Original Article

Identifying key pathogenic mechanisms and potential intervention targets for recurrence after laryngeal cancer treatment through bioinformatics screening

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Contributions: (I) Conception and design: Both authors; (II) Administrative support: None; (III) Provision of study materials or patients: Both authors; (IV) Collection and assembly of data: Both authors; (V) Data analysis and interpretation: L Liu; (VI) Manuscript writing: Both authors; (VII) Final approval of manuscript: Both authors.

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Background: Laryngeal cancer (LC), a prevalent malignant tumor of the head and neck, is characterized by a high rate of postoperative recurrence and significant treatment challenges upon recurrence, severely impacting patients' quality of life. There is a pressing need for effective biomarkers in clinical practice to predict the risk of LC recurrence and guide the development of personalized treatment plans. This study uses bioinformatics methods to explore potential biomarkers for LC recurrence, focusing on key genes and exploring their functions and mechanisms of action in LC recurrence. The aim is to provide new perspectives and evidence for clinical diagnosis, prognostic evaluation, and targeted treatment of LC.

Methods: Gene expression profiles from the GSE25727 data set in the Gene Expression Omnibus database were analyzed to detect the differentially expressed genes (DEGs) between the tumor tissues of postoperative recurrent and non-recurrent early stage LC patients. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were also conducted. A protein-protein interaction (PPI) network and transcription factor (TF)-DEG-microRNA (miRNA) network were developed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, with key genes selected using the Molecular Complex Detection (MCODE) plugin. A Gene Set Enrichment Analysis (GSEA) was carried out to investigate the possible mechanisms of the key genes. A retrospective analysis was conducted using the clinical data of 83 LC patients. Immunohistochemical staining was used to examine the transcription level of the key genes in the LC tumor tissues and the factors affecting postoperative recurrence.

Results: A total of 248 upregulated and 34 downregulated DEGs were identified in the GSE25727 data set. The PPI network analysis identified a significant module and five candidate genes (i.e., *RRAGA*, *SLC38A9*, *WDR24*, *ATP6V1B1*, and *LAMTOR3*). The construction of the TF-DEG-miRNA network indicated that *ATP6V1B1* might be regulated by one TF and interact with 17 miRNAs. The KEGG and GSEA analyses suggested that *ATP6V1B1* may influence LC recurrence through the involvement of pro-inflammatory and pro-fibrotic mediators, glutathione metabolism, matrix metalloproteinases, immune regulation, and lymphocyte interactions. The recurrence rate of the 83 LC patients included in the study was 19.3% (16/83). The immunohistochemistry results indicated that ATP6V1B1 was highly expressed in patients with recurrent LC. The univariate and multivariate logistic regression analyses revealed that tumor stage T3 (P=0.04), tumor stage T4 (P=0.01), and a high expression of ATP6V1B1 (P=0.02) were risk factors for recurrence after surgical treatment in LC patients.

Conclusions: The key genes and signaling pathways identified through the bioinformatics screening provide insights into the potential mechanisms of the pathogenesis of LC. *ATP6V1B1* may promote the recurrence of LC by weakening the immune phenotype. Our findings provide a theoretical basis for further research into clinical diagnostics and treatment strategies for LC.

Keywords: Laryngeal cancer (LC); recurrence; ATP6V1B1; differentially expressed genes (DEGs); bioinformatics

Submitted Jun 18, 2024. Accepted for publication Jul 17, 2024. Published online Jul 26, 2024.
doi: 10.21037/tcr-24-1015
View this article at: https://dx.doi.org/10.21037/tcr-24-1015

Introduction

Laryngeal cancer (LC) is an aggressive tumor that develops from the laryngeal mucosal epithelium. It comprises around 13.9% of head and neck malignancies and 2.1% of all malignant tumors worldwide (1,2). In 2019, there were 209,000 new cases of LC worldwide, with a standardized incidence rate that decreased by 2.5% (3). The exact causes of LC remain unclear but it is thought to be associated with environmental factors, viruses, sex hormones, deficiency in trace elements, and radiation exposure (3).

The mainstay of clinical treatment of LC is surgery that aims to excise the lesion as thoroughly as possible while also striving to restore and reconstruct the patient's ability to speak, breathe, and swallow (4,5). However, some patients already exhibit metastasis at the time of surgery, leading to incomplete treatment and an increased risk of postoperative metastasis or recurrence. Recurrence is a leading cause of surgical treatment failure and has a direct effect on the long-term survival of LC patients (6).

Following the refinement of targeted therapy and precision radiotherapy treatment plans, the early initiation of these treatments in LC patients at risk of recurrence has recently been shown to effectively extend their disease-free survival time and reduce the likelihood of disease recurrence and metastasis (7). Research on patient prognosis has always been a hot topic in clinical studies of LC (7). Previous clinical studies have constructed prognostic prediction models based on LC patient clinical data, and laboratory, and radiological findings. However, due to environmental, ethnic, and lifestyle differences, the reproducibility of such studies has been poor.

Recently, with the formation of metagenomics, there has been an increase in studies examining the correlation between individual genes and LC recurrence (8-10). These studies have reported a high correlation between the activation and expression levels of specific human genes and LC. This correlation has been reproducibly demonstrated in patients of the same ethnicity, providing better reference values for early intervention (8-10).

This study employed bioinformatics analysis methods to conduct a comprehensive search of gene expression microarray chips related to LC samples in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). We investigated the specific enriched pathways of pivotal genes to provide evidence of the pathologic mechanisms related to disease recurrence. Finally, we also analyzed the clinical data of LC patients who underwent surgery in the First Affiliated Hospital of Wannan Medical College. The primary aim of the study was to elucidate the clinical significance of crucial genes by immunohistochemically examining their expression in LC tissues, while also evaluating the association between that

Highlight box

Key findings

- Through bioinformatics methods, we successfully identified the key gene *ATP6V1B1* associated with laryngeal cancer recurrence, whose high expression may be closely related to the recurrence of laryngeal cancer, and plays a role by affecting pro-inflammatory and fibrotic mediators, glutathione metabolism, and other mechanisms.

What is known and what is new?

- Metagenomics has revealed a strong correlation between specific gene activation/expression levels and liver cancer recurrence.
- We have systematically explored the molecular mechanisms behind laryngeal cancer recurrence by combining bioinformatics analysis and clinical data for the first time. Through gene expression profiling analysis, protein-protein interaction network construction, and enrichment analysis, we can discover new potential biomarkers and gain a deeper understanding of how these genes affect the progression and recurrence of laryngeal cancer.

What is the implication, and what should change now?

- The significance of this study lies in providing new theoretical basis for the clinical diagnosis and treatment of laryngeal cancer. The discovery of ATP6V1B1 provides a potential new target for developing targeted therapies for laryngeal cancer recurrence. In addition, our study also emphasizes the importance of early intervention and personalized treatment, which is crucial for improving the survival rate and quality of life of laryngeal cancer patients. Therefore, future research should further explore the regulatory mechanism of ATP6V1B1 and develop effective therapeutic methods targeting this gene.

expression and the recurrence of LC in patients. We present this article in accordance with the STREGA reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1015/rc).

Methods

Microarray data information

The present study identified the differentially expressed genes (DEGs) from the tumor tissues of early stage LC patients with and without postoperative recurrence as documented in the GSE25727 data set. These data underwent normalization and filtering preprocessing. Based on annotation information from the GEO database, all the identification numbers were associated with their respective gene symbols, and duplicate gene names were removed using the average method.

Detection of DEGs

The limma package (https://www.bioconductor.org/) was employed to screen for the DEGs across all data sets. The following selection criteria were applied: P<0.05 and log |fold change (FC)| ≥1. The DEGs with a log FC ≥1 and a log FC ≤–1 were considered upregulated and downregulated, respectively.

Functional enrichment analysis

The clusterProfiler package in R (https://www.bioconductor.org/) was used to conduct a functional enrichment analysis of the DEGs. These genes were classified into two high- and low-expression groups, depending on the levels of the crucial genes. The variations in the biological states of these groups were then evaluated using a Gene Set Enrichment Analysis (GSEA, V.4.2.3 https://www.gsea-msigdb.org/gsea/index.jsp). The gene data set needed for this study was obtained from the Molecular Signatures Database v.7.5.1 (https://www.gsea-msigdb.org/gsea/index.jsp). The C2 and C5 subsets of the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases, respectively, were employed to perform the functional enrichment analyses. Screening criteria: false discovery rate (FDR) <25% and P<0.05 are considered significantly enriched.

Construction of the protein-protein interaction (PPI) network and detection of the hub genes

To analyze the PPI network of the DEGs, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://cn.string-db.org/) was employed. Cytoscape v.3.9.1 (https://cytoscape.org/) was used to visualize the positions and relationships of the key DEGs in the PPI network. The Molecular Complex Detection (MCODE) plugin in Cytoscape was employed to filter central clusters, which aided in the identification of potential functional modules and activated signaling pathways.

Construction of the transcription factor (TF)-DEG-microRNA (miRNA) network

The interaction data for the TF-DEG, and miRNA-DEG analyses were obtained from the MiRTarBase v.8.0 (https://mirtarbase.cuhk.edu.cn/) and ENCODE databases (https://www.encodeproject.org/). The regulators of the miRNAs and TFs that underwent reciprocal changes were identified in the DEGs, and the respective TFs and miRNAs were isolated. Finally, an integrated TF-DEG-miRNA network was established and displayed using Cytoscape.

GSEA

An online functional analysis was conducted *via* Metascape (https://metascape.org/) to identify the DEGs for a further functional analysis. A GSEA was used to reveal whether the abundance of a whole set of genomes significantly increased or decreased in biological processes. Identifying shared signaling pathways and regulating network modules helps to understand the underlying mechanisms of biological differences between different samples.

Clinical data of LC patients

The clinical information of the LC patients who were surgically treated at the First Affiliated Hospital of Wannan Medical College between January 2020 and December 2023 was included in this retrospective analysis. To be eligible for inclusion in this study, the patients had to meet the following inclusion criteria: (I) be aged ≥18 years; (II) have positive detection results for LC based on the standard

surgical treatment; (III) have histopathological result of squamous cell carcinoma with negative margins; (IV) have attended postoperative follow-up through outpatient visits, including laryngoscopy and imaging examinations, for which the follow-up period ended on December 31, 2023. Patients were excluded from the study if they met any of the following exclusion criteria: (I) had insufficient clinical data; (II) had concomitant other tumors; (III) had uncertain results as to postoperative recurrence; and/or (IV) were lost to follow up. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of the First Affiliated Hospital of Wannan Medical College (No. LLSC-2020-003) and informed consent was taken from all the patients.

Observational indicators and follow-up

The general clinical data of the patients were gathered, including their gender, age, body mass index (BMI), smoking status, comorbidities, tumor location, tumor-regional lymph node-metastasis (TNM) staging, differentiation degree, initial surgical approach, and postoperative radiotherapy and chemotherapy (yes/no). The follow-up period for all the study subjects began on the day of surgery and lasted until the occurrence of recurrence, or the follow-up cut-off date. Based on recurrence, the study subjects were divided into recurrence and non-recurrence groups.

Tissue microarray construction and immunohistochemistry

The tissue was fixed overnight at 4 ℃ in 10% formalin, and paraffin-embedded, and the sections were then mounted onto slides. The sections were cleared using xylene for 10 minutes. After thoroughly cleaning the slides with deionized water for 5 minutes, the sections were immersed in an 80% solution of alcohol (100%) for 2 minutes. These sections were kept in a solution of 90% methanol/3% H_2O at 25 ℃ for 15 minutes to inhibit endogenous peroxidases. Next, the sections were blocked in a blocking solution (host serum diluted with tris buffered saline (TBS) at a 1:10 ratio) for 1 hour at 37 ℃. The sections were covered with ATP6V1B1 antibody (A6876, ABclonal, Wuhan, China) diluted in blocking solution (ratio: 1:50) and kept for 1 hour at 37 ℃. Prediluted universal secondary antibody [Alexa Fluor 594-conjugated AffiniPure Goat Anti-Rabbit Immunoglobulin G (IgG) (H+L) # AS074, Wuhan Fine Biological Technology Co., LTD, Wuhan, China] was used to counterstain the tissue sections that were then incubated at 37 ℃ for 30 minutes. Hematoxylin was used to stain the sections, and the sections were sealed with nail polish.

Grading scores were assigned based on the percentage of the positive-stained cells: 0 (negative), 1 (≤25%), 2 (26–50%), 3 (51–75%), and 4 (>75%), and the staining intensity: 0 (negative or no stain), 1 (weak), 2 (moderate), and 3 (strong). These two scores were multiplied to yield the final score for each specimen. The arithmetic mean of these scores was calculated to determine the cut-off value for the high and low expression of the key genes.

Statistical analysis

The statistical analysis was conducted using SPSS 24.0 software (https://www.ibm.com/spss). The categorical data were expressed as the case (percentage), and were compared using the χ^2 test. The quantitative data were expressed as the mean ± standard deviation, and were analyzed using the *t*-test. GraphPad Prism 7.0 software (https://www.graphpad-prism.cn/) was employed to graph the data and statistical analysis results. For the DEG analysis, the *t*-test was employed to calculate the P values and adjusted P values. The P values were adjusted using the FDR, and a P value <0.05 was considered statistically significant.

Results

Data normalization

We analyzed the DEGs of the tumor tissues of early stage LC patients with and without postoperative recurrence from the GSE25727 data set in the GEO database. To address any technical and systematic variability, the data underwent preprocessing, normalization, and cross-comparability adjustments. To determine the biological variability among each sample, a principal component analysis (PCA) was performed. The PCA plot illustrates the different expressions of the gene profiles (*Figure 1A*), and the boxplot displays the range of gene expression for each sample (*Figure 1B*). The raw data were robustly normalized to ensure the reliability of the data for the downstream analysis.

Detection of DEGs

All the DEGs were detected using the limma program in R

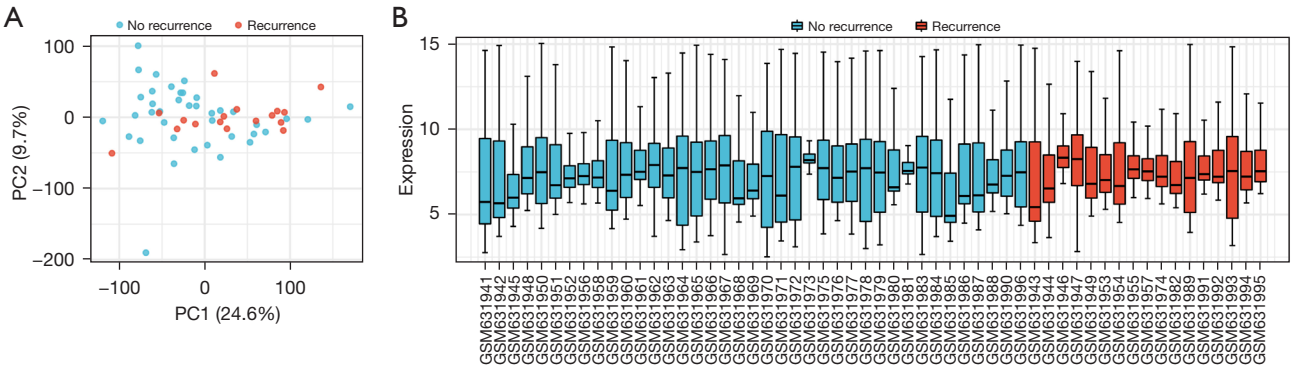

Figure 1 Distribution characteristics of normalized sample expression. (A) PCA results illustrating the variation in the expression levels of each sample; (B) boxplot showing the expression levels of all the samples. PCA, principal component analysis.

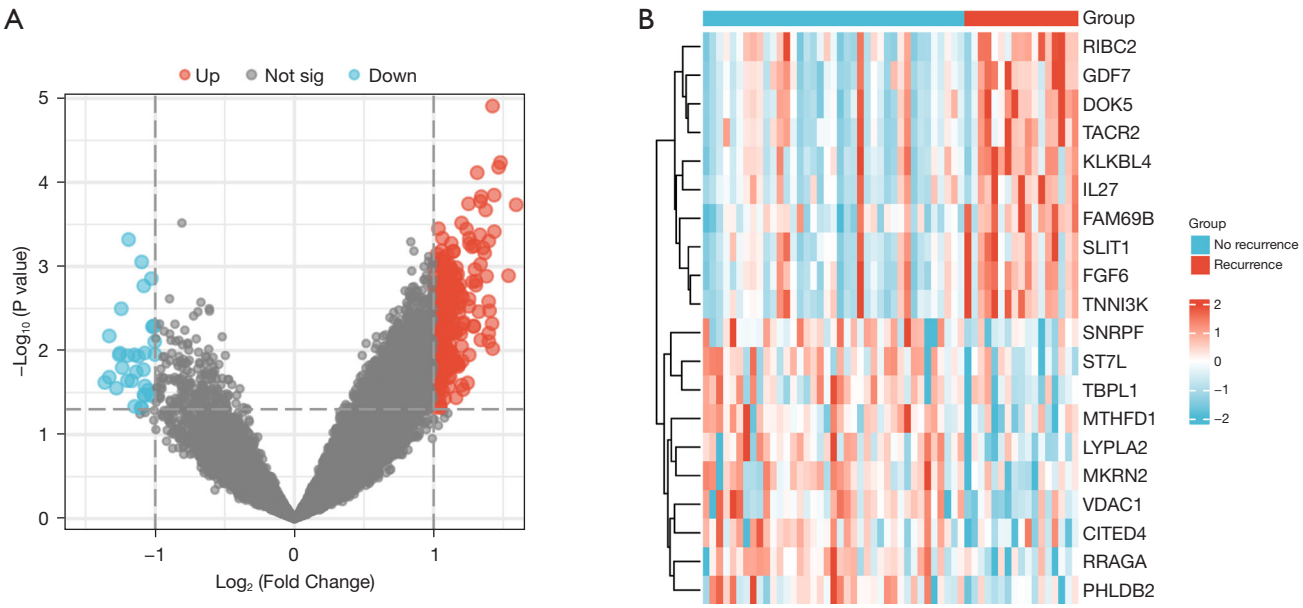

Figure 2 Heat and volcano maps of the DEGs in the data set. (A) The limma package in the R language was used to analyze the volcano map of all the DEGs in the GSE25727 data set; (B) a heatmap of the DEGs, of which 248 were upregulated and 34 were downregulated. DEG, differentially expressed gene.

as per the following criteria: |log (FC)| ≥1, and P<0.05. In total, 248 upregulated genes and 34 downregulated genes were detected. *Figure 2A* shows a volcano plot of the DEGs, while *Figure 2B* shows the considerable variations in the gene expression levels.

Functional enrichment analysis of the DEGs

A GO enrichment analysis was carried out using clusterProfiler (*Figure 3A,3B*) to investigate the biological roles of the known DEGs. The DEGs in the cellular component (CC) category were shown to be enriched in various structures, such as the photoreceptor cell cilium, non-motile cilium, inactive cilium, nucleomorph, and mitochondrial nucleomorph. In terms of their molecular functions (MFs), the DEGs were primarily involved in glycosaminoglycan binding and olfactory receptor activity.

A KEGG pathway enrichment analysis was conducted to identify the main pathways associated with the DEGs (*Figure 3C*). These genes were found to be considerably

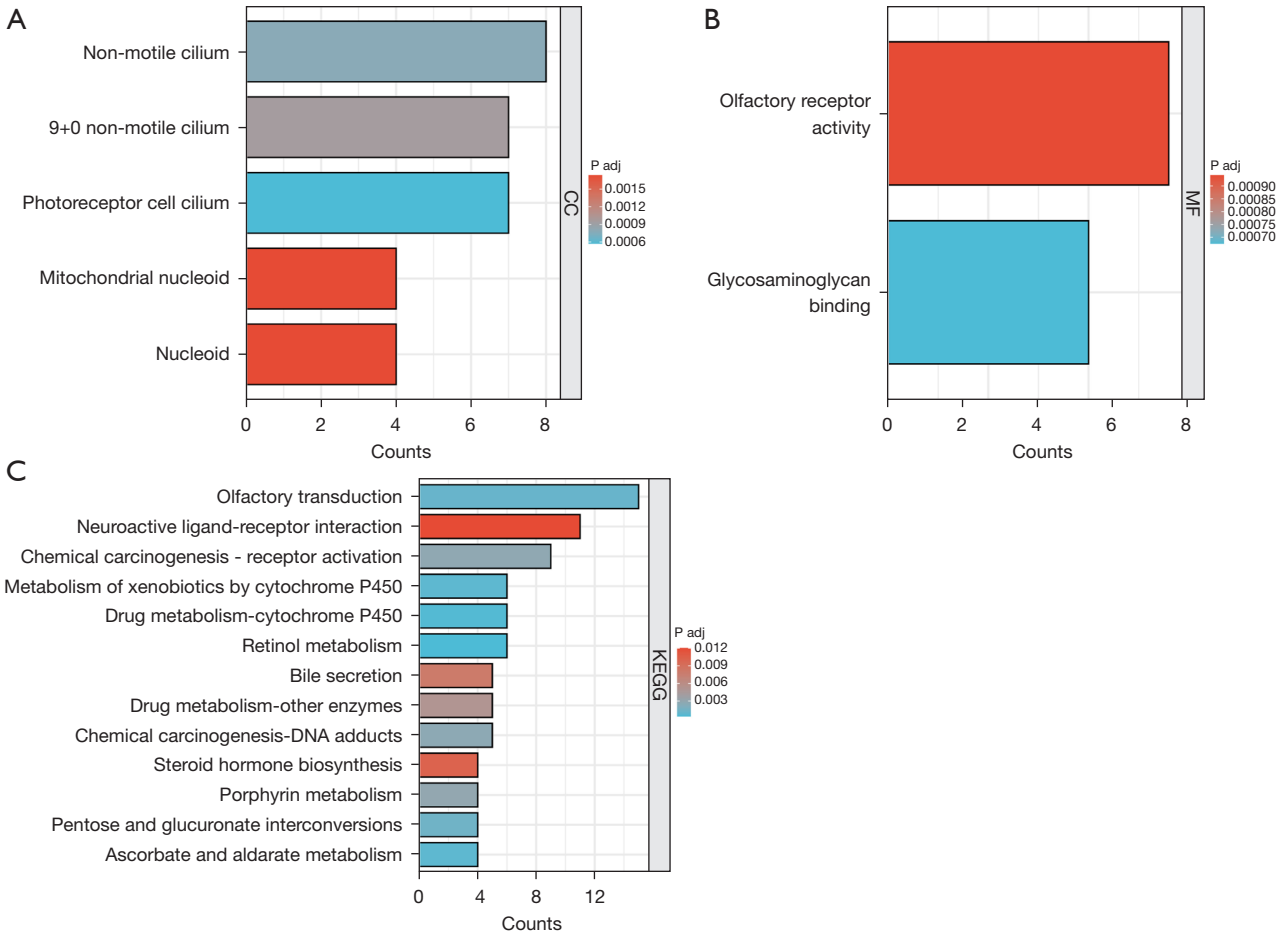

Figure 3 The outcomes of the KEGG and GO enrichment analyses of the DEGs. (A) Enrichment CC results; (B) enrichment MF results; (C) enrichment KEGG signaling pathway results. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; DEG, differentially expressed gene; CC, cellular component; MF, molecular function.

higher in the pathways associated with retinol, ascorbate and aldarate, olfactory transduction, pentose and glucuronate interconversions, porphyrin metabolisms, bile secretion, steroid hormone biosynthesis, and the interaction of neuroactive ligand-receptors. *Table 1* details the five most enriched DEGs.

Construction of the PPI network of the DEGs

The PPI network, which was constructed using the STRING database, demonstrates the functional connections between the proteins encoded by the DEGs (*Figure 4A*). Most of the proteins encoded by the DEGs were highly interconnected with other proteins. In addition, based on module analysis using MCODE, the most important modules were selected from the PPI network, including five genes (*RRAGA*, *SLC38A9*, *WDR24*, *ATP6V1B1*, and *LAMTOR3*) (*Figure 4B*). As per the GEO database, the expression changes of these genes also varied considerably between the two groups (*Figure 4C*).

Development of the TF-DEG-miRNA network

To examine the functional activities of the DEGs, we explored the possible regulatory connections between the DEGs and TFs, as well as those between the DEGs and miRNAs. A network analysis of the DEGs was conducted using the miRTarBase database, and the miRNA-DEG pairs were identified. Based on the genotypic coordinates and TF binding site data presented by ENCODE, potential regulatory associations between the DEGs and TFs were detected (*Figure 5A*). The results revealed that *ATP6V1B1*

Table 1 Top five results of the GO and KEGG enrichment analyses

Ontology	ID	Description	GeneRatio	BgRatio	P value	p. adjust
CC	GO:0097733	Photoreceptor cell cilium	7/230	121/19,594	0.0006	0.0006
	GO:0097730	Non-motile cilium	8/230	166/19,594	0.0008	0.0008
	GO:0097731	9+0 non-motile cilium	7/230	132/19,594	0.0010	0.0010
	GO:0009295	Nucleoid	4/230	44/19,594	0.0017	0.0017
	GO:0042645	Mitochondrial nucleoid	4/230	44/19,594	0.0017	0.0017
MF	GO:0005539	Glycosaminoglycan binding	10/227	234/18,410	0.0007	0.0007
	GO:0004984	Olfactory receptor activity	14/227	430/18,410	0.0009	0.0009
KEGG	hsa00830	Retinol metabolism	6/114	68/8,164	0.0004	0.0004
	hsa00982	Drug metabolism-cytochrome P450	6/114	72/8,164	0.0005	0.0005
	hsa00980	Metabolism of xenobiotics by cytochrome P450	6/114	78/8,164	0.0007	0.0007
	hsa00053	Ascorbate and aldarate metabolism	4/114	30/8,164	0.0007	0.0007
	hsa04740	Olfactory transduction	15/114	439/8,164	0.0011	0.0011

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CC, cellular component; MF, molecular functions.

may interact with 17 miRNAs and is primarily regulated by one TF (*Figure 5B*).

Signaling pathway prediction using a GSEA

Metascape was used to conduct the GSEA and functional analysis of *ATP6V1B1* online. Our findings suggest that *ATP6V1B1* may influence the occurrence and development of LC by participating in pathways that affect pro-inflammatory and pro-fibrotic mediators, glutathione metabolism, matrix metalloproteases, immune regulation, and interactions between lymphocytes (*Figure 6*).

General clinical data of LC patients in the postoperative recurrence and non-recurrence groups

This study included 83 LC patients, of whom 16 (19.28%) experienced postoperative recurrence, and 67 (80.72%) did not experience postoperative recurrence. Based on the arithmetic mean calculated from the immunohistochemistry results, specimens with a score <6 were defined as having a low expression of ATP6V1B1. The immunohistochemical results of the tissues are shown in *Figure* 7.

There were no substantial differences between the two groups in terms of gender, age, BMI, history of hypertension, history of diabetes, smoking history, primary site, M stage, histopathological type, and type of initial surgery (P>0.05). However, compared to patients in the non-recurrence group, those in the recurrence group had higher T stages, higher N stages, and lower tissue differentiation, and higher levels of ATP6V1B1 (P<0.05). Further, a significant difference was observed between the two groups in terms of postoperative radiotherapy and chemotherapy (P<0.05) (*Table 2*).

Risk factor analysis of the postoperative recurrence of LC

The variables with a P value ≤0.1 were incorporated as covariates in the univariate logistic regression. The results showed that tumor T3 stage (P=0.04), tumor T4 stage (P=0.01), and a high expression of ATP6V1B1 (P=0.02) were risk factors for recurrence after surgical treatment in patients with LC (*Table 3*).

Discussion

In China, LC is commonly treated with a comprehensive strategy that prioritizes surgery, radiotherapy, and chemotherapy. However, recurrence after the surgical treatment of LC is relatively common in clinical practice

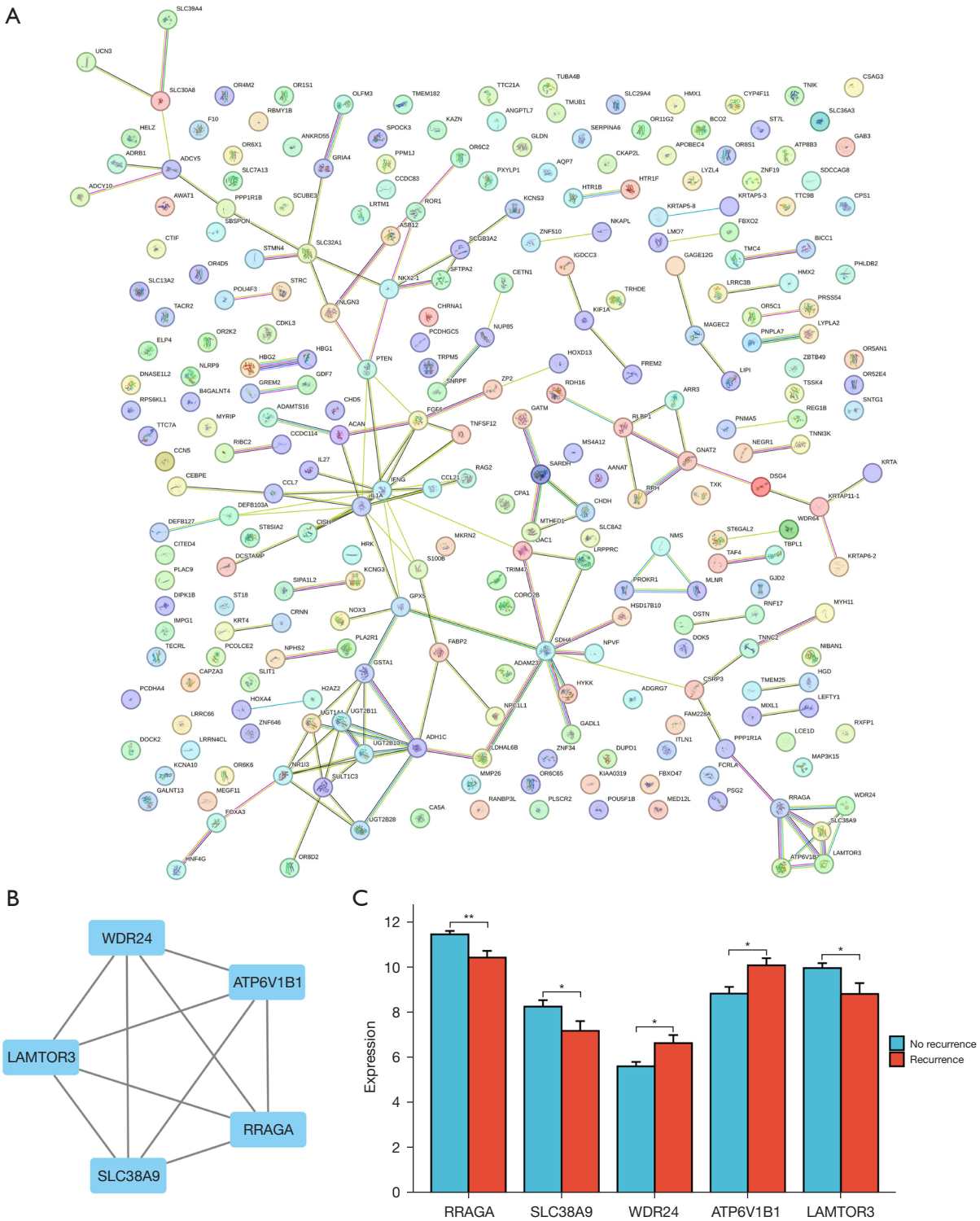

Figure 4 The most important modules of the PPI network and DEGs. (A) The PPI network of the DEGs constructed via STRING. (B) The most important modules obtained from the PPI network using the MCODE module in Cytoscape. (C) Changes in the expression of the five DEGs between the two groups of samples based on the microarray GSE25727 data set. *, P<0.05; **, P<0.01. PPI, protein-protein interaction; DEG, differentially expressed gene; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; MCODE, Molecular Complex Detection.

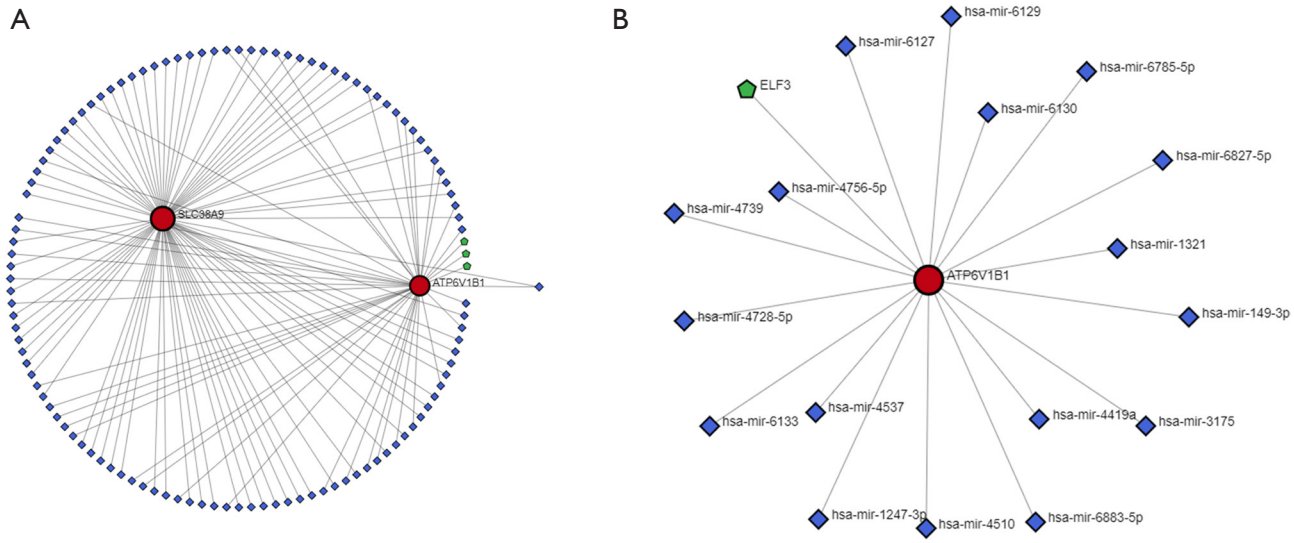

Figure 5 Development of the TF-DEG-miRNA network. (A) Construction of the TF-DEG-miRNA integrated regulatory network. (B) Comprehensive regulatory network analysis of *ATP6V1B1*. TF, transcription factor; DEG, differentially expressed gene; miRNA, microRNA.

and severely affects patient prognosis. This may be related to the complex anatomical and physiological structure of the larynx, along with its rich blood circulation and lymphatic drainage. A domestic study reported that after recurrence, the 3-, 5-, and 10-year survival rates of LC patients were 68.9%, 53.6%, and 35.7%, respectively (11). Despite significant advances in the treatment of LC in recent years, its recurrence rate remains high at 16–40% (12,13).

In recent years, owing to the ongoing advancements in molecular biology methods, gene chip technology, and second-generation sequencing technology, the exploration of DEGs at the transcriptome level and the analysis of key genes have emerged as crucial methodologies for investigating the risk factors and molecular mechanisms underlying LC recurrence. Moreover, there is increasing evidence that the abnormal expression of certain genes plays a significant role in the occurrence and development of LC (14). The identification of crucial genes implicated in the pathogenesis, prevention, and treatment of LC is contingent on the detection of the DEG profiles associated with the disease.

In this study, through the analysis of the GSE25727 data set and the use of bioinformatics techniques, we identified 248 upregulated and 34 downregulated DEGs. The functional enrichment analyses of the GO and KEGG data revealed that these genes were involved in various pathways, such as retinol metabolism, the biosynthesis of steroid hormones, ascorbate and aldarate metabolism, interconversions between pentose and glucuronate, porphyrin metabolism, bile secretion, and neuroactive ligand-receptor interactions. Moreover, these pathways have been shown to be related to the development and advancement of LC in related studies. For example, Baek *et al.* (15) found that ascorbate induces tumor cell death in laryngeal squamous cell carcinoma Hep2 cells through the generation of reactive oxygen species, the activation of protein kinase C, and cytosolic calcium signaling. Min *et al.* (16) demonstrated that INPP4B-mediated tumor resistance is related to the regulation of glucose metabolism through hexokinase 2 in LC cells. Wang *et al.* (17) confirmed through transfection experiments that Wnt1-induced signaling protein 1 regulates glucose metabolism and cisplatin resistance in LC by modulating the expression of glucose transporter 1. Based on research that the Warburg effect promotes radioresistance in various malignancies, Zhong *et al.* (18) speculated that targeting the inhibition of the hexokinase-II signaling pathway in LC could enhance its radiosensitivity.

The construction of the PPI network of the DEGs and the TF-DEG-miRNA network revealed that only ATP6V1B1 was subject to TF regulation and the potential miRNA interaction. *ATP6V1B1* encodes a non-catalytic subunit of the V1 complex of Vacuolar-type H+-ATPase (V-ATPase) (19), a multisubunit enzyme composed of

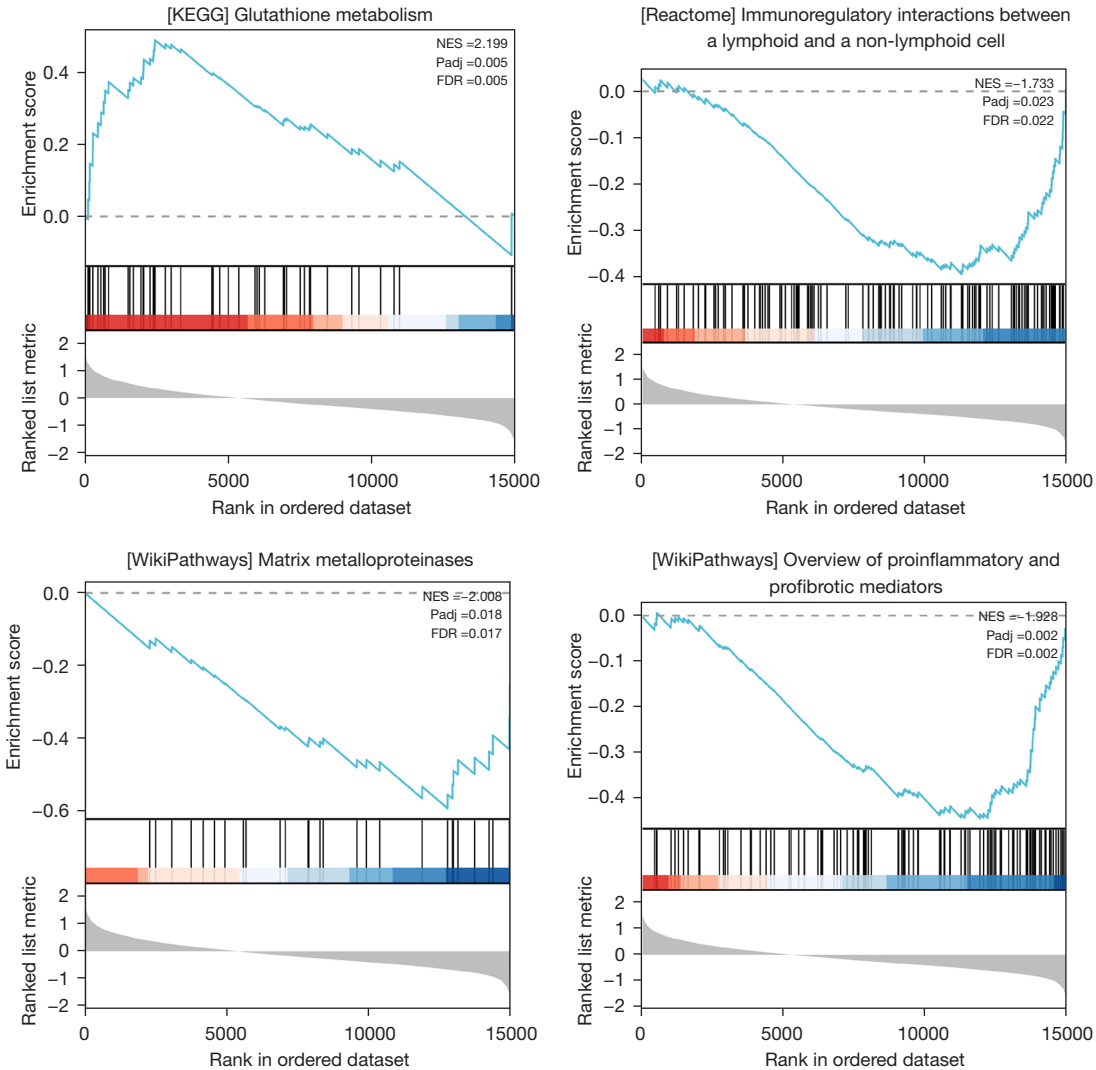

Figure 6 Statistically significant potential pathways were identified in the GSEA. Gene sets from the MSigDB categorized under biological processes were used. An analysis was performed with 1,600 random sample permutations. GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, normalized enrichment score; FDR, false discovery rate.

peripheral (V1) and integral (VO) complexes that hydrolyzes ATP and translocates protons. V-ATPase acidifies and maintains the pH of intracellular compartments. In certain cell types, V-ATPase targets the plasma membrane and is responsible for acidifying the extracellular environment. Studies have shown that the proton pump properties of V-ATPases help cancer cells transfer excess intracellular hydrogen ions to the outside, reversing the transmembrane proton concentration gradient, avoiding the apoptosis of cancer cells, and creating a highly acidic extracellular environment crucial for cancer metastasis and invasion (20).

There has been no research on *ATP6V1B1* in LC specifically, but some studies of other cancers, such as the study by Han *et al.* (21) have shown that the high expression of *ATP6V1B1* is related to a poor prognosis and platinum chemotherapy resistance in epithelial ovarian cancer through immunohistochemistry, RNA sequencing, and analysis of public data sets. Nishie *et al.* (22) established trastuzumab-mediated antibody-dependent cellular cytotoxicity-resistant cells and compared the expression of intracellular pH regulation genes with wild-type cells. It was discovered that cancer cells could develop resistance to antibody-dependent cellular toxicity if the intracellular environment became more acidic due to the downregulation

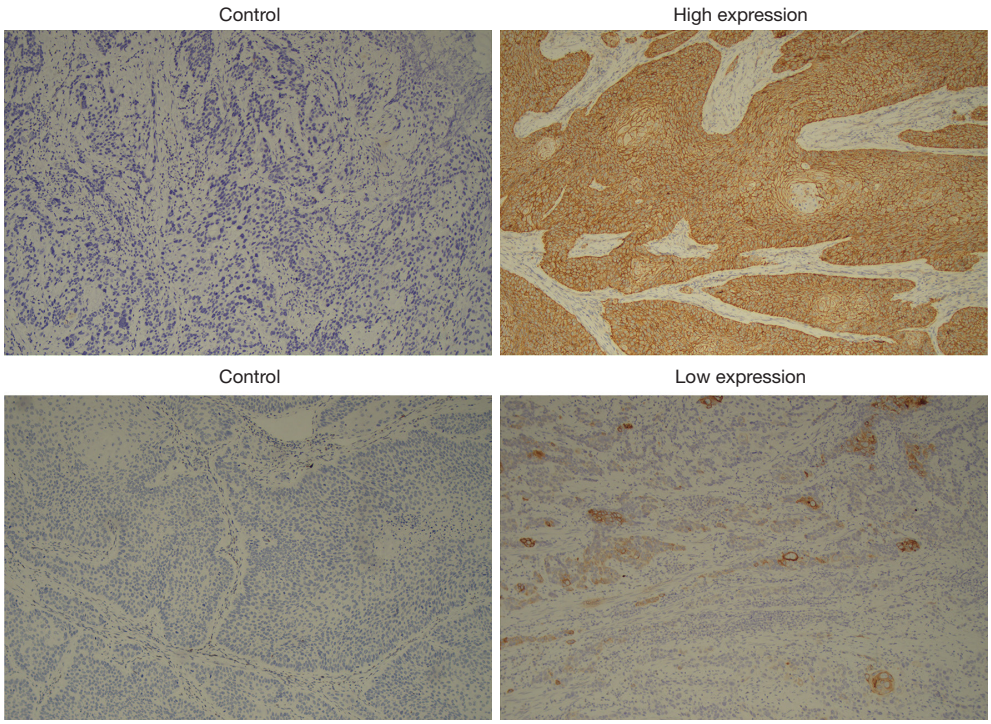

Figure 7 Immunohistochemical staining results (×100).

Table 2 General clinical information of patients with and without postoperative recurrence of LC

Characteristics	Recurrence group (n=16)	No-recurrence group (n=67)	t/χ^2	P
Age (year), mean ± SD	63.27±5.28	64.08±4.99	0.5770	0.57
Male/female	14/2	55/12	0.2696	0.60
BMI (kg/m^2), mean ± SD	20.88±2.64	21.09±2.15	0.3356	0.74
History of hypertension, n			0.0093	0.92
Yes	6	26		
No	10	41		
History of diabetes mellitus, n			0.0563	0.81
Yes	2	7		
No	14	60		
Smoking, n			0.3098	0.58
Yes	13	50		
No	3	17		
Primary site, n			0.0526	0.82
Supraglottic	5	19		
Glottic	11	48		

Table 2 (*continued*)

Table 2 (*continued*)

Characteristics	Recurrence group (n=16)	No-recurrence group (n=67)	t/χ^2	P
T stage, n			18.88	<0.001
T1	1	36		
T2	1	10		
T3	4	10		
T4	10	11		
N stage, n			9.130	0.01
N0	9	57		
N1	2	6		
N2	5	4		
M stage, n			0.0283	0.87
M0	15	62		
M1	1	5		
Tissue differentiation, n			12.66	0.002
High	3	11		
Medium	6	50		
Low	7	6		
Primary surgical procedure, n			3.715	0.16
Partial laryngectomy	9	46		
Total laryngectomy	2	13		
CO_2 laser therapy	5	8		
Postoperative radiotherapy, n			4.212	0.04
Yes	5	40		
No	11	27		
Postoperative chemotherapy, n			4.270	0.04
Yes	4	36		
No	12	31		
ATP6V1B1 expression, n			11.11	<0.001
High	12	20		
Low	4	47		

LC, laryngeal cancer; SD, standard deviation; BMI, body mass index.

of ATP6V1B1.

The GSEA results suggest that *ATP6V1B1* may influence LC recurrence through its involvement in pathways affecting pro-inflammatory and pro-fibrotic mediators, glutathione metabolism, matrix metalloproteinases, immune regulation, and interactions between lymphocytes. Multiple studies suggest that immune regulation plays a role in the recurrence of LC tumors, such as the study by Gong *et al.* (23), who showed that genomic instability and compromised immune responses are key characteristics of immune surveillance escape and relapse after early LC surgery. This study also revealed a weakened immune

Table 3 Results of the logistic regression analysis of the LC risk factors

Characteristics	EXP(B) (95% CI)	P
T stage		
T1	1.00	
T2	1.956 (0.052–73.725)	0.72
T3	26.269 (1.223–564.076)	0.04
T4	42.803 (2.499–733.112)	0.01
N stage		
N0	1.00	
N1	2.431 (0.188–31.376)	0.50
N2	2.893 (0.314–26.666)	0.35
Tissue differentiation		
High	1.00	
Medium	1.348 (0.122–14.889)	0.81
Low	19.024 (0.966–374.653)	0.053
Postoperative radiotherapy		
No	1.00	
Yes	0.588 (0.096–3.603)	0.57
Postoperative chemotherapy		
No	1.00	
Yes	0.273 (0.043–1.742)	0.17
ATP6V1B1 expression		
High	1.00	
Low	11.272 (1.541–82.430)	0.02

LC, laryngeal cancer; CI, confidence interval.

phenotype in recurrent tumors. Such findings provide valuable insights into the management of these high-risk patients. Chatzopoulos *et al.* (24) assessed tumor-infiltrating lymphocyte density in entire LC tumor tissue sections from a morphological perspective, and found that high cytotoxic T lymphocytes 8+ ($CD8^+$) tumor patients had a 77% lower risk of recurrence and a 74% lower risk of death than low $CD8^+$ tumor patients in lymph node-positive patients. Zhou *et al.* (25) also found that a high density of $CD163^+$ cells around the tumor was associated with poorer overall survival after adjusting for tumor stage, relapse, and lymph node metastasis. These findings highlight different patterns of tumor-related immune cell penetration in LC, with the density and location of tumor-related immune cell penetration being related to the clinicopathological characteristics of LC. In summary, the functions of *ATP6V1B1* can be delineated as follows: The tumor microenvironment typically exhibits low oxygen and low pH conditions. V-ATPase, encoded by the *ATP6V1B1* gene, may influence tumor cell growth and invasion by modulating the acid-base balance within this environment (26). Tumor cells display unique metabolic characteristics, including enhanced glycolysis and active glutamine metabolism. The tumor microenvironment hosts various immune cells, such as T cells and NK cells, which exert anti-tumor effects through cytokine secretion and direct cytotoxicity. V-ATPase can indirectly affect immune cell activity and function by regulating lysosomal acidification, which impacts antigen presentation and cytotoxic efficacy (27,28). Additionally, the growth and metastasis of tumors are heavily reliant on angiogenesis, a process in which V-ATPase plays a crucial role (29,30). ATP6V1B1 may regulate V-ATPase activity, thereby influencing blood vessel formation and remodeling in the tumor microenvironment, facilitating tumor growth and metastasis. Furthermore, V-ATPase is integral to intracellular metabolism, participating in amino acid transport and metabolite excretion (31). Consequently, *ATP6V1B1* affects tumor cell metabolic pathways and survival by modulating V-ATPase activity. Combining these research results, it appears that *ATP6V1B1* may promote LC recurrence by weakening the immune phenotype of LC.

A total of 83 patients with LC were ultimately included in the present study. The patients had a recurrence rate of 19.3% (16/83). The immunohistochemistry results suggest that ATP6V1B1 is highly expressed in patients with recurrent LC, which aligns with the results of the bioinformatics analysis. A review of the clinical data of these LC patients revealed that T stage and ATP6V1B1 expression were risk factors for LC recurrence. Specifically, the risk of recurrence increased significantly in T3 and T4 stage patients, as well as in patients with high ATP6V1B1 expression. Therefore, close follow up is recommended for such LC patients after surgery.

A meta-analysis of 2007 patients with LC found that postoperative adjuvant radiotherapy reduces the composite risk ratio, is linked with better disease-free survival and local control rates, and thus improves the survival of patients with locally advanced LC undergoing surgery (32). For patients with LC, surgery can remove most tumor cells, while postoperative adjuvant radiotherapy can effectively eradicate subclinical cancer foci. Thus, the use of radiotherapy after

LC surgery can help reduce the recurrence rate. However, due to the heterogeneity of LC patients, the decision to combine surgery with radiotherapy should be carefully considered, weighing its pros and cons.

Conclusions

In summary, the occurrence and recurrence of LC may involve the regulation of multiple factors. The key genes and related signaling pathways identified through our screening contribute to a deeper understanding of the molecular mechanisms of LC. Based on these findings, targeted treatment strategies for LC can be developed in the following aspects:

(I) Targeted therapy: specific therapeutic drugs can be designed for the key genes identified in our study. These drugs can directly target these genes or their products, inhibiting the proliferation and invasion of tumor cells, thereby slowing or preventing cancer progression.

(II) Immunotherapy: our study underscores the importance of immune regulation in LC recurrence. Immunotherapies, such as CAR-T cell therapy and PD-1/PD-L1 inhibitors, can be considered to enhance the patient's immune system's ability to recognize and eradicate tumor cells.

(III) Personalized treatment: by integrating the patient's gene expression profile with clinical information, personalized treatment plans can be formulated. Assessing the expression levels of key genes in the patient's body allows for predicting their response to various treatment strategies, enabling the selection of the most appropriate therapeutic approach.

(IV) Early diagnosis and recurrence monitoring: Our research findings can be utilized to develop biomarkers for early diagnosis and monitoring of recurrence. By detecting changes in the expression of these key genes, it is possible to identify signs of cancer recurrence earlier and implement timely intervention measures. However, the current study also had some limitations, including the absence of relevant *in vitro* and *in vivo* experimental validations and clinical data verification. Future research, including multicenter, large-sample, prospective studies, should aim to further validate and explore these results.

Acknowledgments

Funding: None.

Footnote

Reporting Checklist: The authors have completed the STREGA reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1015/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1015/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1015/prf

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1015/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of the First Affiliated Hospital of Wannan Medical College (No. LLSC-2020-003) and informed consent was taken from all the patients.

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Cite this article as: Liu L, Wu J. Identifying key pathogenic mechanisms and potential intervention targets for recurrence after laryngeal cancer treatment through bioinformatics screening. Transl Cancer Res 2024;13(7):3826-3841. doi: 10.21037/tcr-24-1015

