Identification of REST regulated genes in prostate cancer via high-throughput technologies

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Abstract
Prostate cancer (PCa) is one of the most commonly diagnosed cancers in the world: males have 17% risk of developing PCa during lifetime. Androgen deprivation therapy (ADT) has been the leading form of PCa therapy [1]. Unfortunately, the effectiveness of this treatment is usually temporary, with a subpopulation of cells surviving. Growing evidence indicates that neuroendocrine differentiation (NED) occurs in 30-100% of disease recurrence after ADT [2, 3]. However, the mechanism is still poorly understood.

Repressor element-1 silencing transcription factor (REST) is responsible for restricting neuronal gene expression to the nervous system. REST was recently shown to be absent in various types of cancer. Blockade of REST function in human LNCaP cells is found to cause NED [4], indicating REST may be one of important determinants of neuroendocrine prostate cancer (NEPC). The aim of this study is to investigate the potential role of REST in PCa progression. We combined state-of-the-art sequencing technologies and bioinformatics with patient-derived next-generation models of PCa to deal with this problem. An integrated data analysis of RNA-seq on mouse xenograft model and expression microarray with RNA interference on human LNCaP cells was performed to identify potential REST targets. Experimentally, we validated most likely targets by QRT-PCR of LNCaP mRNA. In addition, we also estimated the clinical significance of the targets using public datasets. Our results help to elucidate the mechanism that drives development of androgen independent and aggressive NEPC after castration. Furthermore, it could provide a new prognosis factor for PCa and also provide a new target for the PCa treatment.

Key words: REST, prostate cancer, neuroendocrine differentiation, gene expression

Introduction
Prostate cancer (PCa) is the most common non-skin cancer in North American men and a leading cause of cancer death. Approximately, 1 in 7 North American men will be diagnosed with prostate cancer in his lifetime. Many factors contribute to PCa development, including the disruption in the interactions between mesenchymal and epithelial cells, which is mediated through changes in growth factors expression and overexpression of proteases acting on the mesenchyme to increase metastasis [5, 6].
There are several different treatment options for PCa patients. If the cancer is locally confined, treatments such as radical prostatectomy, radiation therapy or active surveillance may be employed. If the cancer at diagnosis has spread well beyond the gland, then androgen deprivation therapy (ADT) is the most common treatment for patients. Unfortunately, the effectiveness of this type of treatment is usually temporary due to the progression of surviving tumor cells which become castration-resistant prostate cancer (CRPC). This extremely painful form of PCa has no curative treatment options and a median life expectancy of around 18 months [7].

Prostate cancer can be broadly divided into two groups at diagnosis, based on the AR expression level [8]. AR and PSA positive adenocarcinoma accounts for over 95% of initial prostate cancer diagnoses, while NEPC which are typically AR and PSA negative (also known as small cell carcinoma) accounts for 2-3% of primary prostate cancer. However, at the late stage of PCa, 30-100% of advanced type show enrichment of neuroendocrine-like cells [3].

Interestingly, blockade of Repressor element 1- silencing transcription factor (REST) function in the human LNCaP cells is found to cause NED [4], indicating REST may be one of important determinants of NEPC. REST has been shown to play critical roles during embryonic development and neurogenesis. However, during tumorigenesis REST shows paradoxical roles in different type of cells. REST acts as a tumor suppressor in human epithelial cancers while it plays an oncogenic role in the development of brain tumors and other non-epithelial cancers. The aim of this study is to investigate the potential role of REST in PCa progression.

**Materials and methods**

**Cell lines and prostate tissue samples**

LNCaP cells were kindly provided by Dr. Leland WK Chung (1992, University of Texas MD Anderson Cancer Center, Houston, TX, USA). LNCaP cells used for sequencing were provided by Pfizer (La Jolla, CA, USA). Surgical samples were collected and snap-frozen (FF) at the Vancouver General Hospital (VGH).

**Mouse xenograft mode**

LTL331 xenograft of human prostate cancer was developed by grafting tumor biopsy specimen (tumor 927) under the renal capsules of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice [9]. To collect PCa tissue after castration, fresh LTL-331 tumor tissues from the 5th generation of grafting were cut into 3mm×3mm×1mm pieces and re-grafted into the subrenal capsules of male NOD/SCID mice.

**Public dataset**

The analyzed data can also be accessed and explored through the Memorial Sloan-Kettering Cancer Center (MSKCC) Prostate Cancer Genomics Data Portal: http://cbio.mskcc.org/prostate-portal/ [10]. This database contains a total of 218 tumor samples and 149 matched normal samples that were obtained from patients treated by radical prostatectomy at MSKCC.
siRNA and shRNA experiments

siRNAs and shRNAs corresponding to the antisense sequence of human REST gene were purchased from Santa Cruz Biotechnology, Inc. REST siRNA (ID: sc-38129) is a pool of 3 target-specific 19-25 nucleotides siRNAs designed to knock down gene expression. REST shRNA Plasmid (ID: sc-38129-SH) is a pool of 3 target-specific lentiviral vector plasmids each encoding 19-25 nt (plus hairpin) shRNAs designed to knock down gene expression.

Microarray and transcriptome sequencing

Total RNA was isolated from the LNCaP cells treated with 2 mg REST siRNA or 2 mg control siRNA for 48 h. An input of 100ng of total RNA was used to generate Cyanine-3 labeled cRNA and samples were hybridized on Agilent SurePrint G3 Human 4x180K Microarrays (Design ID 028679). Transcriptome sequencing of the samples was performed at British Columbia Cancer Agency Michael Smith Genome Sciences Centre (Vancouver, BC, Canada) using the the Illumina Genome Analyser II according to established protocols described in [11].

Data analysis

Statistical analysis was performed using unpaired t-test considering Benjamini–Hochberg corrected p-value of 0.05. Differentially expressed genes with over log2 fold change (FC) of 1.5 greater than or equal to 1.5 (up or down) were considered for the present study. Pathway and functional enrichment analysis was performed using Ingenuity (IPA) Knowledge Base 9 (Ingenuity®Systems, www.ingenuity.com). Survival curves were calculated using the Kaplan–Meier method, where the significance was determined by log-rank test.

Experimental RT-PCR validation

Total RNA was extracted from cultured cells after 48 hours of treatment using Trizol reagent (Invitrogen). Two micrograms of total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Real-time monitoring of PCR amplification of cDNA was performed using DNA primers on ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) with SYBR PCR Master Mix (Applied Biosystems).

Results

Specific and efficient knock-down of REST mRNA in LNCaP cells

We have utilized both siRNA and shRNA to identify genes regulated by REST in the human LNCaP cell model. The efficiency of the RNAi strategy for REST was evaluated by semi-quantitative real-time PCR in LNCaP cells treated with a REST siRNA compared with that treated with controls treated with a scrambled siRNA (Fig. 1).
up-regulation of NE differentiation marker SYP, whereas beta-actin (reference gene) was not changed.

As shown in Fig. 2, the RT-PCR result indicated that all expression levels of the shRNA knockdown samples were lower than the three controls (right side), whereas sample e showed the best silencing efficiency. Thus we continued to use e for the further studies.

High fidelity of mouse xenograft model for neuroendocrine transdifferentiation

The patient-derived LTL331 xenograft retains the histological characteristics of the patient tumor and is strongly PSA positive and AR positive (Fig. 3 A, B). Androgen deprivation by castration of LTL 331 mice resulted decreased tumor volume and serum PSA. In less than 4 months, tumors recurs, accompany with PSA and AR negative (Fig. 3 C, D). This recurrent tumor model is re-named “LTL 331R” and expresses a range of neuroendocrine markers including most common one synaptophysin (SYP), as shown E.

Microarray and RNA-seq analysis for identification of differential expressed genes

Total RNA of LNCaP cells treated with REST siRNA or scramble control for 48 h was isolated for comparative expression analysis using human cDNA microarrays.
On the other side, we performed transcriptome sequencing on patient-derived mouse xenograft models at two different time points: before castration and after neuroendocrine differentiation.

Figure 5 Summary of RNA-seq data on xenograft model. 10381 genes were found to be up-regulated and 7814 genes were down-regulated over log2 fold change of 1.5 after castration on mouse xenograft model.

Interestingly, there was a significant overlap between the genes differentially expressed in the siRNA knockdown of REST in LNCaP cells and those in LTL-331R. Overall, we identified 34 genes up-regulated and 12 genes down-regulated overlapping between these two platforms.

Figure 6 The overlap between genes differentially expressed from microarray data and from RNA-seq data.

Functional categorization analysis

We analyzed our results with functional pathways or disease categories defined by IPA. Cell-to-cell signaling and interaction, molecular transport, small molecule biochemistry and neurological disease were the most significantly represented in our dataset.

Network analysis

To investigate the interaction of these differentially regulated genes with other gene pathways and biological processes, IPA was used to form molecular networks based on functional relationship between gene products referenced in peer-reviewed literature. The top 4 networks among the significant genes up/down regulated in our overlap dataset represent many pathways suspected to be involved in cellular assembly and organization, nervous system development and function, neurological disease, cellular compromise, cell cycle, hereditary disorder, and drug metabolism, lipid metabolism, molecular transport and so on.
Network 1 (Fig. 7) received the highest score (44) and was assembled 18 focus genes that were differentially regulated in REST knockdown condition. In particular, Network 1 contains gene and molecules known to be involved in nervous system development and function (SYN1, UNC13A), neurological disease (BSN, CHRNB2, KCNC1, SYN1), small molecule biochemistry and cell-to-cell signaling and interaction (CHRNB2, SYN1, SYT4, UNC13A). REST acts as one of the hubs in this network, directly interacting with 6 genes (SYT4, SCG3, CHRNB2, KCNH6, SYP, SYN) which significantly up-regulated in our datasets. This network also reveals that REST acts on other 9 genes (PCLO, PHKB, TRPM2, SCN1A, B3GAT1, FGF12, NCAM2, PHF21A, RCOR2) which were not shown in our over-expressed dataset.

Of the genes involved in the top ranked network which were not altered in our experiments, death-associated protein kinase 1 (DAPK1), which is directly regulated by ERK1/2 in the network, plays a crucial role in metastasis of carcinoma cell lines and delay in growth of tumor. The top canonical pathways derived from this network were bladder cancer signaling (DAPK1, ERK1/2,
FGF12, MMP24), Huntington’s disease signaling (Creb, ERK1/2, RCOR2, REST), protein kinase A signaling (Calmodulin, Creb, ERK1/2, PDK2), ERK/MAPK signaling (Creb, ERK1/2, estrogen receptor) which is involved in the development of many cancers, and androgen signaling (Calmodulin, Creb, ERK1/2) which plays a critical role in the development, function and homeostasis of the prostate.

**Experimental RT-PCR validation**

Representative genes BEX1 and SRRM3 were selected from overlap datasets of differentially up-regulated genes for validation via semi-quantitative RT-PCR, using the same LNCaP cell line used in the microarray. The RT-PCR result of BEX1 and SRRM3 are consistent with both microarray and RNA-seq data.

**Kaplan Meier survival analysis**

In order to estimate their clinical significance of the preliminary set of potential REST targets we identified, we utilized the Kaplan–Meier analysis of the survival which measures the fraction of patients living for a certain amount of time after treatment. After obtaining a general view of all gene alterations in a cohort of 216 tumors, we selected a small high priority groups of genes identified by microarray and RNA-seq based on their percentage of alteration and alteration trend. Three genes BEX1, SCG3, and UNC13A were found to be up-regulated in both LNCaP and LTL331 experimental systems. Interestingly, all three of these candidate genes show shortened disease free survival
aggressive subtype of prostate cancer that most commonly evolves from preexisting prostate adenocarcinoma. Remarkably, growing evidence indicates that neuroendocrine differentiation occurs 30-100% of prostate cancer recurrence especially after androgen deprivation treatment.

The unique patient-derived xenograft model of PCa we used here is based on sub-renal capsule xenografting of patient tumor tissue in NOC/SCID mice. The xenograft retains key biological properties of the original malignancies, including histopathological, genomic and transcriptomic characteristics, tumor heterogeneity and metastatic ability. Therefore, this high fidelity model is more clinical research relevant.

It must be stressed that we still need a more comprehensive system to acquire a more detailed understanding of neuroendocrine differentiated prostate cancer. We are still far away from identifying all the regulated genes or molecules involved in this phenotype; it is possible to do so through microarray and RNA-seq, as microarrays give a broad overview whereas RNA-seq detects subtle changes in gene expression and detects alternative splicing, which occurs quite often in cancer progression.

RE1 silencing transcription factor REST is expressed throughout the body and it was recently shown to be absent in various types of cancer including prostate cancer. REST transcriptional complex acts as a master repressor of the neuronal phenotype. Blockade of REST function in the human LNCaP cells is found to cause NED, indicating REST may be one of the important determinants of the neuroendocrine phenotype in PCa.
In our study, an integrated data analysis of RNA-Seq on mouse xenograft model and expression microarray with RNA interference (RNAi) on human LNCaP cells was performed to identify potential REST targets: 34 genes were up-regulated and 12 genes were down-regulated. Afterwards, a functional categorization analysis of the most significant pathways and diseases represented by these significantly different genes was generated using Ingenuity software. To investigate the interaction of these differentially regulated genes with other gene pathways and biological processes, molecular networks were formed based on functional relationships between gene products described in peer-reviewed literature. We found that REST acts as one of the hubs in the most significant network, directly interacting with 6 genes (SYT4, SCG3, CHRN2B2, KCNH6, SYP, SYN) significantly up-regulated in our datasets, as well as other 9 genes (PCLO, PHKB, TRPM2, SCN1A B3GAT1, FGF12, NCAM2, PHF21A RCOR2) which were not shown to be over-expressed in our dataset.

We then extended our research to an external public dataset which contains transcriptomes and copy-number alterations (CNA) in 218 prostate samples (181 primaries, 37 metastases) and 12 prostate cancer cell lines and xenografts. In addition, in order to estimate their clinical significance, we utilized the Kaplan–Meier analysis of survival which measures the fraction of patients living for a certain amount of time after treatment, correlated to the expression of particular genes.

This preliminary study investigated the potential role of REST in prostate cancer progression. It is expected that during the neuroendocrine differentiation, REST may help to repress the expression of several genes not yet required by the differentiation program. And, REST may function different ways at different stages of cell differentiation as changing the expression of associated genes. Thus, further studies are needed to understand these problems.

We identified high consistency between microarray and RNA-seq platforms, thus encouraging the continual use of this method as a tool for differential gene expression analysis. In conclusion, our study contributes to the elucidation of the basic biological mechanism that drives adenocarcinoma to escape AR blockade through NED to androgen independent and aggressive NEPC. Furthermore, REST may serve as a potential target for the treatment of prostate cancer and a novel prognosis factor for prostate cancer.

References


4. Tawadros, T., et al., *IB1/JIP-1 controls JNK activation and


