TITLE PAGE

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.

Rachel M. Hagen, Ph.D[1]; Patricia Adamo, Ph.D[1]; Saima Karamat, M.Sc[2]; Jon Oxley, M.D. FRCPath[2]; Jonathan J Aning M.D.[3]; David Gillatt, M.D[3]; Raj Persad, M.D[3]; Michael R. Ladomery, Ph.D[1*]; Anthony Rhodes, Ph.D[1, 4*#]

1. Faculty of Health & Life Sciences, University of the West of England, Bristol, UK.
2. Department of Cellular Histopathology, North Bristol NHS Trust, Bristol, UK.
3. North Bristol NHS Trust, Bristol, UK.
4. Department of Pathology, University of Malaya Medical Centre, Kuala Lumpur, Malaysia

* Joint senior authors

Page proofs, correspondence, and requests for reprints should be directed to:-

Prof. Anthony Rhodes, Dept of Pathology, Faculty of Medicine, University of Malaya, Lembah Pantai, 50603 Kuala Lumpur, Malaysia. Email: Anthony.Rhodes@uwe.ac.uk, Anthony.Rhodes@ummc.edu.my, Tel: (603) 7949 2064. Fax: (603) 7955 6845

Source of Funding: The authors would like to thank the funding bodies for this work. RMH is supported by the Bristol Urological Institute, North Bristol NHS Trust and the University of the West of England. PA was supported by the Rotary Club of Bristol and the Bristol Urological Institute.

Brief Title: ERG quantification in prostate cancer.

Keywords: FFPE, TMPRSS2-ERG, ERG isoforms, prostate cancer.

DISCLOSURE

The authors declare that there are no conflicts of interests.

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 (72bp) is associated with aggressiveness and progression of disease. Exon 10 (81bp) has also been shown to be alternatively spliced. Within this study we assess whether total ERG protein, 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 (RT-PCR and FISH) and indirect methods for ERG mRNA and protein expression using qPCR 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 up regulation in advanced versus localised 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.

INTRODUCTION

The transcription factor ERG (ETS Related Gene) is overexpressed in 60-80% of prostate cancer cases [1-3] and is often attributed to a fusion between the promoter of the *TMPRSS2* gene and the coding region of *ERG* [4,5]. In benign prostate ERG expression levels are low and are 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 (e.g. *MMP 3/7/9*), osteopontin (*OPN*) and E-cadherin [3, 7, 8].

However, the clinical importance of ERG overexpression and the presence of the TMPRSS2-ERG fusion in prostate cancer is still unclear as there are reports of a positive, negative and zero correlation with development and aggressiveness of prostate cancer.[9-11].

Added complexity of fusion transcripts arise due to alternative splicing. Wild type ERG consists of 17 exons and expresses multiple splice isoforms [12, 13]. A significant finding has been that TMPRSS2-ERG and wild-type ERG variants exhibit differing biological properties [13, 14]. A common alternative splicing event within the central activation exon domain (CAE) of ERG is inclusion/skipping of a 72 bp exon (herein referred to as exon 11). Recent studies *in vitro* suggest that the inclusion of the 72bp exon (exon 11) results in increased cell proliferation and a more oncogenic phenotype [13]. Exon 10 (81bp) can also be alternatively spliced [13].

We hypothesize that the relative proportions of ERG and its variants alter as prostate cancer

progresses. If so the analysis of ERG expression and its splice variants in routine clinical samples of prostate cancer rather than just the genomic fusion, as detected by FISH, may be of value in determining the prognosis.

MATERIALS AND METHODS

Tissue samples

This study utilised tissue samples from fifty-three patients diagnosed between 2000-2009 with clinically localised hormone naïve adenocarcinoma of the prostate (Table 1) (NRES No. 09/H0102/48). Cases were selected based on the availability of cases for review and large enough tumour 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 Mega Tissue Cassettes (Tissue-Tek®, Netherlands). For the study, areas of benign and invasive prostate carcinoma were identified, cored and re-embedded in regular sized cassettes (Tissue-Tek®,). In order to validate the use of RT-PCR to detect the fusion, a total of 20 of the 53 cases were randomly selected for both FISH analysis and RT-PCR. Subsequently, RT-PCR and qPCR for ERG variants was performed on 53 cases.

Fluorescent *in situ* hybridisation (FISH) analysis.

Tri-colour FISH on paraffin-embedded prostate tumour tissue was performed using a break-apart assay designed to detect the microdeletion that occurs between TMPRSS2 and *ERG* at 21q22 (Kreatech, UK) as previously described [16]. Slides were counterstained using DAPI (4',6-diamidino-2-phenylindole), imaged at x100 magnification (Olympus BX41 microscope) and analysed using ISIS software.

Immunohistochemistry

Each of 40 patient samples were stained using two antibodies to ERG (EP111, Dako, UK and EPR3864; Epitomics, USA), in addition to double staining with both ERG EP111 and high molecular weight cytokeratin (34BE12, Dako, UK). All immunohistochemistry, to include antigen retrieval was performed on a BondMax™ instrument (Leica Microsystems, UK), using Bond™ Epitope Retrieval Solution. Nuclear ERG staining was visualised using the Bond™ Polymer Refine Detection system, with a diaminobenzidine chromogen. Cytoplasmic staining for high molecular weight cytokeratin staining was visualised using the Bond™ Polymer Refine Red Detection. Immunohistochemical expression of ERG was assessed as described previously, using a four tier grading system in which the intensity of nuclear staining is recorded as; negative, weakly positive, moderately positive and strongly positive [17]. The specificity of the 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 [17], both giving identical results on 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 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) was used as specified in the manufacturer's instructions with the following modifications: Three 5µm sections were deparaffinised in Histoclear for five minutes at 56°C followed by centrifugation and washing in ethanol. Samples underwent proteinase K digestion at 56°C

overnight. RNA yields were determined by A_{260} measurement using a Nanodrop spectrophotometer (Thermo Fischer Scientific, USA). Reverse transcription was performed using 500ng of RNA and M-MLV Reverse Transcriptase (Promega, UK) as per manufacturers specifications.

RT- PCR analysis

Primers to detect *TMPRSS2:ERG* fusion were as designed by Tomlins *et al* [7]. *TMPRSS2:ERG* forward 5.-TAGGCGCGAGCTAAGCAGGAG-3' and *TMPRSS2:ERG* reverse 5'-GTAGGCACACTCAAACAACGACTGG-3'. PCR was carried out using GoTaq Hot Start Polymerase (Promega) per manufacturers recommendations.

Quantitative realtime PCR

Quantitative realtime PCR was performed using 2x Sybr green master mix (Roche, UK) and primers at 300 nmol concentration on TaqMan7300 Sequence detection System (Applied Biosystems). Primers were designed to span at least one exon boundary using the Primer Express 2.0 software (Applied biosystems, UK) and were purchased from Sigma-Genosys (Haverhill, UK) using standard qPCR cycling conditions (Table 2). Fold changes in expression were calculated by using a standard curve method [18]. Data were normalised 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 the linear equation method (LEM-PCR) was used, as described previously [19]. In brief, 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 *ERG*+72/81 and *ERG*Δ72/81 to the total value of *ERG* calculated. Values were then re-expressed 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's t-test,a Kruskal-Wallis test and the Pearson Chi-Squared Test. Biochemical recurrence after radical prostatectomy was defined as a PSA value greater than or equal to 0.2ng/ml, with a second confirmatory level of PSA of >0.2 ng/ml (33).

RESULTS

ERG mRNA expression is increased in TMPRSS2-ERG fusion positive samples.

We analysed 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 (Figure 1). The RT-PCR reaction detected one, two or no *TMPRSS2-ERG* fusion variants (Figure 1A). RT-PCR and FISH results were highly concordant (18/20), with RT-PCR giving an additional two cases as TMPRSS2-*ERG* positive compared with FISH. Using RT-PCR on all 53 cases within this study we identified 58% (31/53) of samples to have a *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, $p<0.01$(Figure 1C).

Analysis of ERG mRNA and protein expression in prostate cancer cases

ERG protein expression by immunohistochemistry correlated with ERG mRNA expression (Figure 2A and 2B). ERG staining was exclusively nuclear and homogenous in expression and specific to invasive tumour nuclei and the nuclei of PIN. The cells of adjacent normal prostate glands remained unstained Figure 2A).. 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 cases with none or low immunohistochemical staining for ERG (4.59±1.94, p=0.019; Figure 2B). *ERG* mRNA expression was significantly up regulated in both localised (Stage T2, p=0.000416) and advanced cancer (Stages T3A, p=0.00397 and T3B, p=0.04120) cases when compared with benign prostate tissue (Figure 2C). In addition, ERG was significantly up regulated in Stage 3 cancer compared to Stage T2 (p=0.009512).

Quantitative analysis of *ERG* target gene expression in prostate cancer FFPE samples that either have 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=0.0022 (Figure 3A). Increased mRNA expression for Matrix metalloproteinase 7 (*MMP7)* p=0.3483 osteopontin (*OPN)*p=0,0468 and septin 9 *(SEPT9)o=0.00697* mRNA expression was seen in prostate tumours with high *ERG* mRNA expression compared with low *ERG* mRNA expressing tumours (Figure 3B).

Quantitative analysis of ERG expression is associated with seminal vesicle invasion and biochemical recurrence.

Stage 3 disease was significantly associated with biochemical recurrence (p = 0.008). High levels of ERG expression was significantly associated with Stage T3b disease (seminal vesicle invasion, p=0.0045) and biochemical recurrence, with 13/28 of the patients with high levels of ERG in their tumour having biochemical recurrence, compared to just 3/25 of the cases with tumours showing low levels of ERG (p=0.006) (Table 3).

Relative *ERG* CAE splice isoform expression between benign and prostate tumours

In benign tissue the relative proportions of *ERG72/Δ72* and *ERG81/Δ81* were roughly equal (Table 4). However, the percentage of total *ERG* mRNA that was accounted for by *ERGΔ72 (*exon 11 skipped*)* and *ERGΔ81(*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 *ERGΔ72* and *ERGΔ81* in both Stage T3a and T3b advanced cancer cases when compared with T2 localised cancer (Figure 4 and Table 4).

DISCUSSION

For the first time we show that it is possible to reliably detect TMPRSS2-ERG fusion isoforms in routinely collected FFPE clinical samples which have been stored at room temperature for over 10 years. FISH is the gold standard for the detection of the TMPRSS2-ERG fusion on FFPE samples. However, there are limitations to the FISH approach for TMPRSS2-ERG detection, as unlike RT-PCR, it is unable to discern between particular fusion variants of 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, in order to validate the RT-PCR approach against a known standard. We observed highly consistent results for the detection of 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 TMPRSS2-ERG as a biomarker. We hypothesise that these discordances may be attributable to the attention focused on the TMPRSS2-ERG fusion rather than on the downstream signalling effects of ERG. For example the TMPRSS2-ERG fusion can result in non-functional *ERG*

transcripts as a result of inclusion of premature stop codons [20, 21]. Additionally, if there is TMPRSS2-ERG fusion but androgen signalling is absent or disrupted there will be none or little ERG expression in prostate cancer cells [22-24]. As such TMPRSS2-ERG fusion status may therefore not always reflect the levels of ERG present. Thus instead of detecting a TMPRSS2-ERG fusion the accurate measurement of ERG expression may be of more prognostic relevance. Here we show that up-regulation of ERG results in increased expression of genes involved in cell proliferation (septin 9), and metastases (metalloproteinase 7, and osteopontin) (3, 7, 8), and that high levels of 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 detection of overexpression of ERG in clinical cases. Immunohistochemistry has become increasingly utilised as a surrogate marker for the TMPRSS2-ERG fusion status. a [17, 25, 26]. It is important to note that whilst 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 [27-29]. 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 ERG. Like FISH, immunohistochemistry allows for marker expression to be localised in relation to tumour 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 [32]. However, as the retention of the 72bp 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 biopsies, and the possibility of providing guidance to clinicians on the need for radical treatment.

ACKNOWLEDGEMENTS

The authors would also like to thank Jess Broadhurst (University of Bristol) and Martin Figgitt (University of Bristol) for technical assistance with the FISH assay and visualisation. We also thank Dako, UK for provision of the ERG EP111 clone.

REFERENCES

1. Vanaja DK, JC Cheville, SJ Iturria, et al. Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. *Cancer Res.* 2003;63:3877-3882.

2. Petrovics G, Liu A, Shaheduzzaman S, et al. Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. *Oncogene* 2005;24:3847-3852.

3. Iljin K, Wolf M, Edgren H, *et al*. TMPRSS2 fusions with oncogenic ETS factors in prostate cancer involve unbalanced genomic rearrangements and are associated with HDAC1 and epigenetic reprogramming. *Cancer Res.* 2006;66:10242-10246.

4. Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 2005;310:644-648**.**

5. Tomlins SA, Laxman B, Dhanasekaran SM, *et al*. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature* 2007;448:595-599.

6. Oikawa T. ETS transcription factors: Possible targets for cancer therapy. *Cancer.Sci.* 2004;95:626-33.

7. Flajollet S, Tian TV, Flourens A, *et al*. Abnormal expression of the ERG transcription factor in prostate cancer cells activates osteopontin. *Mol.Cancer.Res.* 2011;9:914-924.

8. Gupta S, Iljin K, Sara H, *et al*. FZD4 as a mediator of ERG oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer Res.* 2010;70:6735-6745.

9. Cerveira, N, Ribeiro FR, Peixoto A, *et al*. TMPRSS2-ERG gene fusion causing ERG overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. *Neoplasia* 2006;8:826-832.

10. Demichelis F, Fall K, Perner S, *et al*. TMPRSS2:ERG gene fusion associated with lethal prostate cancer in a watchful waiting cohort. *Oncogene* 2007;26:4596-4599.

11. St John J, Powell K, Conley-Lacomb MK, *et al*. TMPRSS2-ERG fusion gene expression in prostate tumor cells and its clinical and biological significance in prostate cancer progression. *J.Cancer.Sci.Ther.* 2012;4:94-101.

12. Carrere S, Verger A, Flourens A, *et al*. Erg proteins, transcription factors of the ets family, form homo, heterodimers and ternary complexes via two distinct domains. *Oncogene* 1998;16:3261-3268.

13. Wang J, Cai Y, Ren C, *et al*. Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. *Cancer Res.* 2006;66:8347-8351.

14. Wang J, Cai Y, Yu W, *et al*. Pleiotropic biological activities of alternatively spliced TMPRSS2/ERG fusion gene transcripts. *Cancer Res.* 2008;68:8516-8524.

15. Hu Y, Dobi A, Sreenath T, *et al*. Delineation of TMPRSS2-ERG splice variants in prostate cancer. *Clin.Cancer Res.* 2008;14:4719-4725.

16. Chin SF, Daigo Y, Huang HE, *et al*. A simple and reliable pretreatment protocol facilitates fluorescent in situ hybridisation on tissue microarrays of paraffin wax embedded tumour samples. *Mol.Pathol.* 2003;56:275-279.

17. Park K, Tomlins SA, Mudaliar KM, *et al*. Antibody-based detection of ERG rearrangement-positive prostate cancer. *Neoplasia* 2010;12:590-598.

18. Applied Biosystems. Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, 2008.

19. Virtue S, Dale M, Sethi JK, *et al*. LEM-PCR: A method for determining relative transcript isoform proportions using real-time PCR without a standard curve. *Genome* 2010;53:637-642.

20. Soller MJ, Isaksson M, Elfving P, *et al*. Confirmation of the high frequency of the TMPRSS2/ERG fusion gene in prostate cancer. *Genes Chromosomes Cancer* 2006;45:717-729.

21. Clark J, Merson S, Jhavar S, *et al*. Diversity of TMPRSS2-ERG fusion transcripts in the human prostate. *Oncogene* 2007;26:2667-2673.

22. Hermans KG, van Marion R, van Dekken H, *et al*. TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. *Cancer Res*. 2006;66:10658-10663.

23. Saramaki OR, Harjula AE, Martikainen PM, *et al*. TMPRSS2:ERG fusion identifies a subgroup of prostate cancers with a favorable prognosis. *Clin.Cancer Res*. 2008;14:3395-3400.

24. Linja MJ, Savinainen KJ, Saramaki OR, *et al*. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res.* 2001;61:3550-3555.

25. Shah RB. Clinical applications of novel ERG immunohistochemistry in prostate cancer diagnosis and management. *Adv.Anat.Pathol.* 2013;20:117-124.

26. Tomlins SA, Palanisamy N, Siddiqui J, *et al*. Antibody-based detection of ERG rearrangements in prostate core biopsies, including diagnostically challenging cases: ERG staining in prostate core biopsies. *Arch.Pathol.Lab.Med.* 2012;136:935-946.

27. Han B, Mehra R, Dhanasekaran SM, *et al*. A fluorescence in situ hybridization screen for E26 transformation-specific aberrations: Identification of DDX5-ETV4 fusion protein in prostate cancer. *Cancer Res.* 2008;68:7629-7637.

28. Esgueva R, Perner S, LaFargue CJ, *et al*. Prevalence of TMPRSS2-ERG and SLC45A3-ERG gene fusions in a large prostatectomy cohort. *Mod.Pathol.* 2010;23:539-546.

29. Pflueger D, Rickman DS, Sboner A, *et al*. N-myc downstream regulated gene 1 (NDRG1) is fused to ERG in prostate cancer. *Neoplasia* 2009;11:804-811.

30. Jhavar S, Reid A, Clark J, *et al*. Detection of TMPRSS2-ERG translocations in human prostate cancer by expression profiling using GeneChip human exon 1.0 ST arrays. *J.Mol.Diagn*. 2008;10:50-57.

31. Iwamoto M, Higuchi Y, Koyama E, *et al*. Transcription factor ERG variants and functional diversification of chondrocytes during limb long bone development. J.Cell Biol. 2000;150:27-40.

32. Kim E, Goren A, and Ast G. Insights into the connection between cancer and alternative splicing. *Trends Genet.* 2008;24:7-10.

33. Cookson MS, Aus G, Burnett AL et al. Variation in the Definition of Biochemical Recurrence in Patients Treated for Localized Prostate Cancer: The American Urological Association Prostate Guidelines for Localized Prostate Cancer Update Panel Report and

Recommendations for a Standard in the Reporting of Surgical Outcomes. *The Journal of Urology* 2007; 177: 540-545.

TABLE AND FIGURE LEGENDS

Figure 1.

A) RT-PCR was performed on cDNA generated from cancerous regions of prostate from 20 cases using primers directed against exon1 of TMPRSS2 and exon 4 of ERG to detect the TMPRSS2-ERG fusion. PCR products were sequenced to confirm the identity of TMPRSS2-ERG fusion variants. B) Representative image of a fusion positive and a fusion negative case obtained using a triple colour breakapart TMPRSS2-ERG FISH probe. C) Comparison of total ERG mRNA expression in TMPRSS2-ERG positive (n=26) and TMPRSS2-ERG negative (n=14) FFPE cases as determined by qPCR (**p<0.01).

Figure 2.

A) Representative images of immunohistochemistry for ERG, ERG and high molecular weight cytokeratin and H & E staining in ERG_low (top row) and ERG_high (bottom) invasive prostate cancers as determined previously using qPCR. B) Comparison between ERG immunohistochemical staining and average ERG mRNA expression, as determined by qPCR, *p=0.019. C) qPCR analysis comparing ERG mRNA expression in benign (n=12) versus localised Stage pT2 cancer pT2 (n=21) **p=0.000416, and benign tissue versus Stage pT3A (n=19)**$p=0.00397, and benign tissue versus Stage pT3b (n=13) ***p=0.04120 cancers.

Figure 3.

qPCR analysis of ERG target gene expression in prostate cancers with high and low values of ERG mRNA expression. ERG_high was defined as a 2-fold or greater increase in ERG compared with benign tissues. A) Total ERG; on average the ERG_high subset (n=24) had a 16-fold increase in ERG mRNA expression compared with the ERG_low subset (n=16), **p=0.0022, B) ERG-high cancers show increased expression of mRNA for the target genes; Matrix metalloproteinase 7 (MMP7 (p=0.3483), osteopontin (OPN) *p=0.0468 and septin (SEPT9) **p=0.00697.

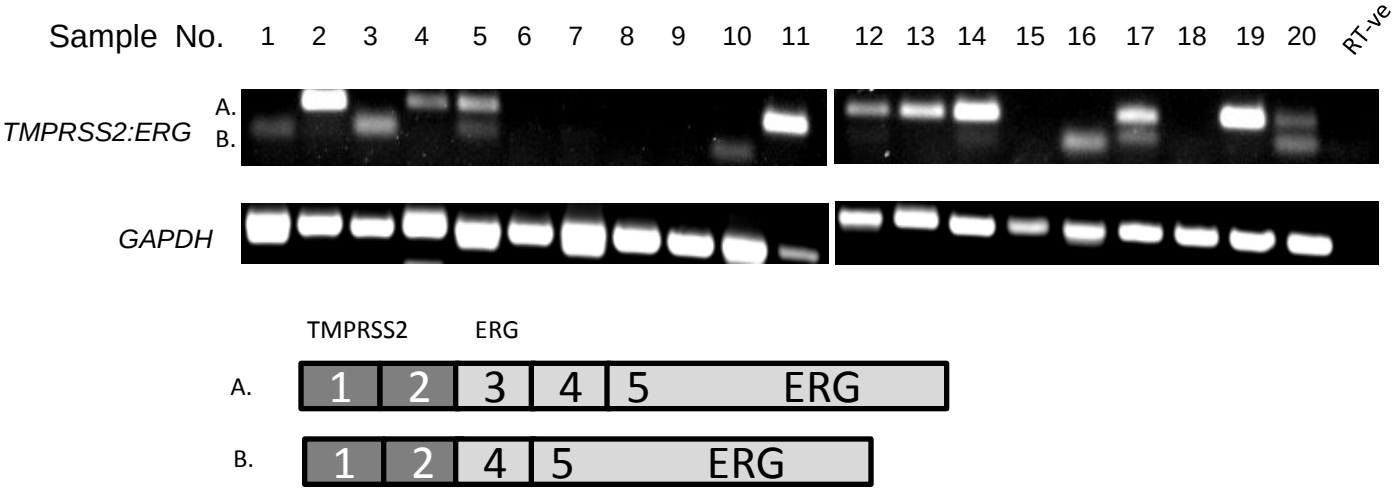
Figure 4.

Relative proportions of ERG variants plus/minus 72bp exon 11of ERG and plus/minus 81bp exon 10 of ERG expressed as percentages of the total ERG present in benign (BN, n=12), localised (pT2, n=21) and advanced (pT3A, n=19 and pT3B, n=13) prostate cancer samples as determined using the LEM-PCR method.

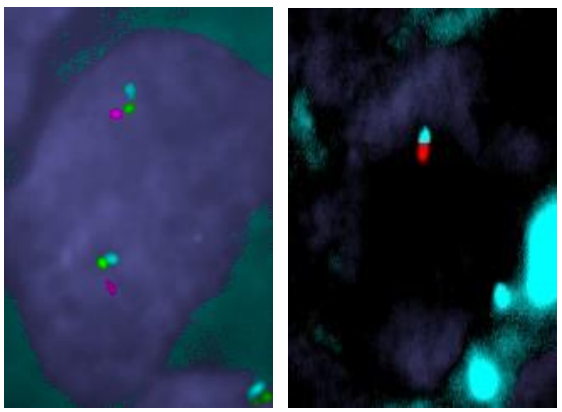
Table 4.

Average values ± standard error mean (SEM) of relative amounts of ERG variants (plus/minus 72/81 bp exon) expressed as percentages of the total ERG present in benign (BN, n=12), localised (pT2, n=21) and advanced (pT3A, n=19 and pT3B, n=13) prostate cancer samples as determined using the LEM-PCR method.

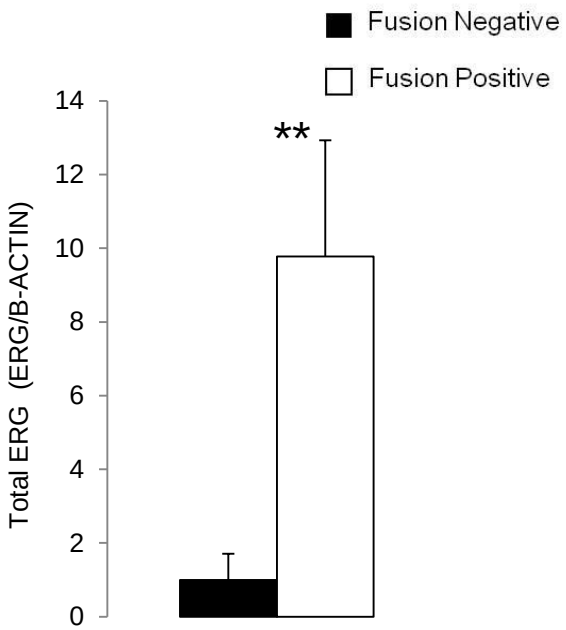
A.

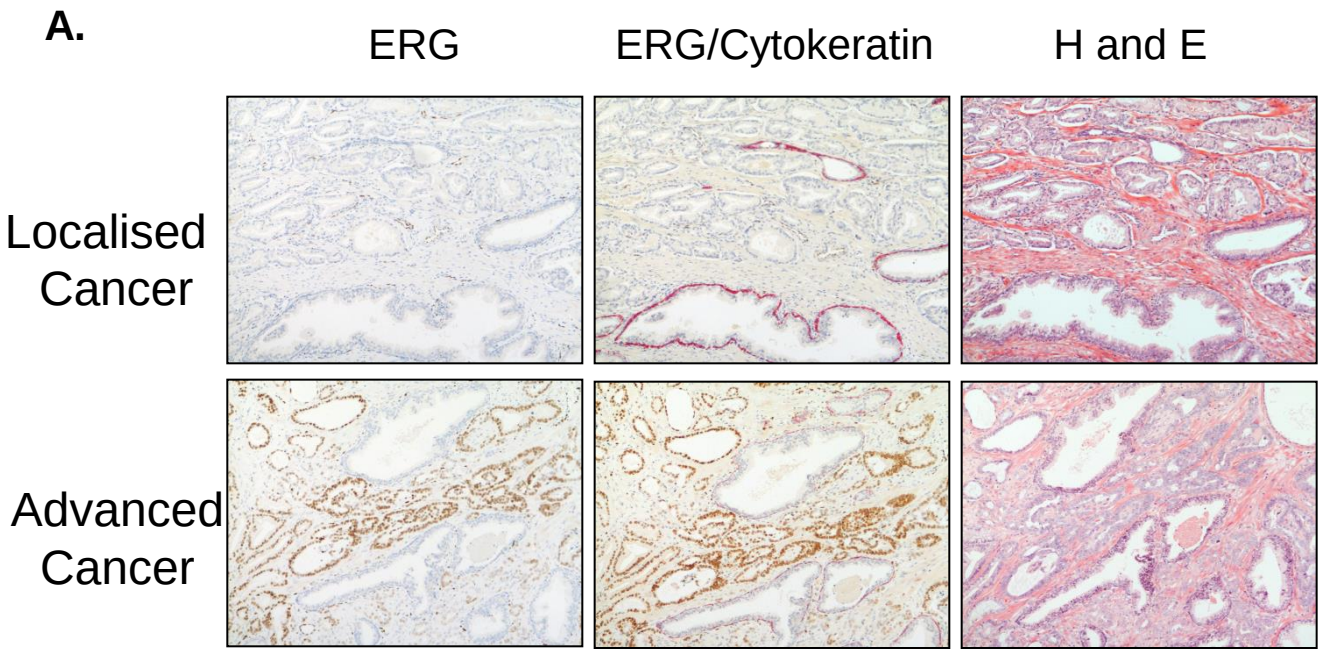

Sample No. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 RT-ve
TMPRSS2:ERG
A.
B.
GAPDH
TMPRSS2
ERG
A.
1 2 3 4 5 ERG
B.
1 2 4 5 ERG

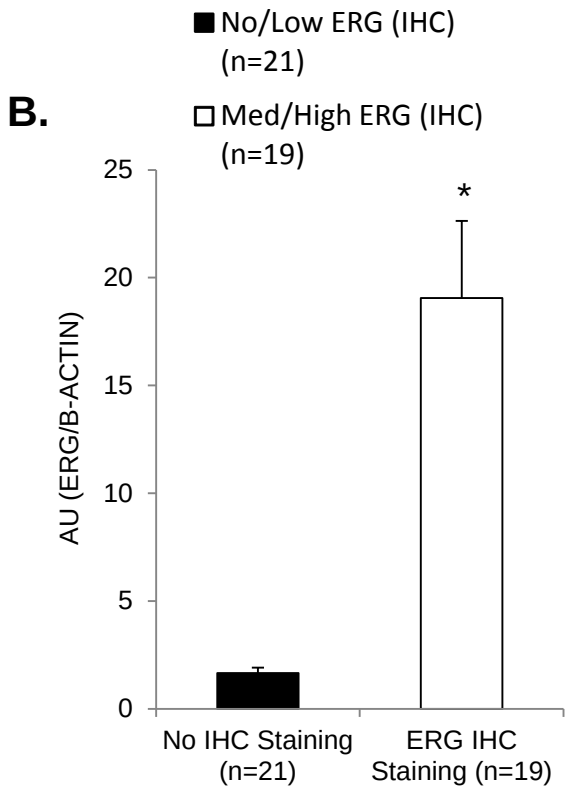
B.

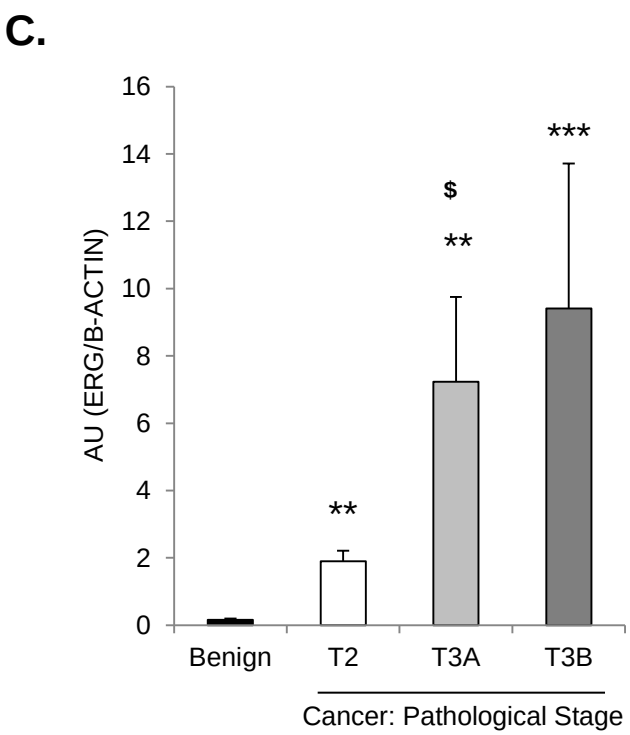

Fusion Negative
Fusion Positive

C.


Fusion Negative
Fusion Positive
Total ERG (ERG/B-ACTIN)
14
12
10
8
6
4
2
0
**


A.
ERG
ERG/Cytokeratin
H and E
Localised Cancer
Advanced Cancer


B.
No/Low ERG (IHC) (n=21)
Med/High ERG (IHC) (n=19)
AU (ERG/B-ACTIN)
25
20
15
10
5
0
*
No IHC Staining (n=21)
ERG IHC Staining (n=19)


C.
AU (ERG/B-ACTIN)
16
14
12
10
8
6
4
2
0
**
$
**
***
Benign
T2
T3A
T3B
Cancer: Pathological Stage

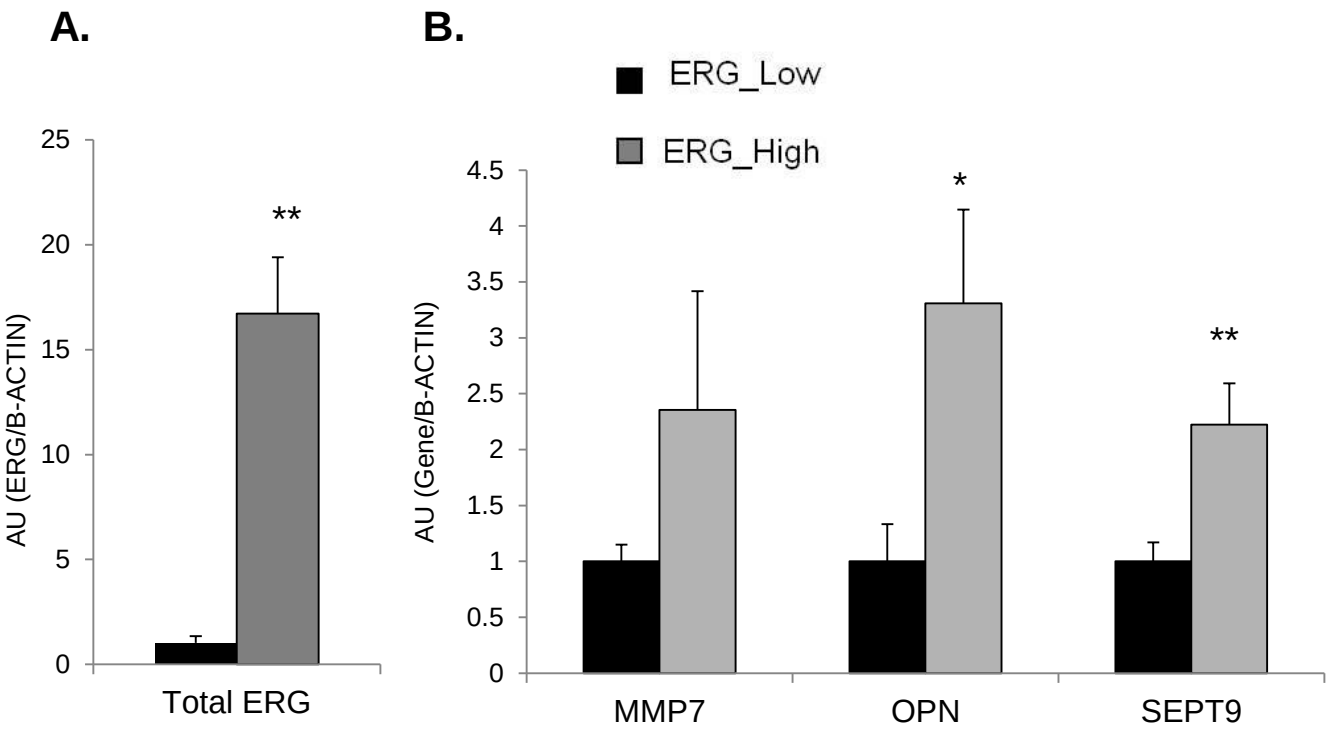
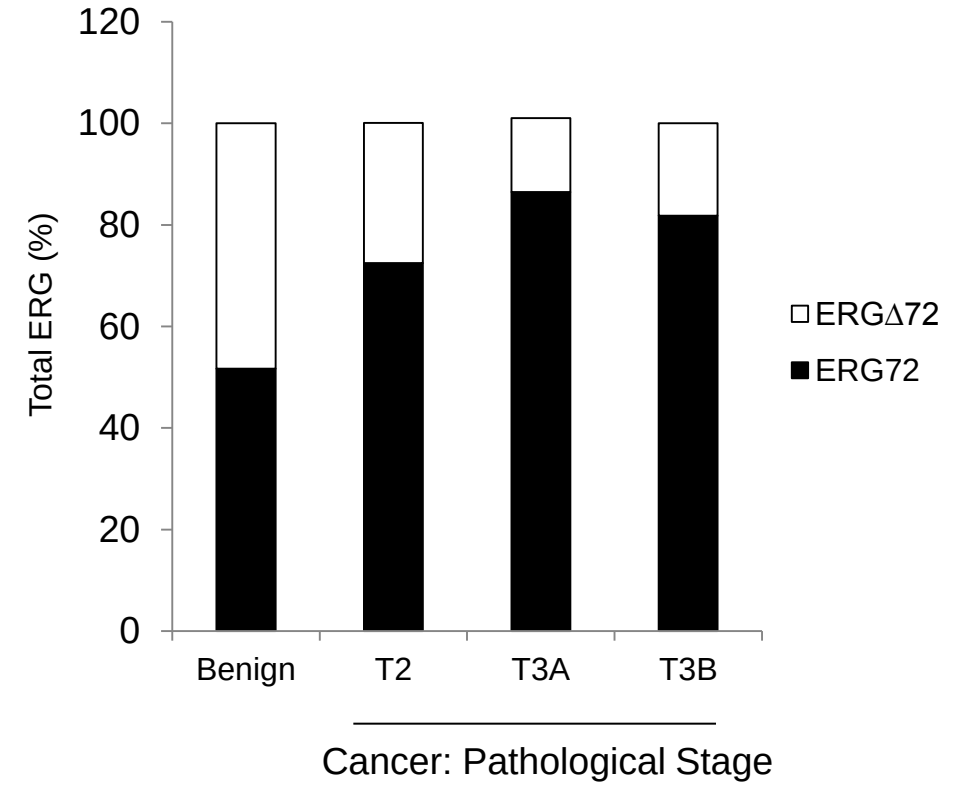

A.
B.
ERG_Low
ERG_High
AU (ERG/B-ACTIN)
AU (Gene/B-ACTIN)
**
*
**
Total ERG
MMP7
OPN
SEPT9

Figure 4


120
100
80
60
40
20
0
Total ERG (%)
Benign
T2
T3A
T3B
Cancer: Pathological Stage
ERGΔ72
ERG72

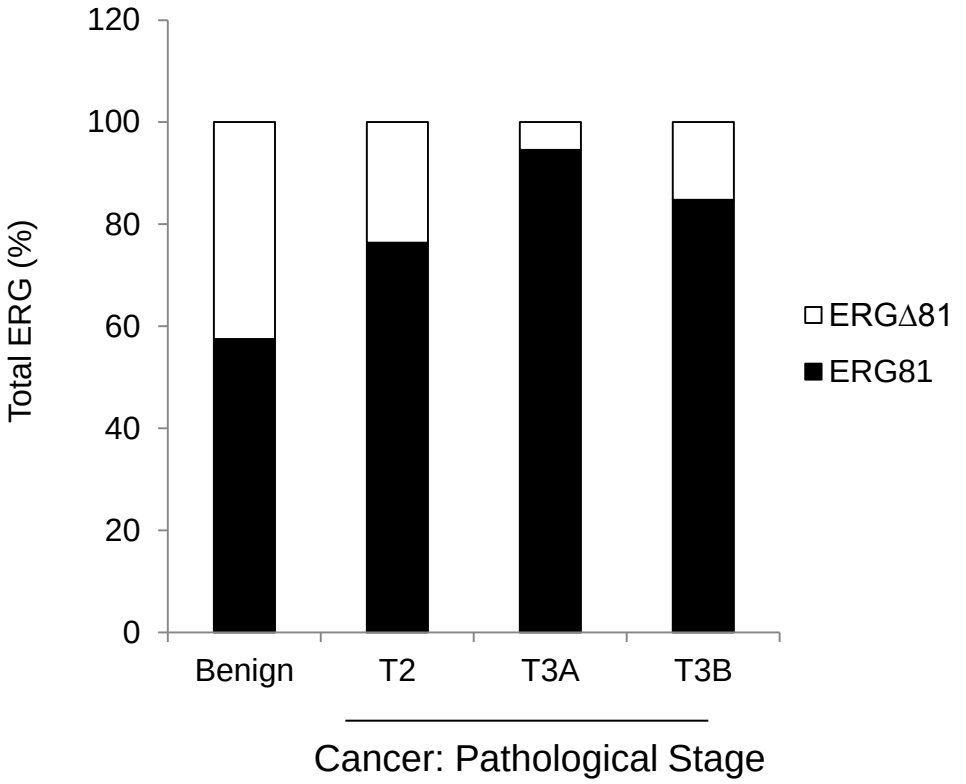

120
100
80
60
40
20
0
Total ERG (%)
Benign
T2
T3A
T3B
Cancer: Pathological Stage
ERGΔ81
ERG81

Table 1. Clinical parameters for patients used in this study

Variable	Localised prostate cancer	Advanced prostate cancer
No. of cases	21	32
Median PSA level ± SD, ng/mL	5.34 ± 2.25	12.80 ± 5.39
Gleason score cat. No.		
3+3 = 6	13	8
3+4 = 7	6	22
4+4= 8+	0	4
Pathologic Stage, No.		
pT2	21	0
pT3A	0	19
pT3B	0	13

Comment [A1]:

Comment [A2R1]: Additional cases analysed to include 13 cases of T3b

Table 2: PCR primer sequences used.

	Forward (5'-3')	**Reverse (5'-3')**
β-Actin	GCATGGAGTCCTGTGGCATCCA	ATCCTGTCGGCAATGCCAGGGTA
Total ERG (exon 16)	CATCTCCTTCCACAGTGCCCA	CTGGATTTGCAAGGCGGCTAC
ERGΔ72bp	AGAAACACAGATTTACCATATGAGC	ACCGGTCCAGGCTGATCTC
ERG 72bp exon	CCTGAAGCTACGCAAAGAATTACA	ACCGGTCCAGGCTGATCTC
ERG 81bp	TCTCCACGGTTAATGCATGC	GAAAATAAAAGCTGCACCCCCT
ERGΔ81bp	TCACATCTCCACTACCTCAGAGA	TTGGGAAAATAAAAGCTGCAC
MMP7	GAACGCTGGACGGATGGT	CATACCCAAAGAATGGCCAAGT
SEPT9	GGAGCGCATCCCCAAGA	CGGACGCCTTTCTCCTCAA
OPN	TGGCTAAACCCTGACCCATCT	TCATTGGTTTCTTCAGAGGACACA

Table 3. Clinico-pathological parameters for patients designated with either low or high ERG mRNA expression.

Variable	*ERG*_low	*ERG*_high
No. of cases	25	28
ERG mRNA expression± SD,	1.03 ± 0.10	16.72 ± 5.34
Median PSA level ± 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	3/25 (12.5%)	13/28 (46%)

Table 4. Relative proportions of ERG with/without the 72bp or 81bp exon in clinical prostate samples

	Average ± SEM		**p-value (ERG72 vs. ERGΔ72)**			
	ERG72 (%)	**ERGΔ72 (%)**	**BN**	**pT2**	**pT3A**	**pT3B**
BN	51.71 ± 8.64	48.29 ± 8.09	0.55	0.0012	0.00009	0.00054
pT2	72.45 ± 7.51	27.64 ± 7.55				
pT3A	86.47 ± 6.23	13.53 ± 6.24				
pT3B	81.82 ± 3.61	18.18 ± 3.61				

	Average ± SEM		**p-value (ERG81 vs. ERGΔ81)**			
	ERG81 (%)	**ERGΔ81 (%)**	**BN**	**pT2**	**pT3A**	**pT3B**
BN	57.52 ± 4.87	42.48 ± 7.12	0.68	0.008	0.015	0.013
pT2	76.37 ± 2.53	23.63 ± 2.75				
pT3A	94.55 ± 3.27	5.45 ± 12.87				
pT3B	84.78 ± 6.14	15.22 ± 5.04				