RESEARCH ARTICLE

Gene Microarray Assessment of Multiple Genes and Signal Pathways Involved in Androgen-dependent Prostate Cancer Becoming Androgen Independent

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Abstract

To study the gene expression change and possible signal pathway during androgen-dependent prostate cancer (ADPC) becoming androgen-independent prostate cancer (AIPC), an LNCaP cell model of AIPC was established using flutamide in combination with androgen-free environment inducement, and differential expression genes were screened by microarray. Then the biological process, molecular function and KEGG pathway of differential expression genes are analyzed by Molecule Annotation System (MAS). By comparison of 12,207 expression genes, 347 expression genes were acquired, of which 156 were up-ragulated and 191 down-regulated. After analyzing the biological process and molecule function of differential expression genes, these genes are found to play crucial roles in cell proliferation, differntiation, cell cycle control, protein metabolism and modification and other biological process, serve as signal molecules, enzymes, peptide hormones, cytokines, cytoskeletal proteins and adhesion molecules. The analysis of KEGG show that the relevant genes of AIPC transformation participate in glutathione metabolism, cell cycle, P53 signal pathway, cytochrome P450 metabolism, Hedgehog signal pathway, MAPK signal pathway, adipocytokines signal pathway, PPAR signal pathway, TGF-β signal pathway and JAK-STAT signal pathway. In conclusion, during the process of ADPC becoming AIPC, it is not only one specific gene or pathway, but multiple genes and pathways that change. The findings above lay the foundation for study of AIPC mechanism and development of AIPC targeting drugs.

Keywords: androgen receptor - androgen independence - prostate cancer - gene expression - microarray - signal pathway

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Introduction

Prostate cance is one of the most common malignant tumors of men. The mortality has reached the second highest position among all male cancers (Jemal et al., 2009). Incidence of prostate cancer in Asia is far lower than in the West, a wide study has been done by investigators to find the tumor-resulting factors, such as environment factors, geographic factors and cytokines, and find the early screening biomarkers; and the overall prevalence is apparently increasing in recent years (Hu et al., 2013; Wang et al., 2013). Althouth the morbidity of prostate cancer in China is far lower than that of western countries, the morbidity in recent years is markedly increasing. Duration and spread of prostate cancer depend on signal transduction of androgen receptor. Therefore, the androgen deprivation therapy is the third standard therapies of prostate cancer except surgery and radiotherapy (Sooriakumaran et al., 2014). After remittent stage of median 18-24 months, androgen-dependent prostate cancer (ADPC) is becoming androgen-independent prostate cancer (AIPC). This indicates the next proliferation stage of prostate cancer cells after remittent stage, e.g. prostate specific antigen (PSA) continually increase, and bone metastasis is extremely easy to occur. And once the bone metastasis occurs, the treatment will be intractable and the mortality increases. Therefore, prostate cancer has become the serious disease to threaten the health of elderly men (Mukherji et al., 2013; Dreicer, 2014; Heidenreich et al., 2014; Zhang and Zhang, 2014).

AIPC transformation mechanism is the hotspot in the research field of prostate cancer. The contents mainly include the amplification, over-expression and mutation of androgen receptor (AR) gene, the abnormity of AR signal pathway (Kahn et al., 2014; Nemes et al., 2014), the alternative pathway of androgen and the abnormity of pro-apoptotic regulatory genes (Shi et al., 2014; Xu et al., 2014). Due to that the biological behavior of prostate cancer is extremely complex, any theory above can not clarify the pathogenetic mechanism of AIPC. The pathogenesis of AIPC needs further in-depth study. The growth of prostate cells depends on the biological characteristics of androgen. Many patients of prastate

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cancer gain a markedly curative effect after undergoing the first androgen deprivation therapy. While almost all of the patients will relapse and their androgen-dependent prostate cancer will transform into highly deteriorated androgen-independent prostate cancer with widespread metastasis. In this case, the conventional androgen deprivation therapy has no longer effect. Once ADPC transform into AIPC, cancer cells are uncontrollable, resulting in the death of prastate cancer patient. The primary reasons for ADPC becoming AIPC are gene expression variation, signal pathway abnormity, and disregulation of proto-oncogene, cancer suppressor genes and growth factors. So far, researchers have discovered a large number of relevant genes and signal pathways of prostate cancer (Cariaga-Martinez et al., 2013; He et al., 2013; Nyquist et al., 2013; Carstens et al., 2014). The tumorigenesis and development of prostate cancer are very complex to involve multi-steps and poly-genes. Therefore, the information from separately single study is very limited and incomplete. The study of AIPC formation mechanism is still a great challenge.

The present study adopts the method of microarray, cytobiology, molecular biology and bioinfromatics to explore the gene expression change of human prostate cancer, and reveal the possible pathogenesis mechanism during ADPC change to AIPC. LNCaP human prostate cancer cells maintain the differentiation feature of human prostate cancer and have extremely strong dependence on androgen, so the present study establish the LNCaP cell model to simulate the process of clinic prostate cancer change from androgen-dependent to androgen-independent.

Materials and Methods

Materials

RPMI-1640 and fetal bovine serum (FBS) were purchased from ExCell Biology Company (Shanghai, China). TRIzol was purchased from Invitrogen Company (Shanghai, China). LNCaP human prostate cancer cells were purchased from Nanjing Kai Ji Biotechnology Development Co. Ltd. (Nanjing, Jiangsu, China). LNCaP human prostate cancer cells were cultured in phenol red-free RPMI 1640 media containing 10% FBS under the conditions of 37°C and 5% CO_2.

Culture and induction of AIPC LNCaP-A or LNCaP-A+F cells

For induction of LNCaP-A cells, LNCaP cells were subcultured in androgen-free RPMI-1640 medium containing 10% dextran/charcoal-stripped FBS by maintaining saturated humidity, 37°C and 5% CO_2. For induction of AIPC LNCaP-A+F cells, LNCaP cells were cultured in androgen-free RPMI-1640 medium containing dextran/charcoal-stripped FBS and flutamide. The first flutamide concentration was 1.0×10^{-7} mol/L. After passaged 20 generations, the concentration of flutamide was elevated up to 5.0×10^{-6} mol/L.

Proliferation and invasion ability of LNCaP-A and LNCaP-A+F cells

To detect cell proliferation, LNCaP, LNCaP-A and LNCaP-A+F cells were digested by trypsin, suspended and inoculated into 96-well culture plate, 5.0×10^3 cells in each well, to incubate for 72h. Ten μl CCK-8 were added to each well at 37°C for 3h, and absorbance at 450 nm (A_{450}) was measured. A_{450} of originally seeded cells and cells 72 h after incubation were measured respectively. Cell growth fold was calculated by formula: Fold of cell growth= (A_{450} 72h/A_{450} seeded) - 1. To detect cell invasion ability, LNCaP, LNCaP-A and LNCaP-A+F cells were digested by trypsin and suspended at a density of 6×10^4/ml, and 1.6 ml suspension were added to Transwell (Corning Costar, Lowell, MA, USA) chamber and cultured for 24 h under the conditions of 5%CO_2, 37°C. Cells to pass through the membrane (Transwell cells) were digested and counted.

Microarray scanning and screening of differential expression genes

Total RNA were extracted from the LNCaP, LNCaP-A, and LNCaP-A+F cells using TRIzol one-step method, and then KLENOW enzyme (Roche, Shanghai, China) labeling method was conducted by random primer. Labeled RNA was dissolved in hybridization solution (3×SSC, 0.2%SDS, 5×Denhart's, 25% formamide) and hybridized overnight at 42°C. After hybridization, the hybridized RNA was cleansed for 5 min in 0.2% SDS, 2×SSC liquid at 42°C, and cleansed for 5 min again in 0.2×SSC liquid in room temperature. Genes with weak fluorescence signal (the data that IV is E or M), redundant data of negative control, inner standard and external standard on the genechip were deleted and then differential expression genes were screened with 2 or 1.5 fold of standard using LuxScan 10KA dual-channel laser scanner (CapitalBio Corp., Beijing, China) equipped with the Molecule Annotation System (MAS) V3.0 1 (CapitalBio Corp., Beijing, China). Then real-time PCR was performed to verify the screening results.

Bioinformatics analysis of differential expression genes

Gene ontology analysis, combined biological process and molecular function analysis, and KEGG pathway analysis for the differential expression genes were performed by Molecule Annotation System (MAS) (CapitalBio Corp., Beijing, China).

Results

Change in proliferation and invasion ability of LNCaP-A and LNCaP-A+F cells

The CCK-8 method was used to measure A_{450nm} for determining cell growth fold of LNCaP , LNCaP-A and LNCaP-A+F cells. The growth speed of LNCaP-A and LNCaP-A+F cells were significantly higher compared with LNCaP cell control ($p<0.01$). There was no difference between LNCaP-A+F and LNCaP-A+F cells ($p>0.05$) (Figure 1A).

Transwell LNCaP-A and LNCaP-A+F cells were counted to determine the invasion ability. The result showed that the number of LNCaP-A and LNCaP-A+F Transwell cells was significantly higher than that of

LNCaP cell control (p<0.01), indicating that the invasion ability of induced androgen-independent LNCaP cells increased significantly. There was no difference between LNCaP-A+F and LNCaP-A+F cells (see Figure 1B).

Screening of differential expressed genes of androgen-independent LNCaP cells

Total RNA was extracted from LNCaP, LNCaP-A and LNCaP-A+F cells, labeled with fluorescence reagents, and scanned. The original data obtained from the experiment were analyzed and totally 12, 207 expression genes were compared. With the standard of difference fold>2.0, 347 differential expression genes were acquired from androgen-independent LNCaP-A cells and LNCaP cell control. Among those genes, 156 were up-ragulated and 191 down-regulated (Figure 2). Respetive 10 up-ragulated genes and 10 down-regulated genes were randomly selected from the differential expression genes to verify the result validation using real-time PCR. The real-time PCR results were found to be consistent with the microarray screening results.

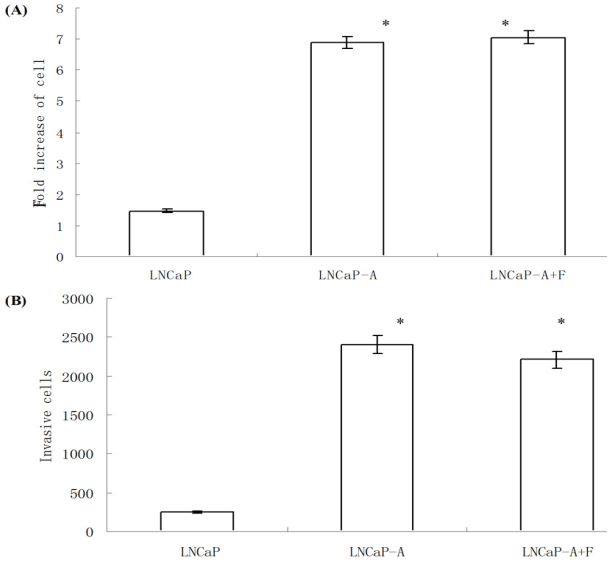

Figure 1. Changes in Proliferation Ability (A) and Invasion Ability (B) of Androgen-independent LNCaP cell (A). Student's t-test was performed for statistical analysis, *p<0.01 *vs* LNCaP cell control

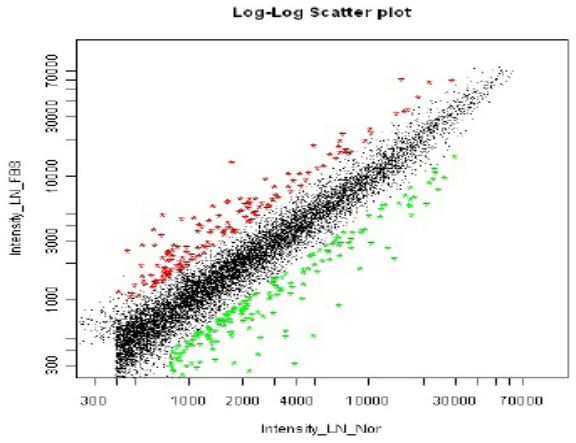

Figure 2. Gene Expression difference between Androgen-independent LNCaP-A Cells and LNCaP Cell Control. X axis is the fluorescence signal strength of LNCaP cell control. Y axis is the fluorescence signal strength of LNCaP-A cells. Red is the up-regulated genes of X or Y. Green is the down-regulated genes of X or Y. Black indicates that there is no gene expression difference between X and Y

Biological process and molecular function analysis of differential expression genes

Biological process analysis towards differential expression genes of androgen-independent LNCaP-A cells and LNCaP cell control was performed using the Molecule Annotation System (MAS). The results showed that differential expression genes were mainly involved in the processes of cell signaling, apoptosis, cell proliferation and differentiation, nucleic acid metabolism, protein metabolism and modification and cell cycle control (Table 1).

The molecular function analysis towards relevant genes showed that these genes mainly served as signal molecules, enzyme molecules, peptide hormones, cytokines, cytoskeletal proteins and adhesion molecules. These results were consistent with the biological process analysis results of genes (Table 2).

KEGG pathway analysis of differential expression genes

The KEGG pathway analysis towards differential expression genes of androgen-independent LNCaP-A cells and LNCaP cell control was performed using the Molecule Annotation System (MAS). The results showed that relevant AIPC cells mainly participated in glutathione metabolism, cell cycle, P53 signal pathway, cytochrome P450 metabolism and nitrogen metabolism. The possible relevant signaling pathways include P53 signal pathway,

Table 1. Biological Cluster Analysis of Specifically Expressed Genes

Go Term	Count	P-Value	Q-Value
Cell cycle	20	2.27E-22	7.68E-22
Regulation of transcription, dna-dependent	25	1.36E-19	3.99E-19
Apoptosis	14	6.43E-14	1.23E-13
Carbohydrate metabolism	12	7.76E-14	1.42E-13
Oxygen transport	5	5.66E-13	9.06E-13
Dna replication	9	1.58E-12	2.40E-12
Cell division	9	2.03E-12	2.88E-12
Oxidation reduction	11	9.04E-12	1.17E-11
Transcription	17	1.69E-10	1.91E-10
Negative regulation of cell proliferation	8	1.85e-10	1.99e-10
Anti-apoptosis	7	3.87E-10	3.87E-10
Mitosis	7	1.53E-09	1.47E-09
Dna repair	7	4.12E-09	3.77E-09
Protein amino acid Phosphorylation	9	1.15E-08	1.01E-08
Negative regulation of survival gene product activity	3	2.16E-08	1.80E-08
Proteolysis	10	3.38E-08	2.75E-08
Sequestering of actin monomers	3	3.46E-08	2.77E-08
Positive regulation of transcription from rna Polymerase Ii promoter	6	8.43E-08	6.62E-08
Response to dna damage stimulus	6	5.14E-07	3.65E-07
Transport	13	7.74E-07	5.32E-07

Table 2. Molecular Function Cluster Analysis of Specifically Expressed Genes

GO Term	Count	P-Value	Q-Value
Protein binding	75	1.11E-56	1.63E-55
Transferase activity	33	4.02E-34	4.43E-33
Zinc ion binding	35	4.14E-32	3.64E-31
Metal ion binding	39	2.77E-28	1.52E-27
Nucleotide binding	30	2.28E-27	1.11E-26
Dna binding	28	2.78E-23	1.11E-22
Atp binding	22	1.97E-21	6.18E-21
Calcium ion binding	15	6.09E-15	1.41E-14
Hydrolase activity, Hydrolyzing O-glycosyl Compounds	8	3.52E-14	7.73E-14
Oxidoreductase activity	13	3.84E-14	8.05E-14
Peptidase activity	12	1.78E-13	3.13E-13
Oxygen transporter activity	5	5.66E-13	9.06E-13
Serine-type Endopeptidase activity	8	1.69E-12	2.48E-12
Transcription factor activity	13	3.13E-12	4.31E-12
Glutathione transferase activity	5	1.15E-11	1.45E-11
Protein homodimer-ization activity	8	7.34E-11	8.97E-11
Dna-directed dna polymerase activity	5	8.74E-11	1.04E-10
Oxygen binding	5	1.03E-10	1.19E-10
Hydrolase activity	16	1.75E-10	1.93E-10
Heme binding	6	1.25E-09	1.22E-09

Table 3. KEGG Pathway Analysis of Specifically Expressed Genes

Pathway	Count	P-Value	Q-Value
Glutathione metabolism	7	2.85E-09	1.78E-07
Cell cycle	9	3.98E-09	1.78E-07
p53 signaling pathway	5	1.56E-05	0.00022
Metabolism of xenobiotics by cytochrome P450	5	1.67E-05	0.000224
Drug metabolism - cytochrome P450	5	1.92E-05	0.000245

cytochrome P450 metabolism, Hedgehog signal pathway, MAPK signal pathway, adipocytokines signal pathway, PPAR signal pathway, TGF-β signal pathway and JAK-STAT signal pathway (Table 3).

Discussion

In the present study, we select the strongly androgen-dependent LNCaP cell lines, which maintain the functional differentiation feature of human prostate cancer, and induce them to make androgen-independent cell model. Then we analyze the gene expression change during the process of ADPC transforming into AIPC to understand the genetic conditions of ADPC transforming into AIPC. By comparison between 12, 207 expression genes, 347 expression genes are acquired. Among them, 156 are up-ragulated and 191 down-regulated.

After analyzing the biological process and molecule function of differential expression genes, these genes are found to play crucial roles in cell proliferation, differntiation, cell cycle control, protein metabolism and modification and other biological process, and these genes serve as signal molecules, enzyme molecules, peptide hormones, cytokines, cytoskeletal proteins and adhesion molecules. The KEGG pathway analysis shows that all relevant genes, NF-κB, IGFR, EGFR, TGF-β, ErbB, VEGF, PLA, Smads, PSA, MMP, BMP2, MYC, ZIC, FOS, Wnt, GnRH and mTOR, play important roles in P53 signal pathway, Hedgehog signal pathway, MAPK signal pathway, PPAR signal pathway, TGF-β signal pathway and JAK-STAT signal pathway.

The increasing expression of PSA genes shows AR signal pathway has not been closed during the process of androgen-dependent LNCaP cell transforming into androgen-independent prostate cancer cell. In signal pathways, the expression of multiple growth factors EGF, IGF, VEGF and their receptors increase, and the expression of DUSP, PLA, NF-κB, MYC and mTOR genes are changed, indicating that the MAPK signal pathway is likely to play an important role in AIPC tranformation process. The expression of NF-κB in LNCaP-A cells increases and the expression of AKT and ERK does not nearly changed, implying that NF-κB is likely to be a key in the signal pathway or there are other pathway to activate AR, without AKT and ERK1/2 signal pathway.

In addition, Smads, TGF-β, BMP2 and MYC genes are primarily involved in TGF-β signal pathway. Smads, as the acceptors of androgen, coordinate with activity factor to interact with androgen receptors, demonstrating that the TGF-β/Smads pathway is likely associated with androgen signal pathway and is mutually participated in the process of AIPC transformation. ZIC, BMP2 and MYC genes regulate androgen receptors to promote the AIPC transformation through the Hedgehog signal pathway.

In the present study, instead of focusing on a single gene or pathway involved in the AIPC transformation, a group of genes to function during the process of ADPC transforming into AIPC is selected. Gene expression conditions after AIPC transformation are obtained through analyzing polygenes and multiple pathways using microarray and bioinformatics. The findings of the present study lay the foundation for the study of AIPC transformation mechanism and development of AIPC targeting drugs.

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