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Comparative RNA-seq analysis reveals potential mechanisms mediating the conversion to androgen independence in an LNCaP progression cell model

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ABSTRACT

The androgen-independent phenotype is an important symptom of refractory prostate cancer. However, the molecular mechanisms underlying this phenotypic conversion remain unclear. Using RNA-seq analysis of androgen-dependent prostate cancer cells (LNCaP) vs. androgen-independent cancer cells (LNCaP-AI-F), we identified 788 differentially expressed genes, 315 alternative splicing events, and eight novel LNCaP-AI-F-specific fusion genes. The fusion genes *EIF2AK1-ATR* and *GLYR1-SLC9A8* were predicted to be damaging and oncogenic. We also observed dramatic changes in androgen receptor (AR)-mediated pathway molecules, including prostate-specific antigen (PSA, a major biomarker of prostate cancer) and AR variants, as well as neuroendocrine-like (NE-like) and tumor stem cell-like characteristics, during androgen-independent phenotype progression. Our findings provide new insights into the regulatory complexities of refractory prostate cancers.

1. Introduction

Prostate cancer is one of the most common malignancies endangering aging males. Androgen ablation, currently serving as the front-line therapy, only provides temporary relief for severe tumors. The emergence of progressive androgen-independent phenotypes is an important sign that prostate cancer is undergoing further deterioration and is also a primary cause of refractory prostate cancer. Once the surviving prostate cancer cells develop into androgen-independent cancer cells, the cancer becomes lethal [1]. Several mechanisms have been proposed to explain androgen-independent phenotype progression, and most of these mechanisms are central to the androgen and androgen receptor (AR) signaling pathway, including intratumoral androgen synthesis, AR amplification/overexpression, AR mutations, AR variants, AR coactivators, ligand-independent AR activation, and calpain proteolysis of AR into androgen-independent isoforms [2–4]. Recently, several advancements involving fusion genes, RNA editing, microRNAs, non-coding RNA, cancer stem cells, and low-PSA or PSA-negative prostate cancer cells have been proposed as contributing mechanisms to androgen-independent phenotype progression [5–11]. Although these interpretations have advanced our understanding of the pathogenesis of prostate cancer, there are still many molecular events that need to be identified in the androgen-independent phenotype conversion process.

RNA-seq is a powerful tool for quantifying gene expression and detecting changes of gene structure at the transcript level. Recently, RNA-seq has been increasingly used to explore the genetic and environmental factors of prostate cancers, such as fusion genes, noncoding RNAs, alternative splicing events, and mutations

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in prostate cancer cell lines and tumors [12]. Using RNA-seq for sequencing microRNA transcripts (isolated from androgen-dependent LNCaP cells vs. androgen-independent LNCaP-AI-F cells), we identified 43 microRNAs, and the associated target genes were involved in signal transduction and cell communication, especially the MAPK signaling pathway [7]. To understand the potential molecular mechanisms underlying androgen-independent phenotype conversion, we performed further RNA-seq on our established LNCaP progression cell model, as previously described [7]. In this study, we report the identification and comparative analysis of the differentially expressed genes (DEGs), alternative splicing (AS), and gene fusion events that occurred during the androgen-independent phenotype conversion process.

2. Materials and methods

2.1. cDNA library preparation and sequencing

The androgen-dependent human prostate cancer cell LNCaP (purchased from ATCC, passage 1) was induced into androgen-independent LNCaP-AI-F cells (passage 110) through chronic culture in an androgen-deprived medium with antiandrogen flutamide [7]. LNCaP-AI-F is actually the LNCaP-AI cell line originally named in our previous study [7]. The LNCaP and LNCaP-AI-F cells were harvested in the exponential phase, and the total RNA of each cell line was isolated with an RNeasy Mini Kit (Qiagen, Shanghai, CHN). For the cDNA library preparation, the RNA samples were treated with RNase-free DNase I (Takara, Otsu, Japan) and then quantified using a NanoDrop 1000 (Thermo-Fisher Scientific, Waltham, MA, USA) and assessed with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The RNA integrity number (RIN) of each sample was >8. Two cDNA libraries were prepared according to the manufacturer's protocol (Illumina, San Diego, CA, USA). Each library was sequenced using the Solexa/Illumina Genome Analyzer II platform and generated 75 nt paired-end (PE) reads. These two raw sequencing data sets were deposited in the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/sra) under accession number SRA053575.

2.2. Identification of differentially expressed genes (DEGs)

We used Cufflinks (http://cufflinks.cbcb.umd.edu/) to identify DEGs and measured transcript abundances with FPKM (fragments per kilobase of exon per million fragments mapped) [13]. Gene ontology (GO) annotation analysis of the DEGs was performed using the DAVID web server (http://david.abcc.ncifcrf.gov/) to highlight the most relevant GO terms and pathways associated with the emergence of the androgen-independent phenotype [14,15]. To obtain a deeper understanding of functional insight, we completed an Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com) on the DEGs against the prostate cancer cell lines.

2.3. Detection of alternative splicing (AS) and gene fusion events

To identify the AS events at the whole genome level, a systematic comparative analysis was performed on both cell lines using a newly released program named SpliceSeq (http://bioinformatics.mdanderson.org) [16]. In addition, RNA-seq has been proven efficacious for detecting fusion events resulting from chimeric transcripts in some cancers [17–20]. In the present study, both TopHat-Fusion (http://tophat-fusion.sourceforge.net/) and FusionHunter (http://bioen-compbio.bioen.illinois.edu/FusionHunter/) were used to detect potential fusion events based on the RNA-seq data [20,21].

2.4. Validation of DEGs, AS and gene fusion events

Five micrograms of total RNA were converted to cDNA with Superscript III (Invitrogen, CA, USA) and were used as PCR templates. The validation of DEGs, AS and gene fusion events was conducted using standard quantitative real-time PCR (qPCR), RT-PCR or Sanger sequencing (the results are summarized in Tables S1–S3, S14 3 and Figs. 2–4). The primers are listed in Table S4 and available upon request.

3. Results

3.1. Overview of the sequencing data

A series of necessary assessments on RNA-seq quality were conducted. Relatively even distribution of the sequencing reads along the gene body represented random mRNA fragmentation in our constructed library (Fig. S1A and B). The nucleotide vs. cycle (NVC) plots indicated a normal nucleotide composition in the sequencing data (Fig. S1C and D). By sequencing two cDNA libraries established from LNCaP and LNCaP-AI-F cells, we obtained ~30.8 million and ~34.3 million of reads output, respectively (Table S5). Among the reads of each cell line, 82.65% vs. 86.83% of them were mapped to human genome hg19 (Table S6). Along with the increase of sequencing reads, the gene coverage slowly increased and reached a flat curve, suggesting sufficient sequencing reads for subsequent analysis (Fig. S1E).

3.2. Characteristic variations of DEGs

Using RNA-seq and comparative analysis, we identified 788 DEGs between the LNCaP and LNCaP-AI-F cells, including 335 (42.5%) upregulated and 453 (57.5%) downregulated genes (Table S7). The DEG validation by qPCR confirmed 91.2% of the selected genes (e.g., 62 out of 68 genes) had a similar expression trend, suggesting that the DEG analysis produced reliable results. In general, the DEGs were classified into 17 categories based on their associated functions in the GO annotation analysis (Table S8). To focus on the DEGs that are closely associated with novel characteristics, cancer and related signaling pathways, we summarized the DEGs in four sections (Table 1). The DEGs in Section 1 are involved in neuroendocrine secretion and NE-like cellular morphological formation and functions. The DEGs in Section 2 are associated with chromatin assembly, lipid and steroid hormone metabolism as well as responsiveness to various stimuli. The DEGs in Section 3 are closely related to cancer cell adhesion, migration, differentiation and proliferation. The DEGs in Section 4 are implicated in several signaling transduction pathways, such as the Notch, SAPK (stress-activated protein kinase), and IGF I signaling pathways.

We identified some representative DEGs that are involved in the following canonical pathways. (1) The Notch pathway-related DEGs were markedly altered, including Notch receptor (*Notch3*), ligand (*JAG1*), Notch regulators (*DTX3*, *DTX4*, *FURIN*, *LFNG*, and *MIB2*), targets (*CFD* and *HES7*), and transcriptional factors or coactivators (*MAML2* and *MESP2*). (2) The DEGs in the SAPK pathway, related to response various stress or stimuli, were mostly decreased, including two SAP kinases (*MAPK11* and *MAPK13*), four regulators (*LYN*, *MAPK8IP2*, *MAPK8IP3*, and *TP73*). (3) The DEGs in the IGF 1 signaling pathway also displayed significant alterations, such as the dramatic downregulation of *IGF1R* (~17-fold).

Interestingly, the IPA analysis of the DEGs revealed an AR- and KLK3 (kallikrein-related peptidase 3, also known as PSA)-centered molecular network that is functionally related to cellular development, cellular proliferation, and organismal development (Fig. 1). AR directly or indirectly interacts with multiple molecules, including *CTNNB1*, *IGFBP3*, *KLK3*, *SERPINB5*, *TMPRSS2*, *UGT2B15* and *UGT2B17*. Among these molecules, the elevation of *UGT2B15* and *UGT2B17*, two recently identified AR target genes, was positively correlated with the upregulated *AR* (~2-fold) in the LNCaP-AI-F cells as previously demonstrated [22]. However, *KLK3*, one of the main targets of AR, was observed to have a marked downregulation (~87-fold), which is contradictory to the expression of its positive regulator *AR*, suggesting that additional molecules or regulators may be involved in controlling *PSA* expression.

To examine the generality and the novelty of our data, we compared 788 DEGs (Table S7) with the reported DEGs datasets from several LNCaP-derived androgen-independent sublines, which were mostly examined using microarrays. Overall, approximately 19.1% (ranging 10.5–25.8%) of the reported DEGs matched our data, whereas 80.9% (ranging 74.2–89.5%) of the reported DEGs were unmatched (Table S9). Thus, many DEGs identified in this study were not detected in previous studies by microarray (examples in Table S10), suggesting a higher sensitivity and specificity in DEGs detection when using RNA-seq.

Table 1
Characteristic variations of DEGs.

GO category	Representative DEGs in LNCaP-AI-F	Gene functional description
(1) Neurotransmitter	ALDH5A1 (↑4); NPY1R (↑8); PRIMA1 (↑9); SLC1A3 (↑12), ABAT (↓4), MAOA (↓2)	Catalyzes GABA; catalyzes neuroactive and vasoactive amines (5-HT); receptor for neuropeptide Y and peptide YY; anchor acetylcholinesterase (ACHE); glutamate transporter
Cell secretion	STXBP5 (↑6), RIMS1 (↑2), NLGN1 (↑7), SLC22A3 (↑3), ADORA1 (↑4); SYT4 (↓29), SCAMP5 (↓6), WDR44 (↓5), RAB27A (↓4)	Exocytosis regulation, vesicle recycling, regulate synaptic vesicle release, synapse function and synaptic signal transmission
Cell projection	ANTXR1 (↑28), CACNA1H (↓210), DDN (↑15), DPYSL3 (↑66), FERMT1 (↑4), MERTK (↓6), MET (↑8), TLN2 (↓7), VGF (↓4), SPRY1 (↓7), TLN2 (↓7)	Compose and regulate activities of the cytoskeleton the neuron projection, dendrite, axon, contractile fiber, play roles in axon guidance, adhesion, neuronal growth, cell spreading and migration
Cytoskeleton	TUBA3D (↓8), TUBA3E (↓51), TUBB3 (↓5), TUBB4 (↑3), TUBB6 (↑26); MAPT (↑2), MAP2 (↓12); MICAL1 (↓12), RHOU (↓7), RND3 (↑10)	Microtubule tubulin alpha and beta family members; microtubule associated proteins; actin regulators; play roles in the regulation of cell morphology and cytoskeleton organization
(2) Chromatin	H1F0 (↑2), H2AFJ (↓12), H2AFZ (↑2), HIST1H2AE (↓6), HIST1H2BG (↓7), HIST2H2AA3 (↓3), HIST2H2BE (↓2)	Histone family members, involved in chromatin/nucleosome assembly or disassembly, play roles in transcription regulation, DNA repair, DNA replication and chromosomal stability
Lipid metabolism	ACSM3 (↓3), ALOX15 (↓2), C5ORF4(↓8), ELOVL3 (↑), ELOVL5 (↓3), ELOVL6 (↑5), ELOVL7 (↓2), ACSL3 (↓2), MECR (↑2); ACAA1 (↓2), ACADVL (↓2), ACOX1 (↓2), ACOX2 (↓124), EHHADH (↑8); ACOT4 (↑31), TYRP1 (↑38); UGT2Bs (↑)	Fatty acid biosynthetic process; fatty acid beta-oxidation; short-chain fatty acid metabolic process; steroid hormone metabolism
Stimulus response	AR (↑2), KLK3 (↓83); ALDH1A3 (↓6), ALDH2 (↓5), ASPHD1 (↓115), C5ORF4 (↓8), GPD2 (↑9), MICAL1 (↓12), NDRG1 (↓5), NXN (↓13); PXDN (↓6), SOD3 (↓160); AQP3 (↓451); PDIA2 (↓8); TFRC (↑4)	Response to androgen, serine proteases; oxidation reduction; response to oxygen levels; water channel; antigen processing and presentation of peptide antigen; hormone response; response to metal ion
(3) Development differentiation	BMP6 (↑6), CYP27B1 (↑14), PBX1(↑4), ZNF675 (↓18); GLI3 (↑6), HES7 (↑13), HOXA5 (↑11), HOXB6 (↑7), HOXB7(↑), LEF1 (↑5), MDK (↓16), PAX1 (↑3), PTCH1 (↓3), TSPY3 (↓13), VANGL2 (↑12), VGF (↓4)	Skeletal system development, differentiation and tissue morphogenesis; involved in cell proliferation and differentiation, and some disturbed genes are associated with cancers
Adhesion junction	FERMT1 (↑4), NRXN1 (↑485), PCDH1 (↓4), PCDH20 (↓6), PCDH7 (↑50), PCDH9 (↑15), PPFIA2 (↑11), PTPRM (↓9), RND3 (↑10), SPON2 (↓2), TNS1 (↑12), CHRNA2 (↓6), HOMER2 (↓5), NLGN1 (↑7), PRIMA1 (↑9), SCAMP5 (↓6), STXBP5 (↑6)	Involved in cell adhesion and cell junction, play roles in cell migration, development, receptor and signal transduction
Proliferation segmentation	AZGP1P1 (↓), CLU (↓24), FGFR3 (↓10), IGF1R (↓17), IGFBP5 (↑30), PTPRM (↓9), SMO (↓36), VEGFB (↓11), RARRES3 (↓23), TESC (↓), TGFA (↑13), PBX1 (↑4), TENC1 (↓3)	Regulate cell proliferation, differentiation and development, play roles in cell growth, adhesion, signal transduction, tumor progression
Cell motion migration	F2RL1 (↓3), FURIN (↓4), ITGA2 (↑4), LAMB1 (↑5), LYN (↓3), WDR44 (↓5), TNS1 (↑12), TENC1 (↓3)	Involved in cell motion, migration, locomotion, play roles in cell adhesion, proliferation, survival, signal transduction
(4) Signaling transduction	NOTCH3 (↑4), JAG1 (↓3), CFD (↓5), HES7 (↑13), DTX3 (↑10); MAPK11 (↓5), MAPK13 (↓), MAPK8IP2 (↓51), MAPK8IP3 (↓3); IGF1R (↓17), IGFBP5 (↑30), IGFBP3 (↓2); BMP6 (↑6); EPHA10 (↓20), EPHA3 (↑135)	Notch signaling pathway; SAP kinase signaling pathway; insulin-like growth factor I signaling pathway; BMP signaling pathway; transmembrane receptor protein tyrosine kinase activity

Notes: "↑": upregulation. "↓": downregulation. The number in parenthesis represents fold change of gene expression level.

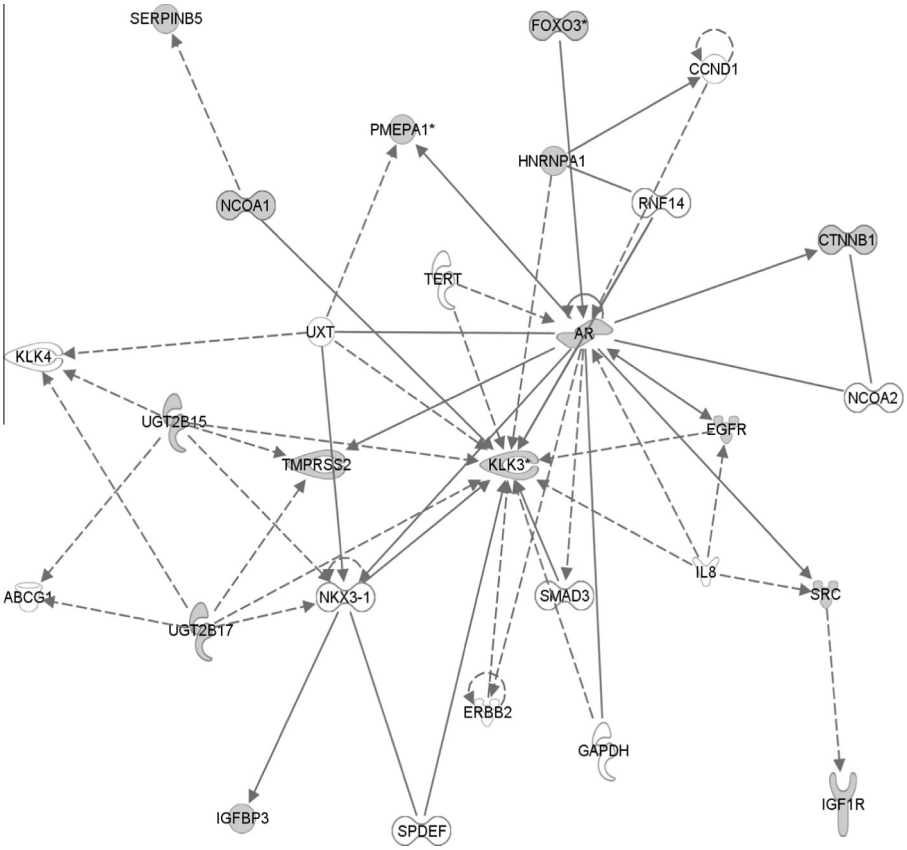

Fig. 1. Top gene network identified by IPA analysis. An illustration of a molecular network functionally associated with cellular development, cellular growth and proliferation, and organismal development. 8 of 15 identified focus molecules are present in DEGs. Solid arrows represent known physical interactions, dotted arrows represent indirect interactions. Grey nodes indicate focus molecules in this network. Node shapes represent different categories of encoded proteins.

Table 2
Summary of AS events in LNCaP vs. LNCaP-AI-F cells.

AS event	LNCaP	LNCaP-AI-F	Differential AS
Alternative promoter (AP)	1728	1758	84
Alternative terminator (AT)	2115	2175	26
Exon skip (ES)	2788	3073	171
Mutually exclusive exon (MEX)	65	72	4
Premature stop (PS)	623	606	43
Total events	5252	5515	315

Notes: the diagram of five AS types see Fig. S2. Alternative promoter, equal to the different 5′-UTR.

3.3. The role of AS in androgen-independent phenotype conversion

We identified 315 genes with differential AS (Table S11) in five different types (Table 2 and Fig. S2). We focused on the alternative terminator (AT) and premature stop (PS) because they often result in truncated proteins [23]. GO annotation indicated the genes with differential AT and PS were involved in 24 different biological functions, ranging from catalytic activity to the tricarboxylic acid cycle (Table S12). Among these genes, some appeared to have potentially carcinogenic effects. For instance, a PS in exon 14 of *MTA1* (metastasis associated 1) was predicted to produce a truncated protein without the C-terminal SH3-binding domain, which thus disrupted its interaction with other proteins. In other examples of AS, such as AP (alternative promoter), we found that exon 2 of *LZIC* (leucine zipper and CTNNBIP1 domain containing) preferentially presents in LNCaP-AI-F cells. This gene has been demonstrated to inhibit the WNT-beta-catenin-TCF signaling pathway in gastric cancers [24].

Of note, we detected AT and AP events in the AR gene (Table S13). The AR1 and AR3 variants, which were derived from the AT and AP events, displayed upregulation in LNCaP-AI-F cells, similar to the upregulation previously described in tumors from patient cohorts [25,26]. In addition, we detected another newly emerged AR transcript, AR2 (also known as AR45), in LNCaP-AI-F cells as previously observed only in normal prostate gland and other tissues [27]. Interestingly, the typical number of CAG repeats in the AR transcripts is 23 in LNCaP cells, but it increases to 28 in LNCaP-AI-F cells (Fig. 2).

3.4. Gene fusion events

In total, we identified 22 gene fusion events, including 8 LNCaP-AI-F-specific fusion events and 14 common fusion events in both cell lines (Tables 3 and S14). All of the events were confirmed by RT-PCR or Sanger sequencing (Tables 3 and S14 and Figs. 3 and 4). Among these fusion events, none was reported in tumors from patient cohorts in previous studies, whereas three fusion events (*FAM117B-BMPR2*, *GPS2-MPP2*, and *RERE-PIK3CD*) were reported in LNCaP or other prostate cancer cells [12,17,18,28–31].

From the sequence analysis of the 8 LNCaP-AI-F-specific fusion events, we were able to predict their possible fusion proteins and potential impact on biological functions (Table 3). With the exception of *ATP5E-ATP5EP2*, most fusion events were partially deleted or formed novel gene products likely to be carcinogenic. The fusion of *GLYR1-SLC9A8* occurred between intron 13 of *GLYR1* and intron 3 of *SLC9A8* (Fig. 3), resulting in a truncated SLC9A8 because of a premature stop codon. GLYR1 (glyoxylate reductase 1) is important for regulating p38 MAP kinase activity and LSD2 demethylase activity

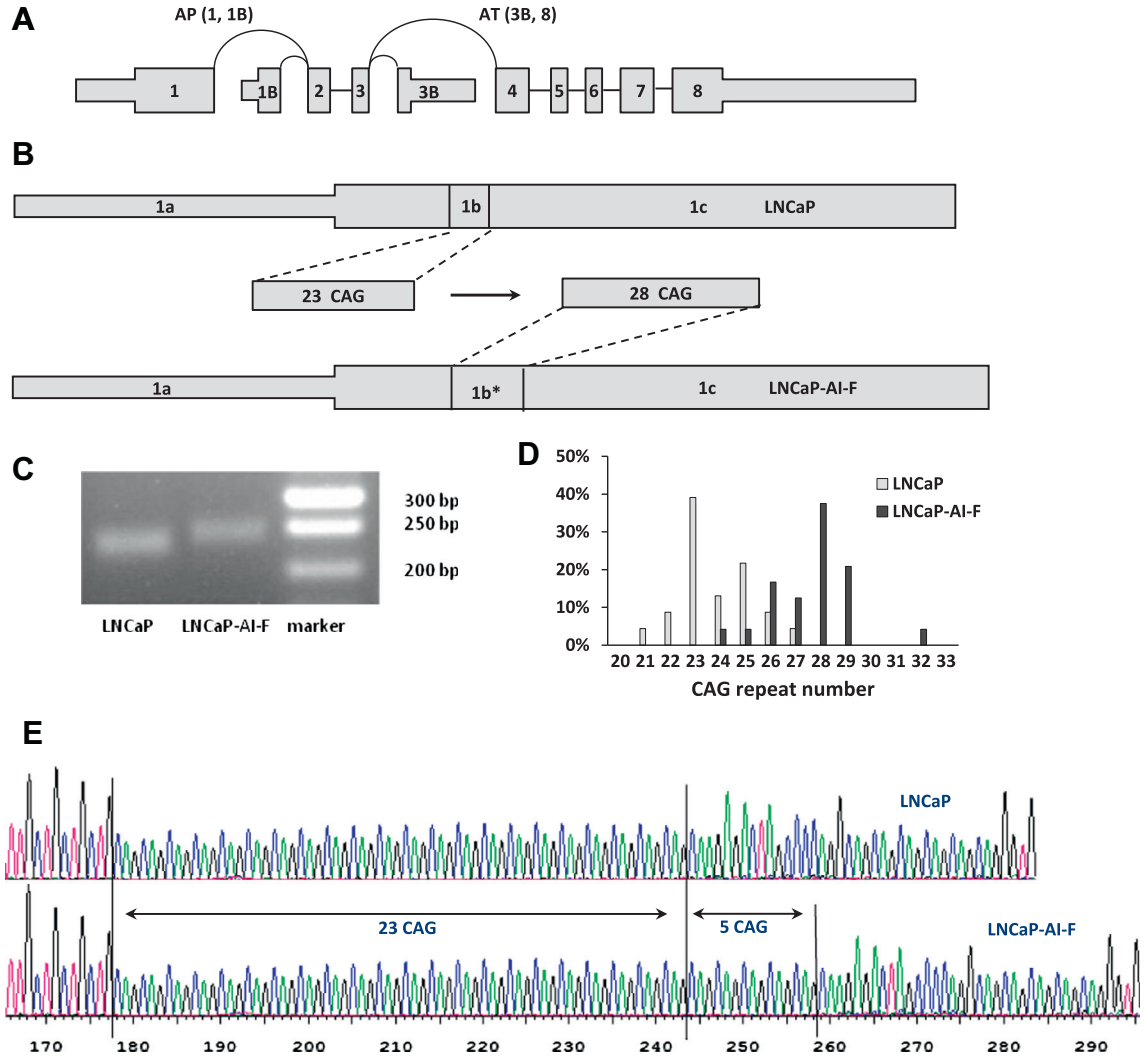

Fig. 2. Splicing events and sequence variations of *AR* gene. (A) Two types AS events (AP and AT) are shown on AR. (B) CAG repeat sequence variations in exon 1b are depicted. (C) Agarose gel graph of PCR products including CAG repeat sequence. (D) In LNCaP cells, the typical CAG repeat is 23 times (range 21–27), whereas in LNCaP-AI-F cells, the typical CAG expansion is 28 times (range 24–32). The proportion of the transcript with different repeat number represents by the corresponding percentage. (E) Two representative CAG repeat Sanger sequence chromatographs form LNCaP and LNCaP-AI-F were shown.

Table 3
Summary of putative LNCaP-AI-F specific gene fusion events.

No.	Fusion gene	Fusion manner	Putative fusion result and impact
1	ATP5E-ATP5EP2	Exon 3-exon 1 chr 20-chr 13 fr	Composed of 5′-UTR, coding region of ATP5E2P and 3′-UTR of ATP5E, protein coding sequence is not altered; negative impact on ATP synthesis
2	CSDA-CSDAP1	Exon 5-exon 1 chr 12-chr 16 rr	Exon 6 (69 aa.) deleted in CSDA
3	EIF2AK1-ATR	Exon 6-intron 46 chr 7-chr 3 fr	Exon 1–2 deleted in EIF2AK1, maybe affect heme-binding domain
4	HNRNPKP1-HNRNPK	Exon 1-exon 16 chr 5-chr 9 rr	No function or exon 1–11 (including two KH RNA-binding domains) deleted in HNRNPK
5	GLYR1-SLC9A8	Intron 13–intron 3 chr 16-chr 20 rf	GLYR1 lost the first 13 exons. SLC9A8 retained the first three exons and premature stop, and lost Na+/H + exchanger domain
6	GPS2-MPP2	Exon 1-exon 3 chr 17-chr 17 rr	N-terminal 11 aa. deleted in MPP2, which may serve as a signal peptide
7	SDCBP-SDCBPP2	Exon 7-exon 3 chr 8-chr 8 ff	Exon 8 (30 aa.) deleted in SDCBP, close to the C-terminal, affects partial PDZ domain
8	SNX9-CYP2C19	Exon 4-exon 6 chr 6-chr 10 ff	A novel protein with SNX9 N-terminal SH3 domain and CYP2C19 C-terminal p450 domain

Notes: eight fusion events have been confirmed by RT-PCR or Sanger sequencing. chr: chromosome. f: Forward strand. r: Reverse strand. UTR, Untranslated region. aa, Amino acid.

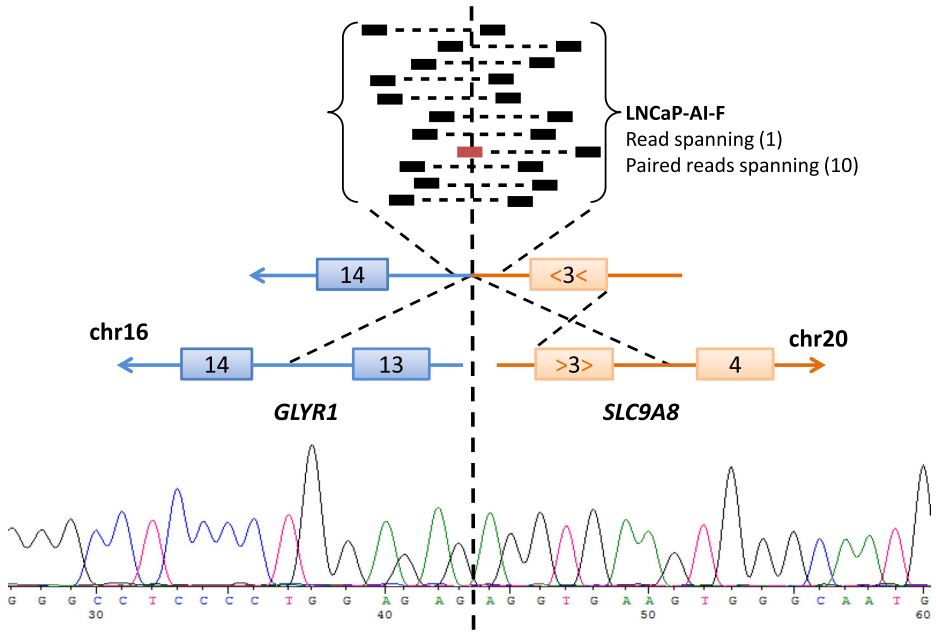

Fig. 3. Characterization of the *GLYR1-SLC9A8* fusion gene. Top panel: RNA-seq reads are represented by black solid bars. Paired reads are denoted by the dotted lines and two-end bars. Reads spanning the fusion junction are highlighted by red solid bars; Middle panel: Schematic of this fusion between intron 13 of *GLYR1* and intron 3 of *SLC9A8*. Transcript directions in the fusion are indicated by arrows. Chromosomal location of each gene in wild type is also depicted. Lower panel: The fusion junction is confirmed by Sanger sequencing. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

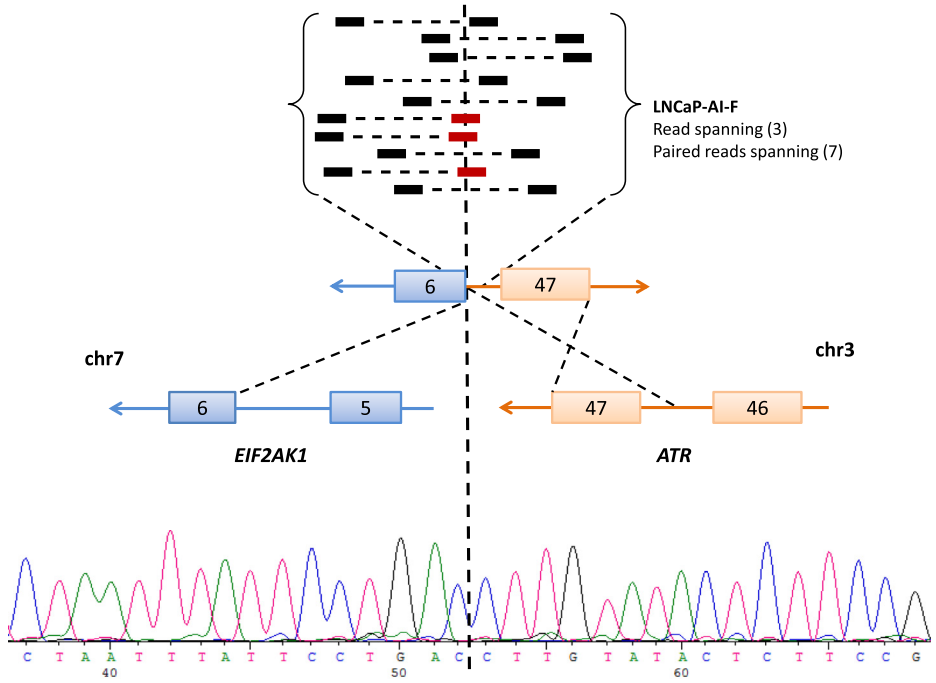

Fig. 4. Characterization of *EIF2AK1-ATR* fusion gene. Top panel: RNA-seq reads are represented by solid bars. Paired reads are denoted by the dotted lines and two-end bars. Reads spanning the fusion junction are highlighted by red solid bars; Middle panel: Schematic of this fusion between exon 6 of *EIF2AK1* and intron 46 of *ATR*. Transcript directions in the fusion are indicated by arrows. Chromosomal location of each gene in wild type is also depicted. Lower panel: the fusion junction is confirmed by Sanger sequencing. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and is closely associated with colorectal cancer. SLC9A8 (solute carrier family 9, subfamily A, member 8) is required for the regulation of intracellular pH homeostasis, cell volume, and ion transport. Loss-of-function in GLYR1 and SLC9A8 may disrupt their responsiveness to stimuli or adverse environmental conditions and thus is potentially implicated in prostate cancer progression. Moreover, we confirmed a fusion event between exon 6 of *EIF2AK1* (eukaryotic translation initiation factor 2 alpha kinase 1) and intron 46 of *ATR* (Ataxia telangiectasia and Rad3 related) (Fig. 4), most likely resulting in a novel *EIF2AK1* transcript without an *N*-terminal heme-binding domain. EIF2AK1 is involved in the downregulation of protein synthesis in response to stress, whereas

ATR is activated by checkpoint signaling upon genotoxic stress. The disruption of either gene has been linked to various cancers, such as ovarian, breast, Hodgkin's lymphoma and endometrioid endometrial cancer [32–36].

4. Discussion

High-throughput RNA-seq technology enabled us to identify the transcript abundance, alternative splicing (AS) and gene fusion events simultaneously. By comparative analysis and necessary validation, we detected 788 DEGs, 315 AS events, and 22 gene fusion events that are potentially involved in the androgen-independent phenotype conversion.

Considerable accumulated evidence suggests that gene fusion events may generate oncogenes and contribute to tumor progression. The eight novel fusion genes identified in this study, such as *GLYR1-SLC9A8* and *EIF2AK1-ATR*, were found to be involved in mediating stress activation and cell survival. Other fusion genes events, such as *CSDA-CSDAP1* and *GPS2-MPP2*, are in need of further characterization. Further studies are required to identify whether clinical prostate cancer samples share these fusion genes.

The development toward a NE-like phenotype is expected to be the most prominent characteristic of LNCaP-AI-F cells. Apart from the NE-like cellular morphology, the expression of multiple neuron-specific and endocrine activity-associated genes was altered [7]. These DEGs are mainly involved in several aspects of neuroendocrine functions (i.e., neurotransmitter, cellular secretion, neuron projection and cytoskeleton; Table 1). For instance, the expression of the neuron-specific microtube-associated protein Tau coding gene *MAPT* was increased 2-fold in LNCaP-AI-F cells. Neurotransmitter release, degradation and transport-related genes, such as *STXBP5* (syntaxin binding protein 5), *RIMS1* (regulating synaptic membrane exocytosis 1), *ALDH5A1* and *SLC1A3*, were found to be upregulated. Notably, the level of *PSA*, a biomarker reported in some NE-like prostate cancer cells, was found to be dramatically decreased in LNCaP-AI-F cells [37]. These results appear to correlate with the NE-like morphology, suggesting that the NE-like phenotype in androgen-independent LNCaP-AI-F cells is displayed not only in terms of the cellular morphology but also by the associated cell structures, functional activities and specific markers [7].

LNCaP-AI-F cells are able to adapt to androgen deprivation and endure various stimuli and responses. We identified a number of DEGs that are related to inhibited stress response capacity from suppressed SAPK signaling to reduced responsiveness to various stresses and stimuli, including androgen deprivation, redox state, oxygen levels, and apoptotic response to DNA damage, among others. As UGT2B15 and B17, members of the UGT2B (UDP glucuronosyltransferase 2 family, polypeptide B) family, are responsible for the inactivation of androgen as well as the removal of other steroid compounds, the overexpression of *UGT2B15* and *B17* in this study, along with the elevation of these two genes in castration-resistant prostate cancer (CRPC) tumors and other LNCaP-derived androgen-independent sublines, such as LNCaP-LNO, LNCaP-SF and LNCaP-abl, suggests that UGT2B15 and B17 play an important role in maintaining the low level of androgens in these tumors or cell lines [22,38–41]. In addition, the HSD3B1 gene, encoding a crucial molecule (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1) in the biosynthesis of all classes of hormonal steroids, was downregulated in LNCaP-AI-F cells. Although the overexpression of *HSD3B1* was previously observed in a minor portion of CRPC tumors, especially those with metastases, low or absent *HSD3B1* expression has been reported in the majority of prostate cancers and CRPC samples [42]. These findings suggest that the elevated *HSD3B1* in CRPC tumors might promote additional androgen biosynthesis from cholesterol or other precursors and contribute to castration-resistant tumor growth [42,43]. However, the potential contribution of intratumoral *de novo* androgen biosynthesis appeared to play a very limited role in prostate cancer progression [43]. Combining the function of HSD3B1 and UGT2B15/17 in androgen metabolism, the loss of *HSD3B1*, together with the overexpressed *UGT2Bs*, suggest that low levels of intracellular androgens and other steroids are associated with the progression of LNCaP-AI-F cells.

DEGs related to differentiation, proliferation, migration and apoptosis in various cancers may confer more aggressive phenotypes to LNCaP-AI-F. The underexpression of *AZGP1* (zinc-alpha 2-glycoprotein 1, ~117-fold decrease in this study) play an important role in lipid metabolism disorders, prostate cancer recurrence and metastasis, and is also in accordance with the progression of androgen-independent phenotypes [44]. We also observed a significant downregulation of *DAPK1* (death-associated protein kinase 1), a positive mediator of cell death and tumor suppressors. The loss-of-function of this gene has been observed in several different tumors. These results imply that the increased cell migration and anti-apoptosis are important properties of androgen-independent LNCaP-AI-F cells.

The AR signaling pathway appears to play a central role in androgen-independent phenotype progression. One of the interesting observations during the androgen-independent phenotype conversion in this study is the increased expression of all three AR variants. In LNCaP-AI-F cells, the increased AR1 (~2-fold) actually is a mutant AR (AR T877A), which is often observed in advanced prostate cancers, especially after flutamide treatment, and the increase of this mutated AR1 contributes to androgen-independent cell growth and survival [25,45]. AR3 is a constitutively active AR, and its transcriptional activity was not dependent on androgens or antiandrogens [26]. The observed elevation of AR3 in LNCaP-AI-F is consistent with previous reports, in which the increased AR3 contributed to the emergence of the androgen-independent phenotype and has been frequently detected in CRPC tumors [26,46–48]. In addition, it has been reported that AR2 (AR45), encoding a NH2-terminally truncated AR protein, is a negative regulator of AR signaling and harbors transcriptional activity under the condition of overexpression of the AR co-activators β-catenin or TIF-2 [27,49,50]. To the newly emerged AR2 in LNCaP-AI-F cells, whether it is also involved in androgen-independent phenotype conversion requires future study [27,49].

Intriguingly, in this study, we observed an increase of *AR* CAG repeat length in LNCaP-AI-F cells. For the general population or the patients with prostate cancer undergoing radical prostatectomy, the shorter CAG repeat length of the AR gene has been linked with an increased risk for the development of prostate cancer or lymph node-positive prostate cancer [51]. Whether the longer CAG repeat in LNCaP-AI-F is associated with the androgen-independent phenotype and the risk of tumor recurrence requires further confirmation in a clinical setting.

Another major player in the AR pathway is KLK3 (PSA), an important marker for the differentiation of epithelial cells of the prostate gland. Prostate cancer cells with low or negative PSA were recently observed to be undifferentiated or low differentiated tumor stem cells [11]. Compared with these tumor stem-like cells, our LNCaP-AI-F cells also shared similar characteristics, including low *PSA* level, resistance to androgen deprivation, suppressed stimuli response, specific fatty acid synthesis and metabolism, anti-apoptosis, early or low development/differentiation, and neural gene expression. This suggests that the LNCaP-AI-F cells have characteristics similar to tumor stem cell-like cells and represent a type of androgen-independent, more aggressive, and low differentiation NE-like prostate cancer cells.

Similar to LNCaP-abl, increased *AR* levels and decreased *KLK3* expression have also been observed in LNCaP-AI-F cells, but the mechanism remained unclear [22]. We speculate that mildly increased *AR* levels can be induced by low levels of intracellular androgen or androgen-independent pathways (e.g., modulatory effects of related microRNAs) [7]. Furthermore, *PSA* levels would be decreased because of androgen deprivation and the addition of antiandrogen but not the change of NF-κB pathway in our case because the levels of related genes remained unchanged. Finally, miR-100, one of the increased microRNAs identified in LNCaP-AI-F cells in our earlier study, was recently demonstrated to be a suppressor that affects *PSA* levels in androgen-independent C4-2 cells [7,52]. Therefore, it is conceivable that these three potential regulatory ways, combined with other unknown factors, may synergistically decrease *PSA* levels in LNCaP-AI-F cells.

Meta-analysis with other studies using androgen-independent sublines (6 microarray reports and 2 RNA-seq papers) confirmed the novelty of our findings (i.e., 19.1% matched and 80.9% unmatched DEGs with our data, Table S9). However, the results of this comparison should be interpreted cautiously for several reasons: (1) different cell line establishment methods (such as androgen deprivation vs. androgen deprivation combined with antiandrogen) as well as different culture media (added with or without androgen) were used for different research purposes; (2) the data were generated by different techniques (Microarray vs. RNA-seq); (3) the analysis and statistics were performed using different strategies and approaches; (4) the cell lines were investigated with different characteristics other than androgen-independent status (such as different metastasis and tumorigenicity capacities) or different progression stages from androgen-dependent to androgen-independent status [53].

Based upon the results of our comprehensive analysis of LNCaP and LNCaP-AI-F cells, we revealed several interesting issues, including eight novel LNCaP-AI-F specific fusion events, several specific cell characteristics, a core network centering around increased *AR* and decreased *PSA*, novel observation of an expanded CAG repeat, as well as changed AR variants, and new features of cancer stem-like cells in the LNCaP-AI-F cells. Our studies highlight several unique characteristics of androgen-independent cells and may enhance the scientific community's understanding of androgen-independent phenotype conversion. Our findings warrant further studies in clinical settings for future efforts in pathogenicity, diagnosis, and treatment of prostate cancers.

Conflict of Interest

All authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.08.044.

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