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Immunohistochemistry for ERG Expression as a Surrogate for TMPRSS2-ERG Fusion Detection in Prostatic Adenocarcinomas

Alcides Chaux, MD¹, Roula Albadine, MD¹, Antoun Toubaji, MD¹, Jessica Hicks, BS¹, Alan Meeker, PhD^{1,2}, Elizabeth A. Platz, ScD, MPH^{2,3}, Angelo M. De Marzo, MD, PhD^{1,2,3}, and George J. Netto, MD^{1,2,3}

¹Department of Pathology, Johns Hopkins Medical Institutions, Baltimore MD

²Department of Urology, Johns Hopkins Medical Institutions, Baltimore MD

³Department of Oncology, Johns Hopkins Medical Institutions, Baltimore MD

Abstract

BACKGROUND—TMPRSS2-ERG fusions have been identified in about one-half of all prostatic adenocarcinomas (PCa). Fluorescence in situ hybridization (FISH) and reverse transcription polymerase chain reaction (RT-PCR) have been the most commonly used techniques in this setting. The aim of the present study is to evaluate the utility of ERG immunoexpression as a surrogate for TMPRSS2-ERG fusion in a large series of PCa cases.

MATERIAL & METHODS—427 radical retropubic prostatectomy tissue samples were used to construct 10 tissue microarrays (TMA). FISH analysis was previously performed using dual-color interphase break-apart probes for the 5' and 3' regions of the ERG gene. ERG expression was evaluated using a commercial rabbit anti-ERG monoclonal antibody (clone EPR3864; Epitomics, Burlingame CA). Each TMA spot was independently assessed and any nuclear staining positivity was considered as indicative of ERG expression.

RESULTS—TMPRSS2-ERG fusions were detected by FISH in 195 (45.7%) of the PCa cases. ERG immunoexpression was found in 192 (45.0%) of the PCa cases and in none of the nontumoral tissue samples. Mean ERG H-scores were significantly higher in tumors harboring FISH-detected TMPRSS2-ERG fusions ($P < 0.00001$) and there was a strong association between ERG immunohistochemical expression and TMPRSS2-ERG status defined by FISH ($P < 0.00001$), with a sensitivity of 86% (95% CI 80–90%) and a specificity of 89% (95% CI 84–93%). Receiver-operating characteristic (ROC) curve analysis showed that ERG immunoexpression had a high accuracy for identifying TMPRSS2-ERG fusions detected by FISH, with an area under the curve (AUC) of 0.87 (95% CI 0.84–0.91, $P < 0.00001$).

CONCLUSIONS—We found that ERG immunohistochemical expression has a high accuracy for defining TMPRSS2-ERG fusion status. ERG immunohistochemistry may offer an accurate, simpler and less costly alternative for evaluation of ERG fusion status in PCa than FISH.

Keywords

prostate adenocarcinoma; TMPRSS2-ERG fusion; ERG expression; fluorescence in situ hybridization; Edel; Esplit

INTRODUCTION

Prostate cancer is the most common malignancy in males with a 2010 estimate of 217,300 new cases and 32,050 deaths in the United States of America.¹⁸ Claims have been made that current serum prostate-specific antigen (PSA)- based screening strategies might lead to the overtreatment of a subset of patients with prostatic adenocarcinoma (PCa),³⁸ promoting the search for clinicopathologic and molecular profiles that may be useful for identifying patients with biologically significant tumors. In 2005 Pretrovics et al, identified ERG, one of the members of the ETS family of transcription factors, as a frequently overexpressed proto-oncogene in prostatic carcinomas.³³ Shortly after, Tomlins et al discovered that the mechanism for ERG overexpression entailed a recurring gene fusion involving the androgen-responsive gene TMPRSS2 and in the ERG gene.⁴⁰ Briefly, they found that TMPRSS2 was fused either with ERG or ETV1 (both members of the ETS family) following inter or intrachromosomal rearrangements, and this led to an overexpression of the ETS-related gene as a consequence of androgen-dependant stimulation of the resulting fusion gene. Thereupon, several studies confirmed that TMPRSS2-ETS fusions are detected in 15–70% of all PCa cases.⁶ Recent studies have found that around 50% of PSA-screened PCa patients show evidence of ERG fusion.^{20,26,39} Among all the members of the ETS family, ERG is the most frequently reported gene that is fused with TMPRSS2, present in more than 90% of all TMPRSS2-ETS gene fusion events.^{6,23} ERG and TMPRSS2 are both located on chromosome 21q and separated by a 3 mega-base (Mb) segment containing a number of other genes; the most common mechanism of TMPRSS2-ERG fusion is deletion of the intervening DNA segment, detected in about two-thirds of all fusion-positive cases, but translocations are also identified.^{29,42,45} In addition, other genes of the ETS family (including ETV1 on chromosome 7p, ETV4 on chromosome 17q, and ETV5 on chromosome 3q) have been identified as well in fusion transcripts; and ETS-related genes can also have other fusion partners besides TMPRSS2, such as SLC45A3, HERV-K17, CI5orf21, CANT-2, and HNRPA2B1, among others.^{6,23,29}

ERG (ETS-related gene-1) is a member of the ETS family of transcription factors involved in several key cellular events such as proliferation, differentiation, stem cell development, angiogenesis, hematopoiesis, migration, oncogenic transformation, and apoptosis.^{6,23} Some studies have found an association between TMPRSS2-ERG fusions in prostate cancer and biochemical recurrence,^{28,31,43,46} metastases,^{8,25} or cancer-related death,^{2,8} suggesting that PCa in which these rearrangements are identified behave more aggressively. In addition, the presence of two or more copies of the TMPRSS2-ERG fusion product has been linked to higher pathological stage, early biochemical recurrence, and poor survival.^{2,14,46} However, other studies reported favorable or no significant prognostic association between TMPRSS2-ERG fusion and outcome^{11,12,14,21,36,42,44,45} or high-risk morphological features.^{10,14} These inconsistent results may be related to differences in study designs, selections of endpoints, population heterogeneity, or technical discrepancies for the gene fusion detection methods. Despite these differences, it is clear that the exquisite specificity for ERG genomic changes for prostate cancer or prostatic intraepithelial neoplasia (PIN) lesions associated with prostate cancer^{6,20,23,27,29,39} indicate that the molecular detection of ETSs family member rearrangements could become useful as tissue-based or bodily fluid-based biomarkers in this disease.

Detection of TMPRSS2-ERG fusions is most commonly carried out using either fluorescence in situ hybridization (FISH) or reverse transcription polymerase chain reaction (RT-PCR), but these methods are costly and require considerable infrastructure and expertise. Until recently, however, ERG protein was not evaluated in prostate cancer as a result of a lack of suitable reagents. Recently, using a commercial rabbit monoclonal antibody in 207 cases of PCa, Park et al found a strong association between ERG protein

expression and ERG gene rearrangement status defined by FISH.³⁰ Classifier performances and receiver-operating characteristic (ROC) curve analyses pointed to a high sensitivity, specificity, and accuracy for ERG immunohistochemistry in defining TMPRSS2-ERG fusion status in this series. In addition, using a different mouse monoclonal antibody, Furusato et al found evidence of ERG expression in 45% of 132 PCa cases studied using whole-mounted sections;¹³ they also analyzed 10 specimens from their series by FISH and found no discrepancies between ERG expression and TMPRSS2-ERG fusion status. Both of these studies show excellent concordance between the ability to detect ERG protein in nuclei of prostate tumor cells and the presence of genomic alterations of ERG as detected by FISH or RT-PCR, suggesting that tissue localization of ERG protein could be highly useful clinically for a number of applications including improving diagnostic accuracy for prostate cancer on needle biopsies. The aim of the present study was to assess ERG protein staining as a surrogate for FISH, by attempting to validate and extend these studies using a larger well-defined cohort of prostate cancer patients that have recently been characterized by FISH for TMPRSS2-ERG rearrangements.⁴¹

MATERIAL AND METHOD

Tissue Microarray (TMA) Construction

The currently analyzed cases are part of a nested case-control study on recurrence that included 4,860 men who underwent radical retropubic prostatectomy (RRP) for clinically localized PCa at The Johns Hopkins Medical Institutions (Baltimore, MD) between 1993 and 2004 and who had not had hormonal or radiation therapy prior to RRP or as adjuvant therapy prior to recurrence. Five-hundred and twenty-four men from this cohort experienced biochemical recurrence (serum PSA >0.2 ng/mL), metastasis, or died of PCa after surgery. For each one of these cases, incidence density sampling was used to select a control who had not experienced recurrence by the date of the case's recurrence and who was matched on age, race, pathological stage, and Gleason sum. A set of 16 TMAs was constructed for the 524 matched cases and controls. Matched pairs were placed on the same TMA, so that a subset of the TMAs could be used depending on sample size calculations. Paired PCa and non-cancer tissues were spotted (0.6 mm) in triplicate from each RRP specimen as previously described by Kononen et al.¹⁹ In RRP specimens with multifocal tumors, only the dominant tumor (with the highest Gleason score and usually largest) was sampled. The first ten of the 16 available TMAs, corresponding to 427 RRP, were used for the current study based on power calculations for the prior FISH study.⁴¹ Consecutive sections of the same TMA were used for FISH analysis and for ERG immunohistochemistry.

Evaluation of TMPRSS2-ERG fusion status by FISH

FISH analysis was performed previously for these samples⁴¹ using dual-color interphase break-apart probes for the 5' and 3' regions of ERG gene, as previously described.^{1,22} Briefly, 4 µm paraffin embedded TMA sections were baked at 56° C for 2 hours then deparaffinized and rehydrated using xylene and graded ethanol, respectively. TMA sections were pretreated using Paraffin Pretreatment Reagent Kit III (Abbott Molecular Inc, IL). BAC FISH probes used were SpectrumGreen d-UTP direct-labeled BAC RP11-95I21 for 5' *ERG*, and SpectrumOrange d-UTP direct labeled BAC RP11-476D17 for 3' *ERG* (Nick transKit, Vysis, Abbott Park, IL). TMAs and BAC FISH probes were co-denatured at 94° C for 5 min and hybridized overnight at 37° C in a humid chamber (StatSpin ThermoBrite, IRIS Inc, MA). FISH interpretation was performed by three urologic pathologists (AT, RA and GJN). Digitally scanned adjacent Hematoxylin and Eosin sections were available for side by side comparison with the FISH image to localize tumor cells. Paired benign prostatic epithelium was also evaluated as a negative control. Nuclei in each TMA spot were classified in one of the following categories: 1) negative for TMPRSS2-ERG fusion: nucleus

with two pairs of juxtaposed red (R) and green (G) signals forming yellow (Y) signals indicating absence of ERG fusion (2Y); 2) ERG signal split (Esplit): nucleus with one juxtaposed red-green signal pair of the non-rearranged ERG allele and additional separate single red and single green signal of rearranged ERG allele (break-apart) reflecting a TMPRSS2-ERG fusion through translocation (1Y1R1G); 3) ERG signal deletion (Edel): nucleus with one juxtaposed red-green signal pair for the non-rearranged allele and a single red signal of a rearranged allele indicating deletion of the 5' ERG probe region (1Y1R). 4) Duplicated ERG signal split (2Esplit): nucleus with one juxtaposed red-green signal pair of the non-rearranged ERG allele and 2 additional separate red and green signals of rearranged ERG allele (1Y2R2G); 5) Duplicated ERG signal deletion (2Edel): nucleus with one juxtaposed red-green signal pair for the non-rearranged allele and 2 red signals of the ERG rearranged allele (1Y2R). 6) Other types of fusion: including Esplit with gene copy number gain (CNG) (2Y1R1G), Edel with CNG (2Y1R) and Esplit/Edel with gene copy number loss (1R1G/1R). In any given TMA spot, a TMPRSS2-ERG fusion was considered to be present when a minimum of 10% of the counted nuclei contained a split or a minimum of 20% of the nuclei contained a deletion. Each case was classified in one or more categories taking into account the type of fusion identified by FISH at each TMA spot.

Evaluation of ERG expression by IHC

ERG expression was evaluated using a commercial rabbit anti-ERG monoclonal antibody (clone EPR3864; Epitomics, Burlingame CA); this novel antibody has been recently validated.³⁰ The protocol for immunohistochemistry was as follows: deparaffinization and hydration of TMA slides; blocking with pre-antibody solution (10 min); applying anti-ERG primary antibody (1:75 for 45 min at room temp); applying Poly-HRP anti-rabbit IgG (30 min); applying DAB (20 min, Sigma Fast DAB tablets, Sigma-Aldrich, St. Louis MO); counter staining with Mayer's hematoxylin (1:5 for 1 min, Dako, Carpinteria, CA); and dehydration, clearing, mounting, and covering.

Each TMA spot was independently assessed by two pathologists (AC and GJN) using an H-score system obtained by multiplying the intensity of the stain (0: no staining; 1: weak staining; 2: moderate staining; 3: intense staining) by the percentage (0–100) of the cell showing that staining intensity (H-score range 0–300). Only nuclear staining in epithelial prostatic cells was evaluated, either in tumoral or benign tissues; because ERG expression is normally observed in lymphocytes and endothelium,^{13,30} these cells were used as internal positive controls for the ERG staining, but were not included in the evaluation of ERG fusion status. Any nuclear staining positivity (H-score >0) was considered as indicative of ERG expression.

Statistical analyses

H-scores were compared between cases with and without evidence of TMPRSS2-ERG fusion using the Mann-Whitney U test. H-scores were compared among the TMPRSS2-ERG fusions categories using the Kruskal-Wallis test, with the Dunn's multiple test for post-hoc pairwise comparisons. The association between the presence of TMPRSS2-ERG fusion and ERG expression was evaluated using Fisher's exact test. Using the presence of any TMPRSS2-ERG fusion detected by FISH as the gold standard, sensitivity and specificity, with their respective 95% confidence intervals (95% CI), were calculated for ERG immunoexpression. ROC curves were generated as follows: using (x) 1-specificity vs. (y) sensitivity charts, H-scores were plotted using the presence of any fusion event as the state variable. The area under the curve (AUC), with its respective 95% CI, was interpreted following the criteria established by Collinson.⁷ In all cases a 2-tailed P-value <0.05 was required for statistical significance. Data were analyzed using PASW Statistics version 18.0 (IBM Corporation, Somers NY).

RESULTS

TMPRSS2-ERG fusions were detected by FISH in 195 (45.7%) of the PCa cases. The most common mechanism was Edel (158 cases), followed by Esplit (108 cases), 2Edel (24 cases) and 2Esplit (6 cases); only 4 cases with fusions not involving any of the aforementioned mechanisms were found. TMPRSS2-ERG fusions were not detected in any of the nontumoral glands, lymphocytes or endothelial cells. ERG immunoexpression was found in 192 (45.0%) of the PCa cases and in none of the nontumoral tissue samples; lymphocytes and endothelial cells were consistently ERG positive. Thus, when comparing benign tissues from matched patients with carcinoma lesions, the specificity of ERG-positive staining for cancer was 100%. Patterns of ERG immunoexpression are depicted in Figure 1. In 64.6% of all positive cases 90% or more of the tumor cells stained positively and in 81% of these positive cases at least 50% of the cells were positive. Focal (5% or less) positivity was observed in only 8.6% of the positive cases. Mean H-scores were significantly higher in tumors harboring any type of TMPRSS2-ERG fusions when compared to tumors with no identifiable chromosomal rearrangements (Table 1); in ERG positive cases the H-score range from 5 to 290 (mean 143.1±69, median 164, interquartile range 103). Although a tendency for higher H-score means in 2Esplit and 2Edel cases was observed, no differences were found among the H-scores regarding the mechanism of fusion, either as a group ($P=0.27$) or by comparing each mechanism against the others ($P>0.05$). The association between ERG immunoexpression and the presence of TMPRSS2-ERG fusion as defined by FISH was statistically significant (Table 2). Regarding classifier performances, immunohistochemistry for ERG expression had a sensitivity of 86% (95% CI 80–90%) and a specificity of 89% (95% CI 84–93%) in detecting any TMPRSS2-ERG fusions. ROC curve analysis showed that ERG expression H-scores measured by immunohistochemistry had a high accuracy for identify TMPRSS2-ERG fusions detected by FISH, with an area under the ROC curve (AUC) of 0.87 (95% CI 0.84–0.91, $P<0.00001$, Fig. 2).

DISCUSSION

In this study we used a large series of cases to evaluate the ERG immunoexpression rate in PCa. We confirmed a strong association between the immunohistochemical expression of ERG in epithelial cells with prostate cancer and the presence of *TMPRSS2-ERG* fusions detected by FISH. These results support the contention that standard immunohistochemistry can validly be used for defining *TMPRSS2-ERG* status in PCa. As mentioned above, two recently published studies have explored the ERG immunoexpression status in PCa patients;^{13,30} our series confirms and expands those previous results. Some of the strengths of our study include the relatively large number of carefully selected tissue samples, the quantitative and qualitative analysis carried out taking into account the various mechanisms of TMPRSS2-ERG fusion and their association with the levels of ERG immunoexpression, and the detailed analysis of the performance of ERG immunohistochemistry as a surrogate for FISH-detected TMPRSS2-ERG fusions. In our study, ERG immunoexpression was not observed in any of the paired tissue samples containing benign glands or in the benign glands found next to malignant glands, when present.³⁰ Nevertheless, Furusato et al found some rare ERG-positive benign glands,¹³ in agreement with previous studies reporting the presence of TMPRSS2-ERG fusion transcripts in isolated benign prostatic glands;^{3,5} because these foci were found in topographical relationship to areas of PCa and/or PIN this unexpected finding might be explained, as suggested in the paper, by the presence of premalignant molecular alterations in those morphologically benign glands.

In a recently published study by Scheble et al³⁷ more than 50 different tumor types in nearly 3,000 tissue samples were evaluated for TMPRSS2-ERG fusion by FISH; the authors did not find ERG fusions in any of the analyzed tumors, suggesting that TMPRSS2-ERG

rearrangements, especially those involving deletion of the DNA segment between both genes, are PCa-specific genomic alterations. These results, along with the ones found in the present and previous studies,^{13,30} suggest that ERG immunoreexpression could be used as a tissue-lineage specific marker for prostatic tumors. Several studies have detected matched TMPRSS2-ERG status between PCa tumor cells and high-grade PIN present in the same specimen^{4,13,16,27,30,32} indicating that ERG fusions appear early in the development of the disease and can be utilized for differentiating preneoplastic lesions from benign prostatic glands or even predict the likelihood of finding cancer in subsequent biopsies.²⁷ In the study by Furusato et al¹³ almost all PIN and none of the prostatic glands with adenosis (atypical adenomatous hyperplasia), sclerosing adenosis, basal cell hyperplasia, or partial atrophy were positive for ERG, which is an indication of the potential utility that this immunostain might have for the differential diagnosis of atypical prostatic glands. Nonetheless, additional studies addressing the potential role of detecting ERG fusion, by immunostains or other techniques, are needed before such application can be considered for standard clinical use.

Our study found that immunohistochemistry for ERG overexpression had high sensitivity and specificity for detecting TMPRSS2-ERG fusions. Nevertheless, 11–14% of cases were “misclassified”, either false positives or false negatives. ERG immunohistochemical expression in the context of no detectable TMPRSS2-ERG fusions could be explained by the presence of fusion of ERG with other genes (such as SLC45A3, NDRG1, and FKBPS), as has been recently described.^{9,15,30,34,35} In our series, CNG were found in only 1 of the 25 cases with ERG expression and no TMPRSS2-ERG fusions, with no identifiable chromosomal rearrangements in the remainder 24 tumors. On the other hand, we observed a tendency for higher H-score means in cases with 2Esplitt and 2Edel cases, suggesting some CNG effect on the level of ERG expression; although more studies are required to properly address this issue, the lack of significance of this tendency could be related to the small number of cases in the 2Esplitt and 2Edel groups. In terms of false negatives, gene rearrangements coding for transcript products that cannot be detected by anti-ERG clone EPR3864 could explain TMPRSS2-ERG FISH-positive cases without ERG overexpression, although this eventually is unlikely because this antibody presumably recognizes an epitope that is retained in all known ERG gene fusion isoforms.³⁰ Another plausible explanation for this event is decrease transcript/protein expression in tumors with TMPRSS2-ERG fusions due to alterations in androgen response signaling or post transcriptional mechanisms.^{17,30} A third explanation of false negative cases could be related to topographical variations in the levels of ERG immunoreexpression within the same tumor, with areas showing intense positivity while others being completely negative, a scenario that is not uncommonly found, especially when using whole-mounted sections;¹³ probably, these topographical variations in ERG expression are related to the previously reported *ERG* rearrangement heterogeneity found in discrete tumor nodules within the same prostate gland.^{3,24} Finally, in both previously mentioned scenarios (ERG overexpression with no TMPRSS2-ERG fusions and TMPRSS2-ERG fusions without ERG overexpression) misclassification due to technical factors, such as poor tissue preservation, could not be ruled out with certainty. Nevertheless, there are several advantages in using immunohistochemistry for defining ERG status: it is less expensive, less technically challenging, and less time-consuming when compared to FISH; it can detect the majority of all ERG fusions, whether or not ERG is fused to TMPRSS2 or other genes such as SLC45A3, NDRG1, or FKBPS,^{9,15,30,34,35} and the criterion for ERG overexpression is straightforward (any positive tumor cell staining, e.g. H-score >0). Considering that the use of TMAs in the present study simulates the amount of tissue available for examination using needle biopsies, our results should be extendable to routine practice.

In summary, we found a strong association between ERG immunohistochemical overexpression and TMPRSS2-ERG fusion detected by FISH; our results suggest that

standard immunohistochemistry has high accuracy for defining ERG fusion status in men with prostate cancer. Taking this into account, immunohistochemistry for ERG immunoexpression may offer an accurate, simpler and less costly alternative for evaluation of ERG fusion status in PCa.

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Figure 1. Patterns of ERG immunoexpression in TMA spots (low-power at left and medium-power at right)

A–B) PCa case with Esplit showing diffuse nuclear positivity in tumor cells. C–D) PCa case with Edel exhibiting moderate to strong nuclear positivity in neoplastic cells. E–F) PCa case with no fusions detected in which neoplastic cells are negative while endothelial cells and lymphocytes are positive.

Figure 2. Receiver-operating characteristic (ROC) curve for ERG immunohistochemistry
The area under the ROC curve (AUC) is 0.87. The curve is plotted to include any fusion event detected by FISH.

TABLE 1

H-score means of ERG immunoexpression according to TMPRSS2-ERG fusion status by fluorescence in situ hybridization (FISH)

FISH	ERG H-SCORE		P-value (against 2Y)
	Median	Interquartile Range	
No chromosomal rearrangement (2Y)	0	0	NA
Esplit (1Y1R1G)	121	141	<0.0001
Edel (1Y1R)	149	125	<0.0001
2Esplit (1Y2R2G)	180	99	<0.0001
2Edel (1Y2R)	161.5	95	<0.0001
Any Fusion *	147	139	<0.0001

* Including Esplit (1Y1R1G), Edel (1Y1R), 2Esplit (1Y2R2G), 2Edel (1Y2R), Esplit with copy number gain (CNG) (2Y1R1G) and Edel with CNG (2Y1R). NA: not applicable.

TABLE 2

Immunohistochemical expression of ERG* according to TMPRSS2-ERG fusion status by fluorescence in situ hybridization (FISH)

FISH	No. cases	No ERG expression (%)	ERG expression (%)	P-value
Esplit				
Present	108	17 (15.7)	91 (84.3)	<0.00001
Absent	319	218 (68.3)	101 (31.7)	
Edel				
Present	158	21 (13.3)	137 (86.7)	<0.00001
Absent	269	214 (79.6)	55 (2.4)	
2Esplit				
Present	6	0 (0)	6 (100)	0.008
Absent	421	235 (55.8)	186 (44.2)	
2Edel				
Present	24	1 (4.2)	23 (95.8)	<0.00001
Absent	403	234 (58.1)	169 (41.9)	
Any Fusion**				
Present	195	28 (14.4)	167 (85.6)	<0.00001
Absent	232	207 (89.2)	25 (10.8)	

* Cutoff point of H-score > 0;

** Including Esplit (1Y1R1G), Edel (1Y1R), 2Esplit (1Y2R2G), 2Edel (1Y2R), Esplit with copy number gain (CNG) (2Y1R1G) and Edel with CNG (2Y1R).