Published in final edited form as:

Cancer Res. 2008 September 15; 68(18): 7629–7637. doi:10.1158/0008-5472.CAN-08-2014.

A fluorescence in situ hybridization screen for E26 transformation-specific aberrations: identification of DDX5-ETV4 fusion protein in prostate cancer

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Abstract

Recurrent gene fusions involving ETS transcription factors *ERG*, *ETV1*, *ETV4*, or *ETV5* have been identified in 40–70% of prostate cancers. Here we employed a comprehensive fluorescence in situ hybridization (FISH) split probe strategy interrogating all 27 ETS family members and their five known 5' fusion partners in a cohort of 110 clinically localized prostate cancer patients. Gene rearrangements were only identified in ETS genes that were previously implicated in prostate cancer gene fusions including *ERG*, *ETV1*, and *ETV4* (43%, 5% and 5%, respectively), suggesting that a substantial fraction of prostate cancers (estimated at 30–60%) cannot be attributed to an ETS gene fusion. Among the known 5' gene fusion partners, *TMPRSS2* was rearranged in 47% of cases followed by *SLC45A3*, *HNRPA2B1*, and *C15ORF21* in 2%, 1% and 1% of cases, respectively. Based on this comprehensive FISH screen, we have made four noteworthy observations. First, by screening the entire ETS transcription factor family for rearrangements, we found that a large fraction of prostate cancers (44%) cannot be ascribed to an ETS gene fusion, an observation which will stimulate research into identifying recurrent non-ETS aberrations in prostate cancers. Second, we identified *SLC45A3* as a novel 5' fusion partner of *ERG*; previously, *TMPRSS2* was the only described 5' partner of *ERG*. Third, we identified two prostate-specific, androgen-induced genes, *FLJ37254* and *CANT1* as

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Disclosure

The University of Michigan has filed a patent on ETS gene rearrangements in prostate cancer, on which R.M., S.A.T., and A.M.C. are co-inventors, and the diagnostic field of use has been licensed to Gen-Probe Incorporated. Gen-Probe has not played a role in the design and conduct of the study, nor in the collection, analysis, or interpretation of the data, and no involvement in the preparation, review, or approval of the manuscript. A.M.C. serves as a consultant to Gen-Probe Inc.

5' partners to *ETV1*. And fourth, we identified a ubiquitously expressed, androgen-insensitive gene *DDX5* fused in frame with *ETV4* leading to the expression of a *DDX5:ETV4* fusion protein.

Keywords

gene fusion; prostate cancer; ETS; DDX5; ETV4; fusion protein

Introduction

ETS (E26 transformation-specific) transcription factors play an important role in cellular proliferation, carcinogenesis and metastasis (1–3). To date, 27 human ETS family members and over 200 ETS target genes have been identified (3). Translocations involving ETS family genes such as *FLI1*, *ERG*, *ETV1*, *ETV4*, and *ETV6* have been described in hematological and mesenchymal malignancies including Ewing's sarcomas, leukemias, lymphomas, fibrosarcomas and mesoblastic nephroma (1,3,4) and in rare secretory breast carcinomas (5).

Recently, recurrent gene fusions involving ETS family genes, *ERG*, *ETV1*, *ETV4*, and *ETV5*, fused to one of five different upstream fusion partners (*TMPRSS2*, *SLC45A3*, *HERV-K_22q11.23*, *C15ORF21* and *HNRPA2B1*) have been identified in a majority of prostate cancers (6–9). The upstream regulatory elements, with their different androgen sensitivity and prostate specificity, are believed to drive aberrant expression of the downstream, ETS genes, contributing to prostate carcinogenesis. Among these gene fusions, the *TMPRSS2-ERG* fusion is the most prevalent, observed in approximately 50–70% of hospital-based, PSA-screened, prostate cancer cohorts (10–15). Emerging data suggests association of *TMPRSS2-ERG* fusion subtypes with a more aggressive phenotype in clinically localized prostate cancer as well in androgen independent metastatic prostate cancer (12,13,16–19) [Reviewed in (20)].

Apart from *ERG*, *ETV1*, *ETV4* or *ETV5* aberrations involving other ETS family genes in prostate cancer are still unknown. In this study, we comprehensively surveyed all 27 ETS family genes and the five known 5' fusions partners for rearrangements using fluorescence in situ hybridization (FISH) analysis on tissue microarray (TMA) from a cohort of localized prostate cancer patients. While confirming the ETS gene rearrangement patterns reported earlier, we noted that more than 40% of prostate cancers are ETS fusion negative, defining a distinct molecular subtype. In addition, we identified novel 5' fusion partners of *ERG* (eg. *SLC45A3*), *ETV1* (eg. *FLJ35294*), and *ETV4* (eg. *CANT1*, and *DDX5*). *DDX5-ETV4* also represents the first fusion protein identified in prostate cancer.

Materials and Methods

Study population, clinical data, and tissue microarray (TMA) construction

A TMA was constructed representing 110 clinically localized prostate cancer patients who underwent radical prostatectomy as a primary therapy between 2004 and 2006 at the University of Michigan Hospital. Three cores (0.6 mm in diameter) were taken from each representative tumor focus and morphology was confirmed by three pathologists (B.H., R.B.S. and R.M.). Detailed clinical, pathological, and TMA data were maintained on a secure relational database as previously described(21). Patient demographics are shown in Supplementary Table 1. This radical prostatectomy series was part of the University of Michigan Prostate Cancer Specialized Program of Research Excellence Tissue Core. This study was approved by the Institutional Review Board at the University of Michigan Medical School.

Fluorescence in situ hybridization (FISH) and FISH-based screening strategy

A previously validated FISH-based split probe strategy was utilized to investigate known and novel gene aberrations in prostate cancer (7–9,21). Interphase FISH was performed as described (7,21). Bacterial artificial chromosomes (BACs) (listed in Supplementary Table 2) were obtained from the BACPAC Resource Center (Oakland, CA), and probes were prepared as described (7,9,21). The integrity and correct localization of all probes were verified by hybridization to metaphase spreads of normal peripheral lymphocytes. Slides were examined using an ImagingZ1 microscope (Carl Zeiss, Oberkochen, Germany). FISH signals were scored manually (100x oil immersion) in morphologically intact and non-overlapping nuclei by three pathologists (B.H., R.B.S., and R.M.), and a minimum of 50 cancer cells from each site were recorded. Cancer sites with very weak or no signals were recorded as insufficiently hybridized. Cases lacking tumor tissue in all three cores were excluded.

A flowchart of FISH-based high-throughput screening strategy is shown in Figure 1. For detection of *ERG*, *ETV1*, *ETV4*, and *ETV5* rearrangements in which breakpoints have been characterized, BACs for split probes were used as previously described (6–9,21). For the remaining ETS family genes with unknown rearrangement status in prostate cancer, split probes flanking approximately 1 Mb regions of interest were utilized for initial screening on the prostate cancer TMA (Figure 1A, B). Subsequently, ETS gene-specific probes tightly flanking the gene of interest (approximately 200 Kb) were used to confirm ETS aberration on tissue sections from prostate cancer cases that were identified on initial screening (Figure 1C).

We further evaluated this TMA for rearrangements of all 5' partners known at the time this study was started by split probe FISH strategy. For cases rearranged for both ETS gene and known 5' partners, a previously validated fusion probe strategy was utilized to confirm potential gene fusions (7,8,21) (Figure 1D). For cases with ETS gene rearrangement only, frozen tissues were obtained to identify 5' fusion partner by RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) (Figure 1D).

Cell line studies

LNCaP, an androgen sensitive prostate cancer cell line, was maintained in RPMI with 10% FBS. For androgen stimulation experiments, cells were placed for two days in phenol red-free RPMI, supplemented with 5% of charcoal-treated FCS before being treated with 1% ethanol or 10nM R1881 for the following time points 0, 3, 12, 24, and 48 hours. Cells were treated for 16 hours with androgen and crosslinked for chromatin immunoprecipitation (ChIP) analysis. Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Quantitative Real- time Reverse Transcriptase PCR (Q-PCR)

Q-PCR was carried out according to standard protocols as previously described (7). All oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Supplementary Table 3. Samples were normalized by the mRNA level of the housekeeping gene GAPDH. Androgen stimulation reactions were performed in triplicate, and all other reactions were performed in duplicate.

RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE)

RLM-RACE was performed as previously described to identify unknown 5' partners of aberrant ETS genes in prostate cancer (7). First-strand cDNA was amplified with gene specific reverse primers *ETV1_exon4-5r* (7), *ETV4_exon7r* (8) and 5' gene racer primers (Invitrogen) using Platinum Taq High Fidelity enzyme (Invitrogen) following the touch-down PCR protocol according to manufacturer's instructions. PCR amplification products were cloned into pCR4-

TOPO TA vector (Invitrogen) and sequenced bidirectionally using vector primers as described (7).

Chromatin immunoprecipitation (ChIP) analysis

ChIP experiment was carried out as previously described (22) using 5 µg of anti-androgen receptor (AR) antibody (Upstate, Lake Placid, NY) at 16 hours after R1881 or ethanol treatment of hormone-deprived cells. The input whole-cell extract DNA and the ChIP-enriched DNA were amplified by ligation-mediated PCR, and subjected to qPCR assessment of target promoters. ChIP enrichment was evaluated as a percentage of input DNA.

Tissue-specific expression

To determine tissue-specific expression of 5' fusion partners, we interrogated the International Genomics Consortium's expO data set (<https://expo.intgen.org/expo/public/downloaddata.jsp>), consisting of expression profiles from 630 tumours of 29 distinct types, using the Oncomine database (<http://www.oncomine.org>) as previously described (7).

Cloning and expression of DDX5-ETV4

Full length *DDX5-ETV4* fusion cDNA was cloned into Gateway cloning system's entry vector pENTR-D-TOPO (Invitrogen) using 5'RLM-RACE product from case no.85 with 5' primer containing a Kozak sequence-FLAG tag-start codon-*DDX5* N-terminal nucleotide sequence (5'-CACC-ATG-GATTACAAGGATGACGACGATAAG-TCGGGTATTTCGAGTGACCGAGACCGCGGC-3') and 3' primer corresponding to the C-terminal nucleotides of *ETV4*, until the stop codon (CTAGTAAGAGTAGCCACCCTTGGGGCCA). Expression constructs were generated by recombination of pENTR-D-Topo-*DDX5-ETV4* with pCDNA3.2-DEST vector (Invitrogen), using LR Clonase II (Invitrogen). Human embryonic kidney (HEK) 293 cells were transiently transfected with pCDN3.2-FLAG-*DDX5-ETV4* expression construct using FuGENE6 transfection reagent (Roche). Lysates from the transfected cells were resolved by SDS-PAGE, and transferred onto Polyvinylidene Difluoride membrane (GE Healthcare). The membrane was incubated for one hour in blocking buffer [Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk] and incubated overnight at 4°C with rabbit polyclonal antibody against FLAG tag at 1:1000 dilution (Cell Signaling Technology). Following a wash with TBS-T, the blot was incubated with horseradish peroxidase-conjugated secondary antibody and the signals visualized by enhanced chemiluminescence system as described by the manufacturer (GE Healthcare). The blot was re-probed with GAPDH (Abcam) for confirmation of equal loading. Prostate tissue lysates were processed similarly for immunoblotting with *ETV4* polyclonal antibody (Abnova).

Results and Discussion

We generated a comprehensive profile of the rearrangement status of all 27 ETS family genes and all five of the known 5' fusion partners in prostate cancer using FISH split probe hybridizations on a TMA comprised of 110 cases of clinically localized prostate cancers. A flow chart of our systematic ETS FISH screen is described in Figure 1.

A matrix representation of gene rearrangements for the 27 ETS transcription factors and the five 5' fusion partners is shown in Figure 2. *ERG*, as expected, was the most commonly rearranged ETS gene in prostate cancer and *TMPRSS2* was the most commonly rearranged 5' fusion partner (7,9,21). In the cohort represented on this TMA, *ERG* was rearranged in 43% (43/99) of cases, of which 63% (27/43) were fused through deletion of its 5' end to *TMPRSS2*, which is similar to previous reports (11,21). Overall, 98% (42/43) of the *ERG* positive cases harbored *TMPRSS2* as the 5' fusion partner. Interestingly, one of the *ERG*

positive cases was negative for *TMPRSS2*. Upon further inspection of our FISH screen (Fig. 2), this case harbored rearrangement of the 5' partner *SLC45A3* (case no. 102). We proceeded to confirm the genomic fusion of *SLC45A3:ERG* using a fusion assay with probes 5' to *SLC45A3* and 3' to *ERG* (Fig. 3), thus implicating *SLC45A3* as a novel 5' fusion partner of *ERG*. Previous to this finding, it was thought that *ERG* exclusively partnered with *TMPRSS2* possibly due to their co-localization on chromosome 21. *SLC45A3* was previously identified to be the 5' fusion partner of *ETV1* and *ETV5* (6,21).

In contrast to *ERG*, *ETV1* was rearranged in approximately 5% (5/99) of patients (case nos. 4, 51, 54, 72, 91, Fig. 2) which is in line with our previous study (7). *ETV1* has been shown to be fused to a number of genes including *TMPRSS2*, *SLC45A3*, *HERV-K_22q11.23*, *C15ORF21* and *HNRPA2B1* (7). In the 110 cases represented in this study, case nos. 4 and 72 harbored rearrangements in *ETV1* in addition to rearrangements of *SLC45A3* and *HNRPA2B1*, respectively. We utilized fusion probe FISH assays to confirm *SLC45A3:ETV1* (a probe 5' to *SLC45A3* and 3' to *ETV1*) and *HNRPA2B1:ETV1* (a probe 5' to *HNRPA2B1* and 3' to *ETV1*) fusions in these two cases, respectively. Of note, case no. 72 was the second case of *HNRPA2B1:ETV1* reported in the literature so far (7), suggesting *HNRPA2B1:ETV1* fusion is recurrent in prostate cancer. Case no. 4 which harbored *SLC45A3:ETV1* fusion was identified to be the same case as reported earlier (7). In the *ETV1* positive case nos. 51, 54, 91 no rearrangement in the known 5' partners were identified by FISH. In case no. 54, using RLM-RACE we identified exons 1 to 4 of *ETV1* to be replaced by 263 base pairs of the 5' sequence of a prostate EST gene *FLJ35294* (17p13.1) (Fig. 4A). For *ETV1* positive case nos. 51 & 91, we could not perform RACE as frozen tissue was not available, they would presumably harbor uncharacterized 5' fusion partners.

ETV4 rearrangements were present in 5% (5/100) of patients in our cohort (case nos. 40, 46, 53, 64, 85, Fig. 2). Case nos. 64 and 85 had rearrangement through translocation (split) while deletion of the probe 5' to *ETV4* was identified in case nos. 40 and 46. In contrast, case no. 53 revealed deletion of the probe 3' to *ETV4* (the significance of which is unclear). Of note, case nos. 40 and 64 also showed an aberration for *TMPRSS2* when screened by *TMPRSS2* split probe. These two cases were confirmed to harbor *TMPRSS2:ETV4* fusion using a fusion probe assay (employing probes 5' to *TMPRSS2* and 3' to *ETV4*). Interestingly, in case no. 40, instead of split signals, a 5' end deletion was detected using probes 5' and 3' to *ETV4*, suggesting that the *TMPRSS2:ETV4* fusion in this case occurred through an unbalanced translocation. RLM-RACE further revealed that exon 1 of *ETV4* was replaced with exons 1–2 of *TMPRSS2*. Thus, this case is different from the previously reported *TMPRSS2:ETV4* fusion case where a novel upstream exon of *TMPRSS2* was involved in the fusion (8). *ETV4* was rearranged in case nos. 46 and 85, whereas no genetic aberration in the known 5' partners was observed by FISH. To characterize the *ETV4* transcript in these two cases, we performed RLM-RACE. Interestingly, in case no. 46, we identified exon 5 of *ETV4* fused to exon 1a of the *CANT1* gene located on 17q25.3, identical to the fusion sequence reported before (23) (Fig. 4A). In case no. 85, another novel 5' partner gene, DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (*DDX5*) was identified by RLM-RACE (Fig. 4A). Sequence analysis of *DDX5-ETV4* revealed that the fusion transcript is comprised of exons 1–3 of *DDX5*, fused in frame to exons 5–13 of *ETV4* (Fig. 4A). Further, we confirmed genomic fusions of *FLJ35294:ETV1*, *CANT1:ETV4* and *DDX5:ETV4* by fusion probe FISH assay (Fig. 5).

No case with *ETV5* rearrangement was identified in the present cohort, consistent with our previous report suggesting that this gene fusion is relatively rare (6). Among the remaining 23 ETS transcription factors, a small proportion of prostate cancer cases revealed aberrant 5' or 3' deletions for ETS genes using our FISH screening strategy (Fig. 2). We found single cases of deletions in the 5' end of *ETV3*, *ELF1*, and *SPIC*, and the 3' end of *ETV3*, *ELF2* and *ELK3*. Although FISH screening with a ~1Mb split probe approach yielded split signals for

the *ELK3* locus in case no.12 and *ELF4* locus in case no.21, further FISH analysis by narrowing down the region identified the breakpoint location 220 Kb downstream of *ELF4* gene and 140 Kb upstream of *ELK3* gene. Of note, BACs used to detect *ELK4* rearrangement were the same as utilized to detect *SLC45A3* aberration because of proximity (~20 Kb) of the two genes. Split signals were observed in four cases (nos. 4, 39, 67 and 102). Of these, case no. 4 and no. 102 were identified to harbor *SLC45A3:ETV1* and *SLC45A3:ERG* fusions, respectively. None of these cases harbored fusions between *ELK4* and *SLC45A3* (data not shown). Thus, many of these singleton deletions are likely non-specific, non-recurrent lesions.

Further, we screened our 110 patient TMA for all of the known 5' partners of ETS aberrations in prostate cancer and found *TMPRSS2* to be rearranged in 47% of cases (48/102). *SLC45A3* was rearranged in 2% (2/89), followed by *HNRPA2B1* in 1% (1/99), and *C15ORF21* in 1% (1/88) of cases. *HERV-K_22q11.23* rearrangement was not identified in this cohort. Notably, as shown in Figure 2, FISH analysis revealed several cases with 5' partner rearrangements without ETS gene aberrations, indicating these ones may harbor non-ETS gene fusion partners.

Next, we sought to characterize the 5' fusion partners of *ETV1* and *ETV4* identified in this study. By Q-PCR, *ETV1* or *ETV4* over-expression was confirmed in case nos. 54, 46, and 85 which harbored *FLJ35294:ETV1*, *CANT1:ETV4* and *DDX5:ETV4* fusions, respectively (Fig. 6A). As it is possible that aberrant ETS expression was driven by the regulatory elements of *FLJ35294*, *CANT1* and *DDX5*, we explored their tissue specificities and androgen regulation in prostate cancer. Similar to *TMPRSS2*, *SLC45A3*, *FLJ35294* and *CANT1* showed marked overexpression in prostate cancer compared to other cancer types using the International Genomic Consortium's expOdata set accessed in Oncomine (7) (Fig. 4B, $P=2.8 \times 10^{-7}$ (*FLJ35294*); $P=6.8 \times 10^{-7}$ (*CANT1*)). By contrast, *DDX5* exhibited high expression in all tumor types (Fig. 4B) suggesting that it may have a house-keeping function rather than prostate specificity.

By Q-PCR, expression of endogenous *FLJ35294* (11.6-fold at 48 hours, $P=0.0006$) and *CANT1* (3.8-fold at 24 hours, $P=0.0014$) were significantly induced by treatment with synthetic androgen R1881 in the LNCaP prostate cancer cell line (Fig. 6B). Furthermore, ChIP analysis demonstrated androgen receptor (AR) occupancy of the promoter region in the *FLJ35294* (9-fold enrichment, $P=0.005$) and *CANT1* (40-fold enrichment, $P=0.0014$) genes (Fig. 6C). By contrast, the expression of *DDX5* was not affected by R1881 stimulation (Fig. 6B) and AR was not recruited to the promoter region of *DDX5* (Fig. 6C). Therefore, the *FLJ35294-ETV1* and *CANT1-ETV4* fusions can be categorized as class II gene fusions in prostate cancer which include rearrangements involving fusions from prostate-specific androgen-induced 5' partner genes (21); whereas *DDX5-ETV4* may represent a class IV gene fusion, in which non-tissue-specific promoter elements drive ETS gene expression.

Like the previously reported prostate cancer gene fusions, *FLJ35294-ETV1* and *CANT1-ETV4* fusions contain no predicted translated sequence (exons) from the 5' partner. However, unlike almost all previously reported gene fusions in prostate cancer, the *DDX5:ETV4* fusion transcript codes for a putative fusion protein, with an N-terminal 102 amino acid stretch of *DDX5* protein fused to 416 C-terminal amino acids of *ETV4* (Fig. 6D). We confirmed that a fusion protein is expressed by this chimeric transcript by cloning the full length cDNA from case no. 85 into an expression vector encoding an in-frame N-terminal FLAG epitope tag. Upon transient transfection into human embryonic kidney (HEK) 293 cells, we detected the expression of the *DDX5-ETV4* fusion protein across independently derived clones (Fig. 6D). To assess whether case no. 85 expresses the *DDX5:ETV4* fusion protein we subjected tissue extracts to immunoblot analysis using an antibody to the C-terminal end of *ETV4* (Fig. 6D). As expected, only the *DDX5:ETV4* positive case expressed the aberrant 57kDa fusion protein.

To our knowledge, this is the first report of characterization and expression of a fusion protein in prostate cancer.

In this study we employed a systematic and comprehensive approach to screen for recurrent gene rearrangements in the ETS family of oncogenic transcription factors. While we used this approach to delineate ETS aberrations in prostate cancer, one could employ this FISH screen in other epithelial tumors or sarcomas. Gene rearrangements, amplifications, and deletions of the ETS family are all captured by this assay. If the FISH evaluation processes were automated, one could envision scaling up this approach to cover all of the genes in the genome, which would help in discovery of recurrent gene fusions in cancer (which often are difficult to detect by array comparative genomic hybridization based technologies).

Combining the comprehensive FISH analyses carried out in this study with the published literature, we note that only *ERG*, *ETV1*, *ETV4*, and *ETV5* gene fusions have been identified in prostate cancer. Notably, in this study, 44% (47/106) of prostate cancer cases do not harbor any ETS aberration, indicating a substantial fraction of prostate cancers can be clearly designated as being “ETS gene fusion negative”. This observation will stimulate research into identifying recurrent non-ETS aberrations or non-gene fusion mechanisms in prostate cancers. This observation is also consistent with our previous work and that of others, which indicate that ETS fusion positive and ETS fusion negative cancers have distinct transcriptional signatures across profiling studies (7,24). Interestingly, using a meta-Cancer Outlier Profile Analysis (COPA) strategy, we have recently identified *SPINK1* overexpression in a subset of ETS rearrangement negative prostate cancers (~11% of total cases) (25).

In addition to ETS gene fusions, loss of *PTEN* activity is another common molecular aberration in prostate cancer (26–28). Yoshimoto et al (29) have suggested that *TMPRSS2:ERG* fusion may be accompanied by *PTEN* deletion, and *TMPRSS2:ERG* fusion and *PTEN* deletion together are a predictor of PSA recurrence in prostate cancer. These findings suggest that these two leading genetic events may be concomitant in a subset of cases, and these aberrations may cooperate to promote prostate cancer progression.

Among the known 5' fusion partners, we have shown the observed prevalence of chromosomal rearrangements in prostate cancer to be *TMPRSS2* >> *SLC45A3* > *HNRPA2B1*, *CANT1*, *FLJ37254*, *C15ORF21*, *HERV-K_22q11.2*. Recently, *SLC45A3* was identified as a 5' fusion partner of *ETV1* and *ETV5* (6,7). Our current findings confirmed *SLC45A3* as a recurrent 5' partner occurring in approximately 2% of prostate cancer cases. More importantly, this is the first study which shows that *SLC45A3* can serve as an alternative 5' partner of *ERG*, as all previously reported *ERG* fusions involved *TMPRSS2* as the 5' partner. Thus, these findings suggest that other androgen-regulated genes can also serve as 5' fusion partners of *ERG*. This study also identified cases with aberrations in 5' fusion partners not associated with fusion to the ETS family genes, suggesting the possibility that other novel non-ETS genes are involved in chromosomal rearrangements in prostate cancer.

The ETS gene fusions that we have identified so far are all mutually exclusive from one another and thus are presumably ‘drivers’ for their respective tumor, even though some of them are relatively rare in our cohort. One limitation of our current study is that our surgical case series may not be representative of general prostate cancer patient population. In a recent study, Demichelis et al observed a significant lower frequency of *TMPRSS2:ERG* fusions in a Swedish population-based prostate cancer cohort (17). Therefore, additional studies with larger population-based cohort may further define prevalence of ETS gene fusions in prostate cancer.

In a previous study, we identified several 5' fusion partners involved in ETS fusions which are differentially regulated by androgen (androgen-induced, androgen-repressed and androgen

insensitive) that define distinct classes of ETS gene rearrangements (7). In the present study, we identified two prostate-specific, androgen-induced genes *FLJ37254* and *CANT1* partnering *ETV1* and *ETV4*, respectively. While this study was in preparation, Hermans *et al.* reported *KLK2* and *CANT1* genes as novel fusion partners of *ETV4* in prostate cancer (23). Interestingly, the *CANT1:ETV4* fusion transcript in our study contained the same alternative 1st exon of *CANT1* as described by Hermans *et al.*, which they reported was prostate specific. These observations also suggest that *CANT1:ETV4* fusion is a recurrent aberration in prostate cancer.

To date, almost all of the gene fusions identified in prostate cancer have been chimeric mRNA transcripts with no associated fusion protein. Here we report the first fusion protein in prostate cancer comprised of the 102 N-terminal amino acids of *DDX5*, DEAD (Asp-Glu-Ala-Asp) box polypeptide 5, fused to the C-terminal 419 amino acids of *ETV4*. This fusion protein appears to be expressed at high levels and presumably activates an ETS oncogenic program. How the N-terminal 102 amino acids of *DDX5*, which appear to contain neither the DEAD nor the helicase domain, might affect *ETV4* is unclear and would be best addressed in future studies. Interestingly, *DDX5* is known to interact with a large number of signaling molecules including Steroid receptor co-activator (SRC1), estrogen receptor alpha (ESRA), GSK3b etc. (Ref: www.hprd.org) Presumably, like *BCR* in the *BCR-ABL* fusion protein confers aberrant *ABL* kinase activity in chronic myelogenous leukemia, the N-terminal domain of *DDX5* may confer novel activity to *ETV4* or bring about novel signaling protein interactions with *ETV4*, in a subset of prostate cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jill Granger for editorial review of this manuscript and Michele LeBlanc, Julie Kim for technical support. Supported in part by Department of Defense (PC040517 to R.M., W81XWH-06-1-0224), Department of Defense (PC060266 to J.Y.), the National Institutes of Health (Prostate SPOR P50CA69568, R01 CA102872), the Early Detection Research Network (U01 CA111275-01) and the Prostate Cancer Foundation. A.M.C. is supported by a Clinical Translational Research Award from the Burroughs Wellcome Foundation. S.A.T. is supported by a Rackham Predoctoral Fellowship is a Fellow of the Medical Scientist Training Program. S.A.T. is supported by the GPC Biotech Young Investigator Award from the Prostate Cancer Foundation. Fusion sequences identified in this manuscript are deposited in Gene Bank, under the accession numbers EU693077, EU693078, EU693079.

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Figure 1. Schematic approach for the comprehensive FISH screen of ETS aberrations in prostate cancer

A FISH-based high-throughput screening strategy was utilized to evaluate all 27 ETS transcription factors. **A**, A split probe FISH approach was utilized with ~1 Mb probes flanking ETS breakpoints and applied to a prostate cancer TMA for screening genetic rearrangements involving ETS family genes. For the detection of *ETV1*, *ETV4*, and *ETV5* rearrangements, in which breakpoints have been already characterized, BACs for split probes were used as described previously (6–9,11). The ETS gene is indicated by the blue box, with the direction of transcription indicated by the arrow. 5' and 3' BACs are indicated in ovals, indicating the probe color in the accompanying images. **B**, Possible outcomes from FISH-based screening of

prostate cancer. Green and red arrows show individual signals, while yellow arrows indicate co-localized signals in 4'6-diamidino-2-phenylindole stained nuclei. A representative case lacking any ETS rearrangement indicated by two pairs of co-localized green and red signals (yellow arrows). A translocation (split) is indicated by one pair of split 5' and 3' signals. A deletion is indicated by loss of one 5' or 3' signal. **C**, If the 1Mb split probes suggested an aberration, probes tightly flanking the gene of interest (100~200 Kb) were applied on tissue sections to confirm aberrations identified by the initial screening. **D**, For cases rearranged for both ETS gene and known 5' partners, a fusion probe FISH assay was performed to confirm potential gene fusion; in cases with only ETS gene rearrangement, RLM-RACE was utilized to identify novel 5' partners of ETS genes.

Figure 2. Summary matrix of ETS and 5' fusion partner aberrations in prostate cancer
Rearrangement status of the 27 ETS family genes and their known 5' fusion partners were assessed in 110 clinically localized prostate cancer by FISH analysis in triplicate tissue cores. Patient case numbers are shown on the left of the heatmap. Each row represents a single patient and each column represents FISH evaluation for ETS family gene or a known 5' fusion partner. Color key signifies respective aberrations or availability. (*, BACs used for detection of *ERG* gene rearrangement were also utilized to detect *ETS2* gene aberration because of proximity of the two genes. Similarly, **, BACs used for detection of *EHF* aberrations were also utilized to detect *ELF5* gene rearrangements; ***, BACs used for detection of *FLI1* were also utilized to detect *ETS1*; ****, BACs used for detection of *ELK4* were also utilized to detect

SLC45A3 aberrations. #, Due to the very close chromosomal locations of *ELK4* and *SLC45A3*, FISH split probes could not distinguish rearrangements between the two genes individually in Case nos. 39 and 67).

Figure 3. Identification of *SLC45A3* as a novel 5' fusion partner for *ERG*

A, Schematic of BACs located 5' and 3' to *SLC45A3* and *ERG* used as probes for interphase FISH. Chromosomal coordinates are from the March 2006 build of the University of California Santa Cruz (UCSC) Genome Browser. BACs are indicated as numbered rectangles. Genes are shown with the direction of transcription indicated by the arrowhead and exons indicated by bars. **B**, FISH was performed using BACs as indicated with the corresponding fluorescent label on formalin-fixed paraffin-embedded tissue sections for split signal assay of the *SLC45A3*, *ERG* respectively and fusion of the 5' to *SLC45A3* and 3' to *ERG*. Split signals are indicated by red and green arrows, and fused signals are indicated by yellow arrows. For all assays, at least 50 nuclei were evaluated.

Figure 4. Identification of 5' fusion partners *FLJ35294*, *CANT1* and *DDX5* in prostate cancer
A, Schematic of *FLJ35294-ETV1* (PCa_54), *CANT1-ETV4* (PCa_46) and *DDX5-ETV4* (PCa_85) fusions identified by 5'RACE analysis. The numbers in the boxes represent exons. The numbers above the boxes indicate the last base of each exon. Untranslated regions are represented in lighter shades (*FLJ35294* is a predicted transcript with an uncharacterized gene structure). #, Evidence for exon 1a, b and c for *CANT1* gene is based on EST sequence alignments from the March 2006 build of the UCSC Genome Browser; EST accession number is DB045820. **B**, A plot of expression of *FLJ35294*, *CANT1* and *DDX5* genes in prostate cancer (red) compared with 28 other cancer types (blue) based on the International Genomic Consortium's expOdata set accessed in the Oncomine dataset (11). The significance value of

expression in prostate cancer versus all other tumors is indicated. Box and whisker plots show the median and 10th and 90th percentiles in normalized expression units.

Figure 5. Confirmation of *FLJ35294:ETV1*, *CANT1:ETV4* and *DDX5:ETV4* fusions by FISH
A, Schematic of BACs located 5' and 3' to *ETV1*, *ETV4*, *FLJ35294* and *CANT1* used as probes for interphase FISH. Chromosomal coordinates are from the March 2006 build of the UCSC Genome Browser. BACs are indicated as numbered rectangles. Genes are shown with the direction of transcription indicated by the arrowhead and exons indicated by bars. **B–D**, FISH was performed using BACs as indicated with the corresponding fluorescent label on formalin-fixed paraffin-embedded tissue sections for **B**, split signal assay of *ETV1* and *ETV4*, **C**, split signal assay of the 5' fusion partners, and **D**, fusion of the 5' partners and *ETV1* or *ETV4*. Split signals are indicated by red and green arrows, and fused signals are indicated by yellow arrows. For all assays, at least 50 nuclei were evaluated.

Figure 6. Androgen-regulation of the *FLJ35294* and *CANT1* genomic locus and characterization of the *DDX5:ETV4* fusion protein in prostate cancer

A, Expression of *ERG* (purple), *ETV1* (green) and *ETV4* (blue) was determined by Q-PCR in benign prostate and prostate cancer cases positive for either *FLJ35294:ETV1*, *CANT1:ETV4*, *DDX5:ETV4* or *SLC45A3:ERG*. The expression levels of *ERG*, *ETV1* and *ETV4* exons were normalized to that of *GAPDH* and they are means of triplicate experiments. **B**, Androgen directly regulates *FLJ35294* and *CANT1* expression but not *DDX5* expression. LNCaP cells were hormone-deprived for 48 hours, treated with R1881 or ethanol control, and assayed for *FLJ35294*, *CANT1* and *DDX5* transcript expression by Q-PCR. The effect of androgen was evaluated against a control sample at each corresponding time-point after treatment. The levels

of *FLJ35294*, *CANT1* and *DDX5* were normalized to that of *GAPDH* ($n = 3$, mean $\pm$ SEM). **C**, Androgen receptor (AR) is physically recruited to the *FLJ35294* and *CANT1* genomic locus but not the *DDX5* locus. AR enrichment values at the respective loci were assessed by chromatin immunoprecipitation (ChIP) analysis. ChIP was performed using an anti-AR antibody. ChIP-enrichment was determined as the percentage of input DNA ($n = 3$, mean $\pm$ SEM). A 3' exon region of *KIAA0066* was used as a negative control. **D**, Characterization of the *DDX5-ETV4* fusion protein in prostate cancer. Upper panel, a schematic diagram of the *DDX5-ETV4* fusion protein indicated by amino acids 1 to 102 of *DDX5* fused to amino acids 65 to 484 of *ETV4*. A partial Q motif of *DDX5* is retained in the fusion protein along with the ETS domain of *ETV4* as indicated. Middle panel, detection of FLAG-tagged *DDX5-ETV4* fusion protein by immunoblot analysis of HEK 293 cells transiently transfected with pCDNA3.2-*DDX5-ETV4* expression construct. The cDNA was cloned from the patient specimen that harbored the *DDX5:ETV4* fusion. Lower panel, detection of endogenous *DDX5-ETV4* fusion protein (57 kDa) in a tissue lysate derived from the patient harboring the *DDX5-ETV4* gene fusion (PCa_85). A lower band of 54 kDa was seen in all of the prostate lysates and may presumably represent endogenous *ETV4* (not shown). GAPDH is used as the loading control.