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From Cell Biology to Tissue Engineering

Clinical significance and EZH2, ERG and SPINK1 protein expression in pure and mixed ductal adenocarcinoma of the prostate

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Summary. Background: Although ERG and SPINK1 molecular alterations have been studied in acinar and ductal adenocarcinoma of the prostate, EZH2 expression has not been previously evaluated in ductal adenocarcinoma. Methods: We collected cases of pure and mixed ductal adenocarcinoma of the prostate and evaluated clinical significance and EZH2, ERG, and SPINK1 protein expression. Results: We investigated 61 ductal adenocarcinomas, 22 pure and 39 mixed ductal/acinar. Except for tumor growth pattern, none of the clinical parameters studied significantly differed between pure and mixed tumors. Thirty-five percent of ductal adenocarcinomas were organ confined, 15% displayed seminal vesicle invasion. Lymph node and distal metastasis occurred in 13% and 24% of cases, respectively; 34% of patients experienced biochemical failure, 7% died of disease. Ninety-eight percent of tumors expressed EZH2; in 80% of cases >50% of tumor cells were positive. ERG and SPINK1 were expressed in 20% and 36% of cases, respectively. There was no difference in protein expression between pure and mixed ductal adenocarcinomas. ERG expression tended to be lower, and SPINK1 higher than reported for acinar tumors. Biochemical failure, metastasis and death did not differ between EZH2, ERG, and SPINK1 positive and negative patients, nor between <50% versus >50% expression of SPINK1 and EZH2, respectively. Conclusions: Pure and mixed ductal adenocarcinomas have similar clinical behavior and molecular alterations. Higher EZH2 and SPINK1 protein expression, compared to acinar prostatic adenocarcinoma, might account for the more aggressive clinical course of ductal adenocarcinoma.

Key words: Ductal adenocarcinoma, Prostatic Neoplasms, Transcriptional Regulator ERG, Enhancer of Zest Homolog 2 Protein, SPINK

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Introduction

Ductal adenocarcinoma of the prostate is an uncommon aggressive subtype of prostate cancer (Meeks et al., 2012). The presence of papillary fronds with a true vascular core is a distinctive architectural pattern of ductal carcinoma. Ductal adenocarcinoma may also grow as solid nests, cribriform glands, or as simple glands lined by stratified columnar epithelium with cytological and architectural features reminiscent of high-grade prostatic intraepithelial neoplasia (PIN), and

Abbreviations. EPE, extraprostatic extension; ETS, E26 transformation specific; EZH2, Enhancer of Zest Homologue 2; FISH, fluorescence in situ hybridization; GG, Grade Group; GS, Gleason Score; mRNA, messenger RNA; PIN, high-grade prostatic intraepithelial neoplasia; PSA, prostate-specific antigen; PSM, positive surgical margin; SPINK1, serine peptidase inhibitor, Kazal type 1; SVI, seminal vesicles invasion; TMPRSS2, transmembrane protease serine 2; TURP, transurethral resection of prostate.

referred to as 'PIN-like ductal adenocarcinoma (Tavora and Epstein, 2008; Epstein, 2010).

Ductal adenocarcinoma of the prostate may be pure, but is often associated with classical acinar adenocarcinoma intimately coexisting in the same tumor nodule (mixed ductal/acinar tumors) (Epstein, 2010; Samaratunga et al., 2010). Pure ductal and mixed ductal/acinar adenocarcinomas with a ductal component comprising more than 10% of the tumor volume are more likely to present at advanced clinical stage, are associated with more aggressive disease and seem less hormonally responsive than acinar adenocarcinomas (Bostwick et al., 1985; Christensen et al., 1991; Brinker et al., 1999; Samaratunga et al., 2010; Amin and Epstein, 2011; Meeks et al., 2012).

In the last decade prostate cancer has been subdivided based on genetic alterations (Barbieri and Tomlins, 2015). Recurrent gene fusions between the androgen-regulated transmembrane protease serine 2 (*TMPRSS2*) gene and members of the *ETS* transcription factor family have been identified in the majority of conventional acinar prostate cancers. Variable frequency of ETS rearrangements has been reported for ductal adenocarcinoma (Han et al., 2009; Seipel et al., 2013; Morais et al., 2015; Vinceneux et al., 2017). Several studies have shown an association between ETS fusion status and features of aggressive prostate cancer, while others have found no such associations, or even the opposite (Petrovics et al., 2005; Perner et al., 2006; Demichelis et al., 2007; Mehra et al., 2007; Nam et al., 2007; Rajput et al., 2007; Attard et al., 2008; Cheville et al., 2008; Saramaki et al., 2008; Gopalan et al., 2009; Hermans et al., 2009; Spencer et al., 2013). The serine peptidase inhibitor, Kazal type 1 (SPINK1) is a protein, regulated by androgens, overexpressed specifically in a subset of ETS-negative prostate cancers; its overexpression has been associated with decreased biochemical recurrence-free survival (Paju et al., 2007; Tomlins et al., 2008; Lippolis et al., 2013). Only two studies have reported SPINK1 expression in ductal adenocarcinoma of the prostate (Han et al., 2009; Vinceneux et al., 2017). Aberrant expression of Enhancer of Zest Homologue 2 (EZH2) is regarded as a potential marker of aggressive cancer with poor prognosis. EZH2 overexpression has been correlated with aggressive disease in acinar prostate cancer (Melling et al., 2015); however, at the best of our knowledge, its expression has not been previously characterized in ductal adenocarcinoma.

The aim of our study was to investigate the clinical significance and EZH2, ERG, and SPINK1 protein expression in a large series of well characterized pure and mixed ductal adenocarcinomas of the prostate with long follow-up.

Material and methods

The surgical pathology files of our institute were queried for cases containing 'ductal prostate cancer' in the diagnosis. This cohort study was approved by the Cleveland Clinic Institutional Review Board (Cleveland Clinic IRB #13-773). All hematoxylin and eosin slides were carefully reviewed by an experienced genitourinary pathologist (CM-G) to confirm presence and percentage of ductal adenocarcinoma component, Gleason score (Epstein et al., 2005), and pathologic stage (Magi-Galluzzi et al., 2011). Only cases where the ductal adenocarcinoma component comprised more than 10% of the tumor volume on radical prostatectomy or transurethral resection of the prostate were included. Tumors with solely ductal features were classified as pure ductal adenocarcinomas; tumors with ductal and acinar adenocarcinoma intimately coexisting in the same tumor nodule were classified as mixed ductal adenocarcinoma. Patients' age, Gleason Score (GS) and Grade Group (GG), tumor volume, growth pattern, pathologic stage, margin status, lymph node metastasis, biochemical failure, distant metastasis and death were recorded in an Institutional Review Board approved database. Radical prostatectomy tumor volume was calculated as previously described (Chen et al., 2003). Follow-up information was obtained from electronic medical records.

For each case, a representative section of tumor was selected for immunohistochemistry and four-micrometer thick sections were obtained from each selected formalin-fixed paraffin-embedded tissue block. Immunohistochemical staining for EZH2, ERG, and SPINK1 was performed using the streptavidin-biotin detection system. Appropriate positive and negative control slides were stained in parallel. The antibody source, dilution, time, and antigen retrieval method are EZH2 (Clone 6A10 Leica UK, 1:50, 60 min EDTA pH8, Ventana OptiView), ERG (Clone EPR3864 Abcam UK, 1:100, 32 min EDTA pH8, Ventana OptiView), and SPINK1 (M01 Clone 4D4 Abnova Taiwan, 1:50, 120 min Citrate pH6, Ventana UltraMapRed). ERG staining was performed on Benchmark Ultra, Ventana Medical Systems, (Tucson, AZ), whereas SPINK1 and EZH2 staining was performed on Discover XT from the same manufacturer. EZH2 and ERG were scored as positive or negative based on nuclear expression, SPINK1 based on cytoplasmic expression. Staining intensity (weak, moderate, strong) was recorded for all antibodies. For EZH2 and SPINK1, percentage of positive cells (≤50%

Table 1. Histopathologic features of pure and mixed ductal adenocarcinoma of the prostate.

Parameter, n (%)	Pure Ductal Adenocarcinoma (n=22)	Mixed Ductal Adenocarcinoma (n=39)
Growth pattern		
Papillary	10 (45)	11 (28)
Cribriform	2 (9)	1 (2)
Papillary and cribriform	6 (27)	27 (69)
Papillary with PIN-like features	4 (18)	0 (0)

vs. >50% of cells) was also recorded. Cases with less than 5% positive cells and weak staining intensity were considered negative. For mixed ductal adenocarcinoma, the pattern of staining was recorded for both ductal and acinar components.

Pearson's Chi-square and Fisher's exact tests were used to assess differences in categorical outcomes as a function of pure and mixed ductal adenocarcinomas. One way ANOVA methods were used to test for possible differences in continuous outcomes as a function of adenocarcinoma type. Multinomial analysis of odds of change in tumor stage as a function of expression of immunohistochemistry markers was performed. The time to event analysis Kaplan Meier curves were drawn, and the curves compared using the Wilcoxon and Log-Rank tests. The p-values were not adjusted for multiple comparisons.

Results

A total of 61 ductal adenocarcinoma were included in the study: 22 (36%) cases were pure and 39 (64%) mixed acinar/ductal adenocarcinomas. Patients mean age was 63 years (range: 44-76). In all cases, except one, the diagnosis was confirmed on radical prostatectomy specimens. For the patient without radical prostatectomy, the diagnosis of ductal adenocarcinoma was confirmed on transurethral resection of the prostate [TURP] and biopsy of the prostatic urethra. Preoperative transrectal prostate needle biopsy results were available for 57 patients: in 4 cases (2 pure and 2 mixed) the diagnosis of ductal adenocarcinoma was made on needle biopsy; in additional 14 cases (8 pure and 6 mixed) either ductal features or intraductal carcinoma were identified on the initial needle biopsy.

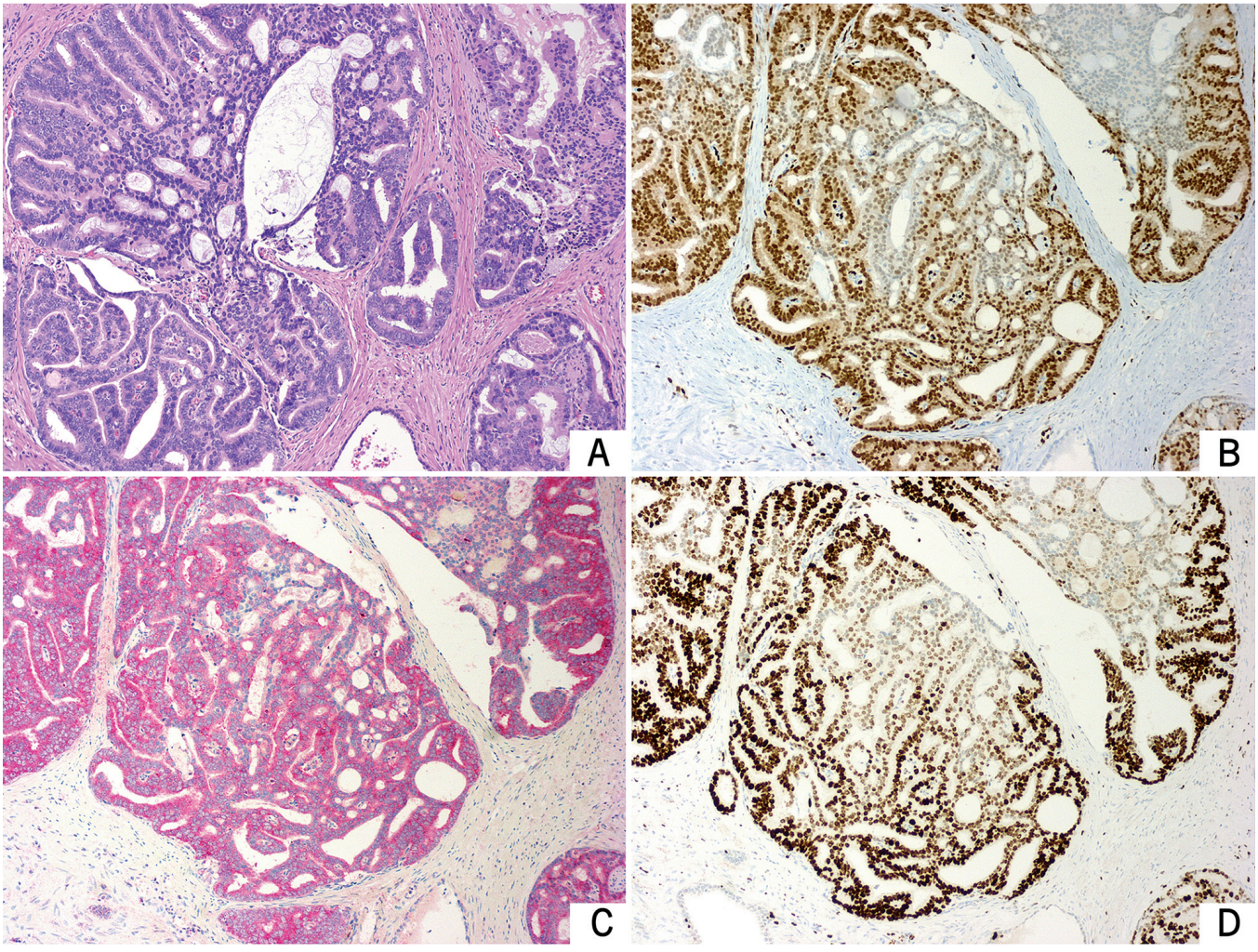

Fig. 1. A. Ductal adenocarcinoma with papillary and cribriform pattern. Tumor consists of pseudostratified columnar cells; papillae with true vascular cores are noted (Hematoxylin and Eosin). **B.** Ductal adenocarcinoma with diffuse and strong ERG nuclear expression by immunohistochemistry. **C.** Ductal adenocarcinoma with diffuse and strong SPINK1 cytoplasmic expression by immunohistochemistry. **D.** Ductal adenocarcinoma with diffuse and strong nuclear expression of EZH2 by immunohistochemistry. x 200.

Pathologic parameters

The pattern of growth was papillary in 21 (34%), cribriform in 3 (5%), papillary and cribriform in 33 (54%) (Fig. 1A), and papillary with PIN-like ductal adenocarcinoma features in 4 (6%) cases (Table 1). The growth pattern was significantly different between pure and mixed ductal adenocarcinomas (p=0.001, Fisher's Exact test): papillary pattern was common (45%) in pure cases compared to a combination of papillary and cribriform architecture (69%) in mixed tumors. PIN-like ductal pattern was seen only in 4 cases of pure ductal adenocarcinomas and was the prominent pattern in 2 cases, both of which were very small (5 mm and 7 mm in maximum diameter, respectively). Mean tumor volume for pure ductal adenocarcinomas was 2.29 cc. Nineteen (86%) pure ductal adenocarcinomas were Gleason score ≥8 (Grade Group 4-5). Two cases with predominant PIN-like ductal features were graded as Gleason score 7 (Grade Group 2), since the recently described PIN-like pattern of ductal adenocarcinoma has been graded as Gleason pattern 3 and reported to behave similarly to Gleason score 6 (Grade Group 1) (Tavora and Epstein, 2008; Epstein, 2010; Rais-Bahrami et al., 2017).

Pathological characteristics of tumors are compared in Table 2 (Table 2). In 14 of 21 (67%) ductal adenocarcinomas at least one separate (>5 mm away) nodule of acinar cancer was noted in the prostatectomy specimen (data not shown). Pure ductal adenocarcinoma was the index tumor in all cases except two: in one case the ductal adenocarcinoma was confined to the prostate, but the separate acinar carcinoma showed extraprostatic extension; in the second case, both acinar and ductal tumors showed extraprostatic extension, but the acinar tumor had higher Gleason score.

The ductal component of mixed ductal adenocarcinomas ranged from 15% to 95% (mean 46%) of the tumor. Mean tumor volume was 3.38 cc. In 27 (69%) mixed tumors at least one separate nodule of acinar prostate cancer was noted in the prostatectomy (data not shown). Mixed ductal adenocarcinoma was the index tumor in all cases.

There was no difference in clinicopathological parameters between pure and mixed tumors (Table 2).

Disease progression on follow-up (Table 2)

The two small adenocarcinomas with predominant PIN-like ductal features and the patient diagnosed on TURP were excluded from the disease progression analysis. Follow-up information was available for 46 patients. The mean follow-up was 68 months (SD ±61). Four patients received neoadjuvant hormonal therapy; 6 combined adjuvant radiotherapy, hormonal and chemotherapy; 7 adjuvant radiotherapy; 6 adjuvant hormonal therapy; 3 adjuvant radiotherapy and hormonal therapy; 2 hormonal therapy and chemotherapy. There was no difference in disease progression between pure and mixed ductal adenocarcinomas (Table 2).

Table 2. Pathologic parameters and disease progression of patients with ductal adenocarcinomas of the prostate.

	Pure Ductal Adenocarcinomas	Mixed Ductal Adenocarcinomas	All Ductal Adenocarcinomas	p value (pure vs. mixed)
Age (years), mean (SD)	64 (±8)	62 (±5)	63 (±6)	0.36[a]
Tumor volume (cc), mean (range)	2.29 (0.03-8.64)	3.38 (0.20-18.48)	3.0 (0.03-18.48)	0.27[a]
GG, n (%)				0.075[c]
GG2 (GS 3+4)	2 (9)	3 (8)	5 (8)	
GG3 (GS 4+3)	1 (4)	7 (18)	8 (13)	
GG4 (GS 4+4)	15 (68)	14 (36)	29 (47)	
GG5 (GS 4+5)	4 (18)	15 (38)	19 (31)	
Pathologic stage, n (%)				0.11[c]
Organ-confined	11 (50)	10 (26)	21 (35)	
EPE	8 (36)	22 (56)	30 (50)	
SVI	2 (9)	7 (18)	9 (15)	
Lymph node metastasis, n (%)	0/17 (0)	7/36 (19)	7 (13)	0.082[b]
PSM, n (%)	5 (24)	14 (36)	19 (32)	0.47[c]
*Follow-up (months), mean (SD)	64 (±55)	70 (±65)	68 (±61)	0.75[a]
*Biochemical failure (%)	7/16 (44)	8/28 (29)	15/44 (34)	0.31[c]
*Months to biochemical failure, mean (SD)	39 (±44)	41 (±63)	40 (±53)	0.95[a]
*Distant metastasis, n (%)	6/17 (35)	5/29 (17)	11/46 (24)	0.17[c]
*Months to metastasis, mean (SD)	48 (±44)	50 (±23)	49 (±35)	0.92[a]
*Death of disease (%)	0/17 (0)	3/29 (10)	3/46 (6)	0.28[b]

*The two small adenocarcinomas with PIN-like ductal features and the patient diagnosed on TURP were excluded from the analysis. GG, Grade Group; GS, Gleason Score; EPE, extraprostatic extension; SVI, seminal vesicles invasion; TURP, transurethral resection of prostate; PSM, positive surgical margin. [a]:ANOVA; [b]: Fisher's Exact test; [c]: Pearson's chi-square test.

Staining pattern

All except one ductal adenocarcinomas expressed some degree of EZH2 nuclear staining (Fig. 1D); staining intensity was strong in most cases (97%). In 80% of cases >50% of cells were positive (Table 3). The acinar and ductal components of all mixed adenocarcinomas showed concordant EZH2 expression.
ERG protein expression (Fig. 1B) was detected in 12 (20%) cases: 2 pure and 10 mixed adenocarcinomas. The ductal component showed either moderate or strong staining intensity (Table 3). In 10 of 39 (26%) mixed ductal adenocarcinomas, the acinar component expressed ERG; intensity of staining was moderate in 5 and strong in 5 cases each. Concordant ERG expression in acinar and ductal components of mixed tumors was seen in 37 of 39 cases (95%); in two cases the acinar component was ERG positive, but the ductal component was negative.

SPINK1 staining (Fig. 1C) was seen in 22 (36%) cases: 5 pure ductal and 17 mixed adenocarcinomas. In 9 (15%) cases, staining was strong and present in more than 50% of the cells (Table 3). In 13 of 17 (76%) mixed tumors, the acinar component also expressed SPINK1; staining was moderate in 10 and strong in 3 cases. Concordant SPINK1 expression in acinar and ductal components of mixed tumors was seen in 31 of 39 (79%) cases: in two cases the acinar component was positive, but the ductal one was negative; in 6 cases the ductal component was positive, but the acinar one was negative. Six (10%) cases, five mixed and one pure, showed co-expression of SPINK1 and ERG; however SPINK1 protein expression was strong and detected in >50% of cells only in 3 (5%) cases. There was no significant difference in protein expression between pure and mixed ductal adenocarcinomas.

The relationship between EZH2, ERG and SPINK1 protein expression and disease progression is summarized in Table 4 (Table 4a,b). There was no difference in tumor stage, rate and time to progression (biochemical failure, metastases and death) on comparison of tumors by protein expression as - tumors positive versus negative for EZH2, ERG, and SPINK1 respectively; percent expression (<50% versus >50%, negative versus <50%, negative versus >50%) for EZH2 and SPINK1 respectively; and combination of expression (EZH2>50%/ERG-/SPINK1>50% versus EZH2>50%/ERG-/SPINK1-).

Discussion

Ductal adenocarcinoma of prostate is an uncommon variant of prostatic carcinoma with adverse prognosis. The frequency of pure ductal and mixed ductal/acinar adenocarcinoma is approximately 1% and 5%, respectively (Epstein, 2010; Morgan et al., 2010; Amin and Epstein, 2011; Meeks et al., 2012). Although Samaratunga and colleagues concluded that any percentage of ductal component increases the risk of extraprostatic extension (Samaratunga et al., 2010), other studies have suggested that pure ductal adenocarcinomas and mixed ductal/acinar carcinomas with ductal component greater than 10% of tumor volume, are more likely to present at advanced clinical stage (Bostwick et al., 1985; Christensen et al., 1991; Brinker et al., 1999; Samaratunga et al., 2010; Amin and Epstein, 2011; Meeks et al., 2012).

We evaluated 61 ductal adenocarcinomas of prostate, with a ductal component comprising more than 10% of the tumor, with the aim to investigate expression and potential prognostic significance of protein biomarkers likely to be associated with aggressive behavior. In our cohort, incidence of pure ductal and mixed ductal/acinar adenocarcinoma was approximately 0.7% and 1.2%, respectively, of all prostate cancer cases. Only 35% of the cases were organ-confined; 15% had seminal vesicle involvement. Similarly, other investigators have reported that approximately 1/3 of ductal adenocarcinomas included in their studies were organ confined, compared to 2/3 of acinar tumors; seminal vesicle involvement was significantly more frequent in ductal than acinar adenocarcinomas (Amin and Epstein, 2011) and ranged

Table 3. EZH2, ERG, and SPINK1 protein expression in ductal adenocarcinomas of the prostate.

Immunohistochemistry		Pure Ductal Adenocarcinomas (n=22)	Mixed Ductal Adenocarcinomas (n=39)	All Ductal Adenocarcinomas (n=61)
EZH2	n (%)	22 (100)	38 (97)	60 (98)
	Moderate	1	1	2
	Strong	21	37	58
	≤50% positive cells	4	7	11
	>50% positive cells	18	31	49
ERG	n (%)	2 (9)	10 (26)	12 (20)
	Moderate	1	5	6
	Strong	1	5	6
SPINK1	n (%)	5 (23)	17 (43)	22 (36)
	Moderate	4	9	13
	Strong	1	8	9
	≤50% positive cells	4	9	13
	>50% positive cells	1	8	9

from 19% to 31% (Amin and Epstein, 2011; Vinceneux et al., 2017). We detected lymph node metastasis in 13% of cases, in keeping with a recent study (Vinceneux et al., 2017). Previous investigators have reported similar rate of lymph node metastases for ductal (3%) and acinar (1.8%) adenocarcinomas, but a threefold higher risk of distant metastases in ductal (11%) versus acinar (4%) carcinoma (Morgan et al., 2010; Meeks et al., 2012). In our cohort, with a mean follow-up of 68 months, distant metastasis and biochemical failure occurred in 24%, and 34% of patients, respectively; 3 patients (7%) died of disease (Table 2). Our disease specific mortality is in keeping with 12% mortality reported for ductal prostatic adenocarcinoma in a SEER study (Meeks et al., 2012). When we compared pure and mixed ductal adenocarcinomas for all clinicopathological variables previously mentioned, we found no significant difference, except for growth pattern.

Only few studies have assessed the molecular relationship of ductal and acinar adenocarcinoma of the prostate. A gene expression profiling study conducted on 5 ductal and 11 acinar adenocarcinomas matched for Gleason grade detected no global differences in gene expression, suggesting a strikingly similarity between the groups (Sanati et al., 2009). A stringent analysis of the data identified only 10-30 gene transcripts with significant differential expression between groups (Sanati et al., 2009).

ERG, the most common fusion partner of *TMPRSS2* (Perner et al., 2007), is detected in approximately 50% of prostate-specific antigen (PSA) - screened prostate cancers (Perner et al., 2006). There has been extensive research on significance of *TMPRSS2-ERG* fusion in acinar prostate cancer prognosis with conflicting results (Petrovics et al., 2005; Perner et al., 2006; Mehra et al., 2007; Nam et al., 2007; Rajput et al., 2007; Attard et al., 2008; Cheville et al., 2008; Saramaki et al., 2008; Gopalan et al., 2009; Hermans et al., 2009; Bismar et al., 2012). To date, only few studies have reported on the frequency of *TMPRSS2-ERG* gene fusion in ductal adenocarcinoma of the prostate. Lotan and colleagues studied 40 ductal and 38 acinar carcinomas, and found ERG gene rearrangement by fluorescence in situ hybridization (FISH) to be less frequent in ductal (11%) compared to stage matched acinar adenocarcinomas (45%) (Lotan et al., 2009). Han and colleagues detected *TMPRSS-ERG* fusion by FISH in half of 18 ductal adenocarcinomas (Han et al., 2009). ERG protein expression by immunohistochemistry correlated with TMPRSS2-*ERG* rearrangement in numerous studies (Lotan et al., 2009; Falzarano et al., 2011; Bismar et al., 2012). Seipel et al. evaluated 60 ductal adenocarcinomas and 46 Gleason Score matched acinar carcinomas and detected ERG protein expression in 38% of ductal and 31% of acinar carcinomas (Seipel et al., 2014). Morais and colleagues assessed ERG status by immunohistochemistry in 37 ductal adenocarcinomas, 18 synchronous acinar adenocarcinomas, and 34 stage- and grade-matched acinar adenocarcinomas (Morais et al., 2015). In their cohort, ERG expression was less in ductal adenocarcinomas (11%) and their synchronous acinar tumors (6%), compared to matched pure acinar adenocarcinomas (50%) (p=0.0005 and 0.002, respectively) (Morais et al., 2015). Vinceneux and colleagues recently reported ERG expression in 40% of ductal adenocarcinomas (Vinceneux et al., 2017). We detected ERG protein expression in 20% cases, a rate within the range previously reported by other

Table 4a. Prognosis by immunohistochemistry expression in ductal adenocarcinoma**.

IHC	BCF	Metastasis	Death
EZH2+ (>50%)	14	10	2
EZH2+ (<50%)	1	1	1
EZH2-	0	0	0
ERG+	3	3	1
ERG-	12	8	2
SPINK1+ (>50%)	4	3	0
SPINK1+ (<50%)	2	1	0
SPINK1-	9	7	3
ERG+/ SPINK1+	1	1	0
ERG-/ SPINK1-	7	5	2
EZH2>50%/ERG-/SPINK1-	5	4	1
EZH2>50%/ERG-/SPINK1>50%	3	2	1

IHC immunohistochemistry; + positive; - negative; BCF biochemical failure; **The two small adenocarcinomas with PIN-like ductal features were excluded from the analysis.

Table 4b. Prognosis by immunohistochemistry expression in ductal adenocarcinoma**.

	BCF (n=15)	No BCF (n=29)	Metastasis (n=11)	No Metastasis (n=35)	Death (n=3)	No Death (n=43)
EZH2>50%	14 (93%)	23 (79%)	10 (91%)	28 (80%)	2 (67%)	36 (84%)
ERG+	3 (20%)	6 (67%)	3 (27%)	6 (67%)	1 (33%)	8 (19%)
ERG-	12 (80%)	23 (79%)	8 (72%)	29 (83%)	2 (67%)	35 (81%)
SPINK1>50%	4 (27%)	8 (27%)	3 (27%)	10 (28%)	0 (0%)	13 (30%)
SPINK1-	9 (60%)	17 (59%)	7 (64%)	20 (57%)	3 (100%)	24 (56%)
ERG+/SPINK1+	1 (7%)	3 (10%)	1 (9%)	3 (8%)	1 (33%)	4 (9%)
ERG-/SPINK1-	7 (47%)	12 (41)	5 (45%)	18 (51%)	2 (67%)	18 (42%)
EZH2>50%/ERG-/SPINK1-	5 (33%)	11 (38%)	4 (36%)	13 (37%)	1/3 (33%)	16 (37%)
EZH2>50%/ERG-/SPINK1>50%	3 (20%)	4 (14)	2 (18%)	7 (20%)	1/3 (33%)	8 (19%)

investigators for ductal adenocarcinoma of prostate and considerably lower than prostatic acinar adenocarcinoma.

SPINK1 is a protein overexpressed in a subset of E26 transformation specific (ETS) translocation-negative prostate cancers (Paju et al., 2007; Tomlins et al., 2008; Lippolis et al., 2013). Although in most studies SPINK1 protein has been detected in 5%-9% of cases (Bhalla et al., 2013; Grupp et al., 2013; Lippolis et al., 2013; Flavin et al., 2014), a wider range of expression has been reported by various investigators (Paju et al., 2007; Smith and Tomlins, 2014). Studies on SPINK1 expression in ductal adenocarcinoma are limited. Han et al. found SPINK1 overexpression in 2 (6%) cases of 31 of *TMRPSS2:ETS* negative prostate cancers, both cases being ductal adenocarcinomas (Han et al., 2009). Vinceneux and colleagues detected SPINK1 protein expression in 9% of 45 ductal adenocarcinoma (Vinceneux et al., 2017). We detected SPINK1 in 36% of cases, a rate much higher than 10% reported by most studies for conventional prostate cancer using the 4D4 antibody, and also higher than 6%-9% reported for ductal adenocarcinoma (Smith and Tomlins, 2014). However, only in 15% of cases more than half of the tumor cells were strongly positive for SPINK1 (Table 3). SPINK1 expression by immunohistochemistry is heterogeneous, and much of the variability in percent cells positive and prognostic importance stems from varying criteria used to determine SPINK1 positivity and use of different antibodies. Tomlins and colleagues have reported variable staining intensity in 36% of prostate cancers (Tomlins et al., 2008). However, as percentage of positive cases was far greater than in other cohorts, the authors defined as positive only cases (9%) showing at least one tissue microarray core with >80% of SPINK1 positive cells (Tomlins et al., 2008). Contrary to earlier reports of ERG and SPINK1 being mutually exclusive (Tomlins et al., 2008), recent studies have reported co-expression of ERG and SPINK1 in up to 4% of prostate cancers (Bhalla et al., 2013; Grupp et al., 2013; Flavin et al., 2014; Smith and Tomlins, 2014). We detected co-expression of SPINK1 and ERG in 6 ductal adenocarcinomas, although only in 3 cases the protein expression was strong and in more than 50% of tumor cells.

SPINK1 expression has been linked to Gleason Score (Paju and Stenman, 2006), but its prognostic significance remains uncertain. Three large independent studies have found no association with SPINK1 expression by immunohistochemistry and clinicopathological parameters, disease progression, or prostate cancer mortality (Grupp et al., 2013; Lippolis et al., 2013; Flavin et al., 2014). Tomlins et al. have considered SPINK1 protein expression an independent predictor of biochemical recurrence after radical prostatectomy (Tomlins et al., 2008). Jhavar and co-authors reported that prostate cancers with high SPINK1 messenger RNA (mRNA) levels had significantly worse PSA failure ratio; however, they failed to find a link between immunohistochemically detected SPINK1 protein expression and poor survival (Jhavar et al., 2009).

EZH2 overexpression in prostate cancer has been associated with aggressive disease, metastases, and poor prognosis (Melling et al., 2015). EZH2 seems expressed at relatively low levels in conventional prostate cancer (Hoogland et al., 2014). Some investigators have reported EZH2 immunoreactivity in 80-90% of prostate tumors, but in most cases the percentage of malignant cells expressing EZH2 did not exceed 10% (Ugolkov et al., 2011). Greater EZH2 expression has been reported in tumors with Gleason score ≥8, positive margins and extraprostatic extension (van Leenders et al., 2007); staining intensity and percent positive cells seem to be associated with Gleason score and nodal status (Matsika et al., 2015). At the best of our knowledge, this is the first study reporting on EZH2 expression in ductal adenocarcinoma of prostate. We detected EZH2 protein in all ductal adenocarcinomas except in one case. In 80% cases more than half tumor cells were positive.

There was no difference in grade, stage, biochemical recurrence, metastasis or death when tumors were compared by EZH2, ERG and SPINK1 expression grouped as positive versus negative, percent expression or by combination of expression.

This study has several limitations. All the patients in our cohort, except one, underwent radical prostatectomy. Since patients with more advanced disease may have not been considered for surgery, one needs to be aware of a potential selection bias. Another limitation is that since pure ductal adenocarcinoma is generally associated with low PSA, biochemical failure may not be the most useful parameter for surveillance in these patients. In addition, the comparison of our IHC findings with other studies performed on TMA is not ideal due to the heterogeneous expression of some of the proteins.

Conclusion

We found no significant difference between pure and mixed ductal adenocarcinomas in terms of clinicopathological parameters and protein expression. These findings support the notion that mixed ductal/acinar adenocarcinomas with >10% ductal features behave similarly to pure ductal adenocarcinomas. We detected EZH2 protein expression in most ductal adenocarcinomas; interestingly, in contrast to what has been reported for acinar prostate cancer, we found EZH2 staining in >50% of tumor cells in most ductal adenocarcinomas. ERG protein expression was less common in ductal adenocarcinoma (20%) compared to rates reported for acinar adenocarcinoma (~50%); on the other hand, a higher percentage of ductal adenocarcinomas expressed SPINK1. The higher EZH2 and SPINK1 protein expression, compared to acinar prostate cancer, may partly explain the more aggressive behavior of ductal adenocarcinomas.

Larger studies on the prognostic significance of

molecular markers involved in pathways common to EZH2, ERG, and SPINK1 are necessary to better elucidate their impact on the clinical behavior of prostate cancer variants.

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