Molecular characterisation of ERG, ETV1 and PTEN gene loci identifies patients at low and high risk of death from prostate cancer

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BACKGROUND: The discovery of ERG/ETV1 gene rearrangements and PTEN gene loss warrants investigation in a mechanism-based prognostic classification of prostate cancer (PCa). The study objective was to evaluate the potential clinical significance and natural history of different disease categories by combining ERG/ETV1 gene rearrangements and PTEN gene loss status.

METHODS: We utilised fluorescence in situ hybridisation (FISH) assays to detect PTEN gene loss and ERG/ETV1 gene rearrangements in 308 conservatively managed PCa patients with survival outcome data.

RESULTS: ERG/ETV1 gene rearrangements alone and PTEN gene loss alone both failed to show a link to survival in multivariate analyses. However, there was a strong interaction between ERG/ETV1 gene rearrangements and PTEN gene loss (P<0.001). The largest subgroup of patients (54%), lacking both PTEN gene loss and ERG/ETV1 gene rearrangements comprised a ‘good prognosis’ population exhibiting favourable cancer-specific survival (85.5% alive at 11 years). The presence of PTEN gene loss in the absence of ERG/ETV1 gene rearrangements identified a patient population (6%) with poorer cancer-specific survival that was highly significant (HR = 4.87, P<0.001) in multivariate analysis, 13.7% survival at 11 years) when compared with the ‘good prognosis’ group. ERG/ETV1 gene rearrangements and PTEN gene loss status should now prospectively be incorporated into a predictive model to establish whether predictive performance is improved.

CONCLUSIONS: Our data suggest that FISH studies of PTEN gene loss and ERG/ETV1 gene rearrangements could be pursued for patient stratification, selection and hypothesis-generating subgroup analyses in future PCa clinical trials and potentially in patient management.

Keywords: ERG/ETV1 gene rearrangements; fluorescence in situ hybridisation; PTEN gene loss; prostate cancer; survival

Prostate cancer (PCa) is the most commonly diagnosed male cancer and the second commonest cause of male cancer related mortality in the Western world (Ferlay et al, 2007). The clinical behaviour and molecular pathology of PCa is highly variable. There is an urgent need to dissect this inter-patient heterogeneity with robust molecular biomarkers to accelerate the successful conduct of clinical trials for this disease, optimise patient treatment and minimise late drug development attrition (Betensky et al, 2002; Attard et al, 2008a). Critically, identifying patient subgroups that require less treatment from those that should be targeted with more aggressive therapy is a key goal.

PTEN loss and ETS gene rearrangements are proposed to be critically important and common molecular events in prostate carcinogenesis (Trotman et al, 2003; Tomlins et al, 2005, 2008a; Carver et al, 2009; King et al, 2009). In particular, recent publications have addressed the relationship between the two events in mouse models demonstrating cooperation (Carver et al, 2009; King et al, 2009). Deletion of all or part of the tumour suppressor gene PTEN is a frequent event. Other classes of alterations including mutations and post-translational modifications occur less frequently (Whang et al, 1998; Verhagen et al, 2006; Yoshimoto et al, 2007, 2008). Previous studies have examined the prognostic significance of PTEN deletions by fluorescence in situ hybridisation (FISH) with small patient cohorts and biochemical recurrence as the outcome (Yoshimoto et al, 2007, 2008). Similarly the clinical implications of the ETS gene rearrangements (Tomlins et al, 2005, 2007) are not yet fully
understood (Clark and Cooper, 2009), with their presence reported as associated with both poor (Demichelis et al., 2007; Nam et al., 2007; Attard et al., 2008b) and good prognosis (Petrovics et al., 2005; Gopalan et al., 2009; Hermans et al., 2009). We reported that deletion of the intermediate region between TMPRSS2 and ERG combined with duplication of the TMPRSS2–ERG fusion sequences is predictive of poor cancer-specific survival (Attard et al., 2008b), an observation supported by other studies (FitzGerald et al., 2008; Yoshimoto et al., 2008; Gopalan et al., 2009). The effect of PTEN loss on PCa survival and its relationship to the ETS gene rearrangements is largely unknown.

To evaluate the natural history of the different disease variants identified by PTEN, ERG and ETV1 FISH we evaluated a retrospective cohort of conservatively managed men (Cuzick et al., 2006). Immunohistochemistry (IHC) was not carried out because of concerns regarding potential interobserver interpretation variability (Kirkegaard et al., 2006); lack of uniformity regarding tissue processing as samples were taken from different hospitals (Cuzick et al., 2006); and previous studies reporting conflicting results with IHC (Bedolla et al., 2007; McCall et al., 2008; Sircar et al., 2009). Rearrangements involving ETV4 and ETV5 have also been reported, but these are rare and therefore unlikely to be used widely in a clinically applicable test (Kumar-Sinha et al., 2008).

PATIENTS AND METHODS

Patient cohort

Tissue microarrays (TMAs) were constructed from unselected transurethral resection of prostate (TURP) specimens from patients who received no initial treatment in a cohort of conservatively managed men with PCa (Cuzick et al., 2006). Ethical approval for the collection of the cohort was obtained from the Ethics Review Committees at every collaborating hospital.

TMAs and FISH studies

Tissue microarrays were constructed as previously described (Attard et al., 2008b). Areas of ‘cancer’ and ‘normal’ were identified on the basis of histopathological examination of haematoxylin and eosin and p63/AMACR-stained sections that flanked the TMA slice used for FISH studies (Figure 1). To assess the frequency of PTEN gene loss in TMA cancer cores, FISH procedures were carried out as described previously for rearrangement at the ERG and ETV1 loci (Attard et al., 2008b, c). The same (or an immediately adjacent) slice was stripped and rehybridised for PTEN. We selected PTEN BACs that map to the minimum region of PTEN deletion in PCa as previously described in xenografts and cell lines (Hermans et al., 2004). We used two, overlapping, DIG-labelled BAC probes to the 5' end of the PTEN locus, RP11-765C10 and RP11-959L24, and a commercially available CY-3 labelled DNA chromosome 10 centromere probe (chromosome 10 (p11.1 Bq11.1 Abbott Molecular, Des Plaines, IL, USA) (Figure 2A). Representative images of nuclei with normal, heterozygous and homozygous PTEN patterns are shown in Figures 1 and 2B.

Establishing PTEN cut-offs

Evaluation of the FISH results in each core containing cancer was independently carried out by two operators (A-R and G-A) who were unaware of clinical data. Signals were scored in at least 200 non-overlapping nuclei. We assumed that part of some nuclei can be lost during slicing and therefore not all nuclei in a section

Figure 1  Haematoxylin and eosin (H & E), fluorescence in-situ hybridisation (FISH) and P63/alpha-methylacyl-CoA racemase (AMACR) on adjacent slides (A) a prostate cancer gland with H & E staining; (B) ETV1 FISH in the same gland on an adjacent slide. Cartoon and magnified images of two nuclei are also shown. The upper nucleus has four paired (ploidy) ETV1 probes and the lower nucleus has two paired ETV1 probes indicating wild-type ETV1; FISH for ERG also showed paired probes indicating wild-type ERG (image not shown); (C) PTEN FISH in the same gland on an adjacent slide. Cartoon and magnified images of one nucleus is shown. The nucleus has four (ploidy) chromosome, 10 centromeric probes (in red) and two PTEN probes (in green) indicating heterozygous loss of PTEN; and (D) the same prostate cancer gland on an adjacent slide, which has absent P63 staining and AMACR positivity.
Clinical Studies

Table 1. Relationship of PTEN status with demographics and tumour characteristics

| Variable                  | Normal PTEN (n = 266) | PTEN loss (n = 56) | P-value* |
|---------------------------|-----------------------|-------------------|----------|
| Mean age ± s.d. (years)   | 69 ± 5                | 70 ± 4            | 0.18     |
| Classes of age (years)    |                       |                   |          |
| ≤ 65                      | 51                    | 8 (14)            |          |
| > 65 – 70                 | 72                    | 13 (15)           |          |
| > 70 – 73                 | 72                    | 14 (16)           | 0.12     |
| > 73 – 76                 | 71                    | 21 (23)           |          |
| Gleason score             |                       |                   |          |
| ≤ 7                       | 146                   | 5 (3)             |          |
| = 7                       | 65                    | 18 (22)           | <0.001   |
| > 7                       | 55                    | 33 (38)           |          |
| Baseline PSA (ng ml⁻¹)    |                       |                   |          |
| ≤ 4                       | 104                   | 9 (8)             |          |
| > 4 – 10                  | 55                    | 7 (11)            |          |
| > 10 – 25                 | 51                    | 14 (21)           | <0.001   |
| > 25 – 50                 | 31                    | 16 (34)           |          |
| > 50 – 100                | 25                    | 10 (29)           |          |
| Clinical stageb           |                       |                   |          |
| T1                        | 81                    | 6 (7)             |          |
| T2                        | 50                    | 14 (22)           | <0.001   |
| T3                        | 19                    | 19 (50)           |          |
| Cancer in biopsyc         |                       |                   |          |
| ≤ 6                       | 74                    | 2 (3)             |          |
| > 6 – 20                  | 75                    | 5 (6)             |          |
| > 20 – 40                 | 38                    | 10 (21)           | <0.001   |
| > 40 – 75                 | 31                    | 13 (30)           |          |
| > 75 – 100                | 45                    | 26 (37)           |          |

*Test for trend in PTEN loss group, (except for mean age). *Restricted to patients for whom clinical stage is available. *Restricted to patients for whom extent of disease is available.

RESULTS

General

PTEN gene status was established from 678 cancer cores from 322 patients. Tissue cores can hybridise with variable efficiency for different FISH probes. Moreover, cores can sometimes be lost in the rehybridisation process. Therefore, ERG and ETV1 gene status was available from 308 of the 322 patients with a PTEN score (662 TMA cancer cores). Demographic and characteristics for tumours stratified for PTEN status alone are shown in Table 1 and according to PTEN and ERG/ETV1 gene status shown in Table 2. The median follow-up was 100 months (3–197 months). A competing risk analysis demonstrated that after 11 years of follow-up, 59% of men had died: 25% from PCa and 34% from other causes; only 22% were alive without progression. Results are described for both cause-specific prostate survival and overall survival.

PTEN gene loss alone is not a significant predictor of clinical outcome in multivariate analysis

There was no statistically significant difference in outcome between the tumours with heterozygous and those with homozygous
PTEN loss (data not shown). Therefore, the analyses presented consider PTEN loss tumours as one group. Of the 322 patients with a PTEN score, 266 (83%) had a normal PTEN score and 56 (17%) had PTEN loss. There were significant associations between PTEN loss and Gleason score (< 0.001), clinical stage (< 0.001), baseline PSA (< 0.001) and cancer in biopsy (proportion of TURP chips with disease or linear proportion of needle biopsy containing disease) (< 0.001), but no association with age (Table 1). Univariate Cox analysis demonstrated that compared to cancers with PTEN, tumours with PTEN loss had significantly worse cause-specific and overall survival (HR = 2.17, 95% CI = 1.39–3.40, P = 0.001 and HR = 1.58, 95% CI = 1.20–2.08, P = 0.001). However, on multivariate Cox analysis ERG/ETV1 gene rearrangement status alone was not a significant independent prognostic factor for either cause-specific or overall survival (HR = 0.93, 95% CI = 0.58–1.50, P = 0.78 and HR = 1.04, 95% CI = 0.77–1.41, P = 0.80). We next considered PTEN and ERG/ETV1 gene status together (Table 2). The 308 patients were stratified into those that had an ERG or ETV1 gene rearrangement in their cancers (122 patients, 40%) and those that did not (186 patients, 60%). The patients were then further stratified as to whether they had PTEN loss or not. There was a significant association between PTEN score and ERG/ETV1 status (< 0.001) with 66% of PTEN loss tumours also having an ERG/ETV1 gene rearrangement compared with 34% of normal PTEN tumours (Table 3). When the patients were stratified according to their ERG/ETV1 gene status there was a significant interaction with PTEN status (test for heterogeneity (Q(1df) = 20.7, P < 0.001 and Q(1df) = 20.9, P < 0.001 in multivariate analysis for cause-specific survival and overall survival, respectively). Cox analyses were therefore conducted using the group with normal ERG/ETV1 and normal PTEN as the reference.

PTEN gene loss with no ERG/ETV1 rearrangement identifies a poor prognosis group

Univariate analysis demonstrated that patients with normal ERG/ETV1 and PTEN loss had a significantly worse cause-specific and overall survival (HR = 9.37, 95% CI = 4.68–18.76, P < 0.001, Figure 3B and HR = 3.14, 95% CI = 1.89–5.23, P < 0.001 respectively). In multivariate analysis cases with normal ERG/ETV1 and

### Table 2: Relationship of PTEN and ERG/ETV1 status with demographics and tumour characteristics

| Variable | Normal PTEN* ERG/ETV1 (n = 147) | Normal PTEN† rearranged ERG/ETV1 (n = 85) | PTEN loss* rearranged ERG/ETV1 (n = 19) |
|----------|--------------------------------|--------------------------------|--------------------------------|
| Mean age (years) | 69 ± 5 | 69 ± 6 | 71 ± 4 | 70 ± 5 |
| Classes of age (years) | 65 | 65–70 | 70–73 | 73–76 |
| < 65 | 30 | 18 | 2 | 6 |
| > 65–70 | 45 | 11 | 5 | 8 |
| > 70–73 | 48 | 23 | 4 | 10 |
| > 73–76 | 44 | 23 | 8 | 13 |
| Gleason score | < 7 | 107 | 29 | 4 | 1 |
| ≥ 7 | 37 | 27 | 4 | 14 |
| Baseline PSA (ng ml⁻¹) | ≤ 4 | 77 | 21 | 7 | 2 |
| > 4–10 | 35 | 17 | 1 | 6 |
| > 10–25 | 31 | 17 | 7 | 7 |
| > 25–50 | 11 | 19 | 3 | 13 |
| > 50–100 | 13 | 11 | 1 | 9 |
| Clinical stage | T1 | 58 | 18 | 2 | 4 |
| T2 | 27 | 19 | 5 | 9 |
| T3 | 9 | 8 | 4 | 15 |
| Cancer in biopsy (%) | ≤ 6 | 58 | 8 | 2 | 0 |
| > 6–20 | 54 | 22 | 3 | 2 |
| > 20–40 | 24 | 13 | 4 | 6 |
| > 40–75 | 16 | 15 | 2 | 11 |
| > 75–100 | 16 | 26 | 8 | 18 |

*Restricted to patients for whom clinical stage is available. †Restricted to patients for whom extent of disease is available.
PTEN loss had a significantly higher risk of dying from PCa (HR = 4.87, 95% CI = 2.28 – 10.41, \(P < 0.001\)). The cancer-specific survival at 11 years was 43.7%. Similar results were found for overall survival (HR = 2.40, 95% CI = 1.40 – 4.11, \(P < 0.001\)). Gleason grades of these tumours (Table 2) demonstrate that in 21% of the normal ERG/ETV1 and PTEN loss tumours, Gleason grade was < 7 supporting a reclassification of this low Gleason grade patient subgroup.

**ERG/ETV1** gene rearranged tumours with and without PTEN loss form two intermediate prognostic groups

In univariate analysis men with rearranged ERG/ETV1 and normal PTEN exhibited significantly worse cause-specific and overall survival (HR = 2.99, 95% CI = 1.70 – 5.25, \(P < 0.001\), Figure 3B and HR = 1.80, 95% CI = 1.30 – 2.47, \(P < 0.001\) respectively) compared with men with normal ERG/ETV1 and PTEN status. Similar results were observed for cases with rearranged ERG/ETV1 and PTEN loss (HR = 3.92, 95% CI = 2.06 – 7.48, \(P < 0.001\), Figure 3B and HR = 1.77, 95% CI = 1.16 – 2.70, \(P = 0.008\), respectively). In multivariate analysis, men with rearranged ERG/ETV1 and normal PTEN had a marginally higher risk of dying from PCa (HR = 1.82, 95% CI = 1.01 – 3.26, \(P = 0.04\)), but no effect was observed in cases with rearranged ERG/ETV1 and PTEN loss (HR = 0.98, 95% CI = 0.49 – 1.97, \(P = 0.96\)). Similar results were found for overall survival (HR = 1.37, 95% CI = 0.98 – 1.92, \(P = 0.06\) and HR = 0.84, 95% CI = 0.52 – 1.35, \(P = 0.48\), respectively). The cancer-specific survival at 11 years for the rearranged ERG/ETV1 and normal PTEN group was 59.8% and for the rearranged ERG/ETV1 and PTEN loss group was 41.0% (Figure 3B).

No ERG/ETV1 gene rearrangement and no PTEN gene loss identifies a good prognosis group

The largest group of patients (\(n = 167\), 54%) comprised those who had neither an ERG/ETV1 gene rearrangement nor PTEN loss. This group (mean age 69 ± 5 years) had a greater cause specific survival (85.5% at 11 years) and overall survival when compared with the three other groups (Figure 3B). In this good prognosis group, 60 out of 167 (36%) had a Gleason grade \(\geq 7\). Prostate cancer-specific deaths in this cohort were not confined to the higher Gleason grades (Table 2). Of these 20 men who died from PCa at 11 years, 5 (25%) had a Gleason score of < 7; 7 (35%) had a Gleason score of 7; and the remaining 8 (40%) had a Gleason score of \(> 7\).

**DISCUSSION**

We present the first large series in which PTEN and ERG/ETV1 gene status have been analysed together. We have identified patient subgroups with high and low risk of death from PCa based on PTEN and ERG/ETV1 status. There was no difference in outcome between tumours with heterozygous and those with homozygous PTEN loss and the PTEN loss tumours were therefore considered as one group. This may be because in heterozygous tumours by FISH, the other allele is lost by an alternative mechanism (Whang et al, 1998; Verhagen et al, 2006). The ‘good prognosis’ group (54%) lacked an ERG/ETV1 gene rearrangement and PTEN gene loss (85.5% PCa survival at 11 years). The PCa-specific deaths in this group did not only occur in the patients with the higher Gleason grades, but across the Gleason grades. These results highlight some inadequacy of Gleason grading in determining which patients require more intensive therapy for their PCa.

We also identified a patient group with a significant ‘poor prognosis’. Patients lacking an ERG/ETV1 gene rearrangement but with PTEN gene loss had the worst cause-specific survival of 13.7% at 11 years. A proportion of patients in this group had a Gleason score of \(\leq 7\). These data are also potentially of clinical importance as they identify a patient group who could be targeted to receive more intensive neoadjuvant and adjuvant therapy when other clinicopathological parameters recommended a more conservative approach. This requires testing in prospectively designed studies. Agents that specifically target the PI3K–AKT–mTOR pathway are undergoing investigation in clinical trials (Yap et al, 2008) and future studies should specifically evaluate these agents in this subgroup.

One previous study (82 patients) reported that the absence of PTEN loss and no ERG gene rearrangement is associated with a longer time to biochemical recurrence and PTEN loss plus an ERG gene rearrangement was associated with the shortest time to biochemical recurrence (Yoshimoto et al, 2008). Our results are similar in that those tumours that had neither an ERG gene rearrangement nor PTEN loss were in a good prognostic group. However, we did not show that the combination of the two alterations was associated with poor outcome, and rather that it is those tumours with PTEN loss but no ERG/ETV1 gene

### Table 3  Summary results of multivariate Cox analyses for prostate cancer survival

|                       | Normal PTEN | PTEN loss | All       |
|-----------------------|-------------|-----------|-----------|
| **Normal ERG/ETV1**   | n = 167     | n = 19    | HR = ref  |
| HR = ref              |             |           |           |
| GS < 7: 64%           | 95% CI = 2.28 – 10.41 | HR = ref |           |
| GS = 7: 22%           |             |           |           |
| **Rearranged ERG/ETV1** | n = 85    | n = 37    | HR = ref  |
| HR = 1.82             |             |           |           |
| 95% CI = 1.01 – 3.26  | 0.04        |           |           |
| P = 0.04              |             |           |           |
| GS < 7: 34%           |             |           |           |
| GS = 7: 32%           |             |           |           |
| **All (308 with ERG/ETV1 status)** | HR = ref |             |           |
| HR = 1.12             |             |           |           |
| 95% CI = 0.68 – 1.86  |             |           |           |
| P = 0.65              |             |           |           |
| **All (322)**         | HR = ref    |           |           |
| HR = 1.19             |             |           |           |
| 95% CI = 0.73 – 1.96  |             |           |           |
| P = 0.49              |             |           |           |

**Abbreviations:** CI = confidence interval; GS = Gleason score; HR = hazard ratio.
rarentation that do particularly badly. These differences may be a consequence of different study sizes or endpoints used.

**ERG/ETV1** gene rearrangement positive and negative tumours have been reported to have distinct chromosomal aberrations, expression signatures and morphological features (Kumar-Sinha et al., 2008), thus suggesting that they represent different PCA classes. Our results support this hypothesis because PTEN loss was only a significant independent prognostic factor for overall survival, when analysed in the **ERG/ETV1** gene non-rearranged tumours. These results also raise the question of whether there are other molecular abnormalities that are mutually exclusive of **ERG/ETV1** gene rearrangements that may contribute to the worse outcome of patients with non-**ERG/ETV1** gene rearranged/PTEN loss tumours. In this respect, the recent observation that a proportion of **ETS**-gene rearranged negative cancers overexpress SPINK1 protein is of particular interest (Tomlins et al., 2008b). SPINK1 expression was linked to poorer outcome (Tomlins et al., 2008b) and SPINK1 has been shown, when overexpressed in colorectal and breast cancer cells, to function as an autocrine growth factor that can stimulate the PI3K pathway (Gouyer et al., 2008).

**CONCLUSIONS**

In conclusion, these data suggest that molecular characterisation of **PTEN**, **ERG** and **ETV1** gene status might be used in future to determine the risk of PCA death. This has implications both for potentially deciding which patients should be conservatively or aggressively treated and also for stratification of patients in clinical trials. At present clinical trial patients are stratified by clinicopathological features alone. Our results suggest that an imbalance in numbers of patients with different **PTEN** and **ERG/ETV1** gene status in different study arms could falsely influence trial outcome and needs to be accounted for.

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