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Comparative Expression Analysis Reveals Relationships between *SPINK1*, *TUBB3*, and *EZH2*, and Prostate Cancer Molecular Biomarkers in the Cancer Genome Atlas (TCGA) Data

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Abstract

Background: Despite the recent discovery of molecular subtypes in prostate cancer (PCa) expressing or not gene fusions involving E26 Transformation-Specific (ETS) transcription factors, including ERG (for v-ets avian erythroblastosis virus E26 oncogene homolog), little is known on molecular alterations associated, and cooperative events at play during initiation and progression of PCa.

Objective and methods: Using RNA-Seq data from The Cancer Genome Atlas (TCGA) collection of surgically managed primary prostate adenocarcinomas, we investigated the relations between gene expression of the candidate prognostic markers *SPINK1*, *TUBB3* (class III beta-tubulin), *EZH2*, and known PCa molecular markers. 484 cases were included in the analysis.

Results: Clustering analysis consistently showed *TUBB3* associating with *EZH2*, and *SPINK1* with *PTEN* and *TFF3*, but not with *ERG*, *ETV1*, *CHD1*, *AR* or *SPOP* expression. Positive and negative correlations were found among these PCa markers. Notably, in tumors highly expressing *SPINK1* or *TUBB3*, a subset of cases showed substantial *EZH2* expression, while *EZH2* expression was highly correlated with *AURKA* expression (r=0.7178; p <0.0001), an oncogenic target in cancer. Interestingly, we found that high expression of *EZH2* was strongly associated with reduced *SPOP* expression (r=-0,455; p <0.0001). Moreover, tumors expressing *SPINK1* and *TUBB3* often appeared to have reduced expression of *RB1*, *AR*, and *REST* as possible signs of neuroendocrine differentiation.

Conclusions: Despite substantial heterogeneity among the PCa cases, the current study suggests that significant associations and overlaps exist between PCa molecular alterations and expression of candidate PCa prognostic markers. A better understanding of these alterations and their cooperative role should help refine PCa subtypes, identify aggressive subgroups among those, and improve PCa management and therapy response.

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Introduction

Prostate cancer (PCa), as many other cancers, is characterized by extensive clinical and molecular heterogeneity [1]. Over the past 10 years, with the advent of high throughout methods, our understanding of the PCa genome has significantly changed, while revealing considerable inter-tumor (between tumors of the same type), and intra-tumor (within tumors, different subclones) heterogeneities [2-8]. Based on the molecular alterations identified, different molecular PCa subclasses or subtypes have emerged with the attempt to correlate those PCa subtypes to clinical features, disease progression and response to therapy. Approximately 50% of PCAs harbor a gene fusion between ERG, an ETS transcription factor, and an androgen-regulated gene (TMPRSS2 ~ 90%, SLC45A3, NDRG1, HERPUD1, or others <10%) [9,10]. ERG expression is routinely used as a surrogate marker of these alterations [11]. Trefoil factor 3 (TFF3) represents a highly specific

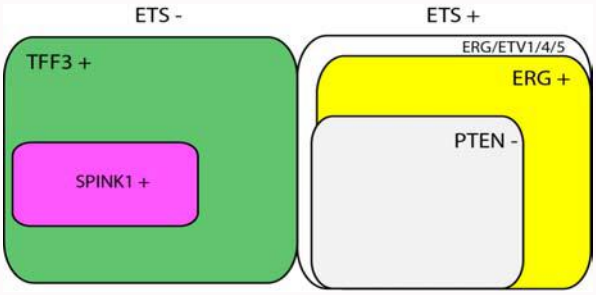

Figure 1: Schematic representation of the PCa molecular subtypes deriving from previous studies. Tumors without or with ETS fusions are shown on the left and right, respectively. In the ETS positive tumors, the vast majority harbor genomic rearrangements leading to ERG overexpression, thus representing an ERG positive PCa subtype. *PTEN* loss is commonly found in this subtype. ETS negative PCas generally express TFF3 as a molecular cancer biomarker, and a subgroup of these PCas express *SPINK1*. This subgroup may reflect a subset of disease with more aggressive behavior.

molecular biomarker of cancer in the prostate. Detectable in 40% to 60% of PCa cases [12,13], it appeared to be inversely correlated with ERG expression in most instances [14] (Figure 1). Inactivation of tumor suppressor phosphatase and tensin homolog (PTEN) is also commonly found in PCa and could be associated with cancer progression [15,16]. Several studies have found that PTEN alterations are enriched in ERG-over expressing PCa. Moreover, ERG overexpression and alterations of PTEN could cooperate, leading to more aggressive disease [17,18]. Despite these significant advances, it remains a challenge to link this molecular classification with clinical features to improve prognostic estimation, and treatment decisions in routine clinical practice [12,13,19-27].

SPINK1 (previously referred to as TATI, or tumor-associated trypsin inhibitor) is expressed in various diseases including cancer [28]. Tomlins et al. [29] identified SPINK1 as a candidate marker for a group of PCa devoid of ETS gene fusions associated with aggressive disease features and adverse outcomes. In other studies, a correlation between SPINK1 expression and adverse prognosis was not observed [25,30,31]. Nevertheless, in a recent survey, the prognostic value of SPINK1 was confirmed in a well-annotated cohort [14]. We proposed that SPINK1 overexpression emerges from a subgroup of PCa with ERG negative/TFF3 (trefoil factor 3) positive pattern [14] (Figure 1).

It is to note that various experimental studies have shown that ERG, TFF3 and SPINK1 are all associated with increased cell motility and/or invasive behavior in PCa models supporting their role in PCa progression [29,32,33].

Elevated βIII-tubulin (encoded from TUBB3 gene) expression was previously identified as significantly associated with tumor aggressiveness in PCa patients with presumed localized disease [34]. In this study, βIII-tubulin expression was found to be an independent marker of disease recurrence after local treatment. Recently, Tsourlakis and colleagues examined a large European cohort and confirmed this finding [35]. Additionally, increased βIII-tubulin expression is associated with the emergence of Castrate Resistant PCa (CRPC) [36,37], and with lower survival for patients receiving docetaxel-based chemotherapy [34].

The polycomb group protein enhancer of zeste homolog 2 (*EZH2*) is known to be increased in metastatic PCas. Clinically localized PCa that express elevated levels of EZH2 show a poorer prognosis [38], suggesting that EZH2 has a potential role in disease progression and patient prognosis [39-41]. Moreover *EZH2* expression is found elevated in Neuroendocrine PCa, a higly aggressive form of human PCa [42,43]. In the era of precision medicine [44,45], these findings underscore the potential utility of decrypting the relationships at play between *SPINK1*, *TUBB3*, *EZH2* expression and other PCa molecular alterations in order to improve our definition of molecular PCa subclasses and find best therapeutic solutions for the management of patients.

In the present work, we examined publically available gene expression data from primary PCa cases of The Cancer Genome Atlas (TCGA), and studied relationships between gene expression of *TUBB3, SPINK1, EZH2* and expression of other known molecular PCa biomarkers including *ERG*, *ETV1*, *PTEN*, *CHD1*, *TFF3*, *MYC*, *RB1*, *MYC*, *AURKA*, and *SPOP* [2,3,6,7,42, 46-49].

Methods

RNA-Seq gene expression analysis, clustering and statistical analysis

Human samples analyzed consisted of primary of prostate adenocarcinomas from The Cancer Genome Atlas (TCGA) project collection (http://cancergenome.nih.gov/). TCGA RNA-Seq expression data and sample information were accessed before June 2016 from cBioPortal [50] and the TCGA public access data (http://tcga-data.nci.nih.gov/). Only cases with available expression data, and analyzed for mutational landscape were considered. The cohort consisted of men surgically managed for localized or locally advanced disease. Of note, about 16% (41 of 260 cases; NA for the remaining cases) also received adjuvant treatments consisting of hormone therapy, radiotherapy, or a combination of those. Available patient cohort characteristics are shown in Table 1 (n=484).

To explore expression levels and associations of the different genes, gene expression levels (RSEM) were subjected to correlation and unsupervised clustering analyses using Cluster and TreeView softwares after transforming the RSEM into Log_2 (RSEM+1). Genes

Table 1: Clinico-pathological characteristics of the TCGA studied cohort (n=484).

Age , year	
mean	61
median (range)	61 (41-78)
Pre-Operative PSA, ng/mL*	7.4 (1.6-87)
Pathological Gleason Score, no. (%)	
6	44 (9.1)
7 (3+4)	144 (30)
7 (4+3)	98 (20)
8	63 (13)
9	132 (27.3)
10	3 (0.6)
Pathological Stage, no. (%)	
pT2	183 (38.3)
pT3a	155 (32.4)
pT3b	130 (27.2)
pT4	10 (2.1)
Lymph nodes, no. (%)	
negative	318 (80.5)
positive	77 (19.5)

*from 187 cases

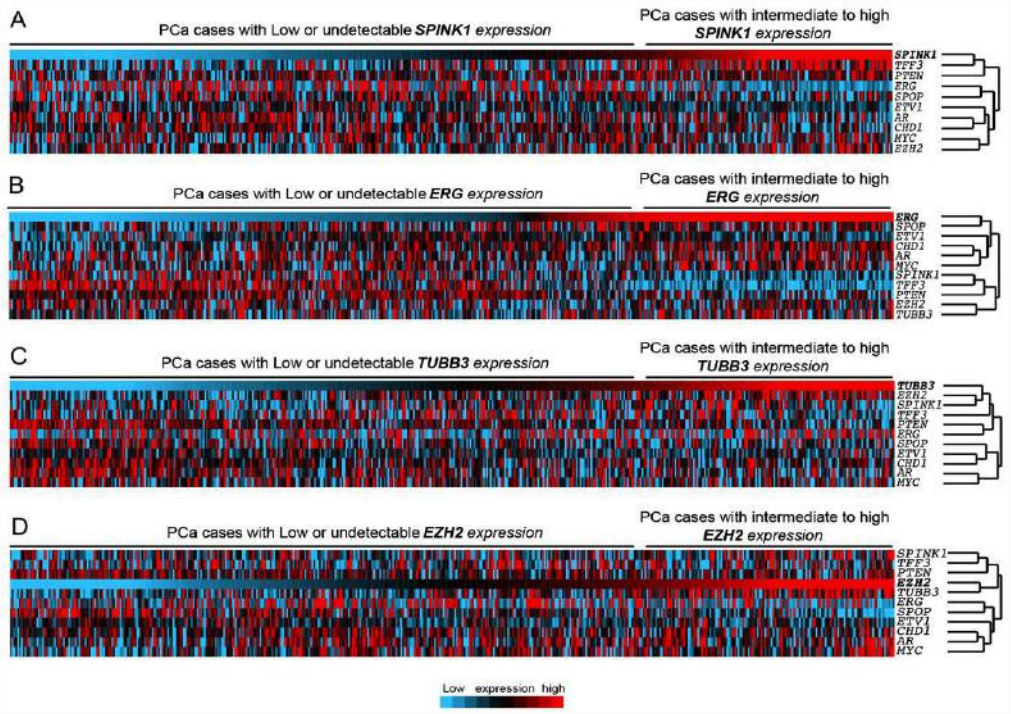

Figure 2: Hierarchical cluster analysis and heatmap generated using *SPINK1*, *ERG*, *TFF3*, *ETV1*, *AR*, *CHD1*, *PTEN*, *SPOP*, *ERG*, and *EZH2* expressions across TCGA prostate adenocarcinomas (n=484). In the heatmap, each column represents a different case, and each row represents a marker. blue to red: lowest to highest expression. PCa cases are ordered with respect to expression of *SPINK1* (A), *ERG* (B), *TUBB3* (C), or *EZH2* (D).

analyzed included *ERG, ETV1, PTEN, CHD1, TFF3, MYC, SPOP, SPINK1, EZH2, TUBB3, RB1*, and *AURKA*. Pearson's coefficient was determined to assess correlations between expression levels. For differential expression analysis, an unpaired t test or a non-parametric Mann-Whitney U test was applied as appropriate. All p values were two-sided and values of $p < 0.05$ were considered statistically significant.

Results

In the group of ETS negative PCa, *SPINK1* expression is positively correlated with *TFF3* and *PTEN* expression levels

Expression data were retrieved from TCGA collection, tumor samples ordered by *SPINK1* expression, and clustering analysis was performed for gene expression of PCa molecular biomarkers including *ERG, TFF3, ETV1, AR, CHD1, PTEN, SPOP, ERG*, and *EZH2* (Figure 2a). Expectedly, *SPINK1* expression clustered with *TFF3* expression, and seems inversely correlated with *ERG* and *ETV1* expression. *SPINK1* and *TFF3* also clustered with *PTEN* expression. *PTEN* expression appeared to be especially elevated in a number of cases expressing high levels of *SPINK1*. By contrast, it was reduced in the cases expressing high levels of *ERG*, as also evidenced by an additional heatmap with tumor classified with respect to *ERG* expression (Figure 2b).

To substantiate these results, we computed correlation scores between *SPINK1* expression and each molecular marker (Table 2). *SPINK1* and *TFF3* were significantly correlated ($r=0.36$, $p < 0.0001$) and both inversely correlated with *ERG*, *CHD1*, and *AR* (Table 2). Moreover, ERG negatively correlated with *PTEN* ($r=-0.2911$; $p < 0.0001$). This likely reflects an enrichment of *PTEN* deletion in ERG+PCa subtype as described previously [17,51,52].

A subset of tumors expressing *SPINK1* concomitantly expresses high levels of *EZH2*

Interestingly, we also noted in the heatmap a relative enrichment of *EZH2* expression in tumors expressing high levels of *SPINK1* (Figure 2a), and based on the correlation analysis, EZH2 expression was positively correlated with *SPINK1* expression ($r=0.1554$, $p=0.0006$). This data suggests that at least a subset of *SPINK1* expressing tumors also expresses high levels of *EZH2*. Importantly, there was however no correlation between *EZH2* and *TFF3* (Table 2), neither with ERG. We previously described the presence of *SPINK1* expression characterizes an aggressive subtype in the group of ERG-/TFF3+PCa tumors [14]. Our observation here suggests that *EZH2* overexpression preferentially arises from ERG-/TFF3+/SPINK1+PCas rather than in ERG-/TFF3+/SPINK1-PCas, which could coincide with more aggressive forms of the disease.

A subset of tumors expressing *TUBB3* concomitantly expresses high levels of *EZH2*

We then investigated how these markers could cluster with *TUBB3* (encoding for Class III β-tubulin), another candidate marker for aggressive PCa disease [34], also assumed to be an early marker for reduced AR signaling [36] and NE differentiation [37,53]. A Heatmap of the same set of genes in tumors classified by TUBB3 expression revealed a marked enrichment of *EZH2* in tumors overexpressing *TUBB3* (Figure 2c), further highlighted by a positive correlation coefficient ($r=0.32$; $p < 0001$; Table 2). The analysis also revealed *TUBB3* and *PTEN* expression patterns inversely correlated ($r=-0.3159$; $p < 0001$), with *AR* and *SPOP* also following this trend ($r=-0.26$ and -0.20, respectively; $p<0001$). Intriguingly, *SPINK1* expression did not appear to be associated with *TUBB3* expression. *SPINK1* expression was correlated with *PTEN* expression, when

Table 2:

Correlation with SPINK1 expression				Correlation with TFF3 expression			
Gene	**Pearson r**	**95% CI**	**P Value**	**Gene**	**Pearson r**	**95% CI**	**P Value**
TFF3	0.3659	0.2861 to 0.4407	<0.0001	*ERG*	-0.6027	-0.6566 to -0.5428	<0.0001
ERG	-0.3166	-0.3947 to -0.2341	<0.0001	*CHD1*	-0.4702	-0.5369 to -0.3977	<0.0001
CHD1	-0.2485	-0.3304 to -0.1630	<0.0001	*SPINK1*	0,3659	0.2861 to 0.4407	<0.0001
PTEN	0.1925	0.1052 to 0.2769	<0.0001	*AR*	-0.2216	-0.3048 to -0.1352	<0.0001
ETV1	-0.1587	-0.2444 to -0.07052	0.0005	*ETV1*	-0.2144	-0.2979 to -0.1277	<0.0001
AR	-0.1561	-0.2419 to -0.06793	0.0006	*PTEN*	0.156	0.06782 to 0.2418	0.0006
EZH2	0.1554	0.06714 to 0.2412	0.0006	*SPOP*	-0.1091	-0.1963 to -0.02012	0.0164
SPOP	-0.06134	-0.1497 to 0.02796	0.1779	*MYC*	-0.1399	-0.2263 to -0.05141	0.002
TUBB3	0.05186	-0.03746 to 0.1404	0.2548	*TUBB3*	-0,04352	-0.1322 to 0.04581	0.3393
MYC	0.0372	-0.05212 to 0.1259	0.4141	*EZH2*	0.01475	-0.07449 to 0.1038	0.7461
Correlation with ERG expression				**Correlation with PTEN expression**			
Gene	**Pearson r**	**95% CI**	**P Value**	**Gene**	**Pearson r**	**95% CI**	**P Value**
TFF3	-0.6027	-0.6566 to -0.5428	< 0.0001	*TUBB3*	-0.3159	-0.3939 to -0.2333	<0.0001
SPINK1	-0.3166	-0.3947 to -0.2341	< 0.0001	*ERG*	-0.2911	-0.3706 to -0.2073	<0.0001
CHD1	0.3161	0.2335 to 0.3941	< 0.0001	*SPOP*	0.2027	0.1156 to 0.2867	<0.0001
PTEN	-0.2911	-0.3706 to -0.2073	< 0.0001	*SPINK1*	0.1925	0.1052 to 0.2769	<0.0001
SPOP	0.2367	0.1507 to 0.3191	< 0.0001	*TFF3*	0.156	0.06782 to 0.2418	0.0006
ETV1	-0.1408	-0.2271 to -0.05228	0.0019	*EZH2*	-0.1378	-0.2242 to -0.04924	0.0024
MYC	0.112	0.02308 to 0.1992	0.0137	*AR*	0.13	0.04133 to 0.2166	0.0042
TUBB3	0.1118	0.02288 to 0.1990	0.0139	*MYC*	0.1197	0.03089 to 0.2067	0.0084
AR	0.06206	-0.02724 to 0.1504	0.1728	*ETV1*	0.04871	-0.04061 to 0.1373	0.2848
EZH2	-0.05982	-0.1482 to 0.02949	0.1889	*CHD1*	0.02522	-0.06408 to 0.1141	0.58
Correlation with TUBB3 expression				**Correlation with EZH2 expression**			
Gene	**Pearson r**	**95% CI**	**P Value**	**Gene**	**Pearson r**	**95% CI**	**P Value**
EZH2	0.3246	0.2424 to 0.4021	< 0.0001	*SPOP*	-0,455	-0.5229 to -0.3813	< 0.0001
PTEN	-0.3159	-0.3939 to -0.2333	< 0.0001	*TUBB3*	0.3246	0.2424 to 0.4021	< 0.0001
AR	-0.2655	-0.3464 to -0.1806	< 0.0001	*MYC*	0.2668	0.1819 to 0.3476	< 0.0001
SPOP	-0.2031	-0.2870 to -0.1160	< 0.0001	*SPINK1*	0.1554	0.06714 to 0.2412	0.0006
CHD1	-0.1719	-0.2571 to -0.08405	0.0001	*AR*	0.1381	0.04956 to 0.2245	0.0023
ERG	0.1118	0.02288 to 0.1990	0.0139	*PTEN*	-0.1378	-0.2242 to -0.04924	0.0024
MYC	-0.07149	-0.1596 to 0.01778	0.1163	*CHD1*	-0.08513	-0.1730 to 0.004048	0.0613
ETV1	-0.05646	-0.1449 to 0.03286	0.215	*ERG*	-0.05982	-0.1482 to 0.02949	0.1889
SPINK1	0.05186	-0.03746 to 0.1404	0.2548	*ETV1*	-0.03071	-0.1195 to 0.05860	0.5002
TFF3	-0.04352	-0.1322 to 0.04581	0.3393	*TFF3*	0.01475	-0.07449 to 0.1038	0.7461

Red: significant positive correlation; Blue: significant negative correlation

TUBB3 expression anticorrelated with *PTEN* expression. Moreover, *TUBB3* expression seemed to be more closely associated with *EZH2* than *SPINK1*. This indicates that SPINK1 or TUBB3 expressions may be mutually exclusive under some circumstances; thus representing two distinct subsets of disease. Another possibility might be that a large proportion of TUBB3 expressing tumors is confined to the ERG+ / PTEN^{low} PCa subtype, that is negative for SPINK1, while a smaller fraction is linked to ETS–TFF3+SPINK1 PCa subtype, and both can exhibit an enriched expression of *EZH2*.

EZH2 expression is associated with *AURKA* expression and *SPOP* alterations

We then generated a heatmap classifying tumors with respect to *EZH2* expression (Figure 2d), in conjunction with correlation analysis. This denoted a striking positive correlation between *EZH2* and *AURKA* levels (r=0.7178; p <0.0001), and an inverse correlation between *EZH2* and *SPOP* levels (r=-0.455; p <0001; Table 2). The connection between *EZH2* expression and downregulated levels of *SPOP* is intriguing, especially considering recent work by Barbieri and colleagues who identified inactivating mutation in *SPOP* gene as the most common point mutation in PCa [6]. Further work by this group revealed that this mutation occurs predominantly in the group of *ERG* rearranged PCa tumors [6], and is concomitant with deletions at 5q21 *CDH1* locus [47]. We then sought to determine whether mutation in SPOP gene, is associated with varying levels of *SPINK1, EZH2* and *TUBB3* (Figure 3). When considering all patients,

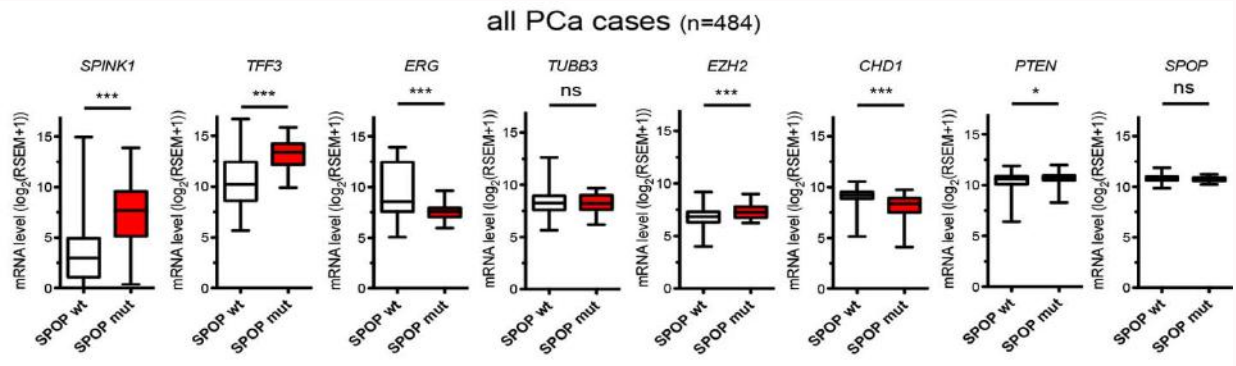

Figure 3: The effect of SPOP mutation on expression of *SPINK1*, *TFF3*, *ERG*, *TUBB3*, *EZH2*, *CHD1*, *PTEN*, and *SPOP* accross 484 PCa samples. Box plots showing the Median, 25th to 75th percentiles. Lower and upper bars correspond to the minimum and maximum values, respectively.

Table 3:

Correlation with *TUBB3* expression				Correlation with EZH2 expression			
Gene	**Pearson r**	**95% CI**	**P Value**	**Gene**	**Pearson r**	**95% CI**	**P Value**
SYP	0.3308	0.2490 to 0.4079	<0.0001	*AURKA*	0.7178	0.6716 to 0.7584	<0.0001
EZH2	0.3246	0.2424 to 0.4021	<0.0001	*TUBB3*	0.3246	0.2424 to 0.4021	<0.0001
RB1	-0.3256	-0.4030 to -0.2435	<0.0001	*ENO2*	-0.2655	-0.3464 to -0.1806	<0.0001
REST	-0.3178	-0.3957 to -0.2353	<0.0001	*SRRM4*	-0.2319	-0.3146 to -0.1458	<0.0001
AR	-0.2655	-0.3464 to -0.1806	<0.0001	*CHGA*	-0.1952	-0.2795 to -0.1079	<0.0001
AURKA	0.2317	0.1455 to 0.3143	<0.0001	*CHGB*	-0.142	-0.2283 to -0.05353	0.0017
ENO2	0.2263	0.1399 to 0.3092	<0.0001	*AR*	0.1381	0.04956 to 0.2245	0.0023
CHGB	0.1169	0.02808 to 0.2040	0.01	*SYP*	0.06937	-0.01990 to 0.1576	0.1275
SRRM4	0.05351	-0.03581 to 0.1420	0.24	*RB1*	-0.06689	-0.1551 to 0.02240	0.1417
MYCN	-0.004543	-0.09366 to 0.08464	0.9206	*REST*	0.0573	-0.03201 to 0.1457	0.2083
CHGA	0.00007283	-0.08908 to 0.08922	0.9987	*MYCN*	0.01582	-0.07344 to 0.1048	0.7285
Correlation with *SPINK1* expression				**Correlation with *TFF3* expression**			
Gene	**Pearson r**	**95% CI**	**P Value**	**Gene**	**Pearson r**	**95% CI**	**P Value**
SYP	0.2342	0.1481 to 0.3167	< 0.0001	*SYP*	0.399	0.3213 to 0.4714	< 0.0001
RB1	-0.2053	-0.2892 to -0.1183	< 0.0001	*REST*	-0.2942	-0.3735 to -0.2105	< 0.0001
REST	-0.1742	-0.2593 to -0.08642	0.0001	*RB1*	-0.2566	-0.3380 to -0.1713	< 0.0001
EZH2	0.1554	0.06714 to 0.2412	0.0006	*CHGB*	-0.236	-0.3184 to -0.1500	< 0.0001
AR	-0.1561	-0.2419 to -0.06793	0.0006	*AR*	-0.2216	-0.3048 to -0.1352	< 0.0001
AURKA	0.06712	-0.02217 to 0.1553	0.1404	*SRRM4*	-0.2085	-0.2923 to -0.1216	< 0.0001
SRRM4	-0.06132	-0.1497 to 0.02798	0.178	*ENO2*	-0.1875	-0.2721 to -0.09997	< 0.0001
CHGA	0.03842	-0.05091 to 0.1271	0.399	*CHGA*	-0.173	-0.2582 to -0.08516	0.0001
CHGB	-0.01088	-0.09994 to 0.07834	0.8112	*MYCN*	-0.1562	-0.2420 to -0.06800	0.0006
ENO2	0.007363	-0.08184 to 0.09645	0.8716	*AURKA*	-0.03589	-0.1246 to 0.05344	0.4309
MYCN	-0.001008	-0.09015 to 0.08815	0.9824	*EZH2*	0.01475	-0.07449 to 0.1038	0.7461

SPOP mutant cases had elevated expression of *EZH2*, *SPINK1* and *TFF3*, but reduction in expression of *ERG* and *CHD1*. There were no significant changes noted for *TUBB3* and *SPOP* expression levels.

SPINK1, *TUBB3* and *EZH2* are associated with various NE features

Previous studies have reported associations of TUBB3 and EZH2 overexpression with aggressive features in localized PCa, or NE features in castrate resistant CRPC tumors [34-36,42]. We asked weather *SPINK1*, *TUBB3*, *EZH2* could also associate with NE features in this cohort of locally managed PCa tumors. Heatmaps were generated with respect to *TUBB3*, *EZH2*, or *SPINK1* expression, and their distribution was studied as above among a panel of NE markers, putative drivers and suppressors of NE phenotype, (NE suppressors (*RB1*, *REST*, *AR*); NE drivers (*AURKA*, *SRRM4*, *MYCN*); *NE* markers (*SYP* (synpatophysin), *ENO2* (NSE), *CHGA* (chromogranin A), *CHGB* (chromogranin B)) (Figure 4). Correlation coefficients were determined as above to assess associations between variables. Notably, *EZH2* highly correlated with *AURKA* gene expression, but with the exception of *TUBB3*, showed no or negative correlation with other NE components. By contrast, tumors with high expression of *TUBB3*, and *SPINK1* to a lesser extent, more frequently exhibited NE

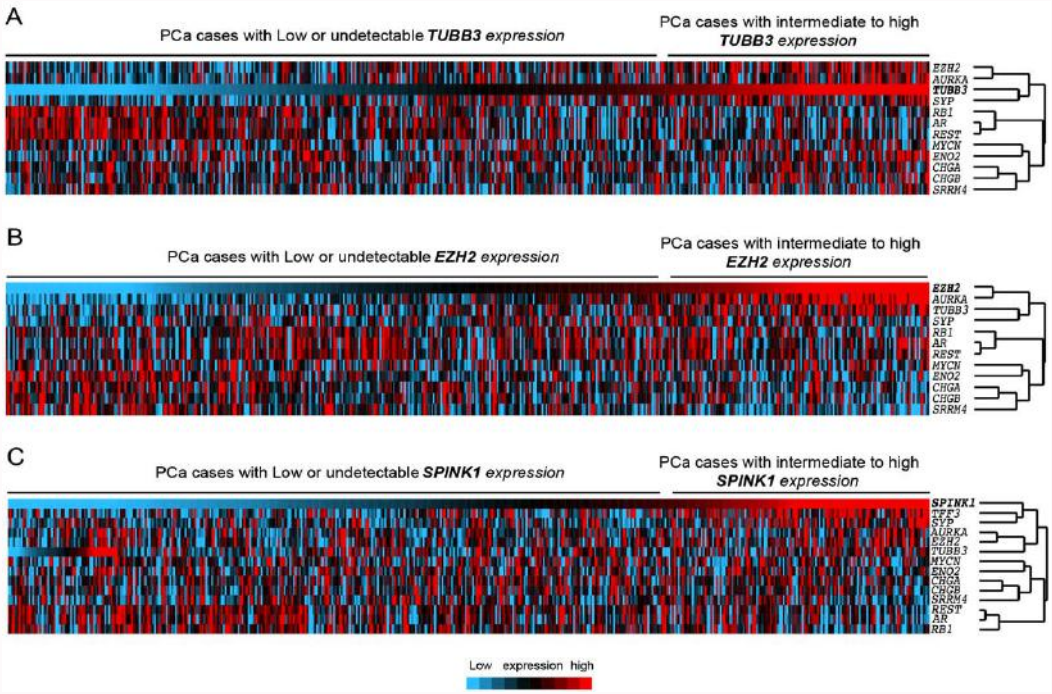

Figure 4: Hierarchical cluster analysis and heatmap generated using expression levels of *SPINK1*, *TUBB3*, *EZH2*, and NE components *AR*, *SYP*, *RB1*, *CHGA*, *CHGB*, *ENO2*, *MYCN*, *AURKA*, *REST*, *SRRM4* across TCGA prostate adenocarcinomas (n=484). In the heatmap, each column represents a different case, and each row represents a marker. blue to red: lowest to highest expression. PCa cases are ordered with respect to expression of *TUBB3* (A), *EZH2* (B), or *SPINK1* (C).

features, as judged by anti-correlations with *REST*, *AR* and *RB1*, and positive correlation with *SYP* (Table 3).

Discussion

We previously proposed SPINK1 and βIII-tubulin expressions as independent prognosticators of disease recurrence in PCa patients primarily managed by prostatectomy [14,34]. Together with EZH2, another potential marker of PCa aggressiveness, these genes may be directly involved in progression, metastatic spread and/or therapy resistance of PCa. One interesting open question regarding these genes is to what extent these genes cooperate or overlap with other known molecular alterations recently characterized in PCa and defining PCa subclasses [1-3,6,7,42,46-49, 54].

In this work, by exploring RNAseq data from the TCGA prostate adenocarciomas, we confirmed on a large series of primary PCas that *SPINK1* positive tumors represent a molecular subgroup of PCa tumors strongly associated with *TFF3* expression, and correlating negatively with ERG expression. We found that these tumors express *PTEN* more often, but less *CHD1* or *AR*. Importantly, a subset of those cases seem to overexpress *EZH2*. Tumors highly expressing *TUBB3* also frequently exhibited higher expression of *EZH2*. Correlation analyses also revealed that EZH2 expression was positively associated with *AURKA* expression, an oncogenic target in cancer, while it was negatively associated with *SPOP* expression, a new putative tumor suppressor in PCa that is frequently mutated [1,6,23]. It is tempting to speculate that a molecular link exists between *SPOP* alterations, *EZH2* and *AURKA* expression. In line with this possibility, our preliminary data already indicate, that in the group of SPINK1 high expressing PCa cases, SPOP mutants displayed higher expression of *EZH2* and *AURKA* compared to *SPOP* wild-type (data not shown).

Altogether these findings should help refine PCa molecular subtypes, and identify subgroups of aggressive PCa. A working model

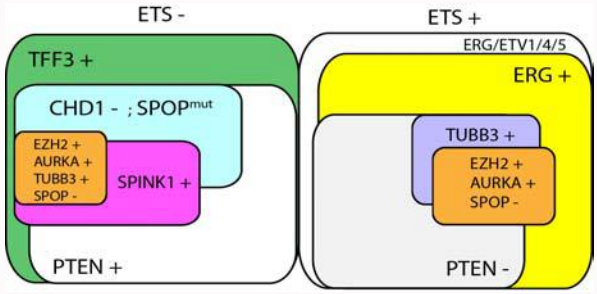

Figure 5: Schematic representation of the PCa molecular subgroups deriving from this study and from previous studies. Tumors without or with ETS fusions are shown on the left and right, respectively. In the *ERG* positive PCa subtype, *PTEN* loss is a commonly found, while it is relatively rare in ETS negative (TFF3+) subtype in which reduction of *CHD1* and inactivating mutations in *SPOP* become more common features. In each PCa subtype, other alterations such as *TUBB3*, *SPINK1*, or *EZH2* overexpression working in parallel or together (likely in association with additional related events such as *AURKA* upregulation and *SPOP* downregulation) may characterize subsets of disease with more aggressive behavior, resistant to therapies, or being able to proliferate or progress more rapidly to metastatic disease. Other important alterations, that are not shown here, are likely involved in initiation, progression, or differentiation of the disease. This includes, but not only, *MYC* amplification, *ETV1* amplification/overexpression; mutations in *TP53*, *CTNNB1*, *ATM*, *BRCA2*, or *FOXA1*; deletion or reduction of *NKX3.1*, *RB1*, *AR*, *REST*.

of the different subgroups is presented in Figure 5.

It remains unclear however weather *SPOP* alterations influences the group of *TUBB3* high PCa. Aside from the apparent relations between *TUBB3*, *EZH2* and *AURKA*, *SPOP* expression was only slightly reduced in *TUBB3* high PCa, and we did not find relationship between *TUBB3* expression and *SPOP* mutation status. We posit that in the groups of *SPINK1* high or *TUBB3* high PCa tumors, also characterized by high *vs.* low expression of PTEN, respectively, a

subset of cases express significant levels of *EZH2* accompanied by substantial *AURKA* expression which could evoke more aggressive features. A thorough assessment of such hypotheses will require further investigations on independent cohorts, and validation by various techniques including Fluorescence in Situ Hybridization (FISH) and immunohistochemistry-based approaches. Our data investigating NE features in this series indicates that *EZH2* is not directly associated with NE differentiation in this disease stage. Hence, *EZH2* is unlikely to be a driver of NE differentiation in primary tumors. However, its concurrent expression with SPINK1 or TUBB3 in some circumstances could be linked to the emergence of NE features. Of therapeutic relevance, many inhibitors directed against AURKA and EZH2 has been developed these recent years [55-57]. Thus, if this hypothesis is confirmed, this could provide a biological rationale for testing the effect of such new-targeted therapies to treat these PCa subgroups. In addition, because *EZH2* and/or *AURKA* upregulation might represent two key events during the transformation of prostate adenocarcinoma towards Neuroendocrine PCa [42,43,58], one could consider targeting these components at an early stage in order to prevent NEPC development and its progression [43,59].

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