Quantitative Analysis of ERG Expression and Its Splice Isoforms in Formalin-Fixed, Paraffin-Embedded Prostate Cancer Samples

Association With Seminal Vesicle Invasion and Biochemical Recurrence

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ABSTRACT

Objectives: The proto-oncogene ETS-related gene (ERG) is consistently overexpressed in prostate cancer. Alternatively spliced isoforms of ERG have variable biological activities; inclusion of exon 11 (72 base pairs [bp]) is associated with aggressiveness and progression of disease. Exon 10 (81 bp) has also been shown to be alternatively spliced. Within this study, we assess whether total ERG protein, messenger RNA (mRNA), and ERG splice isoform mRNA expression is altered as prostate cancer progresses.

Methods: Detection of the TMPRSS2-ERG fusion was done using direct methods (reverse transcription polymerase chain reaction [PCR] and fluorescence in situ hybridization) and indirect methods for ERG mRNA and protein expression using quantitative PCR and immunohistochemistry, respectively. A linear equation method was used to quantitatively determine relative proportions of ERG variants (ERG72/Δ72, ERG81/Δ81) for each sample.

Results: ERG mRNA and protein expression is increased in patients with advanced prostate cancer, with a trend for upregulation in advanced vs localized disease, with higher levels of ERG expression significantly associated with seminal vesicle invasion (stage pT3b) and biochemical recurrence. Genes involved in cell migration and invasiveness (matrix metalloproteinase 7, osteopontin, and septin 9) are increased in prostate cancers that overexpress ERG. In addition, there is a clear indication of increased retention of exons 10 and 11 in prostate cancer.

Conclusions: We propose that analysis of ERG and the relative proportions of ERG variants may be valuable in determining prognosis and development of prostate cancer.

The transcription factor ERG (ETS-related gene) is overexpressed in 60% to 80% of prostate cancer cases and is often attributed to a fusion between the promoter of the TMPRSS2 gene and the coding region of ERG. In benign prostate, ERG expression levels are low and not regulated by androgens; however, in prostate cancer, ERG levels are significantly higher, especially if fused to the TMPRSS2 promoter, which is under the control of androgens. Expression analysis of prostate cancers reveals a wide array of genes potentially regulated by ERG to include genes involved in cell proliferation such as septin 9 (SEPT9) and metastatic pathways such as matrix
metalloproteinases (eg, MMP 3/7/9), osteopontin (OPN), and E-cadherin.\textsuperscript{3,6,7}

However, the clinical importance of \textit{ERG} overexpression and the presence of the \textit{TMPRSS2-ERG} fusion in prostate cancer is still unclear since there are reports of a positive, negative, and zero correlation with development and aggressiveness of prostate cancer.\textsuperscript{8-10}

Added complexity of fusion transcripts arises due to alternative splicing. Wild-type \textit{ERG} consists of 17 exons and expresses multiple-splice isoforms.\textsuperscript{11,12} A significant finding has been that \textit{TMPRSS2-ERG} and wild-type \textit{ERG} variants exhibit differing biological properties.\textsuperscript{12,13} A common alternative splicing event within the central activation exon (CAE) domain of \textit{ERG} is inclusion/skipping of a 72-base pair (bp) exon (herein referred to as exon 11). Recent studies in vitro suggest that the inclusion of the 72-bp exon (exon 11) results in increased cell proliferation and a more oncogenic phenotype.\textsuperscript{12} Exon 10 (81 bp) can also be alternatively spliced.\textsuperscript{12}

We hypothesize that the relative proportions of \textit{ERG} and its variants alter as prostate cancer progresses. If so, the analysis of \textit{ERG} expression and its splice variants in routine clinical samples of prostate cancer rather than just the genomic fusion, as detected by fluorescence in situ hybridization (FISH), may be of value in determining the prognosis.

**Materials and Methods**

**Tissue Samples**

This study used tissue samples from 53 patients diagnosed between 2000 and 2009 with clinically localized hormone-naive adenocarcinoma of the prostate. The study was approved by the UK National Health Service National Regional Ethics Service. Cases were selected based on the availability of cases for review and large enough tumor foci for sampling, excluding any clinically insignificant cases. Whole prostatectomy samples were fixed in neutral buffered saline for 24 hours before processing to paraffin wax and embedding in Tissue-Tek Mega-Cassettes (Sakura Finetek, Leiden, the Netherlands). For the study, areas of benign and invasive prostate carcinoma were identified, cored, and reembedded in regular-sized cassettes (Tissue-Tek). To validate the use of reverse transcription–polymerase chain reaction (RT-PCR) to detect the fusion, we randomly selected 20 of the 53 cases for both FISH analysis and RT-PCR. Subsequently, RT-PCR and quantitative PCR (qPCR) for \textit{ERG} variants were performed on 53 cases.

**FISH Analysis**

Tricolor FISH on paraffin-embedded prostate tumor tissue was performed using a break-apart assay designed to detect the microdeletion that occurs between \textit{TMPRSS2} and \textit{ERG} at 21q22 (Kreatech, Leica Microsystems, Milton Keynes, England), as previously described.\textsuperscript{14} Slides were counterstained using 4',6-diamidino-2-phenylindole (DAPI), imaged at \texttimes100 (Olympus BX41 microscope Olympus, Southend-on-Sea, England), and analyzed using ISIS software (Metasystems, Altussheim, Germany).

**Immunohistochemistry**

Each of the 40 patient samples was stained using two antibodies to \textit{ERG} (EP111 [Dako, Ely, England] and EPR3864 [Epitomics, Burlingame, CA]), in addition to double staining with both \textit{ERG} EP111 and high-molecular-weight cytokeratin (34BE12; Dako). All immunohistochemistry, including antigen retrieval, was performed on a BondMax instrument (Leica Microsystems) by using Bond Epitope Retrieval Solution. Nuclear \textit{ERG} staining was visualized using the Bond Polymer Refine Detection system, with a diaminobenzidine chromogen. Cytoplasmic staining for high-molecular-weight cytokeratin staining was visualized using the Bond Polymer Refine Red Detection. Immunohistochemical expression of \textit{ERG} was assessed as described previously, using a four-tier grading system in which the intensity of nuclear staining was recorded as negative, weakly positive, moderately positive, or strongly positive.\textsuperscript{15} The specificity of the \textit{ERG} antibodies was verified by Western blot analysis. In addition, we compared the staining pattern for both the EP111 clone and the previously validated EPR3864 clone,\textsuperscript{15} both giving identical results in all cases. The EP111 clone was used for comparative analysis with the other variables due to reduced background. Cases with invasive prostatic adenocarcinoma showing strong nuclear positivity by both clones served as positive controls. In addition, occasional endothelial cells and lymphocytes stained positively for \textit{ERG} and served as internal controls. Omission of the primary antibodies served as negative controls.

**RNA Isolation**

For isolation of RNA from FFPE samples, the RNeasy FFPE kit (Qiagen, Hilden, Germany) was used as specified...
in the manufacturer’s instructions with the following modifications: three 5-µm sections were deparaffinized in Histoclear (Thermo Fisher Scientific, Loughborough, England) for 5 minutes at 56°C, followed by centrifugation and washing in ethanol. Samples underwent protease K digestion at 56°C overnight. RNA yields were determined by A260 measurement using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using 500 ng of RNA and M-MLV Reverse Transcriptase (Promega, Southampton, England), as per the manufacturer’s specifications.

**RT-PCR Analysis**

Primers to detect TMPRSS2-ERG fusion were as designed by Tomlinset al.4: **TMPRSS2-ERG** forward 5′-TAGGCCGAGCTAAGCAGGAG-3′ and **TMPRSS2-ERG** reverse 5′-GTAGGCACACTCAAAACAGACTGG-3′. PCR was carried out using GoTaq Hot Start Polymerase (Promega) per the manufacturer’s recommendations. **Quantitative Real-Time PCR**

Quantitative real-time PCR was performed using 2× SYBR Green master mix (Roche Diagnostics, Burgess Hill, England) and primers at a 300-nmol concentration on a TaqMan7300 Sequence Detection System (Thermo Fisher Scientific). Primers were designed to span at least one exon boundary using the Primer Express 2.0 software (Applied Biosystems) and were purchased from Sigma-Genosys (Haverhill, England) using standard qPCR cycling conditions. **Table 2.** Fold changes in expression were calculated by using a standard curve method.16 Data were normalized to the corresponding β-actin value for each sample.

**Quantitative Analysis of ERG Variants Using LEM-PCR**

To assess the relative proportion of ERG splice isoforms, we used the linear equation method–PCR (LEM-PCR), as described previously.17 In brief, messenger RNA (mRNA) expression was quantified using SYBR Green **ERG** primers (Table 2) and two linear equations (benign and cancer) generated. These equations were solved and the contributions of **ERG72/81** and **ERG72/81** to the total value of **ERG** calculated. Values were then reexpressed as percentages.

**Statistics**

Data from experiments are presented as mean ± standard error mean (SEM), with numbers of replicates stated in Figure legends. Statistical significance between variables was tested using the paired two-tailed Student t test, a Kruskal-Wallis test, and the Pearson χ² test. Biochemical recurrence after radical prostatectomy was defined as a prostate-specific antigen (PSA) value greater than or equal to 0.2 ng/mL, with a second confirmatory level of PSA of more than 0.2 ng/mL.18

**Results**

**ERG mRNA Expression Is Increased in TMPRSS2-ERG Fusion-Positive Samples**

We analyzed **TMPRSS2-ERG** gene fusion in cancer samples using both RT-PCR and FISH in an initial 20 cases selected at random to determine concordance between the techniques. **Image 1**, **Image 2**, and **Figure**. The RT-PCR reaction detected one, two, or no **TMPRSS2-ERG** fusion variants (Image 1). RT-PCR and FISH results were highly concordant (18 of 20), with RT-PCR giving an additional two cases as **TMPRSS2-ERG** fusion event. **ERG** mRNA expression determined using real-time PCR was significantly increased in **TMPRSS2-ERG** fusion-positive cancer samples compared with fusion-negative samples (P < .01) (Figure 1).

**Analysis of ERG mRNA and Protein Expression in Prostate Cancer Cases**

**ERG** protein expression by immunohistochemistry...
correlated with ERG mRNA expression. Image 3 and Figure 2. ERG staining was exclusively nuclear and homogeneous in expression and specific to invasive tumor nuclei and the nuclei of prostatic intraepithelial neoplasia. The cells of adjacent normal prostate glands remained unstained (Image 3). On average, cases that had medium or strong staining by immunohistochemistry for ERG had significantly higher levels of ERG mRNA expression (17.99 ± 5.49), as determined by qPCR, than did cases with none or low immunohistochemical staining for ERG (4.59 ± 1.94, P = .019; Figure 2). ERG mRNA expression was significantly upregulated in both localized (stage T2, P = .000416) and advanced cancer (stage T3A, P = .00397 and stage T3B, P = .04120) cases compared with benign prostate tissue. In addition, ERG was significantly upregulated in stage T3 cancer compared with stage T2 (P = .009512).

Analysis of ERG Target Gene Expression in Samples With Low or High ERG Gene Expression

Samples were designated as having high levels of ERG (ERG_high) if they had a 2-fold increase in ERG compared with benign tissues. On average, the ERG_high subset had a 16-fold increase in ERG mRNA expression compared with the ERG_low subset (P = .0022) Figure 4. Increased mRNA expression for MMP7 (P = .3483), OPN (P = .0468), and SEPT9 (P = .00697) mRNA expression was seen in prostate tumors with high ERG mRNA expression compared with low ERG mRNA-expressing tumors (Figure 4B).

Analysis of ERG Expression Is Associated With Seminal Vesicle Invasion and Biochemical Recurrence

Stage 3 disease was significantly associated with biochemical recurrence (P = .008). High levels of ERG expression were significantly associated with stage T3b...
disease (semenal vesicle invasion, \( P = .0045 \)) and biochemical recurrence, with 13 of 28 patients with high levels of \( \text{ERG} \) in their tumor having biochemical recurrence compared with just three of 25 cases with tumors showing low levels of \( \text{ERG} \) (\( P = .006 \)) Table 3 ii.

**Relative \( \text{ERG} \) CAE Splice Isoform Expression Between Benign and Prostate Tumors**

In benign tissue, the relative proportions of \( \text{ERG}72/\Delta72 \) and \( \text{ERG}81/\Delta81 \) were roughly equal Table 4 and Table 5. However, the percentage of total \( \text{ERG} \) mRNA that was accounted for by \( \text{ERG}\Delta72 \) (exon 11 skipped) and \( \text{ERG}\Delta81 \) (exon 10 skipped) was significantly decreased in both T3A and T3B advanced cancer cases compared with benign tissue, and there was a trend for decreased \( \text{ERG}\Delta72 \) and \( \text{ERG}\Delta81 \) in both stage T3a and T3b advanced cancer cases compared with T2 localized cancer (Table 4 and Table 5) Figure 5.

**Discussion**

For the first time, we show that it is possible to reliably detect \( \text{TMPRSS2-ERG} \) fusion isoforms in routinely collected FFPE clinical samples that have been stored at room temperature for over 10 years. FISH is the gold standard for the detection of \( \text{TMPRSS2-ERG} \) fusion on FFPE samples. However, there are limitations to the FISH approach for \( \text{TMPRSS2-ERG} \) fusion detection because, unlike RT-PCR, it is unable to discern between particular fusion variants of \( \text{ERG} \), which was one of the main objectives of this study. We therefore compared FISH and RT-PCR analysis in a random subset of our cohort to validate the RT-PCR approach against a known standard. We observed highly consistent results for the detection of \( \text{TMPRSS2-ERG} \) fusion by FISH and RT-PCR. There are a number of discrepancies within the literature on the clinical relevance and value of using \( \text{TMPRSS2-ERG} \) as a biomarker. We hypothesize that these discordances may be attributable to the attention focused on the \( \text{TMPRSS2-ERG} \) fusion rather than on the downstream signaling effects of \( \text{ERG} \). For example, the \( \text{TMPRSS2-ERG} \) fusion can result in nonfunctional \( \text{ERG} \) transcripts as a result of inclusion of premature stop codons. In addition, if there is \( \text{TMPRSS2-ERG} \) fusion but androgen signaling is absent or disrupted, there will be none or little \( \text{ERG} \) expression in prostate cancer cells. As such, \( \text{TMPRSS2-ERG} \) fusion status may therefore not always reflect the levels of \( \text{ERG} \) present. Thus, instead of detecting a \( \text{TMPRSS2-ERG} \) fusion, the accurate measurement of \( \text{ERG} \) expression may be of more prognostic relevance. Here we show that upregulation of \( \text{ERG} \) results in increased expression of genes involved in cell proliferation (septin 9) and metastases (metalloproteinase 7 and osteopontin) and that high levels of \( \text{ERG} \) expression are significantly associated with seminal vesicle invasion and biochemical recurrence.

Our study also highlights the potential of immunohistochemistry as a high-throughput assay for
the detection of overexpression of ERG in clinical cases. Immunohistochemistry has become increasingly used as a surrogate marker for the TMPRSS2-ERG fusion status.\textsuperscript{15,24,25} It is important to note that while TMPRSS2 is the most common fusion partner for ERG, ERG can also be rearranged and fused with the SLC45A3 and NDRG1 genes. These alternative fusion partners can account for approximately 5% of ERG-overexpressing prostate cancers.\textsuperscript{26-28} As such, using a TMPRSS2-ERG FISH probe in isolation could result in missing a number of prostate cancers that would have significantly elevated levels of

\begin{table}[h]
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\begin{tabular}{|c|c|c|}
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Variable & \textit{ERG\textsubscript{low}} & \textit{ERG\textsubscript{high}} \\
\hline
No. of cases & 25 & 28 \\
ERG mRNA expression, median ± SD & 1.03 ± 0.10 & 16.72 ± 5.34 \\
PSA level, median ± SD, ng/mL & 7.70 ± 1.52 & 7.82 ± 1.12 \\
Gleason score cat. No. & & \\
3 + 3 = 6 & 11 & 10 \\
3 + 4 = 7 & 13 & 15 \\
4 + 4 = 8+ & 1 & 3 \\
Pathologic stage, No. & & \\
pT2 & 12 & 9 \\
pT3A & 10 & 9 \\
pT3B & 3 & 10 \\
Biochemical recurrence, No. (%) & 3/25 (12.5) & 13/28 (46) \\
\hline
\end{tabular}
\caption{Clinicopathologic Parameters for Patients Designated With Either Low or High \textit{ERG} mRNA Expression}
\end{table}
splice sites and intron retention. However, as the retention of the 72-bp exon in ERG increases cell proliferation and invasion in vitro, it is highly possible that the changes in relative proportions of ERG variants may significantly contribute to the progression of prostate cancer. Future studies will focus on addressing the significance of these ERG splice variants in larger cohorts and whether these splice variants may predispose an individual to advanced prostate cancer. If found to be clinically relevant, the technology described readily lends itself to the testing of ERG and its variants on smaller needle biopsy specimens and the possibility of providing guidance to clinicians on the need for radical treatment.

ERG. Like FISH, immunohistochemistry allows for marker expression to be localized in relation to tumor morphology. However, in comparison to FISH, it is relatively inexpensive, technically less demanding, and readily assessed under the light microscope.

The added complexity of alternative splicing of the ERG transcript may also influence the prognostic properties of ERG. Our results suggest for the first time that there is increased retention of both the 72-bp and 81-bp exon as prostate cancer progresses. Bioinformatic studies have shown that alternative splicing is highly deregulated in cancer and that one consequence may be a reduction in exon skipping and an increase in the use of alternative 5' and 3' splice sites and intron retention. However, as the retention of the 72-bp exon in ERG increases cell proliferation and invasion in vitro, it is highly possible that the changes in relative proportions of ERG variants may significantly contribute to the progression of prostate cancer. Future studies will focus on addressing the significance of these ERG splice variants in larger cohorts and whether these splice variants may predispose an individual to advanced prostate cancer. If found to be clinically relevant, the technology described readily lends itself to the testing of ERG and its variants on smaller needle biopsy specimens and the possibility of providing guidance to clinicians on the need for radical treatment.
References


