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.

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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.3–5 In addition, PSA screening often leads to overdiagnosis due to the lack of a clear cutoff point for high sensitivity and specificity.1 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.7–9 For instance, PLA2 group IIa (PLA2G2A) expression and AA turnover rate were found to be increased in tumor tissue compared to those in normal prostate tissue,8,9 in addition, altered expression of COX2 and LOX-5 has been found to be associated with PCa progression.10–14

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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cholangiocarcinoma, and advanced ovarian cancer. A recent study showed that EPHX2 was expressed at similar levels in both clinical PCA samples and normal prostate samples. 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), Broad/Cornell cohort (n = 112), and Memorial Sloan Kettering Cancer Center (MSKCC) cohort (n = 240). The metastatic PCA datasets used in this analysis included the Stand Up To Cancer/Prostate Cancer Foundation (SU2C/PCF) cohort (n = 444), the Metastatic Prostate Adenocarcinoma (MCP) cohort (n = 75). All source data were downloaded from the cBio Cancer Genomics Portal (http://cbioportal.org). Differential expression of EPHX2 in normal prostate and PCA samples was evaluated in multiple PCA gene expression datasets, including the TCGA cohort, Taylor cohort (GSE21032), Grasso cohort (GSE35988), Glinsky cohort, and Ross-Adams cohort (GSE70768).

**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 Qingfeng First Hospital and the Qingfeng Affiliated Hospital of Kunming Medical University (Qingfeng, 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 to dilute 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. 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% [w/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 5 min. 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% [w/v] Tween (TBS-Tween) for 1 h 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).

**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. 15 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).
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 recurrent 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. 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. 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 cells. 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...
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, the functional and clinical relevance of this pathway have not been well explored in PCa. By analyzing genetic alterations in the key enzymes of the AA pathway, we found that EPHX2 gene loss frequently occurs in...
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: epoxydihydrolase 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.\textsuperscript{24} showed that EPHX2, together with five other AA pathway genes, was highly expressed in PCa samples compared to 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 to 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.\textsuperscript{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.\textsuperscript{41} However, the exact pathway mediating the effect of androgens on EPHX2 expression remains to be explored.

In a recent study, Vainio et al.\textsuperscript{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.\textsuperscript{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 androgenreceptors, 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.\textsuperscript{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.\textsuperscript{16} The protumorigenic effect of EETs may be mediated through multiple mechanisms.\textsuperscript{16–18} For instance, EETs have been shown to induce GPCR and EGFR transactivation in cancer cells in vitro,\textsuperscript{15} and Panigrahy et al.\textsuperscript{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 stimulatng 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.\textsuperscript{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 preparation 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.

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