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Identification of Mutations, Expression Alterations and Fusion Transcripts by Next Generation RNAseq in Castration-Resistant Prostate Cancer Cell lines with Possible Clinical Relevance

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Abstract

Androgen Deprivation Therapy (ADT) would benefit prostate cancer patients initially but cancer cells can eventually develop castration resistance. In this study, we compared androgen-dependent and androgen-independent cell lines to find potential genes associated with acquired resistance to ADT. Using RNAseq, we found 4397 mutations distributed in 2579 genes, out of which, 1574 mutated genes could also be found in prostate cancer tumor samples collected in Cosmic database (http://cancer.sanger.ac.uk/cosmic). We also discovered 157 and 549 genes which were down and up-regulated respectively in both PC3 and DU145 compared to LNCaP. Network analysis resulted in 3 dominant connection notes: GCR/MCR (NR3C1) and PKA-cat kinase (PRKACB) and PKC family (PRKD1). By ChimeraScan analysis, 48, 117 and 60 chimeric transcripts were discovered in DU145, LNCaP and PC3 respectively. Among them, six predicted fusions expressed specifically in androgen-independent cell lines (DU145 and PC3). Some of these gene mutations and transcription alterations have been reported in tumor samples from prostate cancer patients and may have certain associations with acquired resistance to anti-hormone therapy in prostate cancer. A proportion of mutations are enriched in genes involved in immune response pathways, suggesting new targets to develop effective treatments to overcome castration resistance.

Introduction

Prostate cancer is the third most common cause of death from cancer in men in Europe [1]. Androgen deprivation therapy (ADT), as a first-line therapy, usually leads to a response with suppression of prostate specific antigen (PSA) levels, symptomatic palliation and prolonged overall survival in most patients. However, all patients would eventually become resistant to the treatment and median overall survival after ADT is 48 to 54 months [2,3]. Metastatic castration-resistant prostate cancer (mCRPC) is the main cause of cancer death in prostate cancer patients.

It has been shown that prostate cancer cells can grow and proliferate at low or even near-zero androgen levels when they become castration resistant [4]. A hypersensitive androgen receptor (AR) pathway can be evolved through accumulation of molecular alterations including AR overexpression, gain of function mutations in AR gene, transcriptional activity of the AR altered by coactivators or corepressors, intra-tumoral testosterone synthesis, and ligand-independent activation of AR [5]. Other alterations includes a) changes in growth factor and corresponding receptors, e.g. transforming growth factor a (TGFa), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), insulin-like growth factor-1 (IGF-1) [6-10], b) apoptotic signaling e.g. phosphatase and tensin homolog (PTEN), bcl-2 and Myb [11-13], and c) Wnt/β-catenin signaling [14].

Despite vigorous efforts only a few prostate cancer cell lines have been established. Among them, LNCaP was derived from a lymph node metastasis. It is androgen-dependent and can represent the early stage of metastatic prostate cancer. PC3 and DU145 were derived from bone and brain metastases respectively. They are androgen-independent and can represent the later and rapid progressing metastatic prostate cancer stage. In this article, we compared androgen-independent cell lines (PC3 and DU145) versus androgen-dependent cell line (LNCaP) to discover which genomic changes are possibly connected to the development of castration resistance.

Methods

RNAseq

Total RNA was extracted by TRIzol (Invitrogen, Catalog #15596018) following by phenol/chloroform. RNA Integrity Number (RIN) was used to control RNA quality by Agilent 2100 Bioanalyzer System. PolyA selection from total RNA samples was done in SciLifeLab, Stockholm, and sequencing was conducted on HiSeq 2000 according to manufacturer's instructions.

Analysis of mutations, differentially expressed and fusion genes

RNAseq fastq data was aligned to reference genome hg38 by STAR 2 pass and GATK (base quality score recalibration, indel realignment, duplicate removal and INDEL discovery) was applied to call variants across all 15 samples according to GATK Best Practices recommendations [15-17]. A published TopHat and Cufflinks protocol was used to find differentially expressed genes [18] and ChimeraScan was employed to discover fusion transcripts [18]. Panther (http://www.pantherdb.org) and Thomson Reuters (https://portal.genego.com) were applied for functional enrichment analysis [19].

PCR validation

We used cloned AMV first-strand synthesis kit (Life Technologies, Catalog # 12328) and PCR to validate expressions and fusions. Forward primer for fusion transcripts was designed upon fusion sequence given by RNAseq. The forward primer located in 5' gene and reverse primer on the 3' gene. PCR was done by Platinum Taq DNA polymerase (Life Technologies, Catalog #10966018), followed by Sanger sequencing in Eurofins Genomics.

Results

Mutations acquired in castration resistant cell lines

We used triplicates of each cell line (Du145, PC-3 and LNCaP) for whole transcriptome RNA-sequencing and compared "hormone resistant" (PC-3 and DU145) and "hormone sensitive" (LNCaP) cell lines to find mutations acquired after hormone treatment. Only mutations, which were present in PC-3 and Du145 but absent in LNCaP triplicates were chosen as "stably acquired mutations".

We found, in 2579 genes, 4397 mutations which were consistently mutated after cell lines acquired resistance to hormone treatment (S1). All 2579 mutated genes distributed relatively even in all chromosomes without preference. GO term analysis showed that binding (GO: 0005488) and catalytic activity (GO:0003824) are two most widely distributed groups among all mutated genes (Figure 1A), and the two most enriched pathways are 1) Immune response B cell antigen receptor (BCR) pathway, and 2) development positive regulation of STK3/4 (Hippo) pathway and negative regulation of YAP/TAZ function.

Gene	Full name	COMIC mutation rate in Pca %	COSMIC mutation rate in all cancers %	Protein Expression in PCa Tissue (Human Protein ATLAS)	Possibly Involved in immune response	Possibly Involved in EMT*
K-RAS	KRAS Proto-oncogene	3.0	19.2	Up-regulated	Yes[15]	Yes[16]
PLCG1	Phospholipase C gamma 1	1.2	1.5	No alteration	Yes[17]	Yes[18]
NOTCH1	Translocation-associated Notch protein TAN-1	1.4	5.7	No alteration	Yes[19]	Yes[20]
NCOR2	Nuclear receptor corepressor 2	1.4	1.8	No alteration	Yes[21]	Yes[22]
JAK1	Janus kinase 1	1.3	1.3	Up-regulated	Yes[23]	Yes[24]
FASN	Fatty acid synthase	1.1	1.7	Up-regulated	Yes[25]	Yes[26]
PREX1	Phosphatidylinositol-3,4,5-trisphosphate dependent rac exchange factor 1	1.1	1.6	Up-regulated	Yes[27]	Yes[28]
MYLK	Myosin light chain kinase	1.4	1.7	N/A	-	Yes[29]
*EMT: Epithelial-Mesenchymal Transition						

Table 1: Selected mutated genes from 10 enriched pathways in Figure 1B [15-29].

Some mutations among these 10 enriched pathways had a higher mutation rate in patient samples (COSMIC) and many of them are involved in immune response (Figure 1B) (Table 1). Cosmic database shows that there are 22909 genes mutated in 4763 prostate cancer tumor samples in total, and 1574 of the 2579 genes mutated in our cell line study showed also mutations in tumor samples from patients.

Genes with alternative expressions in castration resistant cell lines

By comparison of "PC3 vs LNCaP" and "DU145 vs LNCaP", we aimed to identify down- or up-regulated genes in androgen-independent cell lines (S2). We set a cut-off (expression fold-change more than 3 and expression more than 3) and found 157 and 549 genes which were down- and up-regulated respectively in both PC3 and DU145 compared to LNCaP (S3). We selected 30 genes randomly and 28 out of them were validated by PCR (S4). The most up- or down-regulated genes were shown in Tables 2 and 3.

All up- and down-regulated genes in androgen independent PC3 and DU145 cell lines (706 genes) were analyzed by MetaCore from Thomson Reuters to build network which resulted in 3 dominant connection notes (Figure 1C): GCR/MCR (NR3C1) and PKA-cat kinase (PRKACB) and PKC family (PRKD1).
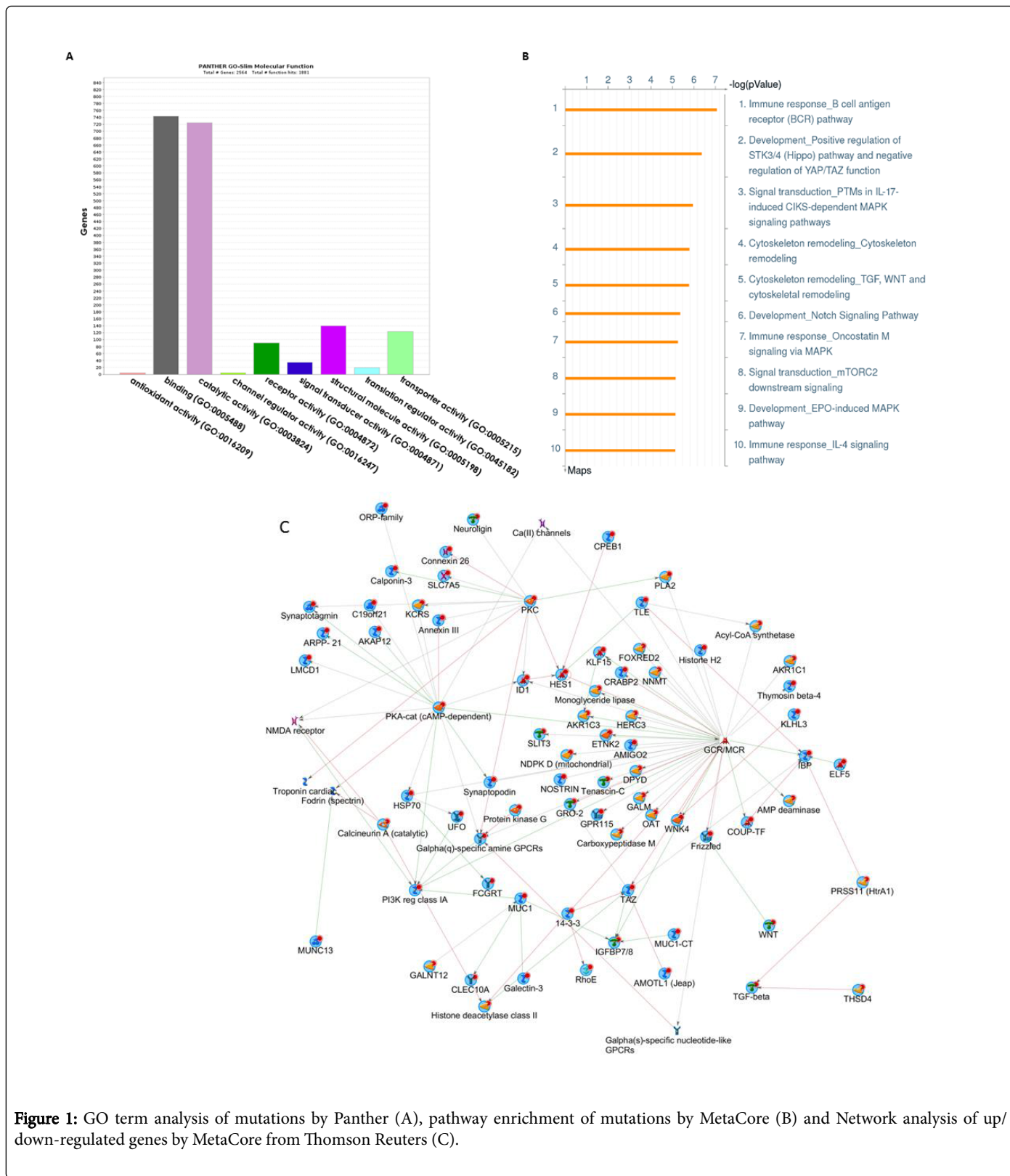
Figure 1: GO term analysis of mutations by Panther (A), pathway enrichment of mutations by MetaCore (B) and Network analysis of up/down-regulated genes by MetaCore from Thomson Reuters (C).

Gene*	Full name	Related pathways or function	Expression level			Stably mutated in our cell lines/Cosmic tumor samples?
			LNCaP	PC-3	DU145	
INPP1	inositol polyphosphate-1-phosphatase	Metabolism	0.007	9.3	13.5	Yes/Yes
GSTP1	Glutathione S-transferase Pi 1	Pathways in cancer	0.6	230.1	363.3	No/Yes
CYP1B1	Cytochrome P450, Family 1, Subfamily B, Polypeptide 1	AhR pathway	0.03	14.2	16.2	No/Yes
MYEOV	Myeloma overexpressed	Myeloma	0.1	45.8	35.5	Yes/Yes
KLF12	Kruppel-like factor 12	Involved in vertebrate development and carcinogenesis	0.01	6.8	3.3	Yes/Yes
GPRC5A	G protein-coupled receptor, Class C, group 5, member A	Cancer pathway and neuroscience	0.2	90.6	58	No/Yes
IGFBP4	Insulin-Like growth factor binding protein 4	IGF binding	0.2	98.7	50.5	No/Yes
CAV2	Caveolin 2	Prostate cancer	0.1	66.3	27.8	Yes/Yes
B3GNT3	UDP-GlcNAc:BetaGal Beta-1,3-N-Acetylglucosaminyltransferase 3	Metabolism	0.04	27	10.1	Yes/Yes
*all the 9 genes were among top 50 in both "PC-3 vs LNCaP" and "DU145 vs LNCaP"						

Table 2: Top 9 up-regulated genes when cells acquired resistance to androgen.

Gene*	Full name	Related pathways or function	Expression level			Stably mutated in our cell lines/Cosmic tumor samples?
			LNCaP	PC-3	DU145	
CSMD1	CUB and Sushi multiple domains 1	benign adult familial myoclonic epilepsy	13.6	0.03	0.004	No/Yes
TMEFF2	Transmembrane protein with EGF-Like and two follistatin-like domains 2	function as both an oncogene and a tumor suppressor depending on the cellular context	122.5	0.3	0.06	No/yes
SPON2	Spondin 2, extracellular matrix protein	ERK signaling	868.4	1.0	0.5	No/yes
UGT2B17	UDP glucuronosyltransferase 2 Family, polypeptide B17	Metabolism	156.3	0.3	0.2	No/Yes
MAGEA4	Melanoma antigen family A4	embryonal development and tumor transformation or progression	126.3	0.2	0.2	No/Yes
UGT2B15	UDP glucuronosyltransferase 2 family, polypeptide B15	UDP-glucuronyltransferase	146.9	0.2	0.2	No/Yes
GLYATL1	Glycine-N-acyltransferase-like 1	Metabolism and biological oxidations	86.1	0.2	0.2	No/Yes
DDC	Dopa decarboxylase (Aromatic L-amino acid decarboxylase)	Metabolism	206.7	0.5	0.7	No/Yes
*all the 8 genes were among top 30 in both "PC-3 vs LNCaP" and "DU145 vs LNCaP"						

Table 3: Top 8 down-regulated genes when cells acquired resistance to androgen.

Fusions associated with castration-resistant prostate cancer

By ChimeraScan analysis, 117, 48 and 60 fusions were discovered in LNCaP DU145 and PC3 (S5) respectively. Venn diagram showed that only one fusion (AF086285-ATP6V1E2) was universally expressed in all 3 cell lines. Six fusions were transcribed commonly in androgen-independent cell lines (PC3 and DU145) but not in androgen-dependent cell line (LNCaP) (Figure 2A) (Table 4). Three of those fusion partners are long non-coding RNA (FLJ39739, LOC100286793

and LOC728855), and a recent study showed that FLJ39739 (LINC01138) was directly AR-targeted lncRNAs and associated with Gleason score and tumor stage [30].

5'	Chr	5' full name	3'	Chr	3' full name
CTSD	11	Cathepsin D	IFITM10	11	Interferon Induced Transmembrane Protein 10
FLJ39739	1	LINC01138 long intergenic non-protein coding RNA 1138 [Homo sapiens (human)]	BC065231	1	Homo sapiens cDNA clone IMAGE:5548407, partial cds
HMGA1	6	High mobility group AT-Hook 1	BTNL8	5	Butyrophilin Like 8
LOC100286793	1	LINC00875 long intergenic non-protein coding RNA 875	BC065231	1	Homo sapiens cDNA clone IMAGE:5548407, partial cds
LOC728855	1	LINC00623 long intergenic non-protein coding RNA 623	BC065231	1	Homo sapiens cDNA clone IMAGE:5548407, partial cds
BC110832	1	Homo sapiens cDNA clone IMAGE: 5770060	BC065231	1	Homo sapiens cDNA clone IMAGE:5548407, partial cds

Table 4: Fusions specifically expressed in androgen-independent cell lines (DU145 and PC3) compared to androgen-dependent cell line (LNCaP).

5' gene	5' chr	Up or down regulation	3' gene	3' chr	Up or down regulation	Verified*	In-frame fusion	Previous publications
MIPOL1	14	no	DGKB	7	no	Yes	No	31
ADCK4	19	no	NUMBL	19	no	Yes	yes	32
GPS2	17	no	MPP2#	17	down	yes	Yes	33
TFDP1	13	no	GRK1#	13	down	yes	Yes	34
SAMD8#	10	up	ADK	10	no	Yes	No	--
SMAGP	12	no	TFCP2#	12	up	yes	No	--
HMGA1#	6	up	BTNL8	5	no	no	No	--
KDM5B	1	no	CR936711	1	no	yes	No	--
BTNL8	5	no	HMGA1#	6	up	no	No	--
RPS24	10	no	AJAP1	1	no	no	No	--
RERE	1	no	PIK3CD#	1	down	yes	No	33
*Marked 'Yes' if PCR found predicted fusions in DU145, PC3 or LNCaP cell lines. #Dramatically up- or down- regulated genes								

Table 5: Fusion transcripts selected and validated by PCR [31-33].

Using "Unique alignment positions" more than 5 as a cut-off provided a total of 25 chimeric transcripts, out of which, 11 selected validations were done by PCR (Table 5). PCR validation found that eight of eleven (72.7%) suspected fusion transcripts could be found in PC3, DU145 or LNCaP cell lines (Figure 2B). Among all the validating fusions, four chimeric transcripts (MIPOL1-DGKB, GPS2-MPP2, RERE-*PIK3CD* and TFDP1-*GRK1*) expressed only in androgen-dependent cell line LNCaP, while three chimeric transcripts (SMAGP-*TFCP2*, KDM5B-CR936711, SAMD8-ADK) expressed only in androgen-resistant cell line (Figure 2C).

Discussion

The development of castration resistance can have different molecular mechanisms as given in the introduction. They can be generally classified into two major categories as AR-dependent and AR-independent. The former requires a functionally normal or hyperactive AR i.e. cancer cells have AR expression. The latter requires no direct AR functional involvement. In line with previous published results, this study showed that PC3 and DU145 cell lines didn't express AR [34-36]. Therefore, possible molecular changes found in either PC3 or DU145 cell lines may represent the AR-independent mechanisms. In this context, the present study identified genes with mutations, expression changes and fusion transcripts in androgen-independent prostate cancer cells as compared to androgen-dependent cancer cells.

Mutations are relatively common in cancer cell lines. They can be cancer specific mutations or random mutations acquired during the cell culture. To discriminate these random mutations, we used more than one cell lines and each cell line with triplicates. Furthermore, molecular changes in cell lines, despite not random, do not always represent molecular changes in tumor samples from patients.
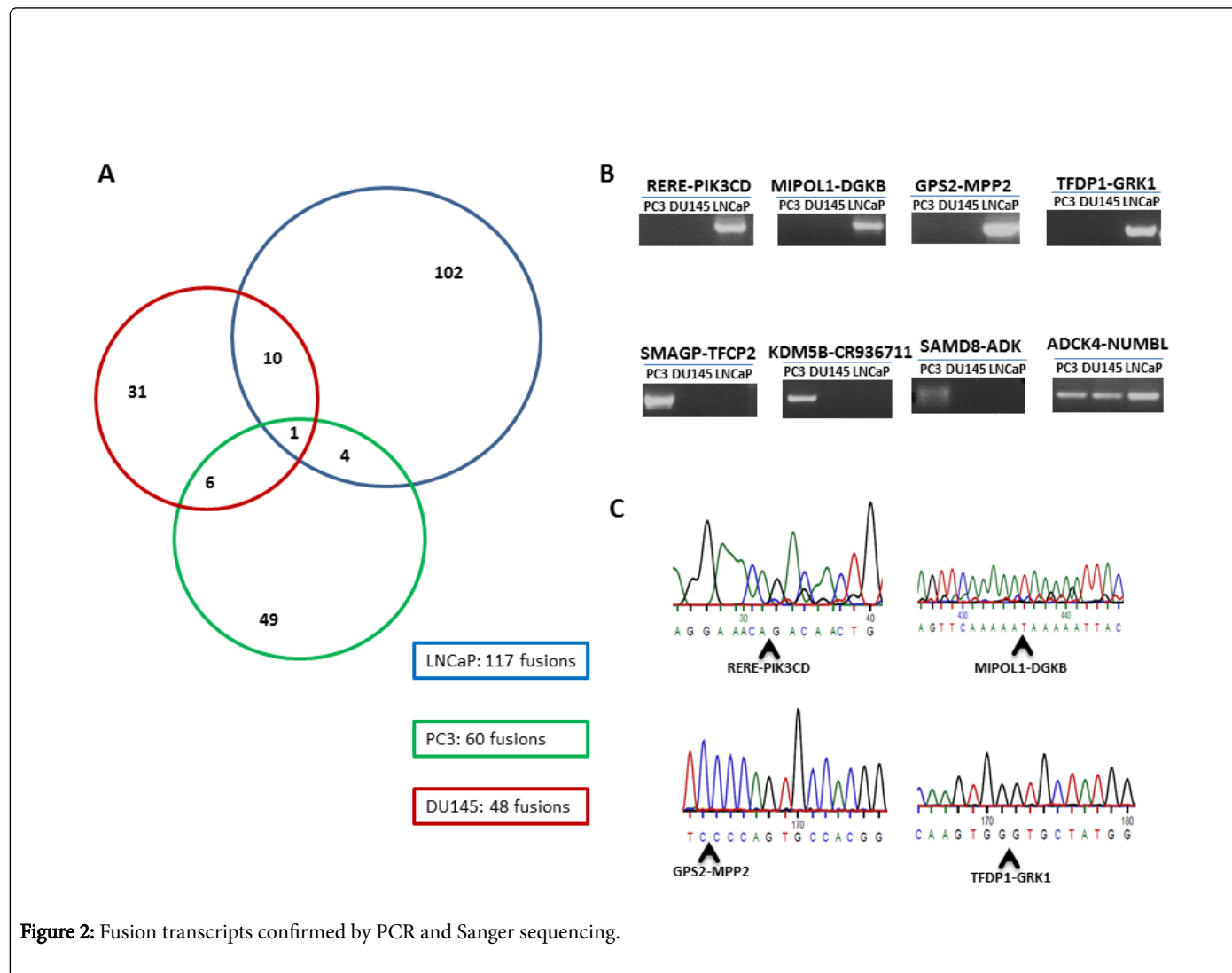
Figure 2: Fusion transcripts confirmed by PCR and Sanger sequencing.

To overcome this limitation, we focused on the importance of those molecular changes that have also been identified in tumor samples from patients in previously published results including the COSMIC database. Our study found 2579 mutated genes which were probably acquired when cells became resistant to hormone treatment. Among them, 1574 (61%) could be found in mutations of Cosmic tumor samples, for instance, SPEG (1.7%) and NCOR2 (1.6%) in tumor samples (COSMIC).

Most interestingly, the most common pathway enriched with mutated genes in androgen-independent cell lines is the Immune response B cell antigen receptor (BCR) pathway, in line with a recent proposed concept epithelial immune cell-like transition (EIT) [37,38]. These findings may indicate that cancer cells can employ cytokine and immune pathways to suppress host's immune activity and escape from control and surveillance by immune system. Immunotherapy combined with hormone therapy could probably become effective treatments for metastatic prostate cancer.

The MetaCore analysis identified NR3C1, PRKACB, PRKD1 and PKD1 as the up-regulated genes with potential functions in hormone resistant cell lines. NR3C1 was significantly up-regulated in PC3 (expression level: 9.5) and DU145 (expression level: 63.8) compared with LNCaP (expression level: 0.2). NR3C1 encodes glucocorticoid receptor (GR), which shares several transcriptional targets with AR.

Previous researches showed that increased GR expression contributed to acquire resistance to ADT in prostate cancer *in vitro* and *in vivo* [39-41]. A phase II trial of enzalutamide (MDV3100) plus the glucocorticoid receptor antagonist mifepristone for patients with metastatic castration resistant prostate cancer (CRPC) has been performed (https://clinicaltrials.gov/ct2/show/study/NCT02012296?view=record).

PRKACB (protein kinase cAMP-dependent catalytic beta) is a member of serine/threonine protein kinase family and a key effector involved in proliferation, apoptosis metabolism and differentiation. In our study, it was down-regulated in PC3 (expression level: 8.9) and DU145 (expression level: 14.7), compared with LNCaP (expression

level: 253.7). PRKD1 (protein kinase D1) is also known as protein kinase C mu (atypical PKC), which is a serine/threonine kinase and can be activated by PKC, involving various functions including adhesion, cell motility, and cell proliferation. PKD1 can interact with androgen receptor (AR) and modulated AR function in prostate cancer [42-44]. In our project, PRKD1 was down-regulated in PC3 (expression level: 4.5) and DU145 (expression level: 5.4), compared with LNCaP (expression level: 100), showing that mRNA level was decreased in androgen-independent phenotype, which is similar to PRKACB above. PKD1 or PRKACB agonists or exogenous PKD1 may probably help to stop or slow down the progression of androgen-independent phenotype.

We also found several fusions which probably associated with resistance to hormone treatment. SMAGP-*TFCP2*, KDM5B-CR936711 and SAMD8-ADK was detected only in PC3 which were one of the androgen-independent cell lines in this study, while some fusions were androgen-dependent cell line (LNCaP) specific, including MIPOL1-DGKB, GPS2-MPP2, TFDP1-*GRK1*, and RERE-*PIK3CD*. SMAGP (Small Cell Adhesion Glycoprotein) could bind as an enhancer with *TFCP2* (Transcription Factor CP2), which activate transcription of genes, such as alpha-globin gene.

MIPOL1-DGKB had been found in LNCaP cell lines in previous study published in 2009 using RT-PCR and FISH [31], and the fusion point is the same with the fusion found in our project and cannot been read-through. GPS2-MPP2 found in our project had the same fusion point with previous study in LNCaP cell line, and was in-frame which could result in the expression a chimeric protein localized differently from wild-type GPS2 and MPP2 in cells and can promoted proliferation and protected against apoptosis [45]. TFDP1-*GRK1* was also mentioned in previous bioinformatics studies without confirmation and function validation [46,47]. RERE-*PIK3CD* was found out of frame in our project. Among all fusion gene partners, *GRK1* and *PIK3CD* expression were top 2 significantly down-regulated genes and *TFCP2* was the most dramatically up-regulated gene [47-52].

Conclusion

Our study discovered mutations, fusion transcripts and genes with altered expression levels in castration-resistant prostate cancer cell lines, adding insights into androgen resistance in prostate cancer at transcriptomic level.

Acknowledgements

Author Contributions

Yuanjun Ma, Chunde Li and Sten Nilsson designed the study plan. Sten Nilsson, Lena Lennartsson, Zhuochun Peng and Chunde Li provided materials. Yuanjun Ma, and Yali Miao performed all experiments. Yuanjun Ma, Johanna Sandgren, Teresita Díaz De Ståhl, and Chunde Li analyzed data together. Yuanjun Ma and Chunde Li wrote the manuscript. All authors revised the manuscript.

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