RNA-seq reveals determinants of sensitivity to chemotherapy drugs in esophageal carcinoma cells

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Abstract: Chemotherapy remains the mainstay of treatment for patients with incurable disease of esophageal carcinoma. Most patients respond poorly to chemotherapy, it is necessary to figure out biomarkers for chemotherapy sensitivity or resistance to perform the individualized therapy. In present work, the sensitivities of two ESCC cell lines to 9 chemotherapy drugs were identified and the transcriptome of these two cell lines were investigated by RNA-seq, the correlation between the sensitivity to drugs and expression of some genes was attempted to construct. Eca-1 was more resistant to most of the chemotherapy drugs than Eca-109 cell line. RNA-seq results showed that there is dramatic difference in the basal expression between these two ESCC cell lines. Pathway analysis demonstrated that these differentially expressed genes were mainly enriched in Gαi signaling, calcium signaling, cAMP-mediated signaling, G-protein coupled receptor signaling and actin cytoskeleton signaling pathways. The molecules in Gαi signaling (ADCY1 and SSTR3) and actin cytoskeleton signaling (MYH6 and MYH7) were highly expressed in multidrug-resistant Eca-1 cells, which were validated by quantitative PCR. Activation of these two pathways results in the upregulation of downstream signaling, PKA signaling and Src-STAT3, and downregulation of RAF-ERK signaling, which was validated by immunoblotting experiments. Our work proposed that activation of Gαi signaling or actin cytoskeleton signaling may confer ESCC cells resistance to most chemotherapy drugs. Our work might provide potential biomarkers and therapeutic targets for treatment of EC patients.

Keywords: Esophageal carcinoma, chemotherapy drugs, RNA-seq, pathway analysis

Introduction

Esophageal carcinoma (EC) is one of the most virulent malignant diseases with high mortality due to the advanced nature of the disease at presentation. At least 50% of patients present with metastatic cancer and most patients with localized disease will develop metastases despite potentially curative local therapy [1]. It ranks as the sixth leading cause of cancer-related mortality and the eighth most common cancer worldwide [2-4]. EC affects more than 481,000 people worldwide and the incidence is increasing rapidly [5-7]. The prognosis is poor and the overall 5-year survival ranges from 15% to 28% [3, 8, 9]. Surgery and preoperative chemoradiotherapy are optional treatments for patients with resectable tumors to treat both esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). Cytotoxic chemotherapy remains the mainstay of treatment for patients with incurable disease [10]. The most commonly utilized chemotherapy agents are fluoropyrimidine, taxanes (paclitaxel or docetaxel), and platinum compounds. Although both EAC and ESCC are responsive to chemotherapy, the response rates are low [1, 11, 12], especially for patients with advanced diseases [13, 14]. Regarding that most patients respond poorly to chemotherapy, it is necessary to figure out biomarkers for chemotherapy sensitivity or resistance to perform the individualized therapy.

Previous studies have suggested that several categories of molecules are correlated with the response and/or prognosis of ESCC patients treated with neoadjuvant chemoradiation therapy (CRT): receptor tyrosine kinase (EGFR, MET) [15, 16], tumor suppressors (p53, p21) [17],
cell cycle regulators (Cyclin D1, CDC25B, 14-3-3sigma) [18], DNA repair molecules (p53R2, BRCA1, ERCC1, MLH1) [18-20], cytokines-related (IL6, sIL6R) [21, 22], drug resistance proteins (MRP2) [23], angiogenic factors (VEGF) [18], molecules involved in cell proliferation/invasion/metastasis (Ki-67, COX-2) [18, 24], PI3K/AKT/mTOR signaling molecules (AKT2, mTOR) [9, 25], wnt/β-catenin signaling molecules (PITX2) [26], and hedgehog signaling molecules (Gli-1) [28]. In addition, several molecules (heat-shock proteins and glucose-regulated proteins, COX7A2, CDK4/6 and Ephrin B3 receptor) [29-32] were supposed to be associated with the sensitivity of EAC cells to chemotherapy. Although so many potential biomarkers for chemotherapy to ESCC and EAC patients have been proposed, few were validated in prospective clinical trials. Furthermore, above biomarkers were mostly deduced through data from DNA microarray, immunohistochemistry, or tissue microarrays. These methods, especially DNA microarray, have many limitations when compared to the next-generation sequencing (NGS) techniques.

RNA-seq (RNA Sequencing), also called “Whole Transcriptome Shotgun Sequencing”, is a technology that uses the capabilities of NGS to reveal a snapshot of RNA presence and quantity from a genome at a given moment in time. This facilitates sequencing of the RNA transcripts in cells, providing the ability to look at alternative gene spliced transcripts, post-transcriptional changes, gene fusion, mutations/SNPs and changes in gene expression [33]. In addition to mRNA transcripts, RNA-Seq can look at different populations of RNA to include total RNA, small RNA, such as miRNA, tRNA, and ribosomal profiling [34]. Moreover, RNA-seq is demonstrated to exhibit a much wider dynamic range and greater precision for 97% of expressed genes [35, 36], compared to microarray-based measurements of gene expression.

In this work, two ESCC cell lines were subjected to several chemotherapy drugs to test the sensitivity to these drugs. And then RNA-seq was carried out in the cell lines and the differentially expressed genes were applied to pathway analysis. Then the expression signatures were linked with drug sensitivity, which was validated by qPCR and immunoblotting.

Materials and methods

Cell culture

The ESCC cell lines, Eca-109 and Eca-1, were used in this work. Eca-109 cell line was purchased from China Center for Type Culture Collection, while Eca-1 cell line is a generous gift from Dr. Yao-Qing Yang, Tumor Cell Biology Research Institute of Tongji University, China. These two cell lines were maintained in DMEM medium (Gibco) supplemented with 10% FBS (HyClone), penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (Life Technologies) in a humidified atmosphere containing 5% CO₂ at 37°C. Cells in the exponential growth phase were used for all the experiments.

Determination of IC50 dose by MTS assay

Eca-109 and Eca-1 cells (1000 cells each well) were grown in 100 μl of DMEM medium containing serum per well in a 96-well plate. After 24 h, the cells were treated with seven or nine doses of 9 chemotherapy drugs (paclitaxel, gemcitabine, docetaxel, topotecan, irinotecan, flouxuridine, and cisplatin, epirubicin, and fludarabine) for 120 h. The seven doses were 1/125-, 1/25-, 1/5-, 1-, 5-, 25-, and 125-fold of reference IC50, respectively. The nine doses were 1/100-, 1/31.6-, 1/10-, 1/3.16-, 1-, 3.16-, 10-, 31.6-, and 100-fold of reference IC50, respectively. The reference IC50 doses for paclitaxel, gemcitabine, docetaxel, topotecan, irinotecan, flouxuridine, and cisplatin, epirubicin, and fludarabine were 0.05, 0.10, 0.005, 0.04, 10, 0.20, 10, 0.2 and 4 μmol/L, respectively. Every treatment was triplicate in the same experiment. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 1 to 4 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer’s protocol. The IC50 calculation was performed with GraphPad Prism 5.0 software.

RNA-seq

Eca-109 and Eca-1 cells (8×10⁴) were grown in 2 ml of DMEM medium containing serum per well in a 6-well plate with duplication. All the samples were homogenized with 1 ml Trizol (Invitrogen, Life Technologies) and total RNAs were extracted according to the manufacturer’s instruction.
Preparation of cDNA followed the procedure described in Trapnell et al. [37]. The cDNA library was size-fractionated on a 2% TAE low melt agarose gel (Lonza catalog # 50080), a narrow slice (∼2 mm) of the cDNA lane centered at the 300 bp marker was cut. The slice was extracted using the QiaEx II kit (Qiagen catalog # 20021), and the extract was filtered over a Microcon YM-100 microconcentrator (Millipore catalog # 42409) to remove DNA fragments shorter than 100 bps. One-sixth of the filtered sample volume was used as template for 15 cycles of amplification using the paired-end primers and amplification reagents supplied with the Illumina ChIP-Seq genomic DNA prep kit. Each library was loaded into its own single Illumina flow cell lane, producing an average of 14.5 million pairs of 51-mer reads per lane (8.4 million purity filtered read pairs), or nearly 1.5 Gb of total sequence for each sample. Transcripts were assembled from the mapped fragments sorted by reference position.

Quantitative real-time PCR (qPCR)

Total RNA above isolated was synthesized to cDNA using PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with mixture of oligo-dT and Random Primer (9 mer). The primers used for qPCR validation were list in Table 1. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control hActb. Gene expression was calculated relative to expression of hActb endogenous control and adjusted relative to expression in Eca-109 cells.

Protein isolation and western blotting

Cell pellets were resuspended in 1×SDS loading buffer (1 mmol/L Na3VO4, 10 mmol/L NaF, 1 mmol/L PMSF) containing protease inhibitors. Lysates (20 μg each lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for GAPDH (Abmart, O80922), AKT (Santa Cruz, sc8312), p-AKT (Santa Cruz, SC7985-R, pS473), ERK (Abclonal, A0228) and p-ERK (Cell signaling, # 9106S, pT202/204) were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) and visualized by chemiluminescence detection system (Millipore, WBKLS0500).

Results

Eca-1 cells display resistance to many cytotoxic drugs compared to Eca-109

Nice chemotherapy drugs were subjected to Eca-109 and Eca-1 cell lines. For each drug, 7 or 9 different doses were used to treat the two cell lines and the IC50 dose was calculated with the aid of GraphPad Prism 5.0 software (Figure 1A and 1B). Interestingly, Eca-1 cells were relatively more resistant to seven cytotoxic drugs (paclitaxel, gemcitabine, docetaxel, topotecan, irinotecan, flouxuridine, and cisplatin) than Eca-109, while there were no dramatically different sensitivities between these cell lines to epirubicin, Eca-109 was more resistant to fludarabine (drug used in the treatment of hematological malignancies) than Eca-1.

RNA-seq showed that hundreds of genes were differentially expressed between Eca-109 and Eca-1 cell lines and Gαi signaling pathway was activated in Eca-1 cells

Total RNAs from Eca-109 and Eca-1 cells were applied for RNA-seq. The raw data were normalized in a standard distribution, and the basal expression difference was analyzed. The results showed that there were 162 lowly-expressed genes and 186 highly-expressed genes in Eca-1 cells, compared to that in Eca-109 cells. The expression difference was higher than 4-fold for these 348 genes between the two cell lines. The top 40 differentially-expressed genes were list in Table 2.

### Table 1. Primers used for qPCR validation

<table>
<thead>
<tr>
<th>gene</th>
<th>forward</th>
<th>reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>CACCATGTACCTTGCGATT</td>
<td>GTACCTGGCGGTCAAGGAGG</td>
</tr>
<tr>
<td>ADCY1</td>
<td>CGTCCCTGCTCTGTCTAATACC</td>
<td>AGGCCACCTGGAAAACACT</td>
</tr>
<tr>
<td>SST3</td>
<td>CCTGCTCTTCTTGGCTCTA</td>
<td>GCCGTTAGAGAGAAGGACATA</td>
</tr>
<tr>
<td>TRPV6</td>
<td>CTGCTGGGGATACATAA</td>
<td>CGAAGTGAGACACCGAGCTA</td>
</tr>
<tr>
<td>IGFBP5</td>
<td>TGACCGAAAAGATTCTACAAG</td>
<td>CGTCAACGTACTCCATGCCT</td>
</tr>
<tr>
<td>MYH7</td>
<td>CTTTGCCGTTATGGCAAGGCTT</td>
<td>AGATGCGCAACTTTCTCTGTTGC</td>
</tr>
<tr>
<td>MYH6</td>
<td>CCAGAAGAGGACAGCAAGAT</td>
<td>TGACTACCTGGTTGCCCACTT</td>
</tr>
<tr>
<td>PAX5</td>
<td>ACTGCTCATCGGAAGTTCAG</td>
<td>TCTCATAATACGCCAGCTT</td>
</tr>
</tbody>
</table>

And then these 348 genes were applied to Ingenuity Pathway Analysis (IPA) online software. The results showed that these genes were mainly enriched in Gαi signaling, calcium signaling, cAMP-mediated signaling, G-protein coupled receptor signaling and actin cytoskeleton signaling pathways (Figure 2A). In Gαi signaling pathway, the downstream PKA and Src-STAT3 signaling were predicted to be activated, while the ERK signaling was predicted to be suppressed in Eca-1 cell line (Figure 2B). These predictions were deduced basing on the highly expression of four genes (ADCY1, CNR1, PTGER3 and SSTR3) and the low expression of CHRM2 in Eca-1 cells, compared to that in Eca-109 cells.

qPCR validation demonstrated that molecules in Gαi signaling and actin cytoskeleton signaling were highly expressed in Eca-1 cells

To validate the RNA-seq data, expression of 7 genes was investigated in Eca-109 and Eca-1 cells by qPCR assay. The relative expression of these 7 genes in Eca-1 was log2 transformed and plotted (Figure 3). The change folds varied to some extent between the RNA-seq data and qPCR data, however, expression trends of most of genes were consistent between two data sets except that of two genes, MYH7 and MYH6. RNA-seq data showed that MYH7 and MYH6 were expressed at a low level in Eca-1, while the qPCR data demonstrated that MYH7 and
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Table 2. Top 40 differentially expressed genes between Eca-109 and Eca-1 cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change (Eca-1 vs Eca-109)</th>
<th>Gene</th>
<th>Fold Change (Eca-1 vs Eca-109)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRT18P1</td>
<td>0.08</td>
<td>CCDC144NL</td>
<td>7.35</td>
</tr>
<tr>
<td>AC097639.8.1</td>
<td>0.09</td>
<td>RP11-203M5.6.1</td>
<td>7.60</td>
</tr>
<tr>
<td>RP11-55L3.2.1</td>
<td>0.10</td>
<td>RP11-450H5.2.1</td>
<td>7.91</td>
</tr>
<tr>
<td>MPPED2</td>
<td>0.10</td>
<td>MKRN4P</td>
<td>8.03</td>
</tr>
<tr>
<td>CTD-2089O24.1.1</td>
<td>0.11</td>
<td>RP11-173E2.1.1</td>
<td>8.16</td>
</tr>
<tr>
<td>RP11-405A12.1.1</td>
<td>0.11</td>
<td>AC104843.3.1</td>
<td>8.24</td>
</tr>
<tr>
<td>SCDP1</td>
<td>0.11</td>
<td>PRKIRP1</td>
<td>8.31</td>
</tr>
<tr>
<td>RP3-342P20.2.1</td>
<td>0.12</td>
<td>RP11-392P7.1.1</td>
<td>8.39</td>
</tr>
<tr>
<td>RP11-64K12.1.1</td>
<td>0.12</td>
<td>WBP1P1</td>
<td>8.48</td>
</tr>
<tr>
<td>RP11-211P0.2.1</td>
<td>0.12</td>
<td>AC006026.9.1</td>
<td>8.57</td>
</tr>
<tr>
<td>HERC2P5</td>
<td>0.13</td>
<td>RP11-297L17.6.1</td>
<td>8.69</td>
</tr>
<tr>
<td>API5P1</td>
<td>0.13</td>
<td>ST13P18</td>
<td>8.79</td>
</tr>
<tr>
<td>RBMXP2</td>
<td>0.13</td>
<td>FTH1P12</td>
<td>8.93</td>
</tr>
<tr>
<td>RP13-395E19.2.1</td>
<td>0.13</td>
<td>ST13P5</td>
<td>9.07</td>
</tr>
<tr>
<td>RP11-798L4.1.1</td>
<td>0.13</td>
<td>TAF1L</td>
<td>9.53</td>
</tr>
<tr>
<td>RP13-98N21.2.1</td>
<td>0.14</td>
<td>SUCLA2P1</td>
<td>9.89</td>
</tr>
<tr>
<td>RP13-98N21.3.1</td>
<td>0.14</td>
<td>CTB-33G10.1.1</td>
<td>10.18</td>
</tr>
<tr>
<td>ESRRAP1</td>
<td>0.14</td>
<td>RP11-244F12.1.1</td>
<td>10.49</td>
</tr>
<tr>
<td>AC139452.2.1</td>
<td>0.14</td>
<td>KCNA7</td>
<td>12.56</td>
</tr>
<tr>
<td>GLULP4</td>
<td>0.14</td>
<td>USP8P1</td>
<td>18.25</td>
</tr>
</tbody>
</table>

MYH6 were highly expressed in Eca-1 cells. Expression trends of 71% (5/7) genes in RNA-seq data were validated by qPCR, suggesting that the RNA-seq data were reliable for further analysis. ADCY1, SSTR3, TRPV6, IGFBP5, MYH7 and MYH6 were validated to be highly expressed, while PAX5 was validated to be low expressed in Eca-1 cells.

Western blotting experiments showed that AKT/ERK signaling was activated in Eca-109 cells

Then the most famous signaling molecules essential for cell growth and survival, AKT and ERK, were investigated in Eca-109 and Eca-1 cells. Interestingly, the phosphorylation of AKT and ERK was activated in Eca-109, compared with that in Eca-1.

Discussion

Chemotherapy remains the mainstay of treatment for patients with incurable disease of esophageal carcinoma. Most patients respond poorly to chemotherapy, it is necessary to figure out biomarkers for chemotherapy sensitivity or resistance to perform the individualized therapy.

In present work, the sensitivities of two ESCC cell lines to 9 chemotherapy drugs were identified and the transcriptome of these two cell lines were investigated by RNA-seq, the correlation between the sensitivity to drugs and expression of some genes was attempted to construct.

RNA-seq results showed that dramatic difference existed in the basal expression between these two cell lines. Pathway analysis demonstrated that these differentially expressed genes were mainly enriched in Gαi signaling, calcium signaling, cAMP-mediated signaling, G-protein coupled receptor signaling and actin cytoskeleton signaling pathways. Gαi signaling pathway has been proposed to mediate the signal transduction from Hedgehog signaling to NF-κB signaling [38, 39], cross-talk to EGFR signaling pathway [40], and regulate cancer cell proliferation by Src-STAT3 signaling [41]. In our data, Gαi signaling was predicted to be activated in Eca-1 cells mainly based on the highly expression of ADCY1 and SSTR3, which was validated by qPCR (Figure 3). Due to the highly expression of ADCY1, downstream PKA and Src-STAT3 signaling were predicted to be activated whereas the RAF-ERK signaling were pre-
Figure 2. Ingenuity pathway analysis (IPA) for those differentially expressed genes between Eca-109 and Eca-1 cells. A: The most significant canonical pathways in which the differentially expressed genes were enriched. The 348 differentially expressed genes (expression difference >4-fold) were applied to Ingenuity Pathway analysis (IPA) software, and the most significant canonical pathways were shown. B: Gαi signaling pathway was predicted to be activated in multidrug-resistant Eca-1 cells.
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Actin cytoskeleton signaling has been associated with the sensitivity of cancer cells to antimicrotubule drugs, such as paclitaxel, docetaxel and vinorelbine [42-44]. In our work, RNA-seq data suggested that actin cytoskeleton signaling was suppressed in Eca-1 cells, based mainly on the lower expression of MYH6 and MYH7. However, subsequent qPCR results showed that MYH6 and MYH7 were actually highly expressed in Eca-1. Therefore, the actin cytoskeleton signaling should be activated in Eca-1 cells. This demonstrates that although RNA-seq has been proposed to be relative more accurate than DNA microarray [35, 36], further validation by qPCR is essential before the data have been used to deduce some conclusions.

Collectively, our work proposed that activation of Gα(i) signaling or actin cytoskeleton signaling may confer ESCC cells resistance to most chemotherapy drugs. Our work might provide potential biomarkers and therapeutic targets for treatment of EC patients.

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Disclosure of conflict of interest
We have no conflict of interest to declare.

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