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The discovery and application of gene fusions in prostate cancer

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Chromosomal rearrangements play a causal role in haematological and mesenchymal malignancies. Importantly, the resulting gene fusions can serve as specific therapeutic targets, as exemplified by the development of imatinib (Gleevec), which specifically inhibits the BCR-ABL gene fusion product that defines chronic myeloid leukaemia. Recently, gene fusions involving the prostate-specific gene transmembrane protease, serine 2 (TMPRSS2) and members of the erythroblastosis virus E26

transforming sequence (ETS) family of transcription factors were identified in most of PSA-screened prostate cancers. In this review, we summarize the identification, characterization and detection of TMPRSS2:ETS gene fusions and their role in prostate cancer development. We also discuss the discovery of additional 5′ partners that define distinct classes of ETS gene fusions based on the prostate specificity and androgen responsiveness of the 5′ partner. Additionally, we also

summarize conflicting reports about associations between gene fusion status and patient outcome. The specificity of ETS gene fusions in prostate cancer suggests that they may have causal roles in prostate cancer and suggest utility in prostate cancer detection, stratification and treatment.

KEYWORDS

prostate cancer, gene fusion, biomarker

INTRODUCTION

Alterations of gene expression and subsequent function, whether through activation of oncogenes or suppression of tumour suppressors, are hallmark contributors to the development of malignancy. These alterations often result from chromosomal translocations or deletions of segments of the genome that result in modified gene expression or fusion of two distinct gene transcripts. Such chromosomal structural rearrangements are common in haematological malignancies and often function as exclusive trigger points in oncogenesis [1–3]. For example, the translocation of the BCR gene from chromosome 22 with the ABL gene of chromosome 9, yielding the 'Philadelphia chromosome', results in the production of a BCR-ABL fusion protein with a constitutively active tyrosine kinase domain that drives the development of chronic myeloid leukaemia. While this is an example of a chromosomal translocation resulting in fusion of two transcripts resulting in a fusion protein, structural rearrangements of the chromosome can drive the development of malignancy through many methods. These

alterations can change gene products directly or inappropriately modify gene expression by associating upstream promoters or regions of epigenetic control (e.g. hypermethylation of upstream promoters) with other genes (Fig. 1).
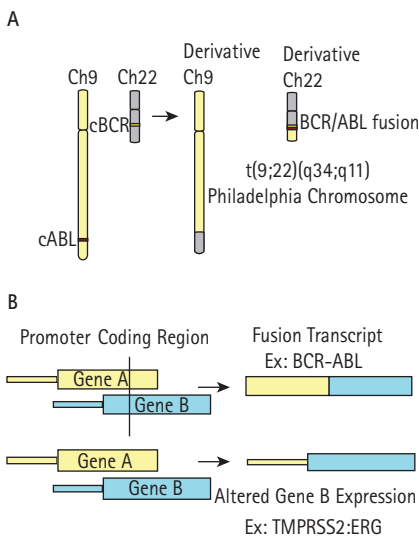
WHY DO FUSIONS MATTER?

The pervasive nature of structural rearrangements in haematological malignancies enables cytogenetics and fusion status to determine tumour subtyping and appropriate therapy decisions [4]. The modern era of targeted drug therapy was bolstered by the success of imatinib (Gleevec) development for targeting the tyrosine kinase domain of the BCR-ABL fusion [5]. Unfortunately, structural rearrangements are historically rare in carcinomas. Besides the obvious explanation that the rearrangements may not exist, the assumption was that chromosomal changes are difficult to discover and document in epithelial-based tumours. Carcinoma cells are difficult to culture and studies based on solid tumour samples detect many nonspecific chromosomal changes. However, recent advances in genomic

profiling, through the use of microarrays, spectral karyotyping (SKY), and competitive genomic hybridization (CGH), has coupled with the emerging field of bioinformatics to uncover many findings not evident with standard analysis techniques. As an example of the power of bioinformatic analysis to detect new associations from previously analysed data, the Chinnaiyan research group developed the Cancer Outlier Profile Analysis (COPA) algorithm to analyse DNA microarray studies, which led to the identification of recurrent chromosomal rearrangements in prostate cancer [6].

COPA ANALYSIS

COPA is based on the three theories: (i) chromosomal rearrangements and amplifications may result in marked overexpression of involved genes, (ii) such alterations are often heterogeneous in a given cancer type, and (iii) the altered gene expression in a subset of samples may be overshadowed when analysing DNA microarray studies using standard analytical approaches (e.g. a two class t-test method). Thus, COPA is designed to detect genes that

FIG. 1. Gene alterations by chromosomal translocations and gene fusion. **A**, The BCR-ABL fusion protein results from reciprocal translocation of the BCR gene from chromosome 22 with the ABL gene from chromosome 9. **B**, Examples of gene fusion methods that may drive oncogenesis. Fusion of the coding region of genes A and B can result in a fusion transcript and associated fusion protein with altered function. Alternatively, fusion of Gene A upstream promoter regions to the coding region of Gene B may alter Gene B expression through transcription factor or epigenetic control.

TABLE 1 COPA analysis identifies outliers in cancer samples

Rank	% of cases	Study	Cancer	Gene	Supporting evidence qPCR	FISH
1	5	Valk et al. [8]	Leukaemia	RUNX1T1	+	+
1	5	Vasselli et al. [9]	Renal	PRO1073	+	
1	10	Ross et al. [7]	Leukaemia	PBX1	+	+
1	5	Lapointe et al. [10]	Prostate	ETV1	+	+
1	10	Tomlins et al. [6]	Prostate	ETV1	+	+
1	10	Tian et al. [11]	Myeloma	WHSC1	+	
1	25	Dhanasekaran et al. [12]	Prostate	ERG	+	+
1	25	Welsh et al. [13]	Prostate	ERG	+	+
1	25	Zhan et al. [14]	Myeloma	CCND1	+	
1	25	Lapointe et al. [10]	Prostate	ERG	+	+
2	10	Tomlins et al. [6]	Prostate	ERG	+	+
4	25	Huang et al. [15]	Breast	ERBB2	+	
9	5	Glinsky et al. [16]	Prostate	ETV1	+	+
9	25	Yu et al. [17]	Prostate	ERG	+	+

have very high expression in only a subset of cancer cases, e.g. gene A is highly overexpressed in only 5% of tumour samples. While the full statistical methodology is outside the realm of this review, for every gene in a given microarray study, the median expression for all samples is centred to 0 and the median absolute deviation is set to 1. This process compresses a biomarker gene profile that has generalized overexpression in cancer compared with normal and accentuates an outlier gene profile that only has overexpression in a subset of cancer cases. As is typical for microarray analysis, the genes are then rank ordered by the extent of their 'outlier' score to enable selection of genes for further characterization and study. Applying this method to multiple cancer datasets ranks genes with known rearrangements at or near the top of the list, e.g. PBX1 ranks number one for outliers from a microarray dataset of leukaemia cases with confirmed fusion of the PBX1 gene [7] (Table 1) [6–17]. The results of COPA analysis of prostate cancer studies identified erythroblastosis virus E26 transforming sequence (ETS) variant gene 1

(ETV1) and v-ETS erythroblastosis virus E26 oncogene like (ERG) as outliers in a fraction of cases with COPA scores in the top 10 for six independent prostate profiling studies [6]. ETV1 (7p21.2) and ERG (21q22.3) are genes from the ETS family of transcription factors and have previously been implicated in oncogenic translocations in Ewing's sarcoma and myeloid leukaemia [18,19]. Furthermore, ETS members are functionally redundant in cancer development, as only one ETS gene is involved in a translocation in each case of Ewing's sarcoma [19]. As further support for a role in prostate cancer oncogenesis, the overexpression of ETV1 and ERG was mutually exclusive and raised the possibility that the overexpression of these genes acts as a trigger point in cancer development.
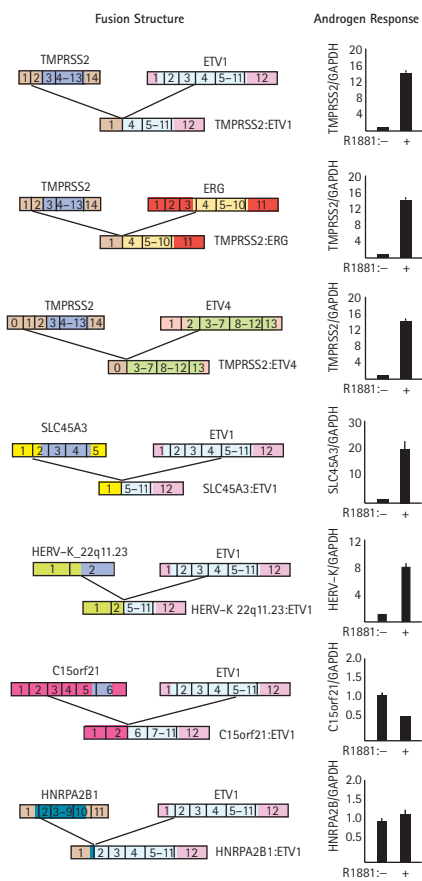
DISCOVERY OF PROSTATE GENE FUSIONS

To determine if structural rearrangements may be responsible for the overexpression of ETV1 and ERG, the RNA from prostate cancer samples was characterized quantitatively using a PCR–based test. Real-time quantitative PCR (qPCR) determines the amount of RNA transcript for a gene of interest by amplifying a segment of the gene transcript and directly measuring the amount of transcript during the amplification process using fluorescent dyes. When applied to prostate cancer, qPCR consistently showed a loss of the 5′ region of ETV1 or ERG for cases with marked overexpression of the 3′ end. Next, RNA ligase mediated rapid amplification

of cDNA ends (RLM-RACE) was used to characterize the 5′ end of the ERG or ETV1 transcripts in such samples. Sequencing determined that the 5′ end of ETV1 or ERG was consistently replaced with the 5′ untranslated region of the prostate specific gene transmembrane protease, serine 2 (TMPRSS2; 21q22.2) (Fig. 2). After documenting the fusion of TMPRSS2 to ETV1 or ERG in cases with overexpression, qPCR using forward primers in TMPRSS2 and reverse primers in ETV1 or ERG was performed to confirm the fusion in a separate group of clinically localized prostate cancer samples. Once again, the overexpression of ETV1 or ERG was mutually exclusive and fusion transcripts could be detected in 95% of cases with overexpression of ETV1 or ERG, but not in benign prostate tissue samples or prostate cancers without ETV1 or ERG overexpression.

CONFIRMATION OF TRANSLOCATIONS

The detection and validation of chromosomal alterations producing gene fusions are typically based on two technologies: the use of PCR-based assays to detect and quantify gene expression and fusion transcripts, and the use of fluorescent in situ hybridization (FISH) based assays to visualize the chromosomal alteration at the genomic level. Similar to designing primers to amplify the gene transcript, FISH probes are selected from genomic DNA sequences that span or abut the segment of genomic DNA potentially involved in a translocation. For example, for FISH-

FIG. 2. *Gene fusions identified in prostate cancer. Gene fusions described to date in prostate cancer are depicted as gene pairs. Exons are represented by numbered boxes and translated and untranslated flanking regions are shown with contrasting colours. The relative response to synthetic androgens R1881 are depicted in relative expression units to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).*

TABLE 2 Frequency of ETS outlier expression and TMPRSS2:ETS expression. The number and frequency of samples with ERG rearrangments (ERG+) or TMPRSS2:ETV1 fusions is indicated

Rearrangement study*	Reference	No. of Samples	ERG+, n (%)	TMPRSS2:ETV1+, n (%)
Cerveira *et al.*	[26]	34	17 (50)	0
Demichelis *et al.*	[25]	111	17 (15.3)	ND
Hermans *et al.*	[27]	11	5	1
Iljin *et al.*	[28]	19	7 (36.8)	ND
Lapointe *et al.*	[23]	63	44 (69.8)	ND
Mehra *et al.*	[20]	65/53	36 (55.4)	1 (1.9)
Mosquera *et al.*	[21]	253	120 (47.4)	ND
Nam *et al.*	[29]	26	11 (42.3)	ND
Perner *et al.*	[30]	24	12 (50)	0
		135	65 (48.1)	ND
Rajput *et al.*	[31]	86	35 (40.7)	ND
Soller *et al.*	[32]	18	14 (77.8)	0
Tu *et al.*	[33]	82	35 (43)	1 (1.2)
Wang *et al.*	[24]	59	35 (59.3)	ND
Winnes *et al.*	[22]	50	18 (36)	0
Yoshimoto *et al.*	[34]	15	6	0
Total+:			477/1051 (45.4)	3/287 (1)

*Assessing TMPRSS2:ETS rearrangements (by FISH or qPCR) are indicated by the last name of the first author; ND, no assessment of TMPRSS2:ETV1 status was made.

based confirmation of the *TMPRSS2:ETV1* gene fusion, probes to the *TMPRSS2* and *ETV1* locus were labelled with distinct fluorescent dyes, and cases with a *TMPRSS2:ETV1* fusion showed a pair of separate signals reflecting the normal 7 and 22 chromosomes, and a single fused signal reflecting the fusion of the *TMPRSS2* and *ETV1* genomic loci. On the other hand, fusion of *TMPRSS2* and *ERG* can occur through either translocation between both chromosome 21s or deletion of the genomic material between *TMPRSS2* and *ERG* as they are located close together on the same chromosome arm. Importantly, both translocations and chromosomal deletions can be detected by specifically designed FISH probes. Although qPCR-based detection may be ideal for quantifying and characterizing

the fusion transcript, FISH-based detection allows screening of large collection of prostate cancer samples through the use of tissue microarrays, where cores from tens to hundreds of prostate cancers are arrayed in a single paraffin block for sectioning. As part of the initial description of the ETS fusions, 23 of 29 cases (79%) of prostate cancer samples from a tissue microarray were shown to harbour either *TMPRSS2:ETV1* fusions (seven cases) or *TMPRSS2:ERG* fusions (16 cases) [6].

Since the initial description of ETS-family-member gene fusions in prostate cancer, the results have been confirmed through many independent centres internationally (Table 2) [20–34]. As more cases are screened and identified, the exact proportion of cases with any specific fusion changes. However, with a total of >500 samples studied, multiple groups have confirmed the largest proportion of fusions involve *TMPRSS2:ERG* (≈50%), with a smaller proportion of *TMPRSS2:ETV1* (1–10%) [20–22]. As characterization continues additional ETS genes have been identified in rearrangements, such as *TMPRSS2*:ETV4, and recently a family of 5′ partners, including *SLC45A3*, *HERV-K_22q11.3*, *HNRPA2B1* and *C15ORF21*, have been identified in ETS fusions (Fig. 2) [23,35].

FUSION FUNCTION AS ONCOGENES?

While fusions have thus far been described in most prostate cancers, the influence of fusion status on the development of cancer remains subject to study and debate. The *TMPRSS2* genes itself is prostate tissue specific and is expressed in both normal and cancerous prostate epithelium. Importantly, the expression of *TMPRSS2* is induced by androgen stimulation in androgen-sensitive prostate cancer cell lines [36,37]. Thus, we hypothesized that the 5′ untranslated region of *TMPRSS2*, which contains the elements needed to drive prostate tissue expression, functions to drive ETS gene overexpression in *TMPRSS2*:ETS-positive prostate cancers. In support of this hypothesis, the expression of *ERG* and *TMPRSS2*:ERG was up-regulated by synthetic androgen and blocked with bicalutamide (Casodex™) in VCaP cells, which express *TMPRSS2*:ERG fusion, but not in LnCaP cells, which harbour an *ETV1* rearrangement [6,38].

The histological development of prostate cancer is often described through a transition from benign epithelium to prostatic intraepithelial neoplasia (PIN) to frank adenocarcinoma and ultimately metastatic disease. Based on several observations, it

FIG. 3. *Classes of chromosomal rearrangements potentially activating ETS oncogenes in prostate cancer. Five general classes of chromosomal rearrangements activate ETS family members in the development of prostate cancer. Classes are determined by the prostate-specificity and androgen-responsiveness of the 5′ fusion partner for the ETS family member. The presence of detectable fusion transcripts the type of genomic sequence of the 5′ partner are noted. Examples of each class are indicated with graphic representation of exon number and fusion location.*

Class	Prostate specific	Androgen response	Fusion transcript	Element type	Example
I	Yes	Induced	Present	Proximal promoter	TMPRSS2:ETV1 TMPRSS2:ERG TMPRSS2:ETV4
IIa	Yes	Induced	Present	Proximal promoter	SLC45A3:ETV1
IIb	Yes	Induced	Present	Retroviral element	HERV-K:ETV1
III	Yes	Repressed	Present	Proximal promoter	C15ORF21:ETV1
IV	No	Unchanged	Present	Proximal promoter	HNRPA2B1:ETV1
V	Yes	Induced	Absent	Prostate specific region	ins(7;14)(p21;q21) (LNCaP) t(7;14)(p21;q21) (MDA-PCa 2B)

appears likely that ETS gene fusions may be a genetic trigger for the PIN to adenocarcinoma transition through the development of invasion. For example, in a FISH-based study of >400 tissue samples, Perner *et al.* [39] identified the presence of the *TMPRSS2:ERG* fusion in 48.5% of clinically localized prostate cancers, 30% of hormone-naive metastases, 33% of hormone-refractory metastases, and in 19% of high-grade PIN lesions that were always found intermingling with cancer foci. However, no benign prostate, BPH or proliferative inflammatory atrophy (PIA) tissue samples harboured *TMPRSS2:ERG* fusions. Furthermore, in an integrative molecular profiling of prostate cancer progression, we found that PIN and prostate cancer had a very similar expression profile, suggesting a limited number of genetic changes needed to drive the PIN to prostate cancer transition. Additionally, the ectopic overexpression of the *ETV1* fusion product in the immortalized benign prostatic epithelial cell line RWPE, markedly increased cell invasion through a modified basement membrane without affecting proliferation. Finally, generation of transgenic mice overexpressing the *ETV1* or *ERG* fusion product resulted in the development of mouse PIN (mPIN; [40–42]). Synthesizing the results of these studies suggests that initial genetic lesions, such as single-copy loss of the tumour suppressors *PTEN* or *NKX3-1*, dysregulate cellular proliferation and drive the benign to PIN transition, and in such a context *TMPRSS2*:ETS gene fusions trigger the

development of invasion and carcinoma. The RWPE-*ETV1* results and the development of mPIN but not carcinoma in transgenic mice suggest that ETS fusions are probably not sufficient for transformation and require pre-existing genetic lesions. Thus, the generation of transgenic mice recapitulating both initial lesions and ETS fusions products will probably provide a highly relevant model for *in vivo* studies of prostate cancer development.

Initially, *TMPRSS2* was the only 5′ partner identified in ETS gene fusions in prostate cancer. As larger cohorts were characterized, while the percentage of *TMPRSS2:ERG*-positive cancers closely matched the percentage of cancers with *ERG* outlier expression, fewer *TMPRSS2:ETV1*-positive cancers were being identified based on the percentage of cases with *ETV1* overexpression. Thus, we used RLM-RACE to characterize additional cancers with *ETV1* outlier expression. Unexpectedly, we discovered a family of 5′ fusion partners involved in *ETV1* gene fusions, including the 5′ untranslated regions from *SLC45A3*, *HERV-K_22q11.3*, *C15ORF21* and *HNRPA2B1* [40]. As these 5′ partners are differentially regulated by androgen as described below (androgen-induced, androgen-repressed and androgen-insensitive), they define distinct classes of ETS gene rearrangements. Thus, as a prospective method of classifying ETS member fusions, our laboratory has suggested a classification schema based on the 5′ partner for ETS members (Fig. 3).

Fusions involving the prostate-specific androgen-induced gene *TMPRSS2* to ETS genes comprise the most frequent class of rearrangements (Class I). ETS fusions with other prostate-specific androgen-induced genes (e.g. *SLC45A3*) and endogenous retroviral elements (e.g. *HERV-K_22q11.3*) define Class IIa and Class IIb rearrangements, respectively. Class III rearrangements represent 5′ prostate-specific androgen-repressed partners, like *C15ORF21*. A strong 5′ ubiquitous promoter drives aberrant ETS expression in Class IV rearrangements. Characterizing prostate cancer cell lines with *ETV1* rearrangements, we found that through distinct mechanisms, the entire *ETV1* locus is rearranged to a 1.5-megabase prostate-specific region at 14q13.3–14q21.1 in both LNCaP cells (cryptic insertion) and MDA-PCa 2B cells (balanced translocation). Thus, rearrangement of the entire ETS gene into a prostate-specific-region defines Class V rearrangements [40].

ASSOCIATION WITH OUTCOMES

Recently, the influence of fusion status on cancer outcome has begun to be addressed. Conflicting data supports the association of *TMPRSS2:ERG* fusion with both improved and worsened patient outcomes. The limitations of small cohort size and varied populations are partially responsible for these findings, and have led to the lack of consensus about whether gene fusions influence the risk of prostate cancer progression or recurrence. Initial screening studies seeking to determine the proportion of cases with gene fusion found no association with PSA recurrence [20,23]. Many of these studies were unable to determine the association with PSA recurrence due to limitations in follow-up and instead reported the association with assumed surrogates of cancer risk such as disease stage and Gleason grade. While some studies have shown correlation with higher stage disease [20], others report either no association with Gleason score [23,39] or an association with lower Gleason score and better survival [22]. On the other hand, a surgical series of 59 patients including 34 cases with PSA recurrence and advanced pathological stage, recently reported an association between certain isoforms of fusion with early recurrence and seminal vesicle invasion [24]. The number of exons from *TMPRSS2* and the alignment of

TMPRSS2 with downstream *ERG* exons determined up to eight isoforms, including the two initially described by Tomlins *et al.* [6] and had a significant impact on disease characteristics. In a separate surgical series of 26 patients including 11 (42%) with *TMPRSS2:ERG* fusion, patients with fusion had a higher rate of biochemical recurrence (80%) than did fusion negative patients (38%) [29]. Additionally, *TMPRSS2:ERG* fusion was significantly associated with a higher likelihood of prostate cancer-specific death in a cohort of 111 men in a watchful-waiting cohort [25]. Although *TMPRSS2:ERG* fusion may be associated with lower disease survival in a European watchful-waiting cohort, the patient population from this cohort differs from the patient cohorts identified through PSA-screening in the USA [20]. The overall proportion of cases with gene fusion is much lower, only 15%, than the proportion noted in screening populations in the USA of >50%, and therefore caution should be used before extrapolating the study conclusions to a population with a different fusion penetrance. Furthermore, these trials reflect small cohorts when compared with the thousands of patients presenting for prostate cancer treatment and results should be confirmed on larger, more inclusive cohorts before fusion status can be utilized to risk stratify patients with prostate cancer.

FUTURE APPLICATIONS

Gene fusion status may eventually be used for pretreatment risk stratification, for enrolment in active surveillance trials, or for guidance during surveillance after therapy. Given the protracted course of prostate cancer progression, supporting data from prospective trials may take years to accumulate. However, due to the specificity of gene fusions for prostate cancer compared with normal, PIA, or BPH nodules, fusion status is currently being developed as a biomarker for prostate cancer presence [39]. The detection of fusion transcripts in urine samples of men with prostate cancer 'opened the door' for trials incorporating prostate cancer fusions in genetic screening tests for prostate cancer [43]. We recently completed a prospective trial designed to predict cancer detection on biopsy by analysing novel urinary markers for prostate cancer, including gene fusion. Urinary expression of seven potential markers, including *TMPRSS2:ERG* fusion, was measured in 236 men presenting

for prostate biopsy or prostatectomy. We generated receiver operator characteristic curves for individual markers and for a multiplexed combination of markers. Incorporating gene fusion status with other urinary prostate cancer markers improved on the testing characteristics of any single marker test, including serum PSA [44]. In a similar analysis, Hessels *et al.* [45] combined fusion status with urinary PCA3 to improve the ability to predict cancer presence in 108 men presenting for biopsy. With most cancers containing gene fusions of one form or another, a test designed to identify fusions may improve prostate cancer detection before biopsy by combining tests for each fusion subtype.

In addition to improved detection, fusion partners potentially determine tumour biology through androgen regulation and may predict response to hormonal treatments. As proposed in the fusion classification schema, sensitivity to androgen stimulation separates fusion pairs with the potential to increase transcription factor signalling through androgen-sensitive promoters or androgen-response elements. Conversely, androgen-repressed fusion partners, such as *C15ORF21*, may further stimulate cancer progression in the androgen-ablation state common with hormonal manipulations. Furthermore, if studies confirm the association of gene fusions with prostate cancer outcomes, the preoperative determination of fusion status would improve patient counselling before therapy.

CONCLUSIONS

The detection and confirmation of gene fusions in prostate cancer may eventually rival the discovery of PSA as a prostate cancer biomarker. Fusion proteins and alterations in gene expression because of fusions may act as trigger points in malignant transformation and progression. While the detection of gene fusions has already changed the classification, detection, and treatment pathways for haematological cancers, the application of gene fusions in prostate cancer is currently in its infancy. Basic science and xenograft experiments have explored the ability of these fusions to influence prostate pathology in cancer development. While early clinical research utilizing surgical cohorts have implicated a link between fusion status

and disease outcome after treatment, future studies are needed before utilizing fusion status as a prognostic marker of cancer outcome. Further applications, including improved diagnostics based on urinary fusion detection, are currently being explored and indicate the potential for prostate cancer gene fusions to change the paradigm of prostate cancer detection, risk-stratification, and treatment.

CONFLICT OF INTEREST

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Abbreviations: **SKY**, spectral karyotyping; **CGH**, competitive genomic hybridization; **COPA**, Cancer outlier profile analysis; **ETS**, erythroblastosis virus E26 transforming sequence; **ETV1**, ETS variant gene 1; **ERG**, v-ETS erythroblastosis virus E26 oncogene like; **qPCR**, quantitative PCR; **RLM–RACE**, RNA ligase mediated rapid amplification of cDNA ends; **TMPRSS2**, transmembrane protease, serine 2; **FISH**, fluorescent *in situ* hybridization; **(m)PIN**, prostate intraepithelial neoplasia; **PIA**, proliferative inflammatory atrophy.