ISL1 Promotes Enzalutamide Resistance in Castration-resistant Prostate Cancer (CRPC) through Epithelial to Mesenchymal Transition (EMT)

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Abstract

Abnormal expression of insulin gene enhancer-binding protein 1 (ISL1) has been demonstrated to be closely associated with cancer development and progression in several cancers. However, little is known about ISL1 expression in metastatic castration-resistant prostate cancer (CRPC). ISL1 has also been recognized as a positive modulator of epithelial-mesenchymal transition (EMT). In this study, we focused on ISL1 which showed maximum upregulation at the mRNA level in the enzalutamide-resistant cell line. Accordingly, we found that ISL1 was overexpressed in enzalutamide-resistant C4-2B cells and its expression was significantly related to EMT. Our findings reveal the important role of ISL1 in androgen receptor (AR)-dependent prostate cancer cell growth; ISL1 knockdown reduced the AR activity and cell growth. ISL1 knockdown using small-interfering RNA inhibited AR, PSA, and EMT-related protein expression in C4-2B ENZR cells. In addition, knock-down ISL1 reduced the levels of AKT and p65 phosphorylation in C4-2B ENZR cells and these suggest that knock-down ISL1 suppresses EMT in part by targeting the AKT/ NF- κ B pathway. Further, ISL1 downregulation could effectively inhibit tumor growth in a human CRPC xenograft model. Together, the present study shows that downregulation of ISL1 expression is necessary for overcoming enzalutamide resistance and improving the survival of CRPC patients.

Introduction

Androgen deprivation therapy (ADT) is the mainstay treatment for advanced prostate cancer (PCa), given the importance of androgens for PCa development. Despite the initial response to ADT, the disease typically progresses to a castration-resistant state, i.e., castration-resistant prostate cancer (CRPC)^{1,2}. Many studies show that androgen receptor (AR) signaling still plays a critical role in CRPC³. The use of AR inhibitor enzalutamide for the treatment of CRPC has been recently approved by the Food and Drug Administration⁴. However, the development of enzalutamide resistance has already been reported in a majority of CRPC patients. Known resistance mechanisms include de novo androgen biosynthesis, expression of AR splice variants, Wnt/ β -catenin pathway activation, and cholesterol biosynthesis⁵⁻⁸. The identification of critical molecular and cellular events associated with tumor progression, invasion, and metastasis to the bone as well as other sites has provided new insights with respect to targeting advanced disease⁹. Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells undergo morphological changes to acquire a motile mesenchymal phenotype. This phenomenon is implicated not only in cancer metastasis but also in the development of therapeutic resistance^{10,11}. Hence, the targeting of EMT can serve as a new potential strategy for the treatment of CRPC through the reversion of the invasive mesenchymal phenotype to a well-differentiated tumor epithelial tumor phenotype.

Insulin gene enhancer-binding protein 1 (ISL1), a LIM homeodomain transcription factor, plays an important role in the development of pancreatic islets of Langerhans during embryogenesis. ISL1-deficient mouse embryos fail to exhibit heart development and the differentiation of the neural tube into

motor neurons^{12–14}. The association between aberrant ISL1 expression and cancer progression is being gradually recognized. For instance, abnormal expression of ISL1 has been demonstrated to be closely associated with cell proliferation and invasion in several cancers^{15,16}. In addition, ISL1 has been known to serve as a novel regulator of cyclin D1, cyclin B, and c-myc genes in cancer^{17,18}.

ISL1 may act as a positive modulator of EMT, a critical regulator of cancer stem cell (CSC) phenotype. This is especially important as CSCs are a subpopulation of neoplastic cells with stem cell-like properties such as the ability to self-renew and undergo metastasis^{19–21}. EMT-inducing transcription factors (EMT-TFs) can be typically classified into three different protein families, namely, the Snail, ZEB1, and basic helix-loop-helix families²². The contribution of EMT to the CSC phenotype is thought to be dependent on the cell type and/or coexisting genetic/epigenetic abnormalities, and abnormal EMT and epigenetic changes are known to be related to cancer metastasis and tumor relapse²¹. Here, we demonstrate that ISL1 is overexpressed in enzalutamide-resistant C4-2B cells and that its expression is significantly related to EMT. Targeting ISL1 expression with a small-interfering RNA (siRNA) resulted in the inhibition of the proliferative and invasive capabilities of enzalutamide-resistant C4-2B cells and colonization abilities of enzalutamide-resistant C4-2B cells in a mouse xenograft model. However, the molecular basis underlying these effects is not well understood, and to the best of our knowledge, has not been investigated in the context of PCa.

Methods

Cell culture and reagents

LNCaP and C4-2B cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The C4-2B MDVR cell line was generated by culturing C4-2B cells in a medium supplemented with 20 μ M enzalutamide. LNCaP and C4-2B cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Gibco BRL) at 37°C in an atmosphere containing 5% CO₂. Tumor spheres were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) supplemented with basic fibroblast growth factor (bFGF; 20 ng/mL; Invitrogen), epidermal growth factor (EGF; 20 ng/mL; BD Biosciences), and N2 supplement (1 $\times$; Invitrogen). Enzalutamide was obtained from Selleckchem, and dihydrotestosterone (DHT) from Sigma. Cells were transfected with siRNAs using Lipofectamine RNAiMax transfection reagent (Invitrogen). The ON-TARGETplus SMART pool siRNA targeting ISL1 as well as the ON-TARGETplus Non-targeting siRNA (siCont) were purchased from Dharmacon (Lafayette, Colorado, USA).

Sphere formation

Cells were resuspended in a stem cell-permissive medium (DMEM/F12 containing 20 ng/mL EGF, bFGF, and N2 supplement (1 $\times$)). Spheres were imaged after 7 days using a microscope.

RNA isolation and reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from cultured cells using RNeasy Purification Kit (Qiagen). Reverse transcription was performed using 1 µg of total RNA with SuperScript® IV First-Strand Synthesis System per the manufacturer's protocol (ThermoFisher). Target gene expression was determined using TaqMan primer and probe sets (Applied Biosystems) and TaqMan Gene Expression Master Mix Reagents. PCR was performed on an Applied Biosystems 7900HT Real-Time PCR System. *ISL 1*, *KLK2*, *KLK3*, *TMRPSS2*, and *IGF1R* primers and probes were designed as per Applied Biosystems (Assay IDs: Hs00158126_m1, Hs00428383_m1, Hs02576345_m1, Hs00237175_m1, Hs00609566_m1). The experiment was performed in triplicates for each sample. All data were normalized to the expression of *GAPDH* (Assay ID: Hs02786624_g1). A comparative threshold method was used to quantify target gene expression.

Library preparation and mRNA sequencing

For control and test RNAs, library construction was performed using SENSE 30 mRNA-Seq Library Prep Kit (Lexogen, Inc., Austria) according to the manufacturer's instructions. In brief, 500 ng total RNA was prepared, hybridized with an oligo-dT primer containing an Illumina-compatible sequence at its 5'-end, and reverse transcribed. After degradation of the RNA template, second-strand synthesis was initiated using a random primer containing an Illumina-compatible linker sequence at its 5'-end. The double-stranded library was purified using magnetic beads to remove all reaction components. The library was amplified to add complete adapter sequences required for cluster generation. The finished library was purified from PCR components. High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, Inc., USA).

mRNA sequencing data analysis

Sense 30 mRNA-Seq reads were aligned using Bowtie2 version 2.1.0²³. Bowtie2 indices were either generated from the genome assembly sequence or the representative transcript sequences to align with the genome and transcriptome. The alignment file was used for assembling transcripts, estimating their abundances, and detecting differential expression of genes. Differentially expressed genes were determined based on counts from unique and multiple alignments by EdgeR within R version 3.2.2 (R development Core Team, 2011) using BIOCONDUCTOR version 3.0²⁴. Read count data were processed based on the global normalization method using the Genowiz™ version 4.0.5.6 (Ocimum Biosolutions, India). Gene clustering was performed using MeV 4.9.0. Clusters and heat maps were visualized using MeV 4.9.0.

Colony formation assay

For the colony formation assay, 1,000 cells were seeded in six-well plates. Cells were cultured for 21 days and stained with 0.1% crystal violet. The cell colonies were imaged and the dye was subsequently extracted using 10% acetic acid. The absorbance was determined by spectrophotometry (570 nm).

Phospho-RTK antibody array

Cells were lysed in NP-40 lysis buffer (1% NP-40, 10% glycerol, 20 mmol/L Tris-HCl, pH 8, 137 mmol/L sodium chloride [NaCl], 2 mmol/L ethylenediaminetetraacetic acid [EDTA], 1 mmol/L sodium orthovanadate, and protease inhibitors). Cell lysates (250 mg) were incubated overnight with receptor tyrosine kinase (RTK) array membranes (ARY-001, R&D Systems), and unbound molecules were washed away. A pan-anti-RTK antibody conjugated to horseradish peroxidase was used to detect phosphorylated tyrosine in activated receptors using the enhanced chemiluminescence (ECL) method.

Cell growth and proliferation assessment

To assess the cell number, an equal volume of 0.4% (w/v) trypan blue was added to each cell suspension, and cell viability was determined based on the ability of live cells to exclude trypan blue. Viable cells were counted using a hemocytometer. Cell proliferation was determined using the Dojindo Cell Counting Kit-8 (CCK-8; Dojindo, Gaithersburg, MD, USA). Toward this, cells were seeded in 96-well plates at a density of 1×10^4 cells in 100 μ L culture medium and allowed to adhere overnight. Cells were treated with 0.1, 1, 5, 10, 20, or 40 μ M enzalutamide for 72 h and then incubated with 10 μ L tetrazolium substrate at 37°C for 2 h. The absorbance at 450 nm wavelength was measured using a microplate reader.

Western blot analysis

Cells (5×10^6) and ground tissue (100 mg) were lysed in 1 mL radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.2], 0.5% NP-40, 1% Triton X-100, and 1% sodium deoxycholate) containing a protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels and the separated protein bands were transferred onto an Immobilon-P membrane (Millipore, Darmstadt, Germany). The membrane was blocked with a solution containing 5% skim milk and 0.1% Tween-20 for 1 h and then probed overnight with the indicated primary antibodies at 4°C. The membrane was then probed with a horseradish peroxidase-conjugated secondary antibody (1:2,000) for 1 h and developed using the ECL-Plus Kit (Thermo Scientific, Rockford, IL). Antibodies against ISL1, β -actin (both Santa Cruz Biotechnology), E-cadherin, N-cadherin (both BD Biosciences), phosphor (p)-protein kinase B (AKT), AKT, Snail, AR, and PSA (Cell Signaling Technology) were used as the primary antibodies. Horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology.

Transwell migration assay

Cells (1×10^5) were loaded into the top chamber of Transwell plates (containing an 8 mm pore size membrane; Corning Costar). FBS (10%) was used as a chemoattractant in the bottom chamber. After incubation for 24 h, cells adhered to the lower surface of the membranes were fixed and stained with 0.005% (w/v) crystal violet. The number of migrated cells was quantified by counting cells in five random fields of each membrane.

Evaluation of the antitumor potential of ISL1 in vivo

Six-week-old female BALB/C nu/nu mice were subcutaneously injected with 1×10^7 human C4-2B cells—expressing the indicated short-hairpin RNA (shRNA)—in their right flanks. For the injection, cells were suspended in 100 μ L of 50% Matrigel (BD, NJ) in complete media. The body weight of mice was measured, and tumor growth was monitored using Vernier calipers for up to 24 days after cell injection. The tumor volume was calculated as follows: tumor volume (mm^3) = length $\times$ (width) $^2 \times 0.5$. At necropsy, the tumors were dissected and weighed. For all tumors, one part was fixed in neutral buffered formalin for immunohistochemistry (IHC), and the remaining part was frozen and stored at -80°C .

Statistical analysis

Data in the graphs represent mean $\pm$ standard deviation (SD) of values from at least three independent measurements. To determine the differences in mean values, a Student's t-test was employed. Intergroup comparisons were performed using the paired two-sample t-test. Differences were considered significant at $p < 0.05$.

Results

Identification of ISL1 in enzalutamide-resistant PCa cells

We established an in vitro model of enzalutamide-resistant PCa, i.e., C4-2B ENZR by culturing C4-2B cells in a medium supplemented with enzalutamide. C4-2B cells have a nearly identical AR status but show higher expression of AR variants^{25,26}. To confirm enzalutamide resistance, we performed cell viability assay after treating the enzalutamide-resistant cell line with different concentrations of the drug (0.1 to 40 μM). As shown in Fig. 1a and b, enzalutamide significantly inhibited the proliferation and clonogenic ability of C4-2B parental cells but had little effect on C4-2B ENZR cells. As the tumor sphere formation is based on the unique property of stem/progenitor cells to survive and grow in a serum-free medium, we performed a tumor sphere formation assay to examine whether enzalutamide resistance enhances the self-renewal of PCa cells. Our data show that enzalutamide resistance enhanced the sphere formation ability of cells in a concentration-dependent manner (Fig. 1c). Furthermore, we performed a phospho-RTK activity array assay (including 42 RTKs) to identify additional RTKs that may be activated in CRPC. As shown in Fig. 2, we observed a substantial increase in the phosphorylation of ErbB family members (epidermal growth factor receptor [EGFR] and ErbB2), insulin R, and IGF-1R in C4-2B ENZR as compared to those in C4-2B parental cells. EGFR phosphorylation appeared to be the strongest among all kinases.

After validation of the enzalutamide resistance, we analyzed global changes in the mRNA expression using quantitative mRNA-sequencing. To identify common pathways underlying the development of enzalutamide resistance, we used the complete transcriptional profile for gene set enrichment analyses (GSEA: www.broadinstitute.org/GSEA) and investigated the predefined oncogenic signatures and hallmarks based on analysis on the Molecular Signature Database (MsigDB). Comparison of the parental and enzalutamide-resistant C4-2B cell lines revealed changes in the expression of several major biological pathways and some EMT-related pathways. Enriched hallmark gene sets included those

involved in EMT, supporting the hypothesis that resistant cells bypass EMT (Fig. 3). We focused on ISL1, which showed maximum upregulation at the mRNA level in the enzalutamide-resistant cell line as compared to that in the parental C4-2B cell line (Fig. 4a). The position of ISL1 on the scatter plot of the two different cell lines has been indicated in Fig. 4B. The change in ISL1 mRNA expression was confirmed by RT-PCR (Fig. 4c).

ISL1 is essential for AR activity and AR-dependent cell proliferation

To test the possible role of ISL1 in AR function in PCa cells, we used siRNA to knockdown ISL1 expression in LNCaP cells, which are known to exhibit AR activity. The knockdown efficiency of ISL1 siRNA was confirmed by RT-qPCR (Fig. 5a) and western blotting (Fig. 5b). Cells expressing the control siRNA and ISL1 siRNA were stimulated with DHT for 24 h, and the expression of AR-target genes was analyzed by western blotting (Fig. 5b) and qRT-PCR (Fig. 5c). The mRNA expression of AR target genes, including KLK3 (PSA), KLK2, TMPRSS2, and IGF1R, increased after DHT stimulation in control siRNA cells; however, ISL1 knockdown prevented the increase in target gene expression. It is well known that AR plays an important role in the growth of PCa cells. To test the possibility that ISL1 may augment AR-mediated PCa cell growth, we measured cell viability at 48 and 72 h using the trypan blue exclusion assay (Fig. 5d). The growth was slower in ISL1 knockdown cells than that in control cells under normal conditions. Thus, ISL1 may play an important role in AR-dependent PCa cell growth. We also tested the colony formation abilities of control and ISL1-knockdown cells. The number of colonies was counted, and the relative numbers were plotted (Fig. 5e). As observed with LNCaP cells, the knockdown of ISL1 expression resulted in a decrease in AR activity and cell growth.

ISL1 depletion suppresses EMT via the AKT/nuclear factor kappa B (NF- κ B) signaling

We determined whether ENZR cells underwent a partial EMT by performing western blotting to evaluate the expression of the markers related to either an epithelial or mesenchymal cell state (Fig. 6a). C4-2B ENZR cells showed an increase in the expression of Snail and vimentin, a loss of E-cadherin, and a gain of N-cadherin expression as compared to control cells. AR and PSA levels were upregulated in C4-2B ENZR cells as compared to those in control cells, suggesting that AR is involved in the development of enzalutamide resistance. Based on our observation that EMT-related proteins and AR are upregulated in enzalutamide-resistant cells, we assessed whether ISL1 plays a role in the induction of AR in ENZR cells and investigated the impact of ISL1 knockdown on enzalutamide resistance. ISL1 knockdown with siRNA resulted in the inhibition of AR, PSA, and EMT-related protein expression in C4-2B ENZR cells (Fig. 6b). Furthermore, the results of transwell migration assay revealed fewer migrating cells in the siISL1 group than those in the siCont group (Fig. 6c). Densitometric analysis of immune-reactive bands in western blotting revealed that ISL1 knockdown reduced p65 phosphorylation (Fig. 6d), which is essential for the nuclear translocation of NF- κ B/p65. To clarify the mechanism underlying the inhibition of NF- κ B signaling by ISL1, AKT expression and phosphorylation were evaluated using western blotting. Knock-

down ISL1 reduced the levels of p-AKT in C4-2B ENZR cells (Fig. 6d). These results suggest that knock-down ISL1 suppresses EMT in part by targeting the AKT/ NF- κ B pathway.

Inhibition of ISL1 helps overcome enzalutamide resistance in vivo

To validate the oncogenic effect of ISL1 in vivo, we established a human CRPC xenograft model by injecting C4-2B cells expressing either control or ISL1 shRNA in nude mice. The shRNA-mediated stable knockdown of ISL1 was confirmed by immunoblotting (Fig. 7a). We compared the increase in tumor volume over 30 days and found that the xenografts from ISL1 knockdown cells showed hardly any increase in size, whereas control shRNA-expressing cells exhibited continuous tumor growth (Fig. 7b). Tumors originating from ISL1 knockdown cells were significantly lower in weight than those from control cells (Fig. 7c). Our results demonstrate that ISL1 downregulation can effectively inhibit tumor growth in a xenograft model of human CRPC.

Discussion

While majority of the patients with metastatic CRPC benefit from enzalutamide treatment, the responders inevitably develop resistance. Hence, studies have been directed to investigate the potential mechanisms associated with the development of enzalutamide resistance. In the present study, we demonstrate that the downregulation of ISL1 serves as an important alternative therapy for CRPC treatment through the targeting of EMT via the negative regulation of the AKT/NF- κ B signaling pathway.

ISL1 serves a major role in multiple tissue types, such as heart, kidneys, skeletal muscle, nervous system, and endocrine organs, and its upregulation is associated with cancer progression and poor prognosis¹⁴⁻¹⁷. Furthermore, ISL1 can influence the expression of genes related with EMT such as ZEB1 and N-cadherin¹⁵. We found that ISL1 was the most highly expressed EMT factor in enzalutamide-resistant cells (Fig. 4a). We analyzed the function of ISL1 in AR signaling in PCa cells and found that cell proliferation decreased and AR signaling was downregulated in ISL1 siRNA-expressing hormone-sensitive PCa cells (Fig. 5).

As epithelial plasticity driver, Snail (a master EMT-TF) is also known to promote the development of resistance against enzalutamide through the regulation of AR activity in PCa²⁷. The loss of epithelial phenotypes, including spindle morphology and intercellular adhesion, and the acquisition of mesenchymal characteristics such as high migration and invasion capacities and reduced cell-extracellular adhesion are the two major events observed during EMT²⁸. The expression of EMT marker genes was downregulated in ISL1-knockdown cells (Fig. 6b). Studies have demonstrated that EMT is associated with CRPC^{29,30}. Sun et al.³¹ found that castration may induce EMT, as is evident from the decreased expression of epithelial markers (including E-cadherin) and increased levels of mesenchymal markers (including N-cadherin, Slug, Zeb1, and Twist1) in human LuCaP35 PCa xenograft tumors as well as in the normal mouse prostate tissue following androgen deprivation. Similar changes have also been

reported in samples from individuals undergoing ADT³¹. EMT is driven by EMT inducing transcription factors (including Snail, Slug, Zeb1, Zeb2, and Twist), some of which have been known to be involved in the development of CRPC²⁸. Shiota et al.³² found that castration-induced oxidative stress may promote AR overexpression through Twist1 overexpression, thereby possibly developing castration resistance. Furthermore, facilitation of castration resistance by Slug in PCa has been reported by Wu et al.³³. Slug, another transcription factor driver of EMT, not only augments the expression of AR but also enhances AR transcriptional activity with or without androgen and acts as a novel coactivator for AR³³. Overall, these aforementioned studies suggest that EMT is responsible for PCa progression and treatment resistance. Accordingly, treatment regimens that could reverse EMT phenotypes may become a viable alternative for CRPC therapy.

Aberrant activation of NF- κ B signaling in PCa has been associated with metastatic progression^{34,35}. In addition, NF- κ B signaling plays an important role in EMT³⁶. The knockdown of ISL1 resulted in reduced p65 phosphorylation (Fig. 6D), which is imperative for the nuclear translocation of NF- κ B/p65. The NF- κ B family, an important class of transcriptional regulators, comprises five members, including RelA (p65), RelB, c-Rel, p50/p105 (NF- κ B1), and p52/p100 (NF- κ B2). NF- κ B binds to the inhibitor κ B (I κ B) protein in the cytoplasm in an inactive state. The I κ B kinase (IKK) complex is activated under pathological conditions and subsequently induces the phosphorylation of I κ B, leading to the degradation of I κ B and translocation of NF- κ B to the nucleus³⁷. Increasing results indicate that the NF- κ B transcription factor family is a crucial mediator of EMT³⁸. Certain studies have shown that NF- κ B binds to the promoters of genes associated with EMT, including those encoding Snail, Slug, and Twist, and increases their transcription^{38,39}. Ozes et al.⁴⁰ reported the involvement of AKT in the activation of NF- κ B by mediating the phosphorylation of IKKA which is responsible for the activation of its downstream target I κ B. In the current study, the knockdown of ISL1 resulted in the inhibition of the phosphorylation of both AKT and p65. These results show that ISL1 knockdown suppresses EMT by negatively regulating the AKT/NF- κ B pathway. Furthermore, the AKT/NF- κ B signaling pathway may drive the progression of CRPC by mechanisms other than EMT induction. Activation of NF- κ B mediated by PI3K/AKT increases the expression of AR via NF- κ B binding to the AR promoter⁴¹. CRPC, previously defined as hormone-refractory PCa, is thought to be androgen dependent⁴², indicating that targeting AR may serve as an effective strategy for CRPC treatment. The present data demonstrate that ISL1 suppressed the phosphorylation of AKT and p65; however, whether the effect of ISL1 on the AR signaling axis occurs through the regulation of the AKT/NF- κ B pathway is unclear and warrants further examination. In addition, a subsequent study is needed to determine the genomic distribution of a critical EMT regulator in CRPC using chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq).

In conclusion, the present study identified that aberrant expression of ISL1 influenced enzalutamide resistance through EMT pathway. The strategy of inhibition of ISL1 holds great promise as a sensitizing strategy to restore the antitumor effects in enzalutamide-resistant cells. Targeting ISL1 may serve as an effective treatment strategy for patients resistant to enzalutamide.

Declarations

Data Availability Statement

Not applicable

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Author contributions

Conceptualization: S.I.S. and J.D.C.; Methodology: M.Y.K.; Software: S.Y.Y.; Validation: S.Y.Y. M.Y.K., and J.D.C.; Formal analysis: S.Y.Y.; Investigation: J.D.C.; Resources: T.J.K.; Data curation: B.C.J.; Writing—original draft preparation: J.D.C.; Writing—review and editing: S.S.J.; Visualization: H.G.J.; Supervision: S.I.S.; Project administration: S.I.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figures

Figure 1

C4-2B MDVR cells are resistant to enzalutamide in vitro. (a) C4-2B parental and C4-2B ENZR cells were treated with different concentrations of enzalutamide for 72 hours, the cells were measured using the CCK8 solution.*, $p < 0.005$. (b) Crystal violet staining for colonies from the same number cells treated with the indicated concentration of enzalutamide. (c) C4-2B parental and C4-2B ENZR cells were seeded in an ultra-low attachment 24-well plate. (d) The average relative number of crystal violet staining colonies is shown in the lower panel. (e) The average relative number of spheroid is shown in the lower panel. Spheroid number was evaluated at 6 days post-seeding.*, $p < 0.005$.

Figure 2

In vitro screening of enzalutamide resistant targets using RTK array analysis. (a) Representative blots of human receptor tyrosine kinase antibody array probed with protein lysates isolated from C4-2B cells carrying parental (upper) and ENZR (lower). (b) Individual RTKs are spotted and identification of phospho-EGFR, p-ErbB2, p-Insulin R and p-IGF-IR are indicated. Positive control spots are located at the corners of the human phospho-RTK array. (c) Spot intensity was quantified by densitometry and the data are presented in histogram format.

Hallmark gene sets are coherently expressed signatures derived by aggregating many MSigDB gene sets to represent well-defined biological states or processes.

Figure 3

Hallmark wheels of genomic/transcriptomic and full integrated datasets, related to Figure 4. Hallmark wheel showed enrichment of hallmark pathways when the transcriptional and genomic information is included.

Figure 4

ISL1 is upregulated in enzalutamide-resistant (ENZR) prostate cancer. (a) Heat map of the most significantly differentially expressed epithelial mesenchymal transition genes from three independent experiments using the parental and enzalutamide-resistant C4-2B cell lines. Expression of transcripts encoding genes involved in EMT (Epithelial mesenchymal transition) was analyzed by GSEA. Genes that were regulated 2-fold between C4-2B parental and enzalutamide-resistant C4-2B cells were enriched and heatmap was generated by TM4 Multi Experiment Viewer (MeV). GSEA, Gene Set Enrichment Analysis. (b) Scatterplot of transcript expression from the parental and enzalutamide-resistant C4-2B cell lines, with the position of ISL1 indicated. (c) The mRNA levels of ISL1 from the parental and enzalutamide-resistant C4-2B cell lines calculated from three different samples by RT-qPCR. Errors bars represent the mean $\pm$ SD of three independent experiments and * denotes $p < 0.005$ vs. the parental cell line.

Figure 5

ISL1 is required for AR activity and AR-dependent cell proliferation. (a) The efficiency of ISL1 knock-down was measured by comparing mRNA levels of ISL1 in siRNA expressing LNCaP cell lines. ISL1 mRNA levels were measured by qRT-PCR. Error bars represent the mean $\pm$ SD of three independent experiments and * denotes $p < 0.05$ vs. the control siRNA (siCont) group. (b) Whole cell extracts were analyzed with anti-ISL1, anti-PSA and anti- β -actin antibodies. For the induction of AR activity, 5 nM DHT(dihydrotestosterone) was added after one day of serum deprivation. Bottom, a Western blotting experiment; top, bands on the Western blot analysis were quantified by densitometry and the data are presented in histogram format. *, $p < 0.05$ (vs. siCont treated DHT). (c) Relative mRNA expression levels of the indicated genes were measured in ISL1 knock-down LNCaP cell lines. Error bars represent mean $\pm$ SD of three independent experiments and * denotes $p < 0.05$ vs. the control siRNA (siCont) group. (d) Cell

viability in siSL1 transfected LNCaP cells. The cells were plated at a density of 1x10⁴ cells in 6 well plate for the period indicated. The detached living and dead cells were collected, stained with trypan blue and counted under a microscope using a hemocytometer. Error bars represent the mean $\pm$ SD of three independent experiments and * denotes p < 0.05 vs. the control siRNA dead cell (siCont) group. (e) Crystal violet staining for colonies from the same number of indicated siRNA expression LNCaP cells. The average relative number of colonies is shown in the lower panel. Error bars represent the mean $\pm$ SD of three independent experiments and * denotes p < 0.05 vs. the control siRNA (siCont) group.

Figure 6

Depletion of ISL1 sensitizes enzalutamide-resistant C4-2B cells and decreases their Epithelial-to-mesenchymal transition (EMT) properties. (a) Parental C4-2B and C4-2B-ENZR cells were subjected to western blot analysis for EMT gene expression. b-actin was used to show equal loading. (b) C4-2B-ENZR cells transfected with siCont or siSL1 and subjected to western blot analysis for the indicated proteins. b-actin was used to show equal loading. (c) Assessment of invasion of C4-2B-ENZR cells using Transwell migration assay. (d) C4-2B-ENZR cells transfected with siCont or siSL1 and the effects on p-p65 (S536) and p-AKT (S473) level were analyzed by western blot analysis; densitometry analysis of immune-reactive bands detected by western blots show that the ISL1 knockdown induced decrease in AKT phosphorylation over the basal was about 2-fold in C4-2B cells.

Figure 7

ISL1 knock-down attenuates tumor growth in enzalutamide-resistant (ENZR) prostate cancer cells. (a) The efficiency of ISL1 knock-down was measured by western blot analysis. b-action was used to show equal loading. (b) Photographs showing tumor formation in nude mice injected with stable cell lines with ISL1 knockdown. Tumor growth curves in relation to the ISL1 suppression in human CRPC C4-2B ENZR xenografts. Tumor size was measured once per week and compared between groups by ANOVA (*, $p < 0.001$) (c) Relationship between tumor weights and ISL1 expression status in human CRPC xenograft model. Tumors were excised and examined at the end of the experiments. Representative graph is shown as vertical box plots and tumor weights are presented as mean (g) SD.

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