Original Article
Differentially expressed genes and microRNAs in bladder carcinoma cell line 5637 and T24 detected by RNA sequencing

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Abstract: Bladder carcinoma is a common malignancy with complicated treatment methods due to its heterogeneity. In this study, we focused on two bladder carcinoma cell lines, 5637 and T24, to compare their differences from the transcriptome level. RNA sequencing was used to generate the transcriptome data of the two cell line and the control cell line SV-HUC-1. Differentially expressed genes (DEGs) and differentially expressed microRNAs (miRNAs) of cell line 5637 and T24 were screened. Their annotation and analyses were conducted using gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) to predict their possible functions and pathways involved. Number of DEGs specific in cell line 5637, specific in cell line T24 and in both the cell lines was 880, 1512 and 1412, respectively. Number of differentially expressed miRNAs of the three categories was 7, 20 and 18, respectively. These DEGs and miRNAs participated in different biological processes and pathways, among which some were further verified by qRT-PCR. Interferon-stimulated genes (ISGs), including STAT1, TMEM173 and OAS3, were down-regulated in cell line 5637 compared to SV-HUC-1. NDOR1 and NDUFV1, genes related to mitochondrial metabolism, were up-regulated in cell line T24. miR-4257, miR-6733 and gene WNT9A and WNT10A were down-regulated in both the cell lines. Thus cell line 5637 might have lower chemotherapy resistance while T24 might exhibit abnormal mitochondrial metabolism. These results uncovered major differences between cell line 5637 and T24, which indicated the two cell lines, should be selectively used in bladder carcinoma research.

Keywords: Bladder carcinoma, differentially expressed genes (DEGs), microRNAs (miRNAs), chemotherapy resistance, mitochondrial metabolism

Introduction

Bladder carcinoma is one of the most common malignancies with an increasing incidence in the world [1]. The majority of the bladder carcinoma patients are non-muscle-invasive cancer [2]. Though surgical cystectomies with adjuvant chemotherapy are efficient to primary bladder carcinoma [3], recurrency has occurred in about 70% of patients and 10-40% of the cases have developed to metastatic diseases ultimately [4]. Moreover, a large proportion of advances bladder carcinoma patients are resistant to chemotherapy [5], characterized by some gene signatures, such as interferon-stimulated genes (ISGs) [6, 7]. Heterogeneity is a characteristic of bladder carcinoma, which means metastasis, treatment response, relapse and clinical outcomes may vary remarkably among different individuals [8-10]. So a better understanding of biological properties and the molecular mechanisms of individual cancer cells is necessary and urgent.

DNA and RNA microarrays and sequencing have been widely used to identify mutations in bladder carcinoma genomic regions and differentially expressed genes [11, 12]. Particularly, RNA sequencing has provided a reliable, quantitative and sensitive method for studying transcriptome and microRNAs (miRNAs) [13]. The development of sequencing methods has facilitated studies on genome and transcriptome landscapes and genetic aberrations of chromatin remodeling genes in bladder carcinoma [10, 14].
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Both the non-muscle-invasive bladder carcinoma cell line 5637 and the muscle-invasive bladder carcinoma cell line T24 have been used in numerous studies. A previous genomic study has compared these cell lines from their gene gains and losses, and pathways and biological functions of the altered genes [11]. But the transcriptome profiles of these cell lines are still unclear. Our study aimed at comparing cell line 5637 and T24 based on their transcriptome information. Their transcriptomes were sequenced using Illumina high-throughput sequencing and the data were analyzed with bioinformatics methods, using a normal bladder cell line SV-HUC-1 as the control. We focused on the differentially expressed genes (DEGs) and differentially expressed miRNAs in these cell lines and predicted their functions and pathways involved. Some DEGs and miRNAs that might participate in cancer-related functions and pathways were further analyzed by real-time quantitative RT-PCR (qRT-PCR). Our results might guide the differential treatments of bladder carcinoma subtypes.

**Table 1. Primers used in qRT-PCR**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>β-actin-forward</td>
<td>TCACCCCTCTGTGCCCATCTACGA</td>
</tr>
<tr>
<td>β-actin-reverse</td>
<td>CAGCGAACCCTGTCATGGAATGG</td>
</tr>
<tr>
<td>STAT1-forward</td>
<td>CAGCTTGAACCAAATTTCCGTGCA</td>
</tr>
<tr>
<td>STAT1-reverse</td>
<td>TGAAGATTAGGCTGCTTTTCCCT</td>
</tr>
<tr>
<td>TMEM173-forward</td>
<td>CCAGAAGCACCCTGGCGTA</td>
</tr>
<tr>
<td>TMEM173-reverse</td>
<td>CGCATTGGGAGGGAGTGAAG</td>
</tr>
<tr>
<td>OAS3-forward</td>
<td>GAAGGTTCGTAGAAGGACGCG</td>
</tr>
<tr>
<td>OAS3-reverse</td>
<td>CCTTGAGACCTTTTACGACC</td>
</tr>
<tr>
<td>U6 snRNA-forward</td>
<td>CTGCGTCTGGCAGCACA</td>
</tr>
<tr>
<td>U6 snRNA-reverse</td>
<td>AGCCTTCACGATTTTGC</td>
</tr>
<tr>
<td>Uni-PCR</td>
<td>AGTGGAAGCAGGGCAAAACACAG</td>
</tr>
<tr>
<td>RT-Stem</td>
<td>GCAGCCGACCTGGCGGAGG</td>
</tr>
<tr>
<td>miR-6733-5p-PCR</td>
<td>GGTGGGAAGAAGACAAACACTCAG</td>
</tr>
<tr>
<td>miR-6733-5p-RT</td>
<td>GTGGATCCAGTTGGAACGCTG</td>
</tr>
<tr>
<td>miR-4257-PCR</td>
<td>GGCCTGCAGAGGAAAGCTG</td>
</tr>
<tr>
<td>miR-4257-RT</td>
<td>GTGGATCCAGTTGGAAGCTG</td>
</tr>
<tr>
<td>NDOR1-forward</td>
<td>CTCTTACCCTGGTGCTAATCC</td>
</tr>
<tr>
<td>NDOR1-reverse</td>
<td>GCAAGTGCTGCACGAGGG</td>
</tr>
<tr>
<td>NDUVF1-forward</td>
<td>AGGATGGAACCGGATTTGCC</td>
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<td>NDUVF1-reverse</td>
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<tr>
<td>WNT9A-forward</td>
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<td>AGTGCGGTCGACCATC</td>
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<tr>
<td>WNT10A-forward</td>
<td>GGCAACCGGTGTCAGTCT</td>
</tr>
<tr>
<td>WNT10A-reverse</td>
<td>CATTCACACCTGCCCATC</td>
</tr>
</tbody>
</table>

**Materials and methods**

**Cell lines**

The human bladder carcinoma cell lines 5637 and T24, and the human uroepithelial cell line SV-HUC-1 were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich, Shanghai, China) medium containing 10% fetal bovine serum (FBS, HyClone, Logan, Utah, USA) in humidified atmosphere with 5% CO₂ at 37°C.

**RNA extraction and sequencing**

Total RNAs from each cell sample were extracted by Trizol (Invitrogen, Carlsbad, CA, USA) according to the manuals. DNA contamination was removed by DNase I (Invitrogen) and examined by agarose gel electrophoresis (AGE). The quality and quantity of RNA samples were detected by AGE and NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). mRNAs were
enriched by oligo(dT) and then digested by RNase III (Invitrogen) into fragments of about 200 nt. The library was constructed using TruSeq Stranded mRNA HT Sample Prep Kit (Illumina, San Diego, CA, USA). As to the miRNA sequencing, miRNAs were isolated and adapters were added. Library was constructed using TruSeq Small RNA Library Preparation Kits (Illumina). Sequencing was performed by Hiseq™ 2000 Sequencing System (Illumina).

**Data analysis**

Clean reads were generated by removing the low-quality reads and adapters. Assembly was conducted using Trinity [15]. The sequencing quality was analyzed by RSeQC [16] and the quality was acceptable if Q20 was more than 95%. Annotation was performed using DAVID Bioinformatics Resources 6.7 [17, 18] to describe the gene ontology (GO) [19] and Kyoto encyclopedia of genes and genomes (KEGG) pathway [20]. miRNAs were blast to miRNA 21 (http://www.mirbase.org/). DEGs and miRNAs were selected according to the threshold within which the absolute value of log2 fold-change (log2 FC) is more than 1.00 [21]. False discovery rate (FDR) control method was used to correct the P values [22] and terms with FDR < 0.05 were considered significant. Then reads per kilobase per million mapped reads (RPKM) was used to indicate the gene expression levels [23]. The pathways were analyzed by Fisher’s exact test and χ2 test. FDR < 0.05 was set as the threshold of significance.

**qRT-PCR and statistical analysis**

The complementary DNAs (cDNAs) of each sample for qRT-PCR analysis were synthesized from 1 μg RNAs using PrimeScript RT-PCR Kit (TaKaRa, Dalian, China). qRT-PCR was conducted on LightCycler 480 (Roche Applied Science, Shanghai, China) with cDNAs of 50 ng (Table 1) and specific primers in the reaction system. All the experiments were performed in triplicate. The relative expression level of genes and miRNAs was normalized by β-actin and U6 small nuclear RNA (snRNA), respectively. Data was calculated by 2^(-ΔΔCt) method and analyzed by Student’s t-test. Differences were considered significant if P < 0.05. Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS) 20 software (IBM, New York, USA).

**Results**

DEGs and miRNAs in cell line 5637 and T24

The qualities of the three sequenced transcriptomes were all acceptable and a total of 12 Gb raw data were obtained, suggesting the RNA-sequencing data were qualified for further analyses. When searching for the DEGs of cell line 5637 and T24 compared to the control cell line SV-HUC-1, we categorized DEGs into three groups: DEGs specific to cell line 5637, DEGs specific to cell line T24 and DEGs found in both the cell lines (including the up-regulated and the down-regulated genes). Results showed that the DEG numbers of the three categories were 880, 1512 and 1412, respectively (Figure 1A and Tables S1-S3). Among the 1412 genes,
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**Figure 2.** GO analysis of DEGs. A. BP, MF and CC ontologies of DEGs specific to cell line 5637. B. BP, MF and CC ontologies of DEGs shared by cell line 5637 and T24. C. BP, MF and CC ontologies of DEGs specific to cell line T24. In each ontology, the first 15 enriched terms were listed. BP: biological process. MF: molecular function. CC: cellular component.

**Figure 3.** KEGG analysis of DEGs. A. Pathway prediction of DEGs specific to cell line 5637. B. Pathway prediction of DEGs shared by cell line 5637 and T24. C. Pathway prediction of DEGs specific to cell line T24. In each category, the first 15 enriched terms were listed.
1360 showed no differential expression between cell line 5637 and T24, including WNT9A and WNT10A, which were both down-regulated in the two cell lines. While 44 were up-regulated and 17 down-regulated in cell line 5637 compared to T24 (Table S3). As to the differentially expressed miRNAs, seven of them were found only in cell line 5637, 20 in cell line T24 and 18 in both the cell lines (Figure 1B and Table S4). Especially, miR-4257 and miRNA-6733 were significantly down-regulated in both 5637 and T24 cell lines, implying their potential roles involved in tumorgenesis and progression.

**GO analysis and pathway prediction**

GO analysis results showed the annotation of genes from three ontologies, that is, biological process (BP), molecular function (MF) and cellular component (CC). DEGs of cell line 5637 were enriched in BP terms like mitotic cell cycle, cell division, and apoptotic process (Figure 2A). In MF terms, they were enriched in protein binding and in CC terms, and in CC terms, they focused on cytosol components. Some of the DEGs shared by the two cell lines fell in the mitotic cell cycle, gene expression, cellular metabolic process and so on (Figure 2B). As to the DEGs in cell line T24, they were predicted mainly in endoplasmic reticulum unfolded protein response, cellular protein metabolic process and regulation of cell proliferation (Figure 2C). A certain number of them were predicted to be perinuclear region components of cytoplasm, differing from DEGs in cell line 5637. These data indicated the DEGs of the three categories exhibited differentiable GO features, implying on one aspect the disparities between cell line 5637 and T24.
KEGG pathway analysis results illustrated the disparities of the three DEG categories from another perspective (Figure 3). The first two terms enriched in cell line 5637-specific DEGs were pathogenic *Escherichia coli* infection and cell cycle (Figure 3A) and the first two terms in cell line T24-specific DEGs were *Salmonella* infection and shigellosis (Figure 3C). While those in the DEGs of both cell lines were Parkinson’s disease and oxidative phosphorylation (Figure 3B). Especially, genes pathways related to cancers were predicted in DEGs of cell line 5637 and T24, such as bladder cancer and small cell lung cancer. As was expected, wingless-type MMTV integration site (WNT) family members WNT9A and WNT10A were predicted in the Wnt signaling pathway. Specific factors in these pathways might indicate the possible relationship between these cell lines and some diseases. Interestingly, we found three ISGs, transmembrane protein 173 (TMEM173), signal transducer and activator of transcription 1 (STAT1) and 2’-5’-oligoadenylate synthetase 3 (OAS3) in DEGs of cell line 5637, which might imply their specific roles in cell line 5637.

**qRT-PCR verification**

To verify the sequencing results, qRT-PCR was used to detect the expression levels of three gene categories and two miRNAs. TMEM173, STAT1 and OAS3 were cell line 5637-specific DEGs and results showed they were all down-regulated in cell line 5637 compared to SV-HUC-1, with significant differences (*P* < 0.05, Figure 4A). NADPH-dependent diflavin oxidoreductase 1 (NDOR1) and NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1), belonging to NADH metabolism, were both cell line T24-specific DEGs and were both significantly up-regulated in cell line T24 compared to SV-HUC-1 (*P* < 0.05, Figure 4B). mir-4257, mir-6733, WNT9A and WNT10A were all differentially expressed in cell line 5637 and T24 and were significantly down-regulated in the two cell lines compared to SV-HUC-1 (*P* < 0.05, Figure 4C, 4D). These results were in accordance with the sequencing results and gave more detailed expression profiles of these genes and miRNAs.

**Discussion**

In this study, the transcriptomes of bladder carcinoma cell line 5637 and T24 are sequenced by Illumina high-throughput sequencing, and compared to the normal bladder cell line SV-HUC-1. DEGs and miRNAs specific to cell line 5637 or T24 and those shared by the two cell lines are analyzed. GO and KEGG analysis results show the possible functions and pathways involving these DEGs and miRNAs, including cell cycle, cellular protein metabolic process, the pathogenic infection pathway and cancer-related pathways. The differential expression of several DEGs and miRNAs are further verified by qRT-PCR. Three ISGs, such as TMEM173, STAT1 and OAS3 are down-regulated in cell line 5637. While the NADH metabolism-related factors, NDOR1 and NDUFV1 were up-regulated in cell line T24. Our data reflect the differences between cell line 5637 and T24, and offer basic transcriptome information of the two cell lines for selectively usage in future bladder carcinoma research.

ISGs, classified to be antiviral response gene, are considered to be DNA damage resistance signature (IRDS). IRDS are associated with radio- and chemotherapy resistance of cancers, and STAT1 is one of the IRDS genes identified [24]. TMEM137 (alias stimulator of interferon genes protein, STING) [25] and OAS3 [26] referred to in this study are also important ISGs. As DEGs results showed, STAT1, TMEM137 and OAS3 were all down-regulated in cell line 5637 compared to the normal cell line SV-HUC-1, which was further verified by qRT-PCR results. While in cell line T24, the three genes were not DEGs. It could be deduced that cell line 5637 might exhibit lower resistance to chemotherapy, which implied 5637-like bladder carcinoma patients might be more sensitive to chemotherapy than T24-like patients. Another group of genes, NDOR1 and NDUFV1, were both up-regulated in cell line T24 compared to cell line SV-HUC-1. Since the two genes are both NAD(P)H-related genes, whose deletions or mutations may cause mitochondrial deficiency [27-29], their up-regulations in cell line T24 might infer the abnormal mitochondrial metabolism. Some DEGs were up-regulated or down-regulated in both cell line 5637 and T24, among which WNT9A and WNT10A were verified. Wnt signaling molecules are related to the process of mitogenic, cell development and differentiation [30]. Abnormal expression of WNT9A and WNT10A may lead to pathological conditions, such as congenital diseases and cancer [31], and down-regulation of WNT9A has been proved to promote the cell.
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proliferation of breast cancer cells MA891 [32]. The expression profiles of WNT9A and WNT10A indicated the Wnt signaling pathways in cell line 5637 and T24 both possessed abnormal status, inferring the aberrant cell cycle progress in bladder carcinoma cells.

miRNAs are important in regulating RNA silencing and post-transcriptional regulation of gene expression [33]. Over one third of metazoan transcriptomes are directly regulated by miRNAs [34]. Though no detailed studies have discussed the specific roles of miR-4257 or miR-6733, researchers have shown miR-4257 may bind to genes involving in mitochondrial apoptosis pathway [35]. So based on the data in this study, we could speculate that the differentially expressed miRNAs might regulate the expression of DEGs in cell line 5637 and T24, especially the DEGs in mitochondrial metabolism, thus causing aberrant patterns of bladder carcinoma.

As discussed above, cell line 5637 and T24 could be differentiated from normal bladder cell line SV-HUC-1 based on DEGs and differentially expressed miRNAs. Disparities were also found between cell line 5637 and T24, both being used as bladder carcinoma cells in many studies. The former might be characterized by its lower resistance to chemotherapy, and the latter could be distinguished by its abnormal mitochondrial metabolism. So the two cell lines should be selectively used in bladder carcinoma research. For example, cell line 5637 may be unsuitable for comparative tests on chemotherapy sensitivity, and cell line T24 is an inadequate experimental material for studying the relationship between mitochondrial metabolism and carcinogenesis. Still, further information is necessary for more accurate comparisons of the two cell lines.

To sum up, this study has compared the transcriptomes of two bladder carcinoma cell lines, 5637 and T24, based on data generated from RNA-sequencing. Analyses on DEGs and differentially expressed miRNAs have revealed important features of the two cell lines, including chemotherapy sensitivity and mitochondrial metabolisms. In order to better distinguish and utilize the two cell lines in bladder carcinoma research, further studies are needed to verify the functional mechanisms of these DEGs and miRNAs.

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Disclosure of conflict of interest

None.

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