ERG rearrangement as a novel marker for predicting the extra-prostatic extension of clinically localised prostate cancer

LI LU*, HAO ZHANG*, JUN PANG, GUO-LIANG HOU, MIN-HUA LU and XIN GAO

Department of Urology, Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510630, P.R. China

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Abstract. Currently, there are no well-established preoperative clinicopathological parameters for predicting extra-prostatic extension (EPE) in patients with clinically localised prostate cancer (PCa). The transmembrane protease serine 2 (*TMPRSS2*)-ETS-related gene (*ERG*) fusion gene is a specific biomarker of PCa and is considered a prognostic predictor. The aim of the present study was to assess the value of this marker for predicting EPE in patients with clinically localised PCa. In total, 306 PCa patients with clinically localised disease, including 220 patients (71.9%) with organ-confined disease and 86 EPE cases (28.1%), were included in the study. Receiver operating characteristic curves and logistic regression were employed to establish the optimal cut-off value and to investigate whether *ERG* rearrangement was an independent predictor for the EPE of clinically localised PCa. A leave-one-out cross-validation (LOOCV) model was implemented to validate the predictive power of *ERG* rearrangement. An increase in *ERG* rearrangements was identified to be associate`d with EPE, and the optimal cut-off for predicting EPE was determined to be 2.25%, with a sensitivity of 70.24% [95% confidence interval (CI), 62.6-78.9%], a specificity of 80.43% (95% CI, 75.4-85.1%), and an area under the curve (AUC) of 0.781 (95% CI, 0.730-0.826). In the LOOCV model, *ERG* rearrangement also demonstrated good performance for predicting EPE (sensitivity, 76.923%; specificity, 71.429%; 95% CI for AUC, 0.724-0.958). In addition, a high Gleason score (≥7) and a cT2c classification upon biopsy were independent factors for EPE.

Introduction

Prostate cancer (PCa) is one of the most prevalent malignant tumours and is the second leading cause of cancer-associated mortality among men in Western countries (1). For men with localised PCa, radical prostatectomy (RP) is considered to be the ideal therapy; however, determining the optimal management strategy for locally advanced PCa remains a challenging issue (2). Traditionally, the malignant properties of PCa are characterised based predominantly on clinical stage, biopsy Gleason score (GS) and serum prostate-specific antigen (PSA) level, individually or collectively. These factors are helpful for guiding treatment decisions; however, they have limited predictive ability (3). Therefore, there is a critical need to develop novel prognostic predictors to improve clinical strategies for the treatment of PCa.

Extra-prostatic extension (EPE) is defined as the presence of cancer extending beyond the prostate gland, and it has long been considered an unfavourable prognostic factor in terms of cancer progression and survival (4-7). Identifying the presence of EPE is likely to reduce the chance of positive surgical margins; furthermore, it may be helpful for identifying patients who require postoperative adjuvant treatment. Traditionally, it has been difficult to assess patients who are at high risk for EPE based on a single preoperative clinicopathological variable or imaging information due to limited sensitivity (8-12). Hence, it is of practical significance to develop a new approach for predicting EPE.

Transmembrane protease serine 2 (*TMPRSS2*)-ETS-related gene (*ERG*) is the most common gene fusion in PCa; however, its prognostic value remains largely elusive (13-15). Our group has previously reported an initial scoring system for assessing *ERG* rearrangements in biopsy samples, based on the use of fluorescence *in situ* hybridisation (FISH), for the diagnosis of PCa and the risk assessment of lymph node metastasis. This proposed system has demonstrated excellent sensitivity and specificity (16,17). In clinical practice, an increase in *ERG* rearrangements was observed to be associated with more aggressive characteristics. Thus, the aim of the current study was to explore the utility of *ERG* rearrangements and other preoperative parameters for predicting EPE in patients with clinically localised PCa.

Materials and methods

Patients and samples. This study included 409 consecutive patients who underwent RP at the Third Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China) between January 2008 and June 2013. Of these patients, 103 without

Correspondence to: Professor Xin Gao, Department of Urology, Third Affiliated Hospital of Sun Yat-Sen University, 600 Tianhe Road, Guangzhou, Guangdong 510630, P.R. China
E-mail: urologyzssy@126.com

*Contributed equally

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complete information prior to biopsy or with suspected metastasis by bone scan, computed tomography scan or magnetic resonance imaging (MRI) were excluded from the study. Finally, 306 cases with clinically localised PCa were enrolled in this retrospective analysis. The diagnosis of PCa was confirmed via transrectal ultrasound (TRUS)-guided needle biopsy preoperatively (median total biopsy cores, 12; range, 10-16). This study was approved by the Institutional Ethics Committee of the Third Affiliated Hospital of Sun Yat-Sen University, and all patients signed informed consent forms prior to the intervention.

Biopsies and corresponding prostatectomy specimens were retrospectively collected from 306 PCa patients for analysis. The selection of slides for FISH analysis was performed by the pathologist conducting the diagnosis. The *ERG* rearrangement was calibrated with a dual-colour break-apart FISH assay (Beijing GP Medical Technologies, Ltd., Beijing, China) as previously described (16-18).

Pathological analysis. Morphological diagnoses were conducted according to the International Union Against Cancer 2009 staging classification guidelines for PCa (19), and histological analyses were performed according to the Gleason grading system (20). EPE was defined as the presence of any malignant cell beyond the prostatic capsule (≥pT3a) according to the criteria described by Epstein (21), or the presence of pathologically confirmed positive lymph node metastasis.

Assessment of ERG rearrangements via FISH. FISH analysis was conducted according to the manufacturer's protocols (Beijing GP Medical Technologies, Ltd.) with certain modifications. Briefly, 3-mm tissue sections were obtained from tissue blocks and mounted on poly-L-lysine-coated slides (Beijing GP Medical Technologies, Ltd.). Following deparaffinisation, the tissue sections were dehydrated in 100, 85 and 70% ethanol for 2 min each. Subsequent to washing in deionised water for 5 min, the sections were boiled in deionised water at 100˚C for 27 min and then digested with Proteinase K (Beijing GP Medical Technologies, Ltd.) at 37˚C for 10 min. The sections on the slides were then dried, and hybridisation was performed as described previously (16,17). Next, the slides were counterstained and mounted with DAPI, examined under an oil objective at 100x magnification using an Olympus fluorescence microscope (BX51; Olympus Corp., Tokyo, Japan) and imaged with a CCD camera (DP70; Olympus Corp.) using the PathFinder CellScan software system (IMSTAR S.A., Paris, France).

According to our scoring system, two yellow (red/green fusion) signals in a cell indicate a normal signal pattern, whereas the presence of one yellow/one green or one yellow/one green/one red signal in a cell commonly represents an abnormal signal pattern indicative of a partial deletion or translocation, respectively, of *ERG*.

During the evaluation of the FISH results, each slide was reviewed and ≥400 epithelial cells were scored, with the strongest abnormal signals in the 'z' axis. *ERG* rearrangement rate in the patient was calculated using the following formula: ERG rearrangement rate (%) = number of cells exhibiting an abnormal signal pattern/number of cancer cells (16,17). All slices were independently assessed by three experienced researchers (Dr Li Lu, Dr Hao Zhang and Dr Guo-Liang Hou) who were blinded to the clinicopathological parameters, and any discordant results were reassessed until a consensus was achieved (Fig. 1A-F).

Table I. Clinicopathological characteristics of the study sample (n=306).

Characteristic	Value
Age, years; median (range)	69 (43-89)
tPSA level, ng/ml; median (range)	12.25 (2.75-45.79)
PV, ml; median (range)	49 (13-105)
TBC, n; median (range)	12 (10-16)
PBC, n; median (range)	3 (1-8)
Clinical T classification, n (%)	
T1b-c	63 (20.6)
T2a	112 (36.6)
T2b	76 (24.8)
T2c	55 (18.0)
Biopsy GS, n (%)	
≤6	75 (57.2)
7	81 (26.5)
≥8	50 (16.3)
Pathological TNM stage, n (%)	
T2a	56 (18.3)
T2b	40 (13.0)
T2c	132 (43.1)
T3a	39 (12.7)
T3b	30 (9.8)
T4	9 (2.9)
Pathological GS, n (%)	
≤6	163 (53.3)
7	79 (25.8)
≥8	64 (20.9)
Seminal vesicle invasion, n (%)	
Negative	265 (86.8)
Positive	41 (13.4)
Lymph node metastasis, n (%)	
Negative	280 (91.5)
Positive	26 (8.5)
Capsule state, n (%)	
Organ-confined	220 (71.9)
Extra-prostatic extension	86 (28.1)

PV, prostate volume; TBC, total biopsy cores; PBC, positive biopsy cores; T, tumour; GS, Gleason score; TNM, tumour-node-metastasis.

Statistical analysis. Clinicopathological parameters were analysed using the χ^2 test or independent samples *t*-test (paired samples *t*-test for comparison between preoperative and postoperative ERG rearrangement rate, and independent samples *t*-test for comparison between EPE group and localised PCa group). Spearman's rank correlation coefficients were

Table II. Comparison of clinicopathological features between the patients with and without EPE.

Feature	Organ-confined	EPE	P-value
Cases, n (%)	220 (71.9)	86 (28.1)	
Age, years; mean ± SD	68.64±7.668	69.22±8.031	0.558
tPSA level, ng/ml; mean ± SD	14.00±8.590	18.05±11.774	0.001
Prostate volume, ml; mean ± SD	50.49±14.339	51.43±13.938	0.604
Percentage of PBC, %; mean ± SD	21.56±10.462	36.41±10.680	<0.0001
Biopsy Gleason scores, n (%)			<0.0001
≤6	151 (86.3)	24 (13.7)	
7	56 (69.1)	25 (30.9)	
≥8	13 (26.0)	37 (74.0)	
Clinical TNM stage, n (%)			<0.0001
Tb-1c	47 (74.6)	16 (25.4)	
T2a-T2b	160 (85.1)	28 (14.9)	
T2c	13 (23.6)	42 (76.4)	

EPE, extra-prostatic extension; SD, standard deviation; tPSA, total prostate-specific antigen; PBC, positive biopsy cores; TNM, tumour-node-metastasis.

calculated to explore the relationship between *ERG* rearrangement and clinicopathological outcome. Receiver operating characteristic (ROC) analysis and binary logistic regression were used to evaluate the predictive values of *ERG* rearrangement and other variables for EPE. The 10-fold leave-one-out cross-validation (LOOCV) approach was used to validate the predictive performance of *ERG* rearrangement. Statistical analyses were performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA) and MedCalc software version 12.7 (Medcalc Software, Ostend, Belgium). LOOCV analysis was performed in R 2.5.1 (http://cran.r-project.org). All tests were two-tailed, and P<0.05 was considered to indicate statistical significance.

Results

Patient characteristics. The characteristics of the 306 patients are summarised in Table I. Pathological examination indicated that a total of 220 patients (71.9%) had organ-confined disease, and 86 (28.1%) showed evidence of EPE in the prostatectomy specimen (EPE group). No significant differences were observed in the comparison of mean *ERG* rearrangements between biopsies and prostatectomy specimens (P=0.796) (Fig. 2A); however, the differences in *ERG* rearrangements in the biopsy specimens were significant between the EPE group and the group with organ-confined disease (P<0.0001) (Fig. 2B).

Table II summarises the clinicopathological characteristics of the patients in the EPE group and the group with organ-confined disease. There was a significantly higher total PSA (tPSA) level (P=0.001) and a higher percentage of positive biopsy cores (PBCs) in the EPE group (P<0.0001), and significant differences were also identified between the stratified biopsy GSs (≤6, 7 and ≥8) and clinical T classifications (T1b-T1c, T2a-T2b and T2c) (P<0.0001). However, there were no differences with regard to age (P=0.558) or prostate volume (P=0.604).

ERG rearrangement for predicting EPE. A significant positive association was identified between the *ERG* rearrangement rate and pathological T classification [r=0.471; 95% confidence interval (CI), 0.379-0.554; P<0.0001; Fig. 2C].

ROC analysis was used to explore the performance of *ERG* rearrangement rates in the biopsy specimens for assessing the risk of EPE. *ERG* rearrangement was expressed as a continuous variable, and the results revealed that the area under the curve (AUC) was 0.781 (95% CI, 0.730-0.826). An optimal cut-off value of 2.25% was established, with a sensitivity of 70.24% (95% CI, 62.6-78.9%) and a specificity of 80.43% (95% CI, 75.4-85.1%) (Fig. 2D).

To investigate the independent risk factors for the EPE of PCa, *ERG* rearrangement and other preoperative variables, including tPSA, biopsy GS and clinical T classification, were included in a logistic regression analysis, and the AUC of each parameter was compared. The results indicated that *ERG* rearrangement had a better predictive value for EPE compared with tPSA (AUC, 0.599; 95% CI, 0.541-0.654; P<0.0001); a slight, non-significant difference existed compared to clinical T classification (AUC, 0.715; 95% CI, 0.660-0.765; P=0.094), and *ERG* rearrangement had a similar predictive value to biopsy GS (AUC, 0.763; 95% CI, 0.711-0.810; P=0.695) (Fig. 2D).

Multivariate logistic regression models revealed that *ERG* rearrangement in the biopsy sample was an independent predictor of EPE [odds ratio (OR), 1.997; 95% CI, 1.277-3.124; P=0.002]. In addition, biopsy GSs of 7 (OR, 2.669; 95% CI, 1.116-6.383; P=0.027) and ≥8 (OR, 39.032; 95% CI, 10.397-146.527; P<0.0001), and a clinical T classification of T2c (OR, 9.103; 95% CI, 3.338-24.824; P<0.0001) were independent predictors of EPE of clinically localised PCa. However, age, tPSA, prostate volume, number of PBCs, percentage of PBCs, biopsy GSs of ≤6 and clinical T1b-1c and T2a-T2b staging were not independent risk factors for EPE (Table III).

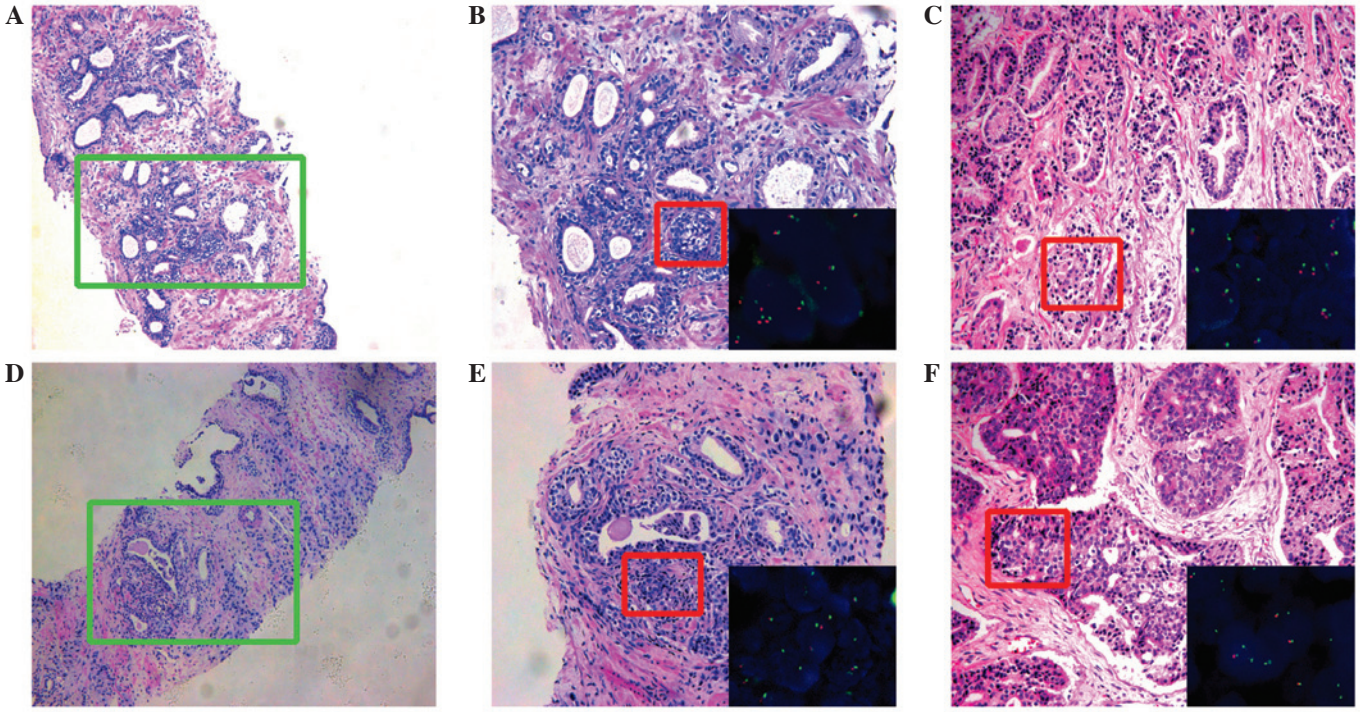

Figure 1. Hematoxylin and eosin staining and the corresponding fluorescence *in situ* hybridization images demonstrating *ERG* rearrangements. (A) Prostatic biopsy tissue with prostate cancer glands (GS 3+4) (magnification, x100). (B) Image of the green boxed area in part A (magnification, x200); inset (lower right) shows *ERG* probe image of the red boxed area in part B (magnification, x1,000). (C) The corresponding prostatectomy tissue from part A with organ-confined disease (GS 3+4) (magnification, x200); inset (lower right) shows *ERG* probe image of the red boxed area in part C (magnification, x1,000). (D) Prostatic biopsy tissue with prostate cancer glands (GS 3+4) (magnification, x100). (E) Image of the green boxed area in part D (magnification, x200); inset (lower right) shows *ERG* probe image of the red boxed area in part E (magnification, x1,000). (F) The corresponding prostatectomy sample shown in part D with extra-prostatic extension, GS 4+4 (magnification, x200); inset (lower right) shows *ERG* probe image of the red boxed area in part F, demonstrating an *ERG* rearrangement (magnification, x1,000). *ERG*, ETS-related gene; GS, Gleason score.

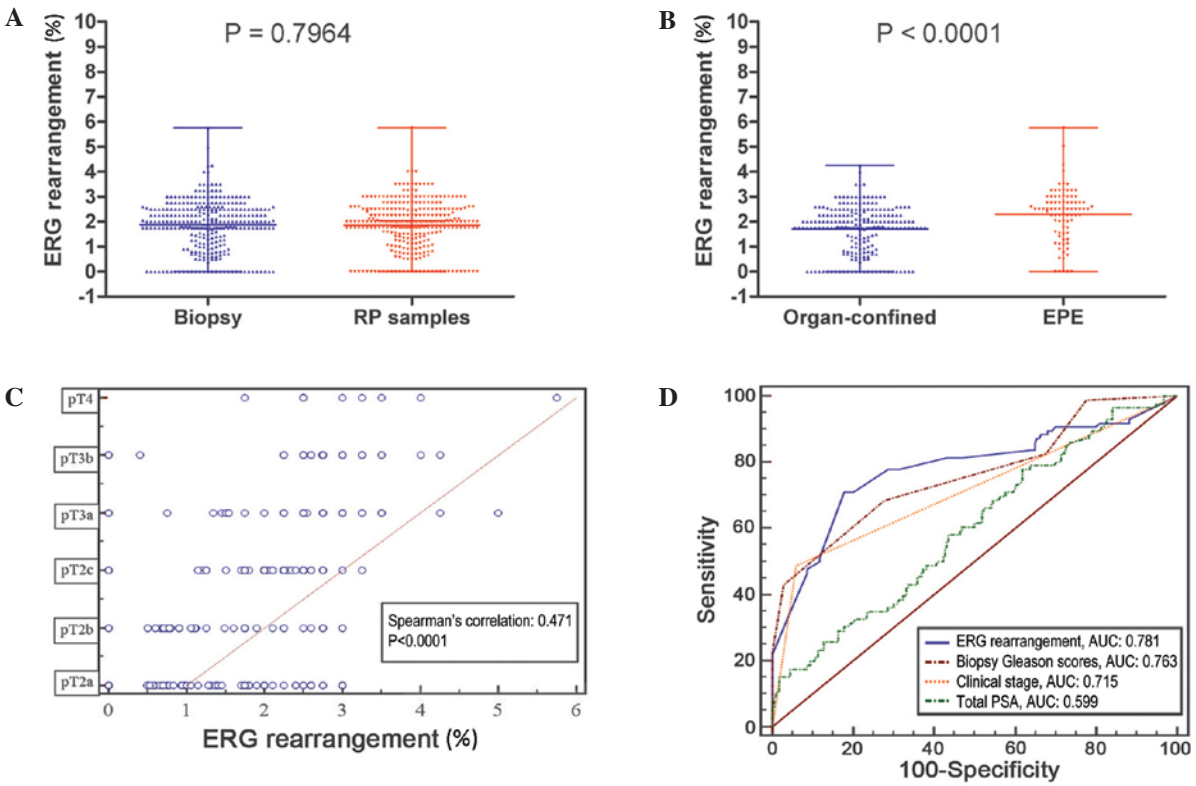

Figure 2. *ERG* rearrangement as a predictor of EPE in prostate cancer. (A) *ERG* rearrangement rates in biopsy and RP samples (P=0.796). (B) Comparison of *ERG* rearrangements in biopsy specimens from the organ-confined group and EPE group (P<0.0001). (C) There was a significant association between the presence of *ERG* rearrangement in the biopsy specimens and pathological stage (r=0.471, P<0.0001). (D) Comparison of AUCs for *ERG* rearrangement and other clinicopathological parameters. *ERG*, ETS-related gene; EPE, extra-prostatic extension; RP, radical prostatectomy; AUC, area under the curve; PSA, prostate-specific antigen.

Table III. Multivariate logistic regression analysis of extra-prostatic extension prediction.

Variable	Odds ratio	95% CI	P-value
Age	0.992	0.945-1.041	0.750
tPSA level	0.971	0.927-1.016	0.203
ERG rearrangement	1.997	1.277-3.124	0.002
Prostate volume	0.997	0.968-1.026	0.966
Number of PBCs	1.956	0.449-8.525	0.372
Percentage of PBCs	70.338	-	0.961
Biopsy Gleason scores			
≤6	-	-	-
7	2.669	1.116-6.383	0.027
≥8	39.032	10.397-146.527	<0.001
Clinical T classification			
T1b-T2b	-	-	-
T2c	9.103	3.338-24.824	<0.001

CI, confidence interval; tPSA, total prostate-specific antigen; *ERG*, ETS-related gene; PBC, positive biopsy core; T, tumour.

Table IV. 10-fold leave-one-out cross validation of *ERG* rearrangement for prediction of extra-prostatic extension.

No.	Sensitivity, %	Specificity, %	AUC	Cut-off (%)
1	54.545	80.000	0.905	2.175
2	62.750	73.684	0.780	2.450
3	64.500	70.000	0.876	2.175
4	63.636	68.421	0.854	2.450
5	70.000	64.286	0.818	2.450
6	76.923	71.429	0.776	2.450
7	77.778	75.000	0.956	2.175
8	90.000	70.000	0.824	2.450
9	90.909	66.667	0.763	2.175
10	92.857	62.500	0.868	2.175
Total	76.923	71.429	95% CI: 0.724-0.958	

ERG, ETS-related gene; AUC, area under the curve; CI, confidence interval.

Validation of the ability of ERG rearrangement to predict EPE. Ten-fold LOOCV was used to validate the power of *ERG* rearrangement for predicting EPE. For all cases that were excluded from the model, a cut-off was assigned using 10-fold cross-validation, and a predictive value was determined for the excluded cases. In this LOOCV model, *ERG* rearrangement performed well for predicting EPE, with a sensitivity of 76.923% and a specificity of 71.429%. The 95% CI for the AUC was 0.724-0.958. Thus, the null hypothesis of ROC <0.5 (random prediction) could not be rejected (Table IV).

Discussion

EPE is defined as the presence of cancer cells outside of the prostatic capsule (22). This terminology was introduced in 1996; subsequently, EPE was confirmed to be an adverse prognostic factor for PCa (22). Several variables, including the Partin table, MRI, TRUS and digital rectal examination findings, and prostate cancer antigen 3 score, have been studied to assess the predictive power of EPE; however, sensitivities have ranged from only 50-70% (8,9,23-25). Thus, there is an urgent need to explore novel and more effective approaches for predicting EPE for determining an appropriate surgical strategy and also for delineating a favourable postoperative adjuvant therapy.

The prostate-specific gene *TMPRSS2* is fused with the transcription factor *ERG* in a large proportion of cases of PCa; however, its biological relationships with the clinicopathological parameters of the disease, such as PSA level, GS, pathological stage and prognosis, are not clear-cut, and the reported results lack consistency (13-15,26). This may be due to differences in study design, detection techniques,

sample origin and the intrinsic mechanism of gene rearrangement (27-29). In our pilot study, no significant association was identified between *ERG* status and tumour stage in a limited cohort of patients (16). However, *ERG* rearrangement was found to be positively correlated with advanced tumour stage in a larger cohort of samples in this study, which is consistent with previous reports (28-31).

Overall, *ERG* rearrangement tends to be positively associated with advanced pathological stage, which has been further verified in a meta-analysis of 34 series (32). For example, Furusato *et al* (33) demonstrated that ERG protein expression is positively correlated with pathological stage, tumour grade and metastatic status in Japanese PCa patients. Paulo *et al* (34) also reported that a higher percentage of patients with locally advanced disease (pT3a) possess *ERG* rearrangements compared to patients with organ-confined disease. Minner *et al* (35) demonstrated that *ERG* fusion is positively associated with pathological stage, and *ERG*-positive patients tended to have higher GSs. The results of the present study revealed that *ERG* rearrangement detection in biopsy specimens was positively correlated with advanced stage and was able to predict EPE, with an optimal cut-off of 2.25% and an AUC of 0.781. In addition, *ERG* rearrangement was an independent factor for EPE. In the LOOCV internal validation model, it was also observed that *ERG* rearrangement was a valuable indicator of EPE, with a 95% CI for the AUC ranging from 0.724 to 0.958. The detection of *ERG* rearrangements may be useful for guiding decisions related to surgical margins, regardless of the statuses of other clinicopathological factors.

In the present study, the clinical stage T2c was determined to be an independent predictor of EPE. The clinical T stage has long been considered an important factor for predicting EPE. For example, the use of a Partin table, including the clinical T stage, tPSA level and biopsy GS, was investigated for the prediction of EPE in 1997 (36). Subsequently, following several updates, other clinical variables were also incorporated into this model, thereby improving its predictive capacity (3,37).

In the current study, it was also observed that biopsy GSs of 7 and ≥8 were positively correlated with EPE, and a high GS was usually associated with advanced-stage PCa. The findings also demonstrated that *ERG* rearrangement had a similar AUC as biopsy GS (0.781 vs. 0.763); furthermore, multivariate logistic regression models revealed that biopsy GSs of 7 and ≥8 were independent factors for EPE, which is consistent with prior publications (12,38-40).

Other preoperative factors investigated in the present study, such as age, tPSA level, prostate volume and percentage of PBCs, were not found to have predictive power for EPE. Although previous studies have reported that these parameters may individually or collectively be used to predict EPE in PCa patients from Western countries (41), considering the discrepancies related to race and study population, it is necessary to further explore the predictive values of these parameters using a larger sample of Chinese patients with clinically localised PCa.

In summary, the current findings demonstrated that approximately 28.1% of patients with clinically localised PCa have EPE, and that *ERG* rearrangements in biopsy samples may be independent factors for predicting ECE, similar to biopsy GSs of ≥7. These results may be useful for determining an appropriate surgical strategy in PCa patients with organ-confined disease. Consequently, the *TMPRSS2-ERG* gene, which is specific to PCa (18), should be studied further with regard to its roles in progression and prognosis, and may be a target for modern personalised therapy for PCa patients.

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