Title Page

ERG rearrangement for predicting subsequent cancer diagnosis in high-grade prostatic intraepithelial neoplasia and lymph node metastasis

Xin Gao†#, Liao-Yuan Li†, Fang-Jian Zhou†, Ke-Ji Xie³, Chun-Kui Shao⁴, Zu-Lan Su⁴, Qi-Peng Sun¹, Ming-Kun Chen¹, Jun Pang¹, Xiang-Fu Zhou¹, Jian-Guang Qiu¹, Xing-Qiao Wen¹, Ming Yang⁵, Xian-Zhong Bai⁶, Hao Zhang¹, Li Ling⁷, and Zhong Chen⁸#

¹Department of Urology, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510630, China
²Department of Urology, Cancer Center, Sun Yat-sen University, Guangzhou, 510060, China
³Department of Urology, Guangzhou First Municipal People’s Hospital, Guangzhou Medical College, Guangzhou, 510180, China
⁴Department of Pathology, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510630, China
⁵Department of Urology, Foshan First Municipal People’s Hospital, Sun Yat-sen University, Foshan, 528000, China
⁶Department of Urology, Cancer Hospital, Guangxi Medical University, Nanning, 530021, China
⁷Department of Medical Statistics and Epidemiology, School of Public Health, Sun Yat-sen University, Guangzhou 510080, China
⁸Division of Medical Genetics, University of Utah School of Medicine, Salt Lake City, UT 84101, USA

† These authors contributed equally to this work.
# To whom correspondence should be addressed:
Xin Gao, Department of Urology, The Third Affiliated Hospital, Sun Yat-sen University, Tianhe Road 600, Guangzhou, 510630, China
Telephone: +86-20-85252660; Fax: +86-20-85252678
Email: billurology@126.com; xingsysu@gmail.com
OR
Zhong Chen, Division of Medical Genetics, University of Utah School of Medicine, Salt Lake City, UT 84101, USA
Telephone: +86-10-67882639; Fax: +86-10-67867396
Email: z.chen2000@yahoo.cn

The running head: ERG rearrangement in prostate biopsies

Abbreviations: AMACR = α-methyl-acyl-CoA; AUC = Area under curves; ERG = v-ets erythroblastosis virus E26 oncogene homolog; FISH=fluorescence in situ hybridization; HGPIN=high-grade prostatic intraepithelial neoplasia; PSA=prostate-specific antigen.

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Translational Relevance

The recently discovered recurrent ERG rearrangement is specific for prostate cancer. Using fluorescence in situ hybridization, our results show that patients with high-grade prostatic intraepithelial neoplasia with an ERG rearrangement rate ≥1.6% on initial prostate biopsy were at a greater risk for subsequent diagnosis of prostate cancer than those with an ERG rearrangement rate <1.6%. Additionally, there were significant associations of ERG rearrangement in preoperative biopsies with pelvic lymph node metastasis. ERG protein expression by immunohistochemistry was highly concordant with ERG rearrangement by fluorescence in situ hybridization. Our findings indicate that measuring ERG rearrangement in routine prostate biopsy samples has a potential translational relevance in determining which patients with high-grade prostatic intraepithelial neoplasia have greater risk for subsequent diagnosis of prostate cancer, and which patients with early prostate cancer have a greater predilection for lymph node metastasis.
Abstract

**Purpose:** We aimed to analyze whether ERG rearrangement in biopsies could be used to assess subsequent cancer diagnosis in high-grade prostatic intraepithelial neoplasia (HGPIN) and the risk of lymph node metastasis in early prostate cancer (PCa).

**Experimental Design:** Samples from 523 patients (361 with early PCa and 162 with HGPIN) were collected prospectively. Based on the cutoff value established previously, the 162 patients with HGPIN were stratified to two groups: one with an ERG rearrangements rate ≥1.6% (n=59) and the other with an ERG rearrangements rate <1.6% (n=103). For the 361 PCa cases undergoing radical prostatectomy, 143 had pelvic lymph node dissection (node-positive, n=56 and node-negative, n=87). All ERG rearrangement fluorescence *in situ* hybridization (FISH) data were validated with ERG immunohistochemistry.

**Results:** 56 (56/59, 94.9%) HGPIN cases with an ERG rearrangements rate ≥1.6% and 5 (5/103, 4.9%) HGPIN cases with an ERG rearrangements rate <1.6% were diagnosed with PCa during repeat biopsy follow-ups (*P*<0.001). There were significant differences in ERG rearrangement rates between lymph node positive and negative PCa (*P*<0.001). The optimal cutoff value to predict lymph node metastasis by ERG rearrangement was established, being 2.6% with a sensitivity at 80.4% (95% CI: 67.6%-89.8%) and a specificity at 85.1% (95% CI: 75.8%-91.8%). ERG protein expression by immunohistochemistry was highly concordant with ERG rearrangement by FISH.

**Conclusions:** The presence of ERG rearrangement in HGPIN lesions detected on initial biopsy warrants repeat biopsies and measuring ERG rearrangement could be used for assessing the risk of lymph node metastasis in early PCa.
Text

Coincident with increased prostate-specific antigen (PSA) testing, there has been a significant increase in both the number of prostate needle biopsy performed and the number of equivocal prostate needle biopsy samples (1, 2). The diagnosis of prostate cancer with needle biopsies is still difficult, not only because the amount of tissue available is small for histological examination, but also only few malignant glands are present among many benign glands, thus increasing the risk of underdiagnosis (3). For instance, the possibility of finding cancer in subsequent biopsies after a diagnosis of high-grade prostatic intraepithelial neoplasia (HGPIN) is 10-39% depending on the time of repeat biopsy and the number of cores (4, 5). In addition, the natural history and aggressiveness of prostate cancer vary widely. A fraction of cases detected by prostate biopsies would have occult lymph node metastases, which lead to cancer-related death, while others remain indolent even if left untreated (6, 7). Thus, identification of robust biomarkers in prostate biopsy samples in parallel with other existing parameters for reliable cancer diagnosis and prediction of lymph node metastasis is urgently needed.

Recently, ERG (v-ets erythroblastosis virus E26 oncogene homolog) rearrangement primarily involving the TMPRSS2-ERG fusion has been identified in prostate cancer (8-12). ERG rearrangement is highly specific for prostate cancer, as most of them being found in prostate cancer and a subset of HGPIN glands that are intermingled with fusion-positive cancer glands (11, 13). Recently, based on a limited sample size, Perner et al. (14) suggested that localized prostate cancer harboring the ERG
rearrangement can result in metastatic spread to regional lymph nodes. However, multicenter data to evaluate the relationship of ERG rearrangement in biopsies with clinical course of prostate cancer are still lacking. In a previous study, we designed a dual-color multi-probe fluorescence in situ hybridization (FISH) method and established the cutoff value for diagnosis of prostate cancer (15). The criteria for FISH positivity were based on the numbers of cells with abnormal signal patterns for ERG rearrangements. For determining the optimal operation point of ERG rearrangements, we counted at least 400 cells to obtain respective proportions of cells with abnormal signal patterns in 85 surgical specimen cases (50 with prostate cancer, 20 with benign prostatic hyperplasia, and 15 with normal prostate). Then, a receiver operator characteristics curve, which compared the sensitivity and specificity of various cutoffs for numbers of cells with abnormal signal patterns, was used to determine the optimal cutoff (an ERG rearrangements rate at ≥1.6%) that should be used to interpret a positive FISH result. We now present a multicenter study to analyze two hypotheses: first, that HGPIN patients with an ERG rearrangements rate ≥1.6% on initial prostate biopsy are at greater risk for subsequent diagnosis of prostate cancer than those with an ERG rearrangements rate <1.6% and, second, that there are significant differences in ERG rearrangement status on initial prostate biopsy between lymph node positive and negative prostate cancer.

Materials and Methods

Study population
From May 2008 through May 2010, samples from prostate biopsies were collected prospectively from men who were admitted for prostate biopsies based on elevated PSA levels, or abnormal digital rectal exam or clinical suspicion of prostate cancer. This study protocol was reviewed and approved by our institutional review boards. All men had received study information, and they had signed their informed consent.

In this study, a total of 523 patients (162 with HGPIN and 361 with early prostate cancer (T1-2) undergoing radical prostatectomy) were included in this analysis (Figure 1). The biopsy paraffin blocks were available for analysis and all corresponding H&E-stained and immunostaining slides were reviewed. The following criteria were used to define prostate cancer patients in whom pelvic lymph node dissection was performed because of a high likelihood of nodal disease: PSA ≥ 20 ng/mL, biopsy Gleason score ≥ 7, or clinical suspicion of lymph node metastases. We performed standard template pelvic lymph node dissection, encompassing all nodal tissue from the medial inferior margin of the external iliac vein down to the internal iliac and obturator vessels (16). For all patients included in this study, follow-up was done quarterly to semiannually for the first 2 years and annually thereafter by clinical evaluation, measurement of serum PSA and other investigations as indicated. The median follow-up was 18 months (range 3-36) for all patients.

Pathologic analysis and immunostaining

All patients underwent 12-core transrectal ultrasound guided biopsies, and 12 paraffin blocks per patient were prepared. The morphologic diagnosis was confirmed on H&E
slides by two independent pathologists (Z.-L.S. and C.-K.S.) who were blinded to the results of \textit{ERG} rearrangement. Gleason score and morphologic features of each case were assessed independent of \textit{ERG} rearrangement evaluation. If necessary, immunostaining was performed using an avidin-biotin complex staining procedure as previously reported (15). In brief, a cocktail of the three antibodies, including a rabbit monoclonal antibody to $\alpha$-methyl-acyl-CoA (AMACR) (P504S, Corixa, Seattle, WA), a mouse monoclonal antibody to high-molecular-weight cytokeratin (34$\beta$E12, DAKO), and a mouse monoclonal antibody to p63 (NeoMarkers, Fremont, CA), was mixed and applied.

All the 162 patients with HGPIN underwent a repeat biopsy 6 months after the initial biopsy regardless of PSA level. In addition, a repeat biopsy was also performed in the 78 HGPIN patients with persistence of increased PSA at any time point during follow-up. The repeat biopsy was done with a 12-core template scheme like the first biopsy.

\textbf{Assessment of \textit{ERG} rearrangement in biopsies via FISH}

The biopsy paraffin blocks were available for analysis and all corresponding H&E-stained and immunostaining slides were reviewed. A representative slide from each patient with suspicious for carcinoma, was selected for evaluation of \textit{ERG} rearrangement status by FISH. The selection of the core for FISH analysis was made by the pathologist performing the diagnosis and the core with the highest proportion of HGPIN or cancer cells was elected. We have previously described a dual-color
break-apart-rearrangement model designed for an ERG probe to detect rearrangements between the ERG gene and a partner gene, such as TMPRSS2, as well as deletion of the gene. Probe labeling and FISH analysis were performed according to the manufacturer’s protocols (GP Medical Technologies, Ltd., Beijing, China) with some modifications (15). Briefly, 3μm tissue sections were obtained from the tissue blocks and mounted on poly-L-lysine coated slides. After the deparaffinage with xylene (15min×2), digestion with proteinase K (7 min), denaturation (100°C, 26 min), gradient rinse with alcohol, and hybridization as described in our previous study (15), the section slides were counterstained and mounted by DAPI, and examined under oil objective original magnification (×100) using an Olympus fluorescence microscope (Olympus Co., Tokyo, Japan) and imaged with a CCD camera using the IMSTAR software system (IMSTAR S.A., Paris, France). With this system, two yellow (red/green fusion) signals in a cell indicated a normal signal pattern, whereas one yellow/one green or one yellow/one green/one red in a cell commonly represented abnormal signal patterns indicative of partial deletion or translocation, respectively.

In each representative slide, firstly, under 10× microscope we searched for the glands of each tissue with a “z” pathway, then under a 100× oil immersion objective using an Olympus BX-51 fluorescence microscope, we screened cells with abnormal signal patterns; subsequently, we scored at least 400 epithelial cells around the area containing the richest abnormal signals. After this systemic examination of representative tissue, the proportion of cells with abnormal signal patterns was scored and represented the final ERG rearrangement status of this patient.
Evaluation of FISH results for each sample was independently performed by two experienced observers (Q.-P.S. and M.-K.C.) who were blinded to the pathologic diagnoses, and any discrepant scores were re-examined to achieve a consensus result. Of 361 specimens diagnosed as prostate cancer by pathologic review, ERG rearrangement status was considered was falsely negative (ERG rearrangement rate <1.6%) in 69 cases. A receiver operator characteristics curve, which compared the sensitivity and specificity of various cutoffs for numbers of cells with abnormal signal patterns for lymph node positive and negative groups of patients, was then used to determine the cutoff value that was applied to interpret a positive result for lymph node metastasis.

**Evaluation of ERG Protein Expression via immunohistochemistry**

ERG protein expression was detected by immunohistochemistry according to the methods described elsewhere (17, 18). In brief, following deparaffinization, 5μm sections were dehydrated and blocked in 1% hydrogen peroxide in methanol for 20 min. Sections were processed for antigen retrieval in EDTA (pH 9.0) for 30 min in a microwave followed by 30 min of cooling in EDTA buffer. Slides were incubated with 1:100 of rabbit monoclonal ERG antibody (clone EPR3864; Epitomics, Burlingame, CA, USA) overnight at 4°C, followed by chromogenic visualization using the EnVision system (DAKO, Glostrup, Denmark). Sections were then counterstained in hematoxylin for 1 min, dehydrated, cleared and mounted. Evaluation of ERG protein expression was scored using a four-tier grading system:
negative (0; no staining), weak (1+; only visible at high magnification), moderate (2+; visible at low magnification) and strong (3+; striking at low magnification). Nuclear reactivity of the antibody in endothelial cells was used as internal control (18, 19). In addition, ERG protein expression for all cases was also assessed by using the automated Ariol imaging system (Genetix Corp, San Jose, CA). Briefly, the investigator set the color and shape characteristics to properly identify cells with positive staining. The software applied the color classifiers to identify regions of positive nuclear staining, excluding objects that were either too light or too dark. The objective ERG protein expression level with Ariol system was defined as the ratio of “ERG nuclear area” to “analyzed tissue area.” (17).

**Statistical analysis**

Clinicopathological data were compared among patients with different *ERG* rearrangement status in biopsy using Student’s t test on the equality of means or the chi-square test or Wilcoxon rank sum tests. Spearman correlations were calculated to explore the relation between continuous variables. Sensitivity and specificity were determined via receiver operating characteristics analysis. Area under curves (AUC) were compared via the method of DeLong et al. (20) SPSS software package 16.0 (SPSS, Chicago, IL, USA) was used for the analyses and a two-tailed *P* value of less than 0.05 was considered statistically significant.
Results

**ERG rearrangement status correlating with subsequent diagnosis of cancer in HGPIN**

Based on the cutoff value established previously by us (15), the patients with HGPIN were stratified into two groups: one with an ERG rearrangements rate $\geq 1.6\%$ and the other with an ERG rearrangements rate $<1.6\%$. The characteristics of these two groups and representative findings are shown in Table 1 and Figure 2. The patients with an ERG rearrangements rate $\geq 1.6\%$ underwent repeat biopsies with a significantly higher PSA (median 14.3 versus 11.6 ng/ml, $P=0.037$). The median time of the persistence of increased PSA after initial biopsy between the two groups was 5 (3-13) versus 15 (12-36) months, respectively ($P<0.001$). Altogether, 61 cases (37.7%) with HGPIN were diagnosed with prostate cancer during repeat biopsy follow-ups; among them, a greater number of patients (56/59, 94.9%) with an ERG rearrangements rate $\geq 1.6\%$ on initial biopsy were diagnosed with prostate cancer during repeat biopsy follow-ups as compared to those (5/103, 4.9%) with an ERG rearrangements rate $<1.6\%$ ($P<0.001$). In a multivariable analysis, ERG rearrangement rate ($<1.6\%$ or $\geq 1.6\%$) is an independent risk factor (HR: 2.45, 95%CI: 2.26-2.96, $P<0.001$) outside of PSA velocity for predicting prostate cancer on re-biopsy in HGPIN patients. All those ERG rearranged cancers detected on repeat biopsies were in the same zone distribution, which matched the ERG rearranged foci on the initial biopsies.
**ERG rearrangement for predicting lymph node metastasis**

For the 361 early prostate cancer cases undergoing radical prostatectomy, 143 had pelvic lymph node dissection with 56 being positive and 87 negative on postoperative pathological examination. In order to evaluate the significance of FISH in assessing the risk of lymph node metastasis in early prostate cancer, only data from the 143 patients who had pelvic lymph node dissection were included for analysis (Table 2).

The pathological characteristics and corresponding FISH images of ERG rearrangement status in prostatic biopsy tissue and lymph node metastasis tissue of a representative case are shown in Figure 3. There were significant differences in ERG rearrangement status between the lymph node positive and negative prostate cancer ($P<0.001$) (Figure 3D). We found that ERG rearrangement is an independent predictor (HR: 1.79, 95%CI: 1.42-2.13, $P=0.02$) of lymph node metastasis outside of age, biopsy Gleason score, clinical T stage, preoperative PSA level and PSA velocity in a multivariate analysis model. In addition, we demonstrated a significant positive association regarding the ERG rearrangement rate in the primary prostate cancer (radical prostatectomy specimens) with the lymph node metastasis ($r=0.55$, $P<0.05$, data not shown).

Receiver operating characteristics analysis was used to directly compare the performance of ERG rearrangement status alone and Kattan nomogram variables for assessing the risk of lymph node metastasis. ERG rearrangement status alone as a continuous variable demonstrated an AUC of 0.822 (95% CI 0.77–0.94) compared to 0.633 (95% CI 0.61–0.77) for Kattan nomogram variables (Figure 3C). The difference in AUC between ERG rearrangement alone and Kattan nomogram
variables reached statistical significance \((P=0.004)\). Therefore, the optimal cutoff value to assess the risk of lymph node metastasis by \(ERG\) rearrangement was then established, being 2.6\% with a sensitivity at 80.4\% (95\% CI: 67.6\%-89.8\%) and a specificity at 85.1\% (95\% CI: 75.8\%-91.8\%). In the 218 patients who underwent radical prostatectomy and did not have pelvic lymph node dissection, 23 (10.6\%) have elevated \(ERG\) rearrangement rates (≥2.6\%).

**ERG protein expression is highly concordant with \(ERG\) rearrangement**

ERG protein expression was assessed by the pathologists and by using the automated Ariol imaging system. We found a significant positive correlation between the \(ERG\) rearrangement rate by FISH and the ERG protein expression in the initial biopsies from all these 523 cases (Spearman correlation = 0.83, \(P<0.001\)). The ERG protein expression was strongly concentrated in the nuclei (Figure 4 and 5). Using arbitrary expression units, ERG protein expression was highly concordant with \(ERG\) rearrangement status in the HGPIN cases (median = 0.045 in the \(ERG\) rearrangement rate <1.6% group vs 0.108 in the \(ERG\) rearrangement rate ≥1.6% group, \(P=0.013\)) using the Wilcoxon rank-sum test (Figure 4C). There were significant differences in ERG protein expression between the lymph node positive and negative prostate cancer (median = 0.149 in the positive group vs 0.116 in the negative group, \(P<0.001\)) (Figure 5C). A significant association in the interpretation of ERG protein expression between manual and automated image analyses has been well established (17). Using the manual four-tier grading system, ERG protein expression was identified in 54/162
(33.3%) HGPIN lesions and 291/361 (80.6%) adenocarcinomas on needle biopsies, which were also consistent with our FISH results based on the 1.6% cutoff value.

Discussion

Because diverse genomic fusion events may lead to ERG overexpression, it could be more practical to capture such alterations by using assays targeting ERG sequences that are retained in all gene fusions involving ERG (11). Many laboratories prefer break-apart probes because the results are easier to interpret and the abnormalities sought are readily recognized. By using break-apart probes normal signals may occasionally appear to be separated slightly and mimic the signal pattern as observed in abnormal cells, which may result in the possibility of a false positive result. Thus, the slightly separated "normal" signal pattern has to be carefully defined by evaluating the distance between two signals in relation to the signal diameter (21). However, in general this phenomenon can be well managed by relatively experienced technologists with the establishment of optimal cutoff values. In addition, abnormal signal patterns are commonly observed rich in some special corners with abnormal nuclei scattered around. Therefore, more nuclei are scored, and more accurate reflection of the real ERG fusion status can be achieved. According to our experience, there were about 400 to 800 epithelial cells in a biopsy specimen suitable for FISH analysis. Different from the methods described by some other authors (50 or 100 cells were counted on a slide) (9, 11), we thoroughly scored at least 400 cells around the area richest in nuclei with abnormal signals on each representative slide, which could
minimize false positive/negative results to the most degree. Additionally, one of the most critical factors affecting a proper interpretation of FISH analysis in diagnostic samples is the establishment of optimal cutoff values for all signal patterns that might appear with a given assay. In our scoring system, the \textit{ERG} fusion status was scored by using the proportion of nuclei with abnormal signals among the whole eligible cells and \textit{ERG} rearrangement status of enrolled patients was quantitatively evaluated as a continuous variable. Subsequently, a receiver operating characteristic curve was used to establish the optimal cutoff value for diagnosis of prostate cancer or predicting pelvic lymph node metastasis. In order to decrease the possibility of false-positive diagnoses to the greatest degree, we maximized specificity over the set of cutpoints so that sensitivity does not fall below this minimum. Therefore, in our previous study, the optimal operation point was established as the proportion of cells with abnormal signal patterns greater than 1.6\% in a count of at least 400 cells for scoring positive \textit{ERG} rearrangements; this cutoff value was also employed in the present study. Noteworthily, the cutoff value at 1.6\% even appeared low, but it was convincing because break-apart probes normally generate very low background due to the design nature of these probes as well as a larger amount of cells and samples used for the establishment of cutoff values (22). In addition, based on the 143 patients undergoing pelvic lymph node dissection (56 positive and 87 negative), the optimal cutoff value by \textit{ERG} rearrangement for assessing the risk of lymph node metastasis was established in the present study, being 2.6\% with a high detection sensitivity and specificity. Thus, our quantitative scoring system made it possible for predicting
lymph node metastasis by evaluating $ERG$ rearrangement status in daily clinical practice. This approach indicated that detection of $ERG$ rearrangement could be a technically reliable adjunctive tool for pathologic analysis of prostate cancer.

While HGPIN is pathogenically associated with prostate cancer, there is a greater disparity in the incidence of prostate cancer after diagnosis of HGPIN, ranging from uninterpretable 2% to 100% (23-25). Although $ERG$ rearrangement is highly specific for prostate cancer, it is also found in a subset of HGPIN glands that are intermingled with fusion-positive cancer glands (11, 13). Some recent studies have evaluated the prevalence of $ERG$ rearrangement in HGPIN lesions (13, 26). They demonstrated that about 20% of HGPIN lesions, which were in close proximity to $ERG$ rearranged invasive prostate cancer tissue, were also positive for the same $ERG$ rearrangement as observed in the tumor tissue. However, the fact that almost all HGPIN biopsies do not contain any invasive prostate cancer focus appears to be a dilemma in diagnosis. Our results demonstrated that HGPIN patients with an $ERG$ rearrangements rate $\geq 1.6\%$ on initial prostate biopsy were at a greater risk for subsequent diagnosis of prostate cancer than those with an $ERG$ rearrangements rate $<1.6\%$. The majority of cancer diagnoses in our cohort with HGPIN were made on the first repeat biopsy with a median interval of 5 months. Our findings emphasized the importance of repeat biopsies within 3 to 6 months post HGPIN diagnosis with an $ERG$ rearrangements rate $\geq 1.6\%$ on initial biopsy. In contrast, for HGPIN diagnosis with an $ERG$ rearrangements rate $<1.6\%$ and serum PSA levels remaining steady, there was no need for repeat biopsies for at least 12 months. Based on the fact that an $ERG$
rearranged HGPIN lesion proves the existence of an *ERG* rearranged prostate cancer focus within the prostate, the presence of *ERG* rearrangement in HGPIN lesions detected on initial biopsy warrants repeat biopsies. In our study, it is of note that all those *ERG* rearranged cancers detected on repeat biopsies were in the same zone distribution, which matched the *ERG* rearranged foci on the initial biopsies; the presence of *ERG* rearranged HGPIN was shown to be indicative of a prostate cancer bearing the same genetic aberration. These results were consistent with previous FISH analyses demonstrating that *ERG*-rearranged HGPIN is nearly always found in close association with *ERG*-rearranged cancer (9, 19, 27, 28). Thus, *ERG* rearranged isolated HGPIN on biopsies would be highly suspicious for unsampled adjacent cancer or for the rapid progression to invasive disease (9, 27, 28). Importantly, these findings could be translated into contemporary clinical practice. For example, in order to attain a high tumor detection rate and a minimal number of biopsy core specimens per patient, it may be feasible to perform repeat *ERG* rearranged HGPIN lesion-directed biopsies in future.

Among adverse pathologic features, the presence of pelvic lymph node metastasis is the strongest predictor of poor outcome (29). A sensitive and reliable means of detecting lymph node metastases in men with prostate cancer is important because patients with a localized disease have the options of treatment including radical prostatectomy, watchful waiting, and radiotherapy (30). However, conventional staging methods such as histopathological procedures and imaging techniques are of limited value in assessing lymph node metastases, because these methods often fail to
detect early low-volume occult cancer metastases (31). Given that the ability of clinical approaches is limited, the focus has shifted to molecular markers (32). Although there has not been a consistent correlation between clinical outcome and ERG rearrangement status observed after treating men with clinically localized prostate cancer with radical prostatectomy (12, 33), some studies following the natural history of prostate cancer in the watchful waiting cohort have demonstrated a significant association of ERG rearrangement with cancer-specific death (34, 35). Recently, Perner et al. (14) suggested that localized prostate cancer harboring the ERG rearrangement could result in metastatic spread to regional lymph nodes in a small sample size. Additionally, Paris et al. (36) also reported a similar finding in prostate cancer where matched primaries and lymph node metastases showed similar copy number profiles that were distinct from primary tumors that failed to metastasize. These investigations demonstrated that the lymph node metastasis and at least one primary prostate cancer focus were characterized by the same ERG rearrangement (14, 37), and suggested that a seeding prostate cancer focus which causes metastasis may not be necessarily associated with the largest tumor volume or the highest Gleason grade, rather the presence of an ERG rearrangement. In the present study, we established an optimal cutoff value at 2.6% by ERG rearrangement to assess the risk of lymph node metastasis in patients with early prostate cancer with high sensitivity and specificity. Our results suggest that pelvic lymph node dissection strategies may be guided by the ERG rearrangement status in preoperative prostate biopsies. It is of note that previous studies have reported associations of ERG rearrangement with both...
more and less aggressive clinical courses (33, 35, 38-40). Evidence from both prostatectomy series (37, 40) and watchful waiting cohorts (35) demonstrated that ERG rearrangement through deletion, particularly in the presence of gain of ERG, might be associated with more aggressive cancer. In addition to the watchful waiting study as reported by Attard et al. (35), in a prostatectomy series Perner et al. also suggested that ERG rearrangement through deletion was significantly associated with a high tumor stage and the presence of pelvic lymph node metastases (37). The mechanism behind the selective metastatic potential associated with ERG rearrangements needs to be further investigated; however, several reports have demonstrated a critical role of ERG overexpression in cell migration and invasion (41). Obviously, prospective trials correlating biopsy, natural history, and outcome following prostatectomy would be needed to further investigate associations between ERG rearrangement status and clinical outcome.

The present study also demonstrated that recurrent ERG gene rearrangements lead to overexpression of the ERG protein. We observed highly consistent results for ERG rearrangements by FISH and for ERG expression by immunohistochemistry. When ERG protein was expressed in tumor cells, most of the tumor nuclei were positive consistent with earlier observations by FISH (13, 17). In our series, a range of ERG expression levels was observed in these clinically localized prostate cancer cases, which were highly concordant with previous FISH results (15). Notably, HGPIN with positive ERG staining was always located adjacent to cancer with similar levels of ERG protein expression, which was consistent with previous FISH results.
demonstrating that ERG-rearranged HGPIN is almost always found in close association with ERG-rearranged cancer (9, 19, 27, 28). Moreover, there were significant differences in ERG protein expression between the lymph node positive and negative prostate cancer patients in our study, which further underscores the utility of ERG rearrangement status in the setting of lymph node metastatic prostate cancer.

When interpreting the results of our study it is important to consider the main characteristics of our study population. In China, screening for prostate cancer using digital rectal examination and PSA is not routinely performed in practice. Most newly diagnosed prostate cancer patients are symptomatic and high risk (42). In our opinion, the lack of several rounds of screening during the last 15 to 20 years might determine the different characteristics of our population. According to the median PSA value, our population could be considered a subscreening population, and our results may be important for those treating similar patient populations.

In summary, our findings demonstrate the significance of ERG rearrangement in subsequent cancer diagnosis among HGPIN patients and in assessing the risk of lymph node metastasis in early prostate cancer. We correlated ERG protein expression well with the presence of ERG rearrangements in prostate biopsies using a combined immunohistochemistry and FISH analysis. Given the most recent advances in understanding ERG rearrangement in prostate cancer, our work may provide new information in the clinical management of this disease.
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References

is specific to prostate cancer and does not occur in any other common tumor. Mod Pathol. 2010;23:1061-7.


## Tables

### Table 1. Characteristics of the 162 HGPIN patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ERG rearrangements rate</th>
<th>ERG rearrangements rate &lt;1.6%</th>
<th>P value</th>
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<tbody>
<tr>
<td>Total</td>
<td>59</td>
<td>103</td>
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<tr>
<td>Median Age (yr, range)</td>
<td>69 (54-83)</td>
<td>68 (51-87)</td>
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<td>PSA Velocity (Mean±SD,ng/ml/year)</td>
<td>1.04±0.19</td>
<td>0.22±0.07</td>
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<tr>
<td>Median PSA (ng/ml)</td>
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<td></td>
<td></td>
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<tr>
<td>Initial biopsy</td>
<td>9.7 (1.8-18.1)</td>
<td>9.8 (0.6-29.1)</td>
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<tr>
<td>Repeat biopsy</td>
<td>14.3 (3.5-31.2)</td>
<td>11.6 (5.6-23.2)</td>
<td>0.037</td>
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<tr>
<td>Median months to PSA elevation</td>
<td>5 (3-13)</td>
<td>15 (12-36)</td>
<td>&lt;0.001</td>
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<td>No. PCa detected on follow-up</td>
<td>56</td>
<td>5</td>
<td>&lt;0.001</td>
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<td>At 6-month re-biopsy</td>
<td>15 (26.8)</td>
<td>4 (80.0)</td>
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<td>At re-biopsy for elevated PSA biopsy</td>
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</tr>
</tbody>
</table>

Abbreviations: HGPIN, high-grade prostatic intraepithelial neoplasia; PSA, prostate-specific antigen; SD, standard deviation; PCa, prostate cancer.
Table 2. Characteristics of the 143 prostate cancer patients with lymph node dissection

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>LN-Pos</th>
<th>LN-Neg</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>56</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Median age (yr, range)</td>
<td>67(49-82)</td>
<td>72(51-85)</td>
<td>0.37</td>
</tr>
<tr>
<td>Median PSA (ng/ml, range)</td>
<td>13.7 (0.1-37.4)</td>
<td>13.5(1.8-42.0)</td>
<td>0.12</td>
</tr>
<tr>
<td>Biopsy Gleason score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>12(21.4)</td>
<td>18(20.7)</td>
<td>0.73</td>
</tr>
<tr>
<td>7</td>
<td>9(16.1)</td>
<td>19(21.8)</td>
<td></td>
</tr>
<tr>
<td>8-10</td>
<td>35(62.5)</td>
<td>50(57.5)</td>
<td></td>
</tr>
<tr>
<td>Clinical T stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1c</td>
<td>4(7.2)</td>
<td>8(9.2)</td>
<td>0.21</td>
</tr>
<tr>
<td>T2a</td>
<td>7(12.5)</td>
<td>10(11.5)</td>
<td></td>
</tr>
<tr>
<td>T2b</td>
<td>6(10.7)</td>
<td>9(10.3)</td>
<td></td>
</tr>
<tr>
<td>T2c</td>
<td>39(69.6)</td>
<td>60(69.0)</td>
<td></td>
</tr>
<tr>
<td>Pathological T stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT2a</td>
<td>1(1.8)</td>
<td>4(4.6)</td>
<td>0.42</td>
</tr>
<tr>
<td>pT2b</td>
<td>3(5.4)</td>
<td>11(12.6)</td>
<td></td>
</tr>
<tr>
<td>pT2c</td>
<td>16(28.6)</td>
<td>29(33.3)</td>
<td></td>
</tr>
<tr>
<td>pT3a</td>
<td>20 (35.7)</td>
<td>21(24.2)</td>
<td></td>
</tr>
<tr>
<td>pT3b</td>
<td>10(17.8)</td>
<td>15(17.2)</td>
<td></td>
</tr>
<tr>
<td>pT4</td>
<td>6(10.7)</td>
<td>7(8.1)</td>
<td></td>
</tr>
<tr>
<td>Post-op Gleason score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5(8.9)</td>
<td>17(19.5)</td>
<td>0.036</td>
</tr>
<tr>
<td>7</td>
<td>12(21.4)</td>
<td>28(32.2)</td>
<td></td>
</tr>
<tr>
<td>8-10</td>
<td>39(69.7)</td>
<td>42(48.3)</td>
<td></td>
</tr>
<tr>
<td>Surgical margin status</td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>Positive</td>
<td>24 (42.9)</td>
<td>32 (36.8)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>32 (57.1)</td>
<td>55 (63.2)</td>
<td></td>
</tr>
<tr>
<td>Median No. of removed nodes (range)</td>
<td>15.7(6-41)</td>
<td>14.9(10-36)</td>
<td>0.17</td>
</tr>
<tr>
<td>Median No. of positive nodes (range)</td>
<td>3.4(1-12)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: PSA, prostate-specific antigen; LN-Pos, lymph node positive; LN-Neg, lymph node negative.
Figure legends

**Figure 1.** Samples from 523 patients (361 with early prostate cancer and 162 with high-grade prostatic intraepithelial neoplasia) were collected prospectively in the analysis. *ERG* = v-ets erythroblastosis virus E26 oncogene homolog; RP = radical prostatectomy.

**Figure 2.** H&E stains and corresponding FISH images of *ERG* rearrangement in prostatic biopsy tissues with HGPIN. **A:** A representative prostatic biopsy tissue with HGPIN. Right upper *ERG* probe image demonstrates one representative nucleus of a prostate cancer gland with one yellow and one green signals, showing an *ERG* rearrangement. **B:** Prostate cancer was confirmed by repeat prostatic biopsy during follow-up (Gleason 3+4). Right upper *ERG* probe image demonstrates the presence of an *ERG* rearrangement. **C:** Another representative prostatic biopsy tissue with HGPIN. Right upper *ERG* probe image demonstrates one representative nucleus of a normal gland with two yellow signals. **D:** Repeat prostatic biopsy tissue with HGPIN. Right upper *ERG* probe image demonstrates a normal gland with two yellow signals. Original magnification of H&E images, ×20 objective. Original magnification of FISH images, oil objective (×100). *ERG* = v-ets erythroblastosis virus E26 oncogene homolog; FISH = fluorescence *in situ* hybridization; HGPIN = high-grade prostatic intraepithelial neoplasia.

**Figure 3.** *ERG* rearrangement in prostate cancer and corresponding lymph node metastasis. **A:** Prostatic biopsy tissue with prostate cancer glands (Gleason 3+4). Right upper *ERG* probe image demonstrates two representative nuclei of a prostate cancer gland. Inset, a nucleus with one yellow and one green signals, showing an *ERG* rearrangement; a nucleus with one yellow, one green and one red signals, showing another type of *ERG* rearrangement. **B:** Postoperative lymph node metastasis tissue of the same case in (A). Left lower *ERG* probe image demonstrates the presence of an *ERG* rearrangement. **A, B:** Original magnification of H&E images,
×20 objective. Original magnification of FISH images, oil objective (×100). C: Receiver operating characteristics analysis comparing univariate ERG rearrangement (continuous) (blue curve) with Kattan nomogram variables (red curve) (P =0.004) in predicting lymph-node metastasis. D: There were significant differences in ERG rearrangement status between the lymph node positive and negative prostate cancer (P <0.001). ERG = v-ets erythroblastosis virus E26 oncogene homolog; FISH = fluorescence in situ hybridization.

Figure 4. ERG rearrangement by break-apart FISH is highly correlated with ERG protein expression by immunohistochemistry in HGPIN. ERG protein expression in biopsies of HGPIN with an ERG rearrangements rate <1.6% (A) and the other with an ERG rearrangements rate ≥1.6% (B). A, B: Original magnification of immunohistochemistry images, ×20 objective. Original magnification of FISH images, oil objective (×100). C: The box plot demonstrates a highly significant association between the automated image evaluation of ERG protein expression and the ERG rearrangement status for the 162 HGPIN cases (P =0.013, Wilcoxon rank-sum test). D: Distribution of ERG rearrangement rates in the 162 HGPIN patients. ERG = v-ets erythroblastosis virus E26 oncogene homolog; FISH = fluorescence in situ hybridization; HGPIN=high-grade prostatic intraepithelial neoplasia.

Figure 5. ERG rearrangement by break-apart FISH is highly correlated with ERG protein expression by immunohistochemistry in biopsies of prostate cancer. ERG protein expression in prostate cancer with negative lymph node (A) and positive lymph node (B). The expression was strongly concentrated in the nuclei. A, B: Original magnification of immunohistochemistry images, ×20 objective. Original magnification of FISH images, oil objective (×100). C: The box plot demonstrates significant differences in ERG protein expression between the lymph node positive and negative prostate cancer (P<0.001, Wilcoxon rank-sum test). ERG = v-ets erythroblastosis virus E26 oncogene homolog; FISH = fluorescence in situ hybridization.
Clinical Cancer Research

ERG rearrangement for predicting subsequent cancer diagnosis in high-grade prostatic intraepithelial neoplasia and lymph node metastasis


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