Award Number: W81XWH-12-1-0267

TITLE: Identification of Large Noncoding RNAs that Contribute to Prostate Cancer Progression

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REPORT DATE: October 2013

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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**Abstract:**
Large noncoding RNAs (lncRNAs) are pervasively transcribed in the genome, however their potential role in human diseases is poorly understood. In this study, we use RIP approach combined with RNAseq to capture lncRNAs from the genome-wide pool bound to androgen receptor in prostate cancer cells. We identify and confirm that PCGEM1 is an AR-binding partner. Next, to better evaluate the clinical relevance of PCGEM1, we examine its expression levels in a series of prostate tumor samples with different stage by quantitative RT-PCR. We found over all PCGEM1 is highly expressed in tumor samples with even higher expression in more aggressive tumor samples. We also explore the potential of PCGEM1 as aggressive prostate cancer biomarkers by detecting and quantify levels of PCGEM1 in situ on clinical specimen together with AR. We found co-localization signal of PCGEM1 and AR is correlated with tumor aggressiveness. Finally, overexpression and knockdown studies reveal that PCGEM1 plays a role in prostate cancer cells growth and drug resistance. More importantly, PCGEM1 is able to activate AR-mediated transcription. Taken together, the remarkable interaction of PCGEM1 with AR and its elevated expression in a significant percentage of tumor tissues suggest specific functions and clinical significance of PCGEM1 in prostate cancer progression.
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Introduction

Prostate cancer is the second leading cause of cancer death among men in the United States. Despite aggressive hormone therapy (androgen deprivation) along with chemotherapy, patients with metastatic tumors develop resistance to therapies and exhibit clinical and biochemical disease progression. Currently the 5-year survival rate for patients diagnosed with metastatic prostate cancer is ~25% (1). Because the majority of prostate cancers are likely to remain as indolent tumors, a great challenge to physicians is to identify those tumors with the potential to metastasize so that these patients will have an option for specific treatment (2).

The androgen receptor (AR) is a key regulator of prostate growth and the principal drug target for the treatment of prostate cancer because, as a nuclear receptor transcription factor, AR controls expression of genes important to prostate cancer cell growth and progression, and the development of castration-resistant prostate cancer (CRPC) (3, 4). While hormone therapy is the primary treatment for advanced prostate cancer, the development of CRPC after androgen deprivation therapy remains the major challenge in the treatment of advanced prostate cancer (5). Evidence indicates that continuous AR signaling constitutes a major mechanism of castration-resistant progression (6). However, it is not fully understood as to how AR signaling is reactivated after castration, leading to castration resistance. This may be attributable to the fact that we still do not have a comprehensive picture of AR-mediated signaling pathway. Therefore, there is a critical need for a better understanding of AR regulatory network.

Transcriptome analyses have suggested that, although only 1–2% of the mammalian genome is protein-coding, whereas the vast majority of transcripts are non-
coding RNAs. Among them are long noncoding RNAs (IncRNAs) with molecular weight of >200 bases in length (7, 8). Many of the identified IncRNAs show spatial- and temporal-specific patterns of expression. Almost every step in the life cycle of genes can be influenced by IncRNAs. Generally, IncRNAs have been implicated in gene-regulatory roles, such as chromosome dosage-compensation, imprinting, epigenetic regulation, cell cycle control, nuclear and cytoplasmic trafficking, transcription, translation, splicing, cell differentiation, and others.

Recent progress suggests that the involvement of IncRNAs in human diseases could be far more prevalent than previously appreciated. For example, HOTAIR was shown to reprogram chromatin state to promote breast cancer metastasis by genome-wide relocalization of protein repression complex 2 (PRC2), leading to epigenetic silencing of metastasis suppressor genes. Similarly, prostate cancer associate transcript 1 (PCAT-1) is a prostate cancer-specific regulator of cell proliferation and serves as a target of the PRC2 (9). Other IncRNAs associated with prostate cancer include ANRIL and PCAT-1. For example, ANRIL, also upregulated in prostate cancer, is required for the repression of the tumor suppressors INK4a/p16 and INK4b/p15 (10, 11). Together, these studies suggest that IncRNAs are an important player in prostate cancer biology and some of these IncRNAs could be valuable biomarkers and therapeutic targets in prostate cancer. However, it remains to be determined as to whether IncRNAs can play a role in prostate cancer progression through interaction with AR.

The purpose of this study is to identify long non-coding RNAs (IncRNAs) that play a role in prostate cancer progression so that such IncRNAs may serve as biomarkers to
distinguish indolent from aggressive prostate cancer or to predict the therapeutic response.

Body

PCGEM1 is an AR-associated IncRNA in prostate cancer cells

Due to the important role of AR in prostate cancer progression and CRPC, we switched our object from identifying PCR2-associated IncRNAs in prostate cancer cells to identifying IncRNAs that contribute to prostate cancer progression through interaction with AR. Native RNA immunoprecipitations (RIP) previously identified RepA, Xist, and Tsix as PRC2-interacting RNAs (12). Here, we utilized a method of capturing the genome-wide pool bound to AR by combining native RIP (12) and RNAseq (13). Briefly, nuclear RNAs immunoprecipitated by α-AR antibodies were isolated from prostate cancer cells (LNCaP, Du145, and PC3) exhibiting various degrees of aggressiveness and non-transformed RWPE1 cells. cDNAs created using strand-specific adaptors, and those from 200–1,200 nt were purified and subjected to Illumina sequencing and bioinformatics analysis (Fig. 1A).
RNA immunoprecipitation will be performed in prostate cancer cells. Cell nuclei will be isolated and nuclear lysate will be prepared, treated with DNase, and incubated with α-AR antibodies or control IgG. RNA-protein complexes will be immunoprecipitated with protein A agarose beads and RNA will be extracted. cDNAs will be generated by reverse transcription. Resulting cDNAs will be amplified by forward and reverse Illumina primers. PCR products from 200-1200 bp will be sequenced.

In pilot experiments, we performed RIP in 10^7 LNCap cells and included control IgG RIPs to assess the specificity of α-AR pull downs. In the LNCap pulldown and its technical and biological replicates, α-AR antibodies precipitated 70–170 ng of RNA from 10^7 LNCap cells and yielded a cDNA smear of >200 nt. Treatment with RNAses eliminated products in this size range and −RT samples yielded no products, suggesting that the immunoprecipitated material was indeed RNA. There was ~10-fold less RNA in immunoprecipitated by IgG (~24 ng). A 500-fold enrichment over a mock RIP control (no cells) was also observed. In the <200 nt size range, control RIPs (IgG pulldowns, mock) were even further depleted of RNA, as these samples were dominated by adaptor and primer dimers. We computationally filtered out adaptor/primer dimers, rRNA, mitochondrial RNA, reads with <18 nt or indeterminate nucleotides, and homopolymer runs in excess of 15 bases. From an equivalent number of cells, control RIPs were significantly depleted of reads. In LNCap libraries, 431,880–2.2 million reads remained after filtering. By contrast, only 6,888 to 83,691 reads remained in controls. The
overwhelming majority of transcripts in the controls were of spurious nature (adaptor/primer dimers, homopolymers, etc.). Therefore, LNCap RIPS exhibited substantial AR associated RNA enrichment and greater degrees of RNA complexity in comparison to control RIPS.

After validation of the RIP system, prostate cancer cell lines exhibiting various degrees of aggressiveness including non-transformed RWPE1 cells, androgen-sensitive LNCap, androgen-independent modest metastatic Du145, and androgen-independent most metastatic PC3 cell are applied for RIP and following RNA-seq. Through preliminary screen, we identified PCGEM1 as the most promising candidate. To further verify the interaction of PCGEM1 and AR, cellular extract was prepared from LNCaP cells and then was subject to RIP assay with AR antibody. The precipitated RNA was then used for quantitative RT-PCR (qPCR) using PCGEM1 specific primer. We observed significant enrichment in AR pull down samples than IgG samples. Further characterization with a different set of primers confirmed this interaction between AR and PCGEM1 (Fig. 1).

![Figure 1](image.png)

**Fig. 1** Association of AR with PCGEM1, as detected by RNA immunoprecipitation with AR antibody (A) and qRT-PCR (B). LNCaP extract was used for this experiment. n = 3; **, p < 0.01.
To further verify PCGEM1 and AR interaction, we performed RNA precipitation using synthetic biotin-labeled PCEGM1 RNA as a probe (Fig. 2A). It is evident that PCGEM1 can interact with AR (Fig. 2B). PCEGM1 consists of three exons with 1603 nucleotides in length. Of interest, the 5’-region of PCGEM1 (PCGEM1-A) seemed to interact with AR better than the full-length PCGEM1 (Fig. 2B), also suggesting that the binding site(s) may reside in this region.

**Fig. 2 Confirmation of AR-PCGEM1 interaction by RNA precipitation and Western blot.** PCGEM1 consists of three exons (E1, E2 and E3). A, Procedure for making RNA probes and precipitation. B, Western blot showing the interactions of full-length (FL) PCGEM1 and the 5’ end region (PCGEM1-A) of AR.

To further examine their interaction, we carried out immunofluorescence (IF) staining and fluorescence in situ hybridization (FISH) to detect AR and PCEGM1 co-localization in LNCaP cells. Thus, we performed FISH with a biotin-labeled antisense PCGEM1 probe, followed by IF with AR antibody. The PCGEM1 probe was modified with locked nucleic acid (LNA), which was purchased from Exiqon, to increase the stability of probe/target complex. FISH signal (red) was revealed by TSA™ Kit #24 with
Alexa Fluor 568 (Invitrogen). To determine the specificity of PCGEM1 signal, we took two approaches. The first one was to introduce 10 x excess amount of blocking oligo which was complementary to the probe in the hybridization buffer. Thus, the blocking oligo served as a competitor to PCGEM1 probe. As shown in Fig. 3A, little PCGEM1 signal was detected in the blocking oligo treated cells. The second approach was to design another probe derived from a different region of PCGEM1. Both PCGEM1 probes revealed the same PCGEM1 distribution pattern. These results demonstrate that the PCGEM1 signal as seen in Fig. 3 is specific. Of interest, while AR was predominantly in the nucleus, PCGEM1 was mainly localized to cytoplasm or nucleoli if it was in the nucleus in the absence of androgen (Fig. 3B). There was a relatively small amount of co-localization of PCGEM1 and AR, especially in “nuclear speckle-like structures” (Fig. 3B). However, in the presence of androgen, more AR moved into the nucleus and the nuclear AR signal became stronger; at the same time, more PCGEM1 was also detected in the nucleus (Fig. 3C). Of particular interest, the co-localization signal became much stronger, especially in the nucleoplasm (Fig. 3C), suggesting that androgen may promote both nuclear localization of AR and PCGEM1, and their interaction.
Fig. 3 Co-localization of AR and PCGEM1 in LNCaP cells. AR was detected by immunofluorescence (IF) staining with AR antibody; PCGEM1 by fluorescence in situ hybridization (FISH). A, Cells were grown in androgen free medium for 2 days and then IF and FISH were performed in presence of blocking oligo. B, Same as in A, but in the absence of blocking oligo. C, The same cells as in B were then cultured with androgen for overnight.

PCGEM1 is highly expressed in prostate tumor samples and associated with tumor cell aggressiveness
To better evaluate the clinical relevance of PCGEM1, we determined its expression in a series of prostate tumor samples with different Gleason scores by qPCR. We total examined 14 normal prostate samples and 89 prostate tumor samples. We found PCGEM1 was significantly highly expressed in tumor samples (Fig 4).

**Fig. 5 Expression of PCGEM1 in clinical samples.** The expression of PCGEM1 was determined by qPCR in normal prostate samples and tumor samples

We further characterized the expression of PCGEM1 based Gleason scores and found PCGEM1 expression maintains similar low level as normal samples in tumor samples with Gleason scores 5/10, then increases in tumor samples with higher Gleason scores (Fig. 6), suggesting that PCGEM1 expression may associate with tumor cell aggressiveness and PCGEM1 may serve as a biomarkers for prostate cancer to distinguish aggressive from indolent disease.
Fig. 6 Expression of PCGEM1 in prostate cancer samples with different Gleason scores.

To explore this possibility, we further evaluate the clinical relevance of PCGEM1 in clinical specimens together with AR. Although FISH for paraffin-imbedded specimens is technically challenging because these specimens often carry a high background of autofluorescence, we were able to manage to reduce the background and obtain specific signals for AR and PCGEM1, respectively (Fig. 7). For example, in the low grade prostate tumor, the distribution patterns of AR and PCGEM1 were similar to what we observed in LNCaP cells in the absence of androgen (Fig. 7, left panels). While AR was seen mainly in the nucleus, PCGEM1 was predominantly in the cytoplasm. There was little co-localization. However, in the high grade prostate tumor, we observed a relatively strong co-localization signal of PCGEM1 and AR (Fig. 7, right panels).
Since the epitope of the AR antibody used here was derived from the N-terminus (Cell Signaling, Cat#5153), it is expected to also recognize AR variants lacking ligand binding domain (LBD) such as AR3-V7 (14), in addition to full length AR. Therefore, it would be of interest to determine whether this increase in co-localization is due to upregulation of alternative AR splicing variants which have been shown to be associated with aggressiveness of prostate cancer and castration resistance (15).

**PCGEM1 promotes prostate cancer growth, colony formation and drug resistance**

*PCGEM1* is expressed as noncoding poly(A) RNA of 1643 nucleotides. *PCGEM1* along with *PCA3 (DD3)* (16) represent a novel class of prostate-specific genes whose functions remain to be defined in prostate biology and cancer. To better evaluate the role of PCGEM1 in prostate cancer cells, we cloned the PCGEM1 in lentivector and infected into LNCaP cells. We first performed MTT assay to compare the growth rate between vector control and PCGEM1 infectant cells. We found that PCGEM1 infectant cells grow significantly faster than vector control cells. In addition,
soft agar assay also showed that overexpression of PCGEM1 also increases colony formation. These results indicate the biological role of PCGEM1 in cell growth regulation.

Fig. 8 PCGEM1 promotes cell growth and colony formation in LNCaP cells. A, MTT assay was performed to monitor the cell growth rate between vector control and PCGEM1 infectant cells. Overexpression of PCGEM1 increases cell growth rate from Day1 to Day4. B, Soft agar assay was used to evaluate the anchor-free cell growth. Overexpression of PCGEM1 promotes colony formation in LNCaP cells.

The impressive promotion of cell growth of PCGEM1 leads us to address another important question as whether PCGEM1 also plays a role in drug resistant. Therefore, we treat the vector control LNCap cells and PCGEM1 infectant cells with clinical used anti-cancer drug doxorubicin (DOX) with different doses and measured cell survive rate by MTT assay. Interestingly, we found PCGEM1 infecting cells are more resistant to DOX than vector control cells, suggesting the role of PCGEM1 in drug resistance. The underlying molecular mechanism of PCGEM1 related cell growth and drug resistance needs further investigation.
Relative cell survive rate

![Graph showing relative cell survive rate vs. drug concentration]

**Fig. 9 Overexpression of PCGEM1 increases drug resistance in prostate cancer cells.** LNCap cells infected with vector control of PCGEM1 virus were treated with doxorubicin with different doses. MTT assay was used to measure evaluate relative cell survive rate.

**PCGEM1 can increase AR-mediated transcription**

After revealing the role of PCGEM1 in cell growth and drug resistance, the next question we want to address is whether PCGEM1 also impacts AR-regulated gene expression. For this purpose, we suppressed PCGEM1 in LNCaP cells and observed downregulation of PSA and TMPRSS2 by over 60% (Fig. 10), both of which are direct targets of AR, suggesting that PCGEM1 can increase AR-mediated transcription. Together, our results suggest that the interaction between AR and PCGEM1 could be important to the AR-regulated gene expression and may have a clinical significance. However, the molecular mechanism of this interaction between AR and PCGEM1 and subsequent gene activation still needs further investigation.
Fig. 10 Suppression of PSA and TMPRSS2 by PCGEM1-siRNA. Experiment was done in LNCaP cells. n = 3; **, p < 0.01.

Key Research Accomplishments

- We identified that PCGEM1 is an AR-associated lncRNA in prostate cancer.
- PCGEM1 is highly expressed in prostate tumor tissues and its expression is associated with prostate cancer aggressiveness.
- We demonstrated that localization of PCGEM1 and AR in prostate cancer cells changes in the present of Androgen.
- Co-localization signal of PCGEM1 and AR in clinical samples indicate that PCGEM1 may serve as a biomarker to distinguish aggressive from indolent disease.
- Overexpression studies reveal the role of PCGEM1 in promoting prostate cancer cell growth, colony formation as well as drug resistance.
Suppression of PCGEM1 impacts AR-regulated gene expression, suggesting the interaction between PCGEM1 and AR has biological function in prostate cancer cells.

Reportable Outcomes
Not yet.

Conclusion
Large noncoding RNAs (lncRNAs) are robustly transcribed in the genome, however their potential role in prostate cancer is poorly understood. In this study, using RIP approach in combination of RNAseq, we captured lncRNAs from the genome-wide pool bound to androgen receptor in prostate cancer cells. We successfully identified and confirmed that PCGEM1 is an AR-binding partner. To better evaluate the clinical relevance of PCGEM1, we examined its expression in a series of prostate tumor samples with different Gleason scores by quantitative RT-PCR. We found over all PCGEM1 is highly expressed in tumor samples and its expression level actually is associated with cancer aggressiveness. We then further explored the potential of PCGEM1 as aggressive prostate cancer biomarkers by detecting and quantify levels of PCGEM1 in situ on clinical specimen together with AR. We found nuclear co-localization signal of PCGEM1 and AR is correlated with tumor aggressiveness, indicating that PCGEM1 may serve as a biomarker to distinguish aggressive from indolent disease. Finally, overexpression studies revealed that PCGEM1 plays a role in prostate cancer cells growth, colony formation and drug resistance. On the other hand,
suppression of PCGEM1 decreases AR-mediated transcription, suggesting PCGEM1 also involves in AR-mediated transcription regulation. Taken together, the remarkable interaction of PCGEM1 with AR and its elevated expression in tumor tissues suggest specific functions and clinical significance of PCGEM1 in prostate cancer progression.
References


Appendices

Acronyms and Symbol Definitions:

AR: Androgen receptor
CRPC: castration resistant prostate cancer
IncRNAs: long noncoding RNAs
PRC2: protein repression complex 2
PCAT-1: prostate cancer associate transcript 1
RIP: RNA immunoprecipitation
qPCR: quantitative RT-PCR
IF: immunofluorescence
FISH: fluorescence in situ hybridization
LNA: locked nucleic acid
DOX: doxorubicin