Two Unique Novel Prostate-Specific and Androgen-Regulated Fusion Partners of ETV4 in Prostate Cancer

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Abstract

Recently, fusion of ERG to the androgen-regulated, prostate-specific TMPRSS2 gene has been identified as the most frequent genetic alteration in prostate cancer. At low frequency, TMPRSS2-ETV1 and TMPRSS2-ETV4 fusion genes have been described. In this study, we report two novel ETV4 fusion genes in prostate cancer: KLK2-ETV4 and CANT1-ETV4. Both gene fusions have important unique aspects. KLK2 is a well-established androgen-induced and prostate-specific gene. Fusion of KLK2 to ETV4 results in the generation of an additional ETV4 exon, denoted exon 4a. This novel exon delivers an ATG for the longest open reading frame, in this way avoiding translation start in KLK2 exon 1. Although wild-type CANT1 has two alternative first exons (exons 1 and 1a), only exon 1a was detected in CANT1-ETV4 fusion transcripts. We show that CANT1 transcripts starting at exon 1a have an androgen-induced and prostate-specific expression pattern, whereas CANT1 transcripts starting at exon 1 are not prostate specific. So, the two novel ETV4 fusion partners possess as predominant common characteristics androgen-induction and prostate-specific expression. [Cancer Res 2008;68(9):3094–8]

Introduction

Prostate cancer is the most common malignancy in men in Western countries (1). Growth of prostate tumors depends on androgen signaling, mediated by the androgen receptor (AR). Metastatic disease is treated by endocrine therapy; however, all tumors eventually become resistant to this therapy. The majority of resistant tumors still contain a functional active AR (2). In part of these tumors, AR is overexpressed due to amplification of a small region of the X chromosome, where AR maps (3).

Most frequent genomic alterations in primary prostate cancers are losses of large fragments of chromosome arms 6q, 8p, 13q, and 16q and gain of 8q (4, 5). In a subset of tumors, a small region of loss of chromosome 21q22 has been detected. This genomic alteration is associated with recurrent fusion of prostate-specific and androgen-regulated TMPRSS2 (6) to the ETS transcription factor gene ERG, which maps at a distance of 3 Mbp from TMPRSS2 on 21q (7).

TMPRSS2-ERG fusion is present in 40% to 70% of clinical prostate cancers (7, 8). The gene fusion is an early event that has also been detected in a proportion of precursor lesions (9), Although in many tumors, TMPRSS2-ERG overexpression is accompanied by loss of the region between TMPRSS2 and ERG, in others, this region has been retained, indicative of different mechanisms of gene fusion (10). At low frequency, fusion of TMPRSS2 to a second ETS gene, ETV1, which maps to chromosome band 7p21, has been reported (7, 10). TMPRSS2 seems the only fusion partner of ERG, but it has recently been shown that ETV1 has more fusion partners (11).

For a third ETS gene, ETV4, only fusion to TMPRSS2 has been described (12). In the present study, we identified in clinical prostate cancer samples two unique novel ETV4 fusion partners: Kallikrein 2 (KLK2) and Calcium-Activated Nucleotidase 1 (CANT1). KLK2, or hGK1, is a well-known prostate marker highly homologous to KLK3 or prostate-specific antigen (13). Like KLK3, KLK2 is prostate-specific and androgen-regulated expressed (13, 14). We show that CANT1 expression is also androgen regulated. CANT1 possesses two alternative first exons, but only one of these is present in the CANT1-ETV4 fusion transcript. In contrast to the majority of CANT1 transcripts, this mRNA is preferentially expressed in the prostate. The novel fusion genes indicate prostate-specificity and androgen-regulation as important characteristics of ETV4 fusion partners in prostate cancer.

Materials and Methods

Samples. Two series of clinical prostate cancer samples were obtained from the tissue bank of the Erasmus University Medical Center. Samples were snap frozen and stored in liquid nitrogen. All samples contained at least 70% tumor cells. Collection of patient samples has been performed according to national legislation concerning ethical requirements. Use of these samples has been approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act (MEC-2004-261). Prostate cancer xenografts were propagated by serial transplantation on male nude mice as described (10, 15).

DNA and RNA isolation. RNA from clinical prostate cancer specimens was isolated using the RNA-Beet kit (Campro Scientific). DNA was isolated using the DNeasy DNA extraction kit (Qiagen). Xenograft RNA was isolated according to the LCI protocol. RNA from the prostate cancer cell line LNCaP cultured in the presence of 10-9M R1881, or in the absence of hormone, was isolated using the RNasea RNA extraction kit (Qiagen).

mRNA expression analysis. Analysis of mRNA expression was performed by reverse transcription-PCR (RT-PCR) or by QPCR. Two micrograms of RNA were reverse transcribed using 400 U M-MLV RT (Invitrogen) and an oligo-dT12 primer. cDNAs of 16 different tissues were purchased from Clontech. RT-PCR products were analyzed on 1.5% agarose gel. QPCR was performed in an ABI Prism 7700 Sequence Detection System (Applied Biosystems), using Power SYBR Green PCR Master Mix (Applied Biosystems), containing 330 nmol/L forward and reverse primer,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Results and Discussion

ETV4 expression was studied by QPCR on two sets of clinical prostate cancer samples. Set 1 was composed of 84 clinical prostate cancer samples (49 primary prostate tumors, 11 lymph node metastases, and 24 recurrent tumors) and set 2 of 36 primary prostate tumors and 29 recurrent tumors. In primary tumor (98), and in recurrent tumor (206), both from set 1, ETV4 overexpression was detected (Fig. 1A). In the second set, we did not detect ETV4 overexpression (data not shown), indicating that overexpression is a rare event, occurring in <2% clinical prostate cancers. Follow-up experiments showed that ETV4 overexpression was caused by fusions to two different genes with unique properties.

First, RT-PCR experiments with TMPRSS2- and ETV4-specific primers excluded TMPRSS2 as fusion partner (data not shown). Next, we performed RLM-RACE using a reverse primer in ETV4 exon 6. Sequencing of the amplified fragments showed KLK2 (sample 98) and CANT1 (sample 206) as novel ETV4 fusion partners. The presence of KLK2-ETV4 and CANT1-ETV4 fusion transcripts in the individual samples was confirmed by RT-PCR (Fig. 1B).

The KLK2-ETV4 mRNA fragment, detected by RLM-RACE, was composed of KLK2 exon 1 linked to a new ETV4 exon (here denoted exon 4a), followed by ETV4 exon 5 and 6 sequences (Fig. 1C). The novel ETV4 exon 4a has a length of 133 bp and delivers the ATG start codon of the longest predicted open reading frame in the KLK2-ETV4 fusion transcript (Fig. 1D). KLK2 maps to chromosome band 19q13 and ETV4 on chromosome band 17q21. Because of the orientations of KLK2 and ETV4, KLK2-ETV4 gene fusion cannot be explained by a single chromosomal translocation.

The CANT1-ETV4 fragment detected by RLM-RACE contained one of the two described exons 1 of CANT1 (here denoted exon 1a). This exon maps ~4 kbp downstream of the other first exon (here denoted exon 1; Fig. 1C). CANT1 exon 1a delivers the ATG start

Figure 1. Expression of ETV4 and characterization of ETV4 fusion transcripts in clinical prostate cancer samples. A, QPCR analysis of ETV4 expression in clinical prostate cancer samples compared with PBGD expression. Overexpression of ETV4 was detected in samples 98 and 206. Met, regional lymph node metastasis. B, confirmation of KLK2-ETV4 (sample 98) and CANT1-ETV4 (sample 206) fusion transcripts by RT-PCR, using ETV4 and fusion partner specific primers. RNA from prostate cancer cell line PC3 was used as a negative control. An RNA Pol II–amplified fragment is shown as a loading control. C, schematic representation of KLK2-ETV4 and CANT1-ETV4 fusion transcripts. Exons are indicated by colored boxes. In both transcripts, ATG start codons and TAG stop codons of longest predicted open reading frames are indicated. D, sequence of ETV4 exon 4a and flanking sequences. The ETV4 exon 4a sequence is underlined. KLK2 intron 1 sequence present in the fusion gene is in red. Splice sites are bold. The ATG start codon in ETV4 exon 4a is depicted in bold and italic.
Figure 2. Characterization of ETV4 fusion genes in clinical prostate cancer samples. A, schematic representation of the ETV4 genomic region on chromosome 17. Distance from the top of chromosome is indicated in Mbp. Blue arrowhead, orientation of ETV4. BAC probes used in split signal FISH are indicated in colors corresponding to FISH staining. Arrows, split signal FISH on frozen tissue sections confirms ETV4 rearrangements in samples 98 and 206. Schematic representation of genomic breakpoints in ETV4, KLK2, and CANT1 as found by long-range PCR and sequencing. Open boxes, numbered exons. Red arrows, positions of genomic breakpoints. Distances in kbp between exons are indicated. C, sequences of CANT1-ETV4 and KLK2-ETV4 fusion points. Red arrow, fusion point.

codon of the predicted ETV4 open reading frame. Remarkably, CANT1-ETV4 fusion transcripts starting at CANT1 exon 1 were not detected, although wild-type CANT1 transcripts starting either at exon 1 or 1a of the nonrearranged second allele were present in the tumor sample and in normal prostate (data not shown). CANT1 and ETV4 map in the same orientation on 17q, at a distance of ≈35 Mbp. However, array-based comparative genomic hybridization showed that the genomic region between ETV4 and CANT1 was not lost (data not shown), indicating that either an internal rearrangement of 17q had occurred or that this region was reintegrated in another part of the genome.

ETV4 rearrangements in samples 98 and 206 were confirmed by split signal FISH with probes flanking ETV4 at both sites (Fig. 2A). Next, the genomic fusion points were mapped by long-range PCR and sequencing (Fig. 2B and C). As expected, the breakpoints of CANT1-ETV4 were located in CANT1 intron 1, downstream from exon 1a, and in ETV4 intron 5, respectively. This latter breakpoint was located in a MER20 repeat, a low copy repetitive element known to be involved in unstable genomic regions prone to chromosomal rearrangement (16). Breakpoints in KLK2 and ETV4 in sample 98 were not in repetitive sequences. The breakpoint in ETV4 intron 4 of the KLK2-ETV4 rearrangement was 2 bp upstream of ETV4 exon 4a (Figs. 1D and 2C). Due to the gene fusion the purine-rich ETV4, intron sequence was replaced by a more pyrimidine-rich sequence of KLK2 intron 1, generating a novel splice acceptor sequence. To confirm that the rearrangement induced ETV4 exon 4a, a splicing assay was performed (Supplementary Fig. S1). ETV4 exon 4a was indeed retained if the KLK2-ETV4 fusion sequence was used, but not if intron 4 sequence of wild-type ETV4 was used in the assay (Supplementary Fig. S1).

KLK2 is a well-known androgen-regulated and prostate-specific gene (13, 14). To establish the specific characteristics of CANT1 expression, QPCR analysis on cDNAs from 11 human prostate cancer xenografts derived from various stages of the disease and with different AR status was performed (10, 15). We compared expression of transcripts starting at CANT1 exon 1 and transcripts starting at exon 1a to KLK2 expression. KLK2 mRNA expression was completely restricted to xenografts with highest AR expression (Fig. 3A). CANT1 transcripts starting at exon 1a showed highest expression in the more differentiated, androgen-dependent xenografts (Fig. 3B). In contrast, transcripts starting at exon 1 were expressed at variable levels in all xenografts. Further analysis of RNA from LNCaP prostate cancer cells, which were in vitro cultured in the presence of the synthetic androgen R1881 or in the absence of hormone, showed that both transcripts starting at CANT1 exon 1 and exon 1a are induced by androgens (Fig. 3C). As expected, KLK2 expression was strongly induced by androgens. Next, we tested the tissue specificity of the CANT1 and KLK2 transcripts in a cDNA panel from 16 different normal tissue samples. KLK2 showed an expected strictly prostate-specific expression pattern (Fig. 3D). Remarkably, transcripts starting at CANT1 exon 1 were ubiquitously expressed, but transcripts starting at exon 1a had a much more restricted expression pattern, with highest expression in the prostate (Fig. 3E).

Both KLK2-ETV4 and CANT1-ETV4 have specific, unique characteristics: KLK2-ETV4 because a novel ETV4 exon is generated (exon 4a) and CANT1-ETV4 because CANT1 exon 1a is
exclusively used in CANT1-ETV4 fusion transcripts. A KLK2-ETV4 fusion protein containing the NH2-terminal KLK2 signal peptide would be secreted and could not function as a transcription factor. However, the start codon in the novel ETV4 exon 4a, which is preceded by an in frame stop codon, prevents the generation of such a fusion protein. Instead, synthesis of a truncated ETV4 protein, starting in ETV4 exon 4a can now be predicted (Fig. 1D). Exclusive usage of CANT1 exon 1a as first exon in CANT1-ETV4 fusion transcripts might have various explanations, including the positions of breakpoints of the specific genomic rearrangement and the prostate-specific expression of transcripts starting at exon 1a.

In prostate cancer, TMPRSS2 is the common fusion partner of the ETS family members ERG, ETV1, and ETV4. Recently, several novel ETV1 fusion partners have been identified: SLC45A3, HERVK-K_22q11.23, C15orf21, and HNRPA2B1 (11), with different expression characteristics. Based on expression profiles, the fusion partners were divided into distinct classes of ETV1 rearrangements, separating prostate-specific, androgen up-regulated genes and down-regulated genes, and ubiquitously regulated genes. Recently, we identified three novel ETV1 fusion partners: FOXP1, HERVK17, and EST14.4 Both HERVK17 and EST14 have a prostate-specific and androgen-induced expression pattern; FOXP1 seems expressed in many tissues. The three ETV4 partners identified, TMPRSS2, KLK2, and CANT1, show identical expression profiles: up-regulation by androgens and prostate-specificity.

The common expression pattern of ETV4 fusion partners indicates a similar mechanism of gene fusion. It is tempting to speculate that the genomic region of prostate-specific genes is unstable in a specific cell type. Based on the stem cell/progenitor cell concept of tumor development (17), progenitor cells of the luminal epithelial prostate cells, in which expression of the prostate-specific fusion partners is expected to be activated, are interesting candidates. In this regard, we could confirm TMPRSS2 induction during development of the mouse prostate.4 Unfortunately, there are no appropriate mouse counterparts of KLK2 and CANT1 (exon 1a).

Figure 3. Characteristics of KLK2 and CANT1 mRNA expression. Expression of (A) KLK2 and AR mRNA, and (B) transcripts starting in CANT1 exon 1 and CANT1 exon 1a, respectively, in 11 human prostate cancer xenografts. PBGD expression is used as control. C, androgen-regulated expression of KLK2 and CANT1 transcripts assessed by QPCR analysis in LNCaP cells grown in the absence or in the presence of the synthetic androgen R1881 (1 nmol/L). Expression relative to PBGD is presented. Tissue-specific expression of (D) KLK2 and (E) CANT1 transcripts. Tissue-specific expression was tested on a cDNA panel from 16 different normal tissues by QPCR analysis and is shown relative to PBGD expression. Columns, mean calculated from two independent experiments; bars, SD.
A second process that might play a role in a common mechanism of gene fusion includes the nuclear compartmen
talization of gene expression. It might be proposed that prostate-
specific genes are all expressed in a limited number of nuclear
compartments, or so-called transcription factories (18, 19). Possibly, ETV4 is expressed in the same compartments. In
accordance with the latter hypothesis, it has recently been shown that IGH and cMYC, which are fusion partners in Burkitt
lymphoma and plasmacytoma, are preferentially expressed in the
same nuclear regions at in vitro B cell stimulation (20).

Taken together, the results from this study show that ETV4 has multiple fusion partners and highlight the importance of
meticulous examination of gene fusions. Importantly, key deter-
minants of ETV4 fusion partners are not chromosomal location or expression level, but their androgen-regulated and prostate-specific expression pattern.

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