The IncRNA DRAIC/PCAT29 Locus Constitutes a Tumor-Suppressive Nexus
Kouhei Sakurai, Brian J. Reon, Jordan Anaya, and Anindya Dutta

Abstract

Long noncoding RNAs (lncRNA) are emerging as major regulators of cellular phenotypes and implicated as oncogenes or tumor suppressors. Here, we report a novel tumor-suppressive locus on human chromosome 15q23 that contains two multi-exonic lncRNA genes of 100 kb each: DRAIC (LOC145837) and the recently reported PCAT29. The DRAIC lncRNA was identified from RNA-seq data and is downregulated as prostate cancer cells progress from an androgen-dependent (AD) to a castration-resistant (CR) state. Prostate cancers persisting in patients after androgen deprivation therapy (ADT) select for decreased DRAIC expression, and higher levels of DRAIC in prostate cancer are associated with longer disease-free survival (DFS). Androgen induced androgen receptor (AR) binding to the DRAIC locus and repressed DRAIC expression. In contrast, FOXA1 and NKX3-1 are recruited to the DRAIC locus to induce DRAIC, and FOXA1 specifically counters the repression of DRAIC by AR. The decrease of FOXA1 and NKX3-1, and aberrant activation of AR, thus accounts for the decrease of DRAIC during prostate cancer progression to the CR state. Consistent with DRAIC being a good prognostic marker, DRAIC prevents the transformation of cuboidal epithelial cells to fibroblast-like morphology and prevents cellular migration and invasion. A second tumor-suppressive IncRNA PCAT29, located 20 kb downstream of DRAIC, is regulated identically by AR and FOXA1 and also suppresses cellular migration and metastasis. Finally, based on TCGA analysis, DRAIC expression predicts good prognosis in a wide range of malignancies, including bladder cancer, low-grade gliomas, lung adenocarcinoma, stomach adenocarcinoma, renal clear cell carcinoma, hepatocellular carcinoma, skin melanoma, and stomach adenocarcinoma.

Implications: This study reveals a novel tumor-suppressive locus encoding two hormone-regulated IncRNAs, DRAIC and PCAT29, that are prognostic for a wide variety of cancer types. Mol Cancer Res; 13(5); 1–11. ©2015 AACR.

Introduction

The growth of prostate cancer cells initially depends on androgen. Therefore, androgen deprivation therapy (ADT) is useful for primary prostate cancer. However, prostate cancer cells progress after ADT to grow in low androgen, a condition called castration-resistant (CR; formerly androgen-independent) state, leading to a tumor recurrence and metastasis (1). Several lines of evidence have shown that the androgen receptor (AR) or androgen-responsive pathways are differently activated in the CR cells so that pathways are active in low or absent androgen (1, 2). In addition, alternative pathways, such as mTOR and insulin-like growth factor receptor 1 (IGF1R) signaling, are activated to mimic the action of androgens and promote prostate cancer cell growth (3). However, the detailed mechanisms by which androgen-dependent (AD) cells become CR remain unclear.

Recent transcriptome analyses have identified a variety of noncoding RNAs as important gene regulators (4–9). Long noncoding RNAs (lncRNA) are defined as RNAs >200 nt in length with no functional open reading frame (10). Our laboratory has identified two novel lncRNAs, APTR (Alu-mediated p21 transcriptional regulator), which recruits PRC2 (polycomb repressive complex 2) to the p21 promoter region to repress the transcription of p21 (4) and MUNC (MyoD upstream noncoding), which can promote myogenesis (6). Some lncRNAs are known to be aberrantly expressed and act as oncogenes or tumor suppressors in cancers, including prostate cancer. The nuclear lncRNAs PCGEM1 and PRNCR1 bind to AR to stimulate AR-mediated gene programs (11). The cytoplasmic lncRNA PCAT-1 suppresses BRC2A through its 3′ UTR (untranslated region) to control homologous recombination (12). However, how these prostate cancer-related IncRNAs are regulated or whether they contribute to prostate cancer progression is largely unknown (11–13).

In our previous work, we performed miRNA screening using AD and CR cells and identified a tumor-suppressive miRNA, miR-99a, that is downregulated in CR cells and repressed by AR (14, 15). We also showed that multiple oncogenes, mTOR, SMARCD1, SNF2H, and IGF1R targeted by miR-99a contribute to prostate cancer progression (14–16). In this study, we report a novel IncRNA designated as DRAIC (downregulated RNA in cancer, inhibitor of cell invasion and migration) that is similarly regulated. AR is recruited to DRAIC locus to repress DRAIC. Conversely, DRAIC is induced by FOXA1 and NKX3-1, which are recruited to the same region as AR at the DRAIC locus and FOXA1 counters the repression of DRAIC by AR.
Interestingly, a tumor-suppressive lncRNA, PCAT29, which was recently reported by Malik and colleagues (13), is located 20 kb downstream of DRAIC locus and we report that it is also regulated by AR, FOXA1, and NKX3-1 just like DRAIC. Functional analyses show that DRAIC inhibits cancer cell migration and invasion. This study indicates that progression of prostate cancer is accompanied by a decrease of FOXA1 and NKX3-1, which leads to the decrease of both the novel tumor-suppressive lncRNAs, DRAIC and PCAT29, thereby increasing prostate cancer migration and invasion and decreasing disease-free survival (DFS). This is the first report of a novel lncRNA cluster, DRAIC/PCAT29 regulated by the same mechanism and suppressing prostate cancer progression. Analysis of publicly available data from The Cancer Genome Atlas (TCGA) revealed that DRAIC is a predictor of good prognosis in at least seven other malignancies.

Materials and Methods

Cell culture

VCap cells were maintained in DMEM. PC3M-luc cells were maintained in MEM-L, glutamine containing MEM non-essential amino acids, MEM vitamin solution, and sodium pyruvate (all are Life technology). Other cells were maintained in RPMI-1640 medium. All medium contain 10% FCS, except when measuring the effect of androgen. For the experiments on androgen responsiveness, LNCaP cells were cultured in phenol red-free RPMI-1640 medium supplemented with charcoal:dextran-striped FBS (HyClone) for 48 hours before the addition of R1881 (PerkinElmer).

Transfection

Transfections of siRNA (50 nmol/L) and plasmid vector were performed with Lipofectamine RNAiMax and Lipofectamine 2000 (Invitrogen), respectively. siRNA sequences are shown in Supplementary Table S1.

Scratch wound-healing assay

Scratches were performed by pipet tip in 6-well plate. After incubation for 24 or 48 hours, the migration of cells into the scratch was imaged. Gap areas were calculated by ImageJ.

Matrigel invasion assay

Cells were seeded into 24-well Matrigel Invasion Chamber (BD Biosciences) at 1 x 10^5 cells in serum-free medium. Ten percent FCS as chemoattractant was added only to the lower compartment. After incubation for 48 hours, the noninvaded cells were removed from the upper surface of the membrane by a cotton swab. The invaded cells were fixed using methanol, stained by crystal violet, and counted per membrane.

RNA isolation, RT-PCR, Western blotting, and ChIP assay

Total RNA and nuclear/cytoplasmic RNAs were extracted using TRIzol total RNA isolation reagent (Invitrogen), PARIS kit (Ambion), respectively. RT-PCR and Western blotting were performed according to standard protocols. Chromatin immunoprecipitation (ChIP) assay was performed with cells cross-linked with 1% formaldehyde and using 5 μg of antibody on Dynabeads according to published protocol (4). All details of the protocols are in Supplementary Information.

ChIP-seq analysis and RNA-seq analysis

Publicly available ChIP-sequencing (ChIP-seq) and RNA-seq data were downloaded and analyzed by standard bioinformatics protocols. Details are described in Supplementary Information.

Kaplan–Meier plot analysis

Publicly available TCGA data at cbioPortal (17) were used to plot Kaplan–Meier plots on tumors divided into two groups based on level of DRAIC expressed as a Z-score (18–20) Only those plots are included that showed a statistically significant (P < 0.05) survival difference between the two groups of patients. Similar trends were seen in other plots of these malignancies but are not included because the P value did not reach significance.

Results

DRAIC is a novel lncRNA decreased in CR cells and repressed by R1881

To identify novel lncRNAs involved in prostate cancer progression, we compared two published RNA-seq datasets (21, 22), (A) LNCap versus C4-2B cells and (B) vehicle versus R1881 (androgen analogue)-treated LNCap cells (Fig. 1A). C4-2B cells are bone metastatic CR cells derived from parental AD, LNCap cells (23). We tried to identify the lncRNAs that are (i) increased in C4-2B compared with LNCap cells and induced by R1881 in LNCap cells or (ii) decreased in C4-2B compared with LNCap cells and repressed by R1881 in LNCap cells.

The 903 and 751 genes were differentially expressed (P < 0.05) in A and B comparisons, respectively (Fig. 1A). Intersection of these genes identified 72 genes that meet (A) or (B) criteria as mentioned above. Among them, there were two lncRNAs, LOC728431 (also known as LINCO1137) and LOC145837. Both were lower in C4-2B than LNCap cells and repressed by R1881 in LNCap cells (Fig. 1A). LOC728431 and LOC145837 are composed of 3 exons at Chr.1p34.3 and 5 exons at Chr.15q23, respectively (Fig. 1B).

qRT-PCR showed that LOC728431 is almost at the same level in LNCap and C4-2B cells and is not drastically decreased by R1881, contrary to the RNA-seq comparisons (Fig. 1C and D). Therefore, we excluded LOC728431 from further analysis.

In contrast, qRT-PCR confirmed that C4-2B cells express lower level of LOC145837 (renamed by us as DRAIC) than LNCap cells and the expression is also decreased in other CR cells (Fig. 1E). In addition, DRAIC was repressed by R1881 in dose- and time-dependent manners (Fig. 1F).

DRAIC is a cytoplasmic and poly-adenylated RNA

DRAIC is a spliced transcript of 1.7 kb that is expressed mainly in the cytoplasm (Supplementary Fig. S1A). The coding potential (calculated by CpAT: http://rna-cpat.sourceforge.net/) of DRAIC is 0.342, which is comparable with those of other cytoplasmic lncRNAs: PCAT1 (ref. 12; 0.693; 2.1 kb RNA) and TINCR (ref. 24; 0.204; 3.8 kb RNA). For comparison, the coding potential of protein coding genes like GAPDH and Orc1 is 0.99. We confirmed the 3’end of DRAIC by 3’RACE using LNCap polyA+ RNA (Supplementary Fig. S1B). There are at least three additional transcript variants of DRAIC (Supplementary Fig. S2A) although RNA-seq data in LNCap cells (vehicle; ref. 21) show that the read counts of these three
**Figure 1.**
LncRNA, DRAIC is downregulated in CR cells and decreased by R1881. A, left: analysis of published RNA-seq datasets. Right, relative expression (FPKM, fragments per kilobase of exon per million fragments mapped) of LOC728431 and LOC145837 (DRAIC) in LNcap cells treated with vehicle or R1881 and C4-2B cells treated with vehicle (B). Top and bottom, LOC728431 and LOC145837 (DRAIC) gene structures and qPCR primer locations, respectively. C, the expression of LOC728431 in a panel of prostate cancer cells cultured in growth medium was measured by RT-qPCR and normalized to GAPDH. Mean ± SD, n = 3. The expression in LNcap is set as 1. D, LNcap cells were treated with R1881 at different doses (left) and times (right) and the expression of LOC728431 was measured by RT-qPCR. The expression in 0 pmol/L or 0 hour is set as 1. The rest as in Fig. 1C. E, the expression of DRAIC in a panel of prostate cancer cells cultured in growth medium was measured by RT-qPCR. The rest as in Fig. 1C. F, LNcap cells were treated with R1881 at different doses (left) and times (right), and the expression of DRAIC was measured by RT-qPCR. The rest as in Fig. 1C and D.

**DRAIC is a clinically relevant lncRNA in a variety of cancers**

To test whether ADT selects for changes in expression of DRAIC as the cancer progresses to CR state, we analyzed published RNA-seq of seven prostate cancer rich tumor biop-mentary Fig. S2B). There is no evidence in the 3' RACE-PCR products, the EST database, or the RNA-seq data of DRAIC being spliced to the PCAT29 gene (13) that is located 20 kb downstream.

**AR is recruited to DRAIC promoter and required for the repression of DRAIC**

We next sought to identify how DRAIC is repressed by androgen. The downregulation of DRAIC by the androgen analogue R1881 was reversed by androgen antagonist, bicalutamide, and by AR knockdown (Fig. 3A and B). We analyzed published AR ChIP-seq data (26) and identified several sites upstream and within DRAIC that are bound by AR in the presence of R1881 (Fig. 3C). AR ChIP-PER confirmed that AR is recruited to regions 1, 2, and 4 by R1881 (second gray bar set as 1.0). Seven malignancies showed statistically significant survival benefit of DRAIC overexpression in either overall survival (bladder cancer, lower grade glioma, lung adenocarcinoma) or DFS (renal clear cell carcinoma, hepatocellular carcinoma, skin melanoma) or both (stomach adenocarcinoma). Additional variants are much less than the ones of DRAIC (data not shown). qRT-PCR with variant-specific primers revealed that their expression patterns are similar to DRAIC (Supplementary Fig. S2B). There is no evidence in the 3' RACE-PCR products, the EST database, or the RNA-seq data of DRAIC being spliced to the PCAT29 gene (13) that is located 20 kb downstream.

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androgen-driven AR recruitment to the DRAIC locus is associated with the repression.

FOXA1 and NKX3-1 occupy the same regions where AR is recruited at DRAIC promoter

AR often colocalizes with other transcriptional factors across the prostate genome (27). Tan and colleagues showed that the binding motifs of FOXA1 and NKX3-1 are highly enriched in AR ChIP-seq samples (27). In addition, FOXA1 has been reported to act as a pioneer factor that opens local chromatin structure to allow AR to be recruited (28–31). We therefore analyzed the published FOXA1 and NKX3-1 ChIP-seq datasets (26, 27) to examine the binding of these transcription factors to the DRAIC locus (Fig. 4A and B). Interestingly, ChIP-seq peaks of these two transcriptional factors overlapped with those of AR at the DRAIC locus (Figs. 4A and B and Fig. 3C). We confirmed by ChIP-PCR that regions 1, 2, and 4 bind to FOXA1 and to NKX3-1 (Fig. 4A and B). Regions 1, 2, and
4 contain several ARE (androgen-responsive element) half-sites and several FOXA1 and NKX3-1-binding sites close to the AREs (Supplementary Fig. S3).

The expression of DRAIC is positively regulated by FOXA1 and NKX3-1

Contrary to our expectation that FOXA1 is a pioneer factor for AR and should repress DRAIC, the expression pattern of FOXA1 and NKX3-1 was similar to that of DRAIC: lower in most CR cells (except C4-2) compared with the AD cells (Fig. 4C and Fig. 1E). RNA-seq data from prostate adenocarcinomas from the cbioPortal (n = 487) show weak but statistically significant positive correlation between the expression of FOXA1 and DRAIC or NKX3-1 and DRAIC (Fig. 4D and E).

In addition, knockdown of FOXA1 or NKX3-1 decreased DRAIC levels (Fig. 4F). The siRNA-resistant forms of FOXA1 or NKX3-1 partially rescued the downregulation of DRAIC induced by the cognate siRNAs, ruling out the possibility of off-target effects of the siRNAs (Fig. 4G). FOXA1 or NKX3-1 therefore has an opposite effect on DRAIC expression compared with AR, suggesting that FOXA1 is not acting as a pioneer factor for AR at the DRAIC promoter.

Knockdown of FOXA1 also decreased NKX3-1 protein and mRNA (Fig. 4F). Indeed, the FOXA1 and NKX3-1 mRNA levels were positively correlated in clinical samples (Fig. 4H). We did not see any significant FOXA1 ChIP-seq peaks (26) at the NKX3-1 locus (data not shown), suggesting that FOXA1 stimulates NKX3-1 expression by an unknown indirect mechanism.

A lncRNA, PCAT29 is regulated by AR, FOXA1, and NKX3-1

Malik and colleagues recently reported a tumor-suppressive lncRNA, PCAT29, whose expression is repressed by AR (13). Interestingly, PCAT29 gene is located 20 kb downstream of DRAIC. We therefore analyzed the published ChIP-seq dataset for AR, FOXA1, and NKX3-1 and identified that these transcriptional factors are also recruited to PCAT29 locus (Fig. 5A–C). The expression pattern of PCAT29 in a panel of prostate cancer cells is similar to that of DRAIC except for C4-2 cells (Figs. 5D and Fig. 1E). Because PCAT29 is not annotated in the level 3 data from TCGA, we used RNA from deidentified prostate cancer samples collected at University of Virginia (Charlottesville, VA) and used in a previous paper to analyze the correlation between DRAIC and PCAT29 (14). qRT-PCR of these lncRNAs showed a positive correlation between the expression of the two lncRNAs (Fig. 5E). From these results, we hypothesized that PCAT29 is regulated by FOXA1 and NKX3-1 in a manner similar to DRAIC. Indeed, siRNA against FOXA1 or NKX3-1 decreased PCAT29 expression (Figs. 5F and Fig. 4F).

Jin and colleagues recently reported that FOXA1 knockdown can shift or increase AR binding to selected sites (30). We analyzed their AR ChIP-seq data and found that shFOXA1 increases AR recruitment at DRAIC/PCAT29 cluster (Fig. 5G). Thus, FOXA1 actually decreases the recruitment of AR to the DRAIC/PCAT29 locus. Consistent with this, R1881 treatment or FOXA1 knockdown independently repress DRAIC and PCAT29, but together they repress both genes even further (Fig. 5H). This result suggests that instead of being a pioneer factor of AR, FOXA1 counters the action of AR at the DRAIC/PCAT29 cluster.
DRAIC represses cellular migration and invasion

Like PCAT29 (13), DRAIC is a marker for good prognosis in prostate cancer (Fig. 2B), and so is expected to repress oncogenic phenotypes. PCAT29 has been reported to repress invasion and metastasis (13). The ability of a panel of prostate cancer cells to invade through Matrigel in a Boyden Chamber assay was anticorrelated with the level of expression of DRAIC in the same cells (Figs. 6A and Fig. 1E), suggesting that DRAIC, like PCAT29,
represses invasion. Transient knockdown of DRAIC by siRNA in LNCap cells unexpectedly decreased cell numbers by about 30% to 50% (Fig. 6B), suggesting that DRAIC has a proliferative function. When DRAIC was stably knocked down by shRNA in LNCap cells, the cell proliferation was similarly decreased (data not shown) but interestingly, the cell morphology was changed from cuboidal to fibroblast-like shape (Fig. 6C). Stable DRAIC overexpression in PC3M-luc cells, in contrast, showed the opposite phenotype, with a change in morphology from fibroblast shape to cuboidal shape (Fig. 6D). In a scratch assay to measure
cell migration and in a Matrigel invasion assay, the migration and invasion of LNCap cells are increased by DRAIC knockdown (Fig. 6E and F). In similar assays, the migration and invasion of PC3M-luc cells are decreased by DRAIC overexpression (Fig. 6G and H). Taken together, these results suggest that DRAIC promotes cell proliferation but inhibits cell migration and invasion. We summarized the similarities and differences between DRAIC and PCAT29 in Fig. 7A.

Discussion

The regulation of the DRAIC and PCAT29 genes is remarkably similar to that we reported for the miR-99 family (14, 15). AR is recruited to the pri-miR-99a promoter and represses transcription in concert with EZH2 (14, 15). Considering that AR is recruited to broad regions around DRAIC (and the transcript variants) and PCAT29 gene, it is conceivable that a chromatin looping mechanism following AR recruitment is involved to produce a large domain with gene suppression.

FOXA1 and NKK3-1 have been variably thought to be tumor suppressive (32, 33) and oncogenic (34, 35). In the regulation described here, the two factors appear to be tumor suppressive in that their levels are decreased in CR cells and they are positive controls for the regulation of DRAIC and PCAT29, both of which decrease migration and invasion and predict good prognosis. We propose a model that FOXA1 and NKK3-1 induce the expression
A Novel lncRNA, DRAIC Represses Migration

of DRAIC/PCAT29 in AD prostate epithelial cells but are down-regulated in CR cells, leading to the decrease of DRAIC (Fig. 7B). Moreover, DRAIC is further repressed in the CR cells by differentially activated androgen-responsive pathways (Fig. 7B).

FOXA1 is well known to be a pioneer factor and stimulates AR-mediated gene regulation (28–31). However, our study clearly shows that FOXA1 counters the repression of DRAIC/PCAT29 by AR (Fig. 5H). Jin and colleagues showed that excess of FOXA1 opens up an excess of chromatin regions and ends up diluting AR across the genome thereby indirectly inhibiting specific AR-binding events (30). Therefore, we need to study whether FOXA1 directly or indirectly represses AR recruitment to DRAIC/PCAT29 cluster. Similarly, NKX3-1 is known to be positively regulated by AR and facilitates regulation of the AR downstream genes by associating with AR (27). However, at the DRAIC/PCAT29 locus, NKX3-1 has the opposite effect of AR on gene expression, suggesting a different mode of action.

Our functional analysis showed that DRAIC represses migration and invasion (Fig. 6) just like PCAT29 (13). However, knockdown of DRAIC represses cell proliferation (Fig. 6B), whereas knockdown of PCAT29 induces proliferation (13), suggesting that all functions of these lncRNA are not identical. This is borne out by the different cellular localization of DRAIC (cytoplasmic) and PCAT29 (nuclear; ref. 13). Future studies will analyze whether DRAIC and PCAT29 synergize with each other in repressing cell migration and invasion in vitro and in vivo.

Although it is tempting to propose that DRAIC represses epithelial-to-mesenchymal transition (EMT), preliminary results suggest that levels of mRNA involved in EMT are unchanged by DRAIC knockdown or overexpression (data not shown). Diverse mechanisms have been proposed by which lncRNAs could regulate many phenotypes at transcriptional and posttranscriptional levels (36). Thus, a detailed analysis is needed to determine the downstream targets of this cytoplasmic lncRNA and the molecular mechanism by which DRAIC regulates cellular migration and invasion.

It will be interesting to investigate in the future whether DRAIC/PCAT29 expression levels are related to the Gleason grade and whether they are useful as independent prognostic biomarkers of prostate cancer. The results reported here highlight that a thorough study of lncRNAs altered during prostate cancer
genesis and progression will be very important for improving our understanding and the therapy of this cancer.

Finally, DRAIC expression predicts good prognosis in a wide range of malignancies from many other tissues, suggesting that it is an important and ubiquitous tumor suppressor. Whether the mechanism by which clinical progression is suppressed is the same in all these tumors, and whether PCAT29 has a similar antiprogession effect in these tumors as in prostate cancer, will be important questions for the future.

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No potential conflicts of interest were disclosed.

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Conception and design: K. Sakurai, B.J. Reon, A. Dutta
Development of methodology: K. Sakurai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Sakurai

References

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