

# Increased gene copy number of *ERG* on chromosome 21 but not *TMPRSS2–ERG* fusion predicts outcome in prostatic adenocarcinomas

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**The role of *TMPRSS2–ERG* gene fusion in prostate cancer prognostication remains controversial. We evaluated the prognostic role of *TMPRSS2–ERG* fusion using fluorescence *in situ* hybridization analysis in a case–control study nested in The Johns Hopkins retropublic radical prostatectomy cohort. In all, 10 tissue microarrays containing paired tumors and normal tissues obtained from 172 cases (recurrence) and 172 controls (non-recurrence) matched on pathological grade, stage, race/ethnicity, and age at the time of surgery were analyzed. All radical prostatectomies were performed at our institution between 1993 and 2004. Recurrence was defined as biochemical recurrence, development of clinical evidence of metastasis, or death from prostate carcinoma. Each tissue microarray spot was scored for the presence of *TMPRSS2–ERG* gene fusion and for *ERG* gene copy number gains. The odds ratio of recurrence and 95% confidence intervals were estimated from conditional logistic regression. Although the percentage of cases with fusion was slightly lower in cases than in controls (50 vs 57%), the difference was not statistically significant ( $P=0.20$ ). The presence of fusion due to either deletion or split event was not associated with recurrence. Similarly, the presence of duplicated *ERG* deletion, duplicated *ERG* split, or *ERG* gene copy number gain with a single *ERG* fusion was not associated with recurrence. *ERG* gene polysomy without fusion was significantly associated with recurrence (odds ratio 2.0, 95% confidence interval 1.17–3.42). In summary, *TMPRSS2–ERG* fusion was not prognostic for recurrence after retropublic radical prostatectomy for clinically localized prostate cancer, although men with *ERG* gene copy number gain without fusion were twice more likely to recur.**

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Prostate cancer remains a major health problem in the United States. At present, at the time of diagnosis, most cases present as localized disease and are treated by radical prostatectomy, radiation therapy, or active surveillance. Recently, calls for adjustment of our current approach to the diagnosis and management of prostate carcinoma have been voiced with concerns for ‘overtreatment’ being raised.<sup>1,2</sup> A marker able to distinguish cases with

the potential to progress would be of particular utility in helping to determine which individuals should pursue active surveillance and those who need more definitive or even adjuvant therapy. Described by Tomlins *et al*, the recurrent fusion between the androgen-regulated gene *TMPRSS2* (21q22.3) and *ETS* transcription factor family member *ERG* (21q22.2) is a common occurrence in prostate carcinoma and has been reported in 15–80% of all cases.<sup>3–8</sup> *TMPRSS2-ERG* fusion is an early event in prostate oncogenesis that results from either a small deletion on chromosome 21 (seen in approximately two-thirds of cases) or through a translocation.<sup>5</sup> In either type of fusion, the *ERG* gene is brought under the control of an androgen-regulated promoter leading to overexpression of the *ERG* protein.

To date, the clinical significance of *TMPRSS2-ERG* fusion as a prognosticator for recurrence or progression remains controversial. Studies addressing the relationship of *TMPRSS2-ERG* fusion status to the natural history of the disease and to prostate cancer progression have so far led to conflicting results.<sup>9–14</sup> Although earlier studies pointed to the presence of *TMPRSS2-ERG* fusion, or a particular subset of, as being a marker of aggressive outcome,<sup>9–11</sup> more recent studies seem to downplay its role as a predictor of aggressive behavior.<sup>13–17</sup> The aim of the current study was to evaluate *TMPRSS2-ERG* fusion status as a prognosticator for recurrence in a nested case-control study in a prostate-specific antigen (PSA) era cohort of men who underwent radical prostatectomy at our institution for clinically localized prostatic adenocarcinoma.

## Materials and methods

The current study was approved by our Institutional Review Board.

### Study Population and Nested Case-Control Design

We developed a case-control study nested in the cohort of 4860 men who underwent radical retropubic prostatectomy for clinically localized prostate cancer at The Johns Hopkins Medical Institutions between 1993 and 2004 and who had not had hormonal or radiation therapy before radical prostatectomy or as adjuvant therapy before recurrence.<sup>18</sup> The study was designed to efficiently evaluate prognostic and risk factors for recurrence after radical prostatectomy. Cases were 524 men who experienced biochemical recurrence (serum PSA  $\geq 0.2$  ng/ml), metastasis, or prostate cancer death after surgery. For each case, we used incidence density sampling to select a control who had not experienced recurrence by the date of the case's recurrence and who was matched on age, race, pathological stage, and Gleason's sum.<sup>19</sup> In this nested design, a man could be initially sampled as

a control and later be sampled as a case once he recurred. Controls who remained at risk for recurrence were eligible to be sampled more than once. The latter method of control sampling makes the odds ratio estimate an unbiased estimate of the hazard ratio that would have been obtained if the entire cohort had been studied. Sampling controls allowed us to test a smaller number of total men than if we had used the entire cohort making for a more time- and cost-efficient approach. Other clinicopathological data were available for these men, including preoperative PSA, clinical stage, and Gleason's sum.

### Tissues and Tissue Microarrays

A set of 16 tissue microarrays were constructed for the 524 matched cases and controls. Matched pairs were placed on the same tissue microarray, so that a subset of these could be used depending on sample size calculations. Paired prostate cancer and non-cancer tissues were spotted (0.6 mm) in triplicate from each radical prostatectomy specimen as described previously by Kononen *et al*.<sup>20</sup> In specimens with multifocal tumors, only the dominant tumor (highest Gleason's sum and usually the largest) was sampled. In all, 10 of the 16 available tissue microarrays were used for the current study based on *a priori* power calculations; 2521 observations for *TMPRSS2-ERG* fusions were available for a total of 631 cases and controls. There were 3470 fusion observations for 631 men, of which 1977 were for carcinoma tissues. Of these, 990 fusion observations were for 302 cases and 987 were for 191 controls. Upon excluding technically inadequate tissue microarray spots, 172 matched sets with complete data were available for the analysis.

### Evaluation of *TMPRSS2-ERG* Fusion Status using Interphase *ERG* Break-Apart FISH Assay

Fluorescence *in situ* hybridization (FISH) analysis was performed using dual-color interphase break-apart probes for the 5' and 3' regions of the *ERG* gene as detailed previously.<sup>14,15</sup> In brief, 4- $\mu$ m paraffin-embedded tissue microarray sections were baked at 56°C for 2 h, and then deparaffinized and rehydrated using xylene and graded ethanol, respectively. Tissue microarray sections were pretreated using paraffin pretreatment reagent kit III (Abbott Molecular Inc., IL). BAC FISH probes used were SpectrumGreen d-UTP direct-labeled BAC RP11-95I21 for 5'*ERG*, and SpectrumOrange d-UTP direct-labeled BAC RP11-476D17 for 3'*ERG* (Nick transKit, Vysis, Abbott Park, IL). Tissue microarray 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 Inc., MA).

Detecting *ERG* gene rearrangement using break-apart probes provides indirect evidence for the

occurrence of *TMPRSS2-ERG* fusions. FISH interpretation was performed by three urologic pathologists (AT, RA, and GJN). Tissue microarray sections were scored using a 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 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, Canada) and a 100 × /1.4 NA oil immersion Neofluar lens. Fluorescence excitation/emission filters were as follows: Cy3 excitation, 546 nm/10 nm BP; emission, 578 nm LP (Carl Zeiss Inc.); DAPI excitation, 330 nm; emission, 400 nm using an XF02 fluorescence set (Omega Optical, Brattleboro, VT); Alexa Fluor 488 excitation, 475 nm; emission, 535 nm using a combination of 475RDF40 and 535RDF45 filters (Omega Optical). Gray-scale images were captured for presentation using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital camera, pseudo-colored and merged.

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 hematoxylin and eosin sections were available for side-by-side comparison with the FISH image to localize tumor cells. Gleason's grade was confirmed in each TMA spot. Paired benign prostatic epithelium was also scored as a negative control.

Each tissue microarray spot was assessed for *TMPRSS2-ERG* fusion as described previously by Attard *et al*<sup>11</sup> with the following minor modifications: (1) class negative for fusion: a nucleus with two pairs of juxtaposed red and green signals forming yellow signals indicating the absence of *ERG* fusion (Figure 1a); (2) class *ERG* signal split: a nucleus with one juxtaposed red-green signal pair of the non-rearranged *ERG* allele and additional separate single red and single green signals of rearranged *ERG* allele (break-apart) reflecting a *TMPRSS2-ERG* fusion through split (Figure 1b); (3) class *ERG* deletion: 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 (Figure 1c).

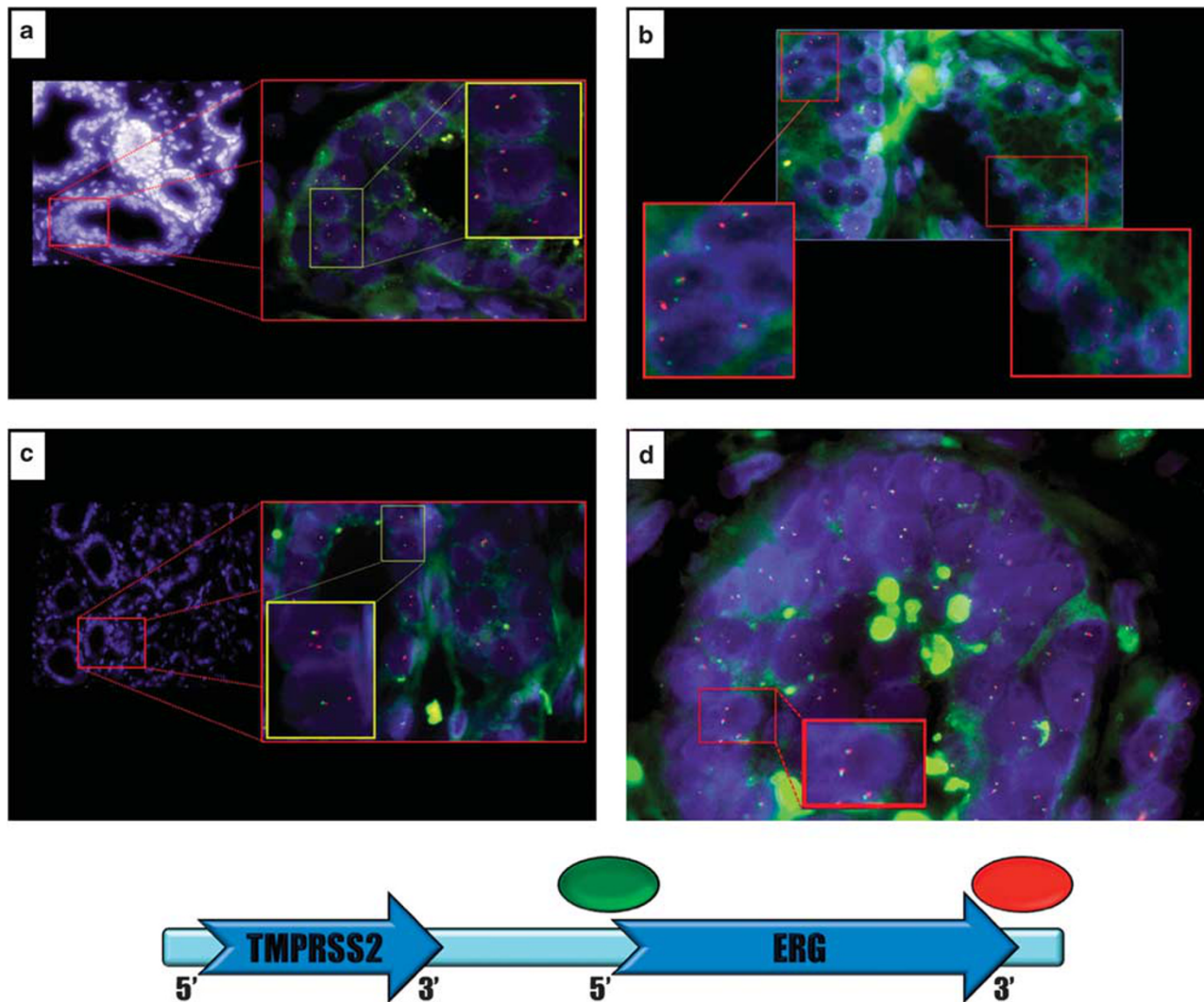
A fusion was considered to be present when a minimum of 10% of the cells counted contained a split or a minimum of 20% of the cells contained a deletion in a given spot. The latter stringent 20% cutoff point for deletion was based on evaluation of a set of 225 consecutive benign FISH-labeled nuclei from consecutive tissue microarray spots where a truncation rate of up to 15% was noted for either red or green signals. A tumor was considered fusion positive if any of its representative spots met the above cutoffs. Analyses were also repeated using the same cutoffs applied to the sum of positive nuclei in a given tumor combining all its representative tissue microarray spots. In addition, to evaluate the

potential effect of 'dosage' of a given fusion type, analysis was also performed using the number of spots that were positive for fusion in each tumor (extent of fusion-positive tissue microarray spots) and using the calculated ratio of fusion-positive spots per total number of analyzed spots per each case (ratio of fusion-positive tissue microarray spots).

A spot with single fusion was classified as harboring a deletion or a split event. Spots with duplicated deletion or split-type fusions were classified as 2+ deletion or 2+ split, respectively. Tumors with two distinct sub-populations of cells with different types of fusion meeting the above cutoffs were assigned to more than one fusion class. In addition, the presence of a copy number gain of a non-rearranged *ERG* gene (>2 copies; presumably due to chromosome 21 polysomy without fusion) was simultaneously assessed in all evaluated nuclei in a given tissue microarray spot. Spots showing copy number gain of an intact *ERG* (>2 copies) were designated as '*ERG* gene copy number gain without fusion' (Figure 1d). Tissue microarray spots with *ERG* gene copy number gain and a second allele showing fusion were designated as either '*ERG* gene copy number gain with single split event' or '*ERG* gene copy number gain with single deletion event'. A tissue microarray spot was deemed technically inadequate for scoring if it lacked a diagnostic target tissue or was of weak non-interpretable probe signal. Spots with overlapping nuclei preventing accurate FISH assessment were also considered technically inadequate.

### Statistical Analysis

Demographic, clinicopathological, and *TMPRSS2-ERG* data were analyzed using Wilcoxon's sign rank test, paired *t*-test, and McNemar's test. We calculated odds ratios of recurrence and 95% confidence intervals by *TMPRSS2-ERG* fusion status using conditional logistic regression taking into account matching factors of age, race, pathologic stage, and Gleason's sum. We estimated the association between fusion status and recurrence for tissue microarray spots with cancer. We modeled *TMPRSS2-ERG* fusion status using the following eight categories: any fusion, fusion due to deletion event, fusion due to split event, duplicated fusion due to 2+ deletion events, duplicated fusion due to 2+ split events, *ERG* gene copy number gain with single deletion event, *ERG* gene copy number gain with single split event, and *ERG* gene copy number gain without fusion. Furthermore, we repeated the statistical analysis for all eight categories based on several permutations of classifying fusion status in a given radical prostatectomy case: each case classified based on fusion status of any individually assessed tumor tissue microarray spot; each case classified based on the combined assessment of all evaluated tumor cells in all represented tissue



**Figure 1** Detection of *TMPRSS2-ERG* fusion events by FISH. (a) No fusion events: Two intact *ERG* alleles are seen in the nuclei of benign prostatic epithelial cells. Two sets of juxtaposed red and green signals with occasional yellow overlap are noted in each nucleus (yellow box). (b) *TMPRSS2-ERG* fusion by split: One intact *ERG* allele and a second rearranged *ERG* allele in prostate adenocarcinoma (right box) and adjacent high-grade PIN epithelial cell (left box). The rearranged *ERG* alleles show split of the red and green signals indicative of an *ERG* fusion by translocation. (c) *TMPRSS2-ERG* fusion by deletion: One intact *ERG* allele and a second rearranged *ERG* allele in prostate adenocarcinoma. The rearranged *ERG* alleles show the absence of a green signal with a remaining red signal seen indicative of *ERG* fusion through deletion. (d) *ERG* gene copy number gain without fusion: increased *ERG* gene copy number in prostate adenocarcinoma (red box). Three sets of juxtaposed red-green signals are seen compared with background nuclei each showing only two sets of juxtaposed red-green signals.

microarray spots; number of tissue microarray spots per case showing fusion and ratio of number of fusion-positive spot(s) divided per total number of tumor spots per case. All analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). Statistical tests were two sided and *P*-values <0.05 were considered to be statistically significant.

**Results**

**Patient Demographics and Clinicopathological Findings**

Patient demographics and clinical data of recurrence cases and matched controls are summarized in

Table 1. As expected, patients in the case group had higher mean preoperative PSA (*P*=0.05) and were more likely to have positive surgical margins (*P*=0.01). The median recurrence time was 2 years after surgery.

**Prevalence of *TMPRSS2-ERG* Fusion**

*TMPRSS2-ERG* fusion and *ERG* gene copy number gain were not detected in paired benign prostate glandular tissue spots in either recurrence cases or controls. Although the overall frequency of *TMPRSS2-ERG* fusion assessed in cancer spots was slightly lower in cases than in controls (50 vs

**Table 1** Demographic and clinicopathological characteristics of 172 prostate cancer recurrence cases and 172 matched controls<sup>a</sup> nested in the Johns Hopkins radical prostatectomy cohort

	Case	Control	P-value <sup>b</sup>
Mean age at surgery (years) ± s.d.	59.5 ± 6.8	59.7 ± 6.3	Matched
<b>Race (%)</b>			Matched
White	79.7	83.7	
Black	12.2	9.3	
Other race/ethnicity	8.1	7	
<b>Preoperative PSA concentration (ng/ml)</b>			
Mean ± s.d.	12.2 ± 11.0	10.2 ± 6.7	0.05
Median (range)	8.8 (0.10–79.1)	8.5 (0.3–35.6)	0.17
< 4	8.7	9.9	
4–10	48.8	54.7	
> 10	42.4	35.5	
<b>Pathological Gleason's sum (%)</b>			Matched
Mean ± s.d.	7.2 ± 0.8	7.1 ± 0.8	
≤ 6	16.3	18.6	
3+4	38.4	45.9	
4+3	22.7	15.1	
> 7	22.7	20.4	
<b>Pathological stage (%)</b>			Matched
pTNM			
pT2N0	16.9	17.4	
pT3aN0	12.2	18.6	
pT3bN0	40.1	33.7	
pT4N0	19.2	18.6	
pT3bN+	5.8	4.7	
pT4N+	5.2	5.8	
Surgical margin positive (%)	36.6	24.4	0.01
<b>Follow-up time (years)</b>			
Mean ± s.d.	2.5 ± 2.0	5.4 ± 2.5	< 0.0001
Median (range)	2 (1–9)	5 (2–11)	< 0.0001

<sup>a</sup>Controls were matched to cases on age, race, pathological stage, and Gleason's sum.

<sup>b</sup>P-values were obtained from paired statistical tests.

**Table 2** *TMPRSS2-ERG* fusion status in prostate carcinoma tissues from 172 recurrence cases and 172 matched controls<sup>a</sup> nested in the Johns Hopkins radical prostatectomy cohort

Fusion status (%)	Case	Control	P-value <sup>b</sup>
Any fusion event	50.0	57.0	0.20
Single fusion due to deletion event	35.5	44.8	0.08
Single fusion due to split event	25.0	30.2	0.31
Duplicated fusion events	11.6	11.0	0.73
<i>ERG</i> gene copy number gain with single fusion event	20.4	19.2	0.48
Any deletion event only	18.0	19.8	0.77
Any split event only	0.6	0.6	1.00
Deletion and split events	25.6	30.8	0.31
<i>ERG</i> gene copy number gain without fusion events	27.9	16.3	0.01

<sup>a</sup>Controls were matched to cases on age, race, pathological stage, and Gleason's sum.

<sup>b</sup>P-values were obtained from paired statistical tests.

57%), the difference was not statistically significant ( $P=0.20$ ; Table 2). We observed no statistically significant difference between prostate carcinoma cases and controls in the prevalence of split, combined deletion and split, or the presence of *ERG* gene copy number gain with *ERG* fusion. The prevalence of deletion events appeared to be lower in cases than in controls ( $P=0.08$ ). In recurrence cases who had *TMPRSS2-ERG* fusion, the prevalence of deletion and split fusion events were 35.5 and 25%, respectively; the latter was almost always of combined deletion and split classes due to the presence of two sub-populations of cells each showing one fusion class (see Table 2). The incidence of *ERG* gene copy number gain without fusion was statistically significantly higher in cases than in controls (28 vs 16%;  $P=0.01$ ).

**Association between *TMPRSS2-ERG* Fusion Status and Prostate Carcinoma Recurrence**

When classifying patients as fusion positive if the criteria for positivity ( $\geq 10\%$  of cells with split or  $\geq 20\%$  with deletion events) were met in at least one tissue microarray spot, the presence of single *TMPRSS2-ERG* fusions or duplicated fusions with or without *ERG* gene copy number gain was not associated with risk of recurrence after radical prostatectomy, with the possible exception of an inverse association for single split fusion (Table 3). This inverse association was also statistically significant when classifying patients as fusion positive only when at least 10% of cancer cells across all tissue microarray spots contained a particular fusion (odds ratio 0.63, 95% confidence interval 0.40–0.99,  $P=0.04$ ; Table 3).

In contrast to the *TMPRSS2-ERG* fusion, the presence of *ERG* gene copy number gain without fusion was associated with an increased risk of recurrence (odds ratio 2.00, 95% confidence interval 1.17–3.47,  $P=0.01$ ); further adjusting for pre-surgery PSA and calendar year of surgery slightly attenuated this association (odds ratio 1.81, 95% confidence interval 0.99–3.31). *ERG* gene copy number gain without fusion remained significantly associated with prostate cancer recurrence upon repeating the analyses based on the combined assessment of all evaluated tumor cells in all tissue microarray spots per radical prostatectomy cases (Table 3), the number of positive spots, and the ratio of number of positive spots to the number of spots assessed (Table 4).

We found no statistically significant associations between the presence of *TMPRSS2-ERG* fusion and the risk of recurrence in White men (137 pairs), younger (<60 years, 79 pairs) or older ( $\geq 60$  years, 88 pairs) men, later-stage (N1 or T3b, 52 pairs) or early-stage (T2 or T3a and N0, 119 pairs) disease, Gleason's sum disease  $\geq 4 + 3$  (42 pairs) vs  $\leq 3 + 4$  (75 pairs), men with poorer (N1 or T3b or  $\geq 4 + 3$ , 80

**Table 3** Association between ERG fusion status and risk of prostate cancer recurrence following radical prostatectomy for clinically localized prostate cancer, 172 cases and 172 matched controls nested in the Johns Hopkins prostatectomy cohort<sup>a</sup>

Fusion status	Criterion for positivity <sup>b</sup>			
	In at least one tissue microarray spot		Across all tissue microarray spots assessed	
	Odds ratio (95% confidence intervals)	P-value	Odds ratio (95% confidence intervals)	P-value
Any fusion event	0.72 (0.45–1.14)	0.16	0.73 (0.46–1.15)	0.17
Fusion due to deletion event	0.75 (0.46–1.24)	0.26	0.68 (0.37–1.21)	0.18
Fusion due to split event	0.64 (0.40–1.03)	0.07	0.63 (0.40–0.99)	0.04
Duplicated fusion due to 2+ split events	1.00 (0.29–3.45)	1.00	1.00 (0.14–7.10)	1.00
Duplicated fusion due to 2+ deletion events	1.08 (0.51–2.29)	0.85	1.67 (0.61–4.59)	0.32
ERG gene copy number gain with single split event	1.06 (0.54–2.10)	0.86	0.30 (0.08–1.09)	0.07
ERG gene copy number gain with single deletion event	1.07 (0.53–2.16)	0.86	1.25 (0.49–3.17)	0.64
ERG gene copy number without fusion events	2.00 (1.17–3.42)	0.01	2.70 (1.31–5.58)	0.01

<sup>a</sup>Estimated from conditional logistic regression taking into account the matching factors age, race, pathological stage, and Gleason's sum.

<sup>b</sup>In all, ≥10% cells were positive for a split or ≥20% for a deletion.

**Table 4** Association between increasing ratio of the number of tissue microarray spots positive for ERG fusion with the number of spots assessed and risk of prostate cancer recurrence following radical prostatectomy for clinically localized prostate cancer, 172 cases and 172 matched controls nested in the Johns Hopkins prostatectomy cohort

Fusion status	Odds ratio (95% confidence intervals) <sup>a</sup>	P-value
Any fusion event	0.62 (0.39–1.00)	0.05
Fusion due to deletion event	0.68 (0.38–1.20)	0.18
Fusion due to split event	0.64 (0.39–1.03)	0.07
Duplicated fusion due to 2+ split events	0.99 (0.21–4.61)	0.99
Duplicated fusion due to 2+ deletion events	1.12 (0.49–2.56)	0.80
ERG gene copy number gain with single split event	0.96 (0.39–2.38)	0.93
ERG gene copy number gain with single deletion event	1.07 (0.44–2.62)	0.88
ERG gene copy number gain without fusion events	2.34 (1.24–4.40)	0.01

<sup>a</sup>Per 1-Unit increase in the ratio of tissue spots positive for ERG fusion to number of spots assessed and estimated from conditional logistic regression taking into account the matching factors age, race, pathological stage, and Gleason's sum.

pairs) and better (T2 or T3a and <4 + 3, 58 pairs) prognosis disease, men with negative surgical margins (89 pairs), and men who recurred <2 years (48 pairs) and ≥2 years (120 pairs) after radical prostatectomy (data not shown). ERG gene copy number gain without fusion was associated with increased risk for recurrence in White men, older but not younger men, men with early- but not later-stage disease, and men with negative surgical margins (Table 5).

**Table 5** Association of ERG gene copy number gain without fusion with prostatic carcinoma recurrence following radical prostatectomy in prognostic subgroups

	Odds ratio (95% confidence intervals)
White men	1.8 (1.02–3.43)
Older men	2.4 (1.14–5.01)
Early stage prostate carcinoma	2.9 (1.46–5.77)
Negative surgical margins	3.4 (1.47–7.95)
Recurrence ≥2 years	2.4 (1.21–4.94)

**Assessment of Chromosome 21 Numerical Alterations**

Chromosome 21 copy number alterations were evaluated in a subset of two tissue microarrays using a second dual-color probe set targeting centromeric (RP11-22D1) and telomeric (RP11-35C4) regions on the long arm of chromosome 21 separated by >27 megabases. Identical FISH processing and scoring parameters to those used with the first probe set for the 5' and 3' regions of ERG gene were used in the 60 evaluable tumors. Using the second probe set targeting centromeric and telomeric regions of the long arm of chromosome 21, we found evidence of chromosome 21 long-arm gains in 24 of 26 (93%) tumors that were originally classified as ERG gene copy number gain without fusion (Figure 2).

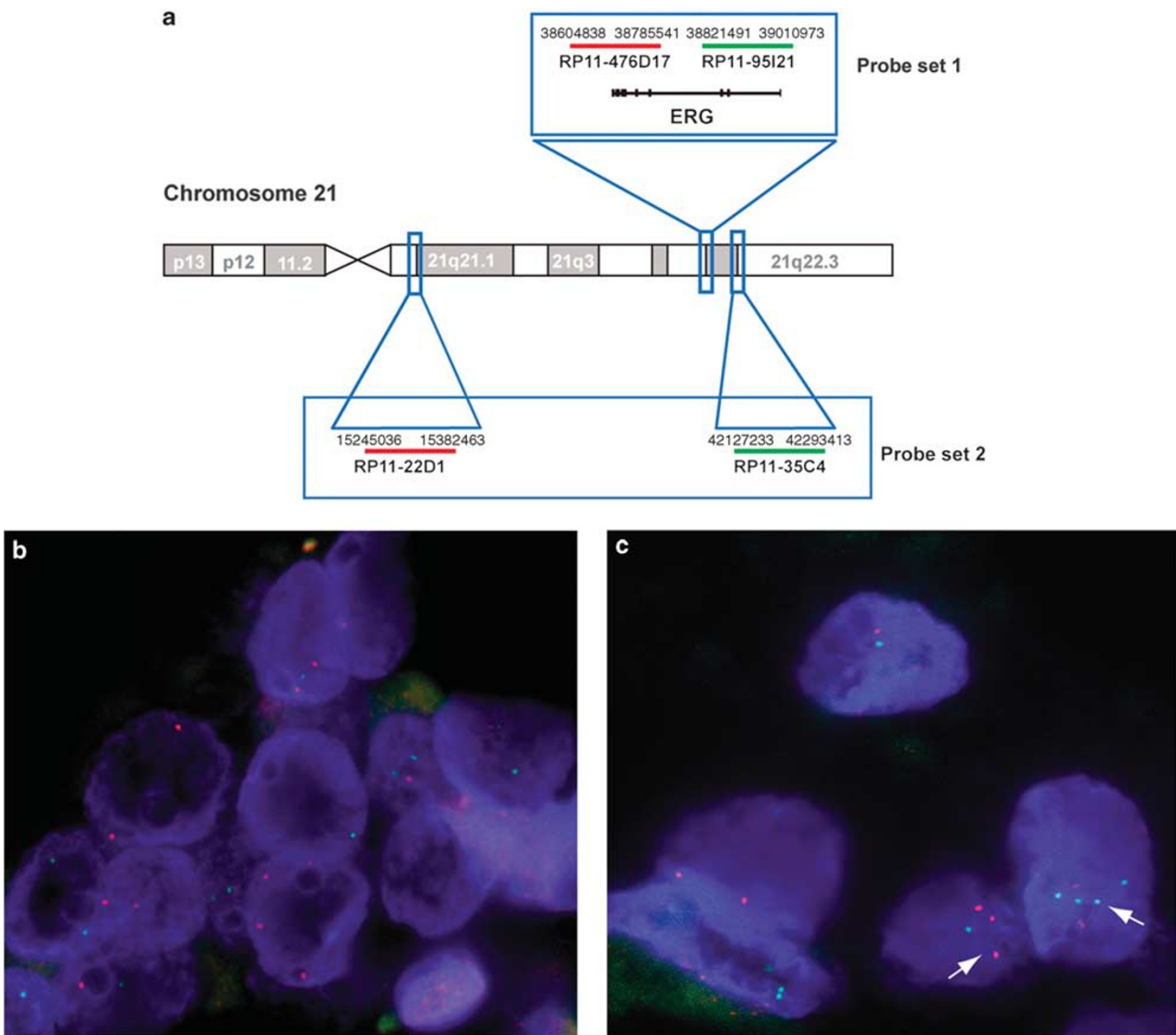
**Discussion**

Although earlier studies linked the presence of TMPRSS2-ERG fusions or a subset of fusion classes with a more aggressive biological behavior of prostate carcinomas,<sup>5-7,9-12,21,22</sup> recent large cohort

studies did not observe such a prognostic role.<sup>13,23-28</sup> In this context, our recent report of a high incidence of *TMPRSS2-ERG* fusions in minute prostatic adenocarcinoma, comparable with that of non-minute prostatic adenocarcinoma, seems to lend support to the lack of prognostic role of fusion given the lack of clinical significance of minute tumors.<sup>15</sup> In our current study conducted among men who underwent radical prostatectomy for clinically localized prostatic adenocarcinoma, *TMPRSS2-ERG* fusions were not associated with recurrence, a finding that contradicts previously reported associations between duplicated *ERG* fusions and

aggressive outcome.<sup>11,13</sup> We could not rule out a possible inverse association between *TMPRSS2-ERG* fusion by a single split event and risk of recurrence. Our results were consistent across methods of classifying the men as fusion positive.

The conflicting evidence on the prognostic significance of *TMPRSS2-ERG* fusion is potentially due to methodological differences and/or differences in examined cohorts. Some of the studies pointing to the association with aggressive behavior were performed on conservatively managed, population-based, watchful waiting cohorts,<sup>9,11</sup> in contrast to the more recent studies assessing large,



**Figure 2** Evaluation of chromosome 21 numerical alterations. (a) Overview of BAC clones used in the study. Probe set 1 flanking the *ERG* genomic locus (RP11-476D17, RP11-95I21) is the set used to determine *ERG* gene rearrangement status in all cases in the current study. Probe set 2 was only used in a subset of cases to further assess chromosome 21 numerical alterations by targeting centromeric (RP11-22D1) and telomeric (RP11-35C4) regions on the long arm of chromosome 21. (b) Prostate adenocarcinoma showing no evidence of numerical chromosome 21 alteration using probe set 2. (c) Prostate adenocarcinoma showing chromosome 21 long-arm copy number gains as indicated by the presence of more than two red and/or more than two green signals per nucleus (white arrows). (d) Comparison of *ERG* gene copy number gain detected using probe set 1 and chromosome 21 numerical alteration assessed using probe set 2 in a subset of two tissue microarrays (60 cases) from our study. A high concordance rate (24/26; 93%) is found between the two sets of probes supporting that *ERG* gene copy number gain are a reflection of chromosome 21 copy number gains.

**d**

	Sample ID	Probe set 1	Probe set 2
1	577118	■	■
2	573115	■	■
3	573120	■	■
4	573111	■	■
5	573113	■	■
6	573117	■	■
7	57724	■	■
8	57728	■	■
9	577216	■	■
10	573215	■	■
11	573212	■	■
12	573211	■	■
13	57727	■	■
14	57728	■	■
15	577311	■	■
16	573314	■	■
17	573315	■	■
18	573311	■	■
19	57331	■	■
20	57743	■	■
21	57744	■	■
22	573420	■	■
23	573414	■	■
24	573412	■	■
25	573413	■	■
26	57344	■	■
27	57793	■	■
28	57799	■	■
29	577910	■	■
30	577913	■	■
31	573917	■	■
32	577117	■	■
33	577114	■	■
34	577116	■	■
35	5731115	■	■
36	5731117	■	■
37	5731114	■	■
38	5771215	■	■
39	5771210	■	■
40	5731216	■	■
41	5731213	■	■
42	573121	■	■
43	577179	■	■
44	5731711	■	■
45	577187	■	■
46	5771811	■	■
47	5731813	■	■
48	5771813	■	■
49	5731817	■	■
50	573195	■	■
51	577207	■	■
52	577205	■	■
53	5772017	■	■
54	5772011	■	■
55	5772013	■	■
56	5731211	■	■
57	57398	■	■
58	5732011	■	■
59	5731217	■	■
60	5731218	■	■

■ Polysomy chr. 21  
 ■ Normal chr. 21 number

Figure 2 Continued.

PSA-screened populations treated by radical prostatectomy for clinically localized prostate carcinoma.<sup>13</sup> Watchful waiting cohorts were often diagnosed by transurethral resection with predominantly transition-zone cancers<sup>9</sup> as opposed to the more recent studies of primarily peripheral-zone cancer diagnosed on needle biopsy. Recent series have shown that in transition-zone prostate cancers, *ERG* fusions are less prevalent (12–13% of all cases) than in tumors originating in the peripheral zone,<sup>29–31</sup> suggesting that the underlying molecular abnormalities might be different between these subsets of prostatic adenocarcinomas. Nevertheless, in our study, *TMRSS2-ERG* fusions were present in 50 and 57% of recurrence cases and controls, respectively, which is consistent with the previous percentages (40–60%) reported in surgical cohort studies assessing *ERG* alterations<sup>3,5,7,10,13,32</sup> using similar FISH break-apart methodology.<sup>5</sup>

In our study population, *ERG* fusion was more likely to be the result of deletion, either homogeneously throughout a given tumor or in association with a sub-population of tumor cells harboring a split event. The preponderance of deletion events is in line with previous observations.<sup>5,24,32</sup> Intronic loss of genomic DNA between *ERG* and *TMRSS2* on chromosome 21q22.2–3 appears to be a main mechanism of *TMRSS2-ERG* fusion. Previous studies have shown the presence of *TMRSS2-ERG* fusion to be homogeneous in a given tumor focus<sup>5</sup> but heterogeneous in the context of multiple cancer foci.<sup>33</sup> Although the identification in the present series of combined deletion and split fusions events within the same focus contradicts these earlier results, our findings are in agreement with those published by Clark *et al*<sup>34,35</sup> who have demonstrated different categories of *ERG* gene alteration to be present either together in a single cancerous region or within separate foci of cancer in the same prostate slice. These results further support the notion that *TMRSS2-ERG* gene fusions may arise independently in different regions of a single prostate or even within the same tumor focus.

Although *TMRSS2-ERG* fusion was not associated with recurrence in this study, *ERG* gene copy number gain without fusion was associated with twice the risk of recurrence. Our findings are consistent with a recent report by Gopalan *et al*.<sup>13</sup> This low-level increase of *ERG* gene copy number is presumably the result of tumor aneuploidy status, potentially leading to chromosome 21 numerical gains. The latter is further supported by our reassessment of chromosome 21 numerical alterations using a second probe set targeting telomeric and centromeric regions at the long arm of chromosome 21, suggesting that chromosome 21 polysomy might be responsible for the detected *ERG* gene copy number gain in this group. *ERG* gene copy number gain was associated with the presence of chromosome 21 long-arm gains in 93% of analyzed cases. Seen in such context, the association of *ERG* gene



copy number gain without fusion with higher likelihood of cancer progression is not surprising given the previous evidence supporting aneuploidy as a negative prognosticator in prostatic adenocarcinomas.<sup>36–38</sup> The lack of association between *ERG* gene copy number gain in the presence of a second allele with *ERG* fusion and cancer progression is counterintuitive and requires further investigation.

In addition to the strong study design, which takes into account clinical features, pathological stage, and Gleason's sum, the detailed analysis of the different fusion classes with multiple approaches to assigning positive fusion status for a given man, represent some of the strengths of our current study. On the other hand, the lack of confirmatory RT-PCR-based analysis or additional molecular methods to further characterize the fusion variants at the exonic level could be viewed as a potential weakness. In this regard, given the previously cited potential prognostic role for specific exonic variant of *TMRSS2-ERG* deletions such as the T1/E4 variant,<sup>27</sup> additional molecular analysis could be warranted in our group of prostate cancer patients. Another plausible limitation of the present series is the effect that *ERG* fusion status heterogeneity might have on the results considering that, when facing multicentric prostate carcinomas, we only sampled the dominant tumor showing the highest Gleason's sum. Indeed, several studies have shown that up to 41% of multicentric *TMPRSS2-ERG* fusion-positive prostatic adenocarcinomas exhibit heterogeneity in different foci of the same gland.<sup>33,39,40</sup> However, this study was not designed to evaluate the heterogeneity of *TMPRSS2-ERG* fusion status within the same tumor but to determine the association between its presence and outcome. Moreover, the rationale for sampling only the dominant tumor is supported by the argument that these high-grade areas are the most likely to dictate prognosis in cases with multicentric lesions.

In summary, our study is the first to evaluate the prognostic role of *TMPRSS2-ERG* fusion in a large nested case-control study of men who underwent radical prostatectomy for clinically localized prostatic adenocarcinoma and that took into account clinicopathological prognostic parameters. We found a comparable prevalence of *TMPRSS2-ERG* fusion in men who did and did not recur, further supporting recent mounting evidence for a lack of prognostic significance of the *ERG* gene fusion by FISH. Copy number increase of *ERG* gene, likely as the result of aneuploidy, was strongly predictive of prostate cancer recurrence in our study.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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