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Microarray and Pathway Analysis of Differential Gene Expression Between Two Kinds of Prostate Cancer Cell Lines Sensitive and Insensitive to Docetaxel

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Abstract: Analyzing Differential Expression Gene (DEG) and identifying candidate gene in prostate cancer cell lines that were resistant to docetaxel, which might be used for the purposes of screening targeted therapy. Gene expression pro?les of the tumors from docetaxel treated mice were performed using Agilent human whole genomic oligonucleotide microarrays. The microarray data were validated and the differential expression genes were analyzed using IPA software. The prostate cancer cell lines PC3 and LNCaP were sensitive to docetaxel but VcaP and CWR22 were resistant to docetaxel cell lines. It indicated that some genes up-regulated or down-regulated in docetaxel resistant cell lines may be candidate genes for targeted therapy, after evaluating by Gene Expression Microarray and signal pathway. The significantly up-regulated genes including Bcl-11A, EPHA4 and CX3CL1 may be the potential candidates for further investigation in docetaxel-resistant tumor cells.

Key words: Prostate cancer; docetaxel, microarray, differential gene expression

INTRODUCTION

Prostate cancer is the second most common cancer which represents a great threat to men's health. Androgen Deprivation Therapy (ADT) involving surgical or chemical castration is the standard treatment for patients with advanced prostate cancer. However, almost all patients with metastatic prostate cancer will initially respond to anti-androgen treatments, the majority will fail hormonal treatments in less than 2 years (Suarez *et al*., 2013). Docetaxel (DTX) improves survival of patients with profiles, such as chemotherapy resistance. In this study we used a gene differential microarray to compare the mRNA expression between two models of docetaxel-resistant and docetaxel-sensitive cell lines and identified a gene involved in resistance to this chemotherapy. Our study has demonstrated new genetic networks and biological pathways in both up-and down-regulated gene expression levels in docetaxel sensitive and docetaxel resistant PCa cell lines.

MATERIALS AND METHODS

Cells culture and prepared for Nude mice inoculation: P3, LNCaP, CWR22 and VCaP prostate cancer cells were provided kindly by Prof. Peter Nelson (Fred Hutchinson Cancer Research Center). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, USA) that was supple- mented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Confluent cells were passaged with trypsin-EDTA (0.05% trypsin and 0.53 mM tetrasodium EDTA) and harvested centrifuged at 1200rpm 5min, resuspended in the medium at 1×10^7/mL single cells, 0.1ml for CB17 Nude mice (purchased from Guangzhou Provincial Medical Experimental Center) subcutaneous injection.

Tumor inoculation and treatment: Four to six weeks old CB17 male Nude mice were used in the experiment. PC3 and VcaP cells ($1x10^6$ cells) were injected subcutaneously

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into both flanks resulting in two tumors per mouse. After tumors have developed surgical castration was performed. The animals were assigned into 4 groups: mice were treated with vehicle (control), DTX (10 mg kg^{-1}), DTX (20 mg kg^{-1}) and castration. Drugs were administered intragastricly in 100 μL volume twice a week for 6 weeks. The diameter of subcutaneously growing tumors was measured with a caliper twice a week until the animals were sacrificed after 6 weeks of treatment. Tumor metastatic Castration Resistant Prostate Cancer (CRPC) and is considered a standard therapy in such cases (Ploussard *et al*., 2010). However, only approximately 50% of patients respond to docetaxel and most of them eventually develop resistance to this therapy (Vainas *et al*., 2012).

Gene expression microarrays technology can be use to simultaneously examine the expression of thousands of genes and determine the molecular characteristics of clinical phenotypes, some of which can be involved in specific cell weight was calculated by the equation:

$$\text{Tumor weight (mg)} = (\text{length} \times \text{width}^2)/2$$

RNA extraction, Labeling, hybridization and scanning of microarray: Total RNAs from xenografts of the treated mice were extracted using Trizol reagent (Takara, Dalian, China) according to the manufactu- rer's instructions and their concentrations were determined by a spectrophotometer (NanoDrop, Nyxor Biotech). All the processes were carried out according to the manufacturers' instructions. Isolated mRNA samples were purified using RNeasy Micro kit (Qiagen) and quality and quantity were assessed on a spectrophotometer. Labelled cRNAs were hybridized onto the Whole Human Genome Oligo Microarray. Images were autogridded and the chemiluminescent signals were quantified, corrected for background and spot and spatially normalized. Differentially expressed genes were identified through filtering the dataset using P-value <0.01 and a signal-to-noise ratio >2 for use in ANOVA statistical analysis.

Pathway enrichment and Network construction: Interception of 400 genes with significant differences from differential gene expression data which has been statistical analysis using the GeneSpring GX software package. The target gene expression data were analysis by IPA and the signal pathway is derived from tumor-related candidate genes in microarray data. In order to find out the differential expression genes closely related with signal pathway, additional filtering (minimum 3-fold change) was applied to extract the most these genes which were analyzed using Ingenui- ty Pathway Analysis software. Those genes with known gene symbols (HUGO) and their corresponding expression values were uploaded into the software. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of these genes were algorithmically generated based on their connectivity.

RESULTS

Microarray analysis: For microarray analysis, Agilent Array platform was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols. Microarray experiment was quali?ed according to quality standards. The experimental system was stable and fiuorescent signal intensity was strong and homogenous. In the four chips, 41 745 probes clearly had signals, representing 92.8% of 45 000 probes (Fig. 1). The scatter plot was used for assessing the gene expression variation between four group arrays.

Screening of DEGs for canonical pathways analysis: A total of 400 DEGs including 200 up regulated genes and 200 down regulated genes were identified in the two sets of data which were analyzed by IPA library of canonical pathways (Fig. 2). The significance of the association between the data set and the canonical pathway was determined. Kyoto encyclopaedia of genes and genomes (KEGG) pathway analysis of gene differential expression

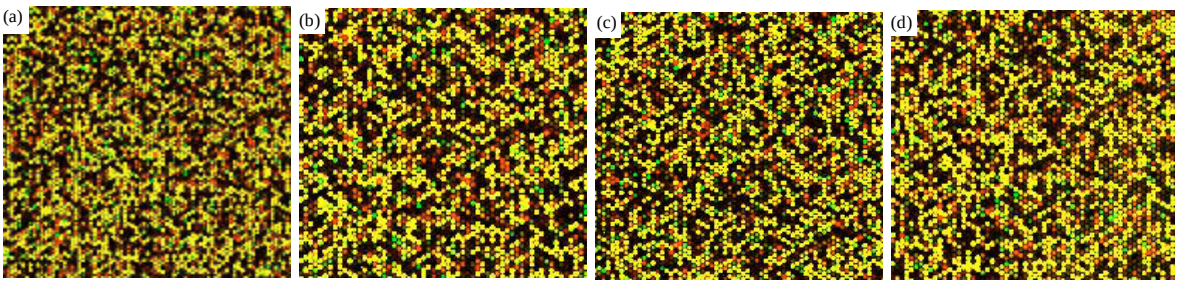

Fig. 1(a-d): Microarray analysis. Gene chip hybridization fiuorescence signal graph in PC3 (A), LNCaP (B), VCaP (C) and CWR22 (D) of human prostate cancer

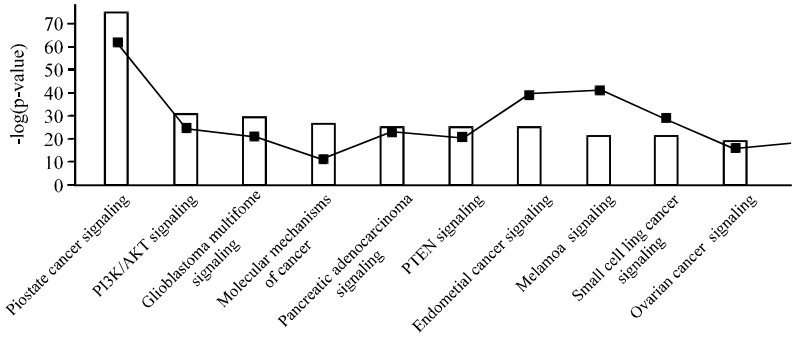

Fig. 2: Pathway function enrichment of differentially expressed genes. The top ten canonical pathways are up-regulated

patterns in docetaxel sensitive and docetaxe resistant from xenograft tissue revealed many enrichment-related pathways including pathways in prostate cancer, (Mohsenzadegan *et al*., 2013) having the potential to discover the diversified and dynamic molecules during tumor progression. Despite enormous efforts made for differential expression detection and biomarker discovery, few methods have been investigated the gene expression level in tumor stage during docetaxel resistant progression. Our study has demonstrated new genetic networks and biological pathways in both up-and down-regulated gene expression levels in docetaxel sensitive and docetaxel resistant PCa cell lines.

Ingenuity network analysis: IPA qualified 400 genes as network and function eligible and analysis showed 12 networks on the basis of this gene selection with a score more than three. Specifically, network 1 (Fig. 3a) was centered on NF-κB pathway. This gene directly interacts with an important complex for cell survival and proliferation, including up-regulated gene such as Bcl-11A and down-regulated genes related to apoptosis 9, CYP27B1 and GPX1. Other down-regulated genes directly related to apoptosis also shown in the network. Network 2 (Fig. 3b) was around the MAPK pathway in which EPHA4 gene associated with formation of tumor blood vessels which was highly up-regulated. Another gene CX3CL1 related to tumor metastasis also highly expressed in docetaxel resistant cell lines.

DISCUSSION

Prostate cancer (PCA) is a heterogeneous disease and many molecular methods have been used in the search to determine the mechanism of the development of the disease and to find new therapeutic and prognostic molecules (Takayama and Inoue, 2013). Microarray technology for gene expression profiling has proven to be successful in a variety of experimental settings down-regulated genes in the docetaxel sensitive and docetaxel resistant PCa cells. In analyzing, often individual genes were found in multiple categories of functions related to cancer development including cell-to-cell signaling and interaction, cell signaling, cell death, cellular growth and proliferation. In addition, it reminds us that there are certain limits to in the analysis since there are many different gene interactions resulting from various cellular/experimental conditions and the edges denoted in the network may not represent the actual causal relationship between genes. In spite of that it is still a useful method to determine similarities and differences obtain useful information for effective treatment and prognosis.

Microarrays analysis was focused on the identification of commonly deregulated genes in the four different cell line models. We use IPA software to analyze 200 up-regulated and 200. One important gene network was identified around the NF-κB gene (Fig. 3a) which is a protein complex that controls the transcript of DNA NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokine, free radicals and ultraviolet irradiation (Gilmore, 2006). Incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases (Brasier, 2006). We observed significantly decreased expr- ession of apoptotic genes around NF-κB including Caspase 9, caspase 3 and caspase 10 in VCaP and CWR22 cells which is consistent with our previous study in docetaxel resistant cell line DU145 (data submitted). However, our study demonstrated that carcinogenesis related gene Bcl-11A was markedly up-regulated in the network around NF-κB pathway. In the present study, Bcl-2 has been reported to be a potent modulator of cellular differentiation and a suitable target for anticancer drug design in PCA (Albrecht *et al*., 2004) however Bcl-11A has not intensely studied.

EPHA4 gene was a highly up-regulated member of the network around the VEGF gene (Fig. 3a) in docetaxel-resistant prostate cancer cell lines. This gene belongs to the ephrin receptor subfamily of the

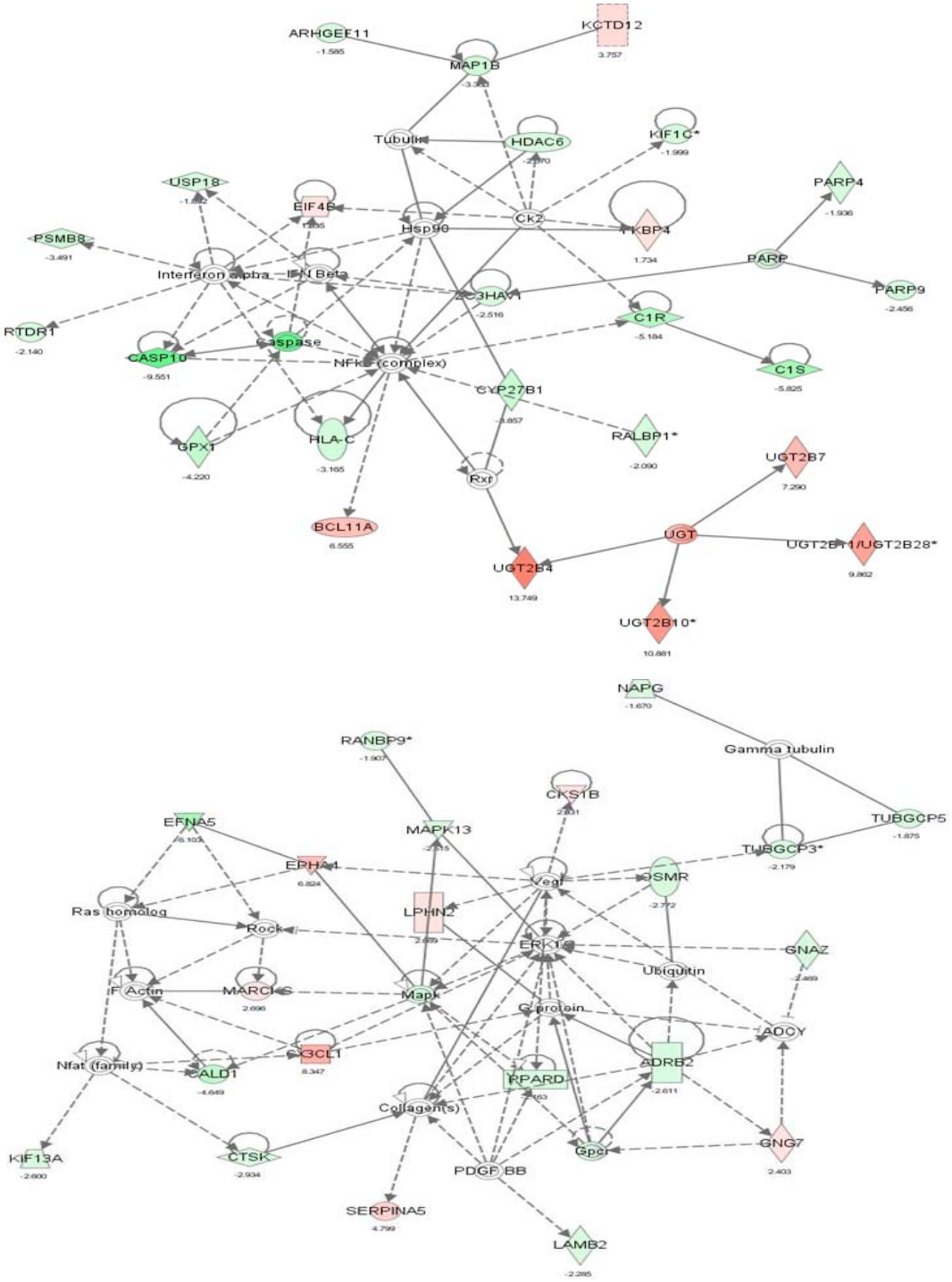

Fig. 3(a-b): Gene network up-regulated and down-regulated in PC3, LNCaP, VCaP and CWR22 prostate cancer cell lines. The intensity of the node color indicated the degree of up or down-regulation. Genes in uncolored notes were not identified as differentially expressed and were integrated into the computationally generated networks on the basis of the evidence stored in the IPA knowledge memory indicating a relevance to this network

protein-tyrosine kinase family. Ephrin ligands and Eph receptors are signaling molecules that are differentially expressed on arteries and veins during development (Kudo *et al*., 2007). The EPHA4 receptor tyrosine kinase regulates a variety of physiological and pathological processes during neural development and the formation of tumor blood vessels. A potential role for EPHA4 in human cancer is receiving increasing attention. Altered expression patterns of EPHA4/ephrins are correlated with tumor behaviors, such as invasiveness, vascularization and metastatic potential. Inhibition of EPHA4 by soluble protein inhibitors such as ephrin-A5-Fc, EPHA4-Fc, or peptides induces axonal regeneration and reduces ischemia-induced apoptotic cell death (Goldshmit *et al*., 2011). Report demonstrated that high levels of EPHA4 RNA expression are correlated significantly with reduced overall survival of cancer patients (Oshima *et al*., 2008).

Another one network was identified around the MAPK (Mitogen-activated protein kinases) genes (Fig. 3b) that was down-regulated in both docetaxel-resistant and docetaxel-sensitive cell lines. However, CX3CL1 (Chemokine C-X3-C motif ligand 1) is highly up-regulated in both cell lines which is immediately linked to the MAPK. For MAPK/ERK pathway, there is a chain of protein in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell (Orton *et al*., 2005). The signal starts when a signaling molecule binds to the receptor on the cell surface and ends when the DNA in the nucleus expresses a protein and produces some change in the cell, such as cell division. When one of the proteins in the pathway is mutated, it can be stuck in the "on" or "off" position, which is a necessary step in the development of many cancers. MAPK/ERK is a crucial regulator of survival and many factors induce cell apoptosis through the inhibition of extracellular signal-regulated kinase. It is generally believed that activation of MAPK/ERK pathway may inhibit the apoptosis of cancer cells through MAPK/ERK activation (Zhang *et al*., 2011).

In summary, this exploratory analysis provides information about potential genes and networks involved in docetaxel resistance and docetaxel sensitive prostate cancer cell lines as well as a basis for forward investigations of the specific mechanism. The identification of docetaxel resistant and sensitive genes may be useful to select patients who may or may not benefit from therapy or to develop targeted therapies to overcome docetaxel resistance. Further clinical validation of these results is needed in patients with prostate cancer.

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