

ETS family-associated gene fusions in Japanese prostate cancer: analysis of 194 radical prostatectomy samples

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The incidence and clinical significance of the *TMPRSS2:ERG* gene fusion in prostate cancer has been investigated with contradictory results. It is now common knowledge that significant variability in gene alterations exists according to ethnic background in various kinds of cancer. In this study, we evaluated gene fusions involving the *ETS* gene family in Japanese prostate cancer. Total RNA from 194 formalin-fixed and paraffin-embedded prostate cancer samples obtained by radical prostatectomy was subjected to reverse-transcriptase polymerase chain reaction to detect the common *TMPRSS2:ERG* T1-E4 and T1-E5 fusion transcripts and five other non-*TMPRSS2:ERG* fusion transcripts. We identified 54 *TMPRSS2:ERG*-positive cases (54/194, 28%) and two *HNRPA2B1:ETV1*-positive cases (2/194, 1%). The *SLC45A3-ELK4* transcript, a fusion transcript without structural gene rearrangement, was detectable in five cases (5/194, 3%). The frequencies of both *TMPRSS2:ERG*- and non-*TMPRSS2:ERG*-positive cases were lower than those reported for European, North American or Brazilian patients. Internodular heterogeneity of *TMPRSS2:ERG* was observed in 5 out of 11 multifocal cases (45%); a frequency similar to that found in European and North American cases. We found a positive correlation between the *TMPRSS2:ERG* fusion and a Gleason score of ≤ 7 and patient age, but found no relationship with pT stage or plasma prostate-specific antigen concentration. To exclude the possibility that Japanese prostate cancer displays novel *TMPRSS2:ERG* transcript variants or has unique 5' fusion partners for the *ETS* genes, we performed 5' RACE using fresh-frozen prostate cancer samples. We identified only the normal 5' cDNA ends for *ERG*, *ETV1* and *ETV5* in fusion-negative cases. Because we identified a relatively low frequency of *TMPRSS2:ERG* and other fusions, further evaluation is required before this promising molecular marker should be introduced into the management of Japanese prostate cancer patients.

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Tomlins *et al*¹ first reported in 2005 that recurrent genomic rearrangements are observed in prostatic

adenocarcinomas, resulting in gene fusion between the untranslated region of the androgen-driven gene *TMPRSS2* and the oncogenic transcription factor genes of the *ETS* family. This gene fusion is now recognized as a frequent event, occurring in almost half of prostate cancer cases.² To date, 14 genes (*TMPRSS2*, *SLC45A3*, *HERV-K*, *KLK2*, *CANT1*, *FOXP1*, *HERVK17*, *EST14*, *FLJ35294*, *C15orf21*, *HNRPA2B1*, *DDX5*, *ACSL3* and *NDRG1*) have been

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identified as 5' fusion partners for the four *ETS* family transcription factor genes (*ERG*, *ETV1*, *ETV4* and *ETV5*), resulting in >20 different fusion genes in prostate cancer.^{2,3,4,5} Among these, approximately 90% occur between *TMPRSS2* and *ERG*.²

The association of the *TMPRSS2:ERG* gene fusion with prostate cancer prognosis has been evaluated in multiple studies but with contradictory results.² Although its clinical significance remains to be clarified, because of its high incidence, *TMPRSS2:ERG* is expected to be a useful marker for the early detection of prostate cancer. Evaluation of *TMPRSS2:ERG* transcripts in urine samples after prostatic massage in combination with other prostate cancer marker RNAs such as *PAC3* are candidates for prostate cancer screening.⁶

It is a well-known paradigm showing importance of ethnicity that the frequency of *EGFR* mutation in lung adenocarcinomas significantly differs between Western and Asian countries.⁷ In this study, we evaluated gene fusions involving the *ETS* family genes in prostate cancer among the Japanese.

Materials and methods

Study Population and Preparation of Prostate Cancer Specimens

The analysis involved 194 Japanese cases of prostate cancer who had radical prostatectomy at the Chigasaki Municipal Hospital (CMH), Chigasaki, Japan (88 cases) and the Kanagawa Cancer Center Hospital (KCCH), Yokohama, Japan (106 cases). None of the patients received preoperative radiation or androgen deprivation therapy. All patients from KCCH and 51 patients from CMH, whose prostatectomy specimens were subjected to whole-mount pathological examination, were consecutive cases from 2003 to 2008 (KCCH) and from 2003 to 2006 (CMH). Patients with small tumors of <5 mm in the maximum diameter were excluded from the study. A total of 37 patients from CMH, whose specimens were not subjected to whole-mount examination, were randomly selected from those operated from 1998 to 2002. The institutional review boards of both hospitals approved this study. Generally, macroscopic tumor location was mapped and, for the index tumor of each case, three serial slices of 10- μ m thickness were prepared from paraffin-embedded specimens, from which total RNA was extracted. For the cases consisting of multiple tumor nodules, which were separated from each other by >5 mm and where tumor size was >5 mm maximum diameter, RNA was extracted from each nodule separately and nodules were each analyzed subsequently as index tumors.

RNA extraction was performed using Recover All total nucleic acid isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA samples were first subjected to primary RT-PCR using 200 ng of sample RNA with the SuperScript III One-Step RT-PCR system (Invitrogen, Carlsbad, CA, USA) in 25 μ l of reaction mixture according to the manufacturer's instructions. Then, 1.5 μ l of the primary reaction was subjected to secondary semi-nested PCR in 25 μ l of the recommended buffer. Next, 5 μ l of the amplified reaction was electrophoresed on a 2.5% agarose gel, followed by staining with ethidium bromide and imaging. The primer sequences are provided in Supplementary Table 1. Representative PCR product bands were recovered and subcloned into the pGem-T-easy plasmid vector (Promega, Madison, WI, USA). Nucleotide sequences were determined to confirm that the bands corresponded to the predicted fusion transcripts. The detailed experimental conditions are provided in the Supplementary Information.

5'-Rapid Amplification of cDNA Ends (RACE)

Each frozen sample was confirmed histologically to contain cancer tissue. Total RNA was extracted using TRIzol reagent (Invitrogen). First, expression of the *ERG*, *ETV1*, *ETV4* and *ETV5* transcripts and the control porphobilinogen deaminase (*PBGD*) was evaluated by conventional RT-PCR, with primers located in exons not involved in reported prostate cancer gene fusions (listed in Supplementary Table 2). Then, 3 μ g of each total RNA was subjected to 5'-RACE with a GeneRacer kit (Invitrogen), according to the manufacturer's protocol. Amplified products were subcloned into pGem-T-easy plasmid vector (Promega) and their nucleotide sequences were determined. For RACE of *ETV5*, the primary RACE PCR gave no products and the nested PCR was performed with the primers given in Supplementary Table 2. The precise experimental conditions are provided in the Supplementary Information.

Statistical Analysis

Statistical difference was evaluated using Yates' χ^2 test or Fisher's test, and *P*-values of <0.05 were considered significant.

Results

Incidence of Fusion Gene Transcript-Positive Prostate Cancer Cases and Clinicopathological Characteristics

To verify the quality and integrity of the RNA samples, we first performed RT-PCR for *PBGD*, a ubiquitously expressed housekeeping gene. All RNAs used in this study gave strong and clear PCR products for *PBGD*. Then, we detected fusion gene

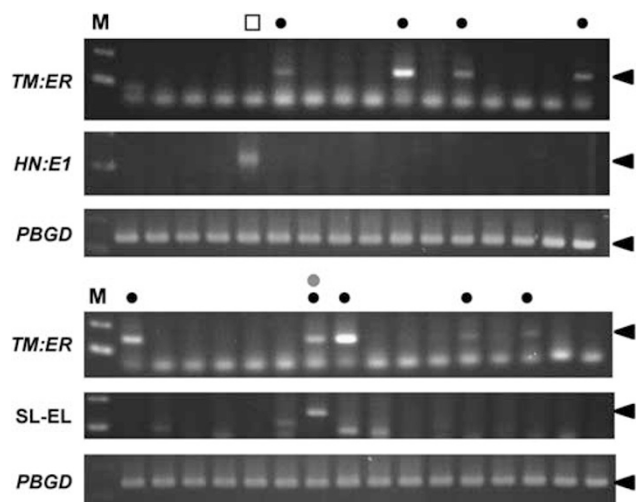


Figure 1 Representative results of RT-PCR to detect fusion transcripts. The upper panel shows a set of 16 cases demonstrating four cases positive for the *TMPRSS2:ERG* transcript (black circles) and one case positive for the *HNRPA2B1:ETV1* transcript (open square). The lower panel shows another set of 16 cases demonstrating five cases positive for the *TMPRSS2:ERG* transcript and one case positive for the *SLC45A3-ELK4* transcript (gray circle). *PBGD* RT-PCR is shown at the bottom of each panel as a positive control for RNA sample integrity. M, molecular size marker; *TM:ER*, *TMPRSS2:ERG*; *HN:E1*, *HNRPA2B1:ETV1*; *SL-EL*, *SLC45A3-ELK4*.

transcripts for two *TMPRSS2:ERG* fusions (the T1-E4 and T1-E5 variants), *TMPRSS2:ETV1*, *SLC45A3:ETV1*, *HERV-K:ETV1*, *CI5ORF21:ETV1* and *HNRPA2B1:ETV1*. Out of 194 radical prostatectomy samples, 54 (28%) were positive for the *TMPRSS2:ERG* T1-E4 transcript. The T1-E5 transcript was present in two cases (1%), and both of these also contained the T1-E4 transcript. Two cases (1%) were positive for the *HNRPA2B1:ETV1* transcript. There was no overlap between the *TMPRSS2:ERG*-positive and *HNRPA2B1:ETV1*-positive cases. No other fusion gene transcripts were identified. For the *SLC45A3-ELK4* fusion transcript, five cases demonstrated identifiable bands after semi-nested PCR. Two of them were also positive for *TMPRSS2:ERG*. Representative gel photographs showing fusion transcripts are shown in Figure 1.

We next evaluated the relationship between the incidence of the *TMPRSS2:ERG* fusion and the Gleason score in all 194 cases. The incidence of gene fusion was significantly lower (8 out of 54 cases, 15%) in those with a high risk Gleason score (≥ 8) than in cases with low or intermediate risk Gleason score (≤ 7 ; Table 1). For the 157 samples on which whole-mount examination was performed, the incidence of the *TMPRSS2:ERG* fusion was evaluated in relation to patient age, plasma prostate-specific antigen (PSA) concentration and pathological tumor (pT) stage. The incidence was significantly lower in patients aged ≤ 59 years, or in those aged ≥ 71 compared with those aged between 60 and 69 years. There was no significant

Table 1 Clinicopathological characteristics of *TMPRSS2:ERG*-positive cases

Characteristics	<i>TMPRSS2:ERG</i> (-), no. of cases (%)	<i>TMPRSS2:ERG</i> (+), no. of cases (%)	Total	P-value
Gleason score^a				
≤ 7	95 (67)	46 (33)	141	0.02 ^b
≥ 8	45 (85)	8 (15)	53	
Total	140 (72)	54 (28)	194	
Age				
≤ 59	17 (94)	1 (6)	18	0.004 ^b
60–69	72 (64)	41 (36)	113	
≥ 70	24 (92)	2 (8)	26	
Total	113 (72)	44 (28)	157	
PT				
pT2 (organ confined)	55 (71)	22 (29)	77	0.99
pT3a (EPE)	43 (72)	17 (28)	60	
pT3b (s.v. invasion)	15 (75)	5 (25)	20	
Total	113 (72)	44 (28)	157	
PSA				
4 to <10 ng/ml	58 (69)	26 (31)	84	0.42
10 to 20 ng/ml	40 (71)	16 (29)	56	
20 ng/ml <	15 (88)	2 (12)	17	
Total	113 (72)	44 (28)	157	

^aGleason score was analyzed against all 194 cases examined. Age, pT and PSA were analyzed against 157 cases for whom the whole-mount examination was performed for the total prostatectomy specimen and the pathological stage was exactly determined.

^bThe statistical difference was evaluated using Yates' χ^2 test and $P < 0.05$ was considered as significant.

correlation with either pT stage or PSA concentration (Table 1). The two *HNRPA2B1:ETV1*-positive cases were high-grade adenocarcinoma with a Gleason score of 8 and 9, respectively.

For the 157 whole-mount cases, the incidence of the *TMPRSS2:ERG* fusion was also separately evaluated for transition zone and non-transition zone cancers. In all, 26 (17%) were recognized as transition zone cancers. The *TMPRSS2:ERG*-positive case was 27% (35 out of 131) in non-transition zone and 23% (6 out of 26) in transition zone cancers, and the incidence was not significantly different between them.

Internodular Differences in the Incidence of *TMPRSS2:ERG*

In all, 11 cases matched our criteria for multiple nodules. Of these, seven cases consisted of two nodules and four cases had three nodules (Table 2). Out of 11 cases, 6 (55%) were positive for *TMPRSS2:ERG* transcripts in at least one nodule. When we evaluated the incidence per nodule, rather than per case, 8 out of 26 nodules (31%) were positive for the fusion transcripts. There was no significant difference between multiple cases of nodules and non-multiple cases in the incidence

Table 2 *TMPRSS2:ERG* fusion transcripts in cases with multiple tumor nodules

Nodules pattern	No. of case	Nodule no.		Fusion positive (%)
○ ○	2	4	Per case	6/11 (55)
○ ●	4	8	Per nodule	8/26 (31)
● ●	1	2		
○ ○ ○	3	9		
○ ○ ●	—	—		
○ ● ●	1	3		
● ● ●	—	—		
Total	11	26		

○ Fusion-negative nodule.

● Fusion-positive nodule.

of *TMPRSS2:ERG*. Internodular heterogeneity was observed in 45% (5/11) of cases.

Detection of *ERG*, *ETV1* and *ETV5* 5-Fusion Partners

We performed 5'-RACE on eight frozen prostate cancer tissues that did not contain any of the fusion gene transcripts examined in this study, together with two positive controls: one positive for *TMPRSS23:ERG* and one positive for *HNRPA2B1:ETV1*. First, we examined *ERG*, *ETV1*, *ETV4* and *ETV5* expression by conventional RT-PCR with primers located in separate exons that have not been reported to be involved in prostate cancer gene fusions. Considerable amounts of *ERG*, *ETV1* and *ETV5* transcripts were detected in all samples, but the levels of *ETV4* were low in all cases (Figure 2a). Therefore, 5'-RACE was performed for *ERG*, *ETV1* and *ETV5* only. The quality of each 5'-RACE template was verified by amplifying approximately 1-kbp fragments of the 5'-end of the β -actin cDNA. For RACE of *ETV5*, the primary RACE PCR gave no products and hence nested PCR was performed with the primers given in Supplementary Table 2. The RACE products were sequenced to verify their identity. The expected 5'-RACE bands derived from rearranged *TMPRSS2:ERG* and *HNRPA2B1:ETV1* fusion transcripts were demonstrated in the positive control samples (Figure 2b, panels for *ERG* and *ETV1*). Major 5'-RACE bands observed in the eight experimental samples were derived from normal non-fusion transcripts of *ERG*, *ETV1* and *ETV5* (Figure 2b). Although extra bands derived from normal but alternatively initiated transcripts were identified with some nonspecific bands of PCR artifacts (Figure 2b, sequencing data not shown), no 5' novel or unexamined fusion partners were identified.

Fusion Transcripts between *SLC45A3* and *ELK4* in Frozen Samples

Expression of fusion transcripts between *SLC45A3* and *ELK4* was evaluated in the frozen prostate

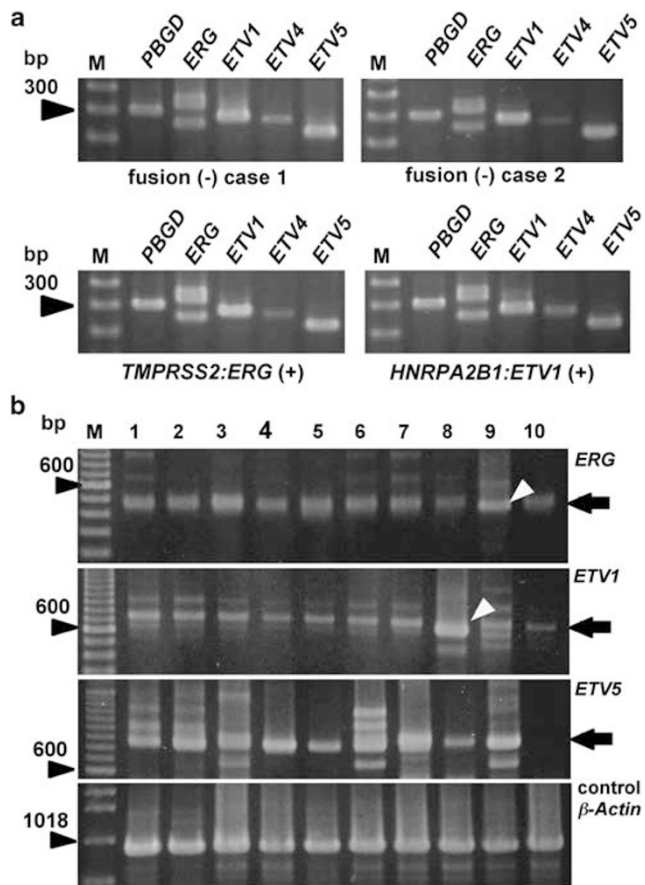


Figure 2 The expression of and 5'-RACE for *ERG*, *ETV1* and *ETV5* transcripts. (a) RT-PCR showing the presence of *ERG*, *ETV1*, *ETV4* and *ETV5* transcripts. The upper two panels show the results for two representative fusion-negative cases, and the lower panels show those for fusion-positive cases as indicated. *PBGD* RT-PCR results are shown as a positive control. (b) 5'-RACE PCR. The upper three panels show 5'-RACE for *ERG*, *ETV1* and *ETV5*, respectively. The bottom panel shows 5'-RACE for β -actin, as a template quality control. Bands indicated by white arrowheads correspond to the 5' ends of the *TMPRSS2:ERG* fusion transcript (lane 9) and the *HNRPA2B1:ETV1* fusion transcript (lane 8), respectively, as confirmed by DNA sequencing. Black arrows in the right side indicate the bands derived from normally initiated non-fusion transcripts of *ERG*, *ETV1* or *ETV5* in each panel. bp, base pairs; M, molecular size marker.

cancer samples used for RACE. After primary RT-PCR, a strong band for the *SLC45A3-ELK4* transcript was obtained in two cases, one of which was also positive for the *HNRPA2B1:ETV1* fusion. After amplification by semi-nested PCR, all but one case showed the *SLC45A3-ELK4* transcript (Figure 3).

Discussion

In this study, we reported the incidence of the *TMPRSS2:ERG* fusion to be 28% in Japanese prostate cancer samples obtained from radical prostatectomy specimens. Although a very high incidence ($\geq 70\%$) has been reported in some small-scale RT-PCR studies, in studies of > 50 cases,

the incidence is 40–60% (mean 53%) when restricted to radical prostatectomy specimens (Table 3). All the prostate cancer patients involved in these other studies were collected in Europe, North America or Brazil, without detailed ethnic information. The Japanese incidence of 28% is relatively low compared with these studies. Only one study, from Mosquera *et al*⁸ that examined 100 prostate biopsy specimens from patients in the United States, reported the *TMPRSS2:ERG* incidence separately in Caucasian and non-Caucasian patients. Although the incidence was 46% (46/100) overall, it was significantly different in Caucasians (44/85, 52%) and in non-Caucasians (2/15, 13%). This may

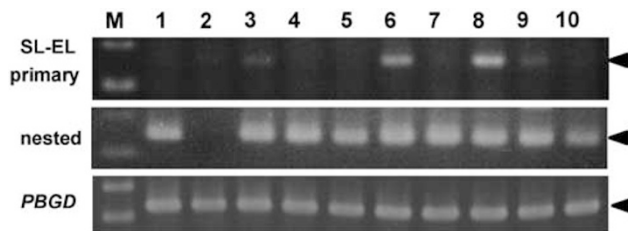


Figure 3 Expression of SLC45A3-ELK4 fusion transcripts in frozen prostate cancer samples with or without other fusion transcripts. The same sample set used for 5'-RACE was evaluated by RT-PCR to detect SLC45A3-ELK4 fusion transcripts. The upper panel shows the primary PCR, the middle panel shows the semi-nested PCR and the lower panel shows *PBGD* RT-PCR for template quality control. The sample numbers shown at the top correspond to those in Figure 2. M, molecular size marker.

imply that the difference in incidence resulted from different genetic backgrounds rather than the effects of lifestyle or diet. Therefore, it is possible that the low incidence among the Japanese is at least partly because of the genetic background of the patients.

Low *TMPRSS2:ERG* incidence can be partly associated with the characteristics of the sample population. In two Swedish watchful waiting cohorts, the incidence was 15% (17/111) and 18% (62/354), respectively.^{9,10} In these studies, the prostate cancer samples were obtained by transurethral resection of the prostate (TURP), representing mainly transition zone carcinomas. The included patients were restricted to those at early clinical stages (T1a-b, Nx, M0). From these studies, the incidence of the *TMPRSS2:ERG* fusion seems to be less frequent in patients at early clinical stages and in prostate cancers obtained by TURP, even among European patients. However, our Japanese prostate cancer samples were obtained by radical prostatectomy, and the low incidence may not be because of the sample characteristics.

So far, 17 types of fusion transcripts have been observed for the *TMPRSS2:ERG* rearrangement.¹¹ Variation in the transcripts is generally caused by alternative splicing of the fusion transcript, and the most common transcript is a recombination between exon 1 of *TMPRSS2* and exon 4 of *ERG*, designated T1-E4. Variants T1-E4 and T1-E5, those evaluated by RT-PCR in this study, are reported to correspond to 89–99% of *TMPRSS2:ERG* fusion transcripts.^{11,12,13}

Table 3 Prevalence of the *TMPRSS2:ERG* fusion in prostate cancer^a

Sample	Incidence (%)	Assay method	Fusion type	Patient residence	Reference
PCa, rp	58/118 (49)	FISH		Germany, USA	19
PCa, rp	35/59 (59)	RT-PCR	8 types	USA	12
PCa, rp	36/101 (36)	FISH		Canada	20
PCa, rp	120/253 (47)	FISH		USA, Canada, Germany	21
PCa, rp/LN	38/63 (60) (70) ^b	RT-PCR/FISH	T1-E4, iso 2	USA, Sweden	22
PCa, rp	30/56 (54)	FISH		USA	23
PCa, rp	115/237 (48)	FISH		USA, Germany	24
PCa, rp	33/74 (45)	RT-PCR/FISH	6 types	USA	25
PCa, rp	81/165 (49)	RT-PCR	T1-E4/T2-E4	Canada	26
PCa, TURP/en	17/111 (15)	RT-PCR/FISH		Sweden ^c	9
PCa, TURP	134/445 (30)	FISH		UK	27
PCa, TURP	62/354 (18)	DASL		Sweden ^d	10
PCa, rp	41/101 (41)	DASL		USA	10
PCa, rp	60/125 (48)	FISH		Brazil	28
PCa, rp	217/521 (42)	FISH		USA	29
PCa, rp	44/75 (59)	FISH		Germany ^e	30
PCa, biopsy	46/100 (46)	FISH		USA	8
44/85 (52) in Caucasian and 2/15 (13) in non-Caucasian population					
Pca, rp	54/194 (28)	RT-PCR	T1-E4/T1-E5	Japan	Present study

PCa, prostate cancer; rp, radical prostatectomy; FISH, fluorescence *in situ* hybridization; LN, lymph node; iso 2, *TMPRSS2* isoform 2; TURP, transurethral resection of the prostate; en, transvesical enucleation of adenoma; DASL, DNA (cDNA)-mediated annealing, selection, ligation, and extension assay.

^aStudies involving > 50 cases are listed.

^bThe incidence was 38/63 (60) for the most common T1-E4 variant and together with the isoform 2, which was 44/63 (70).

^cWatchful waiting cohort.

^dFive Swedish watchful waiting cohorts, partly overlapping with Demichelis *et al*.⁹

^eFamilial prostate cancers.

Therefore, the low incidence among the Japanese is not likely to be because of the assays chosen.

In addition to the most common *TMPRSS2:ERG* fusion, we also evaluated five other fusion transcripts: *TMPRSS2:ETV1*, *SLC45A3:ETV1*, *HERV-K:ETV1*, *CI5ORF21:ETV1* and *HNRPA2B1:ETV1*. Only two cases (1%, 2/194) were positive for *HNRPA2B1:ETV1* transcripts, and no other fusion transcripts were identified. Non-*TMPRSS2:ERG* fusion is reported to be rare, and in total accounts for only 10% of all fusions.² Although we did not evaluate all known non-*TMPRSS2:ERG* fusion transcripts, non-*TMPRSS2:ERG* fusion seems to be less frequent in Japanese prostate cancer patients than in other prostate cancer patients, similar to our findings for *TMPRSS2:ERG*. To exclude the possibility that Japanese prostate cancer exhibits a novel *TMPRSS2:ERG* transcript variant, or has unique 5' fusion partners for the *ETS* genes, we performed 5'-RACE. We did not detect any novel gene fusions.

The combination of *SLC45A3* exon 1 and the *ETS* family gene *ELK4* exon 2 is a known fusion transcript without gene rearrangement that is observed in both normal and malignant prostatic tissues, but is overexpressed in some prostatic cancers.^{14,15} In light of the low incidence of the *TMPRSS2:ERG* fusion, it is possible that there is instead an increased incidence of *SLC45A3-ELK4* overexpression among Japanese prostate cancer patients. In our study, detectable expression of the *SLC45A3-ELK4* fusion transcript in formalin-fixed and paraffin-embedded specimens, which may correspond to overexpression, was rare, and therefore this is probably not a major molecular cause of prostate cancer in the Japanese.

The multifocality of prostate cancer has been recognized both morphologically and genetically.^{16,17} Mehra *et al*¹⁸ first reported that the *TMPRSS2:ERG* fusion could be heterogeneous in multifocal prostate cancer.²⁸ There can be heterogeneity between different tumor foci, not only in the presence or absence of the fusion, but also in the mechanism of the fusion, that is, deletion or translocation. This interfocal or internodular heterogeneity has been confirmed by several follow-up studies.¹¹ In this study, we analyzed 11 multifocal cases and found internodular heterogeneity in 5 of them (45%), demonstrating that the *TMPRSS2:ERG* genetic heterogeneity is not different between the Japanese and the European or the US studies.

The prognostic utility of the *TMPRSS2:ERG* fusion has been evaluated in multiple studies but with contradictory results. We found a positive correlation between the *TMPRSS2:ERG* fusion and a Gleason score of ≤ 7 , but found no relationship with pT stage or PSA concentration. Further studies of clinical outcome, with a larger number of patients, are needed to assess the clinical significance of the fusion gene in Japanese prostate cancer.

In this study, we have raised the possibility that Japanese prostate cancer patients exhibit gene

fusion events involving the *ETS* family genes less frequently than European or North American prostate cancer patients. Further studies with large patient cohorts, and also studies on normal and precursor lesions such as prostatic intraepithelial neoplasia, are needed before an analysis of the *TMPRSS2:ERG* and other fusions should be introduced into cancer screening programs and the management of prostate cancer in the Japanese.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)