Published in final edited form as:
Oncogene. 2008 January 10; 27(3): 253–263. doi:10.1038/sj.onc.1210640.

Duplication of the fusion of *TMPRSS2* to *ERG* sequences identifies fatal human prostate cancer

G Attard[1,2,10], **J Clark**[1,10], **L Ambroisine**[3], **G Fisher**[3], **G Kovacs**[4], **P Flohr**[1], **D Berney**[5], **CS Foster**[6], **A Fletcher**[1], **WL Gerald**[7], **H Moller**[8], **V Reuter**[7], **JS De Bono**[2], **P Scardino**[9], **J Cuzick**[3], and **CS Cooper**[1] **on behalf of the Transatlantic Prostate Group**

1 *Institute of Cancer Research, Male Urological Cancer Research Centre, Surrey, UK*

2 *The Royal Marsden NHS Trust Foundation Hospital, Surrey, UK*

3 *Wolfson Institute of Preventive Medicine, University of London, London, UK*

4 *Ruprecht-Karls-Universitat, Medical Faculty, Laboratory of Molecular Oncology, Heidelberg, Germany*

5 *Department of Histopathology, St Bartholomew's Hospital, London, UK*

6 *Department of Pathology, Royal Liverpool University Hospital, Liverpool, UK*

7 *Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY, USA*

8 *Kings College, Thames Cancer Registry, London, UK*

9 *Department of Urology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*

Abstract

New predictive markers for managing prostate cancer are urgently required because of the highly variable natural history of this disease. At the time of diagnosis, Gleason score provides the gold standard for assessing the aggressiveness of prostate cancer. However, the recent discovery of *TMPRSS2* fusions to the *ERG* gene in prostate cancer raises the possibility of using alterations at the *ERG* locus as additional mechanism-based prognostic indicators. Fluorescence *in situ* hybridization (FISH) assays were used to assess *ERG* gene status in a cohort of 445 prostate cancers from patients who had been conservatively managed. The FISH assays detected separation of 5′ (labelled green) and 3′ (labelled red) *ERG* sequences, which is a consequence of the *TMPRSS2–ERG* fusion, and additionally identify interstitial deletion of genomic sequences between the tandemly located *TMPRSS2* and *ERG* gene sequences on chromosome 21. Cancers lacking *ERG* alterations exhibited favourable cause-specific survival (90% survival at 8 years). We identify a novel category of prostate cancers, characterized by duplication of the fusion of *TMPRSS2* to *ERG* sequences together with interstitial deletion of sequences 5′ to *ERG* (called '2+Edel'), which by comparison exhibited extremely poor cause-specific survival (hazard ratio = 6.10, 95% confidence ratio = 3.33–11.15, $P < 0.001$, 25% survival at 8 years). In multivariate analysis, '2+Edel' provided significant prognostic information ($P = 0.003$) in addition to that provided by Gleason score and prostate-specific antigen level at diagnosis. Other individual categories of *ERG* alteration were associated with intermediate or good prognosis. We conclude that determination of *ERG* gene status, including duplication of the fusion of *TMPRSS2* to *ERG* sequences in 2+Edel, allows stratification of prostate cancer into distinct survival categories.

Correspondence: Professor CS Cooper, Male Urological Cancer Research Centre, Institute of Cancer Research, 15 Cotswold Rd, Belmont, Sutton, Surrey SM2 5NG, UK. E-mail: Colin.Cooper@icr.ac.uk.
[10]Joint first authors.

Keywords

prostate cancer; *ERG* gene; *ERG* gene break point; Gleason score; prognosis

Introduction

Gleason scoring is the sole grading system recommended by the World Health Organization for assessing the aggressiveness of prostate cancer. Although a useful prognostic indicator, as a system based on morphological appearance, it suffers from problems of inter-observer variability (Allsbrook *et al*., 2001a, b; Oyama *et al*., 2005; Melia *et al*., 2006; van der Kwast *et al*., 2006) and of changes in interpretation with time (Smith *et al*., 2002; Chism *et al*., 2003; Albertsen *et al*., 2005). Recently, fusion of the *TMPRSS2* gene to the *ETS* transcription factor gene *ERG* has been reported as a common event in prostate cancer (Tomlins *et al*., 2005, 2006; Hermans *et al*., 2006; Iljin *et al*., 2006; Perner *et al*., 2006; Soller *et al*., 2006; Wang *et al*., 2006; Yoshimoto *et al*., 2006; Clark *et al*., 2007). Less frequently *TMPRSS2* also becomes fused to *ETV1* and *ETV4* (Tomlins *et al*., 2005, 2006; Hermans *et al*., 2006). These discoveries raise the prospect that genetic alteration in *ETS* gene family members may provide an alternative or additional mechanism-based prognostic classification for prostate cancer. In support of this possibility, there are already several indications of correlations of *ERG* gene status with clinicopathological indicators (Perner *et al*., 2006; Wang *et al*., 2006; Demichelis *et al*., 2007; Nam *et al*., 2007). For example, Perner *et al*. (2006) found that the presence of *ERG* rearrangements accompanied by 5′-*ERG* deletion had a significant correlation with higher tumour stage with the presence of metastatic disease involving pelvic lymph nodes. In a watchful waiting cohort of 111 patients, Demichelis *et al*. (2007) reported both a significant association between the presence of a *TMPRSS2–ERG* fusion and prostate cancer-specific death, and a link between the presence of *ERG* alterations and higher Gleason score.

Because of the highly variable natural history of prostate cancer, additional new predictors of cancer aggressiveness are urgently required. Overtreatment of prostate cancers is a particular concern leading to substantial inappropriate morbidity (Yao and Lu-Yao, 2002). This is especially true for many prostrate-specific antigen (PSA) screen-detected cancers, which in the absence of treatment, may never become life threatening. Conversely more conservative approaches to disease detection and management can leave potentially aggressive cancers untreated. Therefore, improved biomarkers are required to allow radical therapies to be targeted to men with potentially lethal cancers, so that the remainder, with more benign-behaving indolent cancers, are spared inappropriate treatment.

To help identify and optimize markers that may be of use in the management of men with prostate cancer, we recently established a retrospective cohort of men whose cancers were conservatively managed (Cuzick *et al*., 2006). Improving on previous studies (Chodak *et al*., 1994; Albertsen *et al*., 1995; Adolfsson *et al*., 1997; Holmberg *et al*., 2002; Johansson *et al*., 2004; Albertsen *et al*., 2005; Bill-Axelson *et al*., 2005), our analyses included centrally assigned Gleason scores determined by modern grading criteria, and allowed comparisons with several additional clinical parameters. In agreement with Johansson *et al*. (2004) and Albertsen *et al*. (2005), we found Gleason score to be an important determinant of cancer-specific mortality, but baseline PSA and to a lesser extent stage of disease added further predictive value. The objective of the current study is to use our cohort of conservatively managed prostate cancer cases to assess the potential clinical significance and utility of different classes of *ERG* gene alteration that can be detected using fluorescence *in situ* hybridization (FISH).

Results

Fluorescence *in situ* hybridization detection of breaks at the ERG gene locus

To assess the frequency of the *TMPRSS2–ERG* fusions in a set of 1062 cores from 445 patients, we used an *ERG* gene 'break-apart' assay similar to that described by Tomlins *et al*. (2005). To increase the sensitivity of this assay, we used three overlapping BAC probes at the centromeric 3′-end (red) and three BAC probes at the telomeric 5′-end (green). The positions of the probes are shown in Figure 1 (lower panel). Using this assay, unrearranged *ERG* loci are visualized in interphase nuclei as immediately adjacent green and red signals (Figure 1a). The *TMPRSS2–ERG* fusion results in the joining of *TMPRSS2* exon 1 or 2 sequences usually to exon 2, 3 or 4 *ERG* sequences (Wang *et al*., 2006; Clark *et al*., 2007). When this fusion has occurred, the 3′-centromeric and 5′-telomeric ends of *ERG* occur as separated red and green signals (Figure 1b; Tomlins *et al*., 2005; Perner *et al*., 2006). Many cancers with alteration in the *ERG* gene FISH pattern also exhibit loss of the lone green 5′-*ERG* signal (Figures 1c–e), consistent with previous findings that the chromosomal region between *TMPRSS2* and *ERG* on chromosome 21 is frequently deleted in cancers with *TMPRSS–ERG* fusions (Iljin *et al*., 2006; Perner *et al*., 2006).

In view of the observed FISH patterns, cancers could be stratified according to whether they (i) had entirely normal *ERG* loci (class N, for Normal, 311 cancers, 70%), (ii) had rearranged *ERG* but had retained both 5′- and 3′-*ERG* sequences (class Esplit, for ERG signal split into separate 3′-red and 5′-green probes, 41 cancers, 9%) or (iii) had retained 3′-*ERG* but had no evidence for the presence of 5′-*ERG* sequences (class 'Edel' for *ERG* 5′ deletion, 93 cancers, 21%). Variation in the precise number of normal (n = 0–5), 3′-*ERG* (red, n = 0–3) and 5′-*ERG* (green n = 0–3) signals was observed leading to considerable diversity in the range of FISH patterns within each category. To record this variation, the interphase nuclei within each core were scored according to the number of each of the three FISH signals: twinned red and green; separate red 3′-*ERG*; separate green 5′-*ERG*. A cancer with the score '2,1,0' (found for five cancers, Figure 2) is, for example, a class 'Edel' cancer, which contains two unrearranged *ERG* alleles, one separate red 3′-*ERG* signal and no green 5′-*ERG* signals. The variation in FISH patterns observed within class N, Esplit and Edel cancers is shown in Figure 2.

To confirm that class Esplit and class Edel *ERG* alterations detected by FISH do indeed correspond to 5′-*TMPRSS2–ERG*-3′ fusions as reported previously (Perner *et al*., 2006), selected cancers were rehybridized using 5′-*TMPRSS2* (green) and 3′-*ERG* (red) FISH probes. These studies (Figure 3) demonstrated that each rearranged 3′-*ERG* signal retained a juxtapositioned 5′-*TMPRSS2* signal, even though intervening sequences between the *TMPRSS2* and *ERG* genes were either missing (class Edel) or located in a different region of the nucleus (class Esplit). Reverse transcription (RT)–PCR studies carried out as described previously (Clark *et al*., 2007) confirmed fusion of 5′-*TMPRSS2* to 3′-*ERG* transcripts in each cancer category.

Tumour demographics and characteristics for the cohort of 445 patients and correlations to clinicopathological parameters are shown in Table 1. There were significant associations between the presence of *ERG* gene alteration and Gleason score ($P < 0.001$), clinical stage ($P = 0.001$) and baseline PSA ($P < 0.001$), but no association with age ($P = 0.765$).

Presence of 'Edel' rearrangements independently predicts poor survival

For the 445 patients, the cancer-specific survival and overall survival for the three categories of FISH pattern (N, Esplit and Edel) are shown in Figures 4a and b. Univariate Cox analysis demonstrates that, compared to cancers retaining only normal *ERG* FISH patterns (class N), Edel cancers had significantly worse cause-specific and overall survival (hazard ratio (HR) =

2.92, 95% confidence interval (CI) = 1.79–4.76, $P < 0.001$ and HR = 1.92, 95% CI = 1.42–2.59, $P < 0.001$, respectively). In contrast, class Esplit cancers did not exhibit significantly worse cause-specific (HR = 1.56, 95% CI = 0.73–3.34, $P = 0.254$) or overall survival (HR = 1.00, 95% CI = 0.61–1.63, $P = 0.987$) compared to class N cancers. A link between the *ERG* gene status and Gleason score in part explains the poorer survival of Edel cancers (Table 1, $P < 0.001$). However, in the multivariate analyses including age, Gleason score and baseline PSA, the presence of Edel still emerged as a significant independent marker of poor cause-specific and overall survival (HR = 1.72, 95% CI = 1.02–2.89, $P = 0.042$ and HR = 1.43, 95% CI = 1.04–1.97, $P = 0.028$, respectively).

The importance of 3′-ERG FISH copy number in determining clinical outcome

We further stratified the 93 Edel cancers according to the number of retained copies of the FISH signal corresponding to 3′-*ERG* sequences. Kaplan–Meier plots are shown in Figures 4c and d. Univariate Cox analyses demonstrate that cancers with two or more copies of the 3′-*ERG* signal (called '2+Edel', 6.6% of all cancers) had much worse cause-specific survival and overall survival (HR = 6.10, 95% CI = 3.33–11.15, $P < 0.001$ and HR = 2.89, 95% CI = 1.86–4.48, $P < 0.001$, respectively) when compared to class N cancers. Edel cancers with a single copy of 3′-*ERG* sequences exhibited intermediate survival, but the difference from class N cancers only reached significance for overall survival (HR = 1.81, 95% CI = 0.97–3.40, $P = 0.064$ and HR = 1.60, 95% CI = 1.12–2.27, $P = 0.010$, for cause-specific and overall survival, respectively), and the differences were not significant in multivariate analyses.

The distribution of Gleason scores for the poor prognosis 2+Edel category can be seen in Table 2. The cancers include a significant proportion of Gleason score 6 and 7 cancers that on the basis of Gleason score alone would have been considered to have a good or intermediate prognosis. Multivariate analyses formally demonstrated that knowledge of *ERG* gene status provides important prognostic information in addition to that provided by Gleason score, age and PSA level at diagnosis. Thus, in these analyses, 2+Edel cancers remained a significant predictor of poorer cause-specific and overall survival compared to class N cancers (HR = 2.66, 95% CI = 1.39–5.11, $P = 0.003$ and HR = 1.84, 95% CI = 1.15–2.94, $P = 0.011$, respectively). In analyses of cause-specific data, the survival of 2+Edel cancers was 25% at 8 years, compared to 90% survival in cancers that lacked *ERG* gene rearrangement. RT–PCR analyses (result not shown) confirmed fusion of 5′-*TMPRSS2* to 3′-*ERG* transcripts in 2+Edel cancers. As expected, hybridization of 2+Edel cancers to a series of FISH probes that spanned the *TMPRSS2* and *ERG* genes demonstrated that (i) both the *TMPRSS2* and *ERG* loci are rearranged, (ii) intervening sequences between the rearranged *TMPRSS2* and *ERG* loci are deleted and (iii) both copies of rearranged 3′-*ERG* sequences had immediately juxtapositioned 5′-*TMPRSS2* sequences (Figure 5).

We also examined the effect of copy number of the 3′-*ERG* FISH signal for class Esplit patients (Figures 6a and b). Univariate Cox analyses demonstrated that class Esplit cancers with two or more FISH copies of 3′-*ERG* sequences (called '2Esplit') cancers did not differ from class N cancers in terms of cause-specific survival (HR = 2.28, 95% CI = 0.55–9.49, $P = 0.259$) or overall survival (HR = 1.55, 95% CI = 0.63–3.79, $P = 0.339$).

We assessed the effect of ploidy status within class N cancers (Figures 6c and d). We failed to find any significant difference in outcome between cancers containing a different number of unrearranged *ERG* loci in agreement with the American College of Pathologists' recommendations that the data on ploidy status is not compelling enough to warrant its routine use as a clinical marker (Bostwick and Foster, 1999).

Discussion

We have detected *ERG* rearrangements in 30% (134/445) of prostate cancers. This is higher than the value of 15% (17/111) reported by Demichelis *et al*. (2007) in a watchful waiting cohort, but lower than the value of 49% (58 of 118) found by Perner *et al*. (2006), who also assessed *ERG* gene alterations using a FISH break-apart assay. In turn all these estimates are lower than those of 55% (16 of 29) reported by Tomlins *et al.* (2006), 59% (35 of 59) reported by Wang *et al*. (2006) and 78% (14 of 18) reported by Soller *et al*. (2006) that were all obtained using PCR-based detection of *TMPRSS2–ERG* fusion transcripts. In view of our observation that incidence of *ERG* gene alterations are linked to Gleason score and to clinical stage, we suspect that these differences may be accounted for, at least in part, by the distinct clinical compositions of the cancer sets examined in each study. For example, the incidence of 15% found by Demichelis *et al.* (2007) in their series of stage T1 cancers is similar to the incidence of 19% in the stage T1 subset of cancers present in our study (Table 1). We found deletions between the *ERG* and *TMPRSS2* genes in 69.5% of cancers containing *ERG* rearrangements, in broad agreement with the value of 60.3% found by Perner *et al*. (2006).

The objective of our study was to assess whether alteration at the *ERG* gene locus could be used as part of a clinically useful, mechanism-based prognostic classification system for prostate cancer. In this respect, we have identified an *ERG* gene alteration called 2+Edel defined by the presence of two or more lone FISH copies of 3′-*ERG* in the absence of sequences 5′ to *ERG*. This alteration, found in 6.6% of cancers in our study, is associated with very poor clinical outcome and provides information on potential cancer aggression in addition to that provided by Gleason score and PSA level at diagnosis. This observation now requires validation in a prospective cohort of patients managed by current protocols, but detection of 2 +Edel could, in principle, provide a more robust marker of prognosis than Gleason score since its interpretation should not vary with observer or time. In contrast, the presence of 1Edel was of only borderline significance as a predictor of poor survival in univariate analysis. The ability of the Edel category of cancers (2+Edel Plus 1Edel) to predict clinical outcome in multivariate analyses was therefore mainly accounted for by the contribution of 2+Edel. We also examined the ability of class Esplit *ERG* alterations with two or more FISH copies of the 3′-*ERG* to predict clinical outcome. We failed to find any evidence of prognostic power, but because of the small number of such alterations it was not possible to formally exclude the importance of this cancer category.

The observation that prostate cancers with two or more FISH copies of 3′-*ERG* have worse clinical outcome than cancers that contain only a single copy of the rearranged 3′-*ERG* is consistent with the view that the high level of overexpression of *ERG* that results from the fusion of 5′-*TMPRSS2* to 3′-*ERG* (Tomlins *et al*., 2005) is responsible for driving cancer progression. The failure to observe worse clinical outcome in class Esplit cancers indicates that the loss of sequences 5′ to *ERG* found in Edel cancers is also critical to cancer aggression. It has been shown by several groups (Hermans *et al*., 2006; Iljin *et al*., 2006; Perner *et al*., 2006) that fusion of the tandemly arranged *TMPRSS2* and *ERG* fusions in prostate cancer is frequently accompanied by loss of the entire intervening chromosome 21 sequence, so a search of this region for genes with a tumour-suppressive effect may be a fruitful avenue of future investigation. Wang *et al*. (2006) have provided evidence that the presence of a particular fusion transcript between exon 2 of *TMPRSS2* and exon 4 of *ERG* that encodes a TMPRSS2–ERG fusion protein may also be associated with aggressive disease. Our studies did not analyse the specific *TMPRSS2–ERG* fusions that are presumed to be encoded by the rearranged *ERG* loci. However, this observation raises the interesting possibility that cancers containing 2+Edel together with this particular fusion may have an especially poor clinical outcome.

Our study agrees with several other studies examining the relationship between *ERG* gene status and clinical indicators in prostate cancer patients. Correlations between the presence of the *TMPRSS2–ERG* fusion and higher Gleason grade and clinical stage (Perner *et al*., 2006; Demichelis *et al*., 2007) have been reported. In a watchful waiting cohort of 111 stage T1 patients, Demichelis *et al*. (2007) found a significant association between the presence of *TMPRSS2–ERG* fusions (which would have included both class Esplit and Edel alterations examined in our study) and prostate cancer-specific death, but multivariate analyses to test independence from Gleason score were not presented. In a series of 26 patients with Gleason 7 cancer who underwent prostatectomy, Nam *et al*. (2007) found that the presence of *TMPRSS2–ERG* fusions was associated with greater probability of biochemical disease relapse. However, many of these studies have limited statistical power and our analyses importantly show that in FISH analyses of *ERG* alterations, it is the presence of '2+Edel' that provides the best predictor of poor clinical outcome, yielding prognostic information in addition to that provided by the most important prognostic factors, Gleason score and serum PSA levels at diagnosis.

New prognostic markers that can be used in the clinical management of prostate cancer are urgently required. The data presented here indicate that detection of 2+Edel could be useful as part of a new mechanism-based prognostic classification for assessing the potential future aggressiveness of every human prostate cancer at diagnosis. 2+Edel may be of particular utility in distinguishing the future clinical behaviour of the aggressive and indolent Gleason 6 and 7 cancers that are otherwise considered together to have a good or intermediate prognosis.

Materials and methods

Patient cohort

Tissue microarrays (TMAs) were constructed from 445 unselected transurethral resection of the prostate specimens taken from patients managed with no initial treatment in a cohort of conservatively managed men with prostate cancer (Cuzick *et al*., 2006). Patients from this cohort who were initially treated with hormone therapy (Cuzick *et al*., 2006) were excluded from this study, because the expression of the fusion gene under investigation of *TMPRSS2–ERG* is controlled by androgens (Tomlins *et al*., 2005). The median age of diagnosis was 70 years (49–76 years) and the median follow-up was 91 months (3–173 months). Most men were diagnosed after the age of 65 years. A competing risk analysis showed that after 10 years of follow-up, 50% of men had died: 17% from prostate cancer and 33% from other causes; only 27% were alive without progression (Cuzick *et al*., 2006). National approval for the collection of the cohort was obtained from the Northern Multi-Research Ethics Committee followed by local ethics committee approval at each of the collaborating hospital trusts. This work was approved by the Clinical Research and Ethics Committee at the Royal Marsden Hospital and Institute of Cancer Research.

Tissue microarrays

TMAs were constructed in 35×22×7mm blocks of Lamb paraffin wax using a manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). Up to four cores of 600 μm diameter were taken from each tumour. Reassignment of areas of 'cancer' or 'normal' in each core was carried out on the basis of histopathological examination of haematoxylin and eosin and p63/AMACR-stained sections that flanked the TMA slice used for FISH studies. The morphological criteria for selection of 'normal' and 'malignant' prostatic epithelium conformed to previously published definitions (Foster, 2000; Foster *et al*., 2000, 2004). 'Hyperplasia', 'dysplasia' and 'PIN' were not scored in this study.

FISH studies

TMA sections (4 μm) were cut onto SuperFrostPlus glass slides (VWR International, Poole, UK). FISH studies were carried out by a similar method to that described by Lambros *et al*. (2006). TMA slices were dewaxed in xylene (55°C, 3 × 5 min), washed in ethanol and then boiled in ethanol for 30 s. The slices were then boiled in pre-treatment buffer (SPOT-light tissue pre-treatment kit, Zymed, South San Francisco, CA, USA) for 15 min, rinsed in water, digested with pepsin solution (Digest All-3, Invitrogen, Paisley, UK) at 28°C for 4.5 min, rinsed in water and drained before hybridization. For the *ERG* 'break-apart' assay, 100 ng of each BAC probe (Biotin-labelled BACs RP11-95G19, RP11-720N21 and CTD-2511E13 corresponding to 3′-*ERG* sequences and DIG-labelled BACs RP11-372O17, RP11-115E14 and RP11-729O4 corresponding to sequences immediately upstream of the *ERG* gene), 5 μg COT-1, 1 μg salmon sperm DNA (Invitrogen) combined in a total volume of 3.5 μl were added to 9.6 μl of hybridization buffer (60% (v/v) formamide, 12% (w/v) dextran sulphate, 2.4 × SSC, 0.14mM EDTA pH 8, 400 μg/ml salmon sperm DNA), mixed and pipetted onto a 22 × 22mm plastic coverslip (Sigma-Aldrich, Poole, UK). The TMA slide was inverted onto the coverslip and sealed in a metal hybridization chamber containing 200 μl 6 × 0.15M NaCl, 0.15M Na_3 citrate; pH 7.0 (SSC). The chamber was heated to 98°C for 9 min and then transferred to 37° C overnight.

Following hybridization, the coverslips were removed and the TMA slices rinsed in 2 × SSC at 42°C, twice for 5 min in 50% (v/v) formamide/2 × SSC at 42°C, twice for 5 min in 2 × SSC at 42°C and for 3 min in SSCT (4 × SSC/0.5% (v/v) Tween-20 (Sigma-Aldrich)) at room temperature. Slices were drained, placed on a rack over a 37°C water bath and flooded with SSCTM (10 ml SSCT/0.5 g skimmed milk powder (Marvel Premier International Foods, Lincs, UK), 0.4 μm filtered) for 15 min and then washed for 3 min in SSCT. A total of 150 μl SSCTM containing 0.75 μl anti-DIG-FITC (Roche, Welwyn Garden City, UK) was pipetted onto the TMA slice under a 22 × 50mm (#1.5 Sigma-Aldrich) glass coverslip, incubated for 37°C for 10 min and then washed three times for 2 min in SSCT at room temperature. This procedure was then repeated with 0.75 μl Streptavidin-Cy3 conjugate (Sigma-Aldrich) in 150 μl SSCTM at 37°C for 10 min. Slides were washed for 2 min in SSCT at room temperature, then twice for 5 min in phosphate-buffered saline, rinsed in 70% (v/v) ethanol and air dried. The TMA slice was mounted in 10 μl Vectashield anti-fade containing 4′,6-diamidino-2-phenylindole (Vector labs, Burlingame, CA, USA) and scanned using an Ariol SL-50 (Applied Imaging, San Jose, CA, USA). Using this method the success rate in obtaining FISH results from TMA cores was greater than 90%. For re-hybridization DAPI and the old probe were removed by washing in 2 × SSC twice for 10 min at room temperature followed by 2 min in 2 × SSC and 4 min in 70% (v/v) formamide/2 × SSC at 68°C. Slides were then washed in water and re-hybridized as described above. The identities of the probes used in additional FISH assays are described in Figure 1.

Evaluation of the FISH results in each core that contained cancer was independently performed by two operators (GA and JCl) who, for each nucleus, scored the number of unrearranged *ERG* loci (twinned red and green signals), separate 5′ *ERG* sequences (labelled green) and separate 3′ *ERG* sequences (labelled red). At least 100 epithelial nuclei per case were evaluated and in each case the modal value, which was almost invariably also the maximum value and usually present in at least 50% of the nuclei, was taken as the score. For example, in cancers judged as having two copies of unrearranged *ERG*, two copies were always observed in at least 80% of nuclei. Lower numbers of signals were observed in a proportion of nuclei: this reflected the fact that some nuclei were sliced and had missing signals, and was not thought to reflect pattern heterogeneity.

Statistical analysis

The primary endpoints for this study were time to death from prostate cancer and time to death from any cause. Univariate and multivariate analysis were performed by proportional hazard (Cox) regression analysis (Cox and Oakes, 1984). All follow-up times commenced at the point of 6 months following diagnosis as in the previous report (Cuzick *et al*., 2006). Associations between categorical data were examined using the χ^2 test and Fisher's exact test when expected cell counts were less than 5. Associations between categorical and numerical variables were assessed using analysis of variance. All *P*-values were two-sided. The following variables, determined as described previously (Cuzick *et al*., 2006), were included in the multivariate analyses: centrally reviewed Gleason score, baseline PSA (last PSA value within 6 months of diagnosis) and age at diagnosis. Clinical stage data, only available for up to 60% of patients and found to be of minimal significance in our previous analysis (Table 1), were not included.

Acknowledgements

This work was funded by Cancer Research UK, National Cancer Research Institute, a specialized program of Research Excellence grant from US National Cancer Institute, Grand Charity of Freemasons, Rosetrees Trust, The Bob Champion Cancer Trust, The Orchid Appeal and David Koch Foundation. Funding bodies had no involvement in the design and conduct of the study, or in collection management, analysis and interpretation of the data or in preparation, review or approval of the manuscript. We thank Christine Bell and Sandra Edwards for help in typing the manuscript and preparing figures and tables.

References

Adolfsson J, Steineck G, Hedlund PO. Deferred treatment of clinically localized low-grade prostate cancer: actual 10 year and projected 15 year follow up of the karolinska series. Urology 1997;50:722–726. [PubMed: 9372882]

Albertsen PC, Fryback DG, Storer BE, Kolon TF, Fine J. Long term survival among men with conservatively treated localized prostate cancer. JAMA 1995;274:626–631. [PubMed: 7637143]

Albertsen PC, Hanley JA, Barrows GH, Penson DF, Kowakczyk PD, Sanders MM, et al. Cancer and the Will Rogers phenomenon. J Natl Cancer Inst 2005;97:1248–1253. [PubMed: 16145045]

Allsbrook WC Jr, Mangold KA, Johnson MH, Lane RB, Lane CG, Amin MB, et al. Interobserver reproducibility of gleason grading of prostatic carcinoma: urologic pathologists. Hum Pathol 2001a; 32:74–80. [PubMed: 11172298]

Allsbrook WC Jr, Mangold KA, Johnson MH, Lane RB, Lane CG, Epstein JI. Interobserver reproducibility of gleason grading of prostatic carcinoma: general pathologist. Hum Pathol 2001b; 32:81–88. [PubMed: 11172299]

Bill-Axelson A, Holmberg L, Ruutu M, Haggman M, Anderson SO, Bratell S, et al. Radical prostatectomy versus watchful waiting in early prostate cancer. N Engl J Med 2005;352:1977–1984. [PubMed: 15888698]

Bostwick DG, Foster CS. Predictive factors in prostate cancer: current concepts from the 1999 college of American pathologists conference on solid tumor prognostic factors and the 1999 world health organization second international consultation on prostate cancer. Semin Urol Oncol 1999;17:222–272. [PubMed: 10632123]

Chism DB, Hanlon AL, Troncoso P, Al Saleem T, Horwitz EM, Pollack A. The gleason score shift: score four and seven years ago. Int J Radiat Oncol Biol Phys 2003;56:1241–1247. [PubMed: 12873667]

Chodak GW, Thisted RA, Gerber GS, Johansson JE, Adolfsson J, Jones GW, et al. Results of conservative management of clinically localized prostate cancer. N Engl J Med 1994;330:242–248. [PubMed: 8272085]

Clark J, Merson S, Jhavar S, Flohr P, Edwards S, Foster CS, et al. Diversity of TMPRSS2-ERG fusion transcripts in the human prostate. Oncogene 2007;26:2667–2673. [PubMed: 17043636]

Cox, DR.; Oakes, D. Analysis of Survival Data. Chapman & Hall; London, New York: 1984.

Cuzick J, Fisher G, Kattan MW, Berney D, Oliver T, Foster CS, et al. Long-term outcome among men with conservatively treated localised prostate cancer. Br J Cancer 2006;95:1186–1194. [PubMed: 17077805]

Demichelis F, Fall K, Perner S, Andren O, Schmidt F, Setlur SR, et al. TMPRSS2:ERG gene fusion associated with lethal prostate cancer in a watchful waiting cohort. Oncogene. 2007epub ahead of publication, 22 Janurary

Foster CS. Pathology of benign prostatic hyperplasia. Prostate Suppl 2000;9:4–14. [PubMed: 11056496]

Foster CS, Bostwick DG, Bonkhoff H, Damber JE, van der KT, Montironi R, et al. Cellular and molecular pathology of prostate cancer precursors. Scand J Urol Nephrol Suppl 2000;205:19–43. [PubMed: 11144897]

Foster CS, Falconer A, Dodson AR, Norman AR, Dennis N, Fletcher A, et al. Transcription factor E2F3 overexpressed in prostate cancer independently predicts clinical outcome. Oncogene 2004;23:5871–5979. [PubMed: 15184867]

Hermans KG, van Marion R, van Dekken H, Jenster G, van Weerden WM, Trapman J. 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. [PubMed: 17108102]

Holmberg L, Bill-Axelson A, Helgesen F, Salo JO, Folmerz P, Haggman M, et al. A randomised trial comparing radical prostatectomy with watchful waiting in early prostate cancer. N Engl J Med 2002;347:781–789. [PubMed: 12226148]

Iljin K, Wolf M, Edgren H, Gupta S, Kilpinen S, Skotheim RI, 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. [PubMed: 17079440]

Johansson JE, Andren O, Anderson SO, Dickman PW, Holmberg L, Magnuson A, et al. Natural history of early localised prostate cancer. JAMA 2004;291:2713–2719. [PubMed: 15187052]

Lambros MB, Simpson PT, Jones C, Natrjan R, Westbury C, Steele D, et al. Unlocking pathology archives for molecular genetic studies: a reliable method to generate probes for chromogenic and fluorescent *in situ* hybridisation. Lab Invest 2006;86:398–408. [PubMed: 16446704]

Melia J, Moseley R, Ball RY, Griffiths DF, Grigor K, Harnden P, et al. A UK-based investigation of inter- and intra-observer reproducibility of gleason grading of prostatic biopsies. Histopathology 2006;48:644–654. [PubMed: 16681679]

Nam RK, Sugar L, Wang Z, Yang W, Kitching R, Klotz LH, et al. Expression of TMPRSS2 ERG gene fusion in prostate cancer cells is an important prognostic factor for cancer progression. Cancer Biol Ther. 2007epub ahead of publication, 2 January

Oyama T, Allsbrook WC Jr, Kurokawa K, Matsuda H, Segawa A, Sano T, et al. A comparison of interobserver reproducibility of gleason grading of prostatic carcinoma in Japan and the United States. Arch Pathol Lab Med 2005;129:1004–1010. [PubMed: 16048389]

Perner S, Demichelis F, Beroukhim R, Schmidt FH, Mosquera JM, Setlur S, et al. TMPRSS2:ERG fusion-associated deletions provide insight into the heterogeneity of prostate cancer. Cancer Res 2006;66:8337–8341. [PubMed: 16951139]

Smith EB, Frierson HF Jr, Mills SE, Boyd JC, Theodorescu D. Gleason scores of prostate biopsy and radical prostatectomy specimens over the past 10 years: is there evidence for systematic upgrading? Cancer 2002;94:2282–2287. [PubMed: 12001128]

Soller MJ, Isaksson M, Elfving P, Soller W, Lundgren R, Panagopoulos I. Confirmation of the high frequency of the TMPRSS2/ERG fusion gene in prostate cancer. Genes Chromosomes Cancer 2006;45:717–719. [PubMed: 16575875]

Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, et al. TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. Cancer Res 2006;66:3396–3400. [PubMed: 16585160]

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 2005;310:644–648. [PubMed: 16254181]

van der Kwast TH, Ciatto S, Martikainen PM, Hoedemaeker R, Laurila M, Pihl CG, et al. Detection rates of high-grade prostate cancer during subsequent screening visits. Results of the european randomized screening study for prostate cancer. Int J Cancer 2006;118:2538–2542. [PubMed: 16353141]

Wang J, Cai Y, Ren C, Ittmann M. Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. Cancer Res 2006;66:8347–8351. [PubMed: 16951141]

Yao SL, Lu-Yao G. Understanding and appreciating over-diagnosis in the PSA era. J Natl Cancer Inst 2002;94:958–960. [PubMed: 12096074]

Yoshimoto M, Joshua AM, Chilton-Macneill S, Bayani J, Selvarajah S, Evans AJ, et al. Three-color FISH analysis of TMPRSS2/ERG fusions in prostate cancer indicates that genomic microdeletion of chromosome 21 is associated with rearrangement. Neoplasia 2006;8:465–469. [PubMed: 16820092]

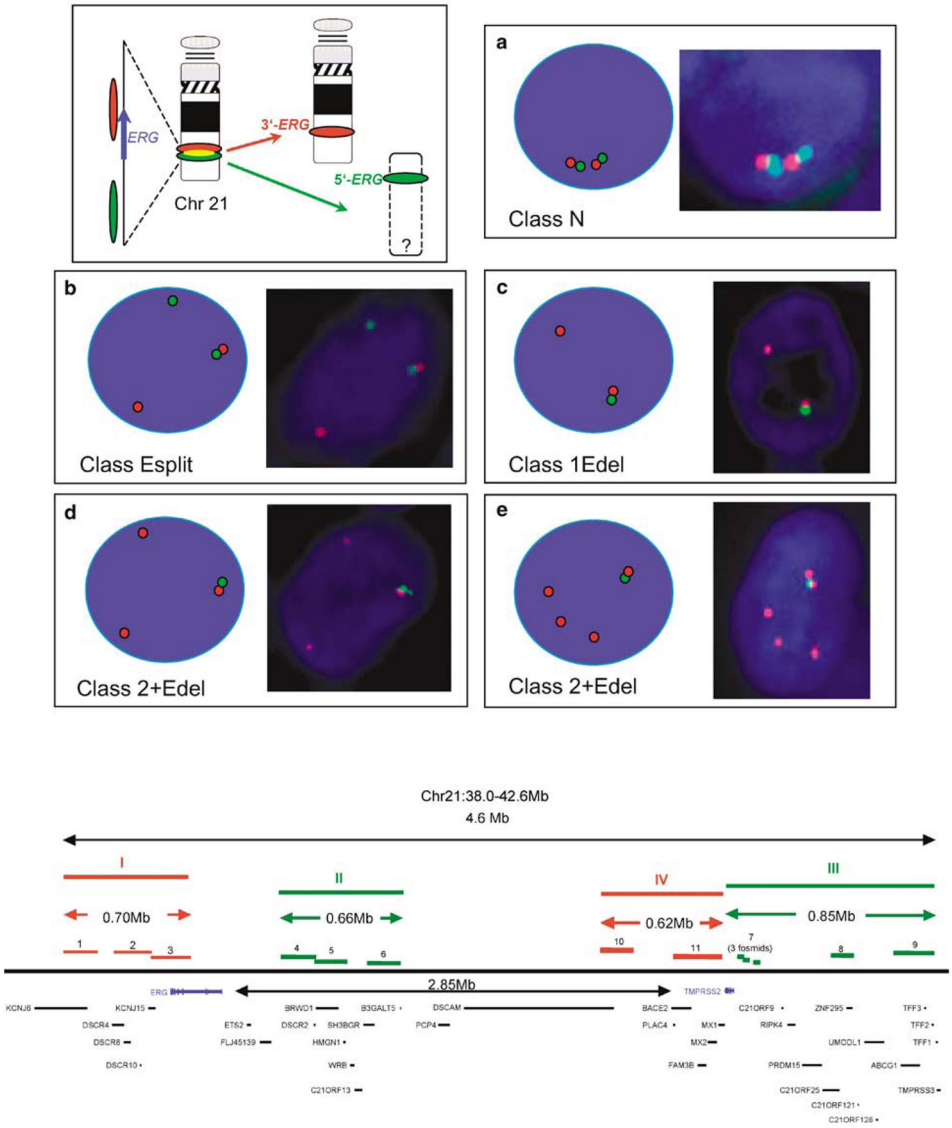

Figure 1.

FISH detection of *ERG* gene breakpoints. Top right: Principle of detection of *ERG* gene status. Interphase nuclei are hybridized to probes that detect sequences 5′ to the *ERG* gene (green) and 3′ to the *ERG* gene (red). The red and green signals are separated when an *ERG* gene rearrangement occurs. (**a**) Signals from normal unrearranged *ERG* loci (class N). (**b**) Signals from rearranged *ERG* gene with retention of separated red (3′) and green (5′) probes (class Esplit). (**c**) Example of a '1Edel' cancer: the rearrangement is associated with deletion of sequences 5′ to *ERG* (green) with retention of a single red 3′-*ERG* signal. (**d** and **e**) Examples of 2+Edel cancers: the rearrangement is associated with deletion of sequences 5′ to *ERG* (green) with retention of two or more red 3′-*ERG* signals. Bottom: Map of the *ERG* and *TMPRSS2* genes showing the position of the BACs and fosmids used as probes in FISH assays. Probe I: 1:RP11-95G19, 2:RP11-720N21, 3:CTD-2511E13; probe II 4:RP11-372O18, 5:RP11-115E14, 6:RP11-729O4; probe III, 7:three pooled fosmids (G248P89444D12, G248P800876A1, G248P8239C5), 8:RP11-35C4, 9:RP11-282I20; probe IV, 10:RP11-114H1, 11:RP11-662D5. Probes I and II correspond, respectively, to sequences

immediately 5′ (green) and 3′ (red) to the *ERG* gene. Probes III and IV correspond, respectively, to sequences immediately 5′ (green) and 3′ (red) to the *TMPRSS2* gene.

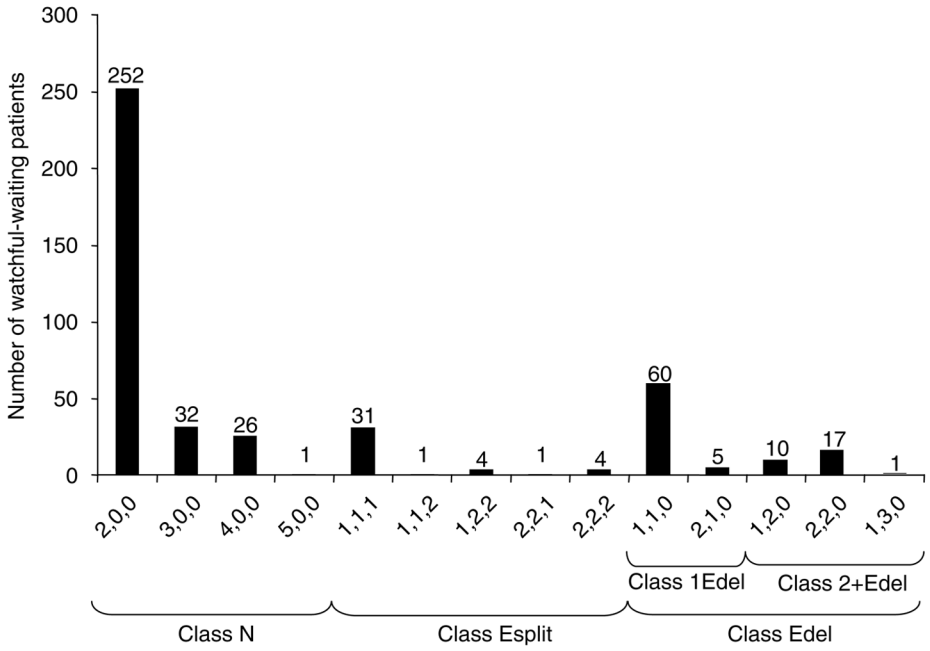

Figure 2.

Distribution of the pattern of *ERG* gene alterations in human prostate cancer. Each cancer is scored for three classes of FISH signals: normal unrearranged *ERG* loci (adjacent red and green signals); separate red signals that correspond to sequences 3′ to a rearranged *ERG* locus and separate green signals that correspond to sequences 5′ to a rearranged *ERG* locus. For example, '2,1,0' means that the cancer contains two normal alleles, one separate red 3′-*ERG* signal and no green 5′-*ERG* signals. Cancers with only normal signals are designated as class N. When the *ERG* locus is split to form both separate 3′-*ERG* (red) and 5′-*ERG* (green) signals, the cancer is designated as class Esplit. Cancers containing separate red 3′ to *ERG* signals, but lacking separate green signals are designated 'Edel' cancers.

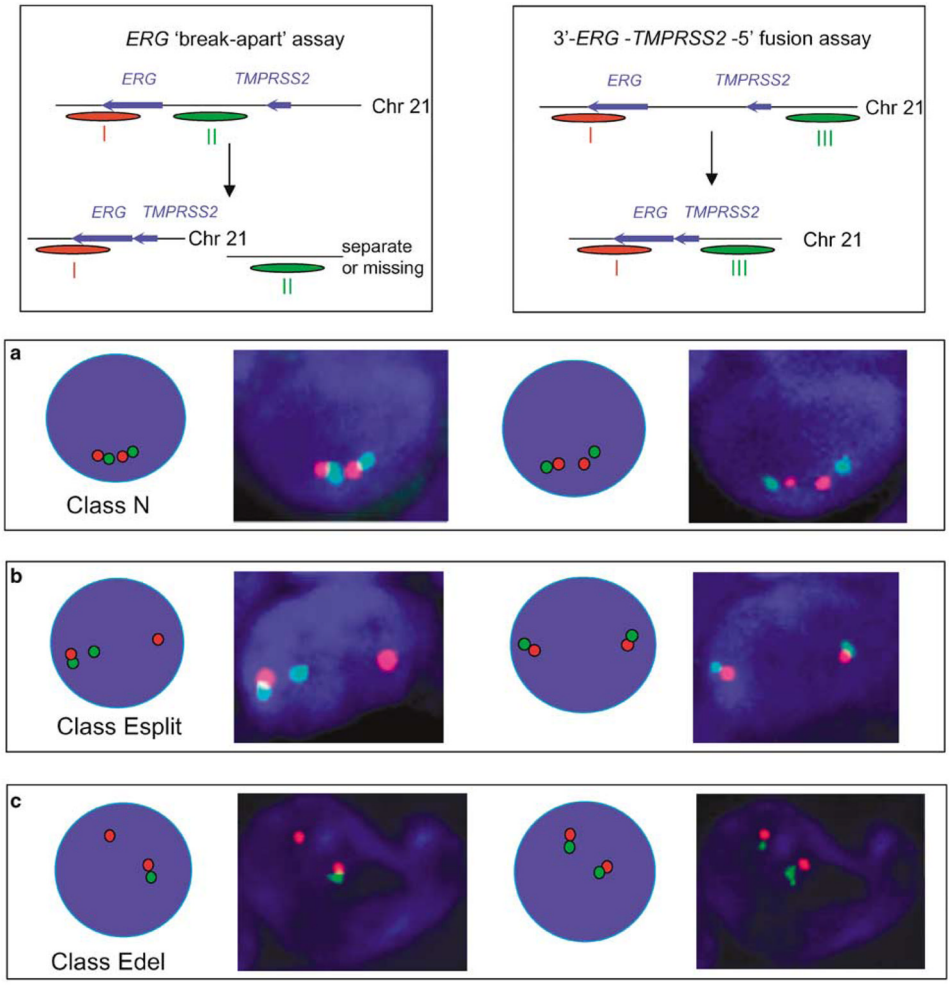

Figure 3.

FISH detection of *TMPRSS2* and *ERG* gene status. (left-hand side): the status of the *ERG* gene was examined using the *ERG* 'break-apart' assay using probes that detected sequences immediately 5′ (green, probe II) and 3′ (red, probe I) to *ERG*. (Righthand side): the same representative cancer cell was re-hybridized with FISH probes that detected sequences 5′ to *TMPRSS2* (green, probe III) and 3′ to *ERG* (red, probe I). The principle of detection is in each case shown at the top and the precise origin of the three separate FISH probes used in these studies (probes I–III) can be seen in Figure 1. Nuclei shown in this figure were from cancers with the following *ERG* status: class N (**a**), class Esplit (**b**) and class Edel (**c**). RT–PCR studies carried out as described previously (Clark *et al*., 2007) confirmed the presence of *TMPRSS2*–*ERG* fusion transcripts only in cancers that contained rearranged *ERG* (result not shown). A comparison of the left- and right-hand panels in this figure shows that rearranged 3′-*ERG* always remains linked to 5′-*TMPRSS2*, and that the sequences examined by FISH located between the *TMPRSS2* and *ERG* loci (probe II) are either missing (class Edel cancers) or positioned elsewhere in the nucleus (class Esplit cancers).

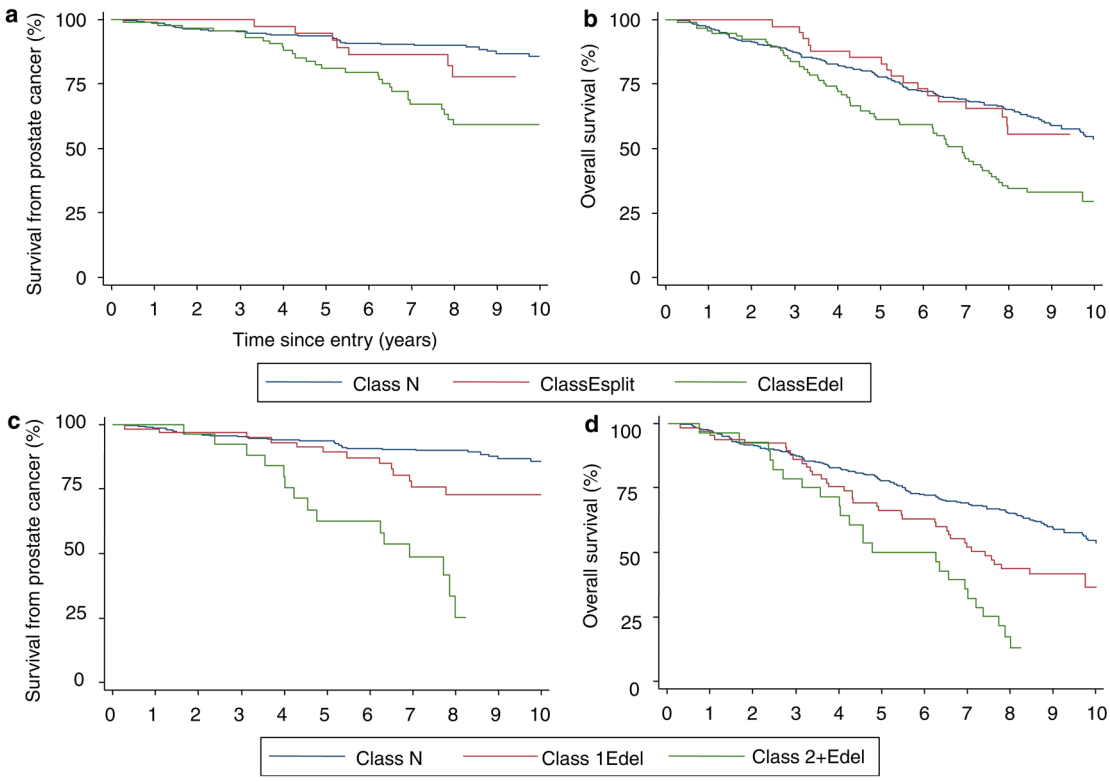

Figure 4.

Kaplan–Meier analysis comparing prostate cancer outcomes for different categories of *ERG* gene alteration. (**a** and **b**) A comparison of class N, class Esplit and class Edel cancers. (**c** and **d**) Outcome stratified according to the copy number of 3′-*ERG* FISH signal in Edel cancers. The Kaplan–Meier curves compare class N cancer with Edel cancer containing a single copy of 3′-*ERG* sequences (1Edel), and with Edel cancer that contain two or more copies of 3′-*ERG* sequences (2+Edel). **a** and **c** are cause-specific survival. **b** and **d** are overall survival.

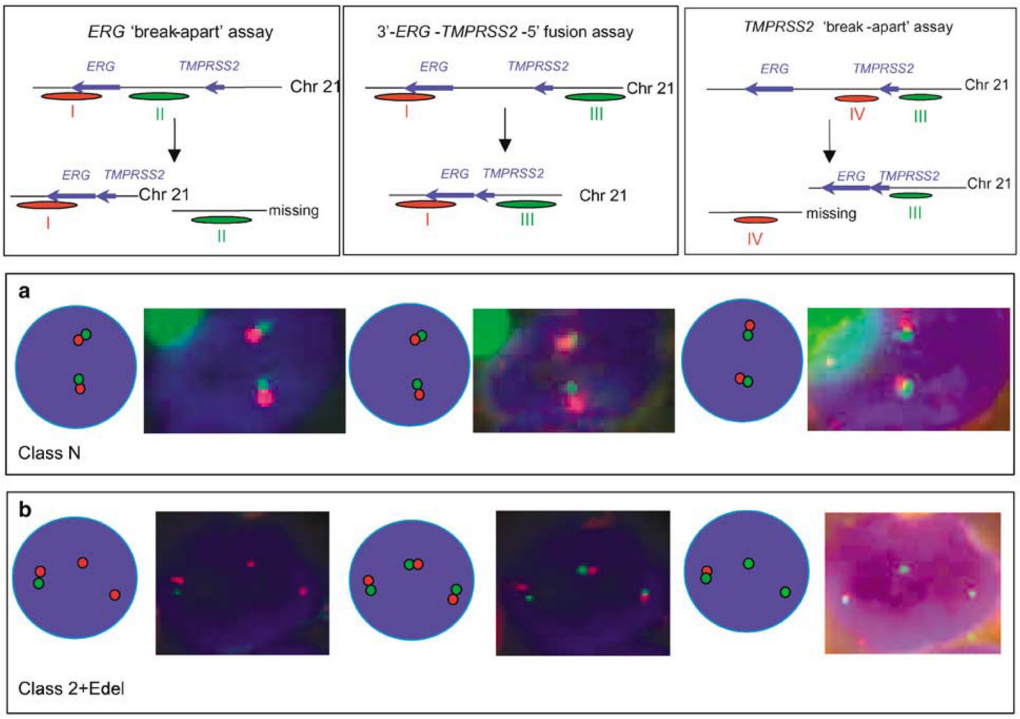

Figure 5.

FISH analysis of the *TMPRSS2* and *ERG* loci in 2+Edel cancers. The 'left' and 'middle' panels in this figure are the same as the 'left' and 'right panels' in Figure 3 showing, respectively, results from the *ERG* locus 'break-apart' assay (left), and from the FISH detection of sequences 5′ to *TMPRSS2* and 3′ to *ERG* (middle). Additionally in the right-hand panel, the status of the *TMPRSS2* gene has been examined using a *TMPRSS2* 'break-apart' assay by re-hybridizing the same representative cancer cell with FISH probes that detected sequences immediately 5′ (green, probe III) and 3′ (red, probe IV) to *TMPRSS2*. The principle of detection is in each case shown at the top and the precise origin of the four separate FISH probes used in these studies (probes I–IV) can be seen in Figure 1. The nuclei shown in this figure were from a class N cancer (**a**) and a class 2+Edel cancer. (**b**) RT–PCR studies carried out as described previously (Clark *et al.*, 2007) confirmed the presence of *TMPRSS2–ERG* fusion transcripts only in the 2+Edel cancer (result not shown). These analyses demonstrate that cancers with a rearranged *ERG* locus also have a rearranged *TMPRSS2* locus, (ii) the rearranged 5′-*TMPRSS2* and 3′-*ERG* sequences remain joined together and (iii) the rearrangement and joining of 5′-*TMPRSS2* and 3′-*ERG* is accompanied by deletion of intervening sequences corresponding to both probes II and IV.

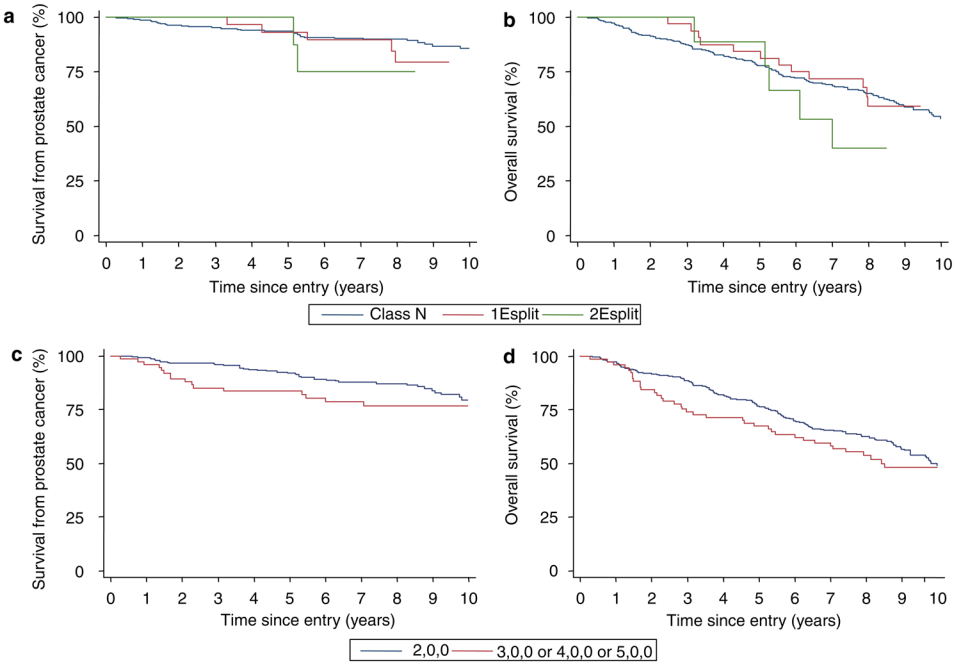

Figure 6.

(**a** and **b**) Kaplan–Meier analysis stratifying prostate cancer outcomes according to copy number of 3′-*ERG* FISH signal: (**a**) class Esplit cancers, cause-specific survival; (**b**) class Esplit cancers, overall survival. (**c** and **d**) Kaplan–Meier analysis comparing prostate cancers outcomes depending on *ERG* ploidy status: (**c**) cause-specific survival and (**d**) overall survival.

Table 1

Relationship of *ERG* gene status with demographics and tumour characteristics

Variable	Class N (*n* = 311)	Class Esplit (*n* = 41)	Edel (*n* = 93)	*P*
Mean age ±s.d. (years)	69±5	69±5	69±5	0.765
Gleason score[a]				
<7	201 (65%)	17 (41%)	25 (27%)	<0.001
= 7	66 (21%)	15 (37%)	32 (35%)	
>7	42 (14%)	9 (22%)	35 (38%)	
Clinical stage[b]				
T1	110 (60%)	6 (27%)	17 (35%)	0.001
T2	56 (31%)	10 (46%)	22 (45%)	
T3	17 (9%)	6 (27%)	10 (20%)	
Baseline PSA				
≤4	144 (46%)	7 (17%)	22 (24%)	<0.001
>4–10	73 (24%)	12 (29%)	17 (18%)	
>10–25	55 (18%)	7 (17%)	25 (27%)	
>25–50	25 (8%)	9 (22%)	24 (26%)	
>50–100	14 (5%)	6 (17%)	5 (5%)	

Abbreviation: PSA, prostate-specific antigen.

[a]Restricted to patients for whom Gleason score is available.

[b]Restricted to patients for whom clinical stage is available.

Table 2

Relationship between *ERG* gene status and Gleason score

Gleason score	*ERG* gene status[a]				
	Class N	1Esplit	2Esplit	1Edel	2+Edel
4	3	0	0	0	0
5	18	1	0	1	0
6	180	14	2	22	2
7	66	9	6	20	12
8	28	4	0	12	6
9	13	4	1	8	7
10	1	0	0	0	0
Unassigned	2	0	0	1	0

[a]Class N: unrearranged *ERG*. Esplit indicates that *ERG* gene is split to form both separate 3-*ERG* (red) and 5′-*ERG* (green) signals: 1Esplit, one copy of 3′-*ERG* signal; 2Esplit, two copies of 3′-*ERG* signal. Edel cancers contain separate red 3′-*ERG* signals in the absence of separate green 5′-*ERG* signals: 1Edel, one copy of 3′-*ERG* signal; 2+Edel, two or more copies of 3′-*ERG* signal.