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Original Article

Expression profiling of genes in androgen metabolism in androgen-independent prostate cancer cells under an androgen-deprived environment: mechanisms of castration resistance

Wu Zhou[1*], Yan Jiang[2*], Lili Ji[1], Lianlian Zhou[3], Meijuan Zhang[1], Mo Shen[1], Jia Zhao[1], Hongxiang Tu[1], Zhongyong Wang[1], Ruihao Wu[1], Yuehui Chen[1], Cui Zhou[1], Kate Huang[4], Zhihua Tao[2]

[1]Department of Medical Laboratory, The First Affiliated Hospital of Wenzhou Medical University, Zhejiang, China; [2]Department of Medical Laboratory, The Second Affiliated Hospital of Zhejiang University School of Medicine, Zhejiang, China; [3]Department of Laboratory Medicine, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Zhejiang, China; [4]Department of Pathology, The First Affiliated Hospital of Wenzhou Medical University, Zhejiang, China. [*]Equal contributors.

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Abstract: Objective: In an earlier study, we obtained androgen-independent prostate cancer cells (LNCaP-AI) and flutamide tolerant- LNCaP-AI (LNCaP-AI+F cells) that could survive in an androgen-free environment without or with flutamide, respectively. In the present study, we investigated the molecular mechanism of the castration resistance. Material and Methods: qRT-PCR was applied to measure the expression levels of genes coding for enzymes in the lysophosphatidylcholine (LPC) metabolic pathway and responsible for cholesterol and androgen metabolism in these cell lines. An ExProfile™ Human PI3K-AKT Signaling Related Gene qPCR Array was also used to investigate the expression levels of genes in the intracellular metabolic pathways in an emasculated hormone environment. Results: Compared with androgen-responsive prostate cancer (LNCaP) cells, phospholipase D1 (PLD1) expression was increased 259- and 410-folds in LNCaP-AI and LNCaP-AI+F cells, respectively, and the PI3K/AKT signaling pathway was enhanced. Additionally, the expression of cholesterol metabolic enzymes and rate-limiting enzymes, such as organic anion-transporting polypeptide 1B3 (OATP1B3) (LNCaP-AI cells: 19.08-fold; LNCaP-AI+F cells: 14.34-fold), low density lipoprotein receptor (LDL-r) (LNCaP-AI cells: 23.34-fold; LNCaP-AI+F cells: 8.78-fold), squalene epoxidase (SQLE) (LNCaP-AI cells: 22.03-fold; LNCaP-AI+F cells: 10.27-fold), and scavenger receptor B1 (SCARB1) (LNCaP-AI cells: 10.27-fold; LNCaP-AI+F cells: 5.75-fold), was significantly increased. Furthermore, the expression of androgen synthetases, such as steroid 17-alpha-monooxygenase (CYP17A1) (LNCaP-AI cells: 28.59-fold; LNCaP-AI+F cells: 4.32-fold), steroid 5 alpha-reductase 2 (SRD5A2) (LNCaP-AI cells: 51.50-fold; LNCaP-AI+F cells: 12.91-fold), aldo-keto reductases (AKR1C1, AKR1C2, AKR1C3) (LNCaP-AI: 8.63-fold; LNCaP-AI+F cells: 7.10-fold), and androgen receptor (AR) (LNCaP-AI: 39.18-fold; LNCaP-AI+F cells: 36.56-fold), was increased significantly ($P < 0.05$). Conclusions: Our results revealed that the survival of LNCaP AI cells in androgen-free medium is due to self-synthesis of androgen, increased cholesterol intake and increased AR expression. These evidences suggest that the castration-resistant LNCaP cells are not androgen-independent but have developed or activated a set of AR-related signaling pathways to increase endogenous androgen biosynthesis and AR expression.

Keywords: Prostate cancer, LNCaP cell, androgen-independent, metabolic pathway

Introduction

Prostate cancer (PCa) is the second leading cause of cancer-related death among men in the United States; the mortality rate is second only to lung cancer. Huggins and Hodges proposed that prostate cancers are androgen dependent, and androgens can affect cellular proliferation and differentiation and may play critical roles in prostate cancer development [1]. Since the publication of this study, androgen ablation has generally been employed as a therapy for patients with prostate cancer that has spread so prostatectomy or irradiation is no longer suitable. However, despite the initial therapeutic efficacy of androgen ablation, the andro-

gen-dependent tumors eventually become androgen independent and grow quickly over a period of 18 to 20 months with an ensuing median survival of 1 to 2 years. To date, the molecular mechanisms underlying the switching from androgen dependence to independence in prostate tumors are unknown. Presently, proposed mechanisms include AR gene amplification, gene mutation, increased AR expression or increased androgen in prostate tumor cells [2-8]. Conversely, cancer cells can also obtain energy by metabolic reprogramming. For example, during the "Warburg effect" period, glycolysis is enhanced in tumor cells for more energy production [9-12]. Vaz et al compared glucose metabolism in androgen-responsive (LNCaP) and androgen-nonresponsive PCa cells (PC3) and found that LNCaP and PC3 cells consumed similar amounts of glucose, whereas PC3 cells presented higher lactate production, suggesting increased levels of oxidative stress in PC3 cells [13]. Recently, Asgari et al investigated 13 different cancer cells and found that, regardless of the significant up- and down-regulation of metabolic genes, the distribution of the metabolic changes was similar in different cancer types, indicating that the Warburg effect is a consequence of metabolic adaptation in cancer cells [14].

In our previous study, we obtained androgen-independent PCa cells (LNCaP-AI) from LNCaP cells by growing LNCaP cells in medium with gradually reduced androgen concentration as well as flutamide-tolerant LNCaP-AI (LNCaP-AI+F) by culturing LNCaP-AI cells in medium with gradually increased reduced flutamide concentration [15]. The expression of a number of genes were profiled in the two cell lines in this study to investigate the molecular mechanisms underlying the androgenic regulation of PCa. The findings would provide new insights on role of androgen in PCa proliferation and help to identify new strategies to treat the cancer.

Materials and methods

Cell culture

Human prostate cancer cell line LNCaP was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and was maintained in DMEM/F12 medium (1:1 mixture, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). Androgen-independent LNCaP cells (LNCaP-AI) and flutamide resistant LNCaP-AI (LNCaP-AI+F) were obtained as previously reported [15]. LNCaP-AI cells were maintained in DMEM/F12 medium (1:1 mixture, Gibco, Grand Island, NY, USA) supplemented 10% charcoal stripped FBS (Gibco, Grand Island, NY, USA), LNCaP-AI+F cells were maintained in the same medium with flutamide (5.0×10^{-6} mol/L, Schering-Plough, Pointe-Claire, Canada). All cells were maintained at 37°C in a humidified incubator with 5% CO_2 and subcultured every 4-6 days.

In this study, androgen-free FBS was used to mimic long-term androgen ablation therapy. In addition, since phenolsulfonphthalein is likely to be a steroid mimic. It was removed from the culture medium for LNCaP-AI and LNCaP-AI+F cell culture. LNCaP cells at the same passage were used as the control.

RNA extraction and reverse transcription

Total RNA was isolated from cells using the RNeasy Plus Mini Kit (QIAGEN, Suzhou, China) according to the manufacturer's instructions. 2 μg total RNA was reversely transcripted into the first strand of cDNA using the Oligo (dT) 18 primer/random hexamers with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer's instructions.

Primers

In our previous study, we found the levels of 23 types of metabolites, particularly, lysophosphatidylcholine (LPC), were higher in LNCaP-AI cells than in LNCaP cells [16]. Therefore, an array of primers were designed for genes (**Table 1**) in LPC metabolic pathways as well as for cholesterol and androgen metabolism. Additionally, ExProfile™ Human PI3K-AKT Signaling Related Gene qPCR Array (GeneCopoeia, Maryland, USA) was also used to investigate the mRNA levels in the intracellular metabolic pathways.

qRT-PCR

qRT-PCR was performed in 20-μL reactions containing 1 μL of reverse transcription product according to the manufacturer's instructions using ABI SYBR Green PCR mix detection kit and the ABI 7500 instrument (Applied Biosys-

Table 1. Primers and their sequences for expression profiling

Gene	Forward	Reverse
AR	AGAGTGCCCTATCCCAGTCCCA	TTCCCTTCAGCGGCTCTTTTG
AKR1C1, 2	GTCCATCGGGGTGTCCAACTT	CATGGTTCTTCTCGGTGGGATC
AKR1C1, 2, 3	TGTGCTCAGGGGCGTTGC	GGTGCCAAATCCCAGGACAGG
AKR1C3	AGCTTTGGAGGTCACAAAATTAGCA	GCCAGTCCAACCTGCTCCTCAT
AKT3	GTTACCTTTCTACAACCAGGACCATG	GGTCCTCCACCAAGGCGTTTA
Ataxin1	GTGGCGGAGCCGTACAGGA	CATCAGGGAGCTTGGTGCTTGT
Ataxin2	GGGGCCGAAACGTGAAGAA	CCAGGGCTCCAGGTCCTTCTC
Ataxin3	TTGCGAAGCTGACCAACTCCTG	CCAGAGCCCTCTGCAAATCCT
Autotaxin	CCACTTTTCATCTGCGAGGGC	CCTTGCTTGGTGGCTGTAATCC
BLC-2	ACATCGCCCTGTGGATGACTG	GGCATGTTGACTTCACTTGTGGC
Catenin	GACGGAGGAAGGTCTGAGGAGC	CTTGAGTAGCCATTGTCCACGCT
CDC37	ATGATGAAGACGAGACGCACCC	CCGTCTTCTCGGGCTTGGTATT
cMYC	GGAGGCTATTCTGCCCATTTG	CGGCTGCACCGAGTCGTA
COX2	CTGGCAGGGTTGCTGGTGG	TTCATCTGCCTGCTCTGGTCAA
c-raf	CTTCTTTGACTATGCGTCGTATGC	GGGCTGAAGGTGAGGCTGATT
CYP11A1	CGCCCTGGGTCGCCTATC	GGGCCACCCGGTCTTTCTT
CYP17A1	GCCATTCCTCCCCAGACACG	GCGATACCCTTACGGTTGTTGGAC
CYP3A4, 5, 7	GAGAAATCTGAGGCGGGAAGCA	AAAGGGGTCTTGTGGATTGTTGAG
DAGK	CTCTGCGGATTGACCCTGTTCC	CACCCTCTGCCCCTGCTTCC
ETS1	CCCTCTGCGCCCTGGGTAA	GGATTCTGGATAGGCTGGGTTGA
EGFR	GAGCGACTGCCTGGTCTGCC	CCTCGGGGTTCACATCCATCTG
HPRT1	TGCAGAAGCTGCTTCCATGTCC	CCCCAGTCTTGGCTGATGACCT
HSD17B2	TGGAGGCTTCCTAACAAATATCGC	GCGGGGAGGTGGTCCAGAAT
HSD17B3	AAGTCTTTCTTGCGGTCAATGGG	CTCTGTGGCAATGGCCTCTAGTTT
HSD3B1, 2	CTCCAGAACAGGACCAAGCTGAC	GGCCCGGCTACCTCTATGCTA
IL6	AATTCGGTACATCCTCGACGGC	GCCAGTGCCTCTTTGCTGCTTT
IL6R	ATTAGCCTGTCCGCCTCTGC	GGGGAACATCCACCAGCAA
ILST	CAGATGAAGGTGGGAAGGATGGT	AGCAAACAGGCACGACTATGGC
KLF4	GCTCCCCAGCAGGACTACCC	CATCCACAGCCGTCCCAGT
K-ras	GTAGTTGGAGCTGGTGGCGTAG	CCTCATGTACTGGTCCCTCATTG
LDL r	GGAGACGTGCTTGTCTGTCACCTG	CGTCTTGGGGGGACAGCCTT
LIFR	CTTTTCGGATTCGTTGTTCTACTG	TCGCAAGTCATGTTGGGGTC
MAPK1, 3	CCTGAGCAATGACCATATCTGCTAC	ACGGGCCAGGCCAAAGTC
ABCC1	CCGACATGACCGAGGCTACAT	GCCAGGAATATGCCCCGAC
ABCC3	TTTAGCCAAGGCAGAGGGTGAG	CGCTGGTCTCAGGGTAGGGG
ABCC4	CGTGTACCAGGAGGTGAAGCC	CAAGGTGCTGTGAGCGGTCTT
MTOR	TTAGAGGACAGCGGGGAAGGC	GCAGGTCCGGTTCCAAGCATC
NAGON	ACCTATGCCTGTGATTTGTGGG	TGTTTCTTGACCGGGACCTTG
ENO2	CCATGCGACTAGGTGCAGAGGT	TTTCCGTGTAGCCAGCCTTGTC
OATP1B3	GTCCCGTTGTTCCCACCTCC	TGAATGACAGGGCTGCCAAGA
POU5F1	GGAGGAAGCTGACAACAATGAAA	CCCCTGAGAAAGGAGACCCA
PCA3	GAAGCACAGAGATCCCTGGGAGAA	AAGCGGGACCAGGCACAGG
PTGES	CCCAGTATTGCAGGAGCGACC	CCCAGGAAAAGGAAGGGGTAGA
PIK3R2	ACAGAGCGTGGGCTGGATG	ACTGCTCGGGCAAGTCGG
PIK3CA	GCAACCGAAAGAATGCGACG	GCAAAATGCAGCAACCTCCTCA
PLA2G1B	TGCTGGACAACCCGTACACCC	CAGCGTTGCGGTCGCAGTT
PLCG1	GGACGCAACCCTGGCTTCTAT	CATTCTGGATGATGGCGCTCTT

PLD1	GACGTGTCTCCCAGCGATCC	TGCTTGAATTTCCTCTTAACTTGCC
Prosaposin	CGCTATGTACGCCCTCTTCCT	CCCCTGGTGCATTCTTTCAGT
PSA	CGCTGGTTGGGTTCTGTGATG	TTGCTGGGACCTCGTGCTTC
SOX2	CCATCACCCACAGCAAATGAC	GCAAAGCTCCTACCGTACCACT
SPHK	ATGCACGAGGTGGTGAACGG	AGAGCCTGCTGGGAGGCTACA
Squalene epoxidase	GGCTCCCAGTTCGCCCTCT	CAAGCACGCCAGCTCCCAC
SCARB1	CGTGATGGTGCCGTCGCT	GAAGGTGATGTTGCTTTTGTGCC
SRD5A1	TGCTGGTGGTGGTTCATACGG	CAGACCAGAGCACATTGATGGC
SRD5A2	GTTTCCTTGGGCTGCGAGCTT	GGCAGAACGCCAGGAGACCTAC
STAT3	CTGGCTGACTGGAAGAGGCG	ACAATGGGGTCCCCTTTGTAGG
UGT2B17	CTGAGCTTCCTTATGTTTCACACATT	TCTGCTCAAAATGAAGCCAAAAT
WNK1	ACTCGGAGTGCCAAAATGCTGC	TTGTCAAAACTGGCTGGCTTCA
WNT1	CGGAATCGCCGCTGGAA	GGCGGAGGTGATAGCGAAGATA

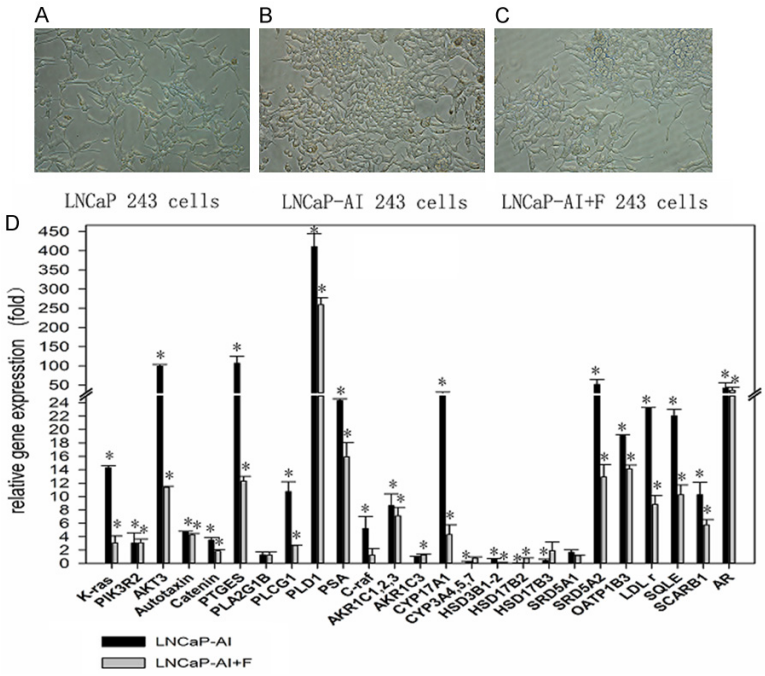

Figure 1. Morphology and gene expression of androgen-dependent and independent prostatic cancer cells. A. LNCaP 243 cells; B. LNCaP-AI 243 cells; C. LNCaP-AI+F 243 cells; D. Relative mRNA levels for genes coding for enzymes in the LPC, cholesterol and androgen metabolism pathways. The black and gray bars represent the relative expression levels in LNCap-AI 243 cells and LNCap-AI+F 243 cells, respectively, compared with the those in LNCap 243 cells; *indicates a statistically significant difference ($P < 0.05$) *vs* the control (LNCap 243 cells).

tems, Carlsbad, CA, USA). The PCR program was as follows: 1 cycle at 95°C for 5 min, followed by 45 cycles at 95°C for 5 sec, 60°C for 60 sec with a final extension at 72°C for 5 min. Real-time fluorescence monitoring and melting curve analysis were performed using the ABI 7500 system according to the manufacturer's recommendations. Negative controls containing no RNA template were included in each experiment. A melting curve was created at the end of the PCR cycle to confirm that a single product was amplified. Data were analyzed using ABI 7500 software version 2.0 to determine the threshold cycle (Ct) above the background for each reaction. The $2^{-\Delta\Delta CT}$ method was used to analyze the fold changes relative to the average value of the corresponding mRNA in LNCaP cells with HRPT as an internal control.

Statistical analysis

The experiments were repeated three times, data were expressed as mean ± standard derivation. The differences between the groups were analyzed by Student's *t*-test using SPSS 13.0 software (SPSS Inc, Chicago, IL, USA).

Results

Generation of LNCaP-AI and LNCaP-AI+F LNCaP cells

LNCaP cells (**Figure 1A**) were changed to LNCap-AI cells (**Figure 1B**) after gradually reducing androgen in the culture medium. The cells became rounder and flatter from spindle-shaped LNCaP cells and grew more consistently.

Table 2. Up- and down-regulated genes in androgen-independent cell lines LNCaP-AI and LNCaP-A+F as compared to androgen-responsive cells

		LNCaP-AI Cell lines			LNCaP-AI+F Cell lines		
Seq ID	Gene	Fold change	*P*-value	Regulation	Fold change	*P*-value	Regulation
NM_004985	K-ras	14.31 ± .22	0.0011	Up	3.54 ± 0.06	0.0173	Up
NM_005027	PI3KR2	3.53 ± 0.39	0.0305	Up	3.08 ± 0.40	0.0262	Up
NM_001206729	AKT3	99.35 ± 2.88	0.0000	Up	11.36 ± 0.12	0.0001	Up
NM_001040092	Autotaxin	4.75 ± 0.05	0.0169	Up	4.27 ± 0.13	0.0159	Up
NM_001098209	Catenin	3.49 ± 0.28	0.0011	Up	1.86 ± 0.12	0.0159	Up
NM_004878	PTGES	106.78 ± 13.15	0.0002	Up	12.30 ± 0.51	0.0002	Up
NM_000928	PLA2G1B	1.29 ± 0.29	0.2504	Up	1.23 ± 0.32	0.6571	Up
NM_002660	PLCG1	10.74 ± 1.03	0.0007	Up	2.66 ± 0.06	0.0067	Up
NM_001130081	PLD1	410.41 ± 23.53	0.0019	Up	259.32 ± 12.79	0.0032	Up
NM_001030047	PSA	24.31 ± 0.20	0.0002	Up	15.90 ± 1.51	0.0003	Up
NM_002880	C-raf	5.22 ± 1.28	0.0162	Up	1.25 ± 0.68	0.8511	Up
NM_001253909	AKR1C3	0.95 ± 0.09	0.4274	Down	1.25 ± 0.10	0.0305	Up
NM_000102	CYP17A1	28.59 ± 0.23	0.0032	Up	4.32 ± 1.03	0.0120	Up
NM_002153	HSD17B2	0.07 ± 0.01	0.0018	Down	0.79 ± 0.02	0.0010	Down
NM_000197	HSD17B3	0.41 ± 0.17	0.0464	Down	1.90 ± 0.91	0.1494	Up
NM_001047	SRD5A1	1.66 ± 0.26	0.1806	Up	1.15 ± 0.07	0.3932	Up
NM_000348	SRD5A2	51.50 ± 9.28	0.0013	Up	12.9 1 ± 1.33	0.0038	Up
NM_019844	OATP1B3	19.08 ± 0.10	0.0000	Up	14.34 ± 0.69	0.0001	Up
NM_000527	LDL-r	23.34 ± 0.31	0.0000	Up	8.78 ± 1.01	0.0011	Up
NM_003129	SQLE	22.03 ± 0.71	0.0002	Up	10.27 ± 1.03	0.0005	Up
NM_001082959	SCARB1	10.27 ± 1.30	0.0046	Up	5.75 ± 0.58	0.0005	Up
NM_000044	AR	39.18 ± 5.39	0.0004	Up	36.56 ± 5.64	0.0005	Up

By gradually decreasing androgen and increasing flutamide concentration, LNCaP cells were transformed into LNCaP-AI+F cells with morphology similar to LNCaP-AI cells (**Figure 1C**).

Gene expression

We analyzed the expression of genes involved in the LPC pathway, PI3K-AKT signaling pathway, cholesterol metabolism and androgen metabolism. The results are shown in **Table 2** and **Figure 1D**. Compared to LNCaP cells, the mRNA levels of phospholipase D1 (PLD1) were 259 and 410 times more in LNCaP-AI and LNCaP-AI+F cells, respectively. Furthermore, Kirsten rat sarcoma viral oncogene homolog (K-ras), phosphatidylinositol 3-kinase regulatory β subunit (PI3KR2), RAC-alpha serine/threonine-protein kinase (AKT) and prostate-specific antigen (PSA) in the PI3K/AKT signaling pathway were significantly up-regulated (**Table 2**). The mRNA levels of cholesterol metabolic enzymes and rate-limiting enzymes such as organic anion-transporting polypeptide 1B3 (OATP1B3), low density lipoprotein receptor (LDL-r), squalene epoxidase (SQLE), and scavenger receptor class B member 1 (SR-B1) were increased. The mRNA levels of androgen synthetases such as steroid 17-alpha-monooxygenase (CYP17A1), steroid 5 alpha-reductase 2 (SRD5A2), aldo-keto reductases (AKR1C1, AKR1C2, AKR1C3), and androgen receptor (AR) were also increased.

Discussion

Most prostate cancers are eventually shifted to be an androgen-independent after androgen-deprivation therapy, making hormone therapies ineffective. Despite many years of intense studies, the precise mechanisms underlying the shifting remain largely unknown. We cultured LNCaP cells under a simulated androgen-free condition without or with flutamide, and obtained LNCaP-AI cells and LNCaP-AI+F cells that could survive without androgen [15]. These cell lines were used to explore the mechanisms underlying the androgen-independence.

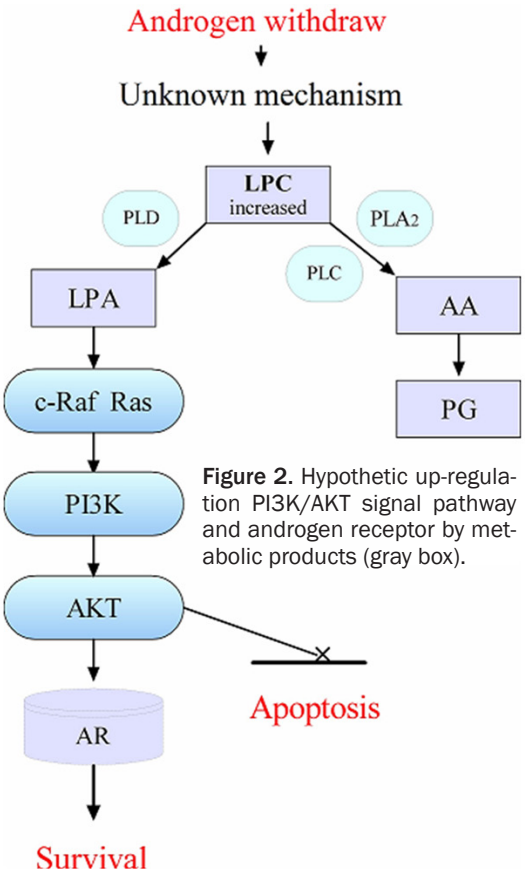

Figure 2. Hypothetic up-regulation PI3K/AKT signal pathway and androgen receptor by metabolic products (gray box).

In our previous study, we observed that the LPC level was remarkably increased in LNCaP-AI cells compared with LNCaP cells [16]. Studies show that LPC might be converted to AA by lipoprotein-associated phospholipase (Lp-PLA2) and then to prostaglandin (PGE) [17] or to LPA by PLD1 [18]. LPA is involved in a wide range of biological processes and can activate the downstream PI3K/AKT pathway, resulting in inhibition of apoptosis through the serine phosphorylation of the pro-apoptotic protein BAD [19]. Another group of AKT substrates named forkhead transcription factors (FKHRs) are translocated from nuclear to cytoplasm to bind with 14-3-3 proteins after being phosphorylated, thus decreasing apoptosis induced by the FasL process [19]. AKT also suppresses apoptosis and promotes the growth of cells via the phosphorylation of other factors such as AR [20]. To get a better picture of expression of genes that may be involved in the LPC-PI3K/AKT pathway, as well as in cholesterol and androgen metabolism, we compared the expression of 160 genes using qRT-PCR in LNCaP-AI cells, LNCaP-AI+F cells and LNCaP cells.

LNCaP-AI cells and LNCaP-AI+F are morphologically different from LNCaP cells, suggesting that there might be some metabolic changes between these cell lines. Compared with LNCaP cells, expression of a number of genes in the PI3K/AKT signaling pathway such as PLD1, K-ras, PI3KR2, AKT and PSA was increased significantly in LNCaP-AI cells and LNCaP-AI+F cells, indicating that the PI3K/AKT signaling pathway is activated probably due increased LPC level, a finding that was consistent with our hypothesis that the increased LPC may be converted to LPA by PLD1, and the resultant LPA is very bioactive and may activate Ras and the PI3K/AKT signaling pathway downstream of Ras (**Figure 2**), as observed in our study.

OATP1B3 is an organic anion transporting polypeptide and is shown to enhance the PCa cell's ability to uptake testosterone [21]. LDL-r is distributed on the surface of cell membrane, promoting the translocation of LDL into the cell. SQLE is a rate-limiting enzyme in sterol biosynthesis [22], and SCARB1 is a cholesterol polymerase [23]. All of these enzymes are associated with the transport and metabolism of cholesterol. Our data showed that the mRNA levels of the four genes were dramatically increased (**Table 2**), suggesting that cholesterol intake and metabolism in the androgen-independent cells may be enhanced in androgen-free environment. Because cholesterol is an important precursor for androgen synthesis, it may be used by the cancer cells to synthesize androgens de novo.

We next analyzed the expression of genes coding for enzymes involved in androgen metabolism and AR. The data reveals that CYP17A1, SRD5A2, AKR1C (AKR1C1, AKR1C2, AKR1C3), and AR were significantly up-regulated (**Figure 1D**). SRD5A1 and SRD5A2 could convert testosterone to DHT [24]. Up-regulation of expression of these genes may increase the intracellular secretion of androgen. Furthermore, because these enzymes are the pivotal enzymes in the classical pathway of androgen biosynthesis [25-29], the results suggest that the classical pathway of androgen biosynthesis may be involved in androgen synthesis under the steroid-deprived condition. However, besides the significant increase in the expression of CYP17A1, SRD5A2, and AKR1C, the expression of hydroxy-delta-5-steroid dehydrogenase (HSD)

3B (HSD3B1, HSD3B2) and HSD17B2 was decreased. The reasons for this down-regulation remain unknown.

Based on the above analysis, we speculate that increased androgen synthesis in LNCaP-AI cells and LNCaP-AI+F cells may be achieved via increased cholesterol intake even if androgen is deprived. PSA is an androgen-regulated gene and is expected to be upregulated in response to increased testosterone levels in LNCaP-AI cells. Our observations that LNCaP-AI had elevated PSA might explain why in two clinical studies patients with relapsed prostate tumors were observed to have elevated PSA after the termination of androgen ablation therapy [30, 31].

Multidrug resistance protein 1 (MRP1) participates in the translocation of chemotherapy drugs, including flutamide, for PCa treatment. Compared with LNCaP cells, there was no difference in expression of MRP1 mRNA either in LNCaP-AI cells or LNCaP-AI+F cells, indicating that the survival of LNCaP-AI+F cells might be independent of flutamide after the cells are cultured in androgen-free medium for long time and is more likely relied on self-synthesized androgen as LNCaP-AI cells do.

In conclusion, LNCaP-AI or LNCaP-AI+F can survive in medium without supplemented androgen irrespective of flutamide. This is achieved either by enhancing the PI3K/Akt signaling pathway by LPC, which inhibits apoptosis and promotes cell survival, or by increasing the androgen synthesis via classical pathways or a "back-door" pathway, simultaneously, which further accelerates the conversation of T into DHT and upregulates the expression of AR to provide androgen for cell survival.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhihua Tao, Department of Laboratory Medicine, The Second Affiliated Hospital of Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou 310009, Zhejiang, PR China. Tel: +86-0571-87783457; Fax: +86-0571-87783777; E-mail: zetaozhihua22@163.com

References

[1] Huggins C and Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. CA Cancer J Clin 1972; 22: 232-240.

[2] Mizokami A, Koh E, Fujita H, Maeda Y, Egawa M, Koshida K, Honma S, Keller ET and Namiki M. The adrenal androgen androstenediol is present in prostate cancer tissue after androgen deprivation therapy and activates mutated androgen receptor. Cancer Res 2004; 64: 765-771.

[3] Steketee K, Timmerman L, Ziel-van der Made AC, Doesburg P, Brinkmann AO and Trapman J. Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer. Int J Cancer 2002; 100: 309-317.

[4] Masiello D, Chen SY, Xu Y, Verhoeven MC, Choi E, Hollenberg AN and Balk SP. Recruitment of beta-catenin by wild-type or mutant androgen receptors correlates with ligand-stimulated growth of prostate cancer cells. Mol Endocrinol 2004; 18: 2388-2401.

[5] Song LN, Coghlan M and Gelmann EP. Antiandrogen effects of mifepristone on coactivator and corepressor interactions with the androgen receptor. Mol Endocrinol 2004; 18: 70-85.

[6] Edwards J, Krishna NS, Grigor KM and Bartlett JM. Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. Br J Cancer 2003; 89: 552-556.

[7] Wang Q, Udayakumar TS, Vasaitis TS, Brodie AM and Fondell JD. Mechanistic relationship between androgen receptor polyglutamine tract truncation and androgen-dependent transcriptional hyperactivity in prostate cancer cells. J Biol Chem 2004; 279: 17319-17328.

[8] Lin HK, Hu YC, Lee DK and Chang C. Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells. Mol Endocrinol 2004; 18: 2409-2423.

[9] Ngo DC, Ververis K, Tortorella SM and Karagiannis TC. Introduction to the molecular basis of

cancer metabolism and the Warburg effect. Mol Biol Rep 2015; 42: 819-823.
[10] Darnell JE Jr. STAT3, HIF-1, glucose addiction and Warburg effect. Aging (Albany NY) 2010; 2: 890-891.
[11] Wei H, Guo L, Li L, Zhou Q and Wu Z. [Mechanism of Warburg effect and its effect on tumor metastasis]. Zhongguo Fei Ai Za Zhi 2015; 18: 179-183.
[12] Lu J, Tan M and Cai Q. The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism. Cancer Lett 2015; 356: 156-164.
[13] Vaz CV, Alves MG, Marques R, Moreira PI, Oliveira PF, Maia CJ and Socorro S. Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile. Int J Biochem Cell Biol 2012; 44: 2077-2084.
[14] Asgari Y, Zabihinpour Z, Salehzadeh-Yazdi A, Schreiber F and Masoudi-Nejad A. Alterations in cancer cell metabolism: the Warburg effect and metabolic adaptation. Genomics 2015; 105: 275-281.
[15] Xu G, Mao Y, Chen B, He J, Tao Z, Lin X, Chen Z and Shen M. [Establishment of an Androgen-independent Prostate Cancer Cell Line Induced by the Combined of Flutamide and Androgen-free Environment]. Chinese Journal of Cell Biology 2009; 31: 373-378.
[16] Lu Y, Zhu X, Chen Y, Guo F, Shen M, Chen Z, Xu W, Chen D and Tao Z. [Metabolomic Profiles in Prostate Cancer Cells Progression]. Chinese Journal of Analytical Chemistry 2011; 39: 305-311.
[17] Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 2001; 294: 1871-1875.
[18] Aoki J, Taira A, Takanezawa Y, Kishi Y, Hama K, Kishimoto T, Mizuno K, Saku K, Taguchi R and Arai H. Serum lysophosphatidic acid is produced through diverse phospholipase pathways. J Biol Chem 2002; 277: 48737-48744.
[19] Datta SR, Brunet A and Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999; 13: 2905-2927.
[20] Ghosh PM, Malik S, Bedolla R and Kreisberg JI. Akt in prostate cancer: possible role in androgen-independence. Curr Drug Metab 2003; 4: 487-496.
[21] Yang M, Xie W, Mostaghel E, Nakabayashi M, Werner L, Sun T, Pomerantz M, Freedman M, Ross R, Regan M, Sharifi N, Figg WD, Balk S, Brown M, Taplin ME, Oh WK, Lee GS and Kantoff PW. SLCO2B1 and SLCO1B3 may determine time to progression for patients receiving androgen deprivation therapy for prostate cancer. J Clin Oncol 2011; 29: 2565-2573.
[22] Stevenson J, Luu W, Kristiana I and Brown AJ. Squalene mono-oxygenase, a key enzyme in cholesterol synthesis, is stabilized by unsaturated fatty acids. Biochem J 2014; 461: 435-442.
[23] Shen WJ, Hu J, Hu Z, Kraemer FB and Azhar S. Scavenger receptor class B type I (SR-BI): a versatile receptor with multiple functions and actions. Metabolism 2014; 63: 875-886.
[24] Tria A, Hiort O and Sinnecker GH. Steroid 5alpha-reductase 1 polymorphisms and testosterone/dihydrotestosterone ratio in male patients with hypospadias. Horm Res 2004; 61: 180-183.
[25] Penning TM and Byrns MC. Steroid hormone transforming aldo-keto reductases and cancer. Ann N Y Acad Sci 2009; 1155: 33-42.
[26] Mohler JL, Gregory CW, Ford OH, 3rd, Kim D, Weaver CM, Petrusz P, Wilson EM and French FS. The androgen axis in recurrent prostate cancer. Clin Cancer Res 2004; 10: 440-448.
[27] Auchus RJ and Miller WL. Molecular modeling of human P450c17 (17 alpha-hydroxylase/17, 20-lyase): insights into reaction mechanisms and effects of mutations. Mol Endocrinol 1999; 13: 1169-1182.
[28] Labrie F, Luu-The V, Lin SX, Simard J, Labrie C, El-Alfy M, Pelletier G and Belanger A. Intracrinology: role of the family of 17 beta-hydroxysteroid dehydrogenases in human physiology and disease. J Mol Endocrinol 2000; 25: 1-16.
[29] Koh E, Noda T, Kanaya J and Namiki M. Differential expression of 17beta-hydroxysteroid dehydrogenase isozyme genes in prostate cancer and noncancer tissues. Prostate 2002; 53: 154-159.
[30] Mathew P. Prolonged control of progressive castration-resistant metastatic prostate cancer with testosterone replacement therapy: the case for a prospective trial. Ann Oncol 2008; 19: 395-396.
[31] Szmulewitz R, Mohile S, Posadas E, Kunnavakkam R, Karrison T, Manchen E and Stadler WM. A randomized phase 1 study of testosterone replacement for patients with low-risk castration-resistant prostate cancer. Eur Urol 2009; 56: 97-103.