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Identification of Differentially Expressed Genes and Signaling Pathways in Esophageal Squamous Cell Carcinoma Using Bioinformatics Analysis

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Abstract

Background: Esophageal Squamous Cell Carcinoma (ESCC) is one of the histological types of esophageal cancers, with more than 80% of esophageal cancers being ESCC. Meanwhile, in Asia, ESCC has higher morbidity and mortality compared with western countries. Due to lack of effective molecular targets and treatments options, the prognosis and 5-year survival rate of ESCC are extremely poor. Therefore, there is an urgent need to identify key pathogenic genes involved in ESCC and reveal potential molecular mechanisms.

Methods: To explore potential therapeutic targets for ESCC, we analyzed three microarray data sets (GSE20347, GSE161533, and GSE38129) derived from the Gene Expression Omnibus (GEO) data base of the National Center for Biotechnology Information (NCBI). We used the GEO2R tool to screen out Differentially Expressed Genes (DEGs) between tumor tissues and normal tissues. Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs were performed *via* the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/). The Search Tool for the Retrieval of Interacting Genes (STRING) database and Cytoscape software were used to construct a Protein-Protein Interaction (PPI) network of these DEGs. Furthermore, we used the online GEPIA database to carry out survival analysis to evaluate the prognostic value of hub genes expression in ESCC patients.

Results: A total of 32 upregulated DEGs and 42 downregulated DEGs were identified in ESCC. Among them, we picked out ten hub genes with a high degree of connectivity. Overexpression of these some hub genes was associated with unfavorable prognosis of ESCC. Particularly, the overexpression of *COL10A1* and *SERPINE1* was observed using the qRT-PCR and indicated poor outcome of ESCC. Simultaneously, low expression of some hub genes was associated with shorter overall survival, such as *ACPP* and *LDHA* genes.

Conclusion: The results in this study might provide some directive significance for further exploring the potential biomarkers for diagnosis and prognosis prediction of ESCC patients. Meanwhile, further study is needed to explore the value of hub genes in the treatment of ESCC.

Keywords: Esophageal squamous cell carcinoma; differentially expressed genes; Gene Ontology; Kyoto Encyclopedia of Genes and Genomes; Protein-protein interaction; Survival analysis

Abbreviations

DEG: Differentially Expressed Gene; GEO: Gene Expression Omnibus; ESCC: Esophageal Squamous Cell Carcinoma; PPI: Protein-Protein Interaction; NCBI: National Center for Biotechnology Information; GO: Gene Ontology; DAVID: Database for Annotation, Visualization and Integrated Discovery; STRING: Search Tool for the Retrieval of Interacting Genes; KEGG: Kyoto Encyclopedia of Genes and Genomes

Introduction

Esophageal Squamous Cell Carcinoma (ESCC) is one of the most common malignancies all over the world, with poor diagnosis and high mortality [1,2]. The incidence rates of ESCC rank fourth and eighth in China and worldwide, respectively [3]. ESCC has strong invasiveness and a fast growth rate, and its five-year survival rate is less than 10%, which makes it a leading cause of cancer-

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related death [4]. The most common clinical symptoms of ESCC include dysphagia and unconscious weight loss [5]. The difficulty of eating in ESCC patients has further increased because of the staging and location of the tumor and the poor adjuvant treatment [6]. Up to date, the molecular mechanism of the occurrence and development of ESCC is still unclear. At present, one of the main researches of ESCC is searching for key genes or specific biomarkers that influence the occurrence and development of ESCC to confirm genetic susceptibility factors in ESCC and clarify their molecular mechanisms [7]. Simultaneously, ESCC is still a disease with poor outcome and limited treatment options [8]. Hence, it is urgent and necessary to explore novel therapeutic targets for ESCC. In the present research, we tried to detect novel indicators of poor prognosis in ESCC patients and endeavor to provide potential therapeutic targets for this challenging disease. In order to detect the Differentially Expressed Genes (DEGs) between ESCC and healthy human esophageal tissue, bioinformatics methods were used to analyze the gene expression profiling data downloaded from the Gene Expression Omnibus (GEO) database. Gene Ontology (GO) functional annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed for the screened DEGs. Then, we established a Protein-Protein Interaction (PPI) network to identify hub genes related to ESCC. The survival analysis of these hub genes was performed using the online data base GEPIA. We believe our data will provide comprehensive biological information about novel differentially genes and enhance the level of understanding regarding the development and progression of ESCC.

Materials and Methods

Data collection

The Esophageal squamous cell carcinoma expression microarray data sets (GSE20347, GSE161533, and GSE38129) were downloaded from the GEO (http://www.ncbi.nlm.nih.gov/geo/) database. All of the data sets were based on platform GPL570 (Affymetrix Human Genome U1332.0 Array). The data sets of GSE20347 included 17 pairs of esophageal squamous cell carcinoma tissues and adjacent normal tissues. The data sets of GSE161533 comprised 28 paired normal and tumor tissues. The data sets of GSE38129 contained 30 pairs of cancerous and matched non-cancerous tissues.

Data processing of DEGs

The GEO2R online tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to identify the DEGs between ESCC tissues and normal tissues, and the adjusted P-value and logFC were also calculated. logFC ≥ 2 and adjusted P<0.05 were used as the cutoff criteria to be considered as DEGs. Finally, a Venn diagram was used to show the intersecting part of each data set using the Venn diagram web tool (bioinformatics.psb.ugent.be/webtools/Venn/).

GO and KEGG pathway enrichment analysis of DEGs

GO analysis is a common useful method for large scale functional enrichment research, which includes in Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). KEGG is a widely used data base which stores a lot of data about biological pathways, genomes diseases, chemical substances, and drugs. For further analyses the potential biological processes, GO and KEGG pathway enrichment analysis were conducted on selected genes *via* the online software the Database for Annotation, Visualization and Integrated Discover (DAVID) tools (https://david.ncifcrf.gov/). P<0.05 and gene counts ≥ 10 were regarded as statistically significant. Finally, the graph of GO analysis was accomplished by the package of ggplot2 in R.

PPI network construction and hub gene identification

We used the online software system Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org/) to analyze the PPI information. To evaluate the potential PPI relationship, the DEGs identified previously were mapped to the STRING database. Confidence score ≥ 0.4 was considered as significant. Subsequently, the PPI network was constructed and visualized by Cytoscape software (www.cytoscape.org/). Then, the plugin cytohubba in cytoscape was used to calculate the degree of each protein node. In this study, the top ten genes were identified as hub genes.

Survival analysis

The GEPIA (http://gepia.cancer-pku.cn/index.html) database was applied to calculate the overall survival and disease-free survival of some DGEs. GEPIA is a newly created online interactive web server which enables users to explore the RNA sequencing expression information of tumors and normal tissues, based on a criterion processing pipeline. GEPIA can also offer many customizable functions such as profiling regarding to pathological stages, cancer types, differential expression analysis, survival analysis, correlation analysis and similar gene detection.

Patients and tissue specimen collection

Eleven pairs of esophageal squamous cell carcinoma tissues and adjacent non-tumor tissues were collected from Mianyang Central Hospital. All specimens were identified by pathology and rapidly stored at -80°C. All of these patients did not receive radiotherapy and chemotherapy before operation. This research was approved by the ethics committee of the Mianyang Central Hospital and written informed consent was acquired from all patients.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted using the tissues total RNA isolation kit (Foregene, Chengdu, China), following the manufacturer's instructions. cDNAs were synthesized from 500 ng total RNA using HiScript III All-in-one RT SuperMix perfect for qPCR reagent kit (Vazyme, Nanjing, China) with the following temperature protocol: 50°C 15 min, 85°C 5s. Quantitative Real-time PCR (qPCR) was performed using the Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). All reactions were performed in triplicate at a volume of 10 μl, containing 1 μl of 10-fold diluted cDNA, and the human *β-actin* gene was used as an internal control. The qRT-PCR amplification was performed using the BIO-RAD CFX96™ Real-Time System and was carried out as follows: 40 cycles at 95°C for 10s and 60°C for 30s, 95°C for 15s, 60°C for 60s, 95°C for 15s. qRT-PCR primers are listed in the Table 1. The relative expression levels of hub genes were calculated using the $2^{-\Delta\Delta CT}$ method.

Results

Identification of DEGs in ESCC

In this study, three expression microarray datasets (GSE20347, GSE161533, and GSE38129) were selected. Among them, GSE20347 included 17 pairs of ESCC samples and normal samples. GSE161533 contained 28 paired ESCC specimens and normal specimens. GSE38129 comprised 30 pairs of ESCC samples and normal samples (Table 2). Based on the cut off criteria of P ≤ 0.05 and logFC ≥

Table 1: The primers of quantitative real-time PCR.

Gene		Primer
COL1A1	Sense	5'CCCCTGGAAAGAATGGAGATG3'
	Antisense	5'AGCTGTTCCGGGCAATCCT
COL5A2	Sense	5'CAGGGTTTACAAGGACAGCAAG3'
	Antisense	5'AGGGCCTTCAAGACCTTTGTG3'
COL10A1	Sense	5'CCAGCACGCAGAATCCATCT3'
	Antisense	5'TATGCCTGTGGGCATTTGGT3'
CXCL8	Sense	5'CATACTCCAAACCTTTCCACCC3'
	Antisense	5'CAAAAACTTCTCCACAACCCTCTG3'
IGFBP3	Sense	5'CCAGGAAATGCTAGTGAGTCGG3'
	Antisense	5'TGAATGGAGGGGGTGGAACT3'
INHBA	Sense	5'CGGGTATGTGGAGATAGAGGATGA3'
	Antisense	5'TCCTGACTCGGCAAACGTGAT3'
MMP10	Sense	5'AGAAAGCTCTGAAAGTCTGGGAA3'
	Antisense	5'TGGGTAGGCATGAGCCAAACT3'
PLAU	Sense	5'CATTGATTACCCAAAGAAGGAGGAC3'
	Antisense	5'AAGGCAATGTCGTTGTGGTGAG3'
POSTN	Sense	5'TTGGCTCATAGTCGTATCAGGG3'
	Antisense	5'GCAGCCTTTCATTCCTTCCAT3'
SERPINE1	Sense	5'CCCCACTTCTTCAGGCTGTT3'
	Antisense	5'GCCGTTGAAGTAGAGGGCAT3'
ACPP	Sense	5'GAGCAGCATTATGAACTTGGAGAG3'
	Antisense	5'GAAAGGCAGGTATAGCAACTGATC3'
CRISP3	Sense	5'CAGTAACCCAAAGGATCGAATGA3'
	Antisense	5'GTGTATAATGTCCAACCACTGCG3'
CRNN	Sense	5'AGAGTTTGCCGATGTGATTGTGA3'
	Antisense	5'GGGCAACTTTAAACACTAAGACCAG3'
ECM1	Sense	5'GCAGCCTTGGTCTTGACCTATT3'
	Antisense	5'GGAGCTGCGTAGCCAACTTCTT3'
EMP1	Sense	5'TGGTCCACATCGCTACTGTTATT3'
	Antisense	5'GAGGGCATCTTCACTGGCATA3'
IL18	Sense	5'TCTGACTGTAGAGATAATGCACCCC3'
	Antisense	5'CATACCTCTAGGCTGGCTATCTTTA3'
KRT4	Sense	5'GAAGCAGCTAGATACCTTGGGC3'
	Antisense	5'CTGTGCGTTTGTTGATCTCCTCT3'
LDHA	Sense	5'GATTCAGCCCGATTCCGTTAC3'
	Antisense	5'GAGTCCAATAGCCCAGGATGTG3'
β-actin	Sense	5'CCTTCCTGGGCATGGAGTC'
	Antisense	5'TGATCTTCATTGTGCTGGGTG'

2,277 DEGs in GSE20347, 231 DEGs in GSE161533, 148 DEGs in GSE38129 were identified. Among the DEGs, 86, 102 and 62 genes were upregulated while 191, 129 and 86 genes were downregulated in GSE20347, GSE161533 and GSE38129 respectively. All DEGs were identified by comparing ESCC samples with normal esophagus samples. Subsequently, Venn analysis was performed to get the intersection of the DEG profiles (Figure 1). Eventually, 74 DEGs were significantly differentially expressed among all three groups, of which 32 were significantly upregulated genes and 42 were downregulated.

Table 2: Statistics of the three micro array databases derived from the GEO database.

Datasets ID	ESCC	Normal	Total number
GSE20347	17	17	34
GSE161533	28	28	56
GSE38129	30	30	60

GEO: Gene Expression Omnibus; ESCC: Esophageal Squamous Cell Carcinoma

Functional enrichment analysis

In this present study, we used the DAVID to perform GO function and KEGG pathway enrichment analysis for DEGs (Figure 2A, 2B). The enriched GO terms were included in BP, MF, and CC ontologies. The results of GO analysis indicated that DEGs were mainly enriched in CCs, including extracellular region, proteinaceous extracellular matrix, extracellular space, extracellular exosome, and extracellular matrix. BP analysis showed that the DEGs were significantly enriched in extracellular matrix organization, and proteolysis. For the molecular function the DEGs were enriched in calcium ion binding. In addition, the results of KEGG pathway analysis showed that DEGs were mainly enriched in pathways in PI3K-Akt signaling, focal adhesion, protein digestion and absorption, transcriptional misregulation in cancer, and ECM-receptor interaction.

PPI networks construction and analysis

We constructed PPI networks to further explore the interaction between the common DEGs by using STRING database and Cytoscape. As presented in Figure 3A, after removing the isolated and partially connected nodes, a total of 70 nodes and 447 edges were involved in the complex PPI network. Among which, 32 DEGs were upregulated and 42 DEGs were down regulated. At the same time, the 17 most significant genes showing significant interaction were *ACPP*, *CEACAM5*, *CLCA4*, *COL10A1*, *COL1A1*, *COL1A2*, *COL5A2*, *CRISP3*, *CRNN*, *CXCL8*, *ECM1*, *EMP1*, *IGFBP3*, *IL18*, *INHBA*, *KRT4*, and MMP1. The top ten genes evaluated by connectivity degree in the PPI network were identified (Figure 3B) and the top ten genes were: C-X-C Motif Chemokine Ligand 8 (*CXCL8*), Periostin (*POSTN*), Matrix Metallopeptidase 1 (MMP1), *MMP10*, Insulin Like Growth Factor Binding Protein 3 (*IGFBP3*), Plasminogen Activator Urokinase (*PLAU*), Serpin Family E Member 1 (*SERPINE1*), Collagen Type I Alpha 1 Chain (*COL1A1*), *COL1A2*, Laminin Subunit Gamma 2 (LAMC2). All of these hub genes were upregulated in ESCC.

Survival analysis

The GEPIA database was used to assess the values of the part DEGs. According to previous research, the expressions of the DEGs were demonstrated in normal esophageal tissue and ESCC. Patients with high expression of *SERPINE1* were associated with shorter overall survival (Figures 4-6). Although p-value is not statistically significant, it was survival significance from the perspective of the picture trend that patients with high expression of *COL10A1*, *COL5A2* and *POSTN* and low expression of *ACPP* were associated with shorter overall survival. Furthermore, we found that patients with high expression of *COL1A1*, *COL5A2*, *COL10A1*, *INHBA* and *POSTN* as well as low expression of *ACPP* and *LDHA* were associated with long disease-free survival.

The part differentially expressed genes were verified within ESCC tissues

In order to verify the results of bioinformatics analysis, them RNA levels of 28 DEGs (10 upregulated genes and 8 downregulated genes)

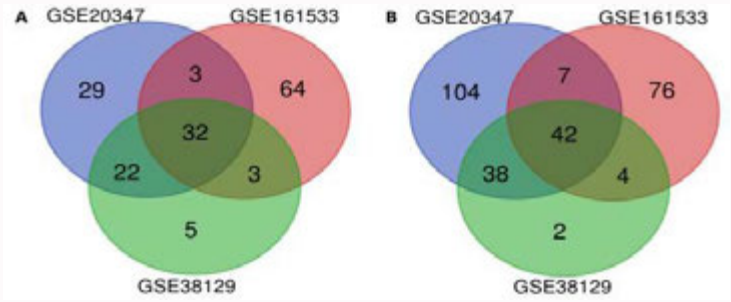

Figure 1: Venn diagram of DEGs common to all three GEO datasets (A) Upregulated genes. (B) Down regulated genes.

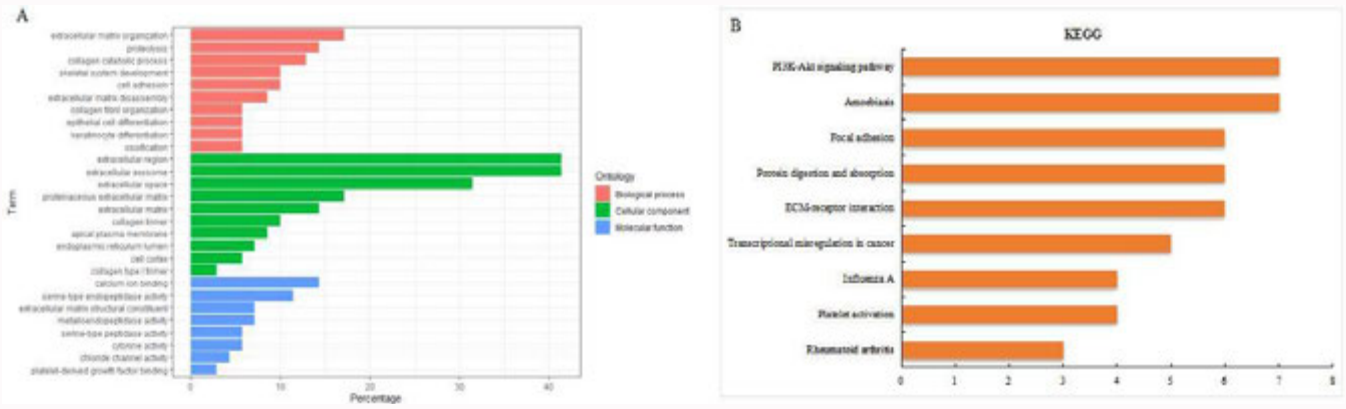

Figure 2: GO and KEGG pathway analysis. (A) The gene ontology annotation analysis of all the differentially expressed genes. (B) The pathway enrichment analysis of all the differentially expressed genes.

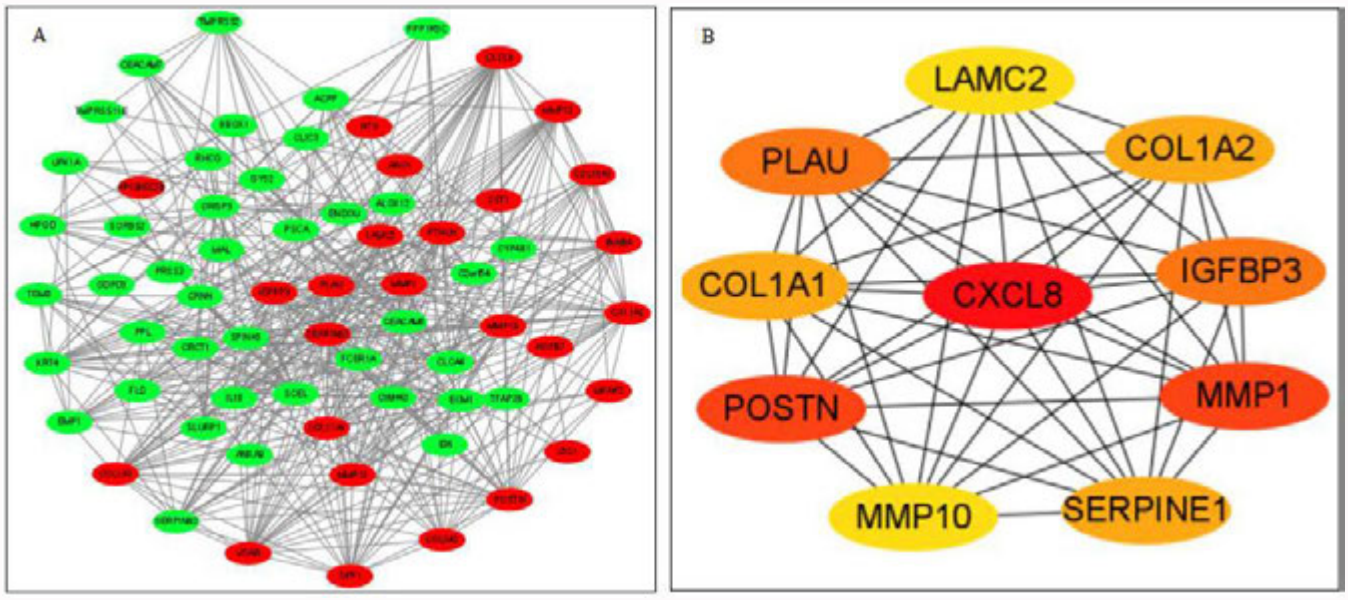

Figure 3: PPI and top ten hub genes analysis. (A) Protein-protein interaction network constructed with the differentially expressed genes. Red nodes represent upregulated genes, and green nodes represent downregulated genes. (B) Top ten hub genes with higher degree of connectivity.

were detected in 11 paired ESCC tissues using qRT-PCR method. As shown in Figure 6, all of the 10 identified upregulated genes were also significantly upregulated in tumor tissues ($p<0.01$), moreover, the 8 identified downregulated genes were also observably downregulated ($p<0.01$) in ESCC tumor tissues, as predicted by the bioinformatics analysis.

Discussion

Although surgery, radiation and chemotherapy techniques have been greatly improved, the prognosis and survival rate of ESCC are still poor. At the same time, the screening and diagnosis of ESCC are also big challenges and difficulties [9]. Recently, with the development

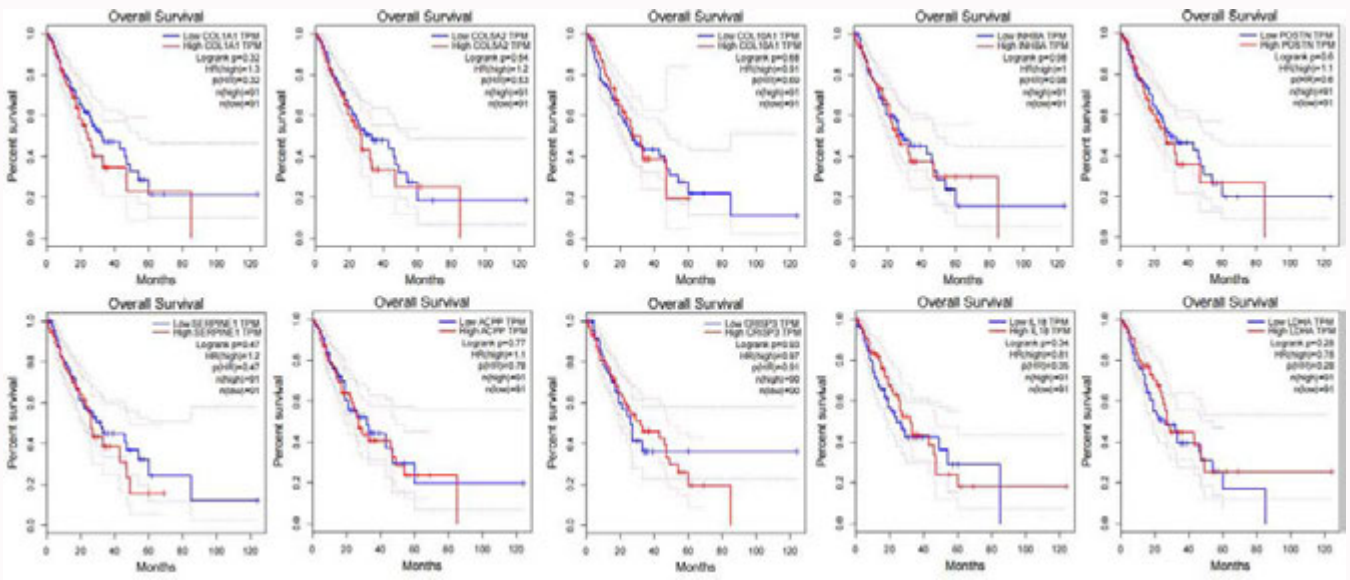

Figure 4: Survival analyses for the differentially expressed gene *ESCC* (upregulated genes: *COL1A1*, *COL5A2*, *COL10A1*, *INHBA* and *POSTN*; downregulated genes: *ACPP*, *CRISP3*, *IL18* and *IL18*). The survival curve comparing the patients with high (red) and low (blue) expression in ESCC.

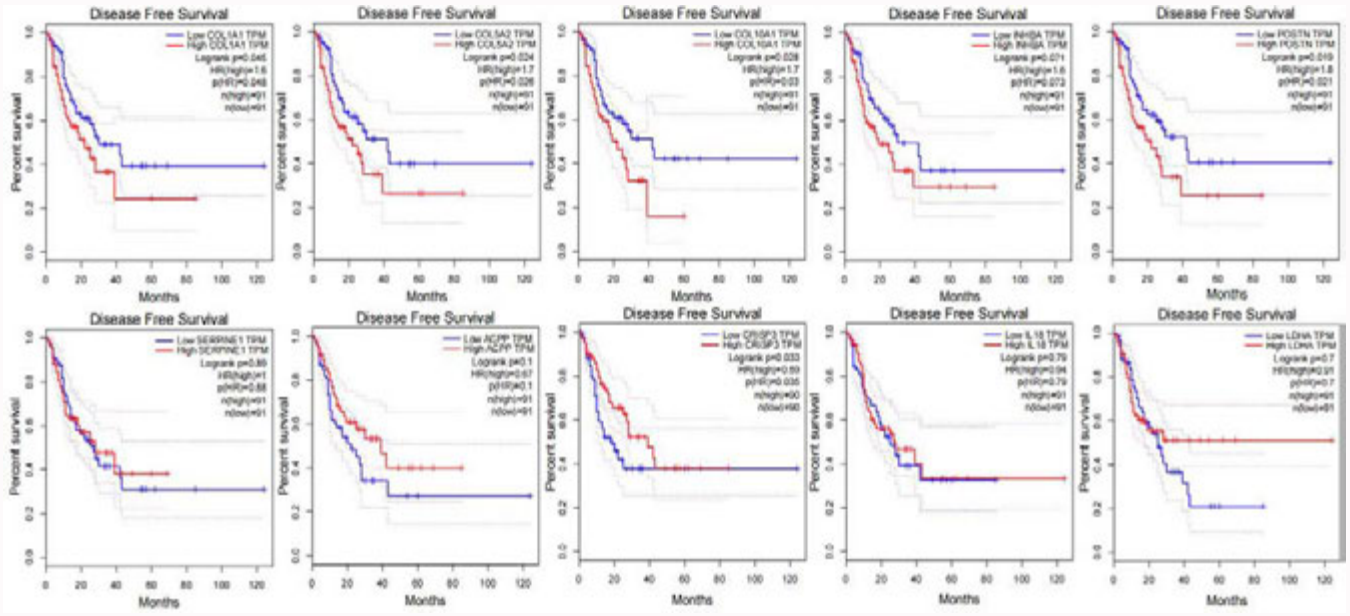

Figure 5: Disease free survival analyses for the differentially expressed gene *ESCC* (upregulated genes: *COL1A1*, *COL5A2*, *COL10A1*, *INHBA* and *POSTN*; downregulated genes: *ACPP*, *CRISP3*, *IL18* and *IL18*). The survival curve comparing the patients with high (red) and low (blue) expression in *ESCC*.

of sequencing technology, the molecularly targeted therapeutics has been carried out, but they have not exhibited beneficial effects on the long-term prognosis of ESCC. So, exploring the potential mechanisms of ESCC occurrence and development will greatly benefit the diagnosis, treatment and prognosis assessment.

In the present study, we integratedly analyzed three microarray data sets from GEO data base with 75 ESCC samples and 75 normal samples *via* multiple bioinformatics tools. Totally, we identified 32 upregulated DEGs and 42 downregulated DEGs between ESCC tissues and normal tissues. The functional enrichment analyses demonstrated that the DEGs were enriched in some biological processes such as ECM organization, proteolysis, collagen catabolic process, skeletal system development, cell adhesion and extracellular matrix disassembly. Previous research has indicated that the altered expression of particular genes may affect ESCC cell metastasis, invasion, apoptosis and proliferation [10-12]. Our results consistent with the previous knowledge that carcinoma cell metastasis and invasion are closely associated with abnormal cell adhesion and endodermal cell division [13-15]. Extracellular Matrix (ECM) is a critical component of the cancer cell niche, which can provide the tissue with the mechanical support and also mediate the cell-microenvironment interactions [16,17]. It is noteworthy that collagens are one of the major proteins found within the ECM and are associated with many aspects of tumorigenesis [18]. Hence, this is consistent with the research results that the activation of these cellular processes through ECM is a major cause of tumorigenesis,

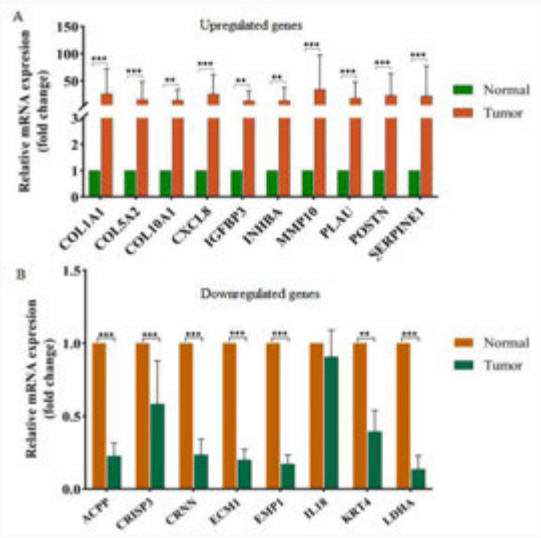

Figure 6: qRT-PCR results for part identified gene biomarkers. Expression of these DEGs was normalized against β-actin expression. The statistical significance of differences was calculated by student's t-test (***P<0.001, **p<0.01).

progression and metastasis [19]. Moreover, KEGG pathway analysis revealed that the DEGs were significantly associated with PI3K-Akt signaling pathway, Amoebiasis, Focal adhesion, Protein digestion and absorption, ECM-receptor interaction and transcriptional misregulation in cancer. The ECM is an important component in regulating tissue homeostasis and is also the first barrier that prevents tumor metastasis [20,21]. The dysregulation of ECM will contribute to cell proliferation, apoptosis, invasion, metastasis, and angiogenesis *via* different signaling pathways. Tumor angiogenesis and destruction of the extracellular matrix are two important conditions for tumor invasion and metastasis [22]. It has been reported that the expression of focal adhesion protein is related to the occurrence, cell differentiation, invasion, lymph node metastasis and prognosis of esophageal cancer and the prognosis of patients with positive expression of focal adhesion protein is worse than that of patients with negative expression [23]. Our findings suggested that these DEGs may be involved in the oncogenes is and progression of ESCC.

A PPI network was constructed to investigate the interrelationship of the DEGs, and ten hub genes were identified, including *CXCL8*, *POSTN*, *MMP1*, *MMP10*, *IGFBP3*, *PLAU*, *SERPINE1*, *COL1A1*, *COL1A2*, and *LAMC2*. All of these genes were upregulated in ESCC. Finally, the online GEPIA data base was applied to predict the relationship between the expression of hub genes and prognosis of ESCC patients. Based on the survival analysis plotter, many of the overexpression genes were related to the unfavorable prognosis of esophageal squamous cell carcinoma patients. However, top three hub genes were selected from this: *CXCL8*, *POSTN* and MMP1.

CXCL8, also known as Chemokine Ligand 8, is a member of the CXC chemokine family and is a major mediator of the inflammatory response [24]. Some research has demonstrated that *CXCL8* is involved in angiogenesis, chemotaxis and inflammatory response of tumor cells [25]. Over expression of *CXCL8* has been reported in many tumors, such as colorectal cancer, breast cancer, pancreatic carcinoma and gastric cancer [26-29].

POSTN, which is a protein coding gene, encodes a secreted extracellular matrix protein that functions in tissue development and regeneration, including wound healing, and ventricular remodeling following myocardial infarction [30]. The encoded protein binds to integrins to support adhesion and migration of epithelial cells. This protein plays a role in cancer stem cell maintenance and metastasis [31]. Gene Ontology (GO) annotations related to this gene include heparin binding and cell adhesion molecule binding. Diseases associated with *POSTN* include myocardial infarction and esophagus carcinoma in situ. Hence, *POSTN* maybe a biomarker that could be used for screening early esophagus cancer, early prognosis and molecular targeted therapy [32,33].

MMP1, also named as fibroblast collagenase, is a member of the peptidase M10 family of matrix metalloproteinases. Proteins in this family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Gene Ontology (GO) annotations related to this gene include calcium ion binding and metallopeptidase activity [34,35].

There are some limitations in this study. First, diagnostic efficiency and prognostic value of the critical genes were only analyzed and validated in the GEO dataset. Therefore, the results need to be verified in other databases. Second, our study was performed merely according to pure bioinformatics analysis. Thus, further experiments are needed to validate the results based on tumor samples and clinical data. Furthermore, *in vivo* and *in vitro* experiments can enhance our understanding of the functional role of the critical genes in ESCC.

In conclusion, esophageal squamous cell carcinoma is one of the most common malignancies all over the world, but the molecular mechanism of the occurrence and development of ESCC has not been clearly studied. Previous researches have mainly analyzed only gene expression data and have not been completely powered to discover hub gene expression data, and also have not been sufficiently powered to discover hub genes and path ways. In this study, our bioinformatics analysis identified 74 DEGs between ESCCs and normal esophageal tissues based on the gene expression datasets obtained from the GEO database. Among them, ten hub genes might be the core genes of ESCC, including *CXCL8*, *POSTN*, MMP1, *MMP10*, *IGFBP3*, *PLAU*, *SERPINE1*, *COL1A1*, *COL1A2*, and *LAMC2*. All of them were upregulated in ESCC, and over expression of some these genes was associated with unfavorable clinical outcome in ESCC patients. In ESCC patients, over expression of *SERPINE1* is an unfavorable prognostic factor. Further study is needed to validate the results of our research. Anyway, *SERPINE1* may be a potential target for ESCC therapy.

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