Original Article

Pathway analysis of esophageal squamous cell carcinoma using iTRAQ-based quantitative proteomic approach

Xuefen Wang¹*, Keming Zhou²*, Bowei Wang¹, Xinling Yan³, Xia Liu¹, Qiaoxin Li¹, Yuqing Ma¹

¹Department of Pathology, First Affiliated Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang, China; ²Hypertension Center of The People’s Hospital of Xinjiang Uygur Autonomous Region, Hypertension Institute of Xinjiang Uygur Autonomous Region, Urumqi 830001, Xinjiang, China; ³Department of Pathology, Urumqi City Hospital of Traditional Chinese Medicine, Urumqi 830001, Xinjiang, China. *Equal contributors.

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Abstract: Purpose: Esophageal squamous cell carcinoma (ESCC) is one of the most common digestive cancers. Many proteins and genes have been found to be associated with ESCC. However, there is rare study on the pathways and biological processes made up of the activities of those multiple genes and proteins. In this study, several key pathways closely related to ESCC were revealed. Furthermore, it was found that Wnt signaling pathway played a very important role in the development of ESCC. Methods: A combination of isobaric tags for relative and absolute quantification (iTRAQ) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis were used to target the proteins, which abnormally expressed. Then, the Clusters of Orthologous Groups (COG) database and The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database were matched against by those proteins’ terms. Finally, after comparing and analyzing, some key pathways were revealed. Findings and conclusions: With the methods, differentially expressed proteins (DEPs) in ESCC were identified. There were a total of 431 kinds of abnormal expressed proteins found from samples. 262 kinds were over-expressed (iTRAQ ratios of ≥1.5), and 169 kinds were under-expressed (iTRAQ ratios of ≤0.67). Based on those data, this subject pointed out several key pathways with a high degree of confidence (p-value<0.0001), including Focal adhesion, DNA replication, ECM-receptor interaction, which were the most closely related to ESCC. The implicated biological processes and signaling pathways could help elucidate the molecular mechanisms of ESCC carcinogenesis and provide new targets for clinical treatment.

Keywords: iTRAQ, esophageal squamous cell carcinoma (ESCC), pathway analysis, Wnt signaling pathway

Introduction

Esophageal cancer (EC) is one of the most lethal digestive malignancies, and it ranks the sixth place of cancer death of the world [1]. The main subtype of EC is esophageal squamous cell carcinoma (ESCC), which is accounting for nearly 90% [2]. About 80% of EC occur in the developing countries, which are with obvious geographic diversity [3, 4]. According to the International Agency for Research on Cancer (IARC) statistics, 456,000 new cases (3.2% of the total) were diagnosed as EC and 400,000 deaths (4.9% of the total) died from EC each year worldwide [5]. Despite the medical care has made great progress over past decades, the overall 5-year survival rate of it is still below 20% [6-8]. Therefore, it is necessary to investigate the pathogenic mechanisms of ESCC.

Isobaric tags for relative and absolute quantitiation (iTRAQ) is a new method of quantitative research on proteomics in recent years. Combined with mass spectrometry, it is an ideal technique for finding biomarkers [9, 10]. The iTRAQ method regarded as a quantitative approach has been used for the identification of biological markers for different cancers including breast cancer [11], renal cell carcinoma [12], oral cancer [13], hepatocellular carcinoma [14], endometrial cancer [15], non-small cell lung carcinoma [16] and nasopharyngeal carcinoma [17].

In this study, we performed quantitative proteomic analysis using iTRAQ method and LC-MS to identify DEPs. The purpose of this subject was to determine the potential key pathways in ESCC by KEGG pathway analysis.
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Materials and methods

Tissue samples

Informed consent was signed by each patient, and the study was approved by the Ethical Committee of the First Affiliated Hospital of Xinjiang Medical University. Tumor tissues and adjacent normal epithelium tissues were obtained from six ESCCs, and each of the adjacent normal epithelium tissue was at least 5 cm away from the edge of ESCC regarded as a control. All HE staining sections of paraffin specimens were confirmed by two independent experienced pathologists. No necrosis was observed under the microscope. The tumor tissues were labeled as 115/118/121, respectively. Meanwhile, their normal tissues were labeled as 113/116/119, respectively. All the tissues were kept at -80°C before use.

Protein extraction and preparation

First, 100 mg fresh tissue were ground into powder in liquid nitrogen, added the right amount of protein lysis buffer, and then, added the final concentration of 1 mm PMSF (Phenylmethanesulfonyl fluoride) and 2 mm EDTA (ethylene diamine tetraacetic acid) in order. Next, after 5 minutes, added final concentration of 10 mm DTT (DL-Dithiothreitol). The tissues were shaken by ultrasonic for 15 minutes and centrifuged at 25000 × g for 20 minutes. Took the supernatant. In order to extract the supernatant, added the pre-cooled acetone, precipitated for 2 hours at -20°C, then centrifuged at 16000 × g for 20 minutes, discarded supernatant and took the precipitation, added the appropriate amount of protein lysis buffer again, repeated the above process. Finally, the supernatant was quantified by Bradford method for protein concentration.

iTRAQ labeling and SCX separation

The protein was digested with Trypsin (Promega, Madison, WI, USA) at 37°C for 16 h. After trypptic digestion, stem peptides were dried by vacuum centrifugal pump with 0.5 mol/l TEAB complex soluble peptides and performed according to the instruction of the iTRAQ labeling. Strong cation exchange (SCX) chromatography was performed with a LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan). With the liquid phase separation column for 4.6 × 250 mm model by Ultremex SCX, the samples finished the liquid phase separation and removed salt, respectively. Finally, freeze dried and reserved at 4°C.

LC-MS/MS proteomic analysis

All steps were carried out according to the instructions. Data acquisition was performed with a Triple TOF 5600 System (AB SCIEX, Concord, ON) comprising a Nanospray III source and a pulled quartz tip worked as the emitter (New Objectives, Woburn, MA). Data was acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 psi, nebulizer gas of 15 psi, and an interface heater temperature of 150°C. The MS was operated with a RP of greater than or equal to 30000 FWHM for TOF MS scans.

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

Only converting into MGF files, raw data files can be used. Protein identification was performed by using Mascot search engine (Matrix Science, London, UK; version 2.3.02) against database. To reduce the probability of false peptide identification, only peptides with significance scores (≥20) at the 99% confidence intervals (CIs) by a Mascot probability analysis greater than “identity” were counted as identified. For protein quantitation, it was required that a protein contains at least two unique peptides. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. Proteins were considered to be differentially expressed if iTRAQ ratios were ≥1.5 or ≤0.67. The Clusters of Orthologous Groups (COG) database was used to classify the function of proteins. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database was used to classify and group these identified proteins and match the corresponding pathway.

We applied the most widely used hypergeometric test to explain the concepts of COG analysis/KEGG pathway analysis. Used COG analysis as an example. With respect to a background set of genes (COG database), the following n denoted as the number of genes in our study and m was the number of genes in the background COG database (http://www.geneontology.org/). Further, let nt and mt be the number of genes annotated to a term in the study set and the background database respectively. The
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**Figure 1.** COG (Cluster of Orthologous Groups) function classification analysis. PIE chart showed the results of COG analysis.

**Table 1.** The most important metabolism and signal transduction pathways in the development of ESCC

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Genes NO.</th>
<th>Patway ID</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal Adhesion</td>
<td>17</td>
<td>ko04510</td>
<td>ECM ITGA ITGB Filamin Parvin Talin PK</td>
<td>ECM caveolin Actinin Paxillin Parivin Filamin MLcp MLC MLCK RAP1</td>
</tr>
<tr>
<td>Arginine and Proline metabolism</td>
<td>5</td>
<td>ko00330</td>
<td>Pcar1 PCS GLSK GLNA P4HA1</td>
<td></td>
</tr>
<tr>
<td>ECM-receptor interaction</td>
<td>9</td>
<td>ko04512</td>
<td>laminin THBS Tenascin Collagen Agrin</td>
<td>vitronectin VWF collagen Agrin</td>
</tr>
<tr>
<td>DNA replication</td>
<td>12</td>
<td>ko03030</td>
<td>SSB MCM2-7 RFA1 RFA2/4 RPA3 PCNA FEN1</td>
<td></td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>9</td>
<td>ko04610</td>
<td>F5 SERPINA1 PLAU SERP1NF2 C4 CIS CIR</td>
<td>A2M CIR</td>
</tr>
</tbody>
</table>

**p-value** $p(t)$ represented the likelihood that a term $t$ meant as many genes as we had observed in our list of genes was calculated as following,

\[
p(t) = \frac{\min(mt, n)}{n} \left( \frac{m}{n} \right) \left( \frac{m - mt}{n - k} \right) \left( \frac{m - mt}{n} \right)
\]

This term of genes/function we studied was reported as an enriched term/function based on COG database if the $p$-value was below a significance threshold of 0.05.

**Result**

**Differentially expressed proteins (DEPs)**

The iTRAQ labeling of peptides derived from six human ESCC tissues and paired adjacent normal tissues. The tumor tissues were labeled as 115/118/121, respectively. Meanwhile, their normal tissues were labeled as 113/116/119, respectively. A total of 342788 spectra were obtained from the ESCC tissues and normal tissues. Removing low score spectrum, there were 60041 unique spectra left and 4999 proteins were matched. There were a total of 431 differen-
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Initially expressed proteins detected from six samples: 262 were over-expressed and 169 were under-expressed.

**COG functional analysis**

All identified proteins were matched against the Clusters of Orthologous Groups (COG) database and classified into 23 categories (Figure 1). In this way, it was clearly known the function of these proteins involved. It was found that the top three functions were as follows: replication, recombination and repair (accounting for 21.33%); lipid transport and metabolism (accounting for 11.17%); signal transduction mechanisms (accounting for 6.87%). Thus, the dysfunctional proteins were mainly involved in the metabolism and pathways during the development of ESCC.

**The most important metabolism and signal transduction pathways**

Through COG functional analysis, it was found that the biochemical metabolic pathways and signal transduction pathways played important roles in the pathogenesis of ESCC. In this study, there were 213 pathways matched for DEPs. Then, the most important biochemical metabolic pathways and signal transduction pathways of DEPs were determined by KEGG pathway analysis with a high degree of confidence (p-values <0.0001), which were shown in Table 1. It was found that there were only up-regulated DEPs in the process of Arginine and proline metabolism and DNA replication. Moreover, DNA replication, which is the process of continuous cell proliferation, was quite active. It was consistent with the biological behavior of the tumor. In addition, the protein of ECM played an important role in Focal adhesion and ECM-receptor interaction.

**The Wnt signaling pathway**

The biological process of cells are involved in the physiological function such as cell proliferation, cell differentiation, cell adhesion, cell migration and cell apoptosis. The interaction between cells is through the cell communication system. Cells communicate with each other and respond to environmental conditions through cell signal transduction pathways. According to the cell communication system, cell signal pathway could be divided into five types including Notch signaling pathway, Wnt signaling pathway, NF-kappa B signaling pathway, Hedgehog signaling pathway, TGF-β signaling pathway. These signaling pathways have been well studied in multiple types of cancers. The abnormality of these signaling pathways can cause abnormal cell metabolism, which would lead to various diseases such as cell differentiation, development and tumor. The process of carcinogenesis is the changes of genetic material in normal cells. Tumor progression is a continuum of dynamic molecular and cellular changes.

The detailed information which altered in these cell signaling pathways of ESCC was shown in Table 2. The result revealed that the number of abnormal proteins in the Wnt signaling pathway was up to 64, ranked the second place of the cell communication system. However, the role of Wnt signaling pathway in ESCC has been little reported.

Through the above analysis, it was found that Wnt signaling pathway was very important during the development of ESCC. Altered proteins in the Wnt signaling pathway of ESCC were listed in the KEGG diagram with pathway ID ko04310 (Figure 2). Proteins in the red boxes were DEPs. As shown in the diagram, it was consisted of the canonical Wnt pathway, the planar cell polarity pathway and the Wnt/Ca2+ pathway. There were 32 DEPs as compared to adjacent non-malignant tissue. The number of DEPs in the canonical Wnt pathway was up to 20, while the number of DEPs in the non-classical pathway was only 12 (shown in the Figure 2). In addition, it was found that Wnt signaling pathway was a complex network of protein interaction. It was influenced by many other tumor-related pathways such as MAPK signaling pathway, P53 signaling pathway, TGF-beta pathway and other pathways. Furthermore, it was associated with Cell cycle, Adherens junction and Focal adhesion. Therefore, Wnt signaling pathway played a very important role in the occurrence of ESCC.

<table>
<thead>
<tr>
<th>Signaling Pathway</th>
<th>Different Protiens</th>
<th>Pathway ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch</td>
<td>12</td>
<td>ko04330</td>
</tr>
<tr>
<td>Wnt</td>
<td>64</td>
<td>ko04310</td>
</tr>
<tr>
<td>NF-KB</td>
<td>83</td>
<td>ko04064</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>7</td>
<td>ko04340</td>
</tr>
<tr>
<td>TGF-B</td>
<td>35</td>
<td>ko04350</td>
</tr>
</tbody>
</table>

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Discussion

ESCC is an upper digestive malignant tumor, with the majority of cases diagnosed at an advanced stage when treatments are not satisfied. When detected early, its survival rates are over 90% [18]. Therefore, novel biomarkers are needed to detect ESCC in its earliest stage.

In the study, there were 213 pathways matched for DEPs. As shown in Table 1, DNA replication was very important for the development of ESCC and there were 12 DEPs including the MCM complex (Mini-chromosome maintenance proteins 2-7), PCNA (proliferating cell nuclear antigen). But, all of them were not driving genes. The expressions of MCM2-7 in ESCC tissues were 1.9-2.6 times higher than in normal adjacent tissues. MCM2-7 as DNA replication licensing factors were involved in the function of Cell proliferation. MCM2-7 as the core of replicative helicase cannot associate with DNA under physiological conditions [19]. MCM2-7 was a critical process, and misleading of this reaction could have fatal consequences for the cell [20]. The expression of PCNA in ESCC tissues was 1.7 times higher than in normal adjacent tissues. PCNA participated in DNA replication and repair as well as interaction with P21 regulating the cell cycle [21]. As we know, DNA replication is a carefully designed process that is central to genome integrity, while misleading of DNA replication would lead to genomic instability, disease or cancer.

The Wnt signaling pathway is a highly conserved pathway. It plays key roles not only in embryonic development but also in cancer biology [22, 23]. Increased Wnt signaling pathway has been involved in many different human cancers [24, 27]. The clinical importance of this pathway has been demonstrated by mutations that could lead to a variety of diseases including breast cancer [24], prostate cancer [28], glioblastoma [29], type II diabetes [30] and other diseases. However, there was little report about the WNT pathway in ESCC. The Wnt signaling pathway could be classified into three major types including the canonical Wnt pathway, the planar cell polar-
ty pathway and the Wnt/Ca2+ pathway [31]. The canonical Wnt signaling pathway also named the Wnt/β-catenin pathway was considered as the most prevalent mechanism in the development of cancer, and its activation was a highly integrated process with complex multiple steps involving in Wnt2, CK1, Axin, APC, β-catenin, TCF, TAK1 and SMAD4. Wnt2, as a member of DEPs, binded with the Fzd receptor on the membrane to initiate the canonical Wnt signaling pathway. CK1, Axin, APC and GSK-3β formed a complex of Axin, priming β-catenin for further phosphorylation by GSK3β and subsequent degradation [32]. Stabilized β-catenin translocated from membrane to nucleus and activated the expression of TCF/LEF (T cell factor/lymphoid enhancing factor)-triggered target genes [33]. TAK1 constituted a part of pro-inflammatory, activating NF-kappa B and MAPK pathways [34]. However, the study of them in ESCC was little reported.

Signaling by the Wnt family is one of the fundamental mechanisms that direct cell proliferation, cell polarity and cell fate determination. Wnt signaling pathway is a complex network of protein interaction. In most cases, it does not play a role independently. It is coordinated with many other pathways such as Notch signaling pathway, MAPK and P53 signaling pathway. These signaling pathways are coordinated through a large number of crosstalk. For example, Wnt signaling pathway links NF-kappa B pathway to MAPK pathway by the protein of TAK1. TGF-β signaling pathway is associated with Wnt signaling pathway through the protein of SMAD4. Therefore, in order to better understand the role of Wnt signaling pathway of ESCC, it is necessary to further study the crosstalk between Wnt signaling pathway and other signaling pathways.

Conclusion

In this study, the purpose was to find out which pathways and processes were likely to be involved in ESCC by KEGG pathway analysis. It was found that the metabolism and signal transduction pathways was the most important process in ESCC. In addition, it was found that Wnt signaling pathway played an important role in the development of ESCC. However, there was little reported research about the Wnt signaling pathway in ESCC. Pathway analysis can help us easily find new therapeutic targets, which could be used as diagnostic, predictive and prognostic markers.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Yuqing Ma, Department of Pathology, First Affiliated Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang, China. Tel: +86-130 7993 8941; E-mail: mayuqing.patho@hotmail.com

References

[8] Pennathur A, Farkas A, Krasinskas AM, Ferson PF, Gooding WE, Gibson MK, Schuchert MJ,


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[34] Dai L, Aye Thu C, Liu XY, Xi J and Cheung PC. TAK1, more than just innate immunity. IUBMB Life 2012; 64: 825-834.