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·*Original Article*·

# Change of the cell cycle after flutamide treatment in prostate cancer cells and its molecular mechanism

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**Abstract**

**Aim:** To explore the effect of androgen receptor (AR) on the expression of the cell cycle-related genes, such as *CDKN1A* and *BTG1*, in prostate cancer cell line LNCaP. **Methods:** After AR antagonist flutamide treatment and confirmation of its effect by phase contrast microscope and flow cytometry, the differential expression of the cell cycle-related genes was analyzed by a cDNA microarray. The flutamide treated cells were set as the experimental group and the LNCaP cells as the control. We labeled cDNA probes of the experimental group and control group with Cy5 and Cy3 dyes, respectively, through reverse transcription. Then we hybridized the cDNA probes with cDNA microarrays, which contained 8 126 unique human cDNA sequences and the chip was scanned to get the fluorescent values of Cy5 and Cy3 on each spot. After primary analysis, reverse transcription polymerase chain reaction (RT-PCR) tests were carried out to confirm the results of the chips. **Results:** After AR antagonist flutamide treatment, three hundred and twenty-six genes (3.93 %) expressed differentially, 97 down-regulated and 219 up-regulated. Among them, eight up-regulated genes might be cell cycle-related, namely *CDC10*, *NRAS*, *BTG1*, *Wee1*, *CLK3*, *DKFZP564A122*, *CDKN1A* and *BTG2*. The *CDKN1A* and *BTG1* gene mRNA expression was confirmed to be higher in the experimental group by RT-PCR, while *p53* mRNA expression had no significant changes. **Conclusion:** Flutamide treatment might up-regulate *CDKN1A* and *BTG1* expression in prostate cancer cells. The protein expressions of *CDKN1A* and *BTG1* play an important role in inhibiting the proliferation of cancer cells. *CDKN1A* has a great impact on the cell cycle of prostate cancer cells and may play a role in the cancer cells in a *p53*-independent pathway. The prostate cancer cells might affect the cell cycle-related genes by activating AR and thus break the cell cycle control. *(Asian J Androl 2005 Dec; 7: 375–380)*

**Keywords:** prostate cancer; LNCaP; *p21*; androgen receptor; *CDKN1A; BTG1;* cell cycle genes; flutamide

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## 1 Introduction

Prostate cancer is a worldwide disease. The incidence of prostate cancer in Europe was 103.5 cases per 100 000 men in the year 2000 [1]. In USA, the inci-

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dence of this disease increased steadily between 1981 and 1989, with a steep increase in the early 1990s. In 1996, 317 000 new cases of prostate cancer have been detected, while 41 400 patients died of this disease in the USA [2]. In China, some well-developed areas such as Shanghai also have seen a dramatic increase in the incidence of prostate cancer due to changes of lifestyles and dietary patterns [3].

Clinical therapy of prostate cancer involves radical prostatectomy followed by adjuvant hormonotherapy or chemotherapy. Administration of antiandrogens provides only partial remission because prostate cancer cells acquire a hormone-independent phenotype and the disease relapses within a few years. Therefore, the development of androgen independency of prostate cancer is the main cause of therapeutic failure. Nevertheless, the underlying molecular mechanisms involved in this situation were not clearly understood [4].

In this study, we used the well-characterized, hormone-sensitive human prostate cancer cell line LNCaP to identify early mechanisms involved in the acquisition of hormone independence.

## 2 Materials and methods

### *2.1 Materials*

LNCaP cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) (Hyclone Inc., Savannah, USA)[5]. Stock solutions of the androgen receptor (AR) antagonist flutamide (Sigma Inc., St. Louis, USA) ($10^{-4}$ mol/L) were made in absolute ethanol, while working solutions were further made in phosphate-buffered saline (PBS) (pH 7.2) [6]. Flutamide was routinely used at a final concentration of $10^{-7}$ mol/L. In the control (non-treated) group, an equal volume of pure ethanol, dissolved in PBS was used to eliminate any effect of the vehicle. After 15 days of treatment, with a change of the medium every 2 days, when the experimental group cells reached 90 % confluence, we isolated mRNA and analyzed it after reverse transcription. Cy3-dCTP and Cy5-dCTP were purchased from Amersham Phamacia Biotech Inc. (Piscataway, NJ, USA) and Oligotex mRNA Midi Kit from Quigen Inc. (Valencia, CA, USA). ScanArray 4 000 laser scanner was from GSI Lomonics (Ottawa, Ontario, Canada). GenePix Pro 3.0 software from Axon Instruments Inc. (Sunnyvale, CA, USA).

### *2.2 Methods*

#### *2.2.1 Analysis of cell cycle changes*

The control cells and cells that had been treated with flutamide for 15 days were analyzed by microscopy, using a microscope (Olympus, Tokyo, Japan) provided with a camera (Olympus, Tokyo, Japan). Additionally, cells were collected and placed into 6-well plates at a density of $1 \times 10^5$/mL, washed with 0.01 mol/L PBS (pH 7.2), fixed in 70 % ethanol for 18 h, resuspended in PBS and stained with propidium iodide (100 μg/mL) for 30 min. Flow cytometer was explored by using blue light Argon-Ion laser (excitation wavelength, 488 nm; laser power, 200 mW; ELITE ESP, Beckman-Coulter, Fullerton, CA, USA) and red fluorescence from the PI/DNA was recorded. Cell cycle analysis was performed using a DNA-Prep Reagent System, with the following settings: one cycle analysis, no apoptosis.

#### *2.2.2 Microarray assay*

mRNA was extracted by Trizol and purified by Oligotex Midi Kit (Quigen Inc., Valencia, CA, USA)[7]. Microarray analysis was performed by using the Human Gene Expression CHIP (version H80s, Biostar, Shanghai, China) containing 8 126 human genes. A fluorescent probe was synthesized by reverse transcription of 100 μg of the above mRNA with 50 U AMV reverse transcriptase (Takara Shuzo, Kyoto, Japan) in the presence of Cy3- or Cy5-dCTP (Amersham, Arlington Heights, USA). Then Cy3- and Cy5-labeled probes were prepared and incubated in the cDNA chip at 42 ºC for 6 h, washed twice with 2 × Standard Saline Citrate(SSC)/0.2 % SDS at 60 ºC for 30 min and then washed again with the same buffer for 5 min. Finally, the chip was washed with 0.05 × SSC at room temperature for 10 min and signals were quantified with the ScanArray 4000 (GSI Lomonics, Ottawa, Canada) and the Quant Array Software (GSI Lomonics, Ottawa, Canada). All the Cy3 fluorescent units were normalized according to the normalized factor [6] and Cy5 fluorescent intensity was counted as 200 if it was below 200 fluorescent units. The expression changes of genes were considered as up-regulated if the Cy5/Cy3 signal ratio was higher than 2.0 and down-regulated if the ratio was lower than 0.5.

#### *2.2.3 Semi-quantitative RT-PCR*

Total RNA was extracted by the Qiagen RNA Isolation Kit (GIBCO Co., New York, USA). For the first-strand cDNA synthesis, 5 mg/mL of RNA (Takara Co.,

Dalian, China) were used. In each reaction, a 100-mL solution containing 3 mmol random hexamers, 25 mmol/L Tris-HCl, 37 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 10 mmol/L DTT, 0.25 mmol/L dNTP, 40 units of RNasin, a RNase inhibitor, 50 U /mL Super Taq DNA polymerase, and 200 units of reverse transcriptase were used. The annealing mixture was incubated at room temperature for 15 min, and then incubated in a water bath at 41 ºC for 60 min. The reverse transcriptase enzyme was inactivated by heating the solution to 95 ºC for 5 min. PCR was then carried out using PCR kit (Perkin-Elmer, Foster City, CA, USA) and primers. The PCR was performed for 30 cycles consisting of denaturation at 94 ºC for 1 min, annealing at 57 ºC for 1 min, and extension at 72 ºC for 2 min. The PCR products were analyzed on 1.5 % agarose gel. The primers used for PCR were as follow: *CDKN1A* sense (5'-GAC ACC ACT GGA GGG TGA CT-3'), *CDKN1A* antisense (5'-TAC AGG TCC ACA TGG TCT TCC-3'); *β-actin* sense (5'-GAT TGC CTC AGG ACA TTT CTG-3'), *β-actin* antisense (5'-GAT TGC TCA GGA CAT TTC TG-3') [8]. And another experiment of *KLK3* (its gene product is *PSA*) expression was also carried out, with the primers set as follow: *KLK3* sense (5'-AGC GTG ATC TTG CTG GGT CG-3'), *KLK3* antisense (5'-CGT CAT TGG AAA TAA CAT GGA GG-3'). Gene primers were synthesized by Beijing Oake Company (Beijing, China).

## 3 Results

### *3.1 Morphological and cell cycle changes induced by flutamide*

Flutamide treatment of LNCaP cells for 15 days resulted in dramatic changes of cell morphology (Figures 1, 2). Antiandrogen-treated cells became smaller, while less mitoses and cell contacts were found. The density of cells also diminished. The results of flow cytometer indicated that (71.47 ± 0.96) % of LNCaP cells sustained at $G_1$ phase after the flutamide treatment while that of control cells was only (66.87 ± 1.50) % (Table 1).

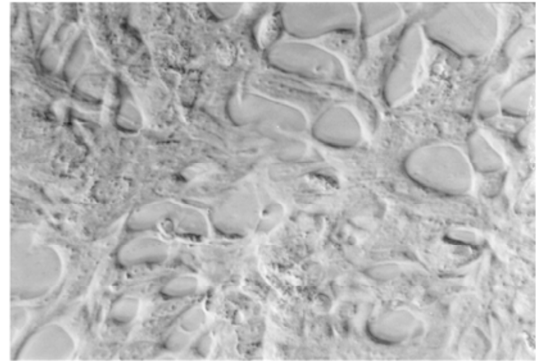

Figure 1. Control group cells under an ordinary phase-contrast microscope without any interference, which had the sense of satiety. Magnification: ×100.

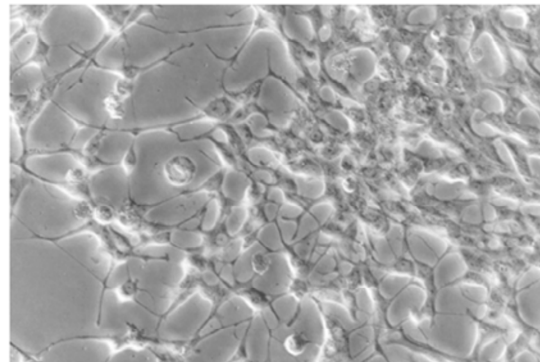

Figure 2. After 15 d of flutamide treatment, cells were not in good condition and few mitoses could be seen in these 'thin' cells. Magnification: ×100.

Table 1. The effect of flutamide on the LNCaP cells checked by flow cytometry. Comparing with the negative control group, more experimental group cells were in $G_1$ phase, less in S phase, indicating the inhibitory effect of flutamide on the LNCaP cells ($P < 0.01$). Statistics by SPLM, Kruskal–Wallis method, $P = 0.0000$, the difference of $G_1$ phase cells between the groups is evident, $^bP < 0.01$.

| Percentage of cells (%) | $G_1$ | $G_2$ | S |
|---|---|---|---|
| Control group | 66.87 ± 1.50 | 8.17 ± 0.23 | 24.96 ± 1.30 |
| Experimental group | 71.47 ± 0.96$^b$ | 6.43 ± 0.96 | 24.03 ± 2.12 |

### *3.2 The changes of gene expression after flutamide treatment*

Detection of RNA purity was assayed by electrophoresis in agarose gels, and absorption spectrometry. As shown in Figure 3, 18S and 28S bands were clean and clear, while absorbance measurements revealed A260/A280 >2.0, indicating that the extracted mRNA was suitable for cDNA microarray assay.

According to the criteria offered by the chip manufacturer (Cy5/Cy3$^*$>2.0, up-regulated; Cy5/Cy3$^*$<0.5, down-regulated), there were 326 genes (3.93 %) which were expressed differentially after the treatment with flutamide. Ninety-seven genes were up-regulated and 219 were down-regulated (Figure 4). It was very inter-

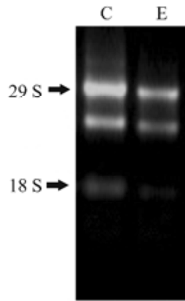


Figure 3. The 18S and 28S bands of both experimental group (E) and control group (C) were clear, which mean the RNA extraction were suitable for cDNA microarray test.

esting that there was an increased expression of eight genes related to cell cycle, namely *CDC10*, *NRAS*, *BTG1*, *Wee1*, *CLK3*, *DKFZP564A122*, *CDKN1A* and *BTG2* (Table 2). Especially *CDKN1A* and *BTG1* who have relatively high Cy3 value. Meanwhile, *p53* and its related genes did not change much after flutamide treatment (Table 3).

The induction of *PSA* mRNA may reflect the ability of AR to stimulate transcription, for the 5'-regulatory region of the *PSA* gene contains multiple androgen response elements [5]. *PSA* was obviously down-regulated (Cy5/Cy3=0.202) in our chip assay, while AR did not change (Cy5/Cy3=0.65).

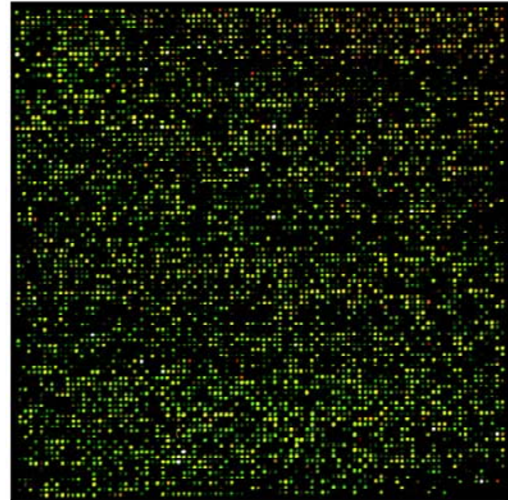

Figure 4. The results of cDNA microarray: the Cy5 red points indicated this gene were up-regulated in this study, Cy3 blue color points indicated down-regulated and yellow ones indicated no change in this hybridization study by cDNA microarray.

Table 2. Cell cycle-related genes product in LNCaP cells after flutamide treatment. After the normalization performed under the help of the 40 housekeeping genes in the chip, Cy3 is turned to Cy3*. Ratio is Cy5/Cy3*. Ratio >2.0 is counted as up-regulated, <0.5 as down-regulated.

| Cy5 | Cy3 | Cy3* | Ratio | Gene explanation |
|---|---|---|---|---|
| 6765 | 3511 | 3341.3 | 2.025 | Homosapien's *CDC10* (cell division cycle 10, S. cerevisiae, homolog) (*CDC10*), mRNA |
| 2418 | 1239 | 1179.1 | 2.051 | Topoisomerase I binding, arginine/serine-rich (*TOPORS*, Gene alias: *TP53BPL*), mRNA |
| 48467 | 24341 | 23164.6 | 2.092 | Homosapien's B-cell translocation gene 1, anti-proliferative (*BTG1*), mRNA |
| 8089 | 3785 | 3602.1 | 2.246 | Homosapien's *Wee1* gene |
| 8700 | 3917 | 3727.7 | 2.334 | Homosapien's *CDC*-like kinase 3 (*CLK3*), transcript variant phclk3, mRNA |
| 4803 | 1520 | 1446.5 | 3.320 | Homosapien's DKFZP564A122 protein (*DKFZP564A122*), mRNA |
| 14296 | 4137 | 3937.1 | 3.631 | Homosapien's cyclin-dependent kinase inhibitor 1A (*p21waf1/cip1*) (*CDKN1A*), mRNA |
| 5657 | 1152 | 1096.3 | 5.160 | Homosapien's *BTG* family, member 2 (*BTG2*), mRNA |

Table 3. p53 and its related genes product in LNCaP cells after flutamide treatment. We found that p53 and its related genes did not change after flutamide treatment. So we postulated that p53 along with all its related genes do not undergo flutamide-induced growth inhibition.

| Cy5 | Cy3 | Cy3* | Ratio | Gene explanation |
|---|---|---|---|---|
| 15796 | 16607 | 15804.4 | 0.999 | Homosapien's tumor protein p53 (Li-Fraumeni syndrome) (*TP53*), mRNA |
| 4849 | 5366 | 5106.7 | 0.950 | Topoisomerase I binding, arginine/serine-rich (*TOPORS*, Gene alias: *TP53BPL*), mRNA |
| 6495 | 5624 | 5352.1 | 1.214 | Homosapien's tumor protein p53-binding protein, 1 (*TP53BP1*), mRNA |
| 4137 | 5875 | 5591.1 | 0.740 | Homosapien's *p53* regulated PA26 nuclear protein (*PA26*), mRNA |
| 7384 | 5031 | 4787.9 | 1.542 | Homosapien's tumor protein p53-binding protein, 2 (*TP53BP2*), mRNA |
| 32517 | 35476 | 33761.4 | 0.963 | Homosapien's *p53*-inducible p53DINP1 (*p53DINP1*), mRNA |

*3.3 Semi-quantitative RT-PCR confirmation*

In the control (non-treated) group, the *CDKN1A* expression in the LNCaP cells was merely faintly detected by RT-PCR. In contrast, in flutamide-treated LNCaP cells, the expression of this gene product was significantly up-regulated (Figure 5). In contrast, *KLK3* (PSA) expression was down-regulated (as expected) after flutamide, indicating an effective blockage of AR by chronic flutamide treatment (Figure 6).

## 4 Discussion

AR plays a great role in regulating the proliferation of both normal and neoplastic prostate cells. The activated DNA-bound AR homodimer complex recruits several kinds of co-regulatory proteins to stimulate or inhibit target gene transcription, thus promotes or represses cell proliferation, apoptosis or angiogenesis [9].

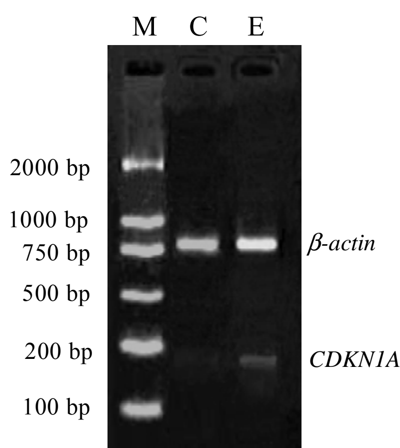


Figure 5. The effect of flutamide on the *CDKN1A* expression on the 15th day. M: DNA marker; C: control group; E: experimental group. The 173 bp band is *CDKN1A*, and the 836 bp band is *β-actin*.

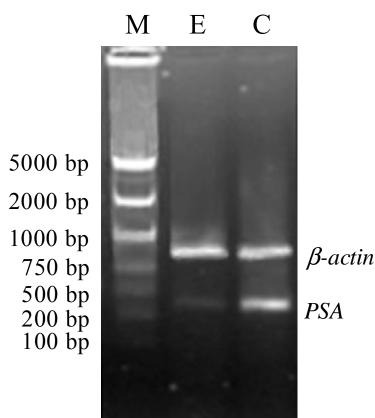


Figure 6. The effect of flutamide on the *KLK3* expression on the 15th day. M: DNA marker; C: control group; E: experimental group. The 337 bp band is *KLK3*, and the 836 bp band is *β-actin*.

In this study, we found that eight cell cycle-related genes, namely *CDC10*, *NRAS*, *BTG1*, *Wee1*, *CLK3*, *DKFZP564A122*, *SLC31A1* and *CDKN1A*, were up-regulated in prostate cancer cells after flutamide treatment. In contrast, *p53* and its downstream genes, such as tumor protein p53-binding protein *TOPORS* (Gene alias: *TP53BPL*), *TP53BP1*, *PA2*, *TP53BP2* and *p53DINP1*, remain unchanged.

*CDKN1A* is an important cell cycle regulator [10]. Its gene product *p21* (*waf1/cip1*) can inhibit the proliferation of cancer cells via both *p53*-dependent and *p53*-independent CDK inhibition. In many tumors, *CDKN1A* could be activated through the *p53*-indpendent pathway [11, 12], in which mitogens as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) may function [13]. Choi *et al.* [12] reported that in prostate cancer cell line PC-3, the *p53*-independent pathway to activate *CDKN1A* may be related with over-expression of *CDC2* and *CDK2*. Our results showed that there was no significant change of *p53* gene and its downstream genes after flutamide treatment; we assumed that the high expression of *CDKN1A* may occur through a *p53*-independent pathway in this experiment. Kokontis *et al.* [14] found that androgen may inhibit androgen refractory prostate cancer cell line LNCaP-104R1 proliferation by a transient *p21* (*waf1/cip1*) induction and following *p27* (*Kip1*) induction as a result of a drop in *c-myc* expression. However, in our research, we found neither *p27* (*Kip1*) nor *c-myc's* expression changed (data not shown), suggesting a different pathway in androgen prolific cell line. Since there have been reports that *CDKN1A* expression was associated with tumor progression to androgen-independent prostate carcinoma [4], we supposed that *p21* may play a key role in molecular events of the initiation of *AIPC* because of its anti-apoptotic effect.

Eder *et al.* [15] found in 2003 that after AR blockage by antisense oligonucleotide, the expression of *IGFBP2*, *PIP5KIA*, *PTOV1* and *S100P* changed. However, in this study, we did not find the same kind of results.

The other genes we discovered in this experiment were also of interest. *BTG1*, for example, may play a

coordinate role in a general transduction pathway that was induced in response to DNA damage [16]. *BTG1* expression was maximal in the $G_0/G_1$ phases of the cell cycle and was down-regulated when cells progress throughout $G_1$ [17]. It affected the proliferation by phosphorylating a putative p34cdc2 kinase site on BTG1, Ser-159, thus modulated CCR4 expression, and then induced the formation of *hCAF-1/BTG1*, which was of great consequence in the signaling events of cell division that lead to changes in cellular proliferation associated with cell-cell contact [18]. However, a shortage of correlated reports hinder further research of this gene on the prostate cancer cells' proliferation and cell contact.

In conclusion, we found that eight cell cycle-related genes may be involved in the process of flutamide-induced cell growth inhibition, especially *CDKN1A* and *BTG1*. *CDKN1A* may function on the prostate cancer cells in a *p53*-independent mode. The clarification of exact mechanism of flutamide's inhibitive effect on prostate cancer cells is of great importance in clinical hormone therapy. Designing a new way with biotechnology to mimic and maximize flutamide's anti-cancer effect while avoiding its notorious hepatotoxicity may be an interesting idea to pursue in the future.

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