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TMPRSS2–ERG gene fusion status in minute (minimal) prostatic adenocarcinoma

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Minute prostatic adenocarcinomas are considered to be of insufficient virulence. Given recent suggestions of *TMPRSS2–ERG* gene fusion association with aggressive prostatic adenocarcinoma, we evaluated the incidence of *TMPRSS2–ERG* fusion in minute prostatic adenocarcinomas. A total of 45 consecutive prostatectomies with minute adenocarcinoma were used for tissue microarray construction. A total of 63 consecutive non-minimal, Gleason Score 6 tumors, from a separate PSA Era prostatectomy tissue microarray, were used for comparison. FISH was carried out using ERG break-apart probes. Tumors were assessed for fusion by deletion (Edel) or split (Esplit), duplicated fusions and low-level copy number gain in normal ERG gene locus. Minute adenocarcinomas: Fusion was evaluable in 32/45 tumors (71%). Fifteen out of 32 (47%) tumors were positive for fusion. Six (19%) were of the Edel class and 7 (22%) were classified as combined Edel + Esplit. Non-minute adenocarcinomas (pT2): Fusion was identified in 20/30 tumors (67%). Four (13%) were of Edel class and 5 (17%) were combined Edel + Esplit. Duplicated fusions were encountered in 5 (16%) tumors. Non-minute adenocarcinomas (pT3): Fusion was identified in 19/33 (58%). Fusion was due to a deletion in 6 (18%) tumors. Seven tumors (21%) were classified as combined Edel + Esplit. One tumor showed Esplit alone. Duplicated fusions were encountered in 3 (9%) cases. The incidence of duplicated fusions was higher in non-minute adenocarcinomas (13 *vs* 0%; $P = 0.03$). A trend for higher incidence of low-level copy number gain in normal *ERG* gene locus without fusion was noted in non-minute adenocarcinomas (10 *vs* 0%; $P = 0.07$). We found a *TMPRSS2–ERG* fusion rate of 47% in minute adenocarcinomas. The latter is not significantly different from that of grade matched non-minute adenocarcinomas. The incidence of duplicated fusion was higher in non-minute adenocarcinomas. Our finding of comparable rate of *TMPRSS2–ERG* fusion in minute adenocarcinomas may argue against its value as a marker of aggressive prostate carcinoma phenotype.
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A recurrent fusion between the androgen-regulated gene *TMPRSS2* (21q22.3) and the *ETS* transcription factor family member *ERG* (21q22.2), initially described by Tomlins *et al*,[1,2] seems to be a common occurrence in prostate carcinoma. *TMPRSS2–ERG* fusion rates of 15–80% have been reported in prostatic adenocarcinoma by various groups,[2–7] making it the most common rearrangement identified in human cancer to date. The fusion can be the result of a small deletion on chromosome 21 (seen in approximately two thirds of cases) or can occur through a translocation.[5] In either type of rearrangement, *ERG* is brought under the control of an androgen-regulated promoter and overexpression of the protein ensues. Studies addressing the clinical significance of *TMPRSS2–ERG* fusion as a prognostic marker have so far led to conflicting conclusions.[8–13] Some of the earlier studies have pointed to a subset of *TMPRSS2–ERG* fusions as being markers of aggressive outcome.[8–10]

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Although the incidence of *TMPRSS2–ERG* fusion has been extensively studied in various prostatic adenocarcinoma cohorts,[8–13] to date, no study has assessed the incidence of *TMPRSS2–ERG* in the unique group of minute (minimal) adenocarcinomas of prostate. Minute prostatic adenocarcinoma is defined as a tumor fulfilling all the following criteria: (i) total volume of 0.5 cm^3 or less; (ii) Gleason Score <7; (iii) tumor is organ confined on radical prostatectomy with no evidence of surgical margin, seminal vesicle or lymph node involvement.[14] Defined as such, it is estimated that around 5% of all prostate adenocarcinoma undergoing radical prostatectomy represent minute prostatic adenocarcinomas. Such tumors are considered to be clinically 'insignificant' tumors lacking the virulence for significant morbidity or mortality.[15,16]

The aim of this study was to assess the incidence of *TMPRSS2–ERG* fusion in a group of minute prostatic adenocarcinomas and to compare such incidence with that of a Gleason score-matched (<7) non-minute prostatic adenocarcinomas of stage pT2N0M0 or pT3N0M0.

Materials and methods

This study is approved by our Institutional Review Board.

Patient Cohort and Tissue Microarray Construction

Minute prostatic adenocarcinoma group
We retrieved all radical prostatectomy specimens for prostatectomies carried out at the Johns Hopkins Hospitals between January 2002 and June 2003, and diagnosed with prostatic adenocarcinoma tumors meeting the above criteria of minute prostatic adenocarcinomas. H&E sections and paraffin blocks were obtained and the diagnosis of minute prostatic adenocarcinomas was confirmed by three urologic pathologists involved in this study (GJN, RA and ML). A total of 45 consecutive minute prostatic adenocarcinoma radical prostatectomy specimens with available archival material were included for tissue microarray construction. In three patients, two separate minute prostatic adenocarcinoma foci were sampled in the tissue microarray. Tumor and paired benign tissue were represented by up to three 1-mm spots using the method previously described by Kononen *et al*.[17]

Non-minute prostatic adenocarcinoma group
Sixty-three consecutive radical prostatectomy specimens from a separate tissue microarray set constructed from 742 parent population-based PSA Era prostatectomies carried out at the Johns Hopkins hospital (1993–2000) were used as our non-minute prostatic adenocarcinoma group.[13] All 63 tumors were of GS <7. They included 30 pT2 and 33 pT3 tumors.

Clinicopathological data
All pertinent clinicopathological data were retrieved from electronic medical records to include patient's age, ethnicity, preoperative PSA, percentages of tumor involvement at biopsies and detailed prostatectomy pathologic findings. The latter included prostate weight, tumor volume, tumor grade, stage and location of tumor.

Evaluation of *TMPRSS2–ERG* Fusion Status with Interphase *ERG* Break Apart FISH Assay

FISH analysis was carried out using dual-color interphase break-apart probes for the 5′ and 3′ regions of *ERG* gene. Briefly, 4 μm paraffin-embedded tissue microarray sections were baked at 56°C for 2 h then dewaxed and rehydrated using xylene and graded ethanol, respectively. Tissue microarray sections were pretreated using Paraffin Pretreatment Reagent Kit III (Abbott Molecular, IL). BAC FISH probes used were Spectrum Green d-UTP direct-labeled BAC RP11-95I21 for 5′ *ERG*, and Spectrum Orange d-UTP direct-labeled BAC RP11-476D17 for 3′ *ERG* (Nick transKit, Vysis, Abbott Park, IL). Tissue microarrays and BAC FISH probes were co-denatured at 94°C for 5 min and hybridized overnight at 37°C in a humid chamber (StatSpin ThermoBrite, IRIS, MA).

FISH interpretation was carried out by two urologic pathologists (RA and GJN). Tissue microarray sections were scored using a $\times 100$ oil immersion lens on an Olympus BX-70 fluorescence microscope (Olympus, Center Valley, PA) equipped with appropriate filters. For photomicrographs, images were captured using a Nikon E400 fluorescence microscope equipped with a Nikon DXM1200 camera (Nikon Instruments, Melville, NY) and the SPOT Advanced digital imaging software (Diagnostic Instruments, Sterling Heights, MI). In each case, a minimum of 50 cells were scored for the presence/absence of *TMPRSS2–ERG* gene fusion through deletion or split. Digitally scanned adjacent H&E sections were available for side-by-side comparison with the FISH image to localize tumor cells. Gleason grade was confirmed for each tissue microarray spot. Paired benign prostatic epithelium was also scored as a negative control.

Each tissue microarray spot was analyzed for *TMPRSS2–ERG* fusion as previously described by Attard *et al*[10] with the following minor modifications: (i) class negative for fusion (N): a nucleus with two pairs of juxtaposed red and green signals forming yellow signals, indicating the absence of *ERG* rearrangement. (ii) class *ERG* signal split (Esplit): a nucleus with one juxtaposed red–green signal pair of the non-rearranged *ERG* allele and additional separate single red and single green signal of rearranged *ERG* allele (break apart) reflecting a *TMPRSS2–ERG* fusion through split. (iii) class *ERG* deletion (Edel): a nucleus with one juxtaposed

red–green signal pair for the non-rearranged allele and a single red signal of a rearranged allele, indicating deletion of the telomeric (green) *ERG* probe region (fusion through deletion). Any case with one of the above *ERG* signal abnormalities in $\geq 10\%$ of the nuclei was scored as a fusion case and classified accordingly. Tumors with a subpopulation of cells with different types of fusions meeting the above cutoff were assigned more than one fusion class.

In addition, the presence of multiple copies of the particular *TMPRSS2–ERG* fusion as well as the presence of a low-level copy number gain of a normal *ERG* (>2 copies) was simultaneously assessed in all evaluated nuclei.

A tissue microarray spot was deemed technically inadequate for scoring if lack of diagnostic tissue, weak probe signal or presence of overlapping nuclei prevented accurate FISH assessment.

Statistical Analysis

Demographic, clinicopathological and *TMPRSS2–ERG* data were analyzed using the Stata 9.2 software package (StataCorp; college Station TX). Equality of population means among groups was tested using Kruskal–Wallis test for non-parametric and one-way analysis of variance by ranks.

Table 1 Demographic and clinicopathological characteristics of 32 patients with clinically insignificant minute prostate cancer

Characteristic	*No of patients* N = *32 (%)*
Age (years)	
<61	21 (66)
61–65	10 (31)
>65	1 (3)
Ethnicity	
American African	3 (9)
Caucasian	28 (88)
Other	1 (3)
Preoperative PSA level (ng/ml)	
<4	8 (23)
4–10	21 (66)
>10	2 (6)
Tumor volume on biopsies	
≤5%	11 (34)
>5%	21 (66)
Multifocality (number of tumors)	
Single	16 (50)
Multifocal	16 (50)
Location	
Apical	13 (41)
Mid	11 (34)
Base	8 (25)

Results

Patient Demographics and Clinical Findings

Patient demographics and clinical data are summarized in Tables 1 and 2. There was no significant difference in mean patient age between minute and non-minute prostatic adenocarcinoma patients. As expected, a higher preoperative serum PSA was present in non-minute prostatic adenocarcinoma patients ($P = 0.002$), similarly, higher incidence of biochemical recurrence was encountered in non-minute prostatic adenocarcinoma patients ($P = 0.001$).

TMPRSS2–ERG FISH Analysis

None of the examined paired, benign, prostate gland tissue spots from 43 minute prostatic adenocarcinoma cases (80 spots) and 63 non-minute prostatic adenocarcinomas showed the evidence of *TMPRSS2–ERG* fusion using our FISH assay.

Minute prostatic adenocarcinoma group
TMPRSS2–ERG fusion was evaluable by FISH in 32 of 45 (71%) samples represented on the tissue microarray.

Fifteen (47%) tumors were positive for the *TMPRSS2–ERG* fusion (Figures 1 and 2; Table 3). Out of the minute prostatic adenocarcinoma tumors that were positive for fusion, 6 (19%) were of the Edel class and seven (22%) were classified as combined Edel + Esplit due to the presence of two subpopulations of cells showing the two fusion classes. None of our minute prostatic adenocarcinomas showed Esplit alone. Duplicated fusion (2Edel or 2Esplit) was not encountered in this group.

Two minute prostatic adenocarcinomas (6%) showed low-level copy number gain of non-rearranged *ERG* allele associated with a single copy *TMPRSS2–ERG* fusion. Low-level copy number gain of a normal *ERG* without *ERG* fusion was not observed in any minute prostatic adenocarcinoma cases.

In two of the three minute prostatic adenocarcinomas cases containing more than one distinct focus of cancer on the radical prostatectomy specimens that were sampled on the tissue microarray, *TMPRSS2–ERG* fusion (Edel) was present in one, but not in the other, focus of prostatic adenocarcinoma.

Non-minute prostatic adenocarcinomas (pT2)
TMPRSS2–ERG fusion was identified in 20/30 (67%) pT2 non-minute prostatic adenocarcinomas. The fusion was due to a deletion in 4/30 (13%) cases. Five additional tumors (17%) were classified as combined Edel + Esplit because of the presence of two subpopulations of cells showing the two fusion

Table 2 Clinical data in minute and non-minute prostatic adenocarcinoma cohorts

	Minute prostatic adenocarcinomas (N = 32)	*Non-minute prostatic adenocarcinomas pT2 (N = 30)*	*Non-minute prostatic adenocarcinomas pT3 (N = 33)*	P-*value*
Race				0.01
White	28	25	26	
Black	3	3	7	
Others	1	2	0	
Mean age (range; years)	55.9 (44–66)	59 (43–68)	55.8 (41–67)	Non-significant
Mean preoperative PSA (range; ng/ml)	5.03 (0.3–11.5)	7.5 (1.4–16)	8.1 (0.6–23)	0.02
Positive margins (%)	0/32 (0%)	0/30 (0%)	12/33 (36%)	0.0001
Prostate weight (range; g)	61.8 (34–126)	67 (27–200)	48.5 (29–99)	0.01
Biochemical recurrence				0.001
Yes	0	2	7	
Non	32	8	5	
NA		20	21	

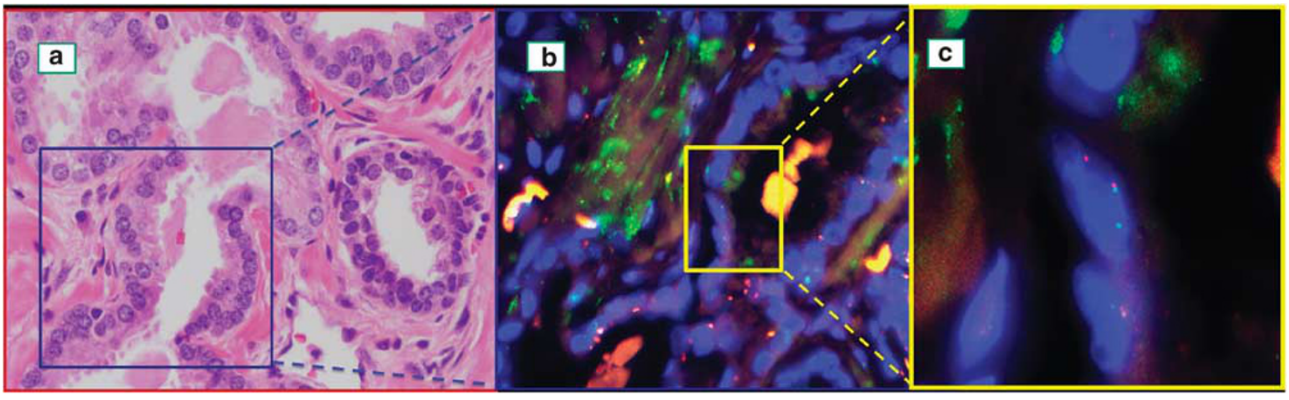

Figure 1 (**a**) Tissue microarray spot showing a focus of minute prostate adenocarcinoma (hematoxylin–eosin stain: × 200). (**b** and **c**) FISH break apart assay for *TMPRSS2–ERG*. *TMPRSS2–ERG* fusion through *ERG* split (Esplit), with one juxtaposed red–green (yellow) signal in each nucleus and the second pair of red and green signals split apart and separated spatially in different regions of the nucleus (original magnification: × 1000).

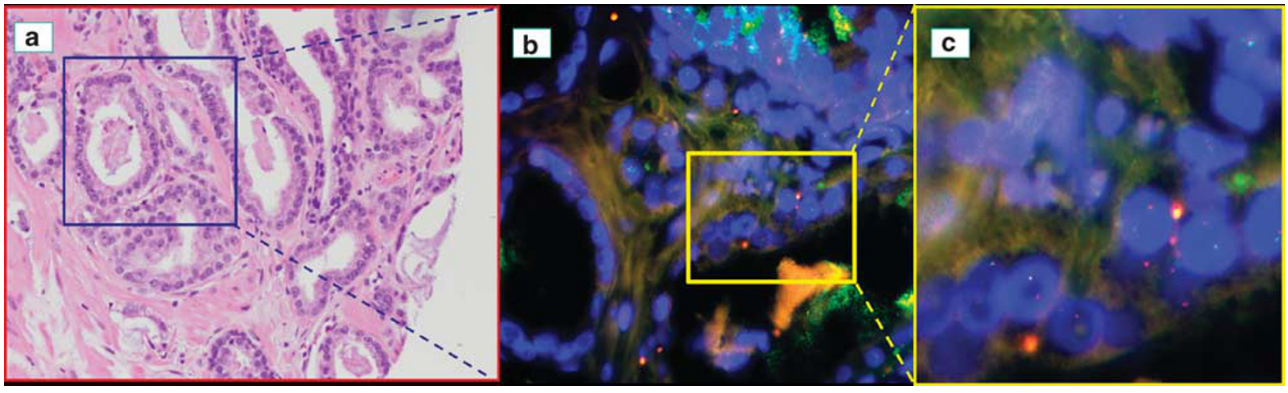

Figure 2 (**a**) Tissue microarray spot showing a focus of minute prostate adenocarcinoma (hematoxylin–eosin stain: × 100). (**b** and **c**) FISH break apart assay for *TMPRSS2–ERG*. *TMPRSS2–ERG* fusion through deletion (Edel), with one juxtaposed red–green (yellow) signal in each nucleus and absence of the second green signal (original magnification: × 1000).

classes. None of pT2 non-minute prostatic adenocarcinomas showed Esplit alone.

Duplicated fusion (2Edel or 2Esplit) was encountered in 5 (16%) cases in this group.

Six (20%) pT2 non-minute prostatic adenocarcinomas showed low-level copy number gain of non-rearranged *ERG* allele associated with a single copy *TMPRSS2–ERG* fusion. In addition, low-level copy

Table 3 *TMPRSS2–ERG* fusion status in minute and non-minute prostate carcinoma cohorts

	Minute prostatic adenocarcinomas	*Non-minute prostatic adenocarcinomas*						
	n = *32 (%)*	*pT2* n = *30 (%)*	P-*value Minute prostatic adenocarcinomas vs pT2*	*pT3* n = *33 (%)*	P-*value Minute prostatic adenocarcinomas vs pT3*	*pT2+pT3* n = *63 (%)*	P-*value Minute prostatic adenocarcinomas vs pT2+pT3*	P-*value Minute prostatic adenocarcinomas vs pT2 vs PT3*
Fusion	15/32 (47)	20/30 (67)	0.1	19/33 (58)	0.39	39/63 (62)	0.16	0.2
Edel	6/32 (19)	4/30 (13)	0.56	6/33(18%)	0.95	10/63 (16%)	0.72	0.82
Esplit	0/32 (0%)	0/30	1	1/33 (3%)	0.32	1/63 (2%)	0.47	0.39
Combined Edel+Esplit	7/32 (22%)	5/30 (17)	0.6	7/33 (21%)	0.94	12/63 (19%)	0.74	0.85
Duplicated Fusion	0/32 (0%)	5/30 (16%)	0.01	3/33 (9%)	0.08	8/63 (13%)	0.03	0.06
Low-level copy number gain of a normal *ERG*+Fusion	2/32(6%)	6/30 (20)	0.1	2/33 (6%)	0.97	8/63 (13%)	0.33	0.12
Low-level copy number gain of a normal *ERG*	0/32 (0%)	3/30 (10%)	0.06	3/33 (9%)	0.08	6/63 (10%)	0.07	0.19

Esplit, class *ERG* split; Edel, class *ERG* deletion; Duplicated fusion, multiple copies of the particular *TMPRSS2–ERG* fusion: low-level copy number gain of a normal *ERG* (>2 copies).

number gain of non-rearranged *ERG* without *ERG* fusion was also observed in 3/30 (10%) of pT2 non-minute prostatic adenocarcinomas.

Non-minute prostatic adenocarcinomas (pT3)
TMPRSS2–ERG fusion was identified in 19/33 (58%) pT3 non-minute prostatic adenocarcinomas. The fusion was due to a deletion in 6/33 (18%) cases. Seven additional tumors (21%) were classified as combined Edel + Esplit because of the presence of two subpopulations of cells showing the two fusion classes. One pT3 non-minute prostatic adenocarcinomas showed Esplit alone.

Duplicated fusion (2Edel or 2Esplit) was encountered in three (9%) cases in this group.

Two (6%) pT3 non-minute prostatic adenocarcinomas showed low-level copy number gain of non-rearranged *ERG* allele associated with a single copy *TMPRSS2–ERG* fusion. In additional, low-level copy number gain of a normal *ERG* without *ERG* fusion was also observed in 3/33 (9%) of pT3 non-minute prostatic adenocarcinomas.

Comparison of *TMPRSS2–ERG* Fusion Incidence Between Minute Prostatic Adenocarcinomas and Non-Minute Prostatic Adenocarcinomas

Detailed comparative analysis of the different groups is shown in Table 3. Although the overall frequency of *TMPRSS2–ERG* fusion was slightly lower in minute prostatic adenocarcinomas compared with non-minute prostatic adenocarcinomas (47 *vs* 62%), the difference was not statistically significant (P = non-significant). The incidence of duplicated fusion was higher in non-minute prostatic adenocarcinoma group (13 *vs* 0%; $P = 0.03$). In addition, a trend for higher incidence of low-level copy number gain of a normal *ERG* without fusion in non-minute prostatic adenocarcinomas (10 *vs* 0%; $P = 0.07$) was noted. We observed no statistically significant difference between minute prostatic adenocarcinomas and non-minute prostatic adenocarcinoma cases in term of the incidence of class Esplit, Edel, combined Espli + Edel or the presence of low-level copy number gain of a normal *ERG* with fusion.

Similar results were obtained when the minute prostatic adenocarcinomas were compared to the subset of stage pT2 non-minute prostatic adenocarcinomas. *TMPRSS2–ERG* fusion incidence was slightly lower in minute prostatic adenocarcinomas cases (47 *vs* 67%), the difference was again not statistically significant (P = non-significant). The incidence of duplicated fusion was higher in the non-minute prostatic adenocarcinoma pT2 group (16 *vs* 0%; $P = 0.01$). The trend for higher incidence of low-level copy number gain of a normal *ERG* without fusion remained in non-minute prostatic adenocarcinoma pT2 tumors (10 *vs* 0%; $P = 0.06$). We observed no statistically significant difference

between minute prostatic adenocarcinomas and non-minute prostatic adenocarcinoma pT2 cases in term of the incidence of the remaining fusion classes.

Relationship of Presence of Fusion and Fusion Class to Biochemical Recurrence

As expected, none of minute prostatic adenocarcinoma patients developed evidence of biochemical recurrence. In non-minute prostatic adenocarcinoma patients, no statistically significant correlation was detected between biochemical recurrence and the presence of fusion (*P*: non-significant). Similarly, we found no association between biochemical recurrence and fusion class, the presence of duplicated fusion nor the presence of low-level increased gene copy number of non-rearranged *ERG* allele (*P* = non-significant).

Discussion

Presently, an accurate prediction of the presence of minute prostatic adenocarcinoma in a prostatectomy specimen cannot be achieved preoperatively. Patients diagnosed with a single small focus of prostatic adenocarcinoma on needle biopsy, especially when the focus is less than one high-power field, are thought to be more likely to harbor a minute prostatic adenocarcinoma on radical prostatectomy.[14] Given that minute prostatic adenocarcinomas are generally considered to be clinically 'insignificant' tumors,[15,16] the need for radical prostatectomy in such patients is frequently brought into question. Finding markers that will help better identify such subset of patients is of great interest. *TMPRSS2–ERG* fusion is a common genetic alteration in prostatic adenocarcinoma. In this study, we aimed at evaluating the incidence of this fusion in a unique set of minute prostatic adenocarcinoma specimens in comparison with non-minute prostatic adenocarcinomas to assess any potential role of the presence of *TMPRSS2–ERG* fusion, or type of fusion class, in differentiating the two groups of patients.

To date, with the exception of a rare study, most large cohort studies have found the incidence of *TMRSS2–ERG* rearrangement in non-minute prostatic adenocarcinoma tumors to be in the 40–60% range.[2,3,5,9,12,18] In our PSA Era cohort of Gleason 6 non-minute prostatic adenocarcinoma tumors, we found a 62% rearrangement rate similar to the rates reported in surgical cohort studies assessing *ERG* gene alterations by similar FISH break-apart assay.[5]

In this study, fusions in minute prostatic adenocarcinoma and non-minute prostatic adenocarcinoma tumors were more likely to be the result of deletion either homogeneously throughout a given tumor or in association with subpopulation of tumor cells harboring a split event. The preponderance of deletion events is in line with prior observations.[5,18,19] Intronic loss of genomic DNA between *ERG* and *TMPRSS2*, on chromosome 21q22.2–3, seems to be the main mechanism of gene fusion.

Although, we observed a slightly lower *TMPRSS2–ERG* fusion rate of 47% in our minute prostatic adenocarcinomas, the difference was not statistically significant compared with non-minute prostatic adenocarcinoma groups. Our latter finding was not totally unexpected given the fact that the presence of fusion has been previously documented in up to 20% of high-grade PIN.[4,20] The occurrence of fusion at the prostatic adenocarcinoma precursor stage suggests that it is a relatively early pathogenic event in prostatic adenocarcinoma oncogenesis. In fact, our finding regarding a high incidence of fusion events in our minute prostatic adenocarcinoma cohort of presumably early prostatic tumors can be seen as additional indirect evidence in support of the early role of *TMPRSS2–ERG* fusion in prostatic adenocarcinoma pathogenesis.

Earlier studies have shown the presence of *TMPRSS2–ERG* rearrangement to be homogeneous in a given tumor focus,[5] but heterogeneous in the context of multiple cancer foci within the same prostate.[21] In this regard, we find our observation regarding incongruous fusion status in two of the three minute prostatic adenocarcinoma tumors to be intriguing and in support of earlier observations in larger volume, more advanced tumors. The variability of fusion status in separate minute prostatic adenocarcinoma tumor foci in the same radical prostatectomy specimen would support the previous suggestion by Clark *et al*[22] that *TMPRSS2–ERG* gene fusions may be arising independently in different regions of a single prostate.

Our *ERG* gene copy number findings of a significantly higher rate of duplicated *ERG* fusions and a trend toward higher rate of low-level copy number gain of a normal *ERG* in non-minute prostatic adenocarcinomas compared with minute prostatic adenocarcinomas are in line with previously shown association between abnormalities in ploidy status and prostatic adenocarcinoma tumor progression.[23–25] Our low-level, increased *ERG* gene copy number suggests that gain of *ERG* copy number is potentially acquired during tumor progression.

Although earlier studies have linked the presence of *TMPRSS2–ERG* fusion, or the presence of a subset of *TMPRSS2–ERG* fusion class, with a more aggressive prostatic adenocarcinoma biological behavior,[5,8,9,11,26] recent large cohort studies failed to reveal a prognostic role for the presence of fusion.[12,27,28] Our finding of a high incidence of *TMPRSS2–ERG* fusions in minute prostatic adenocarcinomas that is comparable with that of non-minute prostatic adenocarcinomas would further lend support to a lack of association between the

presence of fusion and aggressive behavior given the established lack of clinical significance of minute prostatic adenocarcinoma tumors.

In summary, this is the first study documenting the presence of *ERG* rearrangement in minute prostatic adenocarcinomas with a rate comparable with that of a more advanced stage, clinically significant tumors. Our findings can be seen as a indirect evidence supporting *ERG* fusion occurrence as an early event in prostate cancer pathogenesis. Findings of this study could also be interpreted as an additional indirect evidence supporting the lack of association between *TMPRSS2–ERG* and aggressive outcome, bringing into question its value as a marker of aggressive prostatic adenocarcinoma phenotype.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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