Heterogeneity and clinical significance of ETV1 translocations in human prostate cancer

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A fluorescence in situ hybridisation (FISH) assay has been used to screen for ETV1 gene rearrangements in a cohort of 429 prostate cancers from patients who had been diagnosed by trans-urethral resection of the prostate. The presence of ETV1 gene alterations (found in 23 cases, 5.4%) was correlated with higher Gleason Score (P = 0.001), PSA level at diagnosis (P = < 0.0001) and clinical stage (P = 0.017) but was not linked to poorer survival. We found that the six previously characterised translocation partners of ETV1 only accounted for 34% of ETV1 re-arrangements (eight out of 23) in this series, with fusion to the androgen-repressed gene C15orf21 representing the commonest event (four out of 23). In 5’-RACE experiments on RNA extracted from formalin-fixed tissue we identified the androgen-upregulated gene ACSL3 as a new 5’-translocation partner of ETV1. These studies report a novel fusion partner for ETV1 and highlight the considerable heterogeneity of ETV1 gene rearrangements in human prostate cancer.

Keywords: prostate cancer; ETV1; ACSL3; ACSL3:ETV1 fusion

Recently, fusion of the prostate-specific androgen-regulated TMPRSS2 gene to the ETS family transcription factor gene ERG was reported as a common event in prostate cancer (Tomlins et al., 2005, 2006; Clark et al., 2006; Iljin et al., 2006; Perner et al., 2006; Soller et al., 2006; Wang et al., 2006a; Yoshimoto et al., 2006; Hermans et al., 2006). Less frequently TMPRSS2 becomes fused to ETV1 and ETV4. In all these cases a TMPRSS2-ETS chimaeric gene is generated resulting in high-level expression of the fused 3’-ETS gene sequences. The reported incidence of TMPRSS2:ETV1 fusion in these studies (1–2%) was, however, considerably lower than the observed incidence of ETV1 gene overexpression (~10% in prostate cancer). This prompted Tomlins et al. (2007) to search for alternative mechanisms of ETV1 overexpression. They identified five new 5’-fusion ETV1 partners including the prostate-specific androgen-induced gene SLC45A3/Prostein, an endogenous retroviral element HERV-K, a prostate-specific androgen-repressed gene C15orf21, and a strongly expressed housekeeping gene HNRPA2B1. Additionally they found that in the two prostate cancer cell lines LNCaP and MDA-PCa2b, outlier expression of ETV1 was caused through the entire ETV1 gene becoming juxtaposed to sequences at 14q13.3–14q21.1. By characterising the expression of four contiguous genes within this region (SLC25A21, MIPOL1, FOXA1 and TTC6), as well as that of ETV1, in LNCaP cells they demonstrated that this region exhibited prostate-specific expression that was coordinately regulated by androgens in a castration-resistant cell line model without formation of a fusion gene. In that study only single cases of each fusion were reported, with the exception of the juxtaposition of ETV1 sequences to 14q13.3–14q21.1 where two cases were observed. It was therefore not possible to assess the relative importance of the different fusion partners in their small tumour set.

For ERG gene re-arrangements several studies have demonstrated links to clinicopathological indicators (Perner et al., 2006; Wang et al., 2006a; Demicheli et al., 2007; Nam et al., 2007). In a watchful waiting cohort of 111 patients, Demicheli et al. (2007) reported a significant link between the presence of ERG alterations and prostate cancer-specific death. In a series of 165 patients who underwent prostatectomy, Nam et al. (2007) found that the presence of a TMPRSS2:ERG fusion was associated with a greater probability of biochemical relapse. Additionally, we have recently demonstrated that loss of 5’-ERG sequences coupled with duplication of TMPRSS2:ERG fusion sequences predicts extremely poor cancer-specific survival independently of Gleason score and PSA.

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level at diagnosis in a conservatively managed watchful waiting patient cohort (Attard et al, 2008). In contrast very little is known about the clinical significance of alteration at the ETV1 gene locus.

To help identify biomarkers that may be of use in the management of men with prostate cancer, we have established a retrospective cohort of 429 men whose cancers were conservatively managed (Cuzick et al, 2006). Our analyses included centrally assigned Gleason scores determined by modern grading criteria, and allowed comparisons with several additional clinical parameters. In agreement with previous studies (Johansson et al, 2004; Albertsen et al, 2005; Cuzick et al, 2006) we found Gleason score to be an important determinant of cancer-specific mortality, although baseline PSA and to a lesser extent stage of disease added further predictive value. The objective of the current study is initially to use our cohort of 429 conservatively managed prostate cancer cases to assess the potential clinical significance of ETV1 gene alterations and in parallel to assess the relative frequency of each of the known ETV1 fusion partners. As we found these partners to only account for ~34% of all ETV1 translocation events, we undertook 5’-RACE studies to identify novel ETV1 fusion partners in our paraffin-embedded tumour samples.

**Table 1** ETV1 classification and revised Gleason score

| Gleason score | Class N | Class ETV1 Esplit | Total |
|---------------|---------|--------------------|-------|
| 4             | 2       | 0                  | 2     |
| 5             | 11      | 1                  | 12    |
| 6             | 130     | 2                  | 132   |
| 7             | 59      | 11                 | 70    |
| 8             | 27      | 3                  | 30    |
| 9             | 20      | 5                  | 25    |
| 10            | 2       | 0                  | 2     |
| Unknown       | 1       | 0                  | 1     |
| Total         | 252     | 22                 | 274   |

*Revised Gleason score for cancers lacking ERG and ETV1 rearrangements (class N) is compared to cancers with rearrangement of ETV1 (class ETV1 Esplit).

**Table 2** Frequency of detection of known ETV1 translocations

| Class of ETV1 rearrangement | Number of cases (% of total number of ETV1-rearranged cancers) |
|-----------------------------|---------------------------------------------------------------|
| Fusion with C15ORF21        | 4 (18%)                                                       |
| Rearrangement to 4q13.3–14q21.1 | 2 (9%)                                                   |
| Fusion with ACSL3           | 1 (4.5%)                                                     |
| Fusion with HNRPA2B1        | 1 (4.5%)                                                     |
| Fusion with SLC45A5/Protein | 1 (4.5%)                                                     |
| Fusion with HERV-K          | 0                                                             |
| Fusion with TMPRSS2         | 0                                                             |

*The number of cases of ETV1 rearrangement involving each of the six previously described translocation partners namely C15ORF21, 4q13.3–14q21.1, HNRPA2B1, SLC45A5/Protein, HERV-K and TMPRSS2, plus our discovery of translocation with ACSL3 in our series of 23 ETV1-rearranged cancers.

Figure 1 FISH detection of ETV1 gene re-arrangements. Top: Interphase nuclei are hybridised to probes that detect sequences immediately 3’ to the ETV1 gene (probe I, red) and immediately 5’ to the ETV1 gene (probe II, green). The red and green signals are separated when an ETV1 gene rearrangement occurs. (A) Signals from normal un-rearranged ETV1 loci (class N). (B) Rearranged ETV1 gene with separate red (3’) and green (5’) probes (class ETV1 Esplit). Bottom: Map of the ETV1 gene showing the position of the BACs used as probes in FISH assays. Probe I: E1 (RP11-27B1), E2 (RP11-138H16), E3 (CTD-2008I15) labelled with Cy3. Probe II E4 (RP11-905H4), E5 (RP11-621E24), E6 (RP11-115D14) labelled with FITC. The direction of transcription of genes at this locus are indicated by arrows.
RESULTS

Fluorescence in situ hybridisation detection of ETV1 fusions

We have used a fluorescence in situ hybridisation (FISH) ETV1 gene 'break-apart' assay to screen for ETV1 rearrangements on a Tissue Microarray (TMA) consisting of 945 trans-urethral resection of the prostate cancer cores from 429 patients. We used a Tissue Microarray (TMA) consisting of 945 trans-urethral resection of the prostate cancer cores from 429 patients. We used

three overlapping BAC probes at the telomeric 3'–end (red) and three BAC probes at the centromeric 5'–end (green) of the ETV1 gene (Figure 1). Normal ETV1 loci are visualised in interphase nuclei as immediately adjacent green and red signals (Figure 1A, Class ETV1 N). When rearrangements involving the ETV1 gene were present, the 5'–centromeric and 3'–telomeric ETV1 probes separated and were visible as lone red and green signals (Figure 1B, Class ETV1 split). These analyses identified ETV1

![Diagram](image_url)
gene rearrangements in cancer from 23 patients (5.4% of all cancers). An ERG gene break-apart assay, completed as previously described (Attard et al, 2008), demonstrated that an additional 155 cancers (36%) in this series contained ERG gene rearrangements, including one patient who had both ERG and ETV1 rearrangements in distinct foci of cancer in the same prostate, as reported previously (Attard et al, 2008; Clark et al, 2008).
Clinicopathological correlations

Tumour demographics and characteristics comparing patients with only ETV1 gene rearrangements (22 cases) with patients who lacked both ETV1 and ERG gene rearrangements (252 cases) are shown in Table 1. Correlations with clinical parameters demonstrated there were significant associations between the presence of ETV1 gene re-arrangement and Gleason score (\(P = 0.001\)), baseline PSA (\(P < 0.0001\)), clinical stage (\(P = 0.017\)) and age (0.04). However, despite these links to indicators of more aggressive disease there was no evidence for a difference in overall and cancer-specific survival between those cancers harbouring ETV1 gene alteration and those cancers retaining normal ERG and ETV1 loci (Class N) (HR = 1.48, CI = 0.87–2.53, \(P = 0.17\) and HR = –1.48, CI = 0.64–3.46, \(P = 0.39\) respectively) (Supplementary Figure 1).

Heterogeneity of ETV1 fusion partners

We constructed a TMA block containing cores from all of the cancers harbouring ETV1 re-arrangements (23 tumours) and six randomly selected cancers with an ERG gene rearrangement. We used slices of this TMA to carry out break-apart assays for the 5'-fusion partners previously identified by Tomlins et al (2005, 2006, 2007): namely TMPRSS2, SLC45A3, HERV-K, C15orf21 and HNRPA2B1 (Table 2). We also used FISH assays to confirm co-localisation of 3'-ETV1 with 5'-sequences from each of the above partners as previously described by Tomlins et al (2007) (results not shown). To identify tumours with translocation of ETV1 to the androgen-regulated prostate-specific region at 14q13.3–14q21.1 we co-hybridised a TMA slice with a 3'-ETV1 FISH probe (red) and a FISH probe consisting of six BACs spanning the entire region of 14q13.3–q21.1 (green). Co-localisation of the red and green signals was taken as evidence of translocation of ETV1 to this region (Figure 2). The FISH probes used in all of these assays are listed in Supplementary Table 1.

As expected, cancers with rearrangements of the ERG gene had fusions to 5'-TMPRSS2 sequences. In contrast, none of the cancers with rearrangements of the ETV1 gene exhibited fusions involving TMPRSS2 or the HERV-K retroviral sequence. In four cancers 3'-ETV1 exhibited fusion to 5'-C15orf21 sequences, two contained translocation to 14q13.3–14q21.1, one contained fusion to HNRPA2B1 and one contained fusion to SLC45A3/Prostein (Table 2). Thus only eight of the 23 cancers with re-arranged ETV1 genes had known partners. The cancers containing fusion of 5'-C15orf21 to 3'-ETV1 sequences included the previously reported case containing ERG and ETV1 rearrangements in distinct cancer loci (Attard et al, 2008). The recurrent fusions of the prostate-specific androgen-repressed gene C15orf21 to 3'-ETV1 sequences is of particular interest because Tomlins et al (2007) reported that this gene is not androgen driven, implying that tumours containing these fusion genes may exhibit resistance to androgen deprivation therapies. Joining of ETV1 to individual partners was too uncommon to allow survival analysis for specific gene fusions. Of the four cases with a C15orf21:ETV1 fusion, three are still alive and one died of unrelated causes.

Fusion of the ACSL3 gene to ETV1 in human prostate cancer

We performed 5'-RACE to identify novel partners that are fused to 3'-ETV1 sequences. Our studies were severely limited by the small amounts (50–200 ng) of poor quality RNA that could be prepared from the formalin-fixed tissue in this series. As obtainable RT–PCR products from these paraffin tissues were limited to ~100–150 bp and the ETV1 exon breakpoint in each sample was unknown, 5'-RACE–PCR had to be independently initiated from each of the known ETV1 exon breakpoints in each sample, that is, exons 2, 4, 5 and 6. Using this strategy we successfully obtained a 5'-RACE fusion product from one RNA sample that contained an ex6 ETV1 sequence fused to a 51 bp sequence of ACSL3 ex3 sequence identifying ACSL3 as a novel ETV1 fusion partner. The structure of this ACSL3 ex3:ETV1 ex6 fusion is predicted to encode a truncated ETV1 protein as shown in Figure 3A. The presence of the ACSL3:ETV1 fusion was confirmed in this specimen by RT–PCR using 5'-ACSL3 and 3'-ETV1 primers (Figure 3B, C) and co-localisation by FISH of BAC probes corresponding to 5'-ACSL3 sequences (green) and 3'-ETV1 sequences (red) (Figure 3D, panel iii). An ACSL3 break-apart FISH assay screen of the entire TMA containing the 23 cancers with rearrangement of the ETV1 gene failed to identify additional cancers with this particular fusion. Like fusion to TMPRSS2, HNRPA2B1, HERV-K or SLC45A3/Prostein the fusion of 3'-ETV1 sequences to 5'-ACSL3 sequences is not a common one in this panel and we also demonstrated fusion of 5'-ETV1 sequences with 3'-ACSL3 sequences by FISH, indicating that the mechanism underlying formation of this fusion gene is a balanced translocation (Figure 3D, panel iv).

DISCUSSION

We have shown that the presence of ETV1 gene locus rearrangements scored in a FISH-based assay is correlated with Gleason score, associated clinical stage and baseline PSA, but interestingly was not associated with poorer survival. Similar analyses of ERG gene alterations detected by FISH also demonstrate correlation to Gleason score, clinical stage and baseline PSA (Attard et al, 2008). However, in clinical outcome correlations only the presence of a duplication of rearranged ERG together with interstitial deletion of genomic sequences between the tandemly located TMPRSS2 and ERG sequences was correlated with worse cancer-specific death (Attard et al, 2008). In analyses of alteration of the ETV1 gene it was not possible to examine the relationship between survival and duplication of the ETV1 foci because duplications were only found in five of the 23 cases examined (one C15orf21 fusion, one chr14 co-localisation, and three with unknown partners).

Previous studies have each reported single cancers with an ETV1 rearrangement (Tomlins et al, 2005, 2006; Hermans et al, 2006) with the exception of Tomlins et al (2007) who reported four clinical cases. Our study therefore represents the largest single series of primary prostate cancers with an ETV1 rearrangement. Our study confirms previous observations that ETV1 may form a fusion gene with a variety of partners and shows that each individual fusion is relatively rare. Importantly, we show that the known fusion partners, including the novel ACSL3:ETV1 fusion gene, only account for 39% of cancers with an ETV1 rearrangement and it is therefore likely that many new partner genes remain to be identified.

The protein encoded by the ACSL3 gene is an isozyme of the long-chain fatty-acid coenzyme A ligase family that converts free long-chain fatty acids into fatty acyl-CoA esters, and thereby plays a key role in lipid biosynthesis and fatty acid degradation. Insights into the regulation of ACSL3 expression arise from expression array data in which the LNCaP cell line was compared with the androgen-responsive androgen-resistant R1881. In two independent expression array data sets, ACSL3 was upregulated by androgen treatment (Hendriksen et al, 2006; Wang et al, 2006b). One study showed ACSL3 upregulation at time intervals of 2, 4, 6 and 8 h following androgen treatment (Hendriksen et al, 2006) and another study showed ACSL3 upregulation after 16 h (Wang et al, 2006b). Expression of ACSL3 was also elevated in a panel of ‘androgen-sensitive’ (LAPC-4, LNCaP, MDA PCa2a, MDA PCa2b, and 22Rv1) versus ‘androgen-insensitive’ (PPC1, PC3, and DU145) prostate cancer cell lines (Zhao et al, 2005; Tomlins et al, 2007). Expression of TMPRSS2 and SLC45A3 follow the same pattern within these datasets (Zhao et al, 2005). ACSL3 transcription can also be
activated by oncostatin via the ERK-signalling pathway (Zhou et al., 2007) suggesting alternative means of regulation.

These observations raised the question of whether there are any androgen receptor (AR) binding sites capable of explaining the expression levels of ACSL3 and of the other known ETV1 partner genes. A number of groups have recently published AR ChIP-chip studies mapping AR-binding sites within the human genome (Bolton et al., 2007; Massie et al., 2007; Takayama et al., 2007; Wang et al., 2007). Wang et al. (2007) identified a functional AR-binding site 13.5 kb upstream of the TMPRSS2 gene (Wang et al., 2007). The closest AR-binding sites for the other genes involved in ETS gene fusions varied from 60 kb to 1.5 Mb, although in the absence of genome-wide AR ChIP data it is possible that other AR-binding sites occur outside of the current coverage (Supplementary Table 2). Both Massie et al. (2007) and Wang et al. (2007) have proposed mechanisms for AR recruitment to subsets of target sequences through associations between the AR and other transcription factors for example, GATA-2, OCT11, FOXA1 and ETS1.

In conclusion our studies report a novel fusion partner for ETV1 and highlight the wide heterogeneity in the range of the ETV1 fusion partners. Interestingly fusion to the androgen repressed gene C15orf21 was the most common event suggesting the existence of a significant subgroup of cancers that may not respond in a conventional manner to androgen withdrawal therapies.

MATERIALS AND METHODS

Patient cohort and tissue microarrays

TMA s were constructed from 429 unselected transurethral resection of the prostate specimens taken from patients managed with no initial treatment or hormone treatment in a cohort of conservatively managed men with prostate cancer (Cuzick et al., 2006). 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. 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

TMA s were constructed in 35 × 22 × 7 mm blocks of Lamb paraffin wax using a manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). Up to four tumour cores of 600 µm diameter were taken from each prostate. 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 and 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.

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