Observational Study

Medicine®

OPEN

An autophagy-related model of 4 key genes for predicting prognosis of patients with laryngeal cancer

Meng-Si Luo, MS[a], Guan-Jiang Huang, MD[b], Hong-Bing Liu, BS[c,*]

Abstract

Autophagy, a major cause of cancer-related death, is correlated with the pathogenesis of various diseases including cancers. Our study aimed to develop an autophagy-related model for predicting prognosis of patients with laryngeal cancer.

We analyzed the correlation between expression profiles of autophagy-related genes (ARGs) and clinical outcomes in 111 laryngeal cancer patients from The Cancer Genome Atlas (TCGA). Afterward, gene functional enrichment analyses of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed to find the major biological attributes. Univariate Cox regression analyses and multivariate Cox regression analyses were performed to screen ARGs whose expression profiles were significantly associated with laryngeal cancer patients overall survival (OS). Furthermore, to provide the doctors and patients with a quantitative method to perform an individualized survival prediction, we constructed a prognostic nomogram.

Thirty eight differentially expressed ARGs were screened out in laryngeal cancer patients through the TCGA database. Related functional enrichments may act as tumor-suppressive roles in the tumorigenesis of laryngeal cancer. Subsequently, 4 key prognostic ARGs (IKBKB, ST13, TSC2, and MAP2K7) were identified from all ARGs by the Cox regression model, which significantly correlated with OS in laryngeal cancer. Furthermore, the risk score was constructed, which significantly divided laryngeal cancer patients into high- and low-risk groups. Integrated with clinical characteristics, gender, N and the risk score are very likely associated with patients OS. A prognostic nomogram of ARGs was constructed using the Cox regression model.

Our study could provide a valuable prognostic model for predicting the prognosis of laryngeal cancer patients and a new understanding of autophagy in laryngeal cancer.

Abbreviations: ARG = autophagy-related gene, AUC = area under the curve, GO = gene ontology, HADb= Human Autophagy Database, HR = hazard ratios, KEGG = Kyoto Encyclopedia of Genes and Genomes, K–M = Kaplan–Meier, OS = overall survival, ROC = receiver operating characteristic, TCGA = The Cancer Genome Atlas.

Keywords: autophagy-related genes, laryngeal cancer, prognosis, The Cancer Genome Atlas

1. Introduction

Autophagy is a major catabolic system, of which cells can recycle organelles and proteins by degradation in the lysosomes.[1–4] Autophagy selectively targets intracellular microbes, dysfunctional organelles, and pathogenic proteins, and deficiencies in these processes, which may lead to diseases, including cancers, neurodegenerative diseases, and inflammation.[1,5–7] However, the mechanism of autophagy suppressing tumorigenesis is still unclear and inconclusive.[2,5] Because of the complex function of autophagy in cancers, further researches may be focused on the relation of autophagy and tumors, especially biological processes.[1,8] And then applying this knowledge to a well-designed therapeutic strategy could be a new method of cancer therapy.[1,8,9] Even though plenty of researchers have made a lot of effort, the question of whether autophagy is a friend or a foe for cancers cannot be drawn a reliable conclusion for now.[3,10–12]

Editor: Massimo Fusconi.

M-SL and G-JH contributed equally to this paper.

The authors declare that they have no conflicts of interest (including financial and non-financial interests).

Supplemental Digital Content is available for this article.

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

[a] Department of Anesthesiology, Zhongshan Hospital of Traditional Chinese Medicine, Affiliated to Guangzhou University of Chinese Medicine, Zhongshan, Guangdong Province, [b] Department of Otorhinolaryngology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, [c] Department of Otolaryngology-head and neck Surgery, The Second Affiliated Hospital of Nanchang University. No 1, Nanchang, Jiangxi, People's Republic of China.

[*] Correspondence: Hong-Bing Liu, Department of Otolaryngology-head and neck Surgery, The Second Affiliated Hospital of Nanchang University. No 1, Minde Road, Nanchang, Jiangxi 330006, People's Republic of China (e-mail: liuhb1992@163.com).

How to cite this article: Luo MS, Huang GJ, Liu HB. An autophagy-related model of 4 key genes for predicting prognosis of patients with laryngeal cancer. Medicine 2020;99:30(e21163).

Received: 18 March 2020 / Received in final form: 29 May 2020 / Accepted: 4 June 2020

http://dx.doi.org/10.1097/MD.0000000000021163

Laryngeal cancer, one of the most common head and neck cancers worldwide, has a poor prognosis and high mortality.[13–15] The numbers of laryngeal cancer new cases and related deaths in the United States were estimated to be 12,410 and 3760 in 2019.[16] Recently, many studies reported that autophagy inhibition could be a promising therapeutic target for laryngeal cancer, which provided a new route for the clinical management of laryngeal cancer.[17–20] Therefore, exploring the related molecular biomarkers of autophagy would have an attractive value in estimating and treating laryngeal cancer, which may be an important therapeutic trend of laryngeal cancer.

In our study, we analyzed the correlation between expression profiles of autophagy-related genes (ARGs) and clinical outcomes in 111 laryngeal cancer patients from The Cancer Genome Atlas (TCGA). Afterward, gene functional enrichment analyses of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed to find the major biological attributes. Univariate Cox regression analyses and multivariate Cox regression analyses were performed to screen the ARGs whose expression profiles were significantly associated with laryngeal cancer patients overall survival (OS). To facilitate the utility of the selected prognostic ARGs in routine clinical practice, we integrated risk score with clinical factors to improve the prognostic efficiency of laryngeal cancer patients. By using the multivariate Cox regression analysis, the ARGs expressions based on age, gender, grade, stage, T, M, and N were analyzed to determine whether the ARG could be an independent predictor for the patients of laryngeal cancer or not. Furthermore, to provide the doctors and patients with a quantitative method to perform an individualized survival prediction, we constructed a prognostic nomogram. These findings could also provide an effective multi-dimensional biomarker strategy, which would be effective in monitoring autophagy and predicting the prognosis in laryngeal cancer patients.

2. Methods

2.1. Data acquisition

The Human Autophagy Database (HADb, http://www.autophagy.lu/index.html) is the first autophagy-dedicated database, which provides a complete and up-to-date list of human genes involved in autophagy.[21] The list of ARGs was obtained from HADb. ARGs RNA-sequencing (RNA-seq) data and the clinical data of laryngeal cancer were downloaded and extracted from the TCGA database (https://portal.gdc.cancer.gov).[22] The dataset contained 111 patients with solid tumor and 12 patients with non-tumor tissues. Because our study is a bioinformatics study, there is no need of ethics committee or institutional review board to approve the study.

2.2. Differentially expressed ARGs analysis

The "limma" package was applied in R statistical software to estimate differentially expressed ARGs between laryngeal cancer and non-tumor samples. A volcano plot and heat map were drawn using the "ggplot2" package and the "pheatmap" package of R software. Genes that showed at least 1-fold changes and an adjusted *P* value less than .05 were considered as the significant differentially expressed ARGs. The visualization of gene expression of the significant differentially expressed ARGs was drawn the "ggpubr" package of R software. Afterward, gene functional enrichment analyses of GO and KEGG were performed to find the major biological attributes.[23–26] The packages of "clusterProfiler", "org.Hs.eg.db", "enrichplot", "ggplot2", and"GOplot", were applied in R statistical software to conduct enriched GO and KEGG enrichment analyses and the visualization of enrichment terms.

2.3. Construction of risk score formula and ARGs-based prognostic model

Univariate Cox regression analyses were performed to screen ARGs whose expression profiles were significantly associated with laryngeal cancer patients OS. If the number of differentially expressed ARGs was low, we would use all the data of ARGs related to laryngeal cancer. Then, multivariate Cox regression analyses were conducted to remove the genes which might be considered not independent indicators. Subsequently, several prognostic ARGs were selected and the related risk score was developed.[27,28] The formula of risk score is based on a linear combination of the relative expression level of genes multiplied regression coefficients, which represented the relative weight of genes. The risk score formula was built as following: Risk score = sum of coefficients × expression level of ARGs.[27,28] By the risk cutoff value (the median risk score value), patients of laryngeal cancer were separated into high-risk and low-risk groups.[28] The survival curve was plotted by Kaplan–Meier (K–M) method and the difference in the survival rates between high-risk and low-risk groups was assessed using the log-rank test.

By using the multivariate Cox regression analysis, ARGs expressions based on age, gender, grade, stage, T, M, and N were analyzed to determine whether the ARG could be an independent predictor of the patients with laryngeal cancer or not.

2.4. Construction of the nomogram and evaluation of ARGs-based prognostic model

To provide doctors and patients with a quantitative method to perform an individualized survival prediction, we constructed a prognostic nomogram of the ARGs using the Cox regression model.

To further assess the predictive performance of the ARGs-based prognostic model, we calculated the area under the curve (AUC). Furthermore, the predictive accuracy of the ARGs-based prognostic model was compared with other risk factors using receiver operating characteristic (ROC) analysis. Moreover, calibration curves were used to assess the agreements between model predicted outcomes and actual outcomes. Besides, the visualization of high-risk and low-risk groups was drawn using the "ggplot2" package of R software.

2.5. The validation of the autophagy-related model based on TCGA database

The autophagy-related model was validated using the TGGA database. Clinical data on survival and outcome were also downloaded from the TCGA database. The survival curves were plotted by K–M method.

2.6. Statistical analysis

All statistical analyses were performed using SPSS 24.0 (Chicago, IL, USA) and R version 3.5.3 (https://www.r-project.org/). R software was performed to draw plots. Univariate Cox regression

analyses were calculated to evaluate the association between expression profiles and OS, and hazard ratios (HR) were calculated using the Cox regression model. The Multivariate Cox proportional hazards regression model was calculated to construct the ARGs-based prognostic model. ROC curve and the area under the ROC curve for each dataset were performed by the "survivalROC" package in R software. AUC > 0.7 was considered as a good performance. All tests were two-tailed and all statistical significance was defined as $P < .05$.

3. Results

3.1. Differential expression screening and identification of ARGs

Expression values of 222 ARGs were extracted. Based on the criteria of an FDR <0.05 and |log2(Fold Change)|>1, we finally obtained 29 up-regulated and 9 down-regulated ARGs (Fig. 1A, 1B). Moreover, visual scatter plots were exhibited the expression pattern of the 38 differentially expressed ARGs between

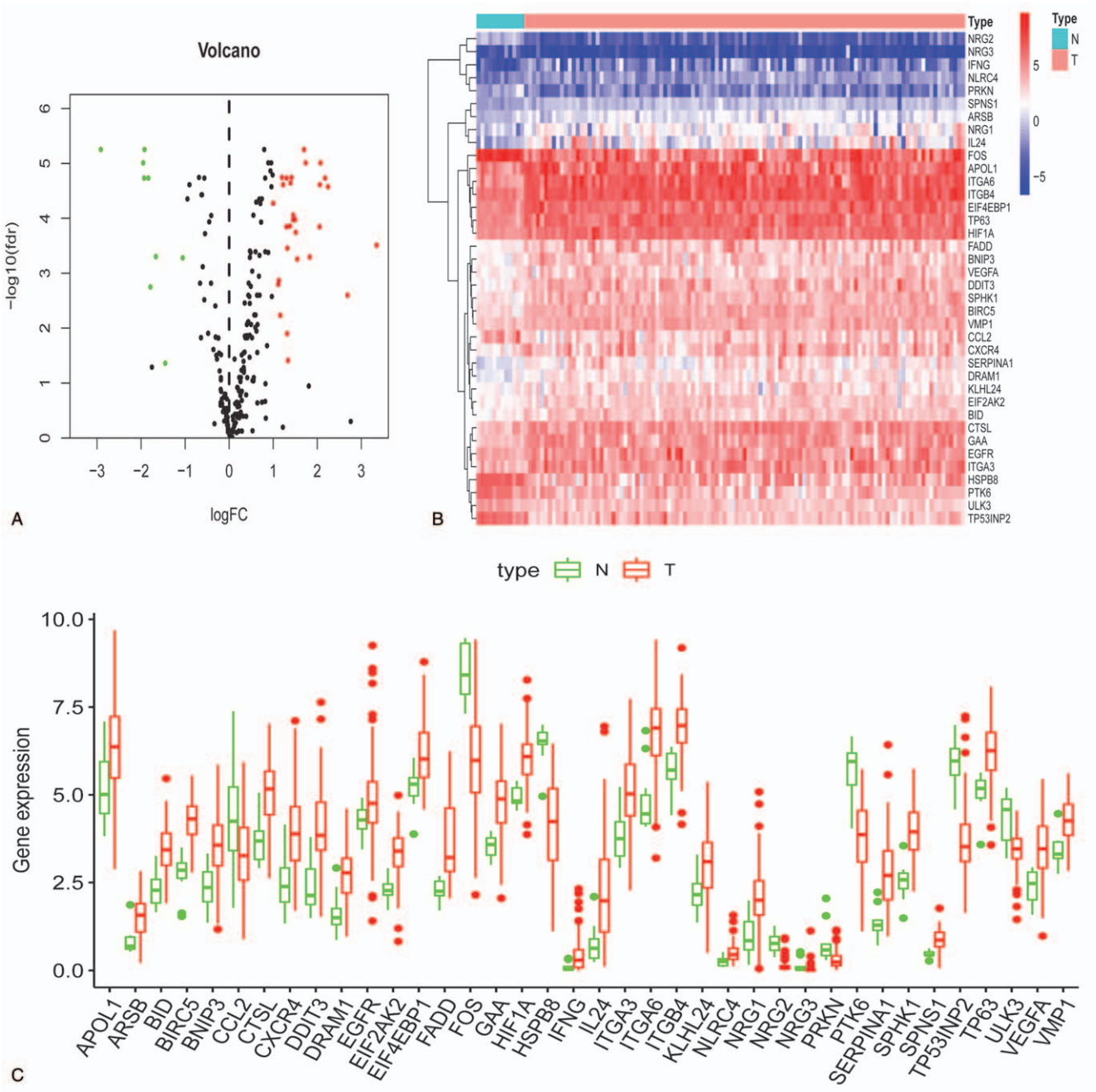

Figure 1. Differentially expressed autophagy-related genes (ARGs) between laryngeal cancer and non-tumor tissues. (A) The volcano plot for the 222 ARGs from The Cancer Genome Atlas (TCGA) database. Red: upregulation; blue:downregulation. (B) Hierarchical clustering of differentially expressed ARGs expression levels. (C) The expression patterns of 38 ARGs.

laryngeal cancer and non-tumor tissues (Fig. 1C). Scatter plots showed expression patterns of ARGs.

3.2. Functional annotation of differentially expressed ARGs

The functional enrichment analysis of 38 differentially expressed ARGs was performed. GO terms function and KEGG pathway enrichment of these differentially expressed ARGs were summarized in Table 1. Using the packages of "clusterProfiler" and "org.Hs.eg.db" in R software, we proved that the top enriched GO terms for biological processes were: neuron apoptotic process, neuron death, and autophagy; for cellular components were: integrin complex, protein complex involved in cell adhesion, and lysosomal lumen; and for molecular function: fibronectin binding, protein tyrosine kinase activity, and receptor-ligand activity. The overview visualization of the GO analysis results is displayed in Figure 2A and B. Moreover, KEGG pathway enrichment analysis for the differentially expressed ARGs showed that they are notably associated with EGFR tyrosine kinase inhibitor resistance, apoptosis, ErbB signaling pathway, and so on. As shown in Figure 2C, the high Z-score of enriched pathways indicated that related pathways were more likely to be increased. The heatmap and circle plot of the relationship between ARGs and pathways were visually displayed (Fig. 2D and E).

3.3. Identification of prognostic ARGs

The relationships between the expression profiles of ARGs and OS were assessed based on the data obtained from TCGA. Using univariate Cox regression analyses, 14 prognosis-related ARGs were selected with the criteria of a $P < .05$ (Table 2, Fig. 3A). To improve the robustness, the further multivariate Cox regression model was conducted (Table 2, Fig. 3B). Therefore, 4 genes including IKBKB, ST13, TSC2, and MAP2K7 were identified (Table 2). The results from K-M analysis indicated that the overexpression of ST13 was strongly correlated with the worse OS of laryngeal cancer patients (HR = 3.685, 95% CI = 1.571–8.645, $P = .003$; Fig. 3B). On the contrary, Figure 3B showed that up-regulated IKBKB, up-regulated TSC2, and up-regulated MAP2K7 indicated laryngeal cancer patients may likely have a longer survival time.

Table 1

GO and KEGG analysis of differentially expressed autophagy-related genes.

Category	ID	Term	*P* value	Genes
GO				
Biological Process	GO:0051402	Neuron apoptotic process	1.80E-08	TP63/CCL2/DDIT3/PRKN/FADD/HIF1A/BNIP3/BID
Biological Process	GO:0070997	Neuron death	1.94E-08	TP63/FOS/CCL2/DDIT3/PRKN/FADD/HIF1A/BNIP3/BID
Biological Process	GO:0006914	Autophagy	2.35E-08	IFNG/PRKN/ULK3/DRAM1/HIF1A/BNIP3/ITGB4/TP53INP2/VMP1/ARSB
Biological Process	GO:0061919	Process utilizing autophagic mechanism	2.35E-08	IFNG/PRKN/ULK3/DRAM1/HIF1A/BNIP3/ITGB4/TP53INP2/VMP1/ARSB
Biological Process	GO:1905477	Positive regulation of protein localization to membrane	1.46E-07	TP63/EGFR/IFNG/PRKN/ITGA3/BID
Biological Process	GO:0048565	Digestive tract development	2.58E-07	ITGA6/TP63/EGFR/PTK6/HIF1A/ITGB4
Biological Process	GO:0055123	Digestive system development	4.33E-07	ITGA6/TP63/EGFR/PTK6/HIF1A/ITGB4
Biological Process	GO:0038128	ERBB2 signaling pathway	4.58E-07	EGFR/NRG1/NRG2/PTK6
Biological Process	GO:0018108	Peptidyl-tyrosine phosphorylation	9.66E-07	EGFR/NRG1/NRG2/EIF2AK2/IFNG/PTK6/VEGFA/IL24
Biological Process	GO:0018212	Peptidyl-tyrosine modification	1.02E-06	EGFR/NRG1/NRG2/EIF2AK2/IFNG/PTK6/VEGFA/IL24
Cellular Component	GO:0008305	Integrin complex	3.18E-05	ITGA6/ITGA3/ITGB4
Cellular Component	GO:0098636	Protein complex involved in cell adhesion	4.18E-05	ITGA6/ITGA3/ITGB4
Cellular Component	GO:0043202	Lysosomal lumen	7.91E-04	CTSL/GAA/ARSB
Molecular Function	GO:0001968	Fibronectin binding	2.61E-05	ITGA3/CTSL/VEGFA
Molecular Function	GO:0004713	Protein tyrosine kinase activity	3.70E-05	EGFR/NRG1/NRG2/EIF2AK2/PTK6
Molecular Function	GO:0048018	Receptor ligand activity	6.27E-05	NRG1/CCL2/NRG2/NRG3/IFNG/VEGFA/IL24
Molecular Function	GO:0005125	Cytokine activity	1.02E-04	NRG1/CCL2/IFNG/VEGFA/IL24
Molecular Function	GO:0002020	Protease binding	1.47E-04	SERPINA1/PRKN/FADD/ITGA3
Molecular Function	GO:0005178	Integrin binding	1.52E-04	EGFR/NRG1/ITGA3/ITGB4
Molecular Function	GO:0030546	Receptor activator activity	2.01E-04	NRG1/NRG3
Molecular Function	GO:0050840	Extracellular matrix binding	2.37E-04	ITGA6/ITGA3/VEGFA
Molecular Function	GO:0031994	Insulin-like growth factor I binding	2.94E-04	ITGA6/ITGB4
Molecular Function	GO:0005126	Cytokine receptor binding	3.24E-04	CCL2/IFNG/FADD/VEGFA/BID
KEGG				
KEGG PATHWAY	hsa01521	EGFR tyrosine kinase inhibitor resistance	1.42E-05	EGFR/NRG1/EIF4EBP1/NRG2/VEGFA
KEGG PATHWAY	hsa04210	Apoptosis	1.45E-05	FOS/BIRC5/DDIT3/FADD/CTSL/BID
KEGG PATHWAY	hsa04012	Erbb signaling pathway	2.04E-05	EGFR/NRG1/EIF4EBP1/NRG2/NRG3
KEGG PATHWAY	hsa05163	Human cytomegalovirus infection	2.51E-05	EGFR/CCL2/EIF4EBP1/FADD/VEGFA/CXCR4/BID
KEGG PATHWAY	hsa05323	Rheumatoid arthritis	3.15E-05	FOS/CCL2/IFNG/CTSL/VEGFA
KEGG PATHWAY	hsa05165	Human papillomavirus infection	3.67E-05	ITGA6/EGFR/EIF4EBP1/EIF2AK2/FADD/ITGA3/ITGB4/VEGFA
KEGG PATHWAY	hsa04066	HIF-1 signaling pathway	6.77E-05	EGFR/EIF4EBP1/IFNG/HIF1A/VEGFA
KEGG PATHWAY	hsa05167	Kaposi sarcoma-associated herpesvirus infection	8.47E-05	FOS/EIF2AK2/FADD/HIF1A/VEGFA/BID
KEGG PATHWAY	hsa04140	Autophagy - animal	2.00E-04	CTSL/HIF1A/BNIP3/TP53INP2/VMP1
KEGG PATHWAY	hsa05418	Fluid shear stress and atherosclerosis	2.14E-04	FOS/CCL2/IFNG/CTSL/VEGFA

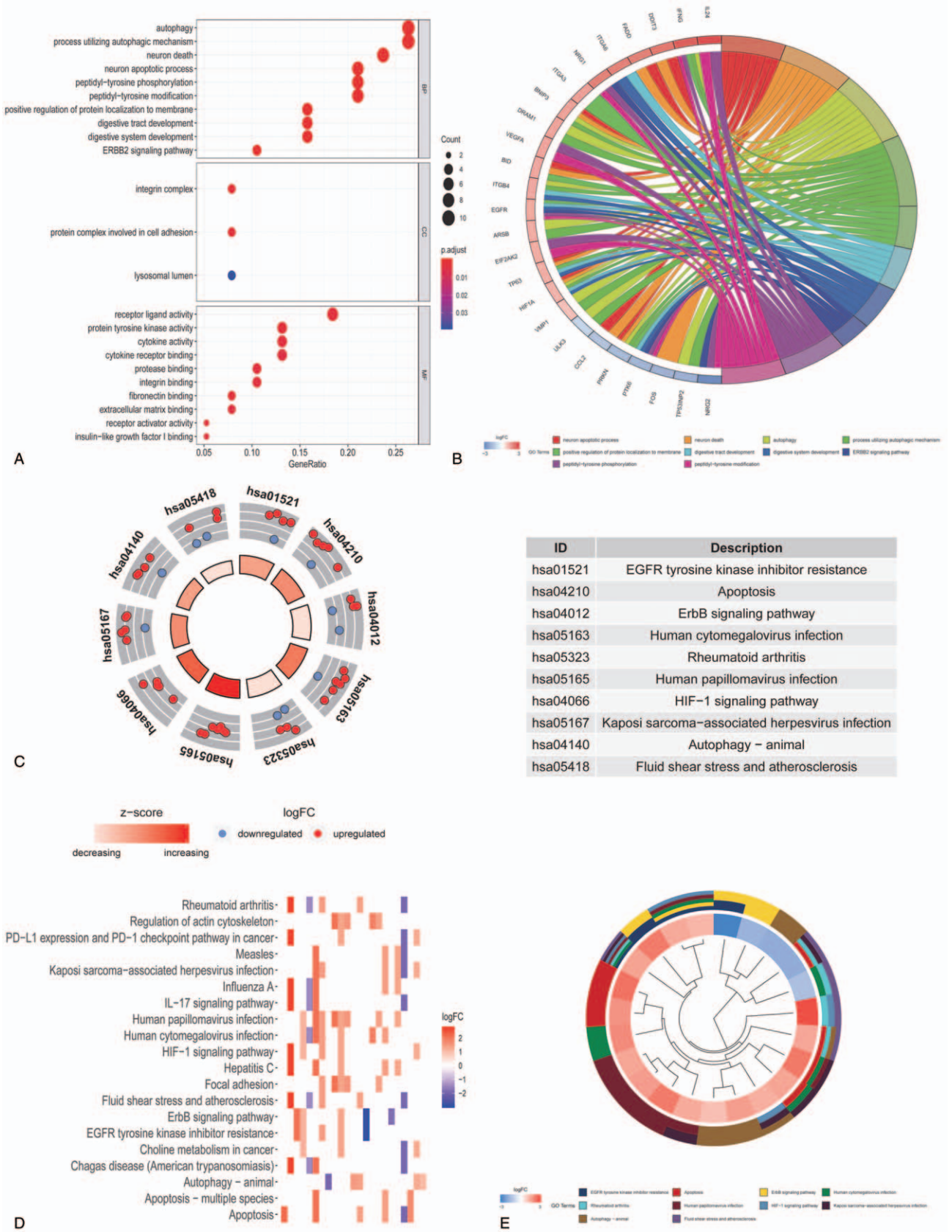

Figure 2. Functional enrichment analyses of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). (A) The bubble plot of enriched GO terms. (B) The circos plot of enriched GO terms. (C) The circular scatter plot of enriched KEGG terms. (D) The heatmap of the relationship between ARGs and KEGG pathways. (E) The circos plot of TOP 10 enriched GO terms.

Table 2

Expression and Cox regression analysis data of the prognosis-related ARGs in laryngeal cancer by TCGA.

	Univariate Cox				Multivariate Cox			
Gene	HR	HR.95L	HR.95H	*P* value	HR	HR.95L	HR.95H	*P* value
IKBKB	0.470	0.251	0.877	.018	0.578	0.290	1.151	.119
ST13	3.241	1.309	8.028	.011	3.685	1.571	8.645	.003
TSC2	0.365	0.169	0.787	.010	0.475	0.193	1.169	.105
MAP2K7	0.360	0.158	0.819	.015	0.458	0.199	1.056	.067
MBTPS2	2.488	1.167	5.302	018				
CLN3	0.373	0.163	0.852	.019				
PEX14	0.426	0.191	0.948	.037				
RPTOR	0.415	0.181	0.951	.038				
ATG16L2	0.594	0.365	0.968	.037				
STK11	0.463	0.250	0.858	.014				
CAPN10	0.378	0.162	0.882	.024				
ERBB2	0.633	0.405	0.990	.045				
RAB24	0.575	0.356	0.929	.024				
HGS	0.468	0.238	0.919	.027				

3.4. Development of risk score formula and ARGs-based prognostic model

To facilitate the utility of the selected prognostic ARGs in routine clinical practice, the following formula was then developed to calculate the risk score for each patient: Risk score= $(-0.548 \times \text{expression}_{IKBKB}) + (1.3 \times \text{expression}_{ST13}) + (-0.744 \times \text{expression}_{TSC2}) + (-0.780 \times \text{expression}_{MAP2K7})$. We found that the coefficient of IKBKB, TSC2, and MAP2K7 is negative, indicating that the expressions of IKBKB, TSC2, and MAP2K7 were related to better OS of laryngeal cancer patients. While the expression of ST13 was related to worse OS. Using the median expression value of risk score, the laryngeal cancer patients were divided into high-risk and low-risk groups.

The performance of the risk score was identified to predict the clinical outcome of laryngeal cancer patients, K–M plots were drawn to analyze the different survival rates between the high-risk and low-risk groups. The result of the K–M analyses indicated that patients in the high-risk group have a worse survival rate than those in the low-risk group ($P=.005$, Fig. 3C). Figures 3D–G showed the risk distribution of patients in the dataset, the number of patients in high- and low-risk groups, the survival time of patients in the dataset, and the heatmap of the 4 ARGs expression profiles in the dataset. Furthermore, univariate analyses and multivariate analyses were performed to assess the correlation between clinicopathological features and OS. After that, Figure 4A, B and Table 3 displayed that gender (HR= 0.401, 95% CI=0.174–0.922, $P=.031$),N (HR=1.631, 95% CI=1.242–2.141, $P < .001$), and risk score (HR=1.731, 95% CI=1.312–2.280, $P < .001$) are very likely associated with patients OS.

Furthermore, the correlations between clinicopathological features and ARGs expression were analyzed (Table 4). The analyses based on independent sample *t* tests indicated that the expression of IKBKB is higher in the elder patients ($P=.010$, Fig. 4C) and higher N ($P=.054$, Fig. 4D); the expression of MAP2K7 is higher in the higher stage group ($P=.002$, Fig. 4E) and higher N group ($P=.005$, Fig. 4F). Besides, the risk score values were higher in the higher stage group ($P=.016$, Fig. 4G).

3.5. Development of a nomogram for predicting prognosis of laryngeal cancer patients

We constructed a nomogram based on OS, which integrated the 4 ARGs (Fig. 5A). The nomogram can be interpreted by the total points assigned to each variable, which is indicated at the top of the scale. The total points can be converted to predict 1-year, 3-year, and 5-year OS for patients. The predictive accuracy of the nomogram is shown in Figure 5B. The AUC at 1-year prediction was 0.656, the AUC at 3-year prediction was 0.779, and the AUC at 3-year prediction was 0.796. Calibration curves for the nomogram revealed no deviations from the reference line and no need for recalibration (Fig. 5C (1-year), 5D (3-year), 5E (5-year)). Figure 5 F–H displayed the expression difference between high-risk and low-risk groups and the visualization of the correlation between risk score and survival time.

3.6. Survival analyses of the autophagy-related model

Based on the TCGA database, we conducted the survival analyses of the autophagy-related model (IKBKB, ST13, TSC2, and MAP2K7). Then, Figure 6 showed that the overexpressing of IKBKB ($P=.047$) and MAP2K7 ($P=.003$) may indicate a better OS. However, there were no statistical differences in the survival analyses of ST13 ($P=.268$) and TSC2 ($P=.094$).

4. Discussion

Laryngeal cancer is a major malignancy of head and neck. The blank area in the knowledge of molecular targeted therapy and molecular biomarkers for laryngeal cancer prognosis monitoring still eagerly needed us to promote a better understanding of the molecular mechanisms.[1,5,18,20] Exploration of the autophagy mechanism would open a new positive outlook for laryngeal cancer.[17,19,29] However, most researches focused on autophagy only analyzed a signal gene or an autophagic inhibitor. Garcia-Mayea et al performed an immunohistochemistry study to identify the putative relevance of autophagy in laryngeal cancer.[17] The expressions of the proteins (LC3, ATG5, p62/SQSTM1, and PTOV1) involved in the process of autophagy were analyzed. Finally, they suggested that autophagy inhibition

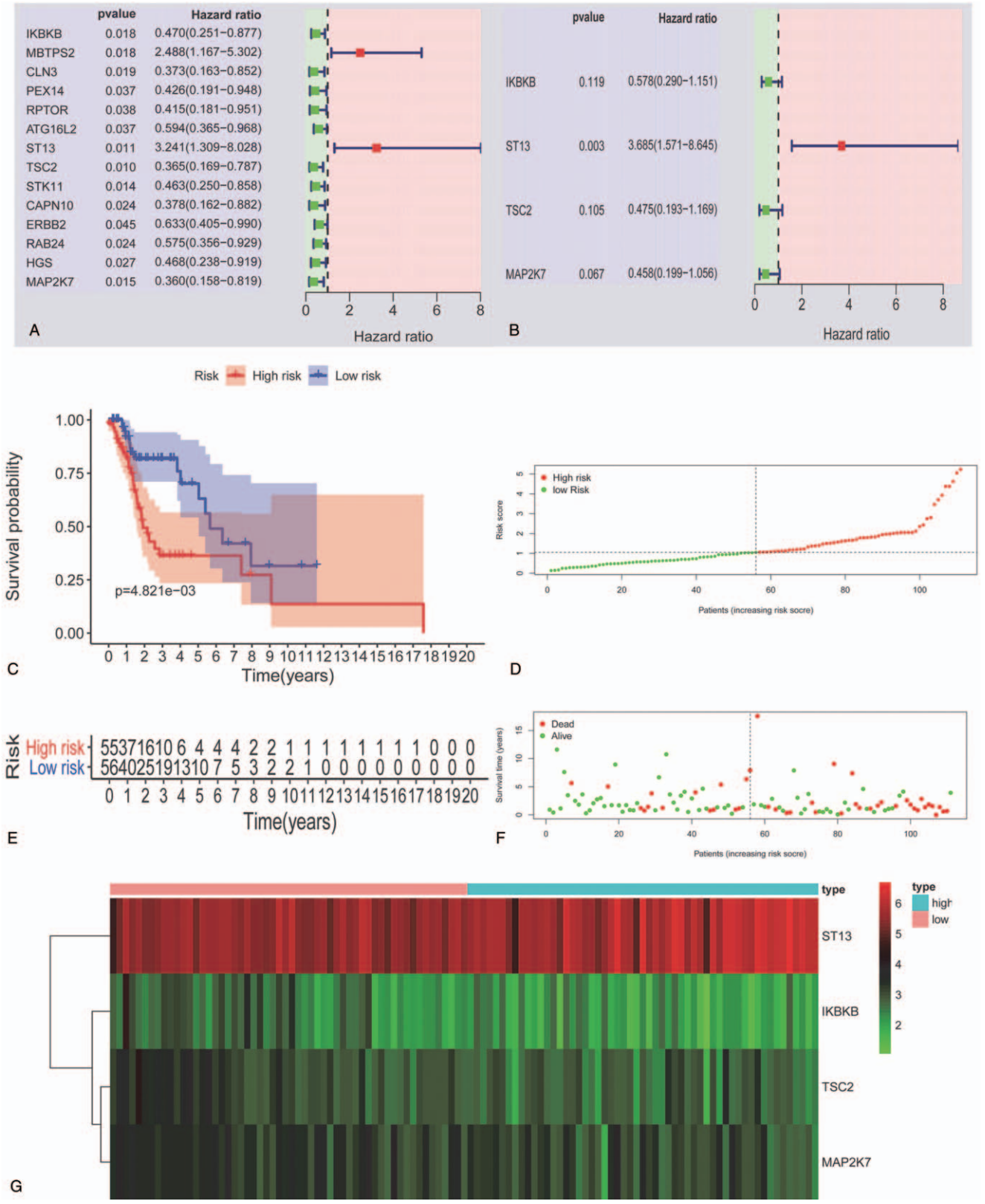

Figure 3. Forest plots of autophagy-related genes (ARGs) and autophagy-related risk score of laryngeal cancer patients. (A) Univariate analyses of ARGs. (B) Multivariate analyses of 4 key ARGs. (C) Kaplan–Meier plot represents that patients in the high-risk group had a significantly worse overall survival (OS) than those in the low-risk group. (D) The risk score distribution of patients in the TCGA dataset. (E) The number of patients in different risk groups. (F) The survival time of patients in the TCGA dataset. (G) The heatmap of the 4 key ARGs expression profiles in the TCGA dataset.

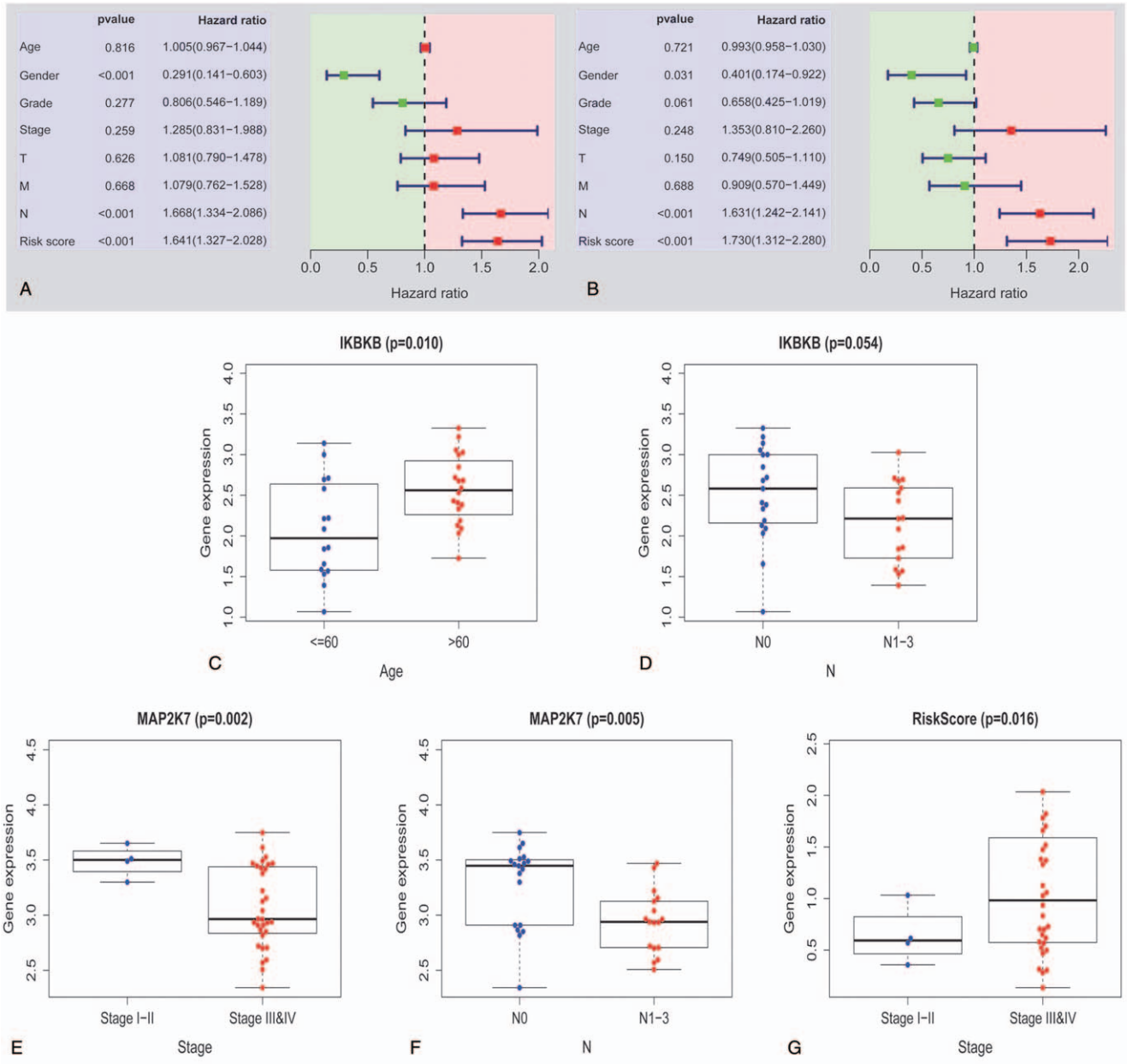

Figure 4. Forest plots of clinical characteristics, receiver operating characteristic (ROC) curve of clinical characteristics, and box plots of the correlation between 4 key ARGs and clinical characteristics. (A) Univariate analyses of clinical characteristics. (B) Multivariate analyses of clinical characteristics. (C-G) Box plots with significant difference were displayed.

Table 3

Univariate and multivariate analyses of OS in laryngeal cancer patients of TCGA.

	Univariate analysis				Multivariate analysis			
Variables	**HR**	**HR.95L**	**HR.95H**	***P* value**	**HR**	**HR.95L**	**HR.95H**	***P* value**
Age	1.005	0.967	1.044	.816	0.993	0.958	1.030	.721
Gender	0.291	0.141	0.603	.001	0.401	0.174	0.922	.031
Grade	0.806	0.546	1.189	.277	0.658	0.425	1.019	.061
Stage	1.285	0.831	1.988	.259	1.353	0.810	2.260	.248
T	1.081	0.790	1.478	.626	0.749	0.505	1.110	.150
M	1.079	0.762	1.528	.668	0.909	0.570	1.449	.688
N	1.668	1.334	2.086	7.36E-06	1.631	1.242	2.141	4.28E-04
Risk score	1.641	1.327	2.028	4.77E-06	1.730	1.312	2.280	1.00E-04

Table 4

t tests of the correlation between 4 key ARGs and clinical characteristics in laryngeal cancer patients of TCGA.

	IKBKB		ST13		TSC2		MAP2K7		Risk Score	
Variables	**t**	***P* value**	**t**	***P* value**	**t**	***P* value**	**t**	***P* value**	**t**	***P* value**
Age	−2.764	.010	−0.210	.835	−0.417	.680	−1.549	.131	1.183	.246
Gender	−0.350	.740	0.420	.690	−0.831	.438	−1.194	.284	0.649	.524
Grade	0.270	.791	−0.051	.960	−0.066	.948	−1.522	.142	−0.234	.820
Stage	0.749	.502	1.123	.320	1.803	.137	4.316	.002	−2.607	.016
T	0.156	.880	0.335	.746	0.222	.830	1.168	.270	−0.207	.840
M	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
N	1.999	.054	1.053	.300	1.730	.093	3.036	.005	−1.076	.290

Only 1 M1 case is in laryngeal cancer patients of TCGA, so t test for M can not be conducted.

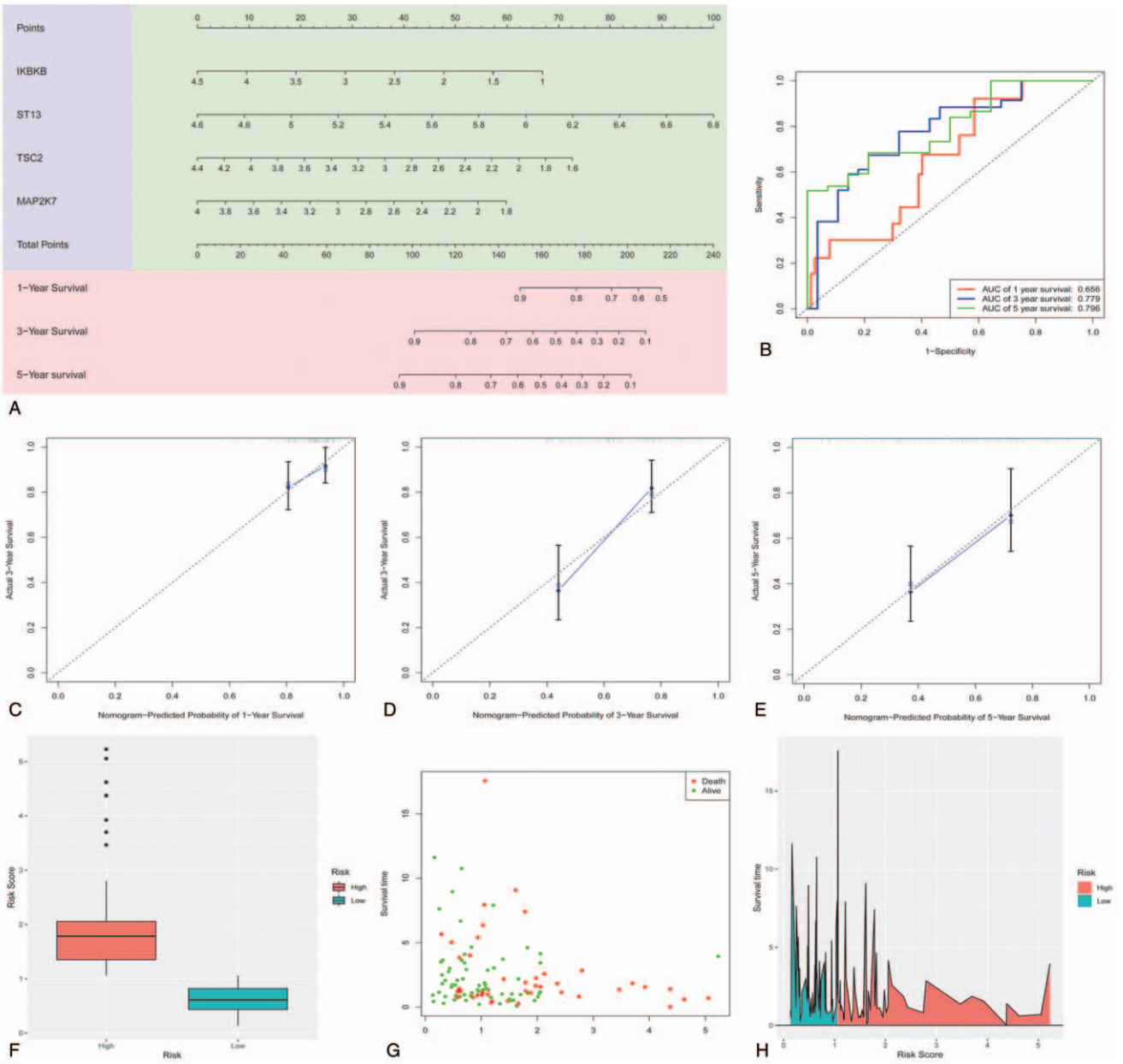

Figure 5. Construction of the nomogram, evaluation of autophagy-related genes (ARGs) prognostic model, and visualizations of risk score in the high-risk group and the low-risk group. (A) A nomogram of the prognostic model using 4 key ARGs. (B) Receiver operating characteristic (ROC) curve of 1-year, 3-year, and 5-year survival. (C-E) Calibration curves of 1-year, 3-year, and 5-year survival. (F-H) Visualizations of risk score in the high-risk group and the low-risk group.

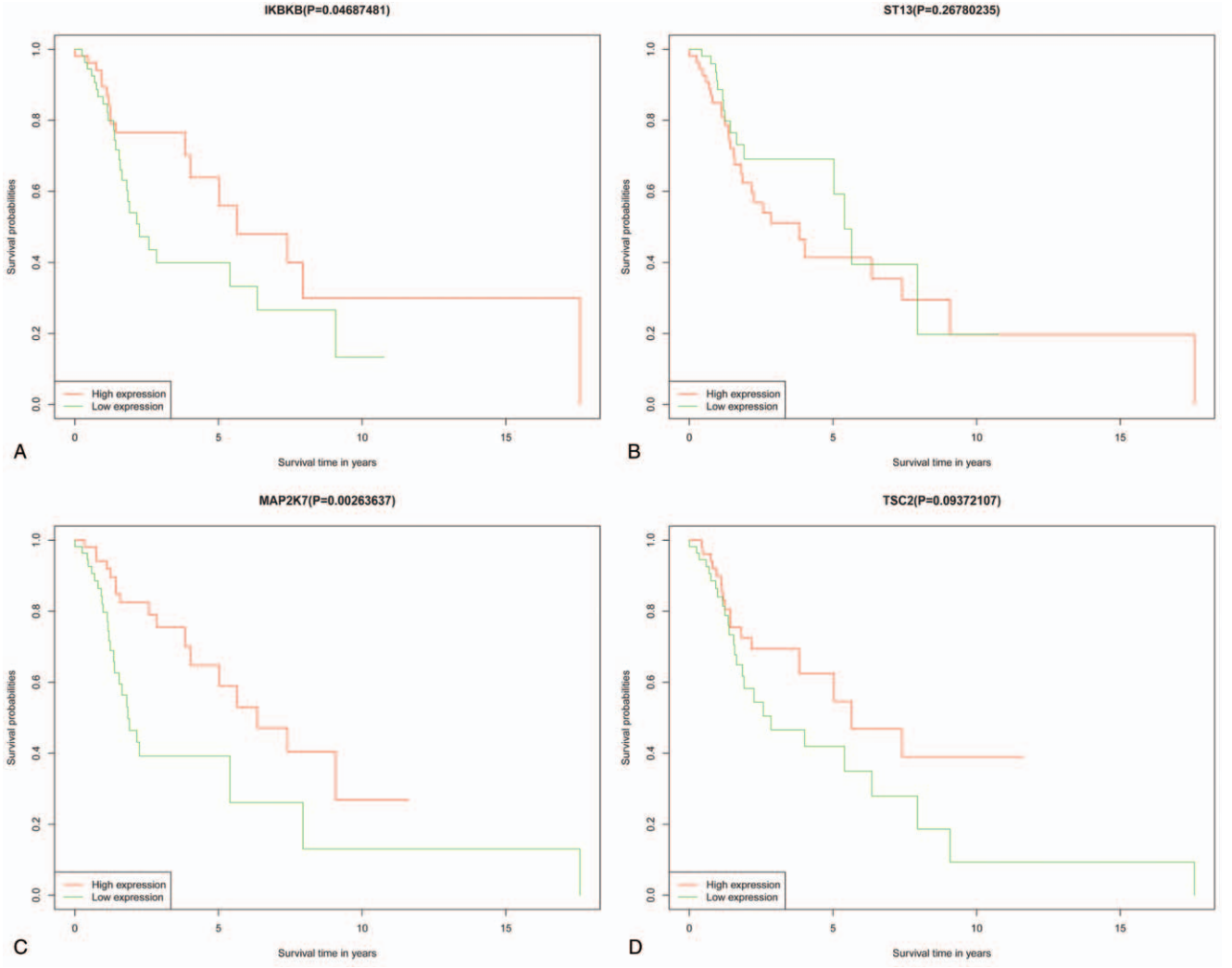

Figure 6. Survival analyses of 4 key autophagy-related genes (ARGs) based on The Cancer Genome Atlas (TCGA) database. (A)IKBKB. (B)ST13. (C)TSC2. (D) MAP2K7.

with hydroxychloroquine could be a promising therapy for laryngeal cancer patients. Ji et al also indicated the protective role of autophagy in laryngeal cancer cells and provided a new attractive therapeutic strategy for laryngeal cancer with autophagic inhibitors.[19] Also, Lin et al and Cao et al both proved the protective role of autophagy in laryngeal cancer.[18,20]

Given understanding advances in high-throughput sequencing, TCGA as the large-scale database provided effective measures for screening prognostic ARGs.[30,31] In our study, we extracted the expression profiles of ARGs from TCGA and then aimed to select molecular biomarkers related to the prognosis of laryngeal cancer. Subsequently, we screened 38 differentially expressed ARGs between laryngeal cancer and non-tumor tissues. Considering these ARGs may be involved in the initiation of laryngeal cancer, GO and KEGG analyses of these genes were performed. Interestingly, the top 3 significant KEGG pathways (EGFR tyrosine kinase inhibitor resistance, apoptosis, and ErbB signaling pathway) of these enriched genes were decreased. Plenty of researches had proved that EGFR tyrosine kinase inhibitor resistance, apoptosis, and ErbB signaling pathway played key roles in various cancers.[32–38] Thus, we indicated that autophagy may likely be considered as a tumor suppressor in the process of laryngeal cancer tumorigenesis. It is acknowledged that autophagy always inhibited tumorigenesis by activation of gene mutations or inactivation of ARGs.[39]

To obtain the autophagy-related molecular biomarkers for laryngeal cancer, we screened all ARGs and then identified 4 key prognostic ARGs, all of which may be the potential therapeutic targets. The result of the univariate survival analysis revealed that 4 ARGs were likely associated with OS in the TCGA database. Further multivariate survival analysis helped us to determine 4 key prognostic ARGs (IKBKB, ST13, TSC2, and MAP2K7) to develop the risk score, which could be an independent prognostic indicator for laryngeal cancer patients. Furthermore, we further analyze prognostic ARGs and clinical characteristics, which would provide an accurate estimation of OS in laryngeal cancer. Thus, univariate analyses and multivariate analyses based on clinicopathological features showed that gender, N, and risk score are very likely associated with patients OS. The analyses based on independent sample *t* tests showed that the expression of IKBKB is higher in the elder patients and higher N; the expression of MAP2K7 is higher in the higher stage group and

higher N group. In addition, risk score values were higher in the higher stage group. To help doctors and patients to perform an individualized survival prediction, a nomogram was constructed to predict the 1-, 3-, and 5-year OS by using this 4 key ARGs and clinicopathological risk variables. These findings could also provide an effective multi-dimensional biomarker strategy, which would be effective in monitoring autophagy and predicting the prognosis in laryngeal cancer patients. Last, we conducted the survival analyses of significant prognostic ARGs (IKBKB, ST13, TSC2, and MAP2K7). Based on the TGGA database, we found that the overexpressing of IKBKB and MAP2K7 may indicate a better OS. However, there were no statistical differences in the survival analyses of ST13 and TSC2. Therefore, the prognostic signature could be converted into a clinical application.

IKBKB, inhibitor of nuclear factor-kappa b kinase subunit beta, is a protein-coding gene that played an essential role in the NF-kappa-B signaling pathway. Krazinski BE et al indicated that IKBKB protein could be associated with clinical relevance in clear cell renal cell cancer, acting as a marker of poor prognosis.[40] Li et al proved that tumor necrosis factor significantly increased phosphorylation of IKBKB.[41] MAP2K7, mitogen-activated protein kinase kinase 7, a protein-coding gene that acts as an essential component of the MAP kinase signal transduction pathway. Shen et al uncovered that MAP2K7 was repressed by KLF4 in T-cell acute lymphoblastic leukemia cells.[42] Hong et al and Min et al proved that MAP2K7 may be a potential target for tumor therapy.[43,44] ST13, st13 hsp70 interacting protein, is a protein-coding gene that suggested that it may be a candidate tumor suppressor gene. Yu et al, Yang et al, and Wang et al all concluded that ST13 may be a novel therapeutic target for colorectal cancer.[45–47] TSC2, TSC Complex Subunit 2, is a protein-coding gene that is related to the pathways of Vesicle-mediated transport and mTOR signaling.[48] Schrader et al and Li et al proved that TSC2 could be a tumor suppressor gene.[49,50] Hence, combined with our results, we concluded that IKBKB, ST13, TSC2, and MAP2K7 may play the tumor suppressor roles, and IKBKB and MAP2K7 may be the most vital.

However, there are indeed several potential limitations in our study. Firstly, owing to the lack of sufficient cases and reliable laryngeal cancer cells (the standard laryngeal cancer cell - Hep-2 cell was challenged), we failed to validate the expression of IKBKB, ST13, TSC2, and MAP2K7. Secondly, our study was based on the data from the TCGA database. Thirdly, the information on several other important clinical outcomes, such as various treatments, and the number of lymph nodes, was unavailable now.

Based on these comprehensive analyses with ARGs expression profiles and clinical outcomes, 4 prognostic ARGs (IKBKB, ST13, TSC2, and MAP2K7) were finally identified. A novel autophagy-related model and a predictive nomogram were conducted to robustly estimate laryngeal cancer patients survival. However, further experiments are still needed to test our conclusion. Supplementary Table 1, http://links.lww.com/MD/E538.

Author contributions

Conceptualization: Meng-Si Luo, Guan-Jiang Huang, Hong-Bing Liu.
Data curation: Meng-Si Luo, Guan-Jiang Huang.
Formal analysis: Meng-Si Luo, Guan-Jiang Huang, Hong-Bing Liu.
Investigation: Meng-Si Luo, Guan-Jiang Huang, Hong-Bing Liu.
Methodology: Meng-Si Luo, Guan-Jiang Huang, Hong-Bing Liu.
Project administration: Hong-Bing Liu.
Resources: Meng-Si Luo, Guan-Jiang Huang, Hong-Bing Liu.
Software: Meng-Si Luo, Guan-Jiang Huang.
Supervision: Meng-Si Luo, Hong-Bing Liu.
Validation: Meng-Si Luo.
Visualization: Guan-Jiang Huang.
Writing – original draft: Meng-Si Luo, Guan-Jiang Huang.
Writing - review & editing: Meng-Si Luo, Guan-Jiang Huang, Hong-Bing Liu.

References

[1] Levine B, Kroemer G. Biological functions of autophagy genes: a disease perspective. Cell 2019;176:11–42.
[2] Clarke AJ, Simon AK. Autophagy in the renewal, differentiation and homeostasis of immune cells. Nat Rev Immunol 2019;19:170–83.
[3] Mizumura K, Cloonan S, Choi ME, et al. Autophagy: friend or foe in lung disease? Ann Am Thorac Soc 2016;13(Suppl 1):S40–7.
[4] Cui Y, Parashar S, Zahoor M, et al. A COPII subunit acts with an autophagy receptor to target endoplasmic reticulum for degradation. Science 2019;365:53–60.
[5] Kondo Y, Kanzawa T, Sawaya R, et al. The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 2005;5:726–34.
[6] Galluzzi L, Green DR. Autophagy-independent functions of the autophagy machinery. Cell 2019;177:1682–99.
[7] Wang Z, Jin J. LncRNA SLCO4A1-AS1 promotes colorectal cancer cell proliferation by enhancing autophagy via miR-508-3p/PARD3 axis. Aging (Albany NY) 2019;11:4876–89.
[8] Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. Nat Rev Cancer 2017;17:528–42.
[9] Bryant KL, Stalnecker CA, Zeitouni D, et al. Combination of ERK and autophagy inhibition as a treatment approach for pancreatic cancer. Nat Med 2019;25:628–40.
[10] Liu L, Liao JZ, He XX, et al. The role of autophagy in hepatocellular carcinoma: friend or foe. Oncotarget 2017;8:57707–22.
[11] Balaji KC. Autophagy: friend or foe for progression and treatment of urothelial carcinoma. J Urol 2014;191:1644–5.
[12] Bhutia SK, Mukhopadhyay S, Sinha N, et al. Autophagy: cancer's friend or foe? Adv Cancer Res 2013;118:61–95.
[13] Corry J, Peters L, Kleid S, et al. Larynx preservation for patients with locally advanced laryngeal cancer. J Clin Oncol 2013;31:840–4.
[14] Strieth S, Ernst BP, Both I, et al. Randomized controlled single-blinded clinical trial of functional voice outcome after vascular targeting KTP laser microsurgery of early laryngeal cancer. Head Neck 2019;41:899–907.
[15] Ho AS, Kim S, Tighiouart M, et al. Association of quantitative metastatic lymph node burden with survival in hypopharyngeal and laryngeal cancer. JAMA Oncol 2018;4:985–9.
[16] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019;69:7–34.
[17] Garcia-Mayea Y, Mir C, Munoz L, et al. Autophagy inhibition as a promising therapeutic target for laryngeal cancer. Carcinogenesis 2019;40:1525–34.
[18] Cao S, Huang Y, Zhang Q, et al. Molecular mechanisms of apoptosis and autophagy elicited by combined treatment with oridonin and cetuximab in laryngeal squamous cell carcinoma. Apoptosis 2019; 24:33–45.
[19] Ji Y, Li L, Tao Q, et al. Deprivation of asparagine triggers cytoprotective autophagy in laryngeal squamous cell carcinoma. Appl Microbiol Biotechnol 2017;101:4951–61.
[20] Lin C, Wang Z, Li L, et al. The role of autophagy in the cytotoxicity induced by recombinant human arginase in laryngeal squamous cell carcinoma. Appl Microbiol Biotechnol 2015;99:8487–94.
[21] Homma K, Suzuki K, Sugawara H. The Autophagy Database: an all-inclusive information resource on autophagy that provides nourishment for research. Nucleic Acids Res 2011;39:D986–90.
[22] Linehan WM, Ricketts CJ. The Cancer Genome Atlas of renal cell carcinoma: findings and clinical implications. Nat Rev Urol 2019;16:539–52.
[23] Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The gene ontology consortium. Nat Genet 2000;25:25–9.

[24] Kanehisa M, Sato Y, Kawashima M, et al. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 2016;44:D457–62.
[25] Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000;28:27–30.
[26] The gene ontology resource: 20 years and still GOing strong. Nucleic Acids Res 2019;47:D330–8.
[27] Wang SS, Chen G, Li SH, et al. Identification and validation of an individualized autophagy-clinical prognostic index in bladder cancer patients. Onco Targets Ther 2019;12:3695–712.
[28] He A, He S, Peng D, et al. Prognostic value of long non-coding RNA signatures in bladder cancer. Aging (Albany NY) 2019;11:6237–51.
[29] Zhong Z, Sanchez-Lopez E, Karin M. Autophagy, inflammation, and immunity: a troika governing cancer and its treatment. Cell 2016; 166:288–98.
[30] Lin P, Guo YN, Shi L, et al. Development of a prognostic index based on an immunogenomic landscape analysis of papillary thyroid cancer. Aging (Albany NY) 2019;11:480–500.
[31] Huang T, Wan X, Alvarez AA, et al. MIR93 (microRNA -93) regulates tumorigenicity and therapy response of glioblastoma by targeting autophagy. Autophagy 2019;15:1100–11.
[32] Zhu YC, Wang WX, Zhang QX, et al. The KIF5B-RET fusion gene mutation as a novel mechanism of acquired EGFR tyrosine kinase inhibitor resistance in lung adenocarcinoma. Clin Lung Cancer 2019;20: e73–6.
[33] Rastogi I, Rajanna S, Webb A, et al. Mechanism of c-Met and EGFR tyrosine kinase inhibitor resistance through epithelial mesenchymal transition in non-small cell lung cancer. Biochem Biophys Res Commun 2016;477:937–44.
[34] Li M, Zhang Z, Li X, et al. Whole-exome and targeted gene sequencing of gallbladder carcinoma identifies recurrent mutations in the ErbB pathway. Nat Genet 2014;46:872–6.
[35] Tebbutt N, Pedersen MW, Johns TG. Targeting the ERBB family in cancer: couples therapy. Nat Rev Cancer 2013;13:663–73.
[36] Mai WX, Gosa L, Daniels VW, et al. Cytoplasmic p53 couples oncogene-driven glucose metabolism to apoptosis and is a therapeutic target in glioblastoma. Nat Med 2017;23:1342–51.
[37] Kuchay S, Giorgi C, Simoneschi D, et al. PTEN counteracts FBXL2 to promote IP3R3- and Ca(2+)-mediated apoptosis limiting tumour growth. Nature 2017;546:554–8.
[38] Maj T, Wang W, Crespo J, et al. Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor. Nat Immunol 2017;18:1332–41.
[39] Wang RC, Wei Y, An Z, et al. Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. Science 2012;338: 956–9.
[40] Krazinski BE, Kowalczyk AE, Sliwinska-Jewsiewicka A, et al. IKBKB expression in clear cell renal cell carcinoma is associated with tumor grade and patient outcomes. Oncol Rep 2019;41:1189–97.
[41] Li W, Li H, Bocking AD, et al. Tumor necrosis factor stimulates matrix metalloproteinase 9 secretion from cultured human chorionic trophoblast cells through TNF receptor 1 signaling to IKBKB-NFKB and MAPK1/3 pathway. Biol Reprod 2010;83:481–7.
[42] Shen Y, Park CS, Suppipat K, et al. Inactivation of KLF4 promotes T-cell acute lymphoblastic leukemia and activates the MAP2K7 pathway. Leukemia 2017;31:1314–24.
[43] Hong L, Pan F, Jiang H, et al. miR-125b inhibited epithelial-mesenchymal transition of triple-negative breast cancer by targeting MAP2K7. Onco Targets Ther 2016;9:2639–48.
[44] Min S, Li L, Zhang M, et al. TGF-beta-associated miR-27a inhibits dendritic cell-mediated differentiation of Th1 and Th17 cells by TAB3, p38 MAPK, MAP2K4 and MAP2K7. Genes Immun 2012;13:621–31.
[45] Yu DB, Zhong SY, Yang M, et al. Potent antitumor activity of double-regulated oncolytic adenovirus-mediated ST13 for colorectal cancer. Cancer Sci 2009;100:678–83.
[46] Yang M, Cao X, Yu MC, et al. Potent antitumor efficacy of ST13 for colorectal cancer mediated by oncolytic adenovirus via mitochondrial apoptotic cell death. Hum Gene Ther 2008;19:343–53.
[47] Wang LB, Zheng S, Zhang SZ, et al. Expression of ST13 in colorectal cancer and adjacent normal tissues. World J Gastroenterol 2005;11: 336–9.
[48] Ma L, Chen Z, Erdjument-Bromage H, et al. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell 2005;121:179–93.
[49] Schrader J, Henes FO, Perez D, et al. Successful mTOR inhibitor therapy for a metastastic neuroendocrine tumour in a patient with a germline TSC2 mutation. Ann Oncol 2017;28:904–5.
[50] Li B, Gordon GM, Du CH, et al. Specific killing of Rb mutant cancer cells by inactivating TSC2. Cancer Cell 2010;17:469–80.