Am J Clin Exp Urol 2014;2(3):249-257
www.ajceu.us /ISSN:2330-1910/AJCEU0002447

Original Article

Significantly higher expression levels of androgen receptor are associated with erythroblastosis virus E26 oncogene related gene positive prostate cancer

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Received September 10, 2014; Accepted September 25, 2014; Epub October 2, 2014; Published October 15, 2014

Abstract: Erythroblastosis virus E26 related gene (ERG) overexpression is correlated with the TMPRSS2-ERG fusion gene, a rearrangement known to be present in about 50% of cases of prostate cancer. Androgen receptor (AR) is a known regulator of the TMPRSS2 gene. Despite knowledge of this relationship, limited data is available on the specific relationship of AR expression to TMPRSS2-ERG fusion (ERG) status in prostate cancer (PCa). We used multiplexed immunohistochemistry, multispectral imaging technology and tissue microarray (TMA) to elucidate this relationship. Two prostate tissue microarrays were created from two cohorts of hormonal naïve patients' prostatectomy specimens: progression TMA (pTMA, from 95 PCa patients) and outcome TMA (oTMA, from 183 PCa patients with at least 5-year follow-up information). Each of the two TMAs were triple-stained with ERG, AR and E-cadherin antibodies and visualized with a different chromogen. We found marked difference in AR expression levels between ERG positive (ERG^+) and ERG negative (ERG^-) prostate cancer. The difference was significant in localized (pT2) prostate cancer. We also found that AR expression levels were significantly higher in PCa tissue compared to benign prostate tissue, with the highest expression levels in ERG^+ metastatic cancer. Neither AR nor ERG expression was associated with clinical outcome. Our findings confirm that TMPRSS2-ERG fusion is AR-dependent and is associated with increased AR expression. Our data suggest that the AR pathway may play an important role in the development of ERG^+ PCa and ERG status may be useful in stratifying PCa patients for hormonal therapy.

Keywords: ERG, TMPRSS2, AR, prostate cancer

Background

Androgen receptor (AR) is known to play a key role in prostate development and is involved in the progression of Prostate cancer (PCa) [1-3]. Transmembrane protease, serine 2 (TMPRSS2) is an androgen-regulated gene [4]. Fusions of TMPRSS2 with ETS transcription factors have been found in prostate cancer [5]. TMPRSS2-ERG fusion is the most common molecular alteration, present in about half of all the PCa [6-9]. Recently, rare fusions of TMPRSS2 with other ETS family members such as ETV1, ETV4, ETV5 and FLI1 have also been reported present in PCa [10-12]. It is believed that TMPRSS2-ERG fusion is an early event in prostate oncogenesis and progression that results from either a small deletion on chromosome 21 or through a translocation [5, 13]. As a result this fusion, the ERG gene becomes androgen-regulated and overexpressed in prostatic epithelium.

Fluorescent in situ hybridization (FISH) is a commonly used assay to detect TMPRSS2-ERG fusions. However, it is cumbersome to perform and quantitate, which hampers clinical application. Recently, an ERG-specific monoclonal antibody EPR 3864 (Epitomics, Burlingame, CA) has been shown to have close correlation with TMPRSS2-ERG fusion status and is considered reliable for detecting TMPRSS2-ERG fusion [14-17]. This ERG antibody makes testing TMPRSS2-ERG fusion relatively easy and more cost-effective.

Since TMPRSS2-ERG fusion first reported by Tomlins and colleagues in 2005, its role in PCa oncogenesis, PCa diagnosis and prognosis have been extensively studied. It is widely accepted that TMPRSS2-ERG fusion is PCa specific [5] and can be used to subtype and stratify PCa [18-21]. However, due to the relative low sensitivity, the value of TMPRSS2-ERG fusion as a PCa diagnostic marker is limited. Conflicting

Table 1. Study Cohorts and TMA Information

TMA (cohort)	Disease/Recurrence Status (#core)	Gleason Score (mean)	Mean age (y)
pTMA (cohort 1)	BPT (96)		62.8
	HGPIN (50)		
	PCa_local (pT2) (86)	6-9 (6.9)	
	PCa_aggr (pT3) (60)	7-9 (7.9)	
	Mets (44)		
	None (250)	5-9 (6.8)	60
oTMA (cohort 2)	Biochemical (76)	5-9 (7.4)	
	Cancer (40)	5-9 (7.6)	
	BPT (96)		

TMA, tissue microarrary; BPT, benign prostatic tissue, PCa_local, localized prostate cancer (pT2); PCa_aggr, aggressive prostate cancer (pT3); Met, metastatic prostate cancer; None, no recurrence; Biochemical, biochemical recurrence; Cancer, cancer recurrence.

data on TMPRSS2-ERG fusion predicting PCa progression and outcome have been published [22-26]. In vitro data suggests that AR can induce TMPRSS2-ERG fusion in PCa cells and in non-malignant prostatic epithelial cells [27]. Using both FISH and conventional quantitative immunohistochemistry to detect TMPRSS2-ERG fusion (ERG status), Minner and colleagues recently reported marked difference of AR expression levels associated with ERG status in human tissue samples [28]. While more insights have been gained, the relationship of AR expression levels and TMPRSS2-ERG fusion and the role of AR in the occurrence of TMPRSS2-ERG fusion in prostate cancer need to be further investigated.

In this study, we explored the AR expression levels in two cohorts of prostate cancer of different ERG status, and examined the prognostic values of ERG status in prostate cancer using multiplexed IHC and multispectral imaging technology.

Materials and methods

Patient cohorts and TMA construction

Two different patient cohorts were used (IRB approval: M-2007-1100-CP003): Formalin-fixed-paraffin-embedded prostatectomy tissu es used in this study were from the archive of Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison. All patients included in this study are Caucasian and were hormonal naïve at the time of surgery. A progression TMA (pTMA) from cohort 1 and an outcome TMA (oTMA) from cohort 2 were constructed. pTMA consists of 336 duplicate cores from prostate tissues of different disease groups. The oTMA consists of 462 duplicate cores from PCa tissues with 5 year outcome information (**Table 1**). The size of each core is 0.6 mm in diameter and arranged 0.2 mm apart vertically and horizontally using a Manual Tissue Arrayer (Beecher Instruments, Sun Prairie, WI; Model MTA-1).

Multiplexed immunohistochemistry (mIHC)

The method was published previously [29]. Briefly, antibodies to AR and ERG and chromogenic multiplexed IHC assays were used detect the target biomarkers. One pTMA and one oTMA sections were stained with the three antibodies and counterstained with hematoxylin. E-cadherin was used to define the epithelial compartment for better tissue segmentation (**Table 2**).

Automated image acquisition and analysis

Briefly, the stained slides were loaded onto the Vectra slide scanner. A scanning protocol was created based on the TMA core number and size. Nuance multispectral image cubes (8-bit) were acquired with 20 x objective lens (0.5 micron/pixel) and using a full CCD frame at 1 x 1 binning (1,360 x 1,024 pixels) for analysis. Nuance 3.0.0 software was used to build the spectral libraries. Each chromogen has its unique spectral characteristics (curve), which are the basis for building the spectral library. Four control prostate tissue slides with one chromogen only (DAB, Warp Red, Vina Green and hematoxylin, respectively) were prepared and scanned to build the spectral library. The spectral library then was used to unmix the signals on the multicolored test slides by recognizing their unique spectral curves for quantitation. By doing this, signal cross-talk was eliminated (**Figure 1**). inForm 1.2 software, the unique pattern-recognition-based image analysis was used to segment tissue compartments (epithelium vs. non-epithelium) and subcellular compartments (nucleus vs. cytoplasm). Then the target signals were quantitated within the selected epithelial nuclear compartment [29].

AR and ERG are located primarily in prostatic nuclei. AR and ERG expression levels were quantitated as optical density (OD) per unit

Table 2. Staining Protocol

1st Ab	pTMA	oTMA	2nd Ab	Chromogen
E-cadherin (mouse mAb, 1:100, Biocare Medical)	x	x	Anti-mouse Ig-HRP	Vina Green
AR (mouse mAb, 1:50, Biocare Medical)	x	x	Anti-mouse Ig-HRP	DAB
ERG (rabbit mAb, 1:200, Epitomics)	x	x	Anti-rabbit Ig-AP	Warp Red
Counterstain	x	x		Hematoxylin

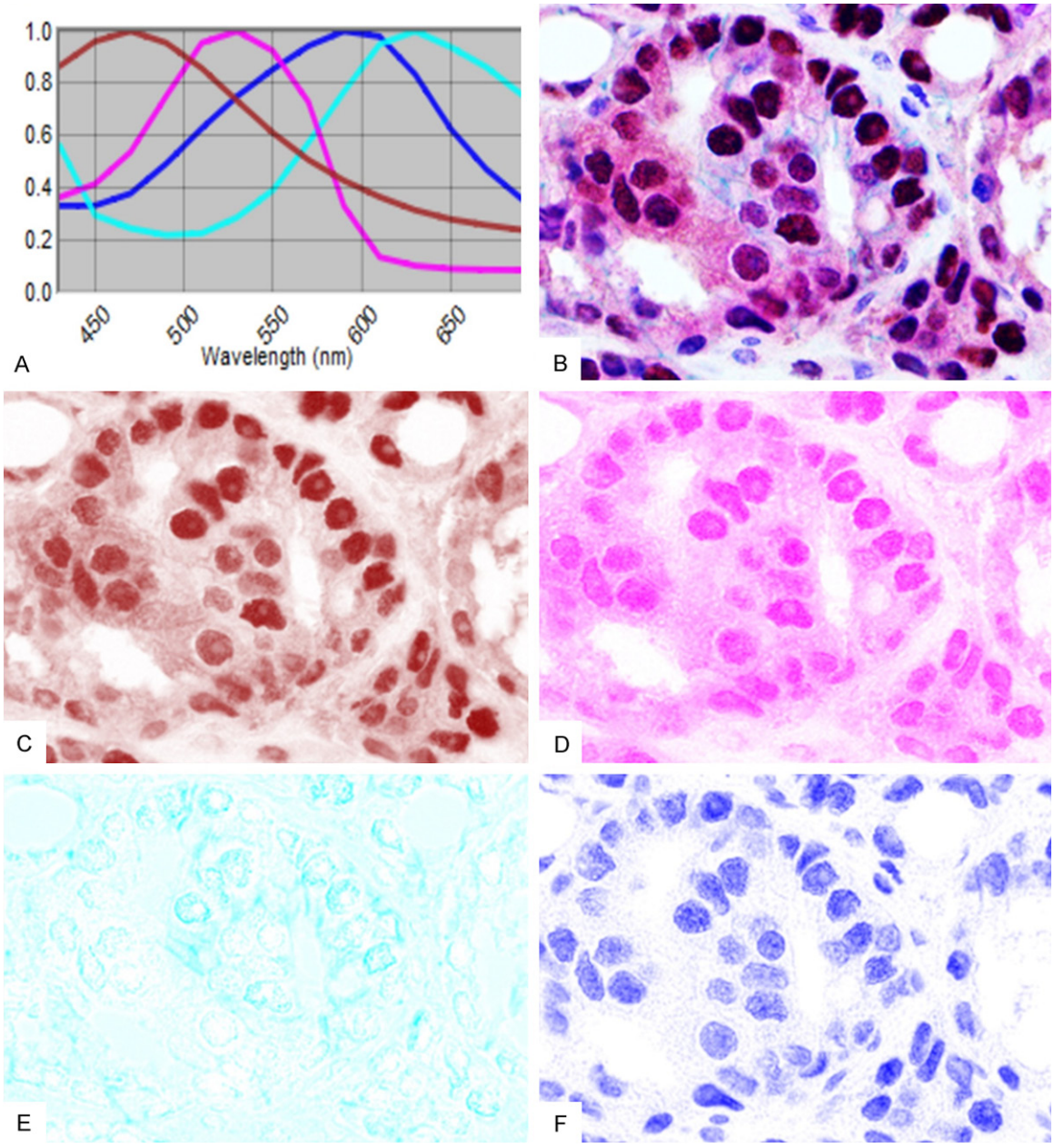

Figure 1. Multiplexed Immunohistochemistry and multispectral imaging. The spectral library of 4 chromogens (DAB, Warp Red, Vina Green and hematoxylin) was built using Nuance software (A). An image of PCa stained with three antibodies (E-Cad, AR and ERG) (B). Images of unmixed signals (DAB, Warp Red, Vina Green and hematoxylin) of PCa with the spectral library (C-F).

nuclear area (pixel). Mean OD/pixel of AR and ERG between the disease groups was compared. Core images with less than 5% epithelial component, significant tissue folding, loss of

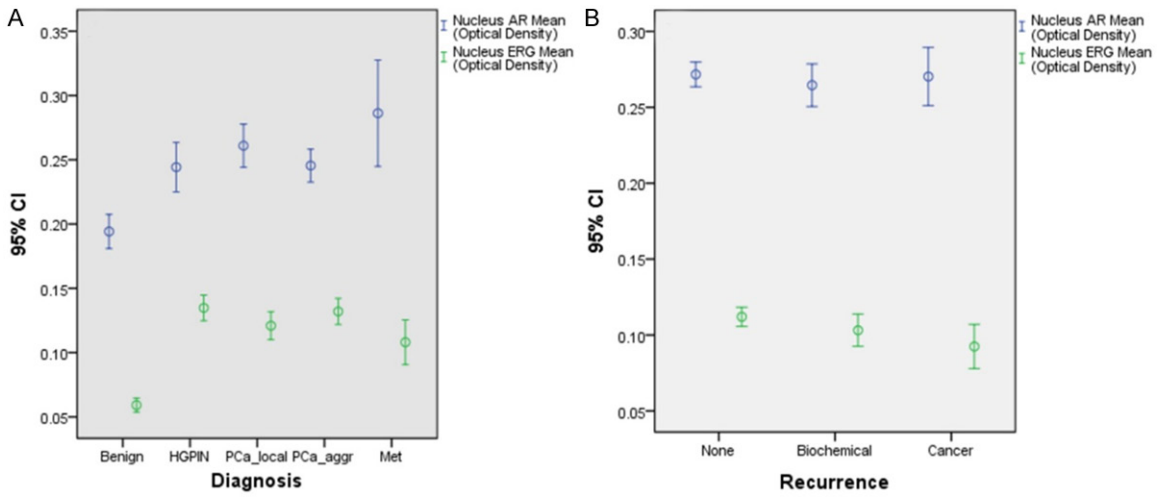

Figure 2. Nuclear AR and ERG expression levels of the prostate tissue samples from the two PCa cohorts. Expression levels of both nuclear AR and ERG are significantly higher in PCa and HGPIN compared to benign prostatic tissue. AR expression levels are significantly increased in PCa tissue compared to benign prostatic tissue ($p < 0.01$). The highest level of AR expression is observed in PCa metastases (mean OD = 0.29) (A). However, neither AR nor ERG expression seems to correlate with patients' outcome (B).

Table 3. Nuclear AR and ERG Expression in Prostate Cancer

Cohort	Diagnosis/Recurrence status	Core #	AR Mean OD ± SD	ERG mean OD ± SD
pTMA	Benign Prostate	85	0.15 ± 0.06	0.06 ± 0.03
	HGPIN	55	0.24 ± 0.07*	0.13 ± 0.04*
	PCa, local	81	0.26 ± 0.08*	0.12 ± 0.05*
	PCa, aggressive	61	0.24 ± 0.05*	0.13 ± 0.04*
	Metastases	41	0.29 ± 0.13*	0.11 ± 0.06*
oTMA	Benign Prostate	73	0.19 ± 0.05	0.07 ± 0.03
	None	226	0.27 ± 0.06*	0.11 ± 0.05*
	Biochemical	75	0.26 ± 0.06*	0.10 ± 0.05*
	Cancer	39	0.27 ± 0.06*	0.09 ± 0.04*

*$p < 0.01$ compared to Benign Prostate; Core #: number of cores analyzed; OD: optical density; SD: standard deviation.

tissue or greater than 5% poorly segmented area were excluded from analysis.

Since the ERG also expressed in endothelial cells and lymphocytes, even after tissue segmentation in which prostatic epithelium and stroma were clearly defined, a few capillary endothelial cells or lymphocytes were present in the tissue cores. Therefore, tissue cores with ≥ 3% positive cells were considered ERG$^+$ [14, 29].

Data analysis

IBM SPSS Statistics 19 was used for statistical analysis and data plotting. To account for multiple associations between tissue categories, which might tend to favor a significant association because of chance, one-way Anova posthoc tests (Tamhane's T2) were used for comparing means. A p-value ≤ 0.05 is considered significant.

Results

Nuclear AR and ERG expression levels in PCa samples from the two cohorts

We found that expression levels of both nuclear AR and ERG are significantly higher in PCa and HGPIN compared to benign prostatic tissue (**Figure 2A**, **Table 3**). AR expression levels are significantly increased in PCa tissue compared to benign prostatic tissue ($p < 0.01$). The highest level of AR expression is observed in PCa metastases (mean OD = 0.29) However, nei-

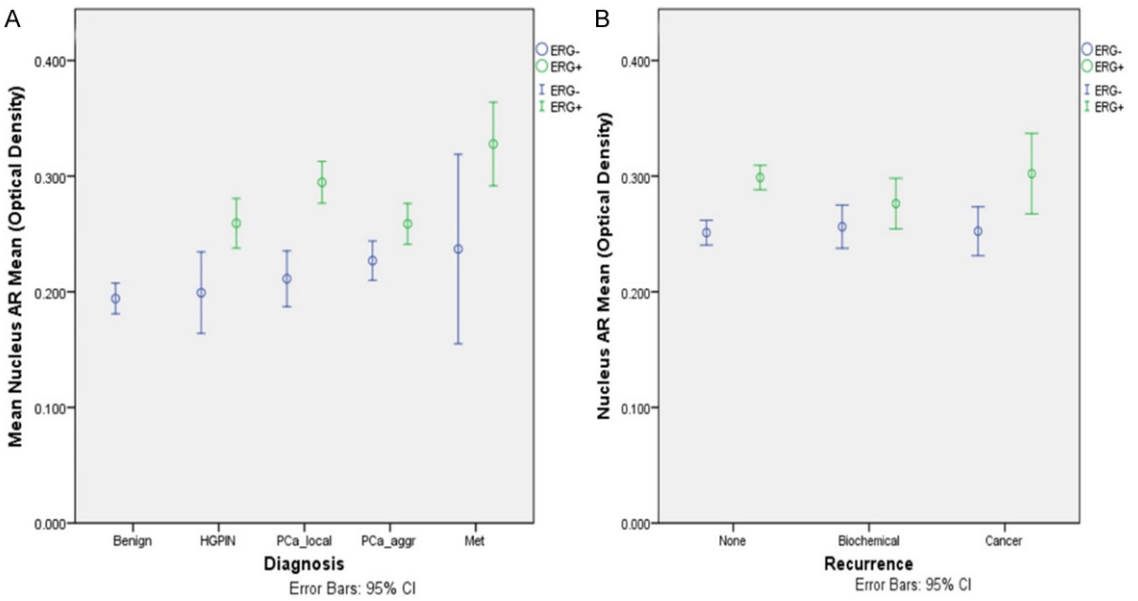

Figure 3. Nuclear AR expression levels of the prostate tissue samples from the two PCa cohorts. AR expression levels in ERG$^+$ PCa overall are much higher than those in ERG$^-$ PCa and the difference is statistically significant only in localized PCa group in cohort 1 (A) and in no-recurrence PCa group in cohort 2 (B) ($p < 0.01$).

Table 4. Nuclear AR Expression in Prostate Cancer Stratified by ERG Status

Cohort	Diagnosis/Recurrence status	Core # (ERG$^+$/ERG$^-$)	AR Mean OD ± SD	
			ERG$^+$	ERG$^-$
pTMA	Benign Prostate	0/84		0.19 ± 0.06
	HGPIN	30/25	0.27 ± 0.07	0.21 ± 0.07
	PCa, local*	41/40	0.30 ± 0.05*	0.21 ± 0.08*
	PCa, aggressive	35/26	0.26 ± 0.05	0.22 ± 0.04
	Metastases	18/23	0.33 ± 0.07	0.25 ± 0.16
oTMA	Benign Prostate	0/72		0.19 ± 0.05
	None*	98/128	0.30 ± 0.05*	0.25 ± 0.06*
	Biochemical	31/44	0.28 ± 0.06	0.26 ± 0.06
	Cancer	14/25	0.30 ± 0.06	0.25 ± 0.05

*$p < 0.001$ ERG$^+$ vs. ERG$^-$; ERG$^+$ PCa = 51% in pTMA cohort, ERG$^+$ PCa = 42% in oTMA cohort.

ther AR nor ERG expression seems to correlate with patients' outcome (**Figure 2B**, **Table 3**).

ERG status (TMPRSS2-ERG fusion) of the PCa patients in the two cohorts

Similar to most published studies, we found 49% of PCa samples express ERG (ERG$^+$) in cohort 1, and 53% of PCa samples are ERG$^+$ in cohort 2.

AR expression levels in ERG$^+$ and ERG$^-$ PCa samples

When further stratifying patients in both cohorts according to ERG status (ERG$^+$ vs. ERG$^-$) we found that AR expression levels in ERG$^+$ PCa overall are much higher than those in ERG$^-$ PCa and the difference is statistically significant only in localized PCa group in cohort 1 and in no-recurrence PCa group in cohort 2 ($p < 0.01$; **Figure 3A** and **3B**, **Table 4**).

Discussion

To the best of our knowledge, we believe this is the first quantitative study on AR expression in PCa with different ERG status using multiplexed IHC and multispectral imaging technology and two dense TMA samples. This quantitative approach allows us to study ERG and AR simul-

taneously on a single TMA section. It removes subjectivity and allows precise quantitation of target proteins either continuously (mean OD/pixel) or categorically (positive vs. negative).

Our data obtained from the two cohorts are concordant with each other and confirm the previous report by Minner and colleagues that there is a marked difference in AR expression levels between ERG^+ and ERG^- PCa [28]. Elevated expression of AR in ERG^+ PCa, then, suggests a dosage effect of AR in the expression of TMPRSS2-ERG fusion.

Mani and colleagues reported that androgen signaling induces spatial proximity of TMPRSS2 and ERG genomic loci, both located on chromosome 21q22, and facilitates the formation of the TMPRSS2 and ERG fusion in LNCaP cells [27]. Bastus and colleaguse reported that treatment with androgen can induce the TMPRSS2-ERG fusion in both malignant and nonmalignant prostate epithelial cells. Although the fusion could be detected in malignant cells following 24-hour treatment, prolonged exposure to androgen was required to detect the fusion transcript in nonmalignant cells. Their data suggested that androgen-induced gene proximity, androgen receptor exon1 CAG repeat length and expression of the PIWIL1 gene were the driving factors. Their experiments demonstrated that fusions can be induced prior to malignant transformation and generation of the fusion is associated with both gene proximity and loss of the ability to prevent double-strand breaks [30]. Other studies also provided evidences that AR and ERG are closely linked in the development of PCa [31-33]. Our finding further supports that AR signaling plays a key role in the formation of TMPRSS2-ERG fusion.

With those data in mind, one might ask: Can ERG status determine and predict cancer patients' responsiveness to androgen deprivation therapy (ADT) or chemoradiation therapy? Can we use ERG status to subtype PCa and to select potentially effective therapy for the patients?

Karnes and colleagues reported that the ability of DNA isotopomerase 2α (TOP2a) and MIB-1 to predict systemic progression in men with high-risk PCa is dependent on ERG status. They also found that the response to adjuvant ADT therapy is associated with ERG status, with more significant treatment effect in ERG^+ patients [34]. Whereas Swanson and colleagues demonstrated that expression of TMPRSS2-ERG gene fusions in PC3 cells increased radiation sensitivity and decreased paclitaxel sensitivity, DU145 type III and VI cells showed a different sensitivity and gene expression change [35]. Using a sensitive, analytically valid reverse transcription polymerase chain reaction assay, Danila and colleagues studied TMPRSS2-ERG fusion in circulating tumor cells (CTCs) as a marker of sensitivity to Abiraterone (AA), an androgen biosynthesis inhibitor shown to prolong life in patients with castration-resistance PCa already treated with chemotherapy. They found that the presence of TMPRSS2-ERG fusion did not predict response to AA treatment [36]. These findings indicate a need for prospective studies to evaluate the relationship of ERG status with sensitivity to adjuvant therapy, particularly to ADT.

Our data showed that AR expression levels are significantly higher in PCa compared to benign prostatic tissue, indicating AR plays an important role in prostate oncogenesis.

Although no clear associations between ERG status and PCa progression are found in our data, AR expression level was higher in metastatic PCa compared to pT2 (localized PCa) and pT3 (aggressive PCa) PCa in both ERG^+ and ERG^- groups (**Table 4**, **Figure 3**) suggesting that AR signaling pathway plays a key role in PCa metastasis. However, due to relatively small number of metastatic PCa samples tested in the present study, further validation of this finding is needed.

Similar to many published studies, we found neither the expression levels of AR or ERG alone, nor ERG status alone, nor the combination of ERG status and AR expression levels were associated with clinical outcome.

Our finding that the prevalence of ERG^+ PCa is 49% and 53% in the two cohorts we studied, respectively, which is in keeping with most published data [9].

We are aware that the prostatectomy and metastatic tumor samples used for constructing the two TMAs encompass 15 years' span. The quality of the older tissue samples may not be the same as the newer tissue samples, which may affect, to some degree, the fidelity this quantitative study intended to accomplish.

In summary, our findings confirm that TMPRSS2-ERG fusion is AR-dependent and is associated with increased AR expression. Our data also suggests that AR pathway plays an important role in the development of PCa, particularly ERG^+ PCa, and that ERG status may be useful in stratifying PCa patients for adjuvant therapies, particularly ADT.

Acknowledgements

This work was done in the TRIP Laboratory, Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison.

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