapies aimed at androgen deprivation result in regression. However, in most cases, invariable relapse to the more aggressive castration-resistant PCa (CRPC), which lacks curative treatment options, occurs. Despite recent advances, the molecular mechanisms involved in PCa development and progression to CRPC are not well understood. Thus, there is a critical need to identify additional screening, prognostic, and disease stratification biomarkers for PCa.[6]

Arachidonic acid (AA) is a common dietary fatty acid stored in cell membranes, where it is liberated by phospholipase A2 (PLA2) in response to extracellular stimuli. Subsequently, AA is converted by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450) epoxygenase pathways to produce biologically active eicosanoids, including prostaglandins (PGs) and hydroxyeicosatetraenoic acids (HETEs). The AA pathway is an important metabolic pathway that plays a key role in normal physiological functions. Dysregulation of the AA metabolic pathway, especially the LOX and COX branches, is implicated in the development and progression of numerous malignancies, including PCa.[7,8] For instance, PLA2 group IIA (*PLA2G2A*) expression and the AA turnover rate were found to be increased in tumor tissue compared to those in normal prostate tissue;[9,10] in addition, altered expression of COX2 and LOX-5 has been found to be associated with PCa progression.[11–14]

Little is known about the implication of the CYP450 branch, which converts AA into epoxyeicosatrienoic acids (EETs), in PCa. EETs are important lipid mediators that actively regulate physiological processes such as proliferation, migration, and inflammation in several tissues. EETs have been shown to exert protumorigenic effects through multiple mechanisms, including activation of G-protein coupled receptor 40 (GPCR40), epidermal growth factor receptor (EGFR), and vascular endothelial growth factor (VEGF) signaling.[15–20] In most tissues, EETs are quickly metabolized to inactive or less-active dihydroxyeicosatrienoic acids (DiHETEs) by soluble epoxide hydrolase (sEH), a bifunctional enzyme encoded by the epoxide hydrolase 2 (*EPHX2*) gene.[21,22] Inhibition of EPHX2 resulted in accumulation of EETs, which in turn promoted tumor growth and metastasis in animal models.[16] In addition, *EPHX2* expression was found to be downregulated in hepatic cancer but upregulated in seminoma,

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Received: 13 November 2019; Accepted: 28 April 2020

cholangiocarcinoma, and advanced ovarian cancer.[23] A recent study showed that *EPHX2* was expressed at similar levels in both clinical PCa samples and normal prostate samples.[24] In the present study, we showed that *EPHX2* gene deletion frequently occurs in PCa and that downregulation of *EPHX2* expression is significantly correlated with disease progression in PCa.

MATERIALS AND METHODS

Gene expression datasets

Copy number alteration (CNA) analysis of AA pathway genes was performed using four primary PCa datasets from the Cancer Genome Atlas (TCGA) cohort (n = 499),[25] Broad/Cornell cohort (n = 112),[26] Fred Hutchinson Cancer Research Center (CRC) cohort (n = 126),[27] and Memorial Sloan Kettering Cancer Center (MSKCC) cohort (n = 240).[28] The metastatic PCa datasets used in this analysis included the Stand Up To Cancer/Prostate Cancer Foundation (SU2C/PCF) dataset (n = 444),[29] the Metastatic Prostate Adenocarcinoma (MCTP) cohort (n = 61),[30] and the Metastatic Prostate Cancer Project (MCP) cohort (n = 75). All source data were downloaded from the cBio Cancer Genomics Portal (http://cbioportal.org).[31] Differential expression of *EPHX2* in normal prostate and PCa samples was evaluated in multiple PCa gene expression datasets, including the TCGA cohort,[25] Taylor cohort (GSE21032),[28] Grasso cohort (GSE35988),[30] Glinsky cohort,[32] and Ross-Adams cohort (GSE70768).[33]

Specimen preparation and immunohistochemistry

Two cohorts of PCa specimens were used in this study. A prostate tissue microarray (TMA) of cohort 1 purchased from Shanghai Outdo Biotech (Shanghai, China) was used to determine the expression of EPHX2 in normal and PCa tissues, as well as its correlation with the Gleason grade. This TMA contained paired normal and malignant prostate tissues from 86 PCa patients. Cohort 2 comprised 12 patients diagnosed with advanced PCa, of which 6 were diagnosed with metastasis. The patients did not undergo chemotherapy or radiotherapy before surgery. All procedures performed involving human participants were in accordance with the ethical standards of the medical ethics committee of the Qujing First Hospital and the Qujing Affiliated Hospital of Kunming Medical University (Qujing, China; Approval No. 201908001). Informed consent was obtained from all patients. All tissue sections were first deparaffinized with xylene and serial ethanol dilutions and were then subjected to heat-induced antigen retrieval using 0.01 mol l^{-1} citrate buffer (pH 6.4). An affinity-purified mouse monoclonal antibody specific for EPHX2 (#sc-166961, Santa Cruz Biotechnology, Dallas, TX, USA) was used at a dilution of 1:500 for 1 h at room temperature. A Supersensitive Detection kit (Biogenex, San Ramon, CA, USA) was used for antigen detection as described previously. Proliferation marker protein Ki67 immunostaining was performed on tissue sections from cohort 2 to assess the correlation between EPHX2 expression and tumor proliferation. Values on a four-point scale were assigned to each immunostained sample to score EPHX2 expression: 0 – no apparent staining; 1 – weak staining; 2 – moderate staining in the majority of cells; and 3 – strong staining in the majority of cells. Images were acquired using a Motic BA600Mot microscopy system (CANY, Shanghai, China).

Cell culture

The human PCa cell lines LNCaP, VCaP, 22Rv1, C4-2B, PC3, and DU145 were purchased from the American Type Culture Collection (Rockville, MD, USA). Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Lonza (Basel, Switzerland). Cells were routinely maintained in a humidified incubator with 5% (v/v) CO_2 and 95% (v/v) air at 37°C in RPMI 1640 medium (LNCaP, C4-2B, 22Rv1, and PC3 cells) or DMEM (VCaP and DU145 cells) containing 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 5 mg ml^{-1} penicillin/streptomycin, and 2 mmol l^{-1} L-glutamine (Lonza). For hormone responsiveness experiments, cells were plated in complete medium containing 10% (v/v) FBS and were then preincubated in medium containing 5% (v/v) charcoal-treated (CT)-FBS for 2 days before induction with the synthetic androgen R1881 (1 nmol l^{-1}; Merck, Kenilworth, NJ, USA) for the indicated time periods. All cell lines were routinely tested and found to be negative for mycoplasma contamination.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions, and 1 μg of RNA was used for cDNA synthesis using Superscript IV (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was used for quantitative polymerase chain reaction (qPCR) analysis using SYBR Green Real-Time PCR master mix (Roche Life Science, Penzberg, Germany) in a LightCycler 480 (Roche Life Science). A standard curve constructed from measurements of serially diluted cDNA was used to calculate the relative amounts of the different cDNAs in each sample. PCR amplification was performed as follows: initial 5 min denaturation step at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 45 s. Melt curve analysis was included in each run. The values were normalized to the relative amounts of the internal standard TATA-box binding protein (*TBP*). All experiments were conducted in triplicate and repeated three times with consistent results.

RNA interference

Small interfering RNA (siRNA) targeting the androgen receptor (AR) and Allstar Negative Control siRNA were purchased from Qiagen (Hilden, Germany). The transfection reagent Lipofectamine RNAiMAX was purchased from Thermo Fisher Scientific and used at a 1:1 ratio with 5 nmol l^{-1} of the appropriate siRNA according to the manufacturer's protocol. Cells were incubated in transfection medium for 48 h. For the generation of stable-knockdown cells, lentiviral pLKO.1 short hairpin RNA (shRNA) vectors targeting human *EPHX2* and a nonsilencing control vector were purchased from Sigma-Aldrich. Lentivirus particles were produced in 293T cells as described previously.[34] C4-2B cells were transduced with the lentiviral particles and were then subjected to puromycin (Invivogen, San Diego, CA, USA) selection (1 μg ml^{-1}) for 7 days. Cells stably expressing shRNA were pooled and maintained in puromycin (0.2 μg ml^{-1}).

Western blotting

Cells were washed once in ice-cold phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (0.1% [w/v] sodium dodecyl sulfate [SDS], 1% [v/v] NP-40, 0.5% [w/v] sodium deoxycholate, 50 mmol l^{-1} Tris-HCl [pH 8.8], and 150 mmol l^{-1} NaCl), and cell lysates were boiled at 95°C for 5min. Protein samples were then resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The blotted membrane was blocked in 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween (TBS-Tween) for 1h and was then incubated with the primary antibody in TBS-Tween containing 5% (w/v) bovine serum albumin (BSA) for 14–16 h at 4°C. The primary antibodies against EPHX2 (1:1000, Santa Cruz Biotechnology), β-actin (1:3000, Sigma-Aldrich), AR (1:1000, Cell Signaling Technology, Danvers, MA, USA), and PSA (1:1000, Santa Cruz Biotechnology) were diluted in 5% BSA, and the

solutions were added to the membrane and incubated overnight at 4°C with constant agitation. An ECL Western blot analysis system (Bio-Rad) was utilized to detect immunoreactive bands according to the manufacturer's instructions.

Statistical analyses

Kaplan–Meier survival analysis was used to analyze the association of *EPHX2* expression with clinical outcomes in PCa cohorts, and the log-rank test was used for significance analysis. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Student's *t*-test was used to compare EPHX2 expression between benign tissue and malignant tissue. $P < 0.05$ was considered statistically significant.

RESULTS

The EPHX2 gene is frequently deleted in PCa

The AA metabolic pathway has three major downstream branches involving several key enzymes. To assess whether this pathway is deregulated during PCa progression, we first used the cBio Cancer Genomics Portal to examine the genetic alterations in 12 key enzymes of the three branches in the TCGA PCa cohort, which comprised 499 primary tumor specimens. The frequency of CNA caused by homozygous deletion was rather low for most of the genes (0%–3%), except for *EPHX2* (14.2%). Collective analysis of multiple PCa datasets indicated that the CNA frequency of the *EPHX2* gene was significantly higher than that of the other AA pathway genes ($P < 0.05$, *t*-test; **Figure 1a**). Pan-cancer CNA analysis showed that genetic alteration of *EPHX2* occurred more frequently in PCa than that in other types of cancer (data not shown). Next, we compared the CNA frequency of *EPHX2* in primary PCa and metastatic PCa. As shown in **Figure 1b**, the CNA frequency of *EPHX2* was comparable between these two stages of PCa. These results indicated that the gene deletion event might occur at the initiation stage of the disease. Consistent with this finding, the CNA frequency of *EPHX2* was not associated with the Gleason pattern of PCa (**Figure 1c**).

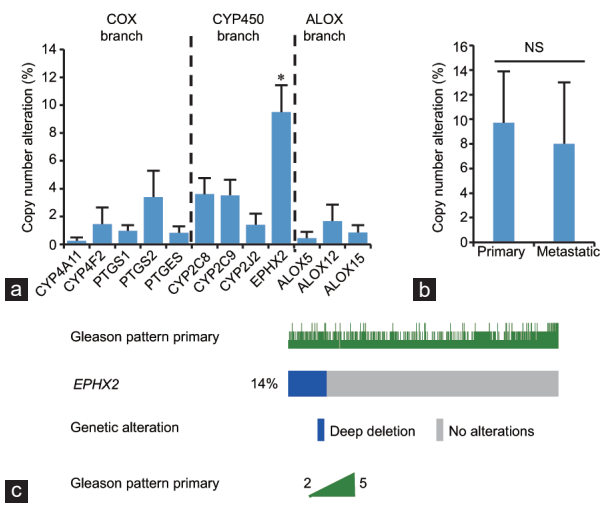


Figure 1: CNA of *EPHX2* occurs frequently in PCa. (**a**) CNA frequency of key genes in the AA metabolic pathway was assessed in the four independent PCa datasets publicly available at the cBioPortal for Cancer Genomics. The *EPHX2* deletion rate was significantly higher than that of the other genes examined. The error bars indicate the standard deviations. Statistical significance between *EPHX2* and the rest genes was determined by Student's *t*-test. $^*P < 0.05$. (**b**) The CNA frequency of *EPHX2* was compared between primary and metastatic prostate tumors. Four primary PCa datasets and three metastatic PCa datasets were used in the analysis. The error bars indicate the standard deviations. Statistical significance was determined by Student's *t*-test. NS: no significant difference, $P > 0.05$. (**c**) The association between *EPHX2* deletion and the Gleason pattern of PCa was analyzed using the cBioPortal for Cancer Genomics. PCa: prostate cancer; *EPHX2*: epoxide hydrolase 2; CNA: copy number alteration; AA: arachidonic acid.

EPHX2 expression is decreased in PCa

We compared *EPHX2* mRNA expression in normal prostate tissues and PCa tissues in several publicly available cohorts. As shown in **Figure 2a**, the mRNA level of *EPHX2* in PCa tissues was significantly reduced compared to that in normal prostate tissues ($P < 0.001$, *t*-test). In addition, a lower expression level of *EPHX2* mRNA correlated with a higher Gleason score (**Figure 2b**), indicating the potential implication of *EPHX2* deregulation in PCa progression.

Next, we sought to determine whether the protein expression of EPHX2 is also deregulated during PCa progression. To this end, we conducted immunohistochemical (IHC) analysis of a TMA comprising paired PCa and normal tissues from 86 cases of PCa. The antibody was first validated by Western blotting using a protein extract from PCa C4-2B cells transfected with siRNA targeting EPHX2 or with scrambled siRNA. As shown in **Figure 3a**, the antibody specifically recognized the EPHX2 protein in the PCa cell extract. IHC analysis showed that the EPHX2 protein was strongly expressed in the cytosol of prostate luminal cells (**Figure 3b**, upper panel), consistent with a previous report.[35] In addition, consistent with the mRNA expression profile in PCa, the protein level of EPHX2 was significantly decreased in PCa tissues compared with normal prostate tissues ($P < 0.05$, *t*-test; **Figure 3c**). Analysis of paired tumor and adjacent normal tissues showed that EPHX2 protein expression was downregulated in 56.9% of the cases in this cohort (**Figure 3d**). However, although EPHX2 protein expression was decreased in high-grade PCa compared with lower-grade PCa ($P < 0.05$, *t*-test), no statistically significant association was found between EPHX2 IHC scores and Gleason scores in this PCa cohort (**Figure 3e**).

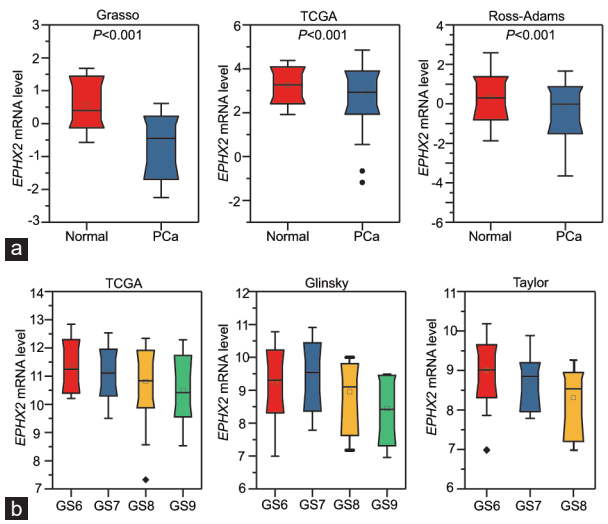


Figure 2: *EPHX2* mRNA expression is associated with PCa progression. (**a**) Boxplots of *EPHX2* expression levels in normal prostate tissue and prostate tumors analyzed in three independent datasets. The thick horizontal lines indicate the medians, the boxes indicate the upper and lower quartiles, and the whiskers indicate the 5th and 95th percentiles. Statistical significance was determined by Student's *t*-test. *P* values are shown. (**b**) The association of *EPHX2* expression with the Gleason grade was analyzed in three independent datasets. The thick horizontal lines indicate the medians, the boxes indicate the upper and lower quartiles, and the whiskers indicate the 5th and 95th percentiles. PCa: prostate cancer; *EPHX2*: epoxide hydrolase 2.

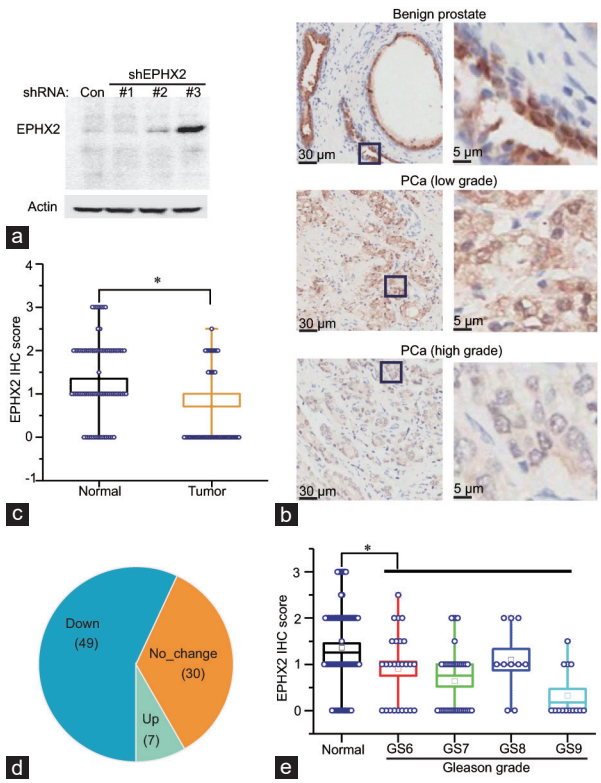


Figure 3: EPHX2 protein expression is decreased in PCa. (**a**) The specificity of the EPHX2 antibody was assessed using Western blot analysis. Whole-cell extracts were prepared from C4-2B cells expressing scrambled shRNA or one of 3 independent shRNAs. Western blot analysis was performed with the indicated antisera. Representative blots for three independent experiments are shown. (**b**) Immunohistochemistry was used to assess EPHX2 expression in normal and malignant human prostate specimens. A tissue microarray (TMA) with paired normal prostate and prostate tumor tissues ($n = 86$) was subjected to IHC analysis. Representative images of normal tissue and for low-grade and high-grade tumors are shown. Images in the right panel are the magnification of the boxed areas in the left panel. Scale bar = 30 μm in left panel and 5 μm in right panel. (**c**) Quantification results of cytosolic EPHX2 staining in the PCa TMA are presented as boxplots. The thick horizontal lines indicate the medians, the boxes indicate the upper and lower quartiles, and the whiskers indicate the 5th and 95th percentiles. Statistical significance between two groups was determined by Student's *t*-test. $^*P < 0.05$. (**d**) Alterations in EPHX2 expression in paired normal and malignant prostate tissues are presented in a pie chart. EPHX2 expression was decreased (Down), unchanged (No_change) and increased (Up) in 48, 30 and 7 cases, respectively. (**e**) Associations of EPHX2 protein expression with the Gleason grade are shown in boxplots. The thick horizontal lines indicate the medians, the boxes indicate the upper and lower quartiles, and the whiskers indicate the 5th and 95th percentiles. Statistical significance between the normal tissue group and each Gleason grade group was determined by Student's *t*-test. $^*P < 0.05$. Con: control; shRNA: short hairpin RNA; IHC: immunohistochemical; PCa: prostate cancer; EPHX2: epoxide hydrolase 2.

EPHX2 deregulation is correlated with the development of advanced PCa and poor prognosis

As the results of the above expression analyses indicate that *EPHX2* downregulation is associated with PCa progression, we assessed whether *EPHX2* expression correlates with PCa metastasis and recurrence. We compared the mRNA levels of *EPHX2* in primary and metastatic tumors from 3 publicly available datasets as described above. As shown in **Figure 4a**, the metastatic PCa tissues expressed lower levels of *EPHX2* mRNA than the primary PCa tissues in all 3 datasets ($P < 0.001$, *t*-test). The difference was significant in the Grasso and Taylor cohorts but not in the Lapointe cohort ($P = 0.31$, *t*-test), probably due to the small number of cases in the Lapointe cohort. A significant reduction in *EPHX2* mRNA expression was also observed in recurred tumors compared with primary tumors ($P < 0.001$, *t*-test; **Figure 4b**). Next, we sought to determine whether EPHX2 protein expression is associated with metastasis. To this end, IHC analysis of EPHX2 was conducted on samples from 12 cases of high-grade PCa, 5 of which were diagnosed with metastasis. EPHX2 staining was negative for 3 of the 5 cases with metastasis but was positive for all metastasis-free cases. These results suggest that downregulation of *EPHX2* at both the mRNA and protein levels is associated with poor outcomes of PCa. Consistent with these findings, lower *EPHX2* mRNA expression predicted worse disease-free survival and biochemical recurrence prognoses for PCa patients in independent cohorts (**Figure 4c** and **4d**). These data showed that the expression level of *EPHX2* negatively correlates with multiple clinical characteristics of advanced PCa and may serve as a potential prognostic biomarker for PCa.

EPHX2 expression is inversely correlated with the Ki67 status

The tumor proliferation index marker Ki67 has been found to be strongly associated with aggressive clinicopathological characteristics.[36,37] To assess the correlation between the expression levels of EPHX2 and Ki67, their protein expression levels were evaluated in consecutive serial tissue sections from 12 cases of high-grade PCa. The expression of EPHX2 was scored on a scale of 0 to 3 based on the intensity of IHC staining, whereas the nuclear Ki67 score was determined as the percentage of positive cells. The protein expression of EPHX2 was inversely correlated with the Ki67 score in the advanced PCa tissues (**Figure 5a**), and Pearson's correlation analysis showed that this inverse correlation was significant ($P < 0.01$, *t*-test; **Figure 5b**). To validate this observation, we assessed the correlation between the mRNA expression levels of *EPHX2* and Ki67 in independent datasets of PCa cohorts. As shown in **Figure 5c**, the Pearson's correlation coefficients indicated a strong inverse correlation in all 5 datasets used in this analysis. The Ki-67 antigen is present in actively proliferating cells but not quiescent cells and is a robust marker for active cell proliferation.[38,39] Therefore, our results suggest that downregulation of *EPHX2* is associated with PCa cell proliferation. Further investigation is required to determine whether deregulation of *EPHX2* contributes to PCa growth.

EPHX2 expression in PCa cells is induced by androgen but is not dependent on AR

EPHX2 mRNA expression has been reported to be significantly correlated with AR expression in PCa tissues.[24] Consistent with this finding, our current study identified EPHX2 expression in four AR-positive PCa cell lines (LNCaP, VCaP, C4-2B, and 22Rv1) but not in two AR-negative PCa cell lines (DU145 and PC3) as shown in **Figure 6a**. These observations suggest the implication of the androgen signaling pathway in the regulation of EPHX2 expression in PCa cells. We thus assessed whether androgens affect EPHX2 expression in PCa cells. *EPHX2* mRNA was significantly increased upon treatment with a synthetic androgen, R1881 ($P < 0.05$, *t*-test; **Figure 6b**). Consistent with this result, Western blot analysis showed that the EPHX2 protein level was increased by androgen treatment (**Figure 6c**). However, the effect of androgen on the EPHX2 protein level appeared to be delayed compared with the effect on the *EPHX2* mRNA level, with only a weak increase in the protein level after 24 h of treatment. To ensure the effect of androgens, PSA was used as a positive control in both the mRNA and protein expression analyses (**Figure 6b** and **6c**). The data indicated that androgens regulate *EPHX2* expression in PCa cells. To determine

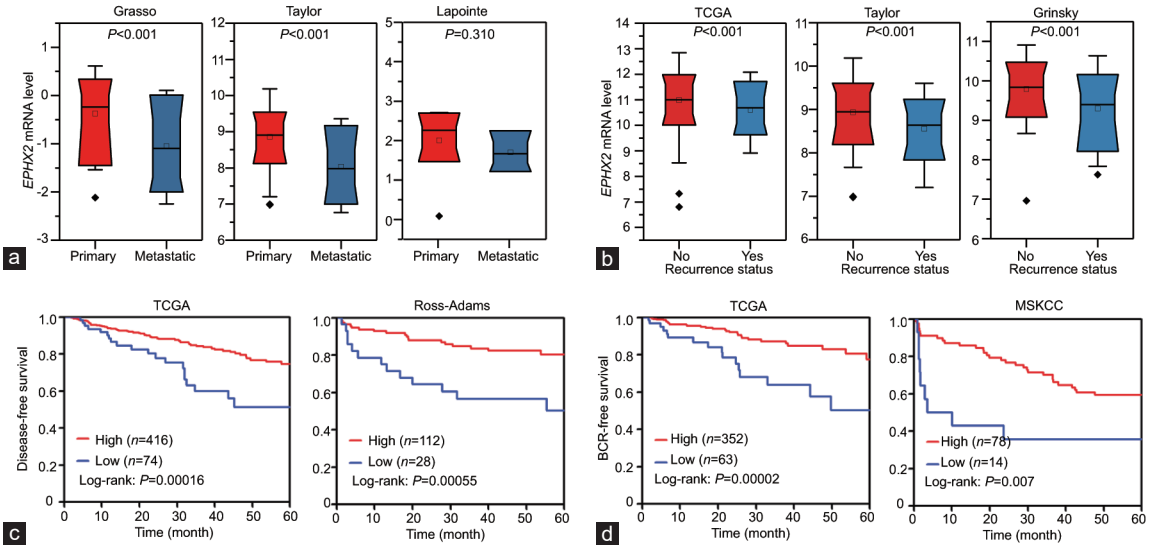
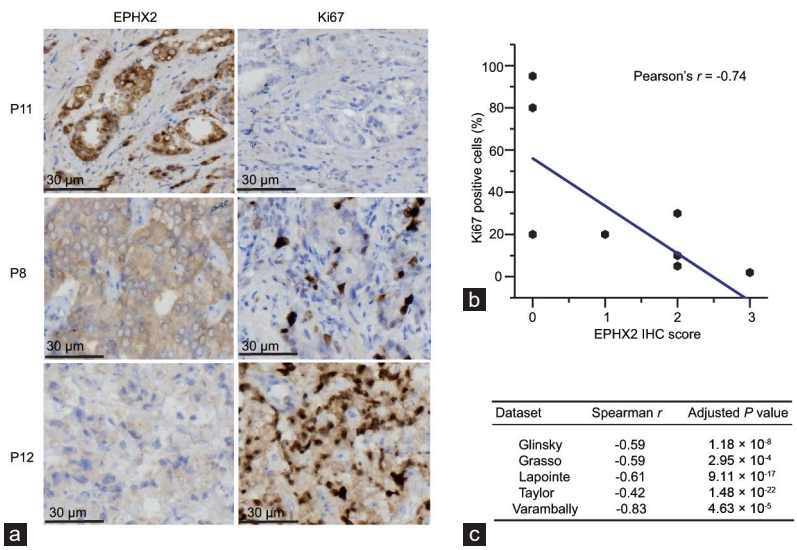


Figure 4: Decreased *EPHX2* expression is associated with metastasis and recurrence of PCa. (**a**) Boxplots of *EPHX2* expression levels in primary localized (Primary) and metastatic (Metastatic) PCa analyzed in 3 independent datasets. The thick horizontal lines indicate the medians, the boxes indicate the upper and lower quartiles, and the whiskers indicate the 5th and 95th percentiles. Statistical significance was determined by Student's *t*-test. *P* values are shown. (**b**) Boxplots of *EPHX2* expression levels in PCa with and without recurrence analyzed in 3 independent datasets. The thick horizontal lines indicate the medians, the boxes indicate the upper and lower quartiles, and the whiskers indicate the 5th and 95th percentiles. Statistical significance was determined by Student's *t*-test. *P* values are shown. (**c**) The Kaplan–Meier plots show a significant association between lower *EPHX2* expression levels (blue line) and shorter disease-free survival times in the Cancer Genome Atlas (TCGA) and Ross-Adams (GSE70768) patient datasets. The log-rank test *P* values are shown. (**d**) The Kaplan–Meier plots show a significant association between lower *EPHX2* expression (blue line) and shorter biochemical recurrence-free (BCR) survival times in the TCGA and the Memorial Sloan Kettering Cancer Center (MSKCC) datasets. The log-rank test *P* values are shown. PCa: prostate cancer; EPHX2: epoxide hydrolase 2.



Dataset	Spearman *r*	Adjusted *P* value
Glinsky	-0.59	1.18×10^{-8}
Grasso	-0.59	2.95×10^{-4}
Lapointe	-0.61	9.11×10^{-17}
Taylor	-0.42	1.48×10^{-22}
Varambally	-0.83	4.63×10^{-5}

c

Figure 5: EPHX2 expression inversely correlates with Ki67 expression in PCa. (**a**) EPHX2 and Ki67 expression levels were assessed by immunohistochemistry (IHC) in consecutive sections from 12 PCa specimens. Representative IHC images of both proteins in the same region are shown. (**b**) The correlation between EPHX2 and Ki67 protein expression in 12 PCa specimens is presented as a dot plot and was evaluated by Pearson's correlation analysis. (**c**) The correlation between *EPHX2* and Ki67 mRNA expression was analyzed in multiple PCa datasets. The Spearman correlation coefficients are presented. PCa: prostate cancer; EPHX2: epoxide hydrolase 2.

whether AR is required for EPHX2 expression in PCa cells, we knocked down AR expression using siRNA in PCa LNCaP and C4-2B cells. Unexpectedly, inhibition of AR expression showed no effect on EPHX2 protein expression in either cell line (**Figure 6d**), indicating that the effect of androgens on EPHX2 expression is independent of AR in PCa cells. The mechanism underlying this observation remains unknown.

DISCUSSION

Although aberrancies in the AA metabolic pathway have been implicated in carcinogenesis,[19] the functional and clinical relevance of this pathway have not been well explored in PCa.[24,40] By analyzing genetic alterations in the key enzymes of the AA pathway, we found that *EPHX2* gene loss frequently occurs in

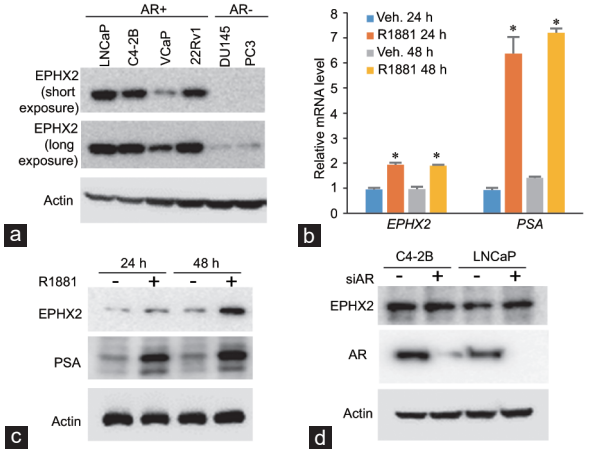


Figure 6: Androgen-mediated regulation of EPHX2 in PCa cells. (**a**) EPHX2 protein expression was determined in AR-positive (AR+) and AR-negative (AR-) PCa cells. Protein extracts were prepared from the indicated PCa cells, and Western blotting was used to determine EPHX2 protein levels. Actin was used as the loading control. (**b**) LNCaP cells were cultured in 5% charcoal-stripped fetal bovine serum (CT-FBS) for 2 days and were then treated with the synthetic androgen R1881 (10 nmol l^{-1}) for the indicated times. RNA was isolated, and qPCR was used to determine *EPHX2* mRNA levels. Prostate-specific antigen (*PSA*) was used as the positive control. Student's *t*-test was used to analyze the statistical significance ($n = 3$). The error bars indicate standard deviations. $^{*}P<0.05$. (**c**) LNCaP cells were cultured and treated as in **b**. Protein extracts were prepared, and Western blotting was used to determine EPHX2 protein levels. PSA and actin were used as the positive control and loading control, respectively. (**d**) LNCaP and C4-2B cells were transfected with either control or *AR*-specific siRNA. Protein extracts were prepared, and Western blotting was used to determine EPHX2 and AR protein levels. Actin was used as the loading control. AR: androgen receptor; qPCR: quantitative polymerase chain reaction; PCa: prostate cancer; EPHX2: epoxide hydrolase 2.

PCa. In addition, our results showed that *EPHX2* downregulation is associated with PCa progression and predicts poor survival prognoses for PCa patients.

Recently, Vainio *et al.*[24] showed that *EPHX2*, together with five other AA pathway genes, was highly expressed in PCa samples compared normal tissues. In contrast, our analysis of multiple gene expression datasets showed that *EPHX2* gene deletion occurred frequently in both primary and advanced PCa and that *EPHX2* expression was decreased in PCa tissues compared with benign prostate tissues. This observation was further confirmed by IHC analysis of EPHX2 protein expression in paired prostate tumor and adjacent benign prostate tissues. *EPHX2* expression correlates with AR mRNA levels in PCa tissues, and *EPHX2* knockdown suppresses AR signaling in PCa cells.[24] However, whether androgen signaling regulates EPHX2 expression in PCa cells remains unclear. Here, we showed that EPHX2 expression was detectable in AR-positive PCa cell lines (*i.e.*, LNCaP, C4-2B, 22Rv1, and VCaP) but nearly undetectable in AR-negative PCa cell lines (*i.e.*, PC3 and DU145). Consistent with this finding, we demonstrated that the synthetic androgen R1881 significantly induced *EPHX2* expression in PCa cells at both the mRNA and protein levels. Unexpectedly, depletion of *AR* did not affect *EPHX2* expression in PCa cells, indicating the AR independence of *EPHX2* expression. Androgens can rapidly affect cellular processes independent of AR, a phenomenon called nongenomic androgen action.[41] However, the exact pathway mediating the effect of androgens on *EPHX2* expression remains to be explored.

In a recent study, Vainio *et al.*[24] showed that *EPHX2* siRNA transfection reduced the viability of LNCaP cells compared with scrambled siRNA-transfected control cells. In addition, *EPHX2* siRNA potentiated the growth-inhibitory effect of flutamide in PCa cells.[24] However, we found that stable silencing of *EPHX2* with shRNA did not significantly affect the growth of PCa cells (**Supplementary Figure 1a**). In addition, we found that *EPHX2* inhibition did not affect the response of PCa cells to antiandrogens, such as enzalutamide and abiraterone (**Supplementary Figure 1b** and **1c**). Our results indicated that *EPHX2* might not be involved in regulating the growth of PCa cells.

The EPHX2 protein catalyzes the rapid hydrolysis of EETs, which are major end products generated by the CYP450 branch of AA metabolism. Pharmacologic inhibition or genetic deletion of *EPHX2* resulted in accumulation of EETs, which in turn promoted tumor-associated angiogenesis and metastasis in animal models.[16] Conversely, a reduction in the EET level by overexpression of the EPHX2 protein or the use of EET antagonists suppressed tumor growth and metastasis.[16] The protumorigenic effect of EETs may be mediated through multiple mechanisms.[16–18] For instance, EETs have been shown to induce GPCR and EGFR transactivation in cancer cells *in vitro*,[15] and Panigrahy *et al.*[16] revealed that VEGF signaling was required for EET-induced tumor-associated angiogenesis. Our work demonstrated that downregulation of *EPHX2* expression occurs frequently in PCa and is significantly associated with poor prognosis and metastasis. Loss of *EPHX2* likely results in accumulation of EETs, thereby stimulating tumor cell proliferation and angiogenesis. Indeed, we observed that the EPHX2 protein levels in advanced PCa tissues were inversely correlated with the nuclear Ki67 index, which is associated with aggressive clinicopathological characteristics of PCa, including the Gleason score and biochemical recurrence and survival.[36,37,42–44] However, in this study, we could not assess the correlation between the expression levels of EPHX2 and angiogenesis markers in PCa tissues. Thus, further investigation is required to assess the potential correlation.

CONCLUSION

The data we presented here highlight the significant correlation of *EPHX2* deregulation with PCa progression. Loss of *EPHX2* was correlated with highly proliferative and metastatic PCa and may serve as an independent biomarker for PCa prognosis. In addition, we found that *EPHX2* expression in PCa cells is regulated by androgens but is independent of AR, although the underlying mechanism is not yet known.

AUTHORS CONTRIBUTIONS

MSL, HZ, and WHL conceived the study; MSL and HZ performed the genomic analysis and drafted the manuscript; CXX, PBX, and WW prepared clinical materials and performed the immunohistochemical analysis; YYY performed the statistical analysis; HQZ and YJ conceived the study and drafted the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

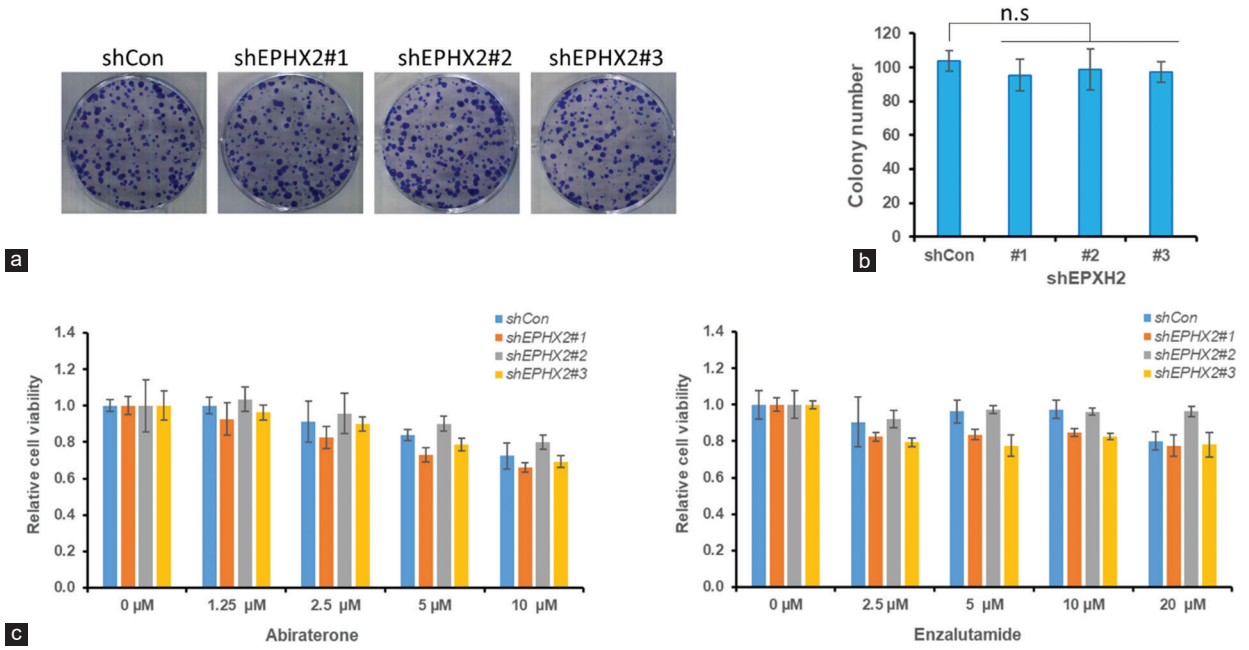
ACKNOWLEDGMENTS

Financial support for this study was supported by Yunnan Fundamental Research Projects (No. 2019FE001 [-277] and 2019FE001 [-005]), the Project for Innovation Team of Yunnan Provincial Science and Technology Department, China (No. 2018HC005), and Qujing Affiliated Hospital of Kunming Medical University (2019YJKT11 and 2020YJKT03).

Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

REFERENCES

1 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, *et al*. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68: 394–424.
2 Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, *et al*. Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *N Engl J Med* 2004; 350: 2239–46.
3 Martin RM, Donovan JL, Turner EL, Metcalfe C, Young GJ, *et al*. Effect of a low-intensity PSA-based screening intervention on prostate cancer mortality: the CAP randomized clinical trial. *JAMA* 2018; 319: 883–95.
4 Schroder FH, Hugosson J, Roobol MJ, Tammela TL, Zappa M, *et al*. Screening and prostate cancer mortality: results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 years of follow-up. *Lancet* 2014; 384: 2027–35.
5 Draisma G, Etzioni R, Tsodikov A, Mariotto A, Wever E, *et al*. Lead time and overdiagnosis in prostate-specific antigen screening: importance of methods and context. *J Natl Cancer Inst* 2009; 101: 374–83.
6 Prensner JR, Chinnaiyan AM, Srivastava S. Systematic, evidence-based discovery of biomarkers at the NCI. *Clin Exp Metastasis* 2012; 29: 645–52.
7 Patel MI, Kurek C, Dong Q. The arachidonic acid pathway and its role in prostate cancer development and progression. *J Urol* 2008; 179: 1668–75.
8 Panagiotopoulos AA, Kalyvianaki K, Castanas E, Kampa M. Eicosanoids in prostate cancer. *Cancer Metastasis Rev* 2018; 37: 237–43.
9 Chaudry AA, Wahle KW, McClinton S, Moffat LE. Arachidonic acid metabolism in benign and malignant prostatic tissue *in vitro*: effects of fatty acids and cyclooxygenase inhibitors. *Int J Cancer* 1994; 57: 176–80.
10 Mirtti T, Laine VJ, Hiekkanen H, Hurme S, Rowe O, *et al*. Group IIA phospholipase A as a prognostic marker in prostate cancer: relevance to clinicopathological variables and disease-specific mortality. *APMIS* 2009; 117: 151–61.
11 Gupta S, Srivastava M, Ahmad N, Bostwick DG, Mukhtar H. Over-expression of cyclooxygenase-2 in human prostate adenocarcinoma. *Prostate* 2000; 42: 73–8.
12 Alabiad MA, Harb OA, Taha HF, El Shafaay BS, Gertallah LM, *et al*. Prognostic and clinic-pathological significances of SCF and COX-2 expression in inflammatory and malignant prostatic lesions. *Pathol Oncol Res* 2019; 25: 611–24.
13 Gupta S, Srivastava M, Ahmad N, Sakamoto K, Bostwick DG, *et al*. Lipoxygenase-5 is overexpressed in prostate adenocarcinoma. *Cancer* 2001; 91: 737–43.
14 Rodriguez-Blanco G, Zeneyedpour L, Duijvesz D, Hoogland AM, Verhoef EI, *et al*. Tissue proteomics outlines AGR2 AND LOX5 as markers for biochemical recurrence of prostate cancer. *Oncotarget* 2018; 9: 36444–56.
15 Maugeri-Sacca M, Barba M, Pizzuti L, Vici P, Di Lauro L, *et al*. The Hippo transducers TAZ and YAP in breast cancer: oncogenic activities and clinical implications. *Expert Rev Mol Med* 2015; 17: e14.
16 Panigrahy D, Edin ML, Lee CR, Huang S, Bielenberg DR, *et al*. Epoxyeicosanoids stimulate multiorgan metastasis and tumor dormancy escape in mice. *J Clin Invest* 2012; 122: 178–91.
17 Jiang JG, Ning YG, Chen C, Ma D, Liu ZJ, *et al*. Cytochrome p450 epoxygenase promotes human cancer metastasis. *Cancer Res* 2007; 67: 6665–74.
18 Cheng LM, Jiang JG, Sun ZY, Chen C, Dackor RT, *et al*. The epoxyeicosatrienoic acid-stimulated phosphorylation of EGF-R involves the activation of metalloproteinases and the release of HB-EGF in cancer cells. *Acta Pharmacol Sin* 2010; 31: 211–8.
19 Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer* 2010; 10: 181–93.
20 Panigrahy D, Kaipainen A, Greene ER, Huang S. Cytochrome P450-derived eicosanoids: the neglected pathway in cancer. *Cancer Metastasis Rev* 2010; 29: 723–35.
21 Wood JN, Bevan SJ, Coote PR, Dunn PM, Harmar A, *et al*. Novel cell lines display properties of nociceptive sensory neurons. *Proc Biol Sci* 1990; 241: 187–94.
22 Tanaka H, Kamita SG, Wolf NM, Harris TR, Wu Z, *et al*. Transcriptional regulation of the human soluble epoxide hydrolase gene *EPHX2*. *Biochim Biophys Acta* 2008; 1779: 17–27.
23 Panigrahy D, Greene ER, Pozzi A, Wang DW, Zeldin DC. EET signaling in cancer. *Cancer Metastasis Rev* 2011; 30: 525–40.
24 Vainio P, Gupta S, Ketola K, Mirtti T, Mpindi JP, *et al*. Arachidonic acid pathway members PLA2G7, HPGD, EPHX2, and CYP4F8 identified as putative novel therapeutic targets in prostate cancer. *Am J Pathol* 2011; 178: 525–36.
25 Cancer Genome Atlas Research N. The molecular taxonomy of primary prostate cancer. *Cell* 2015; 163: 1011–25.
26 Baca SC, Prandi D, Lawrence MS, Mosquera JM, Romanel A, *et al*. Punctuated evolution of prostate cancer genomes. *Cell* 2013; 153: 666–77.
27 Kumar A, Coleman I, Morrissey C, Zhang X, True LD, *et al*. Substantial interindividual and limited intraindividual genomic diversity among tumors from men with metastatic prostate cancer. *Nat Med* 2016; 22: 369–78.
28 Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, *et al*. Integrative genomic profiling of human prostate cancer. *Cancer Cell* 2010; 18: 11–22.
29 Abida W, Cyrta J, Heller G, Prandi D, Armenia J, *et al*. Genomic correlates of clinical outcome in advanced prostate cancer. *Proc Natl Acad Sci U S A* 2019; 116: 11428–36.
30 Grasso CS, Wu YM, Robinson DR, Cao X, Dhanasekaran SM, *et al*. The mutational landscape of lethal castration-resistant prostate cancer. *Nature* 2012; 487: 239–43.
31 Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, *et al*. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2012; 2: 401–4.
32 Glinsky GV, Glinskii AB, Stephenson AJ, Hoffman RM, Gerald WL. Gene expression profiling predicts clinical outcome of prostate cancer. *J Clin Invest* 2004; 113: 913–23.
33 Ross-Adams H, Lamb AD, Dunning MJ, Halim S, Lindberg J, *et al*. Integration of copy number and transcriptomics provides risk stratification in prostate cancer: a discovery and validation cohort study. *EBioMedicine* 2015; 2: 1133–44.
34 Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepfer AM, *et al*. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* 2006; 124: 1283–98.
35 Enayetallah AE, French RA, Barber M, Grant DF. Cell-specific subcellular localization of soluble epoxide hydrolase in human tissues. *J Histochem Cytochem* 2006; 54: 329–35.
36 Berlin A, Castro-Mesta JF, Rodriguez-Romo L, Hernandez-Barajas D, Gonzalez-Guerrero JF, *et al*. Prognostic role of Ki-67 score in localized prostate cancer: a systematic review and meta-analysis. *Urol Oncol* 2017; 35: 499–506.
37 Tollefson MK, Karnes RJ, Kwon ED, Lohse CM, Rangel LJ, *et al*. Prostate cancer Ki-67 (MIB-1) expression, perineural invasion, and gleason score as biopsy-based predictors of prostate cancer mortality: the Mayo model. *Mayo Clin Proc* 2014; 89: 308–18.
38 Miller I, Min M, Yang C, Tian C, Gookin S, *et al*. Ki67 is a graded rather than a binary marker of proliferation versus quiescence. *Cell Rep* 2018; 24: 1105–12.e5.
39 Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, *et al*. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984; 133: 1710–5.
40 Matsuyama M, Yoshimura R. The target of arachidonic acid pathway is a new anticancer strategy for human prostate cancer. *Biologics* 2008; 2: 725–32.
41 Foradori CD, Weiser MJ, Handa RJ. Non-genomic actions of androgens. *Front Neuroendocrinol* 2008; 29: 169–81.
42 Fisher G, Yang ZH, Kudahetti S, Moller H, Scardino P, *et al*. Prognostic value of Ki-67 for prostate cancer death in a conservatively managed cohort. *Br J Cancer* 2013; 108: 271–7.
43 Rubio J, Ramos D, Lopez-Guerrero JA, Iborra I, Collado A, *et al*. Immunohistochemical expression of Ki-67 antigen, cox-2 and Bax/Bcl-2 in prostate cancer; prognostic value in biopsies and radical prostatectomy specimens. *Eur Urol* 2005; 48: 745–51.
44 Richardsen E, Andersen S, Al-Saad S, Rakaee M, Nordby Y, *et al*. Evaluation of the proliferation marker Ki-67 in a large prostatectomy cohort. *PLoS One* 2017; 12: e0186852.



Supplementary Figure 1: Effect of EPHX2 knockdown on PCa cell growth and response to antiandrogens. (**a**) C4-2B cells stably expressing scramble shRNA or EPHX2 specific shRNAs were cultured in RPMI1640 medium supplemented with 10% FBS 2 weeks. Then the cells were fixed with cold methanol and stained with 0.1% crystal violet solution. The assay was performed in triplicates. Left panel, representative images of three independent experiments; right panel, quantification of colonies. Error bars indicate standard deviation. N.S. indicates no significance. (**b**) C4-2B cells stably expressing scramble shRNA or *EPHX2* specific shRNAs were cultured in RPMI1640 medium and treated with 0–20 μM enzalutamide for 2 days. The cell viability was determined with Cell Counting Kit 8 (CCK-8, Sigma-Aldrich). Error bars indicate standard deviation. (**c**) C4-2B cells stably expressing scramble shRNA or *EPHX2* specific shRNAs were cultured in RPMI1640 medium and treated with 0–10 μM abiraterone for 2 days. The cell viability was determined with Cell Counting Kit 8 (CCK-8, Sigma-Aldrich). Error bars indicate standard deviation.