

Analysis of Key Pathways, Genes and Immune Cell Infiltration in Metastatic Esophageal Squamous Cell Carcinoma Based on Bioinformatics

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Research Article

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Analysis of key pathways, genes and immune cell infiltration in metastatic esophageal squamous cell carcinoma based on bioinformatics

[Abstract]: Objective: To seek potential metastatic tumor markers in esophageal squamous cell carcinoma (ESCC) by bioinformatics. **Methods:** Four datasets (GSE70409, GSE26886, GSE161533, GSE17351) were downloaded from GEO database, and the differential expression of mRNA was screened by Limma software package. Gene ontology function and kyoto encyclopedia of genes and genomes pathway enrichment analysis were performed using the database for annotation; Protein–protein interaction network of these DEGs was constructed based on STRING database; then, the hub gene were identified by six algorithms of Cytoscape software. Using GEPIA2 database and OncoLnc database to analyze the survival of hub genes. Immune infiltration was analyzed by TIMER2 database and CIBERSORT deconvolution method. **Results:** A total of 244 differentially expressed genes (DEGs) were screened from 4 data sets of GEO database, including 164 up-regulated genes and 80 down-regulated genes, which were mainly enriched at extracellular region and extracellular space, and involved in biological processes such as cell adhesion and proteolysis related to tumor invasion and metastasis. KEGG results showed that DEGs were mainly enriched in PI3K-Akt signal pathway and extracellular matrix (ECM) receptor interaction signal pathway. Through the protein interaction analysis of DEGs, we found that three hub genes (CXCL8, MMP9 and MMP13) were highly expressed in ESCC. However, the GEPIA2 database shows that only CXCL8 is associated with the overall survival of ESCC patients. The results of immune infiltration showed that macrophages and resting dendritic cells increased significantly, while mast cells and monocytes decreased significantly. **Conclusion:** Three hub genes and immune infiltrating cells may play an important role in ESCC, and are expected to become potential tumor markers of ESCC and be used in the diagnosis and treatment of ESCC.

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Introduction

Esophageal cancer (ESCA) is one of the most common malignant tumors in the world. In the global cancer statistics 2020¹, the incidence of esophageal cancer ranks seventh, and esophageal squamous cell carcinoma (ESCC) accounts for about 90% of esophageal cancer. Due to the absence of typical and early clinical symptoms and the lack of simple and effective early clinical diagnosis methods, esophageal squamous cell carcinoma was often diagnosed in the middle and late stage. Up to now, surgical treatment is still the main treatment for ESCC in the early stage, but the five-year clinical survival rate is still not ideal². So far, the molecular mechanism of the occurrence and development of ESCC has not been fully clarified, and there is an urgent need to find more potential tumor markers for effective evaluation of diagnosis and prognosis.

CXCL8, also known as IL-8, has been shown to up-regulate in many malignant tumors and is related to tumor progression. High levels of CXCL8 in serum and tissues are often associated with distal metastasis and poor prognosis of ESCC³. At present, numerous studies have demonstrated that CXCL8 promotes the progression of ESCC mainly through the recruitment of tumor-associated macrophage⁴ and myeloid-derived suppressor cell⁵. MMP9 and MMP13 belong to the matrix metalloenzyme family. Gu et al⁶ found that MMP9 could be a negative independent prognostic factor of ESCC, which played a role in early ESCC with MMP13.

Some scholars believe that the degree of immune cell infiltration in tumor microenvironment is helpful to analyze the immune status of tumor and evaluate the effect of immunotherapy⁷. Most immune cells can secrete CXCL8, previous studies have found that CXCL8 in bladder cancer⁸ and prostate cancer⁹ can increase the activity of MMP9 and promote further metastasis of tumor cells. Meanwhile, MMP9 and MMP13 are highly expressed in tumors such as ESCC, and MMPs may become an excellent biomarker¹⁰. With the development of gene chip technology and high-throughput sequencing technology, more and more valuable biomolecules¹¹⁻¹³ can be identified by mining big data with bioinformatics technology. Therefore, screening the potential tumor markers of ESCC is of great significance for its early diagnosis, treatment and prognosis.

In this study, differentially expressed genes (DEGs) were screened from Gene Expression Omnibus (GEO). Then, we established a protein-protein interaction network and do gene

enrichment analysis to find the important hub genes. Finally, the immune infiltration analysis was carried out to explore the infiltration of all kinds of immune cells in ESCC, which provided a new idea for the prognosis diagnosis and treatment of ESCC.

Materials and Methods

Data Source

The gene expression datasets (GSE70409, GSE26886, GSE161533, GSE17351) of ESCC were obtained from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). A total of 128 samples were obtained, including 64 cases of ESCC tissues and 64 cases of paracancerous tissues (Tab1).

Data processing of DEGs

Limma package (version: 3.40.2) of R software was used to study the differential expression of mRNAs. The adjusted P-value was analyzed to correct for false positive results in GEO datasets. “Adjusted $P < 0.05$ and $\text{Log}(\text{Fold Change}) > 1$ or $\text{Log}(\text{Fold Change}) < -1$ ” were defined as the thresholds for the screening of differential expression of mRNAs. The overlapping up-regulation and down-regulation DEGs from these four datasets were identified using the Sangerbox online tool (<http://sangerbox.com/Tool>).

Analysis of GO enrichment and KEGG pathway

Gene annotation plays a crucial role in the identification of biological characteristics in transcriptome data. Gene Ontology and KEGG are widely used in functional enrichment analyses; gene functions can be classified into biological process (BP), cellular component (CC), molecular function (MF). KEGG stores a lot of data about genomes, biological pathways, diseases, chemical substances, and drugs. In this study, the DAVID database(<https://david.ncifcrf.gov/>) was used to conduct the Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses in overlapping DEGs. The p value < 0.05 was considered statistically significant.

Protein-protein interaction (PPI) network construction and module analysis

The Search Tool for the Retrieval of Interacting Genes (STRING)¹⁴ is an interacting gene database designed to analyze PPI information. The PPI network of DEGs was constructed by using STRING(<https://string-db.org/>). An interaction score of 0.7 was regarded as statistically

significant. Then the PPI network was built and visualized in Cytoscape software (3.8.2). Next, we use molecular complex detection (MCODE) plugin to determine the significant PPI network modules. In addition, six algorithms¹⁵ in the CytoHubba plugin were used to screen hub genes.

Verification of hub genes in multi-database

The GEPIA2(<http://gepia.cancer-pku.cn/>) is an online public accessed platform, which includes TCGA and GTEx data. Accompany with gene expression data of 32 types of cancers which are derived from 10,882 RNA-seq and 10,546 miRNA-seq data, ENCORI (<http://starbase.sysu.edu.cn/>) allows researchers performing gene analysis. In the present study, expression level of these identified hub genes in ESCC was analyzed at GEPIA2 database and ENCORI database, and the cutoff values were set as $|\log_2FC| = 1.0$ and $p \text{ value} = 0.01$. Meanwhile, the GEPIA2 was applied to analyze expression of hub genes in different stages of ESCC.

Hub Gene correlation and Survival Analysis

We employed GEPIA2 to perform the correlation between hub genes. The survival time of patients with ESCA was analyzed by GEPIA2 in order to explore the correlation between gene expression and prognosis of patients with ESCA. The percentiles of the high expression group and the low expression group were set to 70% and 30%.

Immune infiltration analysis

In this experiment, we estimated the associations between hub gene expression and immune cell population (B cells, CD8+T cells, CD4+T cells, neutrophils, macrophages and DC cells) in Timer2.0 database (<http://timer.cistrome.org/>), $p \text{ value} < 0.01$ was considered to be statistically significant. At the same time, the infiltration and distribution of 22 kinds of immunocytes in ESCC group and normal group were obtained by CIBERSORT deconvolution algorithm.

Results

Identification of DEGs

In this study, 4 gene expression profiles were selected from GEO. After screening the DEGs according the criteria, 1694, 3209, 583 and 1889 genes were identified from GSE70409, GSE26886, GSE161533 and GSE1735, respectively. Among them, the number of up-regulated genes were 941, 1614, 328 and 873 respectively. Meanwhile, the down-regulated genes were

753,1595,255 and 1016, respectively. Then, the intersection of the DEG profiles was get using venn analysis. Finally, we found 164 up-regulated genes (Fig1a) and 80 down-regulated genes (Fig1b).

Gene ontology and pathway functional enrichment analysis

As shown in (Tab2),the results of GO analysis showed that the overlapping DEGs were principally enriched in the biological functions ,especially in the proteolysis, extracellular matrix organization, oxidation-reduction process, cell adhesion and positive regulation of cell proliferation; In terms of cell composition, the DEGs were mainly related to extracellular exosome , extracellular region, extracellular space, endoplasmic reticulum and endoplasmic reticulum membrane; In regard to molecular function, the DEGs were mainly binding to calcium ion and actin, affecting the activities ofserine-type endopeptidase , oxidoreductase and metallopeptidase. Also, KEGG pathway analysis showed that the overlapping DEGs were dramatically concentrated in the PI3K-Akt signal pathway and ECM-receptor interaction.

PPI network construction and hub gene identification

The protein interaction of DEGs was constructed by STRING. The PPI network for DEGs is demonstrated in (Fig2a), which included 103 nodes and 175 edges, with a comprehensive score of more than $0.7 p < 1.0e-16$. In addition, six algorithms in the CytoHubba plugin were used to evaluate the PPI network. And we selected the top 10 genes of each algorithm (Tab3). Based on the intersection of the results of the six algorithms, the three hub genes CXCL8, MMP9 and MMP13 were screened. At the same time, we found that three hub genes appeared in the same module in MCODE analysis (Fig2b) .

Verifying of the hub genes

In order to verify the dependability of the results from the bioinformatics analysis, we next identified the expression levels of three hub genes in the GEPIA2 database and their correlation with tumor clinical stages. The results showed that the transcriptional levels of CXCL8, MMP9 and MMP13 were significantly overexpressed in ESCC tissues when compared with the normal group (Fig3a-c). However, only the expression of MMP9 and MMP13 was positively correlated with tumor stage (Fig4a-c). The online database ENCORI was also used to confirm the significant overexpression of three hub genes in ESCC (Tab4). These results suggest that the expression

levels of CXCL8, MMP9 and MMP13 were higher in ESCC tissues than in normal esophageal tissues, indicating their potential oncogenic effects..

Association of the hub genes with survival time of patients

The online database GEPIA2 were used to confirm the association of the hub genes with the survival curve of patients. The results of total survival analysis of hub genes in ESCA showed that there was a significant correlation between the high expression of CXCL8($P=0.026$) and the overall survival time of patients (Fig5a) . However, there was no significant correlation between MMP9 ($P=0.49$), MMP13 ($P=0.53$) and prognosis of patients (Fig5b-c). Furthermore ,we found that there was a certain correlation between the expression of the three hub genes in ESCA, and the p values were <0.05 (Fig5d-f) .

Association of the hub genes with tumor-infiltrating immune cells

In this study, we attempted to explore the relationship between the mRNA expression of hub genes and immune cell infiltration using TIMER2.0. As shown in (Fig6a-c), each of the hub genes correlated with tumor purity in ESCA. We observed that there was a certain correlation between CXCL8 and the infiltration level of five kinds of immune cells in tumor microenvironment. The expression of CXCL8 was positively correlated with neutrophil, CD8+T cell and dendritic cell infiltration, and negatively correlated with CD4+T cell and macrophage infiltration. Both MMP9 and MMP13 are related to neutrophils, macrophages and dendritic cells (Fig6a-c) . CIBERSORT scores showed that macrophage M0, macrophage M1, myeloid dendritic cell resting and T cell follicular helper were significantly increased in the tumor group. However, B cell, monocyte, CD8+T cell and mast cell activated were significantly decreased in tumor group (Fig7a-b) .

Discussion

Esophageal squamous cell carcinoma is one of the most common malignant tumors, which is often diagnosed as middle and advanced stage. As we all know, invasion and metastasis are the main biological characteristics of malignant tumors and a marker of human cancer¹⁶. The main steps of its occurrence include extracellular matrix degradation, cell adhesion and so on. In our study, DEGs were determined by comprehensive analysis of four data sets, and the results showed that DEGs were mainly enriched in extracellular region and extracellular space, and for functional

analysis, DEGs were mainly concentrated in cell adhesion and proteolysis related to tumor invasion and metastasis. In addition, the results of KEGG signal pathway analysis also showed that DEGs were enriched in the functional pathways of tumor invasion and metastasis, the changes of PI3K-Akt signal pathway and ECM receptor pathway were involved in tumor invasion and metastasis in many kinds of tumors^{17,18}.

In this study, based on six algorithms, three hub genes were screened, among which chemokine CXCL8, also known as interleukin-8, can stimulate neutrophil granules to release and produce superoxide and hydrogen peroxide¹⁹. CXCL8 and its receptor CXCR1/2 can play an important role in tumor progression, invasion and metastasis by interacting with immune cells, cancer stem cells and tumor microenvironment²⁰. In addition, chemokine is a crucial part of tumor microenvironment, which mediates the recruitment of immune cells and promotes the development of lymphoid tissue to regulate the occurrence and development of tumor. It was reported that CXCL8 may be an effective target for immunotherapy in gastric cancer²¹, melanoma²², colorectal cancer²³ and other tumors. Moreover, it has been found that PI3K-Akt signal pathway can induce the expression of CXCL8 in human lung epithelial cells by activating IKK and NF- kappa B protein²⁴. The matrix metalloproteinase (MMP), a member of the methylene zinc protease superfamily, can degrade and modify multiple components of extracellular matrix (ECM) and basement membrane (BM). It not only plays a role in normal physiological processes such as embryonic development and tissue remodeling, but also plays a key role in tumor metastasis, invasion, and diseases of extracellular matrix decomposition²⁵. And according to our research results, CXCL8, MMP9 and MMP13 may play a role in ESCC through interaction, and there is a positive correlation between CXCL8 and MMP9. The overexpression of CXCL8 and MMP9 is associated with the poor prognosis of bladder cancer, and the expression level of both increases with the increase of tumor invasiveness²⁶. The same results were also confirmed in prostate cancer⁹, knockout of CXCL8 in PC-3M-LN4 cells decreased the expression of MMP9, while up-regulation of CXCL8 increased the expression of MMP9. A study on the analysis of gastric cancer microarray data showed that the co-expression of CXCL8 and MMP9 could be an early detection marker of perineural invasion(PNI)²⁷. To sum up, these three hub genes are of great value and significance as tumor markers and prognostic indicators of ESCC.

In our study, CIBERSORT was utilized to analyze and evaluate the immune cell infiltration of ESCC. We found that compared with normal tissues, the levels of macrophage, myeloid dendritic cell resting and T cell follicular helper in ESCC group were higher, while the levels of B cell, monocyte, CD8+T cell and mast cell activated were lower. Chen et al²⁸ found that M0 and M1 macrophage were significantly increased in ESCC, and M1 macrophage was involved in inhibiting the invasion and migration of ESCC cells. In large body of experimental evidence has found that M1 macrophage can produce cytokines such as IL1, IL-12 and TNF- α , which produce anti-tumor effect²⁹. However, with the progression of tumor, M1 can polarize M2 macrophage through PI3K-Akt, STAT6³⁰ and so on, resulting in tumor proliferation and metastasis. Dendritic cells are closely related to the progression of ESCC, and the immunosuppressive environment in tumors may be the cause of dendritic cell dysfunction. Chen et al³¹ found that the number of dendritic cells in ESCA tissue and peripheral blood was significantly lower than that in the control group. Kocian's study³² of colorectal cancer found that high infiltration of resting dendritic cells was closely related to recurrence. Mast cells can secrete IL-10 and TGF- β 1, induce IL-17 production and affect tumor progression. It is worth noting that mast cells can induce CXCL8 and MMP9 to promote tumor by producing related cytokines³³. Monocytes also play a significant role in the development of cancer, a retrospective study by Han et al³⁴ found that a higher preoperative monocyte count can be used as a valuable prognostic indicator for patients undergoing esophagectomy. The decrease of CD8+T cells in ESCC could be related to the inhibition of macrophages. At the same time, Simoni et al³⁵ reported that the infiltrated CD8+T cells were not at all specific for tumor antigens. . In addition, the results in TIMER2.0 database showed that the three hub genes were positively correlated with neutrophils and dendritic cells. In short, immune infiltration analysis provides a certain direction for ESCC immunotherapy.

To sum up, this study uses a variety of bioinformatics methods to systematically analyze the DEGs of ESCC, which provides clues for exploring the mechanism of ESCC. However, this study only analyzes the information data, and further experimental studies are needed to confirm the above results in the future. Overall, these findings suggest that three hub genes may be potential biomarkers in ESCC.

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