Bioinformatics analysis of the gene expression profile in Bladder carcinoma

Jing Xiao and Qiu Yiqing
Department of Urology, The Second Affiliated Hospital of Zhejiang University, Hangzhou, Zhejiang Province, China.

Abstract
Bladder carcinoma, which has the ninth highest incidence among malignant tumors in the world, is a complex, multifactorial disease. The malignant transformation of bladder cells results from DNA mutations and alterations in gene expression levels. In this work, we used a bioinformatics approach to investigate the molecular mechanisms of bladder carcinoma. Biochips downloaded from the Gene Expression Omnibus (GEO) were used to analyze the gene expression profile in urinary bladder cells from individuals with carcinoma. The gene expression profile of normal genomes was used as a control. The analysis of gene expression revealed important alterations in genes involved in biological processes and metabolic pathways. We also identified some small molecules capable of reverting the altered gene expression in bladder carcinoma; these molecules could provide a basis for future therapies for the treatment of this disease.

Keywords: Bladder carcinoma, differential expression, expression profile, small molecular mimic, susceptibility forecast.
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Introduction
Bladder carcinoma is one of the most common malignant tumors and has a high death rate in China (Dai et al., 2011). World-wide, bladder carcinoma has the ninth highest incidence among malignant tumors (Parkin, 2008). Bladder carcinoma results from cumulative, long-term interactions between genetic and environmental elements and is a complex, multifactorial process involving gene mutations and progressive cellular damage. Bladder carcinoma is much more common in men than in women and predisposing factors include cigarette smoking, long-term contact with chemical products and race (Parkin, 2008).

The malignant transformation of bladder cells originates in DNA mutations and alterations in gene expression. Most studies of the etiology of bladder carcinoma have focused on genetic mutations, particularly in oncogenes such as H-Ras, C-Myc, HER-2, BcL-2, erbB-2 and fibroblast growth factor receptor 3 (FGFR3) (Sanchez-Carbayo and Cordon-Cardo, 2007; Cordon-Cardo, 2008), as well as the inactive of anti-oncogenes and the deletion or loss of heterozygosity (LOH) in associated chromosomal regions for genes such as p53, pRb, p21 and p16 (Shariat et al., 2004). The invasiveness and metastasis of bladder carcinoma involve the abnormal expression of growth factors and related receptors such as vascular endothelial growth factor (VEGF) (Wang et al., 2000) and fibroblast growth factor receptor (FGFR) (Knowles, 2008).

A detailed understanding of multi-gene hereditary diseases, such as cancer, is no longer feasible with traditional single gene studies but requires more powerful tools such as large-scale gene expression analysis provided by recent technological advances. The application of such technologies to the study of bladder carcinoma should be useful in identifying new tumor marker genes that will allow the early diagnosis of bladder cancer.

In this study, we used the gene expression profile of GSE27448 to investigate differential gene expression in bladder carcinoma and to search for small molecules that may reverse the altered gene expression. The availability of high-throughput gene expression data and their analysis by computational bioinformatics may be useful in identifying molecules that could provide a basis for developing new therapies for treating bladder carcinoma.

Materials and Methods
Gene expression in bladder carcinoma cells and normal cells
To investigate the differential gene expression in bladder carcinoma compared to normal cells and the possible mechanisms involved in the initiation of bladder cancer, we analyzed the gene expression profile of bladder carcinoma and normal cells available from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/); accession number:
Identification of differentially expressed genes (DEGs)

The original data were classified as bladder carcinoma and control (i.e., normal) groups and were analyzed using R software (v.2.13.0) (R Development Core Team, 2008). Initially, the RMA (robust multichip averaging) method (Irizarry et al., 2003) was applied to normalize the data on different chips, and then Limma (Smyth, 2004), a linear regression model software, was used to compare the differential expression on different classes of chips. A value of p < 0.05 was used as the cut-off criterion to select genes that were differentially expressed in bladder carcinoma relative to the control group.

Gene ontology (GO) analysis of DEGs

To investigate the DEGs at a functional level, DAVID (Database for Annotation, Visualization and Integrated Discovery) was used to cluster the genes according to the Gene Ontology (GO) (Ashburner et al., 2000) categories of cellular component, biological process and molecular function (Huang da et al., 2009a,b).

Pathway enrichment analysis

To gain more insights of the changes at a functional level, we investigated the dysregulated biological pathways in bladder carcinoma. All of the metabolic and non-metabolic pathways available from the canonical KEGG PATHWAY DATABASE were used as DAVID inputs for KEGG PATHWAY cluster analysis (Huang da et al., 2009a,b). A value of p < 0.1 and at least two DEGs contained in a pathway were chosen as the cut-off criteria.

Identification of small molecules

The connectivity map (CMap) database, which contains whole genome expression profiles for small active molecular inferences, consists 6,100 classes of small molecular interference experiments and 7,056 expression profiles (Lamb et al., 2006). We analyzed genes that were differentially expressed between normal and bladder carcinoma cells and contrasted them with genes involved in small molecular interference in the CMap database in an attempt to identify small molecules associated with these DEGs. The DEGs were classified into up-regulated and down-regulated groups and the top 500 significant probes in each group were chosen for GSEA (Gene Set Enrichment Analysis) analysis; these were then compared to the DEGs after small molecule treatment. Finally, the enrichment values were calculated. These values varied between -1 and 1 and reflected the similarity among the genes: values closer to -1 indicated greater similarity between the genes, i.e., the small molecule could imitate the status of normal cells, whereas values closer to 1 indicated that the small molecule could imitate the status of bladder carcinoma cells.

Results

Identification of DEGs

The classic t-test in the Limma package was used to analyze the gene expression profiles of bladder carcinoma and normal cells and identify the DEGs in bladder carcinoma cells. A value of p < 0.05 was used as the significant threshold for DEGs. Based on these criteria, 6562 gene probes (corresponding to 2779 genes) were found to have an altered expression.

GO clustering of DEGs

Functional classification of these 2779 DEGs with the online biological classification tool DAVID and a statistical cut-off criterion of p < 0.001 indicated significant enrichment of these genes in various GO categories. Table 1 shows that in bladder carcinoma DEGs were enriched in cellular components related to the cytoskeleton and loco-
motion (Z discs, I band, myofibrils, contractile fibers, sarcolemma and sarcromere) and signal transduction. There were also changes in cellular components related to protein expression, e.g., the ribonucleoprotein complex, ribosomes, nucleoli, heterochromatin, nuclear lumen and cytosolic large ribosomal subunit.

Table 2 shows the clusters obtained when these DEGs were classified according to biological process (p < 0.01). This analysis revealed inter- and intracellular changes in components related to transportation, such as the regulation of intracellular transport, protein localization, the regulation of nucleocytoplasmic transport, the regulation of intracellular protein transport, negative regulation of intracellular transport and negative regulation of nucleocytoplasmic transport. These changes meant that signal molecules could not be transported to their target site, thus altering intercellular signal transduction. Similar changes were observed in other biological processes, such as intracellular signaling cascades, small GTPase-mediated signal transduction and the protein kinase cascade. Other changes affected protein translation and post-translational modifications, such as amino acid auto-phosphorylation, translation, post-translational elongation, protein complex assembly and protein complex biogenesis. Biological processes related to cell development, e.g., peripheral nervous system development, auditory receptor cell differentiation, mechanoreceptor differentiation and auditory receptor cell development, were also affected.

Table 3 shows the DEGs clustered according to molecular function (p < 0.01). The changes in gene expression affected mainly gene transcription and translation, e.g., nucleoside-triphosphatase regulator activity, purine nucleotide binding and double-stranded RNA binding, and signal transduction, e.g., insulin receptor binding, GTPase regulator activity and GTPase activator activity.

Altered biological pathways in bladder carcinoma cells

The gene expression profile changed significantly during the progression of bladder carcinoma. Some genes changed significantly under pathological conditions. These DEGs were selected for KEGG pathway enrichment analysis and subsequent identification of the altered pathways in bladder carcinoma cells. Based on a threshold value of p < 0.1 and a gene count > 2 we identified eight dysregulated pathways (Table 4). The most significant enrichment involved the complement and coagulation cascades (p = 0.0023). Some of the significant pathways were related to signaling transduction, such as PPAR signaling pathway (p = 0.053) and SNARE interactions in vesicular transport (p = 0.0757).

Identification of related active small molecules

The DEGs were classified into up-regulated and down-regulated gene groups followed by GSEA analysis and matching after small molecule treatment in the CMap.

Table 2 - Clustering of DEGs based on biological process.

| Term                                                                 | p value   |
|----------------------------------------------------------------------|-----------|
| GO:0007422--peripheral nervous system development                     | 5.36E-04  |
| GO:0042491--auditory receptor cell differentiation                    | 0.001356  |
| GO:0046777--protein amino acid autophosphorylation                   | 0.001668  |
| GO:0006412--translation                                             | 0.001726  |
| GO:0007242--intracellular signaling cascade                          | 0.001968  |
| GO:0032386--regulation of intracellular transport                    | 0.002223  |
| GO:0008104--protein localization                                     | 0.003303  |
| GO:0042490--mechanoreceptor differentiation                          | 0.00344   |
| GO:0046822--regulation of nucleocytoplasmic transport                | 0.003457  |
| GO:0033157--regulation of intracellular protein transport             | 0.0035    |
| GO:0010001--glielial cell differentiation                             | 0.0035    |
| GO:0006414--translational elongation                                 | 0.003612  |
| GO:0007264--small GTPase-mediated signal transduction                 | 0.004034  |
| GO:0007155--cell adhesion                                            | 0.004176  |
| GO:0022610--biological adhesion                                      | 0.004267  |
| GO:0030534--adult behavior                                           | 0.004457  |
| GO:0007243--protein kinase cascade                                   | 0.004627  |
| GO:0032387--negative regulation of intracellular transport           | 0.006022  |
| GO:00010604--positive regulation of macromolecule metabolic process  | 0.0064    |
| GO:0046823--negative regulation of nucleocytoplasmic transport       | 0.006632  |
| GO:0016339--calcium-dependent cell-cell adhesion                      | 0.008158  |
| GO:0060117--auditory receptor cell development                       | 0.008276  |
| GO:0043933--macromolecular complex subunit organization              | 0.008799  |
| GO:0006461--protein complex assembly                                 | 0.009174  |
| GO:0070271--protein complex biogenesis                               | 0.009174  |
| GO:0019226--transmission of nerve impulse                            | 0.009828  |

Table 3 - Clustering of DEGs based on molecular function.

| Term                                                                 | p value   |
|----------------------------------------------------------------------|-----------|
| GO:0005158--insulin receptor binding                                 | 2.47E-05  |
| GO:0060589--nucleoside-triphosphatase regulator activity             | 7.39E-04  |
| GO:0030695--GTPase regulator activity                                | 0.001494  |
| GO:0017076--purine nucleotide binding                                | 0.002422  |
| GO:0003725--double-stranded RNA binding                              | 0.002958  |
| GO:0005096--GTPase activator activity                                | 0.004455  |
| GO:0003735--structural constituent of ribosome                       | 0.005429  |
| GO:0000287--magnesium ion binding                                    | 0.006179  |
| GO:0051219--phosphoprotein binding                                   | 0.006982  |
| GO:0022843--voltage-gated cation channel activity                    | 0.007117  |
| GO:0032553--ribonucleotide binding                                   | 0.007602  |
| GO:0032555--purine ribonucleotide binding                            | 0.007602  |
| GO:0031420--alkali metal ion binding                                 | 0.008589  |
database. This analysis identified some small molecules that could reverse the status of bladder carcinoma cells. Table 5 shows the 20 most significant small molecules identified by this procedure.

Thioguanosine (enrichment = 0.865) and tyloxapol (enrichment = 0.857) can partially imitate the carcinoma status of bladder cells, i.e., these small molecules may be strong inducers of bladder cancer. In contrast, adiphenine (enrichment = -0.962) and viomycin (enrichment = -0.947) can imitate the status of normal cells, i.e., they can reverse the abnormal status and are therefore potentially lead molecules for developing new therapeutic drugs for treating bladder carcinoma. Further investigation of these small molecules may clarify their role in the pathogenesis of bladder cancer.

**Table 5 - List of small molecules that can imitate the status of normal cells.**

| CMap name                          | Enrichment | p value |
|------------------------------------|------------|---------|
| Adiphenine                         | -0.962     | 0       |
| Viomycin                           | -0.947     | 0       |
| Trichostatin A                     | 0.253      | 0       |
| Isoflupredone                      | -0.969     | 0.00008 |
| Fludrocortisone                    | -0.693     | 0.00022 |
| Biperiden                          | -0.845     | 0.00026 |
| Thiamphenicol                      | -0.826     | 0.00040 |
| Thioguanosine                      | 0.865      | 0.00046 |
| Finasteride                        | -0.752     | 0.00046 |
| Prestwick-692                      | -0.882     | 0.00048 |
| Genistein                          | -0.471     | 0.00048 |
| Gentamicin                         | -0.875     | 0.00052 |
| Tyloxapol                          | 0.857      | 0.00056 |
| Phthalylsulfathiazole              | 0.813      | 0.00056 |
| Calcium folinate                   | -0.811     | 0.00056 |
| Monensin                           | -0.73      | 0.00073 |
| Trimethobenzamide                  | -0.795     | 0.00074 |
| Atracylloside                      | -0.788     | 0.00082 |
| Etiocholanolone                    | -0.725     | 0.00085 |
| PHA-00745560                       | -0.649     | 0.00088 |

**Discussion**

Bladder cancer involves the proliferation of malignant intrabladder cells and is the fourth most common solid tumor in men and the seventh most common in women worldwide, with 350,000 new cases each year. The American Cancer Society (AGS) statistics indicate that in 2006 there were 61,420 new cases of bladder cancer and 13,060 deaths. In China, bladder cancer is also commonly associated with urinary tract malignant tumors. The morbidity in 2005 was 4/100 in males and 1.5/100 in females. The steady increase in the morbidity of bladder cancer in some Chinese cities in recent years indicates a need for additional research on this disease.

The main biological processes that showed DEGs based on GO cluster analysis were signal transduction, physical immunity and disease initiation. Nearly 2800 genes were differentially expressed in bladder carcinoma compared with normal cells. These DEGs may be important for investigating the mechanism of disease development and may be useful targets for treating bladder carcinoma, hence the potential interest in studying these genes. The results of GO cluster analysis and pathway enrichment analysis of DEGs suggested that the development of bladder carcinoma may involve important changes in cellular signal transduction.

As a signal molecular receptor, SNARE plays a key role in signal transduction (Skalski et al., 2011). Changes in SNARE interactions in vesicular transport directly affect cellular transduction (Fasshauer, 2003; Han et al., 2009). Peroxisome proliferator-activated receptors (PPAR), which are nuclear hormone receptors (Umemoto and Fujiki, 2012), regulate gene expression and changes in the PPAR signaling pathway result in abnormal gene expression. These signaling pathways regulate cell growth (Sertznig et al., 2008), differentiation and proliferation (Peters et al., 2012) by modulating the expression of downstream genes. The changes mediated by these signaling pathways ultimately affect protein synthesis, such as the ribosomal pathway and development (progestosterone-mediated oocyte maturation) (Fauconnet et al., 2002). Such changes are closely related to the onset of bladder carcinoma (Yoshimura et al., 2003).

Based on the DEGs and small molecule interference data we identified a set of small molecules that can imitate the normal cell status, i.e., they can reverse the abnormal gene expression of bladder carcinoma. These small molecules may provide lead compounds for developing new therapies for treating bladder carcinoma. For example, adiphenine inhibits nicotinic receptors, thereby decreasing the frequency of acetylcholine-induced single-channel currents and cluster duration (the latter by 36-fold at 100 μM) while increasing the decay rate (IC50 = 15 μM) of macroscopic currents and accelerating desensitization from the open state; however, prior application of the drug to resting
receptors is required for these effects to be seen. (Spitzmaul et al., 2009). The nicotinic receptor is reported to have an important role in various types of cancer (Russo et al., 2012). Hence, adiphenine is a potential therapeutic antagonist for bladder carcinoma. The small molecules that imitate the normal cell status identified in this study may therefore offer new fields of research in bladder cancer therapy.

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