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ORIGINAL ARTICLE

SPINK1 Overexpression in Localized Prostate Cancer: a Rare Event Inversely Associated with ERG Expression and Exclusive of Homozygous PTEN Deletion

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Abstract SPINK1 is proposed as potential prognostic marker in prostate cancer (PCA). However, its relation to PTEN and ERG in localized PCA remains unclear. The study population consisted of two independent cohorts of men treated by radical prostatectomy for localized PCA (discovery n = 218 and validation n = 129). Patterns of association between SPINK1 and each of ERG and PTEN were evaluated by immunohistochemistry and fluorescence in situ hybridization. Associations between SPINK1 expression and various pathologic parameters and clinical outcome were also investigated. SPINK1 was expressed in 15.3 % and 10.9 % of cases in the discovery and validation cohort, respectively. SPINK expression was observed in 5.56 % of high-grade prostatic intraepithelial neoplasia and 1.1 % of adjacent morphologically benign prostatic glands. SPINK1 and ERG expression were almost exclusive, with only 1.0 % of the cases co-expressing both in the same core sample. SPINK1 interfocal and within-core heterogeneity was noted in 29.2 % and 64.6 % of cases, respectively. SPINK1 expression was not significantly associated with PTEN deletion in the two cohorts (p = 0.871 for discovery cohort and p = 0.293 for validation cohort). While SPINK1 expression did occur with hemizygous PTEN deletion, there was a complete absence of SPINK1 expression in PCA showing homozygous PTEN deletion, which was confirmed in the validation cohort (p = 0.02). Despite SPINK1's association with higher Gleason score (>7) (p = 0.02), it was not associated with other pathological parameters or biochemical recurrence post-radical prostatectomy. We documented absolute exclusivity between SPINK1 overexpression and homozygous PTEN deletion in localized PCA. SPINK1 and ERG expressions are exclusive events in PCA. SPINK1 is not of added prognostic value in localized PCA.

Keywords ERG · PTEN deletion · Homozygous · Hemizygous · SPINK1 · Prostate cancer · Progression · Biomarkers

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Abbreviations

BAC	Bacterial artificial chromosome
CRPC	Castrate resistant prostate cancer
DAB	3,3′-Diaminobenzidine (DAB)
ERG	ETS-related gene
FISH	Fluorescence in-situ hybridization
GS(s)	Gleason score(s)
H&E	Haematoxylin and eosin
HGPIN(s)	High-grade prostatic intraepithelial neoplasia(s)
IHC	Immunohistochemistry

KDal	Kilodalton
PCA(s)	Prostate cancer(s)
PSA	Prostate-specific antigen
PSTI	Pancreatic secretory trypsin inhibitor
PTEN	Phosphatase and tensin homolog
SD	Standard deviations
SPINK1	Serine protease inhibitor Kazal-type 1
TATI	Tumour-associated trypsin inhibitor
TMA	Tissue microarray
TMPRSS2	Transmembrane protease, serine 2
UHN	University Health Network

Introduction

Serine protease inhibitor Kazal-type 1 (SPINK1), also called tumour-associated trypsin inhibitor (TATI) and pancreatic secretory trypsin inhibitor (PSTI), is a 56-amino-acid (6 kDa) protein that is encoded by a four-exon gene [1]. It has been shown to protects against pancreatitis by inhibiting activation of trypsin within the pancreatic parenchyma [2].

SPINK1 is overexpressed in approximately 10 % of localized prostate cancer (PCA) highly mutually exclusive of ERG alterations [3–9]. However, the prognostic significance of SPINK expression remains controversial. While earlier studies suggested an association with aggressive behavior of PCA in radical prostatectomy [4] and hormone-treated PCA cohorts [10], the majority of subsequent studies found no significant association with clinico-pathologic parameters or outcome [3, 8, 11] apart from a recent study showing association of SPINK1 expression with biochemical recurrence after radical prostatectomy [12] and another showing association with improved recurrence free survival [13].

The development and progression of PCA both involve accumulation of multiple genetic aberrations leading to perturbation of several tightly interacting signalling pathways. It is well known that genomic aberrations of the androgen receptor, ERG and PTEN interplay in PCA and are responsible in part for the development of castrate resistant PCA (CRPC) [14]. We recently characterized SPINK1 overexpression in relation to AR, ERG and PTEN in CRPC, where we found SPINK1 overexpression to be positively associated with PTEN deletion in CRPC and is preferentially associated with hemizygous PTEN deletion rather than homozygous deletions (in contrast to its inverse association to ERG rearrangements) [15]. However, the relation of SPINK1 overexpression to specific types of PTEN deletion as well as the heterogeneity of its expression pattern has not been well characterized in localized PCA. To date, studies in localized PCA cohorts have found SPINK1 protein expression to be inversely associated with PTEN gene deletion [11] and positively associated with higher PTEN protein expression [3], in contrast to our results in CRPC. However, these results have not stratified the types of PTEN deletions into hemizygous or homozygous-deletion types which could have differing prognostic impacts in PCA progression with, for instance, earlier onset of biochemical relapse for PCA harboring PTEN homozygous deletion compared to those with hemizygous deleted or intact PTEN gene [16]

The aim of the current study was to clarify the clinical significance of SPINK1 protein overexpression in localized PCA, including its potential association with pathologic parameters and biochemical recurrence using two large radical prostatectomy cohorts for discovery and validation studies. We also assessed SPINK1 heterogeneity within-core and between PCA foci in the same patients and investigated its relationship to ERG protein expression and PTEN deletions.

Material and Methods

Study Population and Tissue Microarray Construction

The first study cohort, used as the discovery cohort, consisted of 218 patients treated by retro-pubic radical prostatectomy as initial monotherapy for localized PCA between 1992 and 2004. The mean follow-up period was 42 months (range 0–142). Clinical and pathological data were obtained with approval from the Institutional ethical review Board. Biochemical relapse was defined by a rise of PSA (prostate-specific antigen) levels of >0.2 ng/ml at two separate occasions following undetectable levels post radical prostatectomy. Gleason scores (GS) were assessed according to the ISUP 2005 modified Gleason score [17]. Prostate samples were embedded on three tissue microarray (TMA) blocks using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). Each block was assembled without prior knowledge of any clinical or pathological staging information. An average of three 0.6 mm diameter cores (range 1–9) per case were sampled per patient to include benign prostatic glands, high-grade prostatic intraepithelial neoplasia (HGPIN) and PCAs representing the predominant two Gleason patterns in the radical prostatectomy. A total of 1267 cores (966 cancer, 112 HGPIN and 189 benign) were assembled into three TMA blocks. After construction, 5 μm sections were cut and stained with haematoxylin and eosin (H&E) to verify the histological diagnosis and Gleason scores.

The second independently constructed cohort from another center, used as the validation cohort, was from UHN (University Health Network, Toronto, Ontario, Canada). It had been assembled specifically to address heterogeneity in localized PCA in radical prostatectomy specimens obtained between 2000 and 2001 as described previously [19]. Where possible, up to three 0.6 mm cores per tumor focus were sampled from different areas within a given focus with a secondary aim of sampling different Gleason patterns within and

between different PCA foci in the same prostatectomy specimen. A total of 724 cores were included in the TMA with on average up to six cores from each prostatectomy specimen represented on the TMA. Non-tumor prostate tissues were also sampled for control purposes. In contrast to the discovery cohort described above, HGPIN was not intentionally sampled for the validation TMA. After construction, 5 μm sections were cut and stained with H&E to verify the histological diagnosis and GSs.

SPINK1 and ERG Protein Expression by Immunohistochemistry

SPINK1 immunohistochemistry (IHC) was performed as per Bismar et al. [15] with 1:25 dilution of SPINK1 monoclonal antibody (WH0006690M1, Clone 4D4, Sigma-Aldrich, St. Louis, Missouri, USA) or 1:50 dilution of ERG rabbit monoclonal antibody (Clone 9FY, Biocare Medical, Concord, California, USA). Ventana iView DAB detection kit (Tucson, Arizona, USA) was used for horseradish peroxidase detection and counter stain. For the evaluation of SPINK1 in the discovery cohort, cytoplasmic staining intensities above benign glands and stroma were interpreted as being positive for SPINK1 expression. SPINK1 positive cases were defined as those with at least one core showing positive SPINK1 immunoreactivity. Figure 1 shows examples of different intensities of SPINK1 IHC staining in malignant prostatic glands with stroma and benign glands as negative controls. In the UHN validation cohort and using a threshold for SPINK1 positivity as staining at or above medium intensity, the rate of SPINK1 positivity was noted in 10.9 % of PCA cases, slightly lower than 15.3 % in the discovery cohort.

ERG IHC was carried out as previously described in Huang et al. [18]. ERG expression in prostate glands was essentially either present or absent and is therefore recorded as binary values (positive vs. negative). ERG protein expression was previously correlated to ERG gene rearrangement as detected by fluorescent in-situ hybridization (FISH) break-apart probe [15] (data not shown). ERG expression as assessed by IHC was consistently and strongly positive in endothelial cells which acted as internal control.

Assessment of PTEN and ERG Gene Alterations

PTEN genomic deletions were assed as described in Bismar et al. [15]. Briefly, PTEN genomic deletions were assessed using four-colour interphase FISH strategy as PTEN deletions are interstitial and are usually restricted to small regions of chromosome 10. The probe design incorporates closely flanking control probes that minimize false positive deletion events caused by nuclear truncation losses of paraffin sectioning using four bacterial artificial chromosome (BAC) clones spanning both flanking PTEN genomic regions (BMPR1A and FAS loci), the PTEN gene locus and a commercially available DNA probe for the centromeric region 10p11.1–q11.1 (Spectrum- Aqua labelled CEP 10; Vysis Inc., Downers Grove, IL, USA). We evaluated the copy number for each probe by counting spots in 100 non-overlapped, intact inter-phase nuclei per tumour tissue core. The establishment of PTEN gene copy number status was defined by considering the adjacent probes (BMPR1A and FAS loci) used for the truncation artefacts, aneusomy, nuclear size and chromatin condensation. Based on hybridization in control cores (data not shown), hemizygous deletion of PTEN were defined as

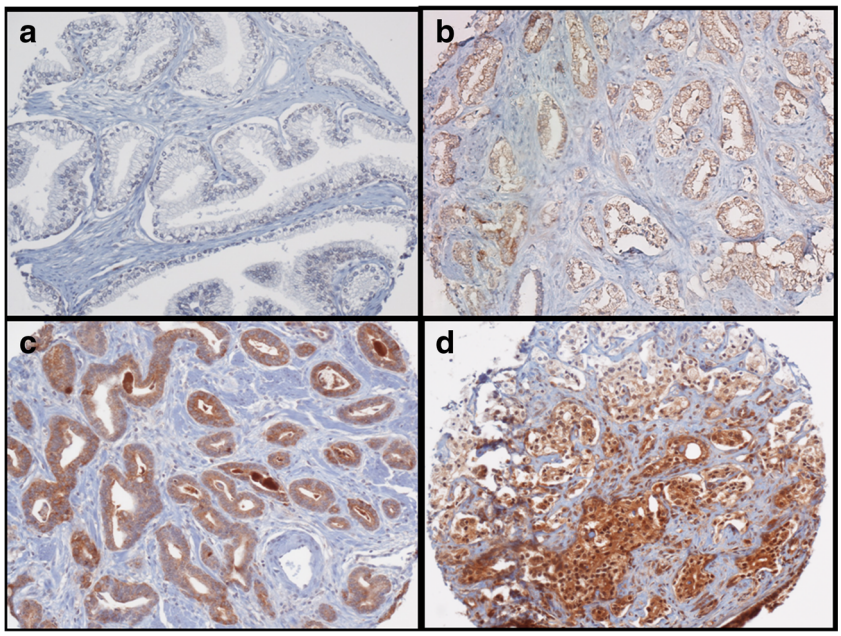


Fig. 1 IHC evaluation of SPINK1 in TMA prostate cancer tissues: **a** Benign prostatic glands and stroma, negative for SPINK1 expression. **b** Gleason score 6 PCA showing weak SPINK1 staining. **c** Gleason score 6 PCA showing moderate SPINK1 staining. **d** Gleason score 7 (4 + 3) PCA showing focal strong SPINK1 staining. (Original magnification 200×)

>20 % [mean ± 3 standard deviations (SD) in non-neoplastic controls] of tumour nuclei containing one PTEN locus signal and by the presence of CEP 10 chromosome enumeration signals and the presence or absence of flanking control probes. PTEN homozygous genomic losses were exhibited by the simultaneous lack of both PTEN locus signals and by the presence of control signals in 20–100 % of cells. For tumours with intra-focal heterogeneity, 100 nuclei were examined in each available focal region (Supplementary Fig. 1).

For the UHN cohort, PTEN andTMPRSS2-ERG gene rearrangement status was determined by FISH as described previously [19].

Statistical Analysis

Patient characteristics were presented as frequencies and percentages for categorical variables, and as means and ranges for continuous variables. Chi-square tests were used to test for associations between SPINK1, ERG and PTEN status as well as pathological parameters (GS, surgical margin status, and pathological stage) unless otherwise specified. The Kaplan-Meier approach along with the log-rank test was used to test the association between SPINK1 expression and biochemical PSA relapse. In all statistical tests, a p value <0.05 was considered significant.

Results

Clinical and Pathological Demographics of the Study Cohorts

The discovery cohort (n = 218) of patients who underwent radical prostatectomy for localized PCA had an average follow-up period of 42 months (range 0–142). In this cohort, complete follow-up data related to PSA biochemical relapse was available for 180 patients. Overall, 59/180 (32.7 %) of patients demonstrated biochemical PSA relapse over the follow-up period.

For the validation cohort, complete follow-up data related to PSA biochemical relapse was available for all 129 patients with evaluable PTEN, ERG and SPINK1 data. Overall, 28/129 (21.7 %) of patients demonstrated biochemical PSA relapse over the study follow up period.

SPINK1 Expression by Immunohistochemistry in Localized Prostate Cancer in Discovery Cohort

SPINK1 exhibited cytoplasmic staining pattern and was detected predominantly in PCA epithelium. In the discovery cohort, 33/215 (15.3 %) of PCA cases exhibited positive SPINK1 expression in PCA epithelium. When SPINK1 staining intensity was assessed on a core-by-core basis, SPINK1 expression was detectable in 65/955 (6.8 %) of PCA cores. Although this cohort was not designed specifically to assess interfocal heterogeneity, interfocal SPINK1 heterogeneity [i.e. different SPINK1 status (negative vs. positive) between different tumor foci] was noted in 7/24 (29.2 %) of PCA cases. Within-core SPINK1 heterogeneity [i.e. different SPINK1 status (negative vs. positive) within the same tumor core was noted in 42/65 (64.6 %) of PCA cores (Fig. 2a,b). SPINK1 was also detected in 5/90 (5.56 %) of HGPINs, however, this rate of expression was not significantly different from that observed in PCA (p = 0.83; Fisher's exact test). Of the 3 SPINK1-positive HGPINs with adjacent PCA, 2 showed discordant negative SPINK1 expression in the adjacent PCA glands (Fig. 2c; representative image) and 1 showed positive SPINK1 expression in the adjacent PCA (Fig. 2d), but the small number of these occurrences did not allow further investigations. SPINK1 was detected rarely in 2/191 (1.1 %) adjacent morphologically benign cores (Supplementary Fig. 2) at a rate that is significantly lower than in PCAs (p = 0.0006; Fisher's exact test) and HGPINs (p = 0.036; Fisher's exact test).

Association of SPINK1 Expression with ERG Expression in Discovery Cohort

It is well documented that ERG expression detected by IHC correlates highly with ERG gene rearrangement status [20, 21]. In the discovery cohort, ERG protein expression was noted in 88/202 (43.6 %) of patients. SPINK1 expression was significantly more frequent in ERG negative cases compared to ERG positive cases [(27/114 (23.7 %) of ERG negative PCA cases vs. 2/88 (2.2 %) of ERG positive cases; p = 0.0001; Fisher's exact test] (Table 3). In other terms, 93.1 % (27/29) of the SPINK1 expressing tumors were ERG negative and 6.9 % (2/29) of the SPINK1 expressing tumors were ERG positive. This preferential SPINK1 expression in ERG negative PCAs was also maintained when the expression was assessed on a core-by-core basis. Specifically, SPINK1 expression was detected in 48/481 (10.0 %) of ERG negative and 2/298 (0.7 %) of ERG positive PCA cores (p = 0.0001; Fisher's exact test). The 2 PCA cores with concomitant expression of SPINK1 and ERG from two separate patients are shown in Fig. 3.

Association of SPINK1 Expression with PTEN Deletion in Discovery Cohort

Based on the strong association between SPINK1 and ERG expression and the previously characterized association between ERG rearrangements and PTEN deletions [19], we also sought to determine whether SPINK1 expression might be linked to PTEN deletion status in the discovery cohort. PTEN deletion status (hemizygous or homozygous) was

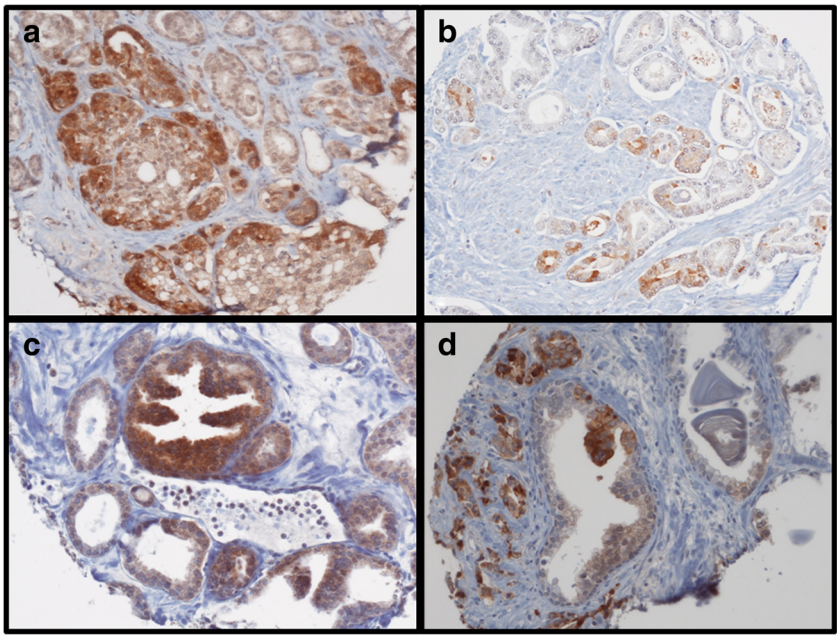


Fig. 2 SPINK1 heterogeneity of expression in malignant prostatic epithelium and HGPIN: **a** and **b** Representative images of within-core heterogeneity of SPINK1 expression in PCA from two different cases. **c** Representative image of a SPINK1-expressing HGPIN with discordant SPINK1 expression in adjacent PCA glands. **d** Heterogeneous SPINK1 expression in HGPIN and adjacent PCA glands expressing SPINK1. (Original magnification 200×)

assessed by FISH analysis and was positive for PTEN deletion in 202/715 (28.3 %) of overall PCA cores. On core-by-core analysis, SPINK1 staining showed no significant association with overall PTEN deletion [(7.2 % (37/513) in wild-type cores vs. 6.4 % (13/202) in cores with any type of PTEN deletion; p = 0.871; Fisher's exact test]. However, significantly, all of the 13 cores positive for both SPINK1 expression and PTEN deletion exhibited hemizygous PTEN deletion, with no homozygous PTEN deletions observed in any SPINK1-positive core (p = 0.021; Fisher's exact test) (Table 1).

Validation of SPINK1 Expression Pattern in Relation to ERG and PTEN Gene Status in the Validation Cohort

Of the 129 patients with complete PTEN, ERG and SPINK 1 data, 10.9 % (14/129) of cases showed at least one SPINK1 positive PCA core on TMA, 37.2 % (48/129) of cases had demonstrable PTEN deletion by FISH (34 hemizygous and 14 homozygous) and 45.7 % (59/129) of cases showed TMPRSS2-ERG rearrangement and/or interstitial deletion by FISH. When SPINK1 staining was assessed in relation to PTEN status,

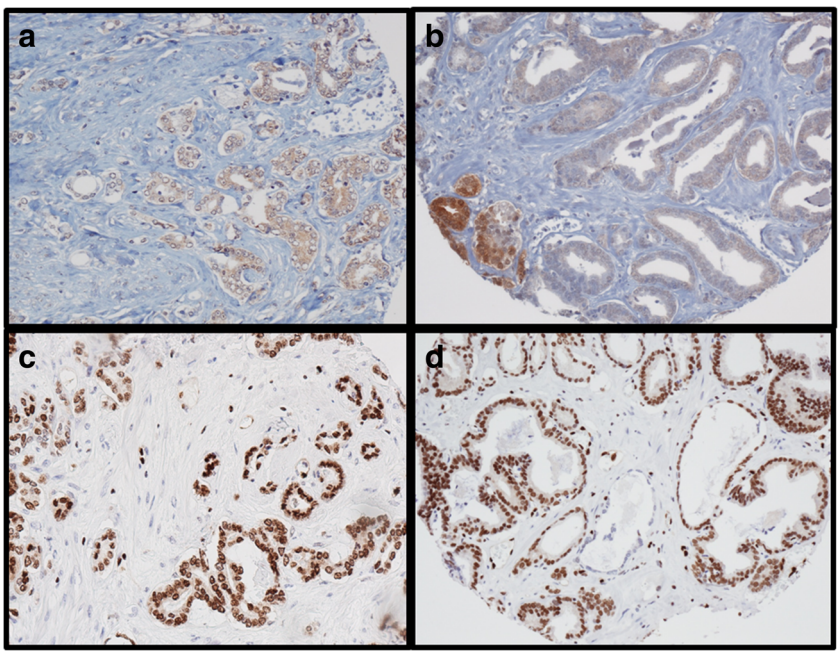


Fig. 3 IHC images of the only two rare TMA cores from two separate cases showing co-expression of SPINK1 and ERG: **a** and **c** represent one of the TMA cores and **b** and **d** the other TMA core. **a** and **b** show SPINK1 expression and **c** and **d** show ERG expression. Note the heterogeneity of SPINK1 expression in contrast to homogenous ERG expression. (Original magnification 200×)

Table 1 SPINK1 expression and *PTEN* genomic aberration status by TMA PCA core diagnosis in the discovery cohort

	PTEN status, n (%)		
	Not deleted	Hemizygous	Homozygous
SPINK1 +	37 (74.0)	13 (26.0)	0 (0) *
SPINK1 -	476 (71.6)	133 (20.0)	56 (8.4)

*No significant association between SPINK1 expression and PTEN deletion (p = 0.871). However, none of the 56/715 PCA-cores with homozygous PTEN-deletion exhibited SPINK1 expression which was significant (p = 0.021)

42.9 % (6/14) of the SPINK1 positive cases had some form of PTEN deletion in at least one of the cores, with hemizygous or homozygous deletion detected in 35.7 % (5/14) and 7.1 % (1/14) of SPINK1 positive cases, respectively. On core-by-core analysis, SPINK1 staining showed no significant association with PTEN deletion overall [(4.6 % (28/604) in wild-type cores vs. 1.9 % (2/106) in cores with any type of PTEN deletion; p = 0.2933; Fisher's exact test]. However, in agreement with the results in the discovery cohort, both of the cores with concomitant SPINK1 expression and PTEN-deletion had PTEN deletions of the hemizygous type. No homozygous PTEN deletions were observed in any SPINK1-positive core.

When assessed for TMPRSS-ERG rearrangement/interstitial deletion status, 14.3 % (2/14) of SPINK1 positive cases were positive for TMPRSS2-ERG rearrangement/interstitial deletion in at least one of the cores. Of note, none of the SPINK1 positive cases showed concomitant SPINK1 expression and TMPRSS2-ERG rearrangement/interstitial deletion within the same core. These observations confirmed the results in the discovery cohort showing that SPINK1 expression occurs exclusively in PCA foci without PTEN homozygous deletion and is preferentially expressed in PCA foci lacking TMPRSS2-ERG gene rearrangements.

This validation cohort was designed to specifically assess PCA tumor heterogeneity within and between PCA foci in a given prostatectomy specimen [19]. 83.3 % (25/30) of all SPINK1 positive cores showed within-core heterogeneity, a value comparable to the 64.6 % in the discovery cohort. 64.3 % (9/14) of the SPINK1 positive cases showed heterogeneous SPINK1 expression status (negative vs. positive) between PCA foci located on the left and right lobes of the prostate (i.e. positivity restricted to either left or right lobe cancer foci).

Expression of SPINK1 in Relation to Pathologic Parameters and Biochemical Relapse in Discovery and Validation Cohorts

The relation of SPINK1 expression to pathologic parameters was assessed in the discovery cohort. There was significant difference in the GSs (GS ≤ 7 vs. >7) of the PCAs between SPINK1 positive and SPINK1 negative PCA cores, with 11.6 % (14/120) of GS > 7 PCAs exhibiting SPINK1 expression versus 6.1 % (51/835) of GS ≤ 7 PCAs (p = 0.032; Fisher's exact test) (Table 2). However, this association was diminished when PCAs were stratified to GS <7 vs. 7 vs. >7 PCA subgroups (p = 0.077 Pearson Chi-Square; data not shown), and was lost when PCAs were stratified into GS <7 vs. 7(3 + 4) vs. 7(4 + 3) vs. >7 PCA subgroups; (p = 0.56 Pearson Chi-Square; data not shown). SPINK1 expression was not associated with any other pathological parameters, including pathologic T stage, and surgical margin status (Table 3). Similarly, there was no association between SPINK1 expression and biochemical relapse post-radical prostatectomy (p = 0.284; Pearson Chi-Square) or time till relapse (p = 0.90; Kaplan-Meier analysis) (Fig. 4).

In the validation cohort, there was no association between SPINK1 overexpression and biochemical failure post-prostatectomy with 21.4 % (3/14) and 22.6 % (26/115) of the SPINK1-positive and SPINK1-negative cases, respectively, exhibiting biochemical failure post-prostatectomy (p = 1.0000; Fisher's exact test). There was also no significant association between SPINK1 expression and PCA-specific death with PCA-specific death observed in 0.0 % (0/14) and 2.6 % (3/115) of SPINK1-positive and SPINK1-negative cases, respectively (p = 1.0000; Fisher's exact test).

Discussion

In the two cohorts studied, 15.3 % and 10.9 % of the PCA cases showed at least one focus of SPINK1 expression, similar to the approximately 10 % rate previously been reported in localized PCA in radical prostatectomy cohorts [22]. The variability in the reported SPINK1 positivity in PCA (range 5–99 %) in IHC studies may be due in part to differences in cohort characteristics (i.e. localized vs. metastasized), antibodies and staining procedures, and IHC scoring criteria in different studies [22].

To date, the majority of reports have shown that SPINK1 and ERG expression, with rare exceptions, tend be exclusive of each other [22]. This is confirmed by our current study with 1.0 % (2/202) of all cases (represented by one TMA PCA core from

Table 2 SPINK1 expression and Gleason score of PCA by TMA core diagnosis

Gleason Score*, n (%)		
	≤7	>7
SPINK1 +	51 (6.1 %)	14 (11.7 %)
SPINK1 -	784 (93.9 %)	106 (88.3 %)

*p = 0.024

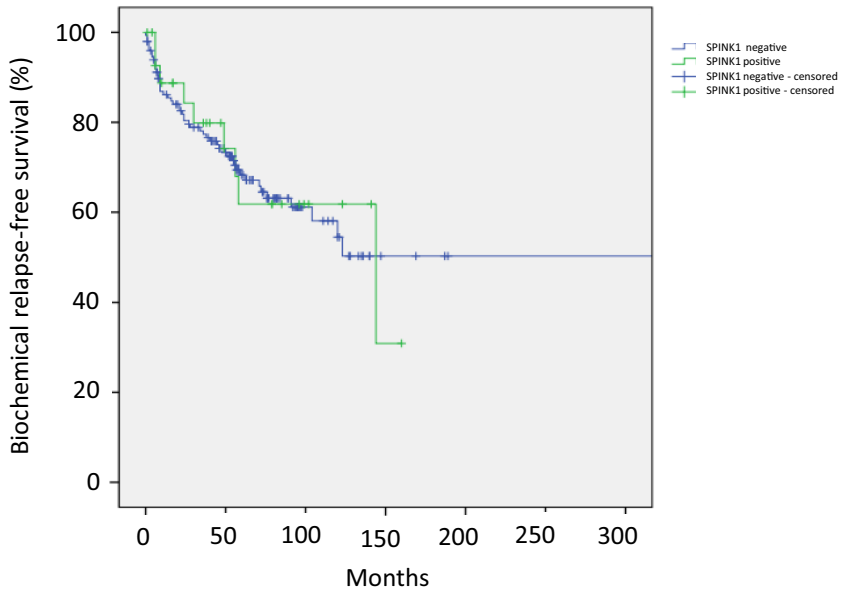
Table 3 Summary of patient characteristics in relation to clinico-pathologic parameters according to SPINK1 expression status

	SPINK – cases	SPINK + cases	p-value
PSA relapse			0.284
Yes	54 (34.2 %)	5 (22.7 %)	
No	104 (65.8 %)	17 (77.3 %)	
ERG expression			0.0001
Negative	87 (50.3 %)	27 (93.1 %)	
Positive	86 (49.7 %)	2 (6.9 %)	
Gleason score			0.555
< 7	59 (31.7 %)	7 (26.9 %)	
7	105 (56.5 %)	14 (53.8 %)	
7(3 + 4)	58 (31.2 %)	9 (34.6 %)	
7 (4 + 3)	47 (25.3 %)	5 (19.2 %)	
> 7	22 (11.8 %)	5 (19.2 %)	
pT			0.314
T2	111 (64.9 %)	23 (74.2 %)	
T3	60 (35.1 %)	8 (25.8 %)	
Surgical margin			0.457
Negative	93 (54.1 %)	19 (61.3 %)	
Positive	79 (45.9 %)	12 (38.7 %)	

each of the 2 case) co-expressing both SPINK1 and ERG-fusion proteins in the discovery cohort (Fig. 3). Of note, in contrast to results of the current and other studies, one study using endocrine-treated PCA specimens found no difference in the frequency of the SPINK1 expression in TMPRSS2-ERG fusion positive and fusion-negative PCAs [10], most likely due to differences in the study cohort (endocrine-treated vs. non-endocrine treated) with other contributors being differences in criterion for SPINK1 positivity and/or the methods of assessing the TMPRSS2-ERG fusion status [22].

Our observation of a small subpopulation of SPINK1 and ERG co-expressing PCA is consistent with some of the studies to date in localized PCA reporting 0–4 % of PCA cases with concomitant SPINK expression and ERG alteration [22]. As demonstrated by some of these studies [3, 23], co-expression of both SPINK1 and ERG is a focal event in PCA and may not be detected in a given study depending on the detection method employed and/or the power of the studies in question [3]. However, it is also possible that these ERG and SPINK1 co-expressing foci may represent collision tumors as discussed in detail by Smith and Tomlins. [22]. Further studies are required to determine the nature and biological significance of this small albeit significant subpopulation of SPINK1/ERG co-expressing PCA.

PTEN deletion has been found to correlate with PCA tumor progression [15] and the degree of PTEN haploinsufficiency may also correlate with PCA biologic behavior and progression. For instance, while hemizygous PTEN deletion is linked to earlier biochemical relapse, homozygous deletion is associated with metastasis and androgen-independent progression (reviewed in Yoshimoto et al. [19]). The relation between SPINK1 expression status and PTEN deletion has not been documented fully. To our best knowledge, only three studies have assessed the relation between SPINK1 expression and



	Total N	N of Events	Censored	
			N	Percent
SPINK1 -	149	50	99	66.4%
SPINK1 +	29	9	20	69.0%
Overall	178	59	119	66.9%

Fig. 4 Kaplan-Meier analysis of biochemical relapse-free survival in prostatectomy-treated patients stratified by SPINK1 expression. $p = 0.90$ (log rank Mantel-Cox)

PTEN deletion with contradictory patterns of association, albeit that one study utilized a CRPC cohort and the other two radical prostatectomy cohorts [3, 11, 15]. Specifically, in PCA treated with radical prostatectomy, protein expression of SPINK1 correlated with higher level of PTEN protein expression [3] and absence of PTEN deletion [11]. In our study, we sought to ascertain the relation of SPINK1 expression to PTEN gene deletion in localized radical prostatectomy cohorts as well as to further subtype the associated PTEN deletion pattern into homozygous and heterozygous deletion types. In both the discovery and validation cohorts in the current study, SPINK1 expression showed no significant association with PTEN-deletion, in contrast to previous results in other radical prostatectomy cohorts [3, 11], perhaps due to a difference in the power of the cohorts secondary to smaller number of patients in our study compared to the other two studies. The present result is also opposite of what we had found in a localized CRPC cohort where SPINK1 expression was positively associated with PTEN-deletion [15]. However, in the present study we also document for the first time in both the discovery and validation cohorts of localized radical prostatectomy PCA that while hemizygous PTEN deletion can occur with SPINK1 expression, none of the SPINK1 positive PCA cores exhibited homozygous PTEN deletion ($p = 0.021$ for the discovery cohort; Fisher's exact test). In the validation cohort, only two cores were positive for SPINK1 and PTEN deletion, both of which were hemizygous (a number too small for meaningful statistical analysis). These suggest that SPINK1 overexpression is an early event in PCA and does not occur in the presence of complete loss of PTEN gene product in localized PCA. However, as we previously documented, SPINK1 can co-exist with complete PTEN loss in locally advanced CRPC. The mechanism for this difference in observation in localized radical prostatectomy and CRPC cohort is unknown. Future studies will be required to elucidate the mechanism and significance of the mutual exclusivity of SPINK1 expression and homozygous PTEN deletion in localized PCA. Specifically, studies will be required to confirm if this mutual exclusivity is lost with PCA progression in other cohorts, and whether detection of PTEN homozygous deletion in SPINK1 expressing PCA has prognostic significance.

Although our previous study suggested that SPINK1 in CRPC does not exhibit intrafocal or interfocal heterogeneity, both our discovery and validation cohorts in the current study demonstrated high levels of within-core (64.6 % and 83.3 %) and interfocal (29.2 % and 64.3 %) heterogeneity of SPINK1 expression, with higher values found in the validation cohort. Our results of prominent heterogeneity in SPINK1 expression down to the level of individual PCA cores and glands (Fig. 2 a, b) are in concordance to previous reports [4, 5, 10, 23]. This high within-core heterogeneity is in contrast to what had been documented for TMPRSS2-ERG rearrangement, which show inter-focal heterogeneity but near homogeneous of expression for a given focus [5, 19, 24]. The heterogeneity of SPINK1 expression is thus more similar to PTEN genomic deletions which demonstrate both intra- and inter-focal heterogeneity [19]. These suggest that SPINK1 expression in PCA may occur as a subclonal event subsequent to ERG-fusion gene expression. However, our observation of SPINK1 expression in 5.56 % of HGPINs (Fig. 2c,d) and the presence of SPINK1-positive PCA glands adjacent to 1/3 of SPINK1-positive HGPINs with adjacent PCA (Fig. 2d) suggest that SPINK1 expression may also occur as an early molecular event independent of ERG gene expression. The finding that there is no significant difference between the frequencies of SPINK1 expression in HGPINs and PCAs also supports the notion that SPINK1 expression can be an early molecular event in PCA progression. Larger studies are required to address the role of SPINK1 in early PCA tumorigenesis.

In this study, we also assessed the association of SPINK1 protein expression to pathological parameters including GS, pathologic T stage and surgical margin status. While there was significant positive association between SPINK1 expression and GS when cases were stratified broadly into low-intermediate- ($GS \leq 7$) vs. high-risk ($GS > 7$) subgroups ($p = 0.024$; Fisher's exact test), this association was diminished when the PCA-cores were stratified further into low- ($GS < 7$) vs. intermediate- ($GS = 7$) vs. high-risk ($GS > 7$) PCA subgroups ($p = 0.077$; Fisher's exact test), and was lost when stratified further into $GS < 7$ vs. 7(3 + 4) vs. 7(4 + 3) vs. >7 PCA subgroups ($p = 0.56$). Moreover, there was no association between SPINK1 and any other pathologic parameters including biochemical recurrence post-radical prostatectomy.

Although a limited number studies had documented SPINK1 protein expression to be significantly associated with biochemical recurrence in localized radical prostatectomy cohort [4], the majority of studies including our current study failed to confirm such an association, and suggest that SPINK1 expression itself likely may not be an independent prognostic marker for PCA progression [3, 8, 11]. Moreover, the high level of heterogeneity of SPINK1 expression is likely to hinder clinical utility of SPINK1 as a prognostic marker if sampling procedures are not adequate.

Conclusion

In summary, our findings suggest that SPINK1 expression may be an early molecular event in PCA development and confirms its predominant mutual exclusivity to ERG protein expression. We also document for the first time a complete absence of SPINK1 expression in PCA harboring homozygous PTEN deletion in two localized PCA prostatectomy cohorts. Future studies will be required to determine the clinical significance of this subpopulation of SPINK1 expressing PCA that have not lost both copies of the PTEN gene, and to fully

characterize SPINK1 and PTEN gene interaction in PCA progression. Such studies may be of potential prognostic and therapeutic importance.

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Compliance with Ethical Standards

Conflict of Interest Statement The authors have no conflict of interest to declare in this study.

References

1. Horii A, Kobayashi T, Tomita N, Yamamoto T, Fukushige S, Murotsu T et al (1987) Primary structure of human pancreatic secretory trypsin inhibitor (PSTI) gene. Biochem Biophys Res Commun 149(2):635–641
2. Stenman UH (2002) Tumor-associated trypsin inhibitor. Clin Chem 48(8):1206–1209
3. Flavin R, Pettersson A, Hendrickson WK, Fiorentino M, Finn S, Kunz L et al (2014) SPINK1 protein expression and prostate cancer progression. Clin Cancer Res 20(18):4904–4911. doi:10.1158/1078-0432.CCR-13-1341
4. Tomlins SA, Rhodes DR, Yu J, Varambally S, Mehra R, Perner S et al (2008) The role of SPINK1 in ETS rearrangement-negative prostate cancers. Cancer Cell 13(6):519–528. doi:10.1016/j.ccr.2008.04.016
5. Bhalla R, Kunju LP, Tomlins SA, Christopherson K, Cortez C, Carskadon S et al (2013) Novel dual-color immunohistochemical methods for detecting ERG-PTEN and ERG-SPINK1 status in prostate carcinoma. Mod Pathol 26(6):835–848. doi:10.1038/modpathol.2012.234
6. Leinonen KA, Saramaki OR, Furusato B, Kimura T, Takahashi H, Egawa S et al. (2013). Loss of PTEN is associated with aggressive behavior in ERG-positive prostate cancer. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 22(12):2333–44. doi:10.1158/1055-9965.EPI-13-0333-T.
7. Jhavar S, Brewer D, Edwards S, Kote-Jarai Z, Attard G, Clark J et al (2009) Integration of ERG gene mapping and gene-expression profiling identifies distinct categories of human prostate cancer. BJU Int 103(9):1256–1269. doi:10.1111/j.1464-410X.2008.08200.x
8. Lippolis G, Edsjo A, Stenman UH, Bjartell A (2013) A high-density tissue microarray from patients with clinically localized prostate cancer reveals ERG and TATI exclusivity in tumor cells. Prostate Cancer Prostatic Dis 16(2):145–150. doi:10.1038/pcan.2013.7
9. Wang C, Wang L, Su B, Lu N, Song J, Yang X et al (2014) Serine protease inhibitor Kazal type 1 promotes epithelial-mesenchymal transition through EGFR signaling pathway in prostate cancer. Prostate 74(7):689–701. doi:10.1002/pros.22787
10. Leinonen KA, Tolonen TT, Bracken H, Stenman UH, Tammela TL, Saramaki OR et al (2010) Association of SPINK1 expression and TMPRSS2:ERG fusion with prognosis in endocrine-treated prostate cancer. Clin Cancer Res 16(10):2845–2851. doi:10.1158/1078-0432.CCR-09-2505
11. Grupp K, Diebel F, Sirma H, Simon R, Breitmeyer K, Steurer S et al (2013) SPINK1 expression is tightly linked to 6q15- and 5q21-deleted ERG-fusion negative prostate cancers but unrelated to PSA recurrence. Prostate 73(15):1690–1698. doi:10.1002/pros.22707
12. Terry S, Nicolaiew N, Basset V, Semprez F, Soyeux P, Maille P et al (2015) Clinical value of ERG, TFF3, and SPINK1 for molecular subtyping of prostate cancer. Cancer 121(9):1422–1430. doi:10.1002/cncr.29233
13. Brooks JD, Wei W, Hawley S, Auman H, Newcomb L, Boyer H et al (2015) Evaluation of ERG and SPINK1 by immunohistochemical staining and Clinicopathological outcomes in a multi-institutional radical prostatectomy cohort of 1067 patients. PLoS One 10(7):e0132343. doi:10.1371/journal.pone.0132343
14. Schrecengost R, Knudsen KE (2013) Molecular pathogenesis and progression of prostate cancer. Semin Oncol 40(3):244–258. doi:10.1053/j.seminoncol.2013.04.001
15. Bismar TA, Yoshimoto M, Duan Q, Liu S, Sircar K, Squire JA (2012) Interactions and relationships of PTEN, ERG, SPINK1 and AR in castration-resistant prostate cancer. Histopathology 60(4):645–652. doi:10.1111/j.1365-2559.2011.04116.x
16. Yoshimoto M, Cunha IW, Coudry RA, Fonseca FP, Torres CH, Soares FA et al (2007) FISH analysis of 107 prostate cancers shows that PTEN genomic deletion is associated with poor clinical outcome. Br J Cancer 97(5):678–685
17. Epstein JI, Allsbrook WC, Jr., Amin MB, Egevad LL, Committee IG. (2005). The 2005 international society of urological pathology (ISUP) consensus conference on Gleason grading of prostatic carcinoma. Am J Surg Pathol, 29(9):1228–1242.
18. Huang KC, Alshalalfa M, Hegazy SA, Dolph M, Donnelly B, Bismar TA (2014) The prognostic significance of combined ERG and androgen receptor expression in patients with prostate cancer managed by androgen deprivation therapy. Cancer Biol Ther 15(9):1120–1128. doi:10.4161/cbt.29689
19. Yoshimoto M, Ding K, Sweet JM, Ludkovski O, Trottier G, Song KS et al (2013) PTEN losses exhibit heterogeneity in multifocal prostatic adenocarcinoma and are associated with higher Gleason grade. Mod Pathol: an official J US Can Acad Pathol, Inc 26(3):435–447. doi:10.1038/modpathol.2012.162
20. Teng LH, Wang C, Begin LR, Dolph M, Yilmaz A, Trpkov K et al (2013) ERG protein expression and gene rearrangements are present at lower rates in metastatic and locally advanced castration-resistant prostate cancer compared to localized disease. Urology 82(2):394–399. doi:10.1016/j.urology.2013.03.029
21. Braun M, Goltz D, Shaikhibrahim Z, Vogel W, Bohm D, Scheble V et al (2012) ERG protein expression and genomic rearrangement status in primary and metastatic prostate cancer–a comparative study of two monoclonal antibodies. Prostate Cancer Prostatic Dis 15(2):165–169. doi:10.1038/pcan.2011.67
22. Smith SC, Tomlins SA (2014) Prostate cancer SubtyPINg biomarKers and outcome: is clarity emERGing? Clin Cancer Res 20(18):4733–4736. doi:10.1158/1078-0432.CCR-14-0818
23. Smith SC, Palanisamy N, Zuhlke KA, Johnson AM, Siddiqui J, Chinnaiyan AM et al (2014) HOXB13 G84E-related familial prostate cancers: a clinical, histologic, and molecular survey. Am J Surg Pathol 38(5):615–626. doi:10.1097/PAS.0000000000000090
24. Gumuskaya B, Gurel B, Fedor H, Tan HL, Weier CA, Hicks JL et al (2013) Assessing the order of critical alterations in prostate cancer development and progression by IHC: further evidence that PTEN loss occurs subsequent to ERG gene fusion. Prostate Cancer Prostatic Dis 16(2):209–215. doi:10.1038/pcan.2013.8