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Prevalence of *TMPRSS2–ERG* and *SLC45A3–ERG* gene fusions in a large prostatectomy cohort

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The majority of prostate cancers harbor recurrent gene fusions between the hormone-regulated *TMPRSS2* and members of the ETS family of transcription factors, most commonly *ERG*. Prostate cancer with *ERG* rearrangements represent a distinct sub-class of tumor based on studies reporting associations with histomorphologic features, characteristic somatic copy number alterations, and gene expression signatures. This study describes the frequency of *ERG* rearrangement prostate cancer and three 5 prime (5′) gene fusion partners (ie, *TMPRSS2, SLC45A3,* and *NDRG1*) in a large prostatectomy cohort. *ERG* gene rearrangements and mechanism of rearrangement, as well as rearrangements of *TMPRSS2, SLC45A3,* and *NDRG1,* were assessed using fluorescence *in situ* hybridization (FISH) on prostate cancer samples from 614 patients treated using radical prostatectomy. *ERG* rearrangement occurred in 53% of the 540 assessable cases. *TMPRSS2* and *SLC45A3* were the only 5′ partner in 78% and 6% of these *ERG* rearranged cases, respectively. Interestingly, 11% of the ERG rearranged cases showed concurrent *TMPRSS2* and *SLC45A3* rearrangements. *TMPRSS2* or *SLC45A3* rearrangements could not be identified for 5% of the *ERG* rearranged cases. From these remaining cases we identified one case with *NDRG1* rearrangement. We did not observe any associations with pathologic parameters or clinical outcome. This is the first study to describe the frequency of *SLC45A3–ERG* fusions in a large clinical cohort. Most studies have assumed that all *ERG* rearranged prostate cancers harbor *TMPRSS2–ERG* fusions. This is also the first study to report concurrent *TMPRSS2* and *SLC45A3* rearrangements in the same tumor focus, suggesting additional complexity that had not been previously appreciated. This study has important clinical implications for the development of diagnostic assays to detect ETS rearranged prostate cancer. Incorporation of these less common *ERG* rearranged prostate cancer fusion assays could further increase the sensitivity of the current PCR-based approaches.
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Since the original description of recurrent gene fusions in prostate cancer involving *TMPRSS2* and *ETV1* or *ERG,*[1] the list of putative recurrent gene fusions continues to grow.[2] As initially observed, these fusions most commonly involve an ETS family member gene fused to a 5 prime (5′) hormonally regulated promoter, most often *TMPRSS2.*[3] Few reports have focused on trying to determine how common a particular gene fusion occurs in a population. Over 20 studies from independent research groups (recently reviewed by Tomlins *et al*[4] and Clark and Cooper[5]) suggest that *ERG* rearrangement can be observed in 40–60% of prostate cancers identified through prostate-specific antigen (PSA) screening. These studies were predominately based on the evaluation of tumor samples from men who underwent surgery for clinically localized disease. Mosquera *et al*[6] recently

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reported a frequency of *ERG* rearrangement in 46% of cases in a prospective PSA-screened prostate cancer biopsy cohort.

There are mounting data suggesting that *ERG* rearranged prostate cancer has several distinct features defining a sub-class of this common epithelial neoplasm. First, mouse model studies overexpressing *ERG* alone,[7,8] or in the context of *PTEN* loss,[9,10] show a neoplastic phenotype. Second, associations between histomorphologic features and *ERG* rearrangement have been observed.[6,11] Third, molecular profiles suggest distinct alterations in pathways, specifically estrogenic signaling, in *ERG* rearranged prostate cancer.[12,13] Fourth, distinct somatic copy number alterations are enriched in *ERG* rearranged prostate cancers.[14] Fifth, if left untreated, *ERG* rearranged prostate cancers are associated with an unfavorable clinical course when compared with non-*ERG* rearranged prostate cancer.[15,16] Finally, Attard *et al*[17] have shown an *ERG* rearrangement status-dependent response to abiraterone acetate, a CYP17 inhibitor. Taken together, *ERG* rearranged prostate cancer seems to represent a distinct molecular and clinical sub-class of prostate cancer.

Most studies have taken a shorthand in defining the *TMPRSS2–ERG* fusion cancers. This is based on the use of a fluorescence *in situ* hybridization (FISH) break-apart assay to identify *ERG* rearrangement as indirect evidence for *TMPRSS2–ERG* gene fusion.[1,18] *A priori* knowledge of possible 5′ partners involved in ETS rearranged prostate cancers led Han *et al*[19] to discover an *ERG* rearranged case to be fused with *SLC45A3* (*Solute carrier family 45, member 3,* also known as Prostein) as the 5′ partner. This was the first study to identify an alternate 5′ fusion partner of *ERG*, other than *TMPRSS2*. Up until then, all cases harboring the *ERG* rearrangement were believed to contain the *TMPRSS2–ERG* fusion. To explore for other potential 5′ partners of *ERG*, we recently evaluated all *ERG* rearranged and *ERG* overexpressing cases from a population of 101 samples and confirmed the recurrent nature of *SLC45A3–ERG* fusions. In addition, we discovered *NDRG1* (N-myc downstream regulated gene 1) as a novel 5′ fusion partner by whole transcriptome sequencing *NDRG1*.[20]

In this study, we analyzed a large prostatectomy cohort for the presence of *TMPRSS2–ERG*, *SLC45A3–ERG*, *and NDRG1–ERG* fusion events to determine their frequency and potential association with clinical parameters.

Table 1 Demographic and clinical characteristics of 540 men (all assessable cases) with clinically localized prostate cancer patients by *ERG* rearrangement status and mechanism

	ERG rearranged, n = *284*		*ERG not rearranged* n = *256*
	Rearrangement through deletion	*Rearrangement through insertion*	
All assessable cases (540)	178 (63%)	106 (37%)	256 (47%)
Age			
≤62	86 (32%)	63 (23%)	120 (45%)
>62	92 (34%)	43 (16%)	136 (50%)
Preoperative PSA (533)			
≤10 ng/ml	130 (24%)	80 (15%)	173 (33%)
>10 ng/ml	47 (9%)	26 (5%)	77 (14%)
pT stage (540)			
pT2	111 (30%)	72 (20%)	186 (50%)
pT3 /T4	67 (39%)	34 (20%)	70 (41%)
Gleason score (540)			
3–6	59 (11%)	36 (7%)	89 (16%)
7	94 (17%)	51 (9%)	115 (21%)
8–10	25 (5%)	19 (4%)	52 (10%)
Biochemical recurrence (515)			
Yes	146 (28%)	86 (17%)	208 (40%)
No	25 (5%)	16 (3%)	34 (7%)
Anti-androgen therapy (538)			
Yes	165 (33%)	102 (20%)	232 (47%)
No	11 (28%)	4 (10%)	24 (62%)

Materials and methods

Case Selection and Pathologic Analysis

The cohort consisted of 614 prostate cancer patients who underwent radical prostatectomy between 1999 and 2005 at the Charité Hospital (Berlin, Germany) (Table 1). The demographics of this cohort were recently described.[21] In brief, patient age ranged from 43 and 74 years (median 62 years). Preoperative PSA levels ranged from 0.8 to 39 ng/ml (median 7.2 ng/ml). In all, 44 patients (7%) had received gonadotropin-releasing hormone analogs at the discretion of the referring urologist before surgery (median 4 weeks, range 2–16 weeks). Clinical follow-up data were reviewed annually. PSA relapse-free survival time was available for 609 patients from this cohort. The median follow-up time of all the cases was 48 months (range 1–108 months) and 89 patients (15%) experienced a PSA relapse after a median time of 13 months (range 1–68). The Gleason scores were distributed as follows: Gleason score 4–6: 217 (35%); Gleason score 7: 291 (48%); and Gleason score 8–10: 106 (17%). Organ-confined carcinoma (pT2) was found in 420 patients; 191 showed extraprostatic tumor extension (pT3).

Tissue Microarray Construction

Formalin-fixed paraffin-embedded tissue blocks from radical prostatectomy specimens were used for tissue microarray construction.[21] Two cores (1.0 mm) of invasive prostate cancer were chosen,

reflecting the primary and secondary Gleason pattern. In addition, normal prostatic tissue was selected for each case.

Assessment of *ERG, TMPRSS2, SLC45A3,* and *NDRG1* Rearrangements Using Two-Color FISH Break-Apart (B/A) Assays

Tissue microarray sections, 4-μm thick, were used for interphase FISH analysis. Rearrangement status was determined using a dual-color break-apart interphase FISH assay as described previously.[1,18,22] In brief, two differentially labeled probes were designed to span the telomeric and centromeric neighboring regions of each locus. The following telomeric/centromeric BAC clones were selected to design break-apart assays to assess for rearrangement status (ie, rearrangement *vs* no rearrangement): *ERG* (RP11-372O17 and RP11-24A11), *TMPRSS2* (RP11-120C17 and RP11-35C4), *SLC45A3* (RP11-131E5 and RP11-249H15), and *NDRG1* (RP11-1145H17 and RP11-185E14). In the case of rearrangement, the assay was also capable of differentiating between two different rearrangement mechanisms (ie, rearrangement through insertion or rearrangement through deletion) as previously described.[18] A nucleus without a gene rearrangement shows two pairs of juxtaposed red and green signals (mostly forming two yellow signals). A nucleus with a rearrangement through insertion shows the split of one red–green (yellow) signal pair, resulting in a single red and green signal for the rearranged allele, and a still combined (yellow) signal for the non-rearranged allele in each nucleus. Finally, a nucleus with a gene rearrangement through deletion shows one juxtaposed red–green signal pair (yellow) for the non-rearranged allele, and a single red or green signal for the allele involved in the rearrangement. Slides were analyzed under an $\times 60$ oil immersion objective using an Olympus (Center Valley, PA, USA) BX-51 fluorescence microscope equipped with appropriate filters and a charge-coupled device camera, and the CytoVision FISH imaging and capturing software (Applied Imaging, San Jose, CA, USA). Evaluation was independently performed by a minimum of two evaluators (RE, CJL, SP, and VS). For each case a minimum of 100 nuclei were assessed.

In cases with co-occurring *ERG* rearrangement and rearrangement of one of the three 5′ partners (ie, *TMPRSS2, SLC45A3,* or *NDRG1),* we defined these cases harboring a fusion of the rearranged genes.

Statistical Analysis

We analyzed significant association between rearrangement status and clinical parameters, including patient age, preoperative PSA serum levels, Gleason score, and pathological stage, using Pearson's chi-square test. We also analyzed associations between biochemical failure and gene fusion status using Kaplan–Meier estimates and log-rank test.

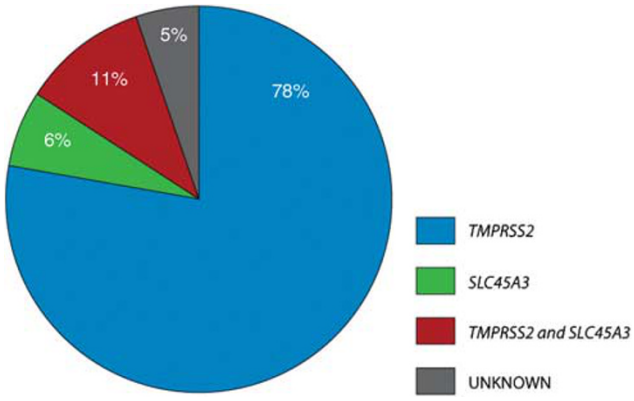

Figure 1 Pie chart summary of study results. Frequencies of the most common 5′ partners of *ERG* rearranged prostate cancer cases are given in percentages (number of rearranged cases/evaluable cases).

Results

We were able to assess 540 out of 614 cases for *ERG* rearrangement status. *ERG* rearrangement was identified in 53% (284/540) of the cases (Figure 1). We then characterized *TMPRSS2* and *SLC45A3* rearrangement status in the *ERG* rearrangement-positive cases as these are known to be the most common fusion partner for *ERG*. Out of 284, 254 cases were assessable for both *ERG* 5′ partners (ie, *TMPRSS2* and *SLC45A3*) in addition to the *ERG* rearrangement. *TMPRSS2* rearrangement was observed in 78% (198/254) of the *ERG* rearranged cases. *SLC45A3* was rearranged in 6% (16/254) of these cases. Interestingly, 11% of the *ERG* rearranged cases (27/254) harbored concurrent rearrangements of *SLC45A3* and *TMPRSS2* (Figures 1 and 2).

Of 27 cases, 7 harbored simultaneous *TMPRSS2* and *SLC45A3* rearrangements within the same tissue microarray core. We evaluated using FISH the original standard tissue block in four of these seven cases. We could confirm that one of them harbored *ERG* rearrangement and coexisting rearrangement of *TMPRSS2* and *SLC45A3* in the same tumor glands (Figure 3). The three remaining cases harbored a mixture of tumor glands with both normal and rearrangement pattern.

ERG with rearrangement through deletion mechanism was observed in 62% (158/254) of the *ERG* rearranged cases. *TMPRSS2* was the fusion partner in 82% (130/158) and *SLC45A3* in approximately 3% (4/158) of these cases, and both 5′ fusion partners were simultaneous rearranged in 10% (16/158) of these *ERG* rearranged cases. *ERG* rearrangement through insertion was detected in 38% (96/254) of cases, with *TMPRSS2* as 5′ fusion partner in 71% (68/96), and *SLC45A3* as 5′ fusion partner in

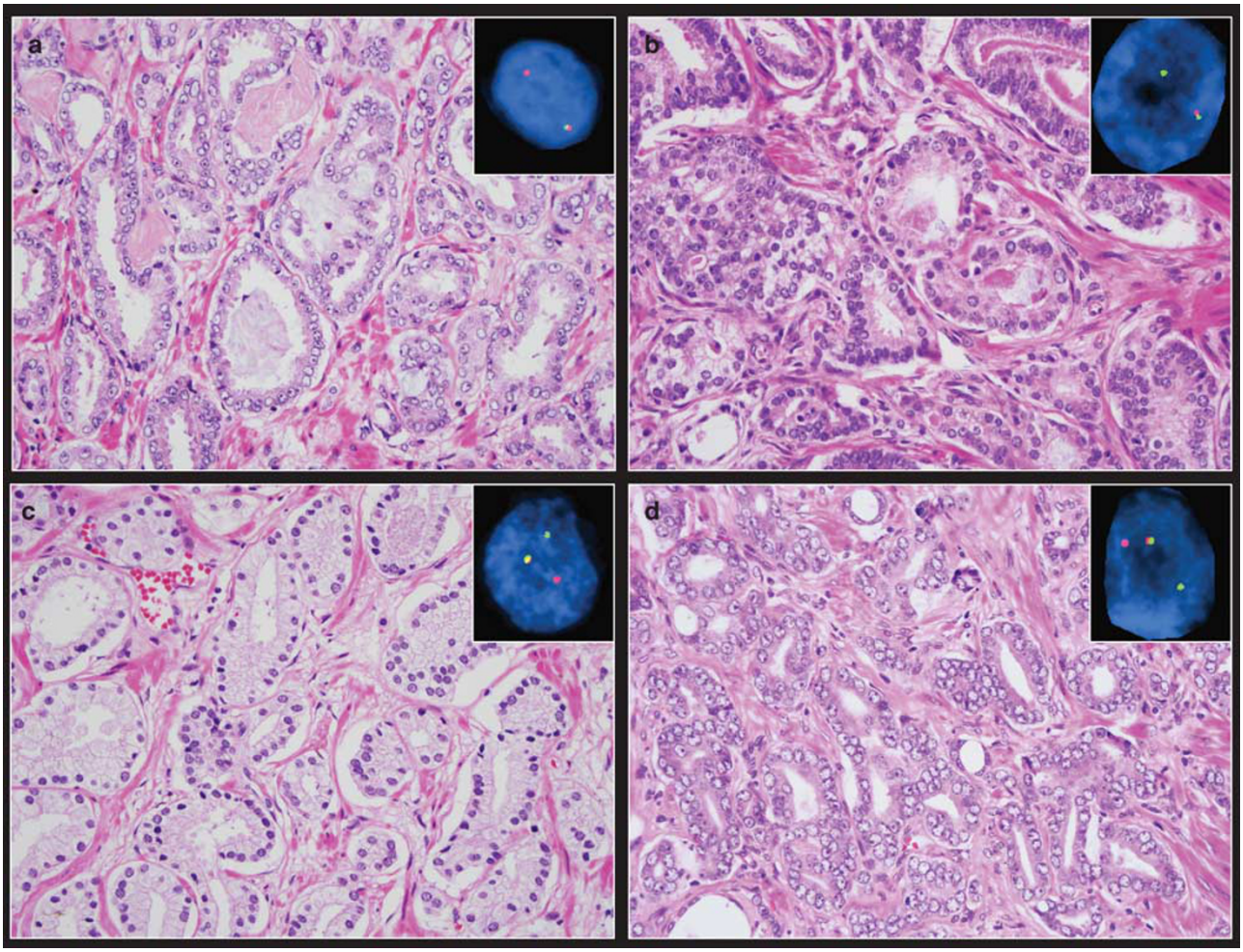

Figure 2 Representative example of four similar prostate cancer cases harboring gene rearrangement. *ERG* rearrangement through deletion (**a**), *TMPRSS2* rearrangement through deletion (**b**), *SCL45A3* rearrangement (**c**), and *NDRG1* rearrangement (**d**). (H&E images are taken at ×40 objective magnification and FISH images are taken at ×60 objective magnification).

12% of cases (12/96) (Figure 4). Simultaneous *TMPRSS2* and *SLC45A3* gene rearrangements were identified in 11% of cases (11/96) (Figure 4).

In the 13/254 (5%) *ERG* rearranged cases without a *TMPRSS2* or *SLC45A3* rearrangement, we next sought for *NDRG1* rearrangement. We could assess *NDRG1* in 10 of these 13 cases and one case showed *NDRG1* rearrangement. The 5′ partner of the remaining *ERG* rearranged cases could not be accounted for by the three 5′ partners. None of the normal prostatic tissue harbored a rearrangement of *ERG, TMPRSS2, SLC45A3,* or *NDRG1*.

Statistical analysis revealed no significant association between any of the assessed gene rearrangements and clinical features such as Gleason grade, stage, or PSA recurrence-free survival (Table 1).

Discussion

Most studies to date are using a FISH break-apart assay to determine *TMPRSS2–ERG* fusion status. Therefore, the prevalence of *ERG* rearrangement is well characterized at approximately 40–60% of PSA-screened prostate cancer cases. This study confirms that in the vast majority of cases, *ERG* rearrangement is due to *TMPRSS2–ERG* gene fusion either through deletion or insertion. As suggested in a recently published study of 101 prostate cancer cases[20] and now shown in a significantly larger population of 540 prostate cancer cases, *SLC45A3–ERG* and *NDRG1–ERG* fusion account for approximately 10% of the *ERG* rearranged tumors. It is yet to be determined whether there are any significant implications to these other less common gene fusions. As suggested by Pflueger *et al*,[20] *TMPRSS2*, *SLC45A3*, and *NDRG1* are all exquisitely driven by both androgen and estrogen. A potential difference is that *NDRG1–ERG* fusions do encode for a chimeric protein product,[20] whereas *TMPRSS2–ERG* and *SLC45A3–ERG* fusions encode for a truncated *ERG* protein. To date, no study has addressed the clinical implications of this observation, but given that expression profiling of *ERG* and

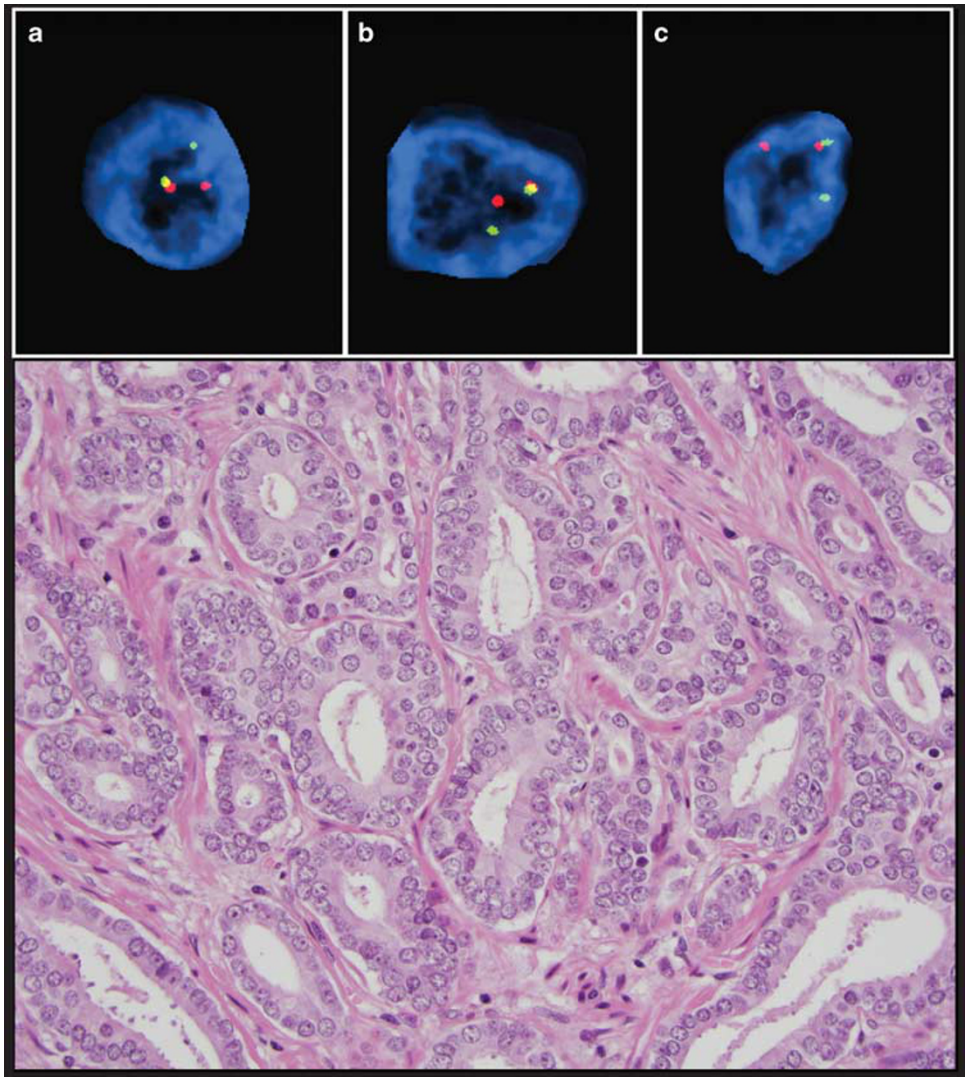

Figure 3 Representative example of a case with simultaneous rearrangements of two 5′ fusion partners. Prostatic tissue with prostatic adenocarcinoma (Gleason $3+3=6$) and FISH images. Within the same gland almost all cells harbor *ERG* rearranged (rearrangement through insertion) (**a**), and both 5′ gene rearrangements, *TMPRSS2* (**b**) and *SLC45A3* (**c**). (H&E images are taken at $\times 40$ objective magnification and FISH images are taken at $\times 60$ objective magnification).

non-*ERG* rearranged prostate cancer samples ($n=455$) did not reveal any clear sub-classes within the *ERG* rearranged category,[13] we can speculate that *ERG* overexpression is the main alteration associated with the molecular phenotype. From a practical perspective, this study suggests that performing the *ERG* and *TMPRSS2* rearrangement assays should help in classifying approximately 90% of the *ERG* rearranged prostate cancer cases.

This study focused only on *ERG* rearranged prostate cancer and did not explore for the frequency of other known ETS rearrangements. Mehra *et al*[23] systematically interrogated all 27 other ETS rearrangements in a cohort of 96 prostate cancer cases and found that after *ERG*, *ETV1* was the most commonly rearranged prostate cancer, suggesting that *TMPRSS2–ETV1* fusion may be in approximately 2% of prostate cancer cases. Attard *et al*[24] examining a cohort of 429 prostate cancer cases found that *ETV1* was rearranged in slightly over 5% of the cases. The Attard study also identified other novel 5′ fusion partners besides *TMPRSS2* for *ETV1*. In a recent unpublished study exploring *ETV1*, *ETV4*, and *ETV5* rearrangements in 88 prostate cancer cases, we identified similar rearrangement frequencies (Svensson *et al*, unpublished data). Therefore, *ERG* and *ETV1* seem to be rearranged in approximately 40–60% and 2–5% of PSA-screened prostate cancer cases, respectively. Other ETS genes, such as *ETV4* and *ETV5,* may have rearrangement frequencies at or below 1–2%. There seems to be a wider range of recurrent 5′ partners besides the originally described *TMPRSS2,*[1] including *SLC45A3* and *NDRG1*. Only large systematic studies will be able to define any clinical significance to the less common rearrangements.

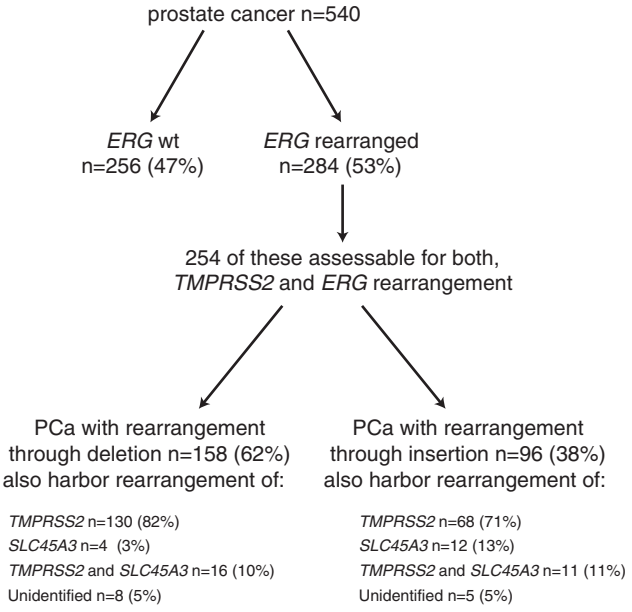

Figure 4 Schematic of *ERG* rearrangement.

Consistent with some of the initial descriptions of *ERG* rearranged prostate cancer cases assessed using FISH,[1,18,25] we found that approximately 53% of cases harbored an *ERG* rearrangement, with 62% of these being rearranged through deletion and 38% being rearranged through insertion. To date, there is no known clinical implication associated with these two types of *ERG* rearrangements. However, we did initially speculate that the region between *TMPRSS2* and *ERG* on chromosome 21 does harbor candidate tumor suppressor genes that might be lost in *ERG* rearrangements through deletion.[18]

This is the first study to systematically evaluate the *SLC45A3–ERG* rearrangement in a large population. Han *et al*[19] first described this fusion and the recurrent nature was confirmed more recently by Pflueger *et al*.[20] By systematically interrogating 540 prostate cancers, we observe a 6% frequency of *SLC45A3–ERG* fusion prostate cancer. As this study has shown and future studies are sure to observe, there is more complexity to ETS rearrangements than initially appreciated. Specifically, in this study, approximately 11% of the individuals harboring *ERG* rearranged showed coexisting rearrangement of two 5′ fusion partners (ie, *TMPRSS2* and *SLC45A3*) and seven of them in the same tissue microarray core. This would suggest different possible gene fusion combinations (*TMPRSS2–ERG*, *SLC45A3–ERG,* and/or other potentially not yet identified fusions). We were able to evaluate four of these seven specific cases harboring multiple rearrangements on the original tissue block and could confirm that one of them harbored *ERG* rearrangement and both 5′ partners *TMPRSS2* and *SLC45A3* rearrangements in the same tumor glands. One important limitation of our study is that we did not have fresh frozen material from these cases to perform assays that would allow us to characterize the nature of these fusions or explore for novel 5′ fusion partners.

This study has potential clinical implications for the development of cancer-specific diagnostic assays. Pioneering work in prostate cancer biomarker development showed that the PCA3 transcript could be used as a prostate cancer diagnostic test in the urine of men at risk of prostate cancer.[26–30] This assay is now being offered for clinical use at several national commercial laboratories. Extending this type of assay, several studies have explored using the *TMPRSS2–ERG* fusion transcript as a prostate cancer-specific urine biomarker, either alone or in combination with other assays.[31–33] The presence of *TMPRSS2–ERG* fusion transcripts is highly specific for prostate cancer when detected.[31,32,34] However, the sensitivity approaches the theoretical limitations of 40–50%, reflecting the prevalence of *TMRPSS2–ERG* fusions in the prostate cancer population. This study suggests that this sensitivity might be improved by incorporating other common recurrent gene fusions, such as *SLC45A3–ERG*, *TMPRSS2–ETV1*, *SLC45A3–ETV1*, and *NDRG1–ERG*. As the next generations of these assays are developed, perhaps incorporations of all known gene fusions would be feasable.

Our study of 540 men with clinically localized prostate cancer did not observe any clinical associations between ETS rearrangement status and pathology or clinical parameters. There is considerable controversy and confusion regarding the clinical implications of ETS rearranged prostate cancer (see Tomlins *et al*[4] for a review on this subject). Associations with cancer-specific death and *ERG* rearrangment prostate cancer were described in two Watchful Waiting cohorts.[15,16] These studies are particular because patients were diagnosed and then followed with prostate cancer without undergoing initial definitive treatment. These studies therefore might represent the natural clinical course of ETS rearranged prostate cancer. A number of other studies have explored for associations between PSA biochemical recurrence and ETS rearranged and have failed to show any consistent findings (see Tomlins *et al*[4] for a review on this subject). We posit that these studies cannot be used as evidence that ETS rearranged prostate cancers do not have a more or less aggressive natural history, as the course of the tumor progression was interrupted by clinical intervention. In addition, PSA biochemical recurrence has not been shown to be an accurate predictor of cancer-specific death.[35] Some have suggested that the frequency of ETS rearranged prostate cancer cases (40–60%) is too high to represent an aggressive sub-population. This is an important observation, given the relative low mortality rate of prostate cancer. More recent works by Carver *et al*[10] and King *et al*,[9] using animal models suggest that *ERG* overexpression alone is insufficient for disease progression and the concurrent

PTEN loss or alteration of the PI3/AKT/PTEN pathway is required. These studies would suggest that ETS rearragements and other molecular concurrent events may best describe an aggressive clinical cancer. Numerous studies are underway to explore this hypothesis.

In summary, we were able to confirm in a large cohort that approximately 80% of prostate cancer cases with *ERG* rearrangement have *TMPRSS2–ERG* gene fusions. We also showed the recurrent nature of *SLC45A3–ERG* and *NDRG1–ERG* fusions in approximately 7% of *ERG* rearranged cases. Interestingly in 11% of the cases, we observed prostate cancer cases in which individuals harbored more than one type of ERG rearrangement and rarely in which two *ERG* fusions seem to occur in the same tumor focus. For the remaining *ERG* rearranged cases we could not confirm the 5′ partner. This study has important clinical implications for the development of diagnostic assays to detect ETS rearranged prostate cancer. Incorporation of these less common *ERG* rearranged prostate cancer fusion assays could further increase the sensitivity of these PCR-based approaches.

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Disclosure/conflict of interest

S Perner and MA Rubin are listed as co-inventors on a patent filed by The University of Michigan and The Brigham and Women's Hospital covering the diagnostic and therapeutic fields for ETS fusions in prostate cancer. The diagnostic field has been licensed to Gen-Probe, Inc. Gen-Probe has no role in the design and conduct of the study, in the collection, analysis, or interpretation of the data, or in the preparation, review, or approval of the article.

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