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Identification of immunocell infiltrates and effective diagnostic biomarkers in laryngeal carcinoma

Xue Han, MM[a], Xiaowen Cheng, MD[b], Kaiqi Dai, MM[a], Wenyu Bao, MM[a], Ran Ding, MM[c], Yufeng Wan, MM[a,*]

Abstract

Laryngeal cancer (LC) is a malignant tumor that occurs in the head and neck. Laryngeal cancer is one of the most common cancers of the neck and head, and its prognosis has always been poor. The incidence of LC increased gradually and showed an early rising trend. Laryngeal cancer is rarely studied in relation to immunity, Malignant tumors will change the state of the human body in various ways to adapt to their own survival and avoid the immune system. This study aims to explore the immune molecular mechanism of laryngeal cancer through bioinformatics analysis. The gene expression data was downloaded for 3 microarray datasets: GSE27020, GSE59102, and GSE51985. CIBERSORT algorithm was performed to evaluate immune cell infiltration in tissues between LC and healthy control (HC). Differentially expressed genes (DEGs) were screened. Functional correlation of DEGs were analyzed by Gene Ontology, Gene Set Enrichment Analysis and Kyoto encyclopedia of genes and genomes. Candidate biomarkers were identified by cytoHubba of Cytoscape. Spearman correlations between the above biomarkers and infiltrating immune cells were explored using *R* software analysis. The immune cell types of LC and HC were significantly different. Twenty-one DEGs were obtained by cross-screening. The function of DEGs is closely related to the number of immune cells. Five central genes (TNNT3, TNNI2, Desmin, matrix metallopeptidase 9 and cytotoxic T lymphocyte antigen 4) were screened. The HUB gene was demonstrated to have the ability to diagnose LC and HC with good specificity and sensitivity. The correlation between immune cells and biomarkers showed that hub gene was positively correlated with macrophages and dendritic cells, and negatively correlated with CD4 + T cell. TNNT3, TNNI2, Desmin, matrix metallopeptidase 9 and cytotoxic T lymphocyte antigen 4 can be used as diagnostic biomarker for LC. Macrophages, dendritic cells and CD4 + T cell may participate in the occurrence and development of LC.

Abbreviations: CTLA4 = cytotoxic T lymphocyte antigen 4, DCs = dendritic cells, DES = desmin, DEG = differential expression analysis, GO = gene ontology, HC = healthy control, ICs = immune checkpoints, LC = laryngeal cancer, MMP9 = matrix metallopeptidase 9, TME = tumor microenvironment.

Keywords: bioinformatics, biomarkers, immune cell, immune cells, infiltration, laryngocarcinoma

1. Introduction

Laryngeal cancer is one of the most common malignant tumors in otolaryngology, which seriously threatens the life and health of men.[1] Smoking, alcohol consumption, and other risk factors have been reported to be associated with laryngeal cancer.[2] A previous study indicated that there were 1,42,000 cases of laryngeal cancer worldwide in 2000.[3] Another study estimated that there are about 12,500 new cases per year.[4] In recent years, the incidence of laryngeal cancer has been younger and gradually increased. Due to the lack of high-efficiency diagnostic markers, most laryngeal cancer patients are only found in clinical stage III and IV, and have distant organ metastases, losing the opportunity for radical surgery. Therefore, the 5-year survival rate of laryngeal cancer is less than 50%.[2,3] In advanced stages, it directly affects the structure and function of organs related to swallowing and speaking, resulting in a decrease in the patient's quality of life.[5,6]

The tumor microenvironment (TME) is composed of multiple cell types, including tumor cells, immune cells, endothelial cells, adipocytes, and fibroblasts, as well as various structures such as blood vessels, lymphatic vessels, and extracellular matrix. It plays an important role in tumor growth, invasion, metastasis, diagnosis and treatment. High levels of T-lymphocyte infiltration

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The datasets generated during and/or analyzed during the current study are publicly available.

[a] Department of Otolaryngology, Head and Neck Surgery, Chaohu Hospital Affiliated to Anhui Medical University, Hefei, China, [b] Department of Clinical Laboratory, The First Affiliated Hospital of Anhui Medical University, Hefei, China, [c] School of Medical Information Engineering, Anhui University of Traditional Chinese Medicine, Hefei, China.

** Correspondence: Yufeng Wan, Department of Otolaryngology, Head and Neck Surgery, Chaohu Hospital Affiliated to Anhui Medical University, Hefei 238000, China (e-mail: windy135@sina.com).*

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in the TME or near the tumor cell parenchyma may have prognostic value.[7,8] Previous studies have found that immune cell infiltration is associated with clinical prognosis in laryngeal cancer patients.[9,10] The poor prognosis is attributed to its hidden early symptoms and the difficulty of early diagnosis. Therefore, the search for efficient early diagnosis markers and precise and safe targeted therapy are particularly critical to improve the poor prognosis and high mortality of laryngeal cancer.

The inflammatory response of the immune system and the factors it produces are thought to play a key role in the development, progression, and response to treatment of laryngeal tumors.[11] In recent years, tumor-infiltrating lymphocytes have emerged as a possible biomarker of immune cells in head and neck squamous cell carcinoma, including CD4 + and CD8+, associated with improved survival rate and treatment response in head and neck cancer.[12,13]

The aim of this study was to evaluate the value of tumor-infiltrating lymphocytes as cellular and biomarkers in tumor tissues of laryngeal cancer patients. Furthermore, this study wished to investigate the prognostic significance and clinical features of these biomarkers.

2. Materials and methods

2.1. Data process

Laryngeal cancer gene expression profiles were screened using GEO (http://www.ncbi.nlm.nih.gov/geo) and UCSC-XENA (https://xenabrowser.net//) databases. UCSC-XENA database collates TCGA clinical data. Inclusion criteria were as follows: mRNA expression profiles by array or high-throughput sequencing; Availability of laryngeal cancer or paracancerous tissue in the dataset; 10 or more laryngeal cancer specimens in the dataset. Four eligible datasets were selected, among which the laryngeal cancer samples of TCGA samples from UCSC-XENA included 111 tumor samples and 12 adjacent cancer samples. The details of GSE27020, GSE59102 and GSE51985 data are shown in Table 1.

The gene expression data were downloaded for 3 microarray datasets: GSE27020, GSE59102, and GSE51985. For TCGA data, the gene expression data of head and neck cancer patients with larynx were extracted from the clinical phenotype. For GEO data, the expression matrix of the probes was extracted and normalized using the robust multi-array averaging algorithm of the affy package of R software. The probe expression matrix was converted to the gene expression matrix using the platform annotation file. For the case where 1 gene corresponds to multiple probes, the average value was taken. After merging the 3 gene expression matrices in the *R* software, the *R* software package SVA was used to eliminate heterogeneity caused by different experimental batches and platforms. Finally, we got a pooled normalized gene expression matrix.

2.2. Assessment of the distribution of immune cell subtypes

The CIBERSORT algorithm was used to evaluate the immune cell infiltration in laryngeal cancer tumor tissue and normal tissue. The algorithm can convert normalized gene expression matrices into components of infiltrating immune cells. After data submission to the CIBERSORT website (http://CIBERSORT.stanford.edu/), LM22 was used as a reference expression label with 1000 permutations. LM22 labeling matrix defines 22 infiltrating immune cell components, including macrophage subsets (*M*0 macrophages, *M*1 macrophages, and *M*2 macrophages), T cells (CD8 + T cells, CD4 + T cells, memory resting CD4 + T cells, memory activated CD4 + T cells, Tfh cells, regulatory T cells and gamma delta T cells), Natural Killer cells (resting and activated Natural Killer cells), mast cells (resting and activated mast cells), B cells (naïve B cells and memory B cells), dendritic cells (DCs) (resting and activated DCs), monocytes, plasma cells, neutrophils, and eosinophils. The *P*-value and root mean square error for each expression file was determined in CIBERSORT. Data with *P*-value < .05 were reserved for subsequent analysis. A complete matrix of immune cell infiltration levels is generated from this file. Results from CIBERSORT were visualized using the R packages corplot, vioplot, ggplot2 and glment.

2.3. Correlation analysis between immune cells and patient survival prognosis

After obtaining the infiltration of different immune cells in the tumor microenvironment, combined with the survival prognosis of TCGA patients, the patients were divided into high and low groups according to the degree of immune cell infiltration, and Kaplan–Meier survival analysis was performed using the survival package in *R* software.

2.4. Screening for differential expression genes

The limma R software package was used to screen the differentially expressed genes (DEGs) between laryngeal cancer (LC) samples and normal samples in the datasets GSE27020, GSE59102 and GSE51985 for validation, and the overlapping parts of the laryngeal cancer differential genes in the 3 datasets were screened by taking the intersection. The threshold was log2FC > 1, and the adjusted *P*-value was < .05. The volcano map, heat map drawing and hierarchical clustering analysis were performed using the R software packages ggplot2 and pheatmap, respectively.

2.5. Functional enrichment analysis

Functional enrichment analysis converted gene names to gene IDs via the R package org. Hs. e.g., db. The gene ontology (GO) of DEGs and the Kyoto encyclopedia of genes and genomes were analyzed using the R package clusterProfiler. Significantly different GO items and signaling pathways were screened by threshold *P* value of .05 and threshold q value of .05. Results were visualized by *R* package enrichment plot and ggplot2.

Table 1

Screening of laryngeal cancer gene expression data

GEO	Platform	Tissue	Samples			Experiment type
			Total	HC	LC	
GSE27020	GPL96	Tumor tissue	59	0	59	Array
GSE59102	GPL6480	Tumor tissue	42	13	29	Array
GSE51985	GPL10558	Tumor tissue	20	10	10	Array

HC = healthy control, LC = laryngeal cancer.

2.6. Screening of key genes and clinical sample validation of key genes

STRING (https://string-db.org) analyzed the PPI network of DEGs with high reliability, passing the filter condition (skey > 0.7). The file string_interactions.tsv was downloaded. Each node gene was scored using cytoHubba in Cytoscape (v 3.7.2), and the key genes were obtained by screening. TIMER (Cistrom.shinyapps.io/TIMER) aims to comprehensively study the molecular characterization of tumor-immune interactions. It offers 6 major analysis modules that allow users to interactively explore the links between immune infiltration and a wide range of factors, including gene expression, clinical outcomes, somatic mutations, and somatic copy number changes. In the present study, we used the gene module to visualize the correlation between key gene levels and immune cell infiltration levels in LC. And the expression levels of key genes in normal and tumor samples of laryngeal carcinoma were displayed by boxplot.

2.7. Ethics approval statement

This study only uses existing database resources, does not belong to clinical research, does not require personal information, and therefore does not require the approval of the ethics committee.

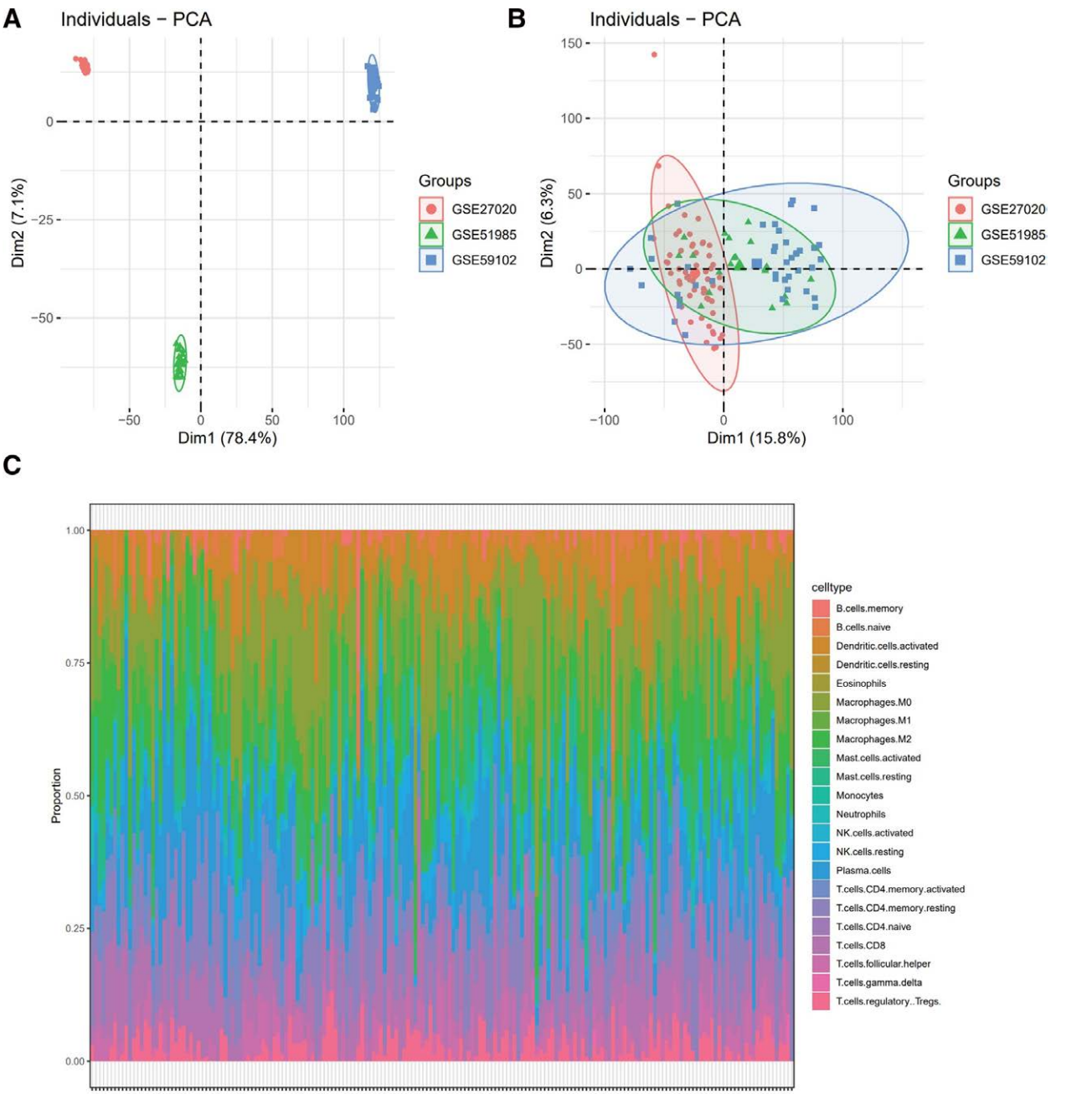

Figure 1. Data preprocessing. GSE59102, GSE51985 and GSE27020 were removed using boxplots and principal component analysis. (A) Batch effects before and (B) after batch correction. (C) Immune cell infiltration in laryngeal carcinoma tumors and adjacent tissues. The composition of the 22 immune cells in each sample is shown with a histogram.

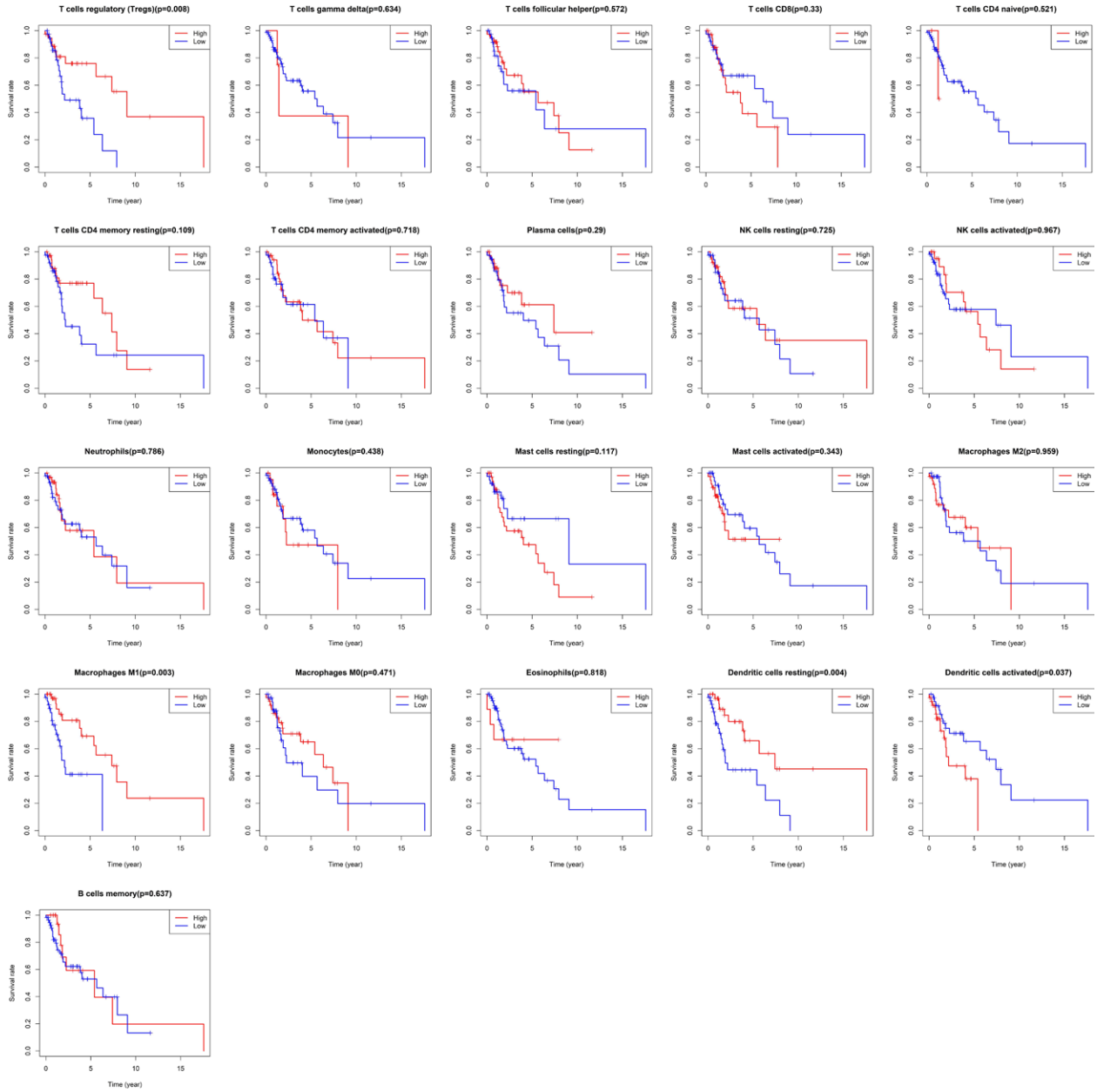

Figure 2. Kaplan–Meier curves of OS (Overall survival) of TCGA laryngeal cancer patients divided into high and low groups according to the degree of immune cell invasion, and *P* values were calculated by inter-type log-rank test. OS = overall survival.

3. Results

3.1. Immune infiltration of tumor tissue and normal tissue in laryngeal carcinoma

Three microarray raw datasets of GEO, including 121 LCs and 23 paracancerous tissues, were selected to study immune cell infiltration. Figure 1A presents the data before batch processing and Figure 1B after batch processing, illustrating that the batch effect of the merged data is successfully removed. A total of 69 LCs were eligible for CIBERSORT analysis ($P < .05$). The composition of the 22 immune cells in each sample was presented as a histogram (Fig. 1C). Colors in the histogram represent the percentage of distinct immune cells in each sample, summed to 1. In the heatmap, immune cells for each sample are shown in normalized absolute abundance.

3.2. Survival analysis of immune cells in tumor and normal tissues of laryngeal carcinoma and prognosis of patients

According to the survival time and survival status of TCGA laryngeal cancer patients, we combined the survival prognosis of TCGA patients with different immune cells in tumor and normal tissues and made a prognosis survival analysis. Different immune cells were divided into high and low levels according to the degree of infiltration. Two lower groups, a dichotomous model was established for these 2 groups, and different survival curves were identified. Figure 2 presents in the survival analysis of 22 immune cells, the number of regulatory T cells, macrophage M1, and DCs were closely related to the survival rate of patients with laryngeal cancer ($P < .05$).

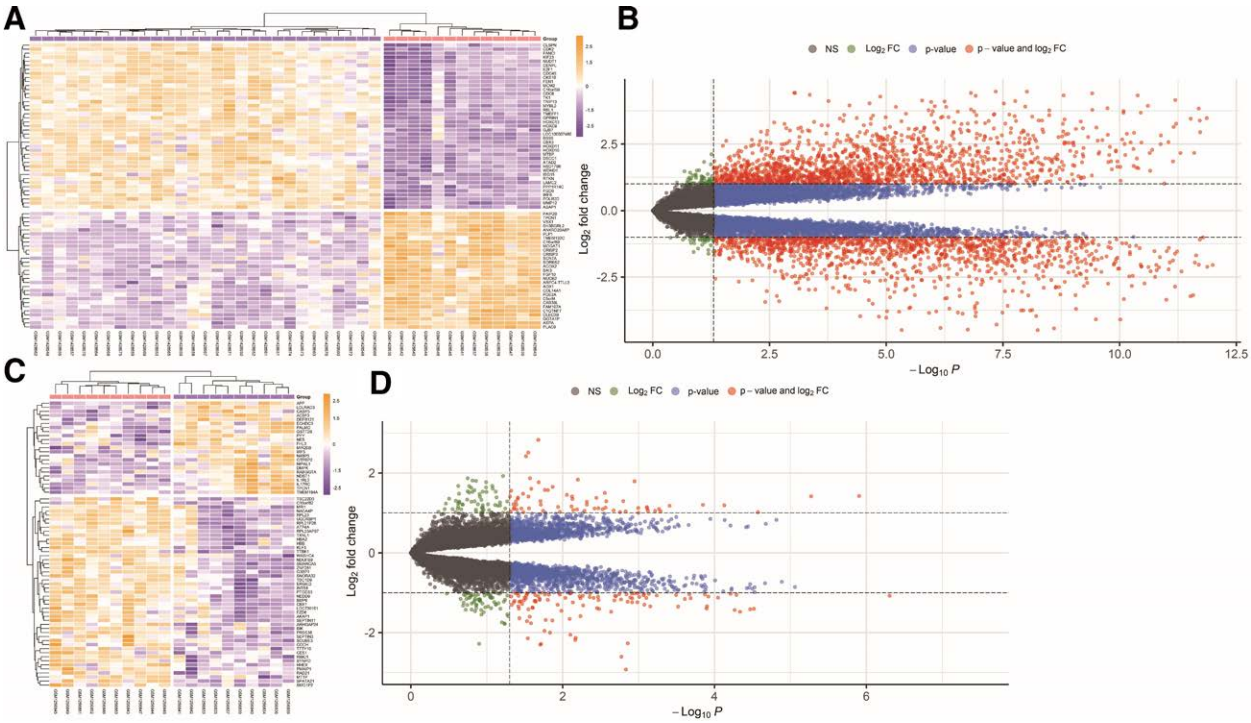

Figure 3. Heat map and volcano map of differential gene expression (A) GSE59102 (B) GSE51985.

3.3. Differential expression analysis

The 2 datasets CSE59102 and GSF51985 downloaded and screened from the GEO database were visualized with the help of R software on the differentially expressed genes of each dataset by heat map and volcano plot, and the results are shown in (Fig. 3A–D). The analysis of CSE59102 yielded 2967 common DEGs, of which 1648 were up-regulated and 1319 were down-regulated, and the analysis of CSE51985 yielded 179 common DEGs, of which 66 were up-regulated and 113 were down-regulated.

3.4. TCGA differential analysis, functional enrichment analysis and pathway enrichment analysis

A total of 123 laryngeal cancer samples from TCGA were differentially analyzed, and PCA principal component analysis was performed on the data using *R* software (Fig. 4A), resulting in 2034 common DEGs, of which 815 were up-regulated and 1219 were down-regulated, and analyzed by heat map and volcano plot The difference analysis results were visualized (Fig. 4B–C), and 21 overlapping genes were obtained by intersecting the difference analysis results of the 3 laryngeal cancers (Fig. 4D), through gene set enrichment analysis enrichment analysis, gene set enrichment analysis enriched (GO: 0002250) adaptive immune response (Fig. 4E), indicating that this metabolic activity is activated during the occurrence and development of laryngeal cancer. In the GO enrichment analysis results, biological processes were mainly enriched in the organization of extracellular matrix and extracellular structure (Fig. 4F). These biological processes mainly represent the metabolic activities of the tumor microenvironment during the occurrence of laryngeal cancer. At the same time, the extracellular matrix mainly relates to immune processes and immune microenvironment; molecular functions are mainly enriched in collagen-containing extracellular matrix, cell-cell junctions (Fig. 4G), and these processes are closely related to tumor division and tumor cell proliferation; the components are mainly related to receptor ligand activity, signaling receptor activator activity, etc. (Fig. 4H), which are related to the occurrence and development of tumors; the pathway enrichment results show that in the process of laryngeal cancer, the interaction of cell cycle and cytokine receptors, DNA replication, ECM-receptor interaction and other cell proliferation, verifying the activation of related immune pathways (Fig. 4I), suggesting that immunity can be used as a targeted therapy pathway in cancer development.

3.5. Analysis of key genes

The STRING online database was used to construct the protein-protein network interaction and visualized by Cytoscape software and scored by Cytohubba to obtain the key network. The 5 genes in the network were TNNT3, TNNI2, desmin (DES), matrix metallopeptidase 9 (MMP9), and cytotoxic T lymphocyte antigen 4 (CTLA4) (Fig. 5A), we investigated the correlation of key genes with immune cells in laryngeal cancer samples, and Figure 5B shows the Pearson correlation value between them. Next, we investigated the scatter plots of the changes in the development of the degree of invasion of regulatory T cells, macrophage M1, DCs and key genes previously associated with survival rate in laryngeal cancer. The number of DCs was positively correlated with the number of CD4 + T cells (Fig. 5C–H). At the same time, we also found that these genes had significant expression differences between tumor and adjacent tissues (Fig. 5I), and the results show that these genes can be used as key markers for judging laryngeal cancer tissue.

4. Discussion

In recent years, with the rapid development of bioinformatics technology, gene chips and high-throughput sequencing technologies have become more and more mature, bringing infinite possibilities for the study of human diseases, especially the pathogenesis of tumors, and for the clinical diagnosis and target of tumors, opening up new avenues for treatment. The analysis of 3 test microarray datasets, GSE27020, GSE59102 and GSE51985, in this study, with the help of a series of

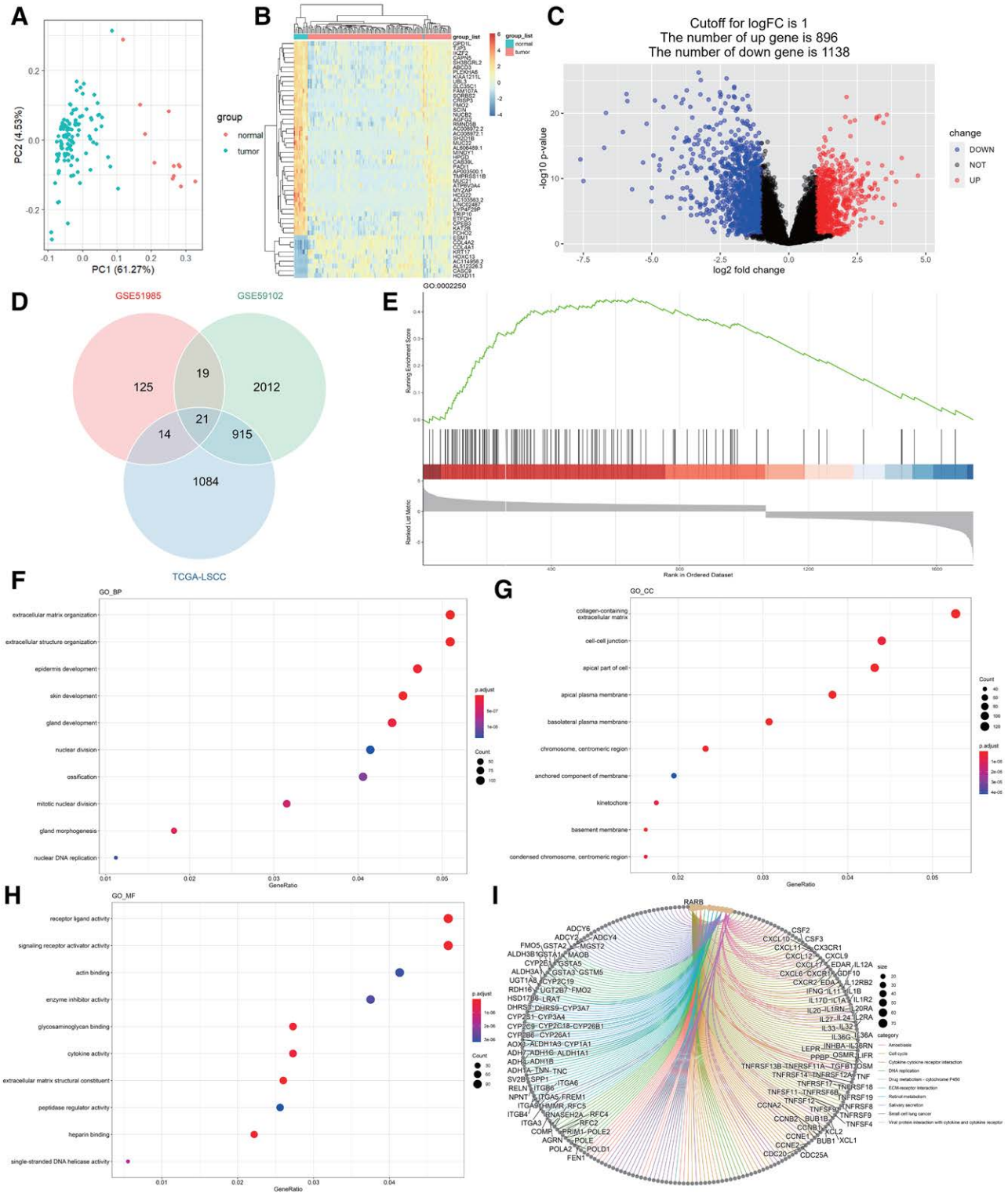

Figure 4. (A) PCA principal component analysis of TCGA laryngeal carcinoma. (B–C) Differential gene expression heat map and volcano plot. (D)VENN graph to take the intersection. (E) GSEA functional enrichment analysis. (F–H) F: biological process; G: cellular composition; H: molecular function; I: KEGG pathway. GSEA = gene set enrichment analysis. KEGG = Kyoto encyclopedia of genes and genomes.

bioinformatics tools, showed that genes such as TNNT3, TNNI2, DES, MMP9 and CTLA4 can be used as key signatures for judging laryngeal cancer tissue. Macrophages, DCs and CD4 + T cells may be involved in the occurrence and development of LC.

TNNT3 and TNNI2 are members of the troponin family and are generally considered to be involved in the regulation of muscle activity. TNNT3 is a risk factor for breast cancer and melanoma.[14,15] TNNI2 encodes a fast-twitch skeletal muscle protein that is present in corneal epithelium and cartilage and acts as an inhibitor of angiogenesis to inhibit tumor growth and metastasis. The expression of TNNI2 in gastric tissue can be used as a specific biomarker for predicting peritoneal metastasis of gastric cancer.[16] DES is a 53.5 kDa protein mainly present

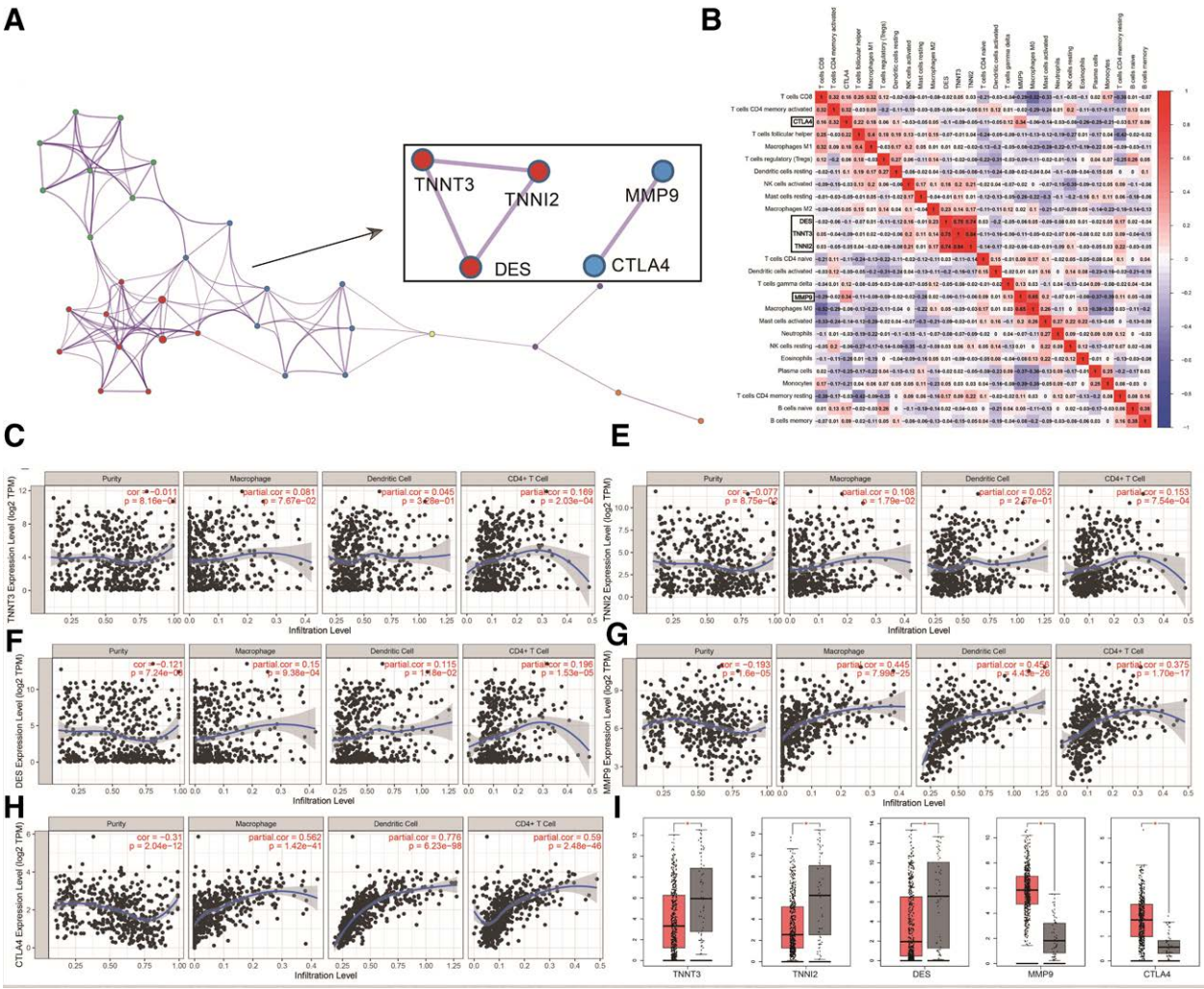

Figure 5. (A) Gene interaction network and core gene network. (B) Correlation between immune cells, core genes. (C–H) Trend plot of core genes and prognosis-related immune cells. (I) The expression of core genes in laryngeal carcinoma tumor tissue and adjacent tissue.

in skeletal muscle, smooth muscle and endothelial cells. DES is present in most rhabdomyomas, leiomyomas, rhabdomyosarcomas, and leiomyosarcoma. DES staining, which identifies pericyte coverage and extent of mature tumor vasculature, can be used as biomarkers to predict the efficacy of antiangiogenic cancer therapy in cancer patients.[17] MMP9 belongs to the family of matrix metalloproteinases, which can degrade collagen fibers and gelatin in the extracellular matrix and change the microenvironment of cells, thereby facilitating tumor invasion and metastasis. Studies have shown that MMP9 is a susceptibility gene for smoking-related laryngeal cancer.[18,19] Immune checkpoints (ICs) are a series of molecules expressed on the surface of immune cells that can regulate the degree of immune activation, and play a "brake" role in the body's immune system. When a tumor occurs in the body, IC is activated, inhibiting the immune function of T cells, and the tumor can escape the immune surveillance of the body and survive. By blocking IC, T cells can be activated and the body's immune response can be enhanced, thereby improving the body's anti-tumor ability.[20] CTLA4 was the first immune checkpoint targeted for cancer therapy. CTLA4 is expressed on activated T lymphocytes and has homology to the CD28 molecule and acts by competitive binding and binding to the B7 molecule on antigen presenting cells. The unique periodic arrangement formed between CTLA4 and B7 ligands provides an abnormally stable signaling complex for the negative regulation of T cell immune responses within the immune synapse.[21] As a negative regulator of T cell activation, blocking CTLA4 can reactivate the immune response of T cells to achieve anti-tumor purposes. Preclinical studies have shown that blocking CTLA-4 reduces tumor growth in mice with melanoma, colon cancer, and many other tumor models.[22,23] CTLA-4 blockers are also used in throat cancer, hepatocellular carcinoma, Hodgkin's lymphoma, prostate cancer, renal cell carcinoma, and other cancers.

The metastasis and recurrence of laryngeal cancer may be due to the abnormal interaction of immune cells with tumor cells and stromal cells in the TME.[24] Infiltrating cells in throat cancer are generally divided into 2 types, those that promote tumor growth and those that inhibit tumor growth. Tumor-associated macrophage infiltration is often associated with poor prognosis and lymphatic metastasis.[25,26] Macrophages in the TME regulate laryngeal cancer stem cells through CD44.[27] Exosomes secreted by laryngeal cancer cells accumulate in the TME and promote endothelial cell angiogenesis.[28] Macrophages also secrete vascular endothelial growth factor to promote new blood vessel formation.[29] DCs often play an antitumor role in the TME.[30–32] Previous studies have shown that several subtypes of tumor-infiltrating T cells, especially CD4 + T cells, have a favorable effect on the prognosis of laryngeal cancer.[12,33,34] High tumor and peritumoral density of CD4 + T cells has also been identified as a positive predictor of overall survival, disease-specific survival and disease-free survival.[35–37]

At present, according to our data analysis, genes such as TNNT3, TNNI2, DES, MMP9 and CTLA4 can be used as diagnostic markers for laryngeal cancer. The correlation between immune cells and biomarkers shows that key genes are associated with macrophages and DCs. There is a positive correlation with the number of CD4 + T cells and a negative correlation with the number of CD4 + T cells, which has important guiding significance for the future clinical treatment of laryngeal cancer. However, we need to collect more datasets for further analysis to validate our findings and further study the biological functions of these key genes. Second, more basic experiments are needed to verify the mechanisms by which key tumor genes and immune cells promote or inhibit tumor cell growth. In the future, more functional studies are needed to better characterize the roles of key genes and immune cells in LC. We strongly recommend further research on this topic to progressively improve the scholarly impact of these genetic biology evidence.

5. Conclusion

TNNT3, TNNI2, DES, MMP9 and CTLA4 can be used as diagnostic biomarker for LC. Macrophages, DCs and CD4 + T cell may participate in the occurrence and development of LC.

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Author contributions

Conceptualization: Yufeng Wan.
Data curation: Xue Han, Kaiqi Dai, Wenyu Bao, Ran Ding, Yufeng Wan.
Formal analysis: Xiaowen Cheng.
Investigation: Xiaowen Cheng, Wenyu Bao.
Software: Ran Ding, Yufeng Wan.
Validation: Kaiqi Dai, Wenyu Bao.
Visualization: Xue Han, Ran Ding, Yufeng Wan.
Writing – original draft: Xue Han.
Writing – review & editing: Xiaowen Cheng, Yufeng Wan.

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