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ORIGINAL ARTICLE – CANCER RESEARCH

Combined loss of TFF3 and PTEN is associated with lethal outcome and overall survival in men with prostate cancer

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Abstract

Background Trefoil Factor 3 (TFF3) has been implicated in Prostate Cancer (PCa) progression. However, its prognostic value and association with other biomarkers have not been fully explored. We assessed the combined value of TFF3 and PTEN in two cohorts: one is managed surgically for localized PCa and the second is managed non-surgically by androgen deprivation therapy for advanced disease.

Design 228 radical prostatectomies (RP) and 318 transurethral resection of prostate (TURP) samples were assessed by immunohistochemistry (IHC) for TFF3 and by IHC and fluorescent in situ hybridization (FISH) for PTEN. Results of biomarkers expression were correlated with various pathological and clinical outcome parameters including biochemical recurrence (BCR) in the RP cohort and cancer-specific mortality (PCSM) and overall survival (OS) in the TURP cohort.

Results TFF3 expression was detected in 131/226 (57.9%) RP samples and 148/318 (46.5%) of TURP cases. In general, TFF3 positivity was less frequently observed with advanced Gleason Groups. TFF3 expression was also assessed in relation to PTEN expression. Only 15–16% of TFF3-expressed cases were present in association with complete loss of PTEN expression in the TURP and localized cohorts, respectively. Loss of TFF3 expression was not related to BCR after RP, but was prognostic in the non-surgical cohort and associated with decrease OS and PCSM (HR 2.31, CI: 1.67–3.18, $p<0.0001$) and (HR 3.99, CI: 2.43–6.56; $p<0.0001$), respectively. Adjusting for Gleason score, combined loss of TFF3/PTEN was most associated with OS (HR 2.33, CI: 1.49–3.62; $p<0.0001$) and PCSM (HR = 3.44, CI: 1.75–6.78, $p<0.0001$).

Conclusion The study documents for the first time significant association for combined status of TFF3 expression and PTEN loss in OS and PCSM in patients not managed by surgical intervention. Prospective assessment of PTEN and TFF3 may provide further insight into molecularly subtyping PCa and aid in stratifying patients at risk for lethal disease.

Keywords *TFF3* · *PTEN* · Protein expression · Immunohistochemistry · Gleason score · Androgen deprivation therapy · Cancer specific mortality · Biochemical recurrence

Introduction

Predicting prostate cancer (PCa) progression remains a major clinical issue facing physicians. Currently, more men with low- and intermediate-risk disease opt for active

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surveillance (AS) as initial treatment modality. However, even with best-implemented nomograms, there is still about 10–15% rate of disease progression per year in addition to similar number of patients leaving AS programs due to anxiety and fear of losing curative window. Even with today's advancement in molecular pathology, none of the biomarkers has been confirmed into routine clinical practice for men with PCa compared to other types of malignancies such as breast, colon and lung cancer. Radical prostatectomy remains a major treatment modality for men with localized disease, along with radiotherapy. However, in the advanced stage, LHRH is considered by many as the main and relatively safe line of therapy to halt disease progression for several years before attempting the more expensive therapies that come with added various side effects. On the other hand, predicting lethal disease and overall survival may allow for better allocation of such therapies in patients who need it the most and avoid harmful side effects in others whose tumors are likely to be of indolent course (Johansson et al. 2004). Our group and others have been heavily investigating clinical biomarkers that could be easy to interpret and can be applied clinically to aid in stratifying men with PCa along other clinical and pathological parameters (Matsui et al. 2012). To date, the only two promising biomarkers that potentially could be relatively easy to implement clinically are ERG and PTEN expression, which can be assessed by immunohistochemistry (IHC) in routine clinical pathology laboratories. ERG expression as detected by IHC is reflective of *ERG* gene rearrangements and is estimated to be present in about half of the clinically significant PCa. ERG expression and/or rearrangements have been widely investigated in several clinical cohorts, and have been shown to have prognostic value in specific clinical scenarios, most notably in men within watchful waiting cohorts or those treated by ADT where outcome is related to PCSM or OS compared to men treated by radical prostatectomy, where outcome is mostly related to BCR (Demichelis et al. 2007; Liu et al. 2011; Bismar et al. 2012a, b; Tomlins et al. 2012; Abou-Ouf et al. 2016; Lee et al. 2015; Teng et al. 2013a, b; Huang et al. 2014a, b; Teng et al. 2013a, b; Dal Pra et al. 2013; Reid et al. 2010; Hoogland et al. 2012).

PTEN, a known tumor suppressor, has been associated with disease progression and metastasis in PCa. It also has been gaining special attention as prognostic marker that could be easily adapted in clinical setting when assessed by IHC (Bismar et al. 2011; Sircar et al. 2009; Bismar et al. 2018).

TFF3 is a member of the trefoil family, which are characterized by having at least one copy of the trefoil motif, a 40-amino acid domain containing *TFF3* conserved disulfide bonds. *TFF3 s* are stable secretory proteins expressed in gastrointestinal mucosa, but without define function yet, but with suggestion that they may protect the mucosa from insults, stabilize the mucus layer and affect healing of the epithelium. This gene is a marker of columnar epithelium and is expressed in a variety of tissues including goblet cells of the intestines and the colon. *TFF3* gene is found in a cluster on chromosome 21. In PCa, TFF3 expression is suggested to be mutually exclusive to ERG expression. However, the association with PTEN expression and the combined prognostic value of TFF3 with PTEN have not been previously characterized.

In this study, we evaluated the prognostic value of TFF3 expression in two large cohorts: the first includes patients with localized PCa treated by radical prostatectomy and the second includes patients treated non-surgically by TURP with/out ADT.

Materials and methods

Study population and Tissue microarray construction

The study cohort consisted of two populations. The first are a group of men ($n = 228$) diagnosed with localized PCa and treated by radical prostatectomy between the years of 2000 and 2005. The second cohort is a group of men diagnosed with PCa by transurethral resection of the prostate (TURP) ($n = 318$). Patients within the TURP cohorts were either not actively treated or treated by androgen deprivation therapy (ADT) with LHRH (luteinizing-hormone-releasing hormone) agonist for disease progression post TURP or prior to the TURP sample in patients with pervious diagnosis of PCa prior to undergoing TURP to relieve symptomatic obstruction due to locally advanced disease. Follow-up information was collected from the Alberta Tumour Registry for overall survival and cancer-specific mortality. Tissue samples of the localized cohort were assembled onto three tissue microarrays (TMAs) with benign, HGPIN and PCa sample (range 2–6) from the two predominant patterns. The samples from the TURP cohort were assembled onto 2 TMA with an average two cores per patient using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). The study was approved by the institutional review board at the University of Calgary, Cumming School of Medicine. Calgary, Alberta, Canada.

TFF3 protein expression by immunohistochemistry (IHC)

TTF3 IHC was carried out on Dako Omnis auto-stainer of (Agilent, Santa Clara, CA, USA) as routine procedure. Four micron FFPE sections were pretreated with citrate pH 6.0 epitope retrieval buffer. Rabbit polyclonal TFF3 antibody (Novus Biologicals, Littleton, CO, USA) was diluted to 1/10 k using Dako antibody diluent and incubated for 20 min followed by 20-min secondary incubation. FLEX DAB + Substrate Chromogen system was used as post incubation detection reagent.

PTEN protein expression was assessed using IHC staining protocol as described by Huang et al. (Huang et al. 2016). PTEN was evaluated by FISH in the localized cohort as previously described (Bismar et al. 2012a, b).

Pathological analysis

Histological diagnoses of individual TMA cores were confirmed by the study pathologists (AHB and TAB) on the initial slides. Gleason scoring was assessed according to the 2014 WHO/ISUP grade groups. In each patient, the predominant two patterns of PCa were sampled and included on the TMAs for analysis. TFF3 expression was assessed using 4-tiered system (0; negative; 1, weak, 2 moderate and 3, high expression). To confirm reliability of the two cohorts, several pathological parameters were confirmed to reflect prognostic value such as surgical margins, pT and Gleason score. Figure 1 shows several examples of variable intensity (protein expression) for TFF3 and PTEN.

Statistical analysis

Descriptive statistics were used to describe the study data. Frequency and proportions were reported for categorical data. Mean and standard deviations were reported for continuous data. Chi square tests were used to compare two categorical variables, Fisher's exact test was used for cell frequency lower than 5. Overall survival (OS) was defined as the time from diagnosis to death; subjects who were alive at the end of the study period were censored. Prostate cancer-specific mortality (PCSM) was defined as death due to prostate cancer; subjects who died due to other reason or who were alive at the end of the study period were censored. Relapse-free survival (RFS) was calculated from the date of treatment to the date of relapse. OS, PCSM and RFS were analyzed using Kaplan–Meier method. Median time and the 95% confidence interval were reported. Log rank tests were used to compare two or more survival curves. Cox's proportional hazard model was used to determine the hazard ratio (HR) and the corresponding 95% confidence interval. Adjusted Cox's model was fitted as well. All statistical analyses were conducted

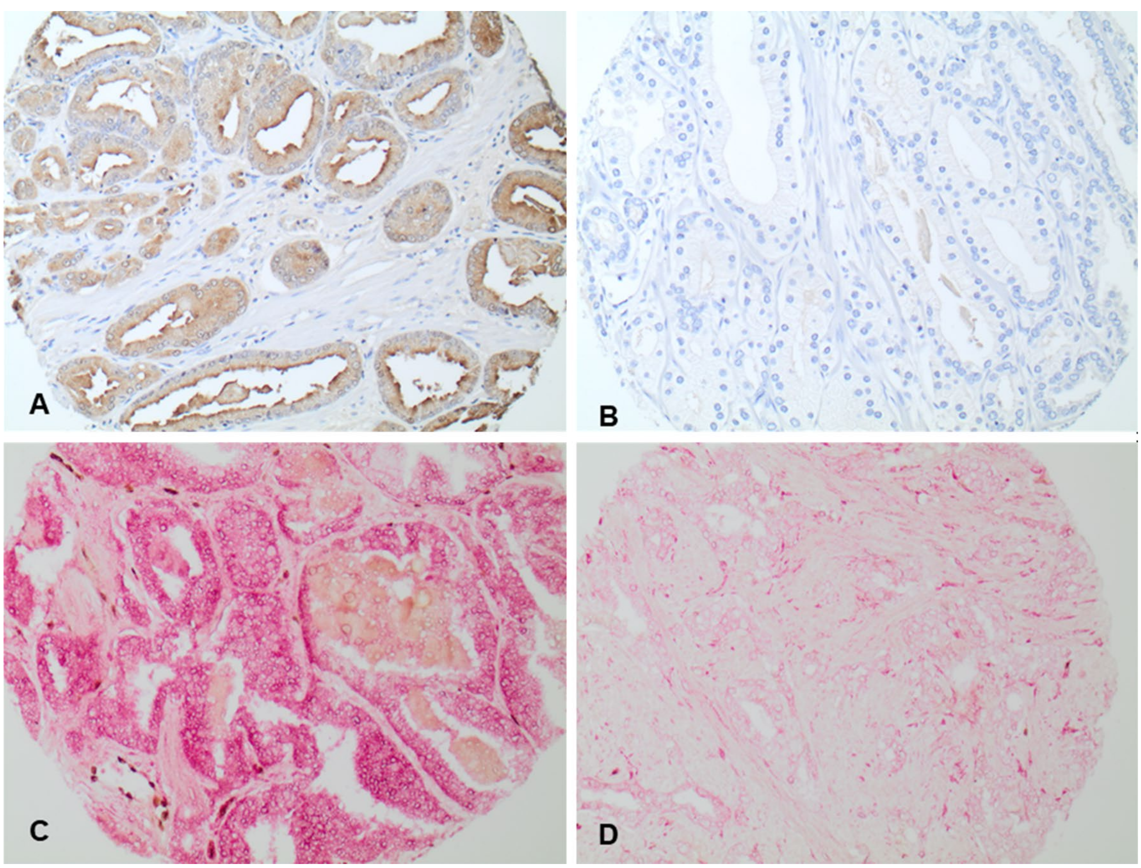

Fig. 1 Representative images of TFF3 and PTEN protein expression in PCa samples using a TFF3 (brown) and PTEN (red). **a**–**d** TFF3 positive, TFF3 negative, PTEN positive (intact) and PTEN low (negative), respectively

Table 1 Gleason score and PTEN status stratified by TFF3 in localized cohort ($n=228$)

Variables	TFF3 negative	TFF3 positive	p value
Gleason score			
≤6	20 (27.8)	43 (32.8)	0.587
3+4	17 (23.6)	32 (24.4)	
4+3	23 (31.9)	44 (33.6)	
8	6 (8.3)	7 (5.3)	
9–10	6 (8.3)	5 (3.8)	
PTEN			
Homozygous deletion	12 (17.6)	18 (15.1)	0.651
Hemizygous deletion/intact	56 (82.4)	101 (84.9)	

Table 2 Gleason score and PTEN status stratified by TFF3 in TURP cohort ($n=318$)

Variables	TFF3 negative	TFF3 positive	p value
Gleason score			
≤ 6	21 (14.7)	65 (51.6)	< 0.0001
3+4	10 (7)	16 (12.7)	
4+3	13 (9.1)	7 (5.6)	
8	13 (9.1)	7 (5.6)	
9–10	86 (60.1)	31 (24.6)	
PTEN intensity			
Absent	82 (56.9)	21 (16.0)	< 0.0001
Present	62 (43.1)	110(84.0)	

using IBM SPSS version 23. A p value < 0.05 was used for statistical significance and two-sided tests were used.

Result

Biomarkers expression in localized prostate cancer

TFF3 positivity was observed in 131/226 (57.9%) cases and although the expression was higher in lower-grade tumors, there was no significant association between TFF3 positivity and Gleason groups. However, TTF1 showed decreased positivity with advanced Gleason grade group with only 9.1% of cases in grade groups 4 and 5 expressing TFF3. Investigating relation to PTEN, TFF3 positivity was more frequently detected in cores without complete loss of PTEN (84.9%) (Table 1).

Biomarkers expression in non-surgical prostate cancer cohort

In the TURP cohort, TFF3 positivity was observed in 148/318 (46.5%) of cases and showed a significant inverse association with Gleason grouping with 51.6% of positive TFF3 cases occurring in Gleason grade group 1. As in the localized cohort, 84% of cases with TFF3 expression occurred in cores showing some sort of PTEN expression (weak, moderate or high) (Table 2).

Biomarkers and clinical outcome analysis

In the localized cohort, neither TFF3 nor PTEN status was associated with BCR post radical prostatectomy, while Gleason group was significantly associated with BCR ($p=0.011$). The risk of BCR was highest for patients with grade group 5 (i.e., Gleason score of 9 or higher; HR 3.7 (CI: 1.32–10.55; $p=0.013$) when compared to patients with a grade group 1 (i.e., Gleason score 6). This was in contrast to the TURP cohort, where each of the PTEN and TFF3 status was significantly associated with OS (HR 2.15; CI: 1.58–2.94; $p<0.0001$) for PTEN negative and (HR 2.31; CI: 1.67–3.18; $p<0.0001$) for TFF3 negative. This significance was also valid for PCSM (Tables 3, 4).

Table 3 C: HR of Gleason score and TFF3 Intensity for BCR in the localized cohort

Variables	Hazard ratio	95% confidence interval	p value
Gleason score (score ≤ 6)			
Gleason score 4+3	0.71	0.32–1.60	0.41
Gleason score 3+4	1.94	0.96–3.90	0.064
Gleason score 8	1.95	0.70–5.43	0.202
Gleason score 9–10	3.74	1.32–10.55	0.013
TFF3 intensity			
Negative	0.91	0.50–1.67	0.77

Table 4 HR of Gleason score and TFF3 Intensity for overall survival in the TURP cohort

Variables	Hazard ratio	95% confidence interval	p value
PTEN			
Negative	2.15	1.58–2.94	< 0.0001
Gleason score (score ≤ 6)			
Gleason score 4+3	2.50	1.31–4.75	0.005
Gleason score 3+4	1.74	0.93–3.26	0.085
Gleason score 8	3.92	2.05–7.49	< 0.0001
Gleason score 9–10	4.04	2.64–6.17	< 0.0001
TFF 3 intensity			
Negative	2.31	1.67–3.18	< 0.0001

Next, we queried the frequency of genetic alterations of TFF3 and PTEN in www.cBioportal.org. Five percent (25/487) of cases in TCGA (PanCancer) dataset exhibited various genetic alterations including amplification, deletion, and mRNA upregulation (Fig. 2a). Twenty-nine percent

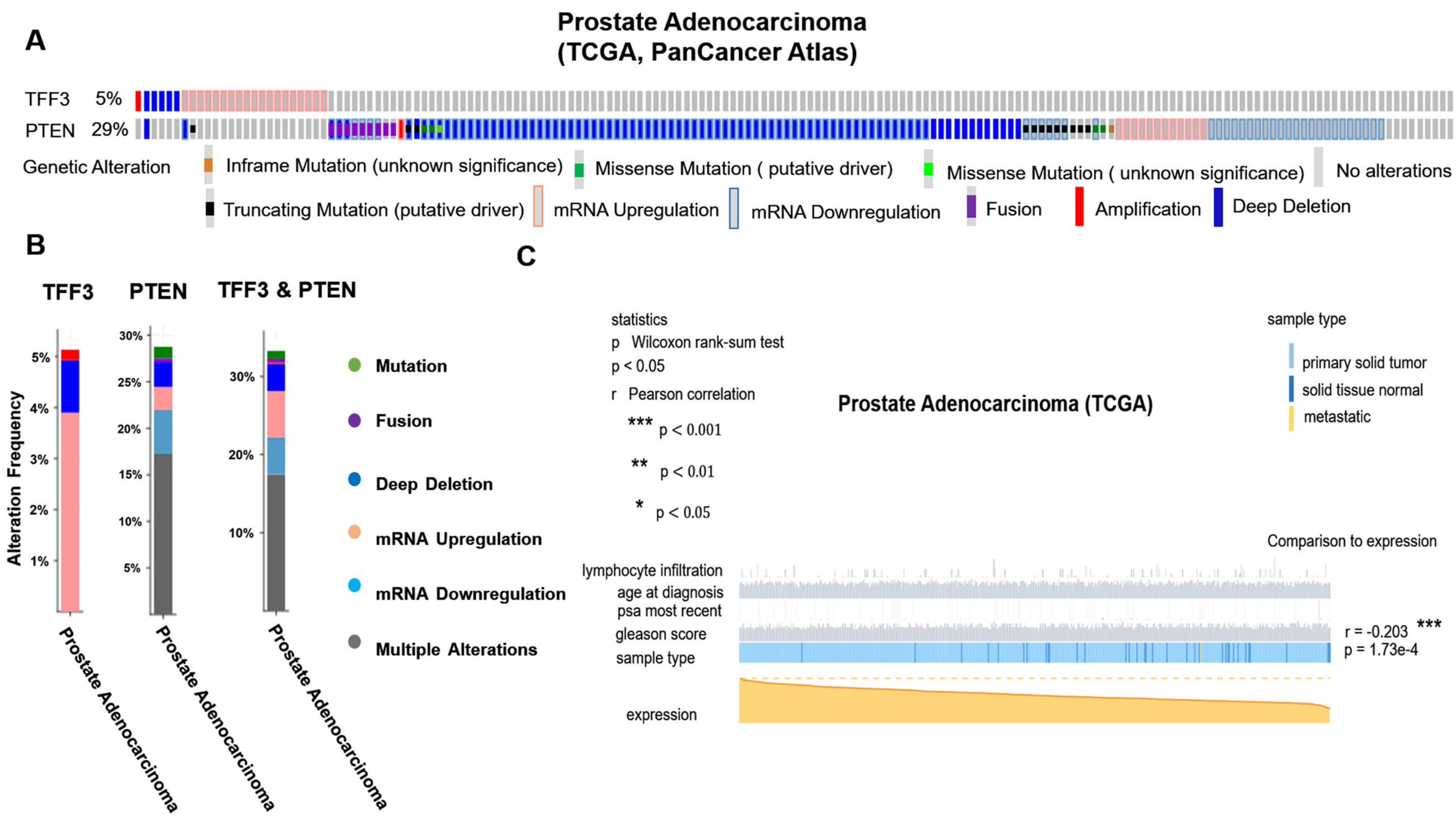

Fig. 2 Genetic alterations of TFF3 and PTEN in prostate cancer. **a** Oncoprint (cBioportal) diagram represents the alterations in TFF3 and PTEN genes in Prostate adenocarcinoma (TCGA, PanCancer Atlas) case set (494 patients). Cancer Study Selected was Prostate Adenocarcinoma (TCGA, PanAtlas). Genomic study selected were (1) Mutations, (2) Putative copy-number alteration from GISTIC, (3) mRNA Expression Z-scores with Z-score thresholds ± 2.0. cBioPortal (http://www.cbioportal.org) data are subjected to scheduled updates. **b** Summary of Alternation Frequency for TFF3, PTEN, and TFF3 and PTEN in TCGA PanCaner Dataset. **c** Visualization of the TCGA data for TFF3 in prostate adenocarcinoma using MEXPRESS (https://mexpress.be/). The 533 patients' samples were ordered by their expression values. Tumor samples tend to have higher TFF3 expression than normal samples. Advanced Gleason score samples significantly correlate with lower expression values

Table 5 Multivariate analysis and HR of the different biomarker combination to overall survival and cancer specific mortality in the TURP cohort

Variables	Overall survival		Prostate cancer-specific survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
PTEN (positive)				
Negative	2.15 (1.58–2.94)	< 0.0001	3.27 (2.12–5.04)	< 0.0001
TFF 3 positive				
Negative	2.31 (1.67–3.18)	< 0.0001	3.99 (2.43–6.56)	< 0.0001
Combination of PTEN and TFF3 (PTEN positive and TFF3 positive)				
PTEN loss and TFF3 negative	3.27 (2.17–4.92)	< 0.0001	7.04 (3.74–13.26)	< 0.0001
PTEN positive and TFF3 negative	2.83 (1.82–4.38)	< 0.0001	4.88 (2.48–9.59)	<0.0001
PTEN loss and TFF3 positive	3.79 (2.15–6.68)	< 0.0001	6.79 (2.80–16.47)	< 0.0001
Combination of PTEN and TFF3 (PTEN positive and TFF3 positive)[a]				
PTEN loss and TFF3 negative	2.33 (1.49–3.62)	< 0.0001	3.44 (1.75–6.78)	< 0.0001
PTEN positive and TFF3 negative	2.23 (1.41–3.51)	0.001	2.99 (1.48–6.03)	0.002
PTEN loss and TFF3 positive	2.57 (1.42–4.66)	0.002	3.40 (1.36–8.48)	0.009

[a]Adjusted for Gleason score

(140/487) of TCGA (PanCancer) samples showed various genetic alterations (Fig. 2a). The alterations in TFF3 and PTEN genes are distributed in a nearly mutually exclusive way across samples ($p = 0.04$). Only three cases that represent 0.61% (3/487) showed co-occurrence between TFF3 and PTEN genetic alterations. In total, 33.26% of 487 cases were genetically altered for both TFF3 and PTEN (Fig. 2b). Since TFF3 has shown to be principally exerting its bioactivity and prognostication in various cancers via its protein level, we asked if there is significant correlation between its expression level and prostate cancer clinical parameters. We used MEXPRESS tool to visualize, and interpret expression and clinical data available in TCGA (http://mexpress.be) (https://doi.org/10.1186/s12864-015-1847-z). Advanced Gleason score TCGA cases showed significant correlations with lower expression values (Fig. 2c).

Investigating the combined value of TFF3 and PTEN status confirmed even more prognostic association in prostate cancer to either one alone in relation to both the OS and the PCSM. Cases with TFF3/PTEN positive showed the best possible clinical outcome compared to other combinations. Patients with TFF3/PTEN negative had the highest association with prostate cancer-specific mortality (HR 7.04; CI: 3.74–13.26, $p < 0.0001$) and remained significant after adjusting for Gleason score (HR: 3.44; CI: 1.75–6.78; $p < 0.0001$). Table 5 shows detailed HR of the different biomarker combination to overall survival and cancer-specific mortality. Figure 3a–c shows survival curves using two and four group biomarker combinations. In this cohort, men within the "high-risk category" had median overall survival time of 25.72 vs. 76.81 months for men with best biomarker group combination ($p < 0.0001$) and median survival time for PCSM of 25.72 months vs. NR (Not reached) for men with best biomarker group combination ($p < 0.0001$). Furthermore, we investigated the prognostic value of TFF3 expression in various cancer types in www.kmplot.com. TFF3 low expression was found to be correlated with worse survival in patients with Cervical Squamous Cell Carcinoma ($p = 0.004$), Head and Neck Carcinoma ($p = 0.03$), and Uterine corpus endometrial carcinoma ($p = 5.6e{-}6$). (Supplementary Fig. 1A, 1B, and 1C).

Discussion

Earlier reports suggested that TFF3 and ERG expressions are mutually exclusive events cooperating in prostate cancer progression. Functionally, it has been shown that ERG expression has a dual regulatory effect on TFF3 expression via directly binding to the ETS binding sites on TFF3 promotor (Rickman et al. 2010). The same study demonstrated that ERG expression has an inhibitory effect on TFF3 in AR-naïve PCa but not in the castration-resistant state.

Although, earlier study had documented that TFF3 promoter is significantly hypo-methylated in PCa tumors associated with higher stage and BCR post RP, no detailed studies have been carried out in regards to TFF3 protein expression relative to its prognostic value to association to other biomarkers implicated in PCa progression. In the current study, we characterize the prognostic value for TFF3 in relation to PTEN expression in relation to BCR and lethal disease in two large cohorts of men, one treated by surgery and one non-surgically where patients were treated by androgen deprivation therapy for disease progression and castrate-resistant disease.

TFF3 was found to be of prognostic significance in relation to overall survival and lethal disease in patients not managed surgically compared to BCR post radical prostatectomy. In patients managed non-surgically, the prognostic value of TFF3 expression showed even stronger association

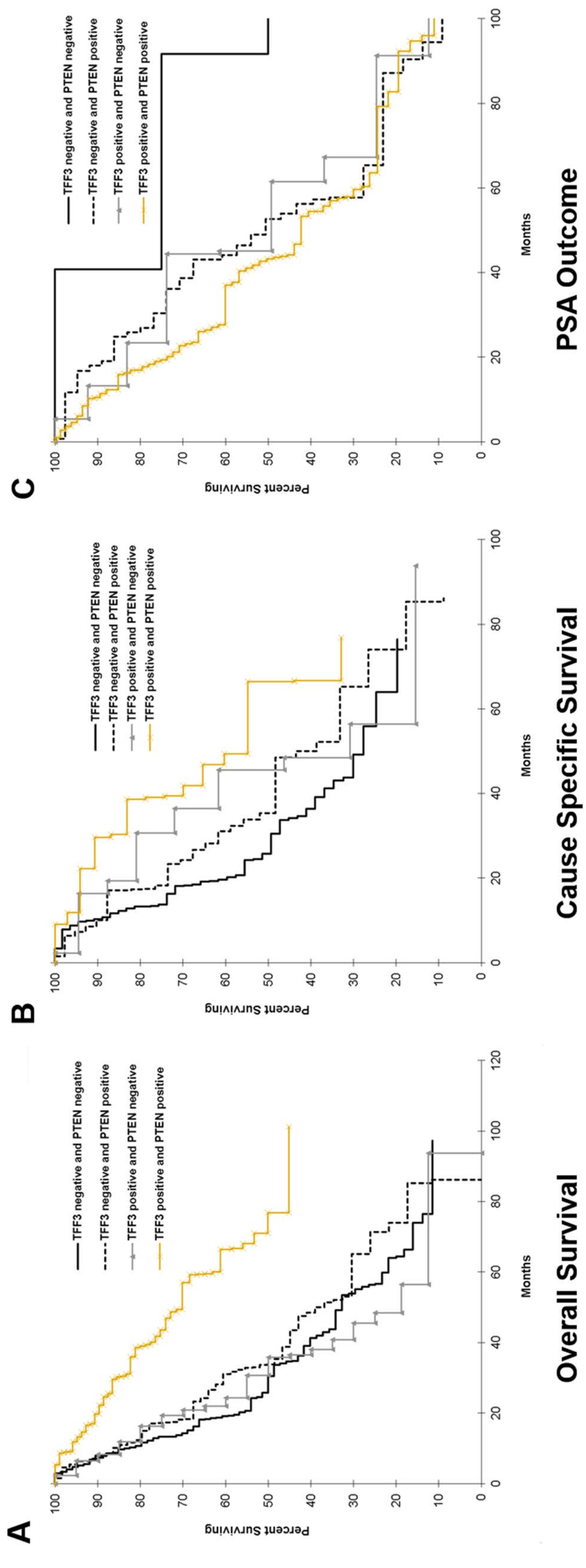

Fig. 3 KM curves for overall survival, Cancer-Specific Mortality and Biochemical Recurrence. **a** KM for overall survival of combined status of TFF3/PTEN expression. **b** KM for cause specific survival of combined status of TFF3/PTEN. C. KM curve for Biochemical Recurrence in Localized Cohort

with lethal disease and overall survival in tumors with concurrent loss of PTEN expression. This is intriguing given the fact that PTEN has been implicated in disease progression, overall survival and prostate cancer mortality. Two issues are worth noting in this study; the first, TFF3 was not associated with BCR while being significantly associated with lethal disease and overall survival in non-surgical cohort. This does not diminish its prognostic power, but contrary, support its powerful endpoint (OS and PCSM) vs. the softer association to post-surgical BCR, since most patients with BCR will not die of PCa. The second point is that TFF3 expression (negative/positive) in association with PTEN loss was still significantly associated with both PCSM and OS, even after adjusting for Gleason score. This is a further argument that TFF3 is one of the potential biomarkers to be assessed in PCa patients for prognostication, specifically those not seeking surgical intervention.

Our results support previously described oncogenic characteristics of TFF3, which documented increased cell proliferation, colony formation and cell migration. Additionally, recent data have implicated TFF3 in radio resistance, suggesting potential value in stratification of such patients. However, such data still need to be validated clinically in retrospective and prospective clinical trials.

Our data on the prognostic value of combined TFF3 and PTEN loss are further supported by previous report investigating some of the mechanisms underlying the tumorigenesis of PTEN loss and the functional significance of one of the P13 K signaling pathways, P110β molecule(Jia et al. 2008). The authors in that study showed that ablation of P110 β significantly hinders tumorigenesis driven by loss PTEN in the mouse prostate. More interestingly, it was shown that, using transgenic mice, the expression of *Tff3* was highly regulated (135.92 folds increase) in MF-p110β TG mice that express a constitutively activated p110β allele in the prostate. However, this *TFF3* up-regulation phenomenon was not demonstrated in PTEN-null mice (Lee et al. 2010). In the light of our paper here and previous P110 β work in mice, TFF3 might poses its unique role in PTEN-loss tumorigenesis.

In conclusion, the data presented here link TFF3 and PTEN in PCa prognosis as defining a lethal signature of PCa and the combination of TFF3/PTEN expression should be further investigated as potential biomarker signature for stratifying patients and perhaps identifying pathways to be targeted for disease progression.

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Compliance with ethical standards

The study was approved by the U Calgary Cumming School of Medicine ethics review board and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest The authors declare no conflict of interest.

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