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Original Article

Identification of Potential Gene in Laryngeal Squamous Cell Carcinoma using Bioinformatics Analysis

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Abstract:

Background: The current treatment results of laryngeal squamous cell carcinoma （LSCC）still remain poor. Lacking molecular targets, radiotherapy and chordectomy are the main treatment for LSCC patients. It is urgent to identify novel diagnostic and prognostic biomarkers for LSCC.The present study aimed to investigate the differentially expressed genes (DEGs) between LSCC samples and normal laryngeal tissue samples, and identify potential core genes associated with the pathogenesis and prognosis of LSCC.

Materials and Methods: To explore potential therapeutic targets for LSCC, we analyzed three microarray datasets (GSE51985, GSE58911, and GSE59102) derived from the Gene Expression Omnibus (GEO) database. The GEO2R tool was used to screen out DEGs between LSCC and normal tissue. Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed using the Database for Annotation, Visualization and Integrated Discovery to identify the pathways and functional annotation of DEGs. Protein–protein interaction (PPI) of these DEGs was analyzed based on the Search Tool for the Retrieval of Interacting Genes database and visualized by Cystoscope software. In addition, we used Texas Cotton Ginners' Association（TCGA）database to express difference analysis of potential hub genes between laryngeal tumor tissues and normal tissues,and the online Kaplan–Meier plotter survival analysis tool to evaluate the prognostic value of hub genes expression in LSCC patients.

Results: A total of 44 upregulated DEGs and 60 downregulated DEGs were identified. Among them, ten hub genes with a high degree of connectivity were picked out. Overexpression of these hub genes was associated with unfavorable prognosis of laryngeal cancer.Especially, CXCL12 overexpression was observed and indicated poor outcome of LSCC.

Conclusion: Our study suggests that CXCL12 was overexpressed in LSCC compared with normal laryngeal tissue, and overexpression of CXCL12 was an unfavorable prognostic factor of LSCC patients. Further study is needed to explore the value of CXCL12 in the treatment of LSCC.

Keywords: laryngeal squamous cell carcinoma, hub genes, CXCL12, bioinformatics analysis

1 Introduction

Head and neck cancer is the sixth most common type of cancer, with an annual incidence of 700,000 patients worldwide [1]. It is reported that 20-30% of cases of head and neck cancer are laryngeal tumors[2]. Laryngeal squamous cell carcinoma (LSCC) is the most common cancer of the head and neck. Smoking , alcohol consumption,air pollution and viral infection are major risk factors involved in LSCC development[3].Although surgery combined with chemoradiotherapy has significantly improved the survival rate of patients[4], recurrence and

metastasis are still common and the prognosis remains poor, particularly in patients at the advanced stage. The five-year survival rates are suggested to be between 52 and 94%, depending on the tumor site, stage and tumor therapy[5].

Great eforts have been made to identify molecular biomarkers for LSCC prognosis. For example, Li et al. found that HOTAIR was highly expressed in LSCC and was an independent prognostic factor of LSCC [6]. Tang et al. identifed SOX2 expression was related to LSCC patient overall survival[7]. Ma et al. suggested that SLC7A11 may be a vital factor for LSCC diagnosis and prognosis[8]. Therefore, the discovery of novel specific markers for early diagnosis and prognosis is urgently needed to improve patient survival. With the development of tumor molecular genetics, many scholars predict the prognosis of tumors using genetics[9].Studies have shown that the up-regulation and down-regulation of the expression of immune-related genes in tumor cells may be correlated with tumor prognosis. Identifying patients with high risk scores enables more targeted clinical treatments. This paper aims to find a differential immune gene model related to prognosis to predict the survival rate of LSCC.

In this study, we tried to detect novel indicators of poor prognosis in LSCC patients and endeavor to provide potential therapeutic targets for this challenging disease.To detect the DEGs between LSCC and healthy human laryngeal tissue, bioinformatics methods were used to analyze the gene expression profiling data downloaded from the GEO database. GO functional annotation analysis and KEGG pathway enrichment analysis were performed for the screened DEGs. Then, we established a PPI network to identify hub genes related to LSCC. The survival analysis of these hub genes was performed using the online database Kaplan–Meier plotter[10].

2. Materials and Methods

2.1 Gene Expression Profile Data.

The gene expression datasets were obtained from the National Center of Biotechnology Information Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). A total of 54 series about human laryngeal cancer were retrieved from the database. After a careful review, three gene expression profiles (GSE51985, GSE58911, and GSE59102) were selected. Among them, GSE51985[11] was based on the Agilent GPL10588 platform (Illumina HumanHT-12 V4.0 expression beadchip), GSE58911[12] was based on the Agilent GPL6244 platform (HuGene-1_0-st Affymetrix Human Gene 1.0 ST Array transcript gene version) and GSE59102[13] were based on the Agilent GPL6480 platform (Agilent-014850 Whole Human Genome Microarray 4x44K G4112F Probe Name version).All of the data were freely available online, and this study did not involve any experiment on humans or animals performed by any of the authors.

2.2 Data Processing of DEGs.

The GEO2R online analysis tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to detect the DEGs between LSCC and normal samples, and the adjusted P-value and |logFC| were calculated. Genes that met the cutoff criteria, adjusted $P<0.05$ and $|logFC|\geq1.0$ were considered as DEGs. Statistical analysis was carried out for each dataset, and the intersecting part was identified using the Venn diagram webtool (bioinformatics.psb.ugent.be/webtools/Venn/).

2.3 GO and KEGG Pathway Analysis of DEGs.

GO analysis is a common useful method for large scale functional enrichment research; gene functions can be classified into biological process (BP), molecular function (MF), and cellular component (CC). KEGG is a widely used database which stores a lot of data about genomes, biological pathways, diseases, chemical substances, and drugs. GO annotation analysis and KEGG pathway enrichment analysis of DEGs in this study was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tools (https://david.ncifcrf.gov/).$P<0.01$ and gene counts≥10 were considered statistically significant.

2.4 PPI Network Construction and Hub Gene Identification.

The Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org/) is designed to analyze the PPI information. To evaluate the potential PPI relationship, the DEGs identified previously were mapped to the STRING database. The PPI pairs were extracted with a minimum required interaction score (low

confidence 0.15) and hided disconnected nodes in the network . Subsequently, the PPI network was visualized by Cytoscape software (www.cytoscape.org/). Nodes with higher degree of connectivity tend to be more essential in maintaining the stability of the entire network. CytoHubba, a plugin in cytoscape, was used to calculate the degree of each protein node. In our study, the top ten genes were identified as hub genes. The gene expression information and clinical information of laryngeal cancer related data were downloaded from the TCGA database (http: //www.tcga.org/). A total of 502 cases of laryngeal cancer related samples were screened, compared with 44 normal tissues, and the clinical data and gene expression information were integrated.

2.5 Survival Analysis of Hub Genes.

The Kaplan–Meier plotter (http://kmplot.com/analysis/) is an online tool. The Kaplan–Meier plotter mRNA for pan-cancer database was applied to evaluate the prognostic values of hub genes in laryngeal cancer patients. In our study, LSCC patients were screened out based on Head-neck squamous cell carcinoma.Split patients selected "Auto select best cutoff".Survival choosed relapse-free survival (RFS) and P<0.01 was considered to indicate a statistically significant result.

3. Results

3.1 Identification of DEGs.

Three gene expression profiles (GSE51985, GSE58911, and GSE59102) were selected in this study.Among them, GSE51985 contained 10 LSCC samples and 10 normal samples,GSE58911 contained 15 LSCC samples and 15 normal samples and GSE59102 included 29 LSCC samples and 13 normal samples (Table 1).

Table 1 Statistics of the three microarray databases derived from the GEO database

Dataset ID	LSCC	Normal	Total number	upregulated genes	downregulated genes
GSE51985	10	10	20	464	330
GSE58911	15	15	30	175	432
GSE59102	29	13	42	1598	1727

Abbreviations: GEO, Gene Expression Omnibus; LSCC, laryngeal squamous cell carcinoma.

Based on the criteria of P<0.05 and |logFC|≥1.0, a total of 794 DEGs were identified from GSE51985, including 464 upregulated genes and 330 downregulated genes. In gene chip GSE58911, 607 DEGs were identified; 175 genes were upregulated, and 432 genes were downregulated. And from GSE59102, 3,325 DEGs including 1,598 upregulated genes and 1,727 downregulated genes were identified. All DEGs were identified by comparing LSCC samples with normal laryngeal samples. The genes with upregulated expression are marked in blue, and the genes with downregulated expression are marked in red. Volcano maps of GSE51985, GSE58911, and GSE59102 are shown in Figure A–C, respectively.Subsequently, Venn analysis was performed to get the intersection of the DEG profiles (Figure 2). Finally, 104 DEGs were significantly differentially expressed among all three groups, of which 44 were significantly upregulated genes and 60 were downregulated.

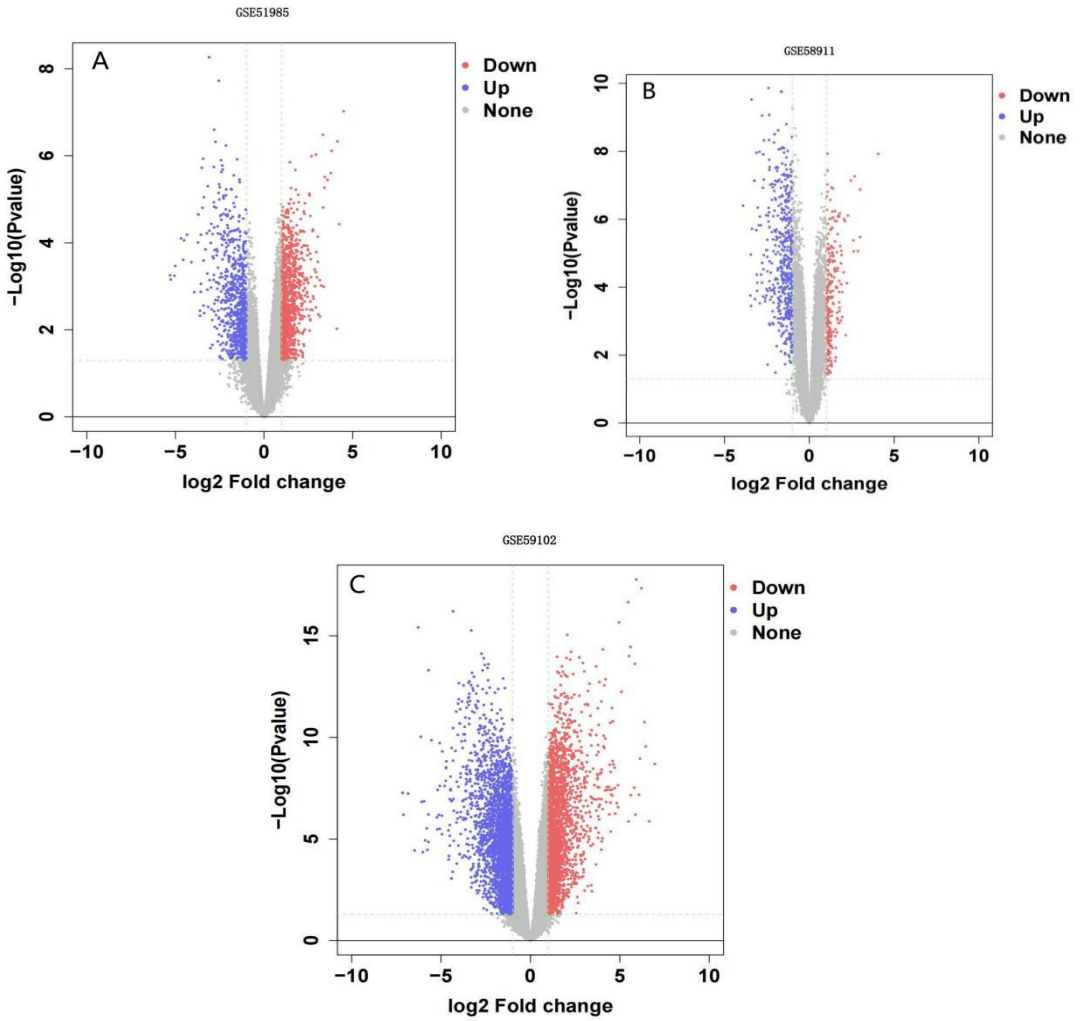

Figure 1. Screening DEGs between LSCC patients and normal laryngeal samples. (A) GSE51985; (B) GSE58911; (C) GSE29102.

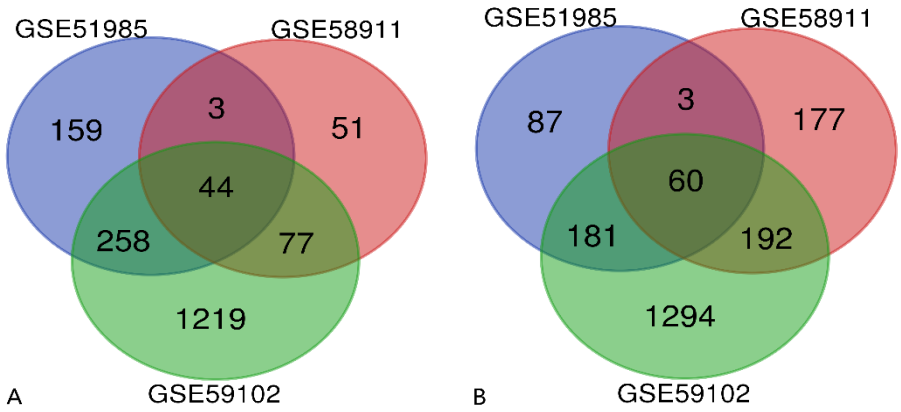

Figure 2 Venn diagram of DEGs common to all three GEO datasets. Notes: (A) Upregulated genes. (B) Downregulated genes.
Abbreviations: DEG, differentially expressed gene; GEO, Gene Expression Omnibus.

3.2 GO and KEGG Pathway Analysis of DEGs

GO function and KEGG pathway enrichment analysis for DEGs were performed using the DAVID (Table 2). The enriched GO terms were divided into BP, CC, and MF ontologies.The results of GO analysis indicated that DEGs were mainly enriched in BPs, including cellular calcium ion homeostasis, positive regulation of peptidyl-tyrosine phosphorylation, positive regulation of transcription from RNA polymerase

II promoter, regulation of cell migration and immune response. MF analysis showed that the DEGs were significantly enriched in protein binding, and protein domain specific binding. For the cell component, the DEGs were enriched in extracellular space, and protein complex. In addition, the results of KEGG pathway analysis showed that DEGs were mainly enriched in pathways in transcriptional misregulation in cancer,chagas disease (American trypanosomiasis), TNF signaling pathway , cytokine-cytokine receptor interaction and proteoglycans in cancer.

Table 2 Significantly enriched GO terms and KEGG pathways of DEGs

Category	Term	Description	Count	FDR
GOTERM_BP_DIRECT	GO:0006874	cellular calcium ion homeostasis	12	0.007145
GOTERM_BP_DIRECT	GO:0050731	positive regulation of peptidyl-tyrosine phosphorylation	11	0.007604
GOTERM_BP_DIRECT	GO:0045944	positive regulation of transcription from RNA polymerase II promoter	42	0.008965
GOTERM_BP_DIRECT	GO:0030334	regulation of cell migration	10	0.009608
GOTERM_BP_DIRECT	GO:0006955	immune response	24	0.009608
GOTERM_CC_DIRECT	GO:0006955	extracellular space	53	4.12E-04
GOTERM_CC_DIRECT	GO:0043234	protein complex	22	0.009594
GOTERM_MF_DIRECT	GO:0005515	protein binding	243	1.55E-06
GOTERM_MF_DIRECT	GO:0019904	protein domain specific binding	17	0.001764
KEGG_PATHWAY	hsa05202	Transcriptional misregulation in cancer	17	0.002944
KEGG_PATHWAY	hsa05142	Chagas disease (American trypanosomiasis)	13	0.002944
KEGG_PATHWAY	hsa04668	TNF signaling pathway	13	0.002944
KEGG_PATHWAY	hsa04060	Cytokine-cytokine receptor interaction	20	0.002944
KEGG_PATHWAY	hsa05205	Proteoglycans in cancer	13	0.007091

3.3 PPI Network Construction and Hub Gene Identification.

Protein interactions among the DEGs were predicted with STRING tools. A total of 104 nodes and 521 edges were involved in the PPI network, and hub gene identification (Figure 2). The top ten genes evaluated by connectivity degree in the PPI network were identified (Table 3).

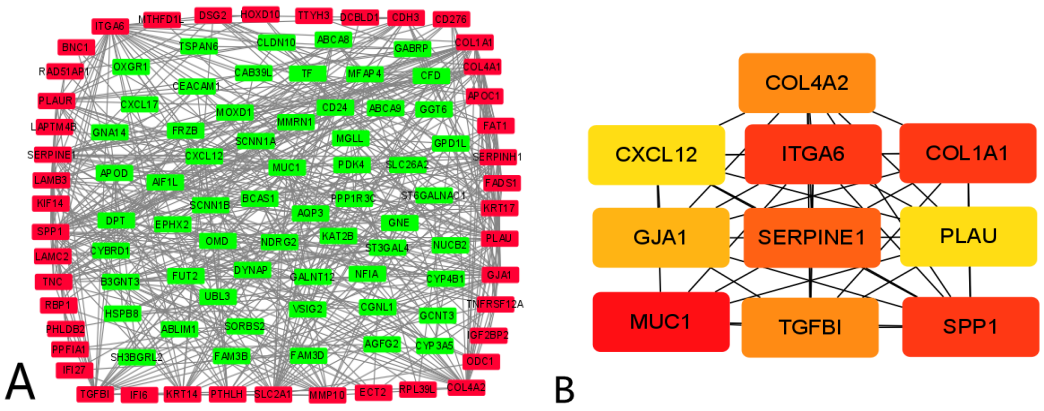

Figure 2 Notes: (A) Protein–protein interaction network constructed with the differentially expressed genes.

Red nodes represent upregulated genes, and green nodes represent downregulated genes. (B) Ten potential hub genes.

Table 3 Top ten hub genes with higher degree of connectivity

Gene symbol	Gene description	Degree
MUC1	Mucin 1, Cell Surface Associated	30
ITGA6	Integrin Subunit Alpha 6	29
SPP1	Secreted Phosphoprotein 1	29
COL1A1	Collagen Type I Alpha 1 Chain	29
SERPINE1	Serpin Family E Member 1	28
COL4A2	Collagen Type IV Alpha 2 Chain	27
TGFBI	Transforming Growth Factor Beta Induced	27
GJA1	Gap Junction Protein Alpha 1	26
CXCL12	C-X-C Motif Chemokine Ligand 12	24
PLAU	Plasminogen Activator, Urokinase	24

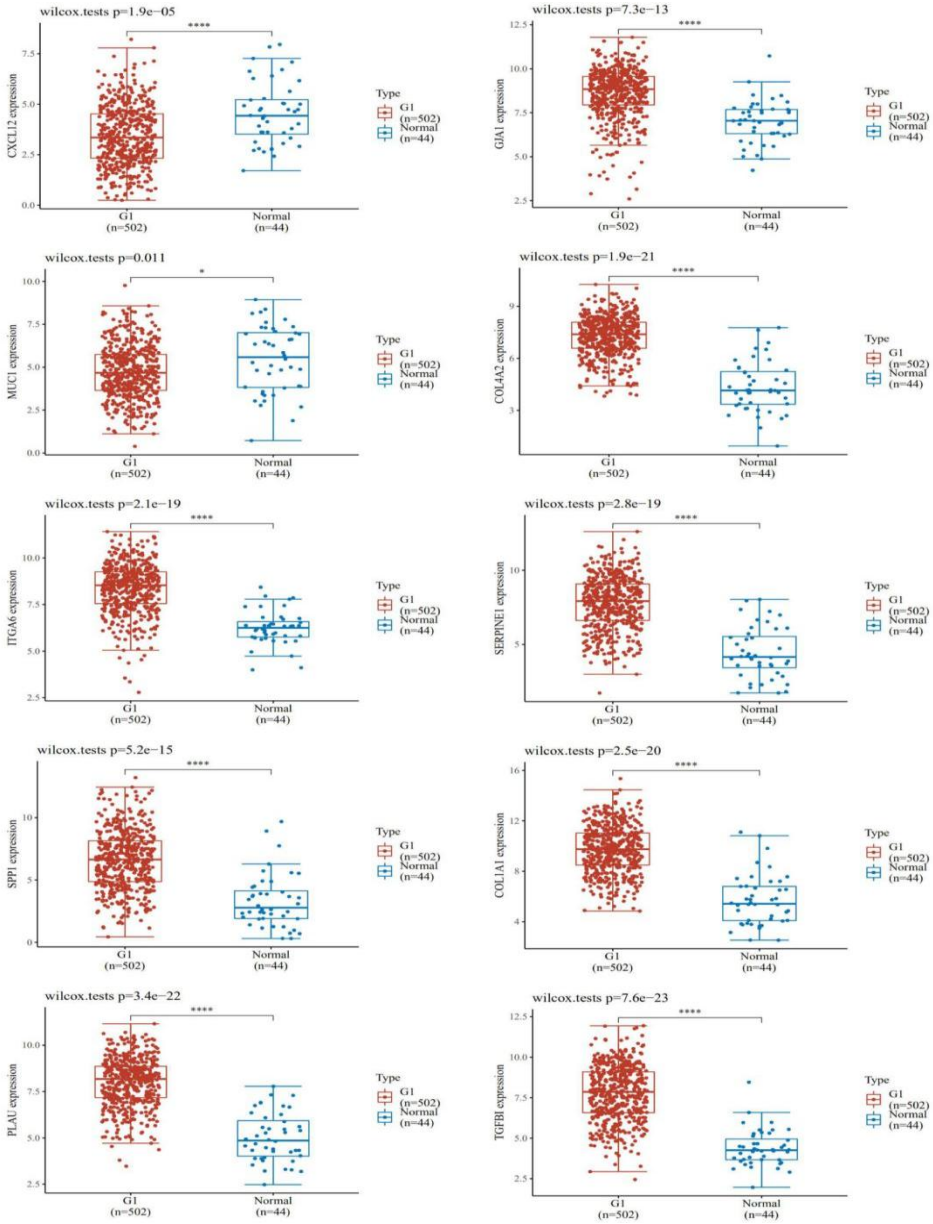

Figure 3 Expression difference analysis of ten potential hub genes between tumor tissues and normal tissues.

3.4 Survival Analysis of Ten Hub Genes.

To investigate the prognostic values of the ten potential hub genes, the Kaplan–Meier plotter bioinformatics analysis platform was used. A total of 54 laryngeal cancer patients were available for the analysis of relapse-free survival. (Figure 4).

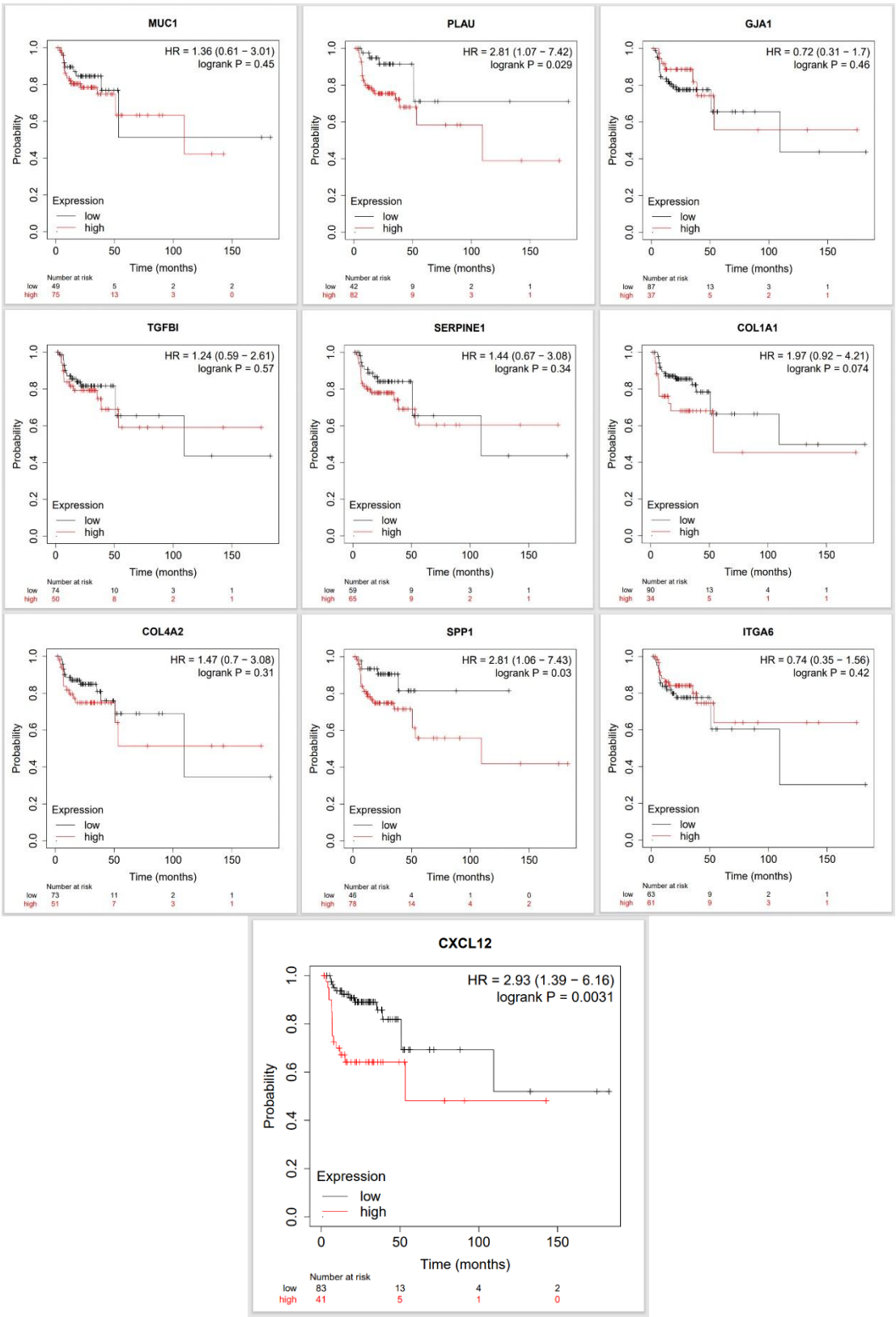

Figure 4 Kaplan–Meier relapse-free survival analyses for the top ten hub genes in laryngeal cancer patients.

However, only overexpression of CXCL12 was an unfavorable prognostic factor of relapse-free survival in LSCC patients (HR=2.93; 95% CI: 1.39–6.16; P=0.0031). There were not enough incidents for overall survival analysis.

4. Discussion

Due to lacking a therapeutic target, patients with LSCC could not benefit from endocrine therapy or HER2-targeted therapy. Radiotherapy and chordectomy are currently the mainstay of adjuvant treatment. In the present study, gene expression and protein–protein expression analysis based on publicly available databases was performed to identify potential key genes correlated with LSCC. After constructing gene networks, we identifed potential biomarkers for LSCC prognosis. Ten hub genes were identified, including MUC1, ITGA6, SPP1, COL1A1, SERPINE1, COL4A2, TGFBI, GJA1, CXCL12, and PLAU.

MUC1 (Mucin 1) is a transmembrane glycoprotein which is expressed in normal epithelial cells, but it was overexpressed and aberrantly glycosylated in majority of carcinomas[14], including laryngeal cancer[15,16]. Accumulating evidence supported the involvement of the oncogenic MUC1 in tumor metastasis.

ITGA6(Integrin Subunit Alpha 6),the gene encodes a member of the integrin alpha chain family of proteins. Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain that function in cell surface adhesion and signaling. For ITGA6 mRNA expression, Pyeon[17] reported a fold change of 2.757 in patients with tongue carcinoma and a fold change of 3.133 in patients with oropharyngeal carcinoma. In Estilo's dataset[18], ITGA6 was overexpressed in tongue squamous cell carcinoma with a fold change of 6.290. According to Talbot's dataset[19], ITGA6 was overexpressed in tongue squamous cell carcinoma with a fold change of 4.086.

SPP1(secreted phosphoprotein 1), also known as Osteopontin (OPN), is located at 4q22.1. Participating in tumorigenesis and metastasis. OSCC is a subtype of HNSCC. Huang et al. [20] detected higher expression of SPP1, PAI and caveolin-1 in OSCC tissues than normal tissues by immunohistochemical staining, indicating that SPP1 overexpression is associated with OSCC carcinogenesis and progression.

GJA1(Gap Junction Protein Alpha 1) ,this gene is a member of the connexin gene family. The encoded protein is a component of gap junctions, which are composed of arrays of intercellular channels that provide a route for the diffusion of low molecular weight materials from cell to cell.

COL1A1(Collagen Type I Alpha 1 Chain),this gene encodes the pro-alpha1 chains of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain. Type I is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis and tendon. Collagen type I α1 (COL1A1) and collagen type I α 2 (COL1A2) expression levels have been reported to predict prognosis in various types of cancer. However, the effect of these biomarkers on hypopharyngeal squamous cell carcinoma (HPSCC) is yet to be fully elucidated[21].

SERPINA1(Serpin Family A Member 1), an inhibitor of serine proteases, irreversibly inhibits trypsin, chymotrypsin, and plasminogen activator [22]. Its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin[22]. A recent study showed a higher abundance of SERPINA1 candidate biomarkers in the saliva of patients with oral squamous cell carcinoma (OSCC), demonstrating that SERPINA1 is related to OSCC development [23].

COL4A2(Collagen Type IV Alpha 2 Chain), a gene plays an important role in the formation of type IV collagen. The role of type IV collagen is essential for maintaining the stability and functionality of the vascular basement membrane [24].

TGFBI(transforming growth factor beta-induced protein) ,also known as betaig-3, is a multi-purpose matrix molecule induced by TGF- β[25]. In the present study, TGFBI was highly expressed in tumor tissues compared with adjacent non-tumor or normal tissues, showing a highest correlation with head and neck squamous cell carcinoma (HNSCC). Further validation of the role and association of TGFB1 and HNSCC development may lead to new targets in HNSCC treatment[26].

CXCL12(C-X-C Motif Chemokine Ligand 12) ,regulates adhesion of tumor cell with laminin,

fibrinogen, stromal cells and endothelial cell by activating various cell surface adhesion molecules. CXCL12, also known as stromal-derived factor 1 (SDF-1), is widely secreted in different tissues by stromal cells, fibroblasts and epithelial cells in six different isoforms, encoded on chromosome 10q11. The effects of CXCL12 on the expression and activity of integrins on cell surfaces may be crucial in adhesion to fibronectin and collagen I in prostate cancer cell. CXCL12 is closely related to angiogenesis which supplying nutrient to tumor cells and giving rise to excretion of tumor cells metabolites efficiently. CXCL12 can stimulate angiogenesis directly or indirectly[27].

PLAU(Plasminogen Activator, Urokinase), one of the major proteolytic enzymes involved in degradation of extracellular matrix, has been demonstrated to play critical roles in tissue remodeling and migration in the developmental as well as tumorigenesis process, whereas SERPINE1, as the most important physiological inhibitor of the PLAU, could in turn reverse this process and regulate the adhesion/ deadhesion balance of cells to the ECM[28].

In view of the prognostic potency of these hub genes for LSCC in database, by the validation of their top degree of genes and change levels of mRNA in microarrays, we selected CXCL12 further detect their protein level by immunostaining. Our clinical analysis showed that CXCL12 were signifcantly changed in the progression of LSCC. They were aberrantly expressed in the epithelium of LSCC and correlated with aggressiveness of LSCC patients, which implied that these signature genes are possibly not only involved in the initiation of tumorigenesis but also late stages of cancer. Therefore, CXCL12 could be potentially utilized as diagnostic and prognostic biomarkers for LSCC. More importantly, by comparing the extent of protein changes, the overexpressed CXCL12 are the most promising markers, and its detection could help to identify tumor cells in tissues[29].

Finally, the Kaplan–Meier plotter online tool was applied to predict the relationship between the expression of hub genes and prognosis of LSCC patients. Based on the Kaplan–Meier plotter, overexpression of all the above genes was related to unfavorable prognosis of laryngeal cancer patients. However, only overexpression of CXCL12 was an unfavorable prognostic factor of LSCC patients.

There are several limitations of our study. First, we did not perform biological experiments to validate our results. Second, the quality of data acquired from public databases cannot be evaluated.

5. Conclusion

Our bioinformatics analysis identified 104 DEGs between LSCC and normal laryngeal tissues based on the gene expression datasets obtained from the GEO database. Among them, ten hub genes might be the core genes of laryngeal cancer, including MUC1, ITGA6, SPP1, COL1A1, SERPINE1, COL4A2, TGFBI, GJA1, CXCL12, and PLAU. All of them were upregulated in laryngeal cancer, and overexpression of these genes was associated with unfavorable clinical outcome in laryngeal cancer patients. In LSCC patients, CXCL12 overexpression is an unfavorable prognostic factor. Further study is needed to confirm the results of our research. Anyway, CXCL12 may be a potential target for LSCC therapy.

Acknowledgment

Disclosure The authors report no conflicts of interest in this work.

Ethical approval All the data used was open access and approved with the ethics approval of The National Cancer Institute's (NCI) Genomic Data Commons (GDC) policy.

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