

Identification of an Autophagy-Related Signature Predicting Overall Survival for Papillary Thyroid Carcinoma

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Abstract

Background: Papillary thyroid carcinoma usually shows an excellent prognosis. However, its recurrence or persistence rate is high. In this study, we used bioinformatics to identify autophagy-related genes (ARGs) and establish a novel scoring system for papillary thyroid carcinoma.

Methods: We collected ARGs sequencing data of patients with papillary thyroid carcinoma from The Cancer Genome Atlas database. Differentially expressed ARGs were identified by the “Limma” package in R language. After univariate and multivariate Cox regression analysis, an ARG signature was developed. The established prognostic signature was evaluated by Kaplan-Meier curve and time-dependent receiver operating characteristic.

Results: A sum of 26 differentially expressed ARGs were identified. Gene set enrichment analysis revealed that several significantly oncological signatures were enriched, such as autophagy, p53 signaling pathway, apoptosis, human cytomegalovirus infection, and platinum drug resistance. After univariate and multivariate analysis, 3 ARGs (*ITPR1*, *CCL2*, and *CDKN2A*) were selected to develop autophagy-related signature. Patients with high risk had significantly shorter overall survival than those with low risk. The areas under the curve indicated that the signature showed good accuracy of prediction.

Conclusions: We established a novel scoring system based on 3 ARGs, which provides a promising tool for the development of personalized therapy.

Keywords

papillary thyroid carcinoma, autophagy-related genes, prognostic signature, RNA-seq

Introduction

Thyroid cancer (TC) is the most common endocrine malignancy. The incidence of TC has been gradually increasing over the past few decades, and its morbidity has also been on a rising tendency worldwide, mainly due to the increasing use of diagnostic equipment.^{1,2} Papillary thyroid carcinoma is the most common and least aggressive type, taking up approximately 75% of total thyroid malignancies.³ Papillary thyroid carcinoma includes several histological subtypes with distinctive architectural tumor features, such as the follicular variant and the tall cell variant.⁴ Although it is a well-differentiated papillary carcinoma with an excellent prognosis, about half of patients with papillary thyroid carcinoma exhibit lymph node metastases, which are reported to be associated with increased risk of recurrence.^{4,5} Additionally, the treatment of papillary thyroid carcinoma has not been significantly improved yet. Therefore, novel molecular biomarkers or prognostic models

are urgently needed to provide accurate treatment and to improve prognosis for patients with papillary thyroid carcinoma.

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Autophagy is an important physiological process for cells to degrade cytoplasmic components and maintain the stability of the intracellular environment.⁶ Abnormal levels of autophagy are associated with the pathogenesis of various diseases, including inflammation, neurodegenerative diseases, and tumors.^{7,8} However, the role of autophagy in tumorigenesis remains controversial, and the underlying mechanism is still rudimentary and inconclusive. In recent years, autophagy has been targeted in the search for new therapeutic strategies, such as therapies that inhibit or stimulate autophagy.^{9,10} However, few studies have used autophagy-associated gene expression profiles as a tool to assess the prognosis of patients with papillary thyroid carcinoma. In addition, while most studies have focused on the selection of treatment options for the general patient, few studies have been able to analyze and address the condition of individual patients.

At present, the most important risk factor for predicting patient survival is based on the tumor–node–metastasis (TNM) classification system.¹¹ However, this provides only limited information for the clinical prognostication since even patients within the same stage display a strong heterogeneity for prognosis and treatment response. Therefore, finding the ideal biomarker or model to predict the prognosis of patients with papillary thyroid carcinoma has been a long-standing goal. Moreover, few have considered the analysis of genetic characteristics to establish a risk stratification system for papillary thyroid carcinoma. Recently, several studies indicated that gene signature can well predict the prognosis of cancer.^{12,13} However, no prior prognostic models for papillary thyroid carcinoma were established based on autophagy-related genes (ARGs). Therefore, the current study aimed to identify differentially expressed ARGs and build a novel prognostic signature to predict the prognosis of patients with papillary thyroid carcinoma.

Materials and Methods

Data Collection

We collected messenger RNA (mRNA) expression data and the corresponding clinical data of papillary thyroid carcinoma tissues and normal tissues from The Cancer Genome Atlas (TCGA) database. The autophagy gene list was downloaded from the Human Autophagy Database (HADb; <http://autophagy.lu/clustering/index.html>). The current study did not require ethics approval as all study data sets were downloaded and analyzed in accordance with the corresponding data policies of previous databases.

Screening of the Differentially Expressed ARGs

All genes were downloaded in fragments per kilobase million format. The differentially expressed genes (DEGs) were identified by Wilcoxon-rank sum test and fold change (FC) methods, with the thresholds of adjusted P value $< .05$ and $|\log FC| > 1$.¹⁴ The heatmap of DEGs was created using “pheatmap”

package of R language (<https://cran.r-project.org/web/packages/pheatmap>).

Functional Enrichment Analysis

Gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted by “clusterProfiler” package in R/Bioconductor to predict the possible function of differentially expressed ARGs. Adjusted P value $< .05$ was regarded as the cutoff value.

Construction of ARG Risk Score

To discover potential protective (hazard ratio (HR) < 1) and hazardous ARGs (HR > 1) that are significantly correlated with the overall survival (OS) of patients with papillary thyroid carcinoma, a univariate Cox proportional hazards model was used to screen out those with a significant P value $< .05$. These candidates were then subjected to a multivariate Cox proportional hazards regression with stepwise selection of variables based on the Akaike information criterion. By weighting the estimated Cox regression coefficients, an autophagy-related signature was constructed. The median risk score is considered as classification cutoff value between high- and low-risk papillary thyroid carcinoma subgroups. Between high- and low-risk papillary thyroid carcinoma subgroups, the differences in OS time were compared and analyzed via Kaplan-Meier analysis and log-rank test. To evaluate the predictive value of the ARG signature, we drew the time-dependent receiver operating characteristic (ROC) curve.¹⁵

Statistical Analyses

All statistical analyses were performed by SPSS version 23.0 (Chicago, Illinois), along with version 3.6.0 of R software (<https://www.r-project.org/>). Survival curves were created by the Kaplan-Meier method and compared using the log-rank test. The Cox regression coefficients were used to establish a risk score signature. The prediction ability of signature was assessed by area under ROC curve in the package “survivalROC” in R language. Data were considered to be statistically significant with P value $< .05$.

Results

Identification of Differentially Expressed ARGs

The mRNA expression data of 510 papillary thyroid carcinoma tissues and 58 normal tissues were collected from the TCGA. A total of 231 ARGs were obtained from the HADb. Based on the cutoff criteria (adjusted P value $< .05$ and $|\log FC| > 1.0$), a total of 26 differentially expressed ARGs (including 15 upregulated and 11 downregulated genes) were identified between papillary thyroid carcinoma and normal thyroid tissues (Figure 1).

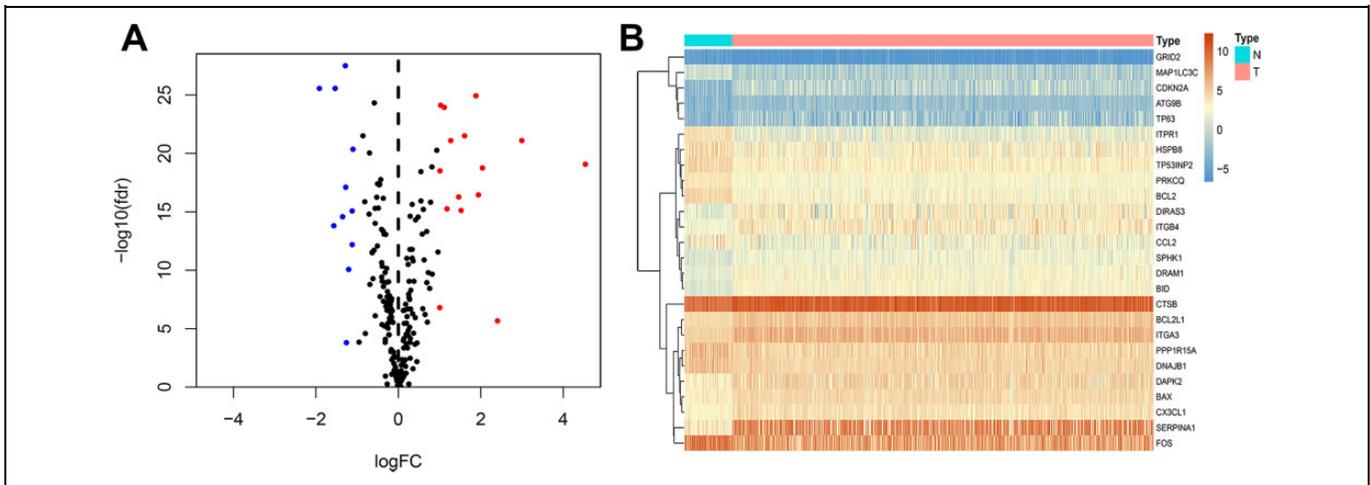


Figure 1. The volcano plot (A) and heatmap plot (B) of 26 differentially expressed autophagy-related genes (ARGs) between papillary thyroid carcinoma (PTC) and normal tissues.

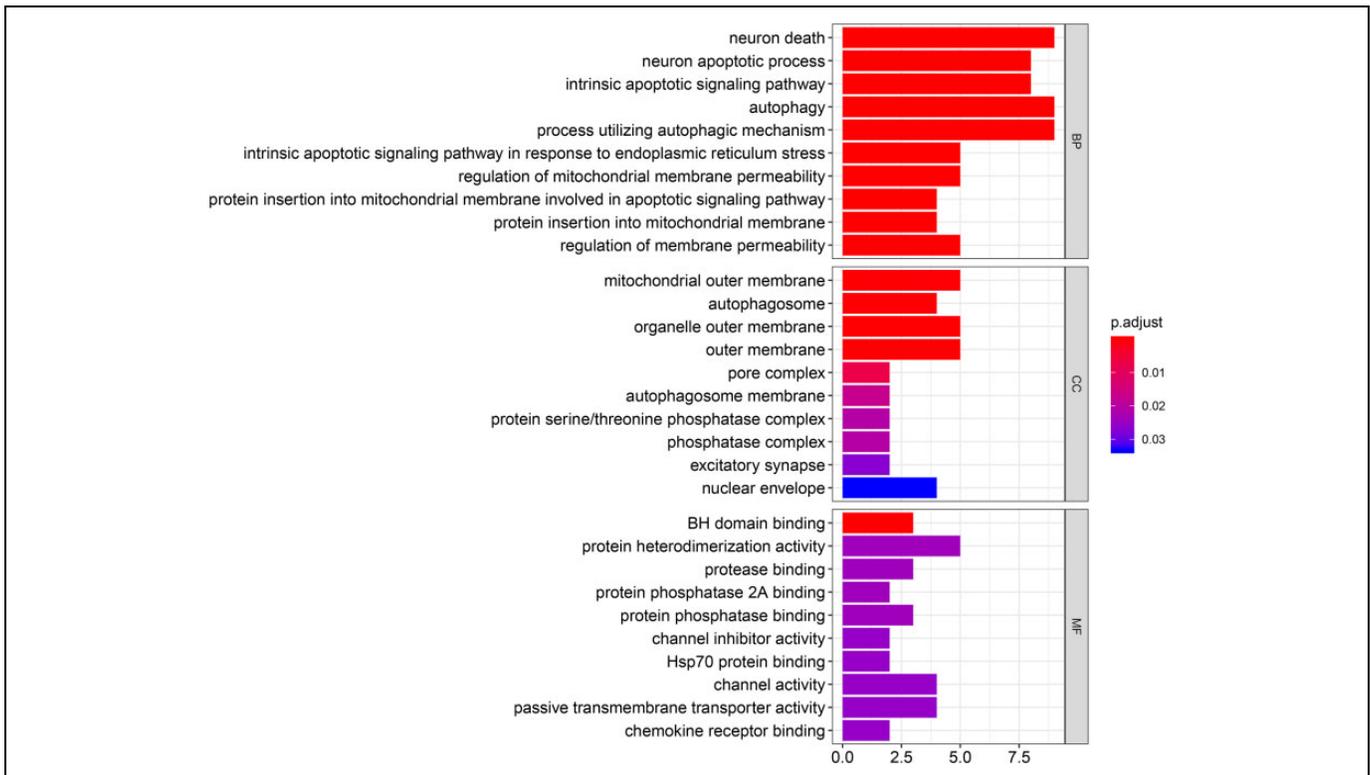


Figure 2. Enrichment of top 10 Gene ontology (GO) terms of differentially expressed autophagy-related genes (ARGs). The node color changes gradually from red to blue in ascending order according to the adjusted P values. The size of the node represents the number of counts.

Functional Enrichment Analysis

The GO functional analysis of the 26 differentially expressed ARGs can be segmented into biological process (BP), molecular function (MF), and cell component (CC). The top 10 results of the GO functional analysis are shown in Figure 2. The differentially expressed ARGs were primarily enriched in BP including neuronal death, autophagy, and process utilizing autophagic

mechanism; in CC including mitochondrial outer membrane, organelle outer membrane, and outer membrane; and in MF including protein heterodimerization activity. Furthermore, we identified certain KEGG pathways positively associated with ARGs such as autophagy, apoptosis, p53 signaling pathway, human cytomegalovirus infection, human immunodeficiency virus 1 infection, and platinum drug resistance (Figure 3).

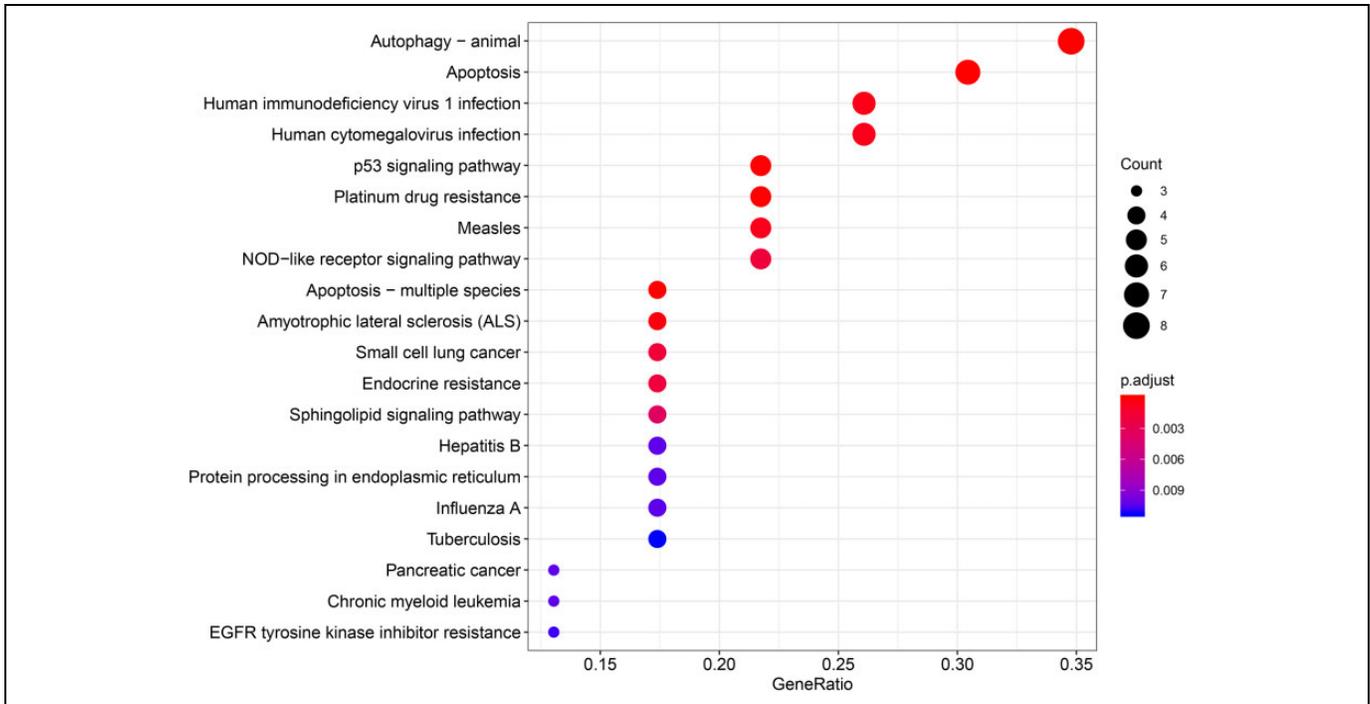


Figure 3. Enrichment of top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of differentially expressed autophagy-related genes (ARGs). The node color changes gradually from red to blue in ascending order according to the adjusted P values. The size of the node represents the number of counts.

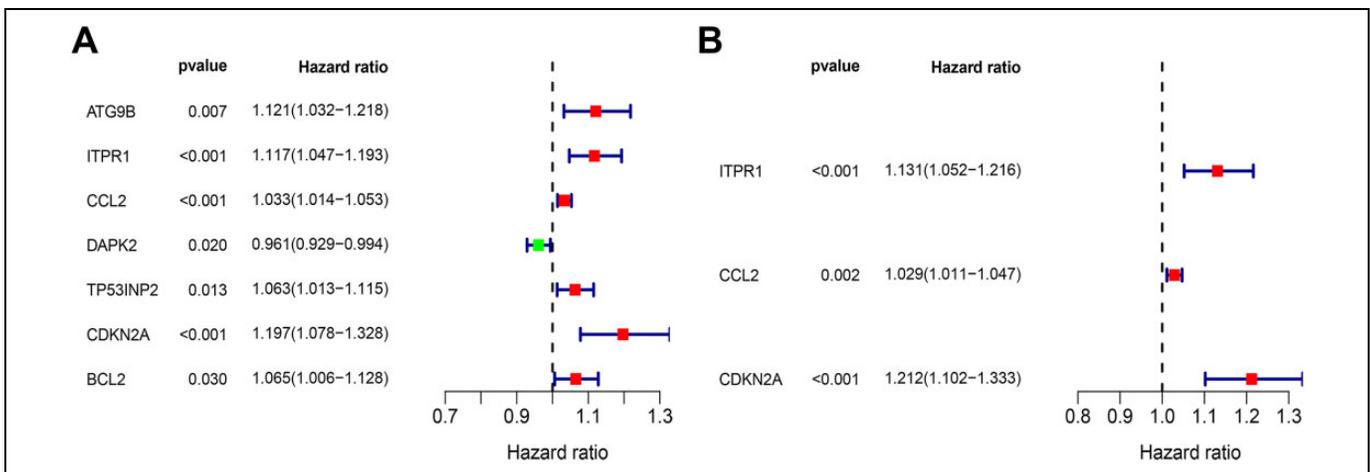


Figure 4. Univariate and multivariate analyses assessing relationship between expression levels of autophagy-related genes (ARGs) and overall survival (OS) in patients with papillary thyroid carcinoma (PTC).

Establishment of 3-ARG Signature

Univariate and multivariate Cox proportional hazards regression analyses were conducted to assess relationship between expression levels of ARGs and OS in patients with papillary thyroid carcinoma (Figure 4A and B). A predictive signature model was established according to 3 ARGs (*ITPR1*, *CCL2*, and *CDKN2A*) selected from further multivariate Cox regression analysis. The risk score for OS was calculated as follows: risk score = $(0.123337693147 \times \text{expression value of } ITPR1) + (0.028524 \times \text{expression value of } CCL2) + (0.192202 \times$

expression value of *CDKN2A*). We then used this model to calculate the risk score for each patient. Using the median of the risk score as the cutoff value, patients were stratified into high-risk ($n = 233$) and low-risk group ($n = 234$), respectively. The Kaplan-Meier curves, risk score, and survival status of these 3 prognostic ARGs are shown in Figure 5. The prognostic power of the autophagy-related signature was evaluated via Kaplan-Meier curve and areas under the curve (AUC) value of the ROC curve. Kaplan-Meier curves showed that patients in the high-risk group had a significantly lower OS than those in

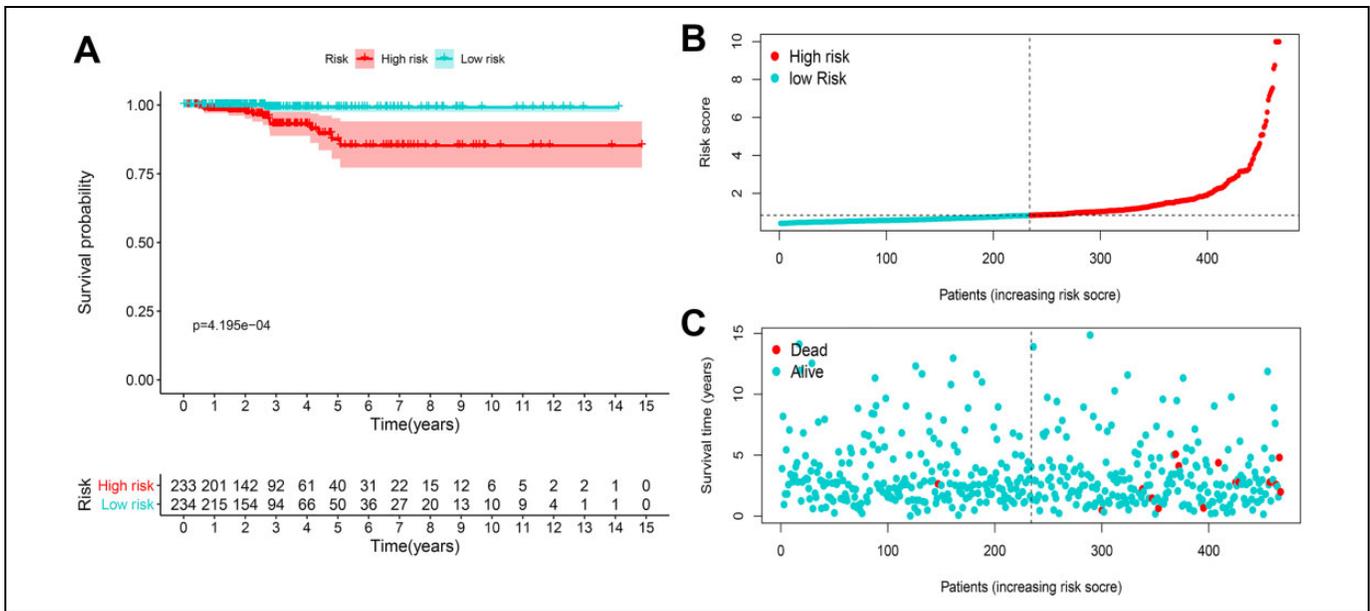


Figure 5. The distribution of Kaplan-Meier curve (A), risk score (B), and survival status (C) of 3 autophagy-related gene signature.

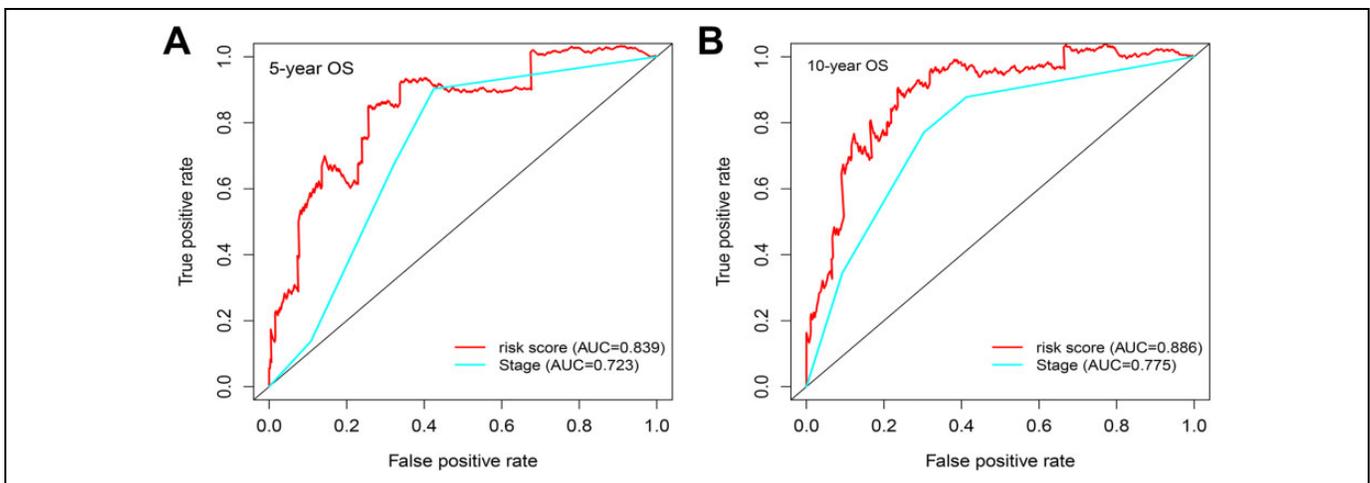


Figure 6. Receiver operating characteristic (ROC) curves of the signature predicting the 5- (A) and 10-year overall survival (OS) rates (B).

the low-risk group ($P < .001$). Areas under the curve value of the signature predicting the 5- and 10-year OS rates were 0.839 and 0.886, respectively (Figure 6). Furthermore, comparison showed that the discrimination of the signature was higher than that of the American Joint Committee on Cancer (AJCC) TNM classification (Figure 6).

Discussion

Although papillary thyroid carcinoma is a well-differentiated papillary carcinoma with an excellent prognosis, its recurrence rate after treatment is relatively high. Moreover, the TNM staging system does not adequately predict the risk of papillary thyroid carcinoma. Therefore, there is urgent need for better prognostic methods to predict the risk of patients with papillary

thyroid carcinoma. In this study, we explored the ARG expression profiles of 467 patients with papillary thyroid carcinoma from TCGA and established a prognostic signature for stratifying the risk of papillary thyroid carcinoma. We identified 26 differentially expressed ARGs between papillary thyroid carcinoma tissues and normal thyroid tissues. Considering that these genes may be involved in the initiation of papillary thyroid carcinoma, we further analyzed the functions of the differentially expressed ARGs through GO and KEGG pathway analysis. The result indicated several significantly enriched oncological signatures, such as autophagy, apoptosis, p53 signaling pathway, human cytomegalovirus infection, human immunodeficiency virus 1 infection, and platinum drug resistance. After univariate and multivariate analysis, ARGs with independent prognostic value were selected to define a

risk-score model using their expression levels weighted by corresponding correlation coefficient in the multivariate Cox analysis. According to median risk scores, the patients with papillary thyroid carcinoma were assigned to either low- or high-risk group. Patients with high-risk score have significantly worse OS than those with low-risk score. The prognostic power of the autophagy-related signature was evaluated via AUC value of the ROC curve. The results showed that the risk-score model can effectively stratify the risk of patients with papillary thyroid carcinoma.

The proposed autophagy-related signature included 3 ARGs (*ITPR1*, *CCL2*, and *CDKN2A*). All genes in the signature were associated with OS of patients with papillary thyroid carcinoma. Inositol 1,4,5-trisphosphate receptor, type 1 (*ITPR1*), a member of IP3 receptor family, encodes an intracellular receptor mediating calcium release from the endoplasmic reticulum and plays a role in inducing autophagy.¹⁶⁻¹⁸ Messai et al¹⁷ reported that *ITPR1* is a new direct target of hypoxia-inducible factors 2 α (HIF2 α), and that targeting *ITPR1* significantly increased the sensitivity of renal cancer cells to natural killer (NK)-mediated lysis. Mechanistically, *ITPR1* overexpression regulated NK-mediated killing via autophagy activation in target cells by NK-derived signal. Silencing *ITPR1* in renal cancer cells inhibited NK-induced autophagy. In addition, in vivo *ITPR1* targeting significantly enhanced NK-mediated tumor regression. *ITPR1*-induced autophagy was also reported in other tumors, including papillary thyroid carcinoma.^{19,20}

Yu et al analyzed the transcriptomics data from 3 microarray data sets and identified *ITPR1* as a key candidate gene in papillary thyroid carcinoma, consistent with our results. *CCL2* (chemokine C-C motif ligand 2), also named monocyte chemoattractant protein-1, is a well-known cytokine, which binds to *CCR2* G-protein-coupled receptors to regulate macrophage recruitment during acute inflammation.²¹ Xu et al²² found that drug-resistant gastric cancer cells can secrete more *CCL2* than drug-sensitive gastric cancer cells. *CCL2* attenuated drug-induced cytotoxicity by activating PI3K–Akt–mTOR signaling to inhibit proapoptotic autophagy. Knockdown of *CCL2* or autophagy induction successfully reversed the drug resistance of gastric cancer cells. Fang et al²³ reported that *CCL2* is overexpressed in luminal B breast cancer cells and increases cell growth and survival by inhibiting necrosis and autophagy. In anaplastic thyroid carcinoma,²⁴ *CCL2* can be secreted after cytokines stimulation, leading to a different modulation. However, it was not assessed whether *CCL2* could be used as a marker in the follow-up of patients with anaplastic thyroid carcinoma. Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) has been reported to induce autophagy in multiple malignancies.^{25,26} However, there are no reports of *CDKN2A*-induced autophagy in papillary thyroid carcinoma.

In our study, functional enrichment analysis of 26 ARGs was performed to further elucidate molecular and biological mechanisms in papillary thyroid carcinoma. The results demonstrated that these ARGs were mainly enriched in autophagy, apoptosis, p53 signaling pathway, human cytomegalovirus infection, human immunodeficiency virus 1 infection,

and platinum drug resistance. Several signaling pathways identified in this study have been investigated in papillary thyroid carcinoma in recent years. Autophagy is a key cellular process that not only protects cells and organisms from stress but also ensures the maintenance and development of various cancers. Several autophagy-related pathways have been reported in TC.²⁷⁻²⁹ Wang et al²⁷ showed that baicalein-induced autophagy of undifferentiated TC cells via the ERK/PI3K/Akt pathway. Xu et al²⁹ indicated that transmembrane protein 21 (TMP21) modulates cell growth of papillary thyroid carcinoma cells by inducing autophagy through activation of the adenosine monophosphate-activated protein kinase/(AMPK) mTOR pathway. P53 is a classic tumor suppressor that often mutates between multiple cancer types. As a key guardian in tumorigenesis, p53 is activated in response to carcinogenesis and regulates a large number of genes involved in cell cycle arrest, DNA repair, and apoptosis.³⁰ Mutations of p53 are highly frequent in colorectal cancer, and p53 mutation status is associated with disease outcome.³¹ Taken together, these differentially expressed ARGs are involved in many important papillary thyroid carcinoma-associated biological functions and pathways.

Our study is the first to establish an autophagy-related signature and to show favorable predictive ability. Our results are of great relevance to clinicians and patients because they allow to predict individual patient outcomes. When patients who were previously diagnosed with a tumor ask for a clinician's help, they are likely more concerned about their individual survival risk at that specific moment, than the risk since diagnosis, as traditionally considered.

Nonetheless, there are still some limitations of this study. First, the proposed signature was established based on data from the TCGA database. Additional validation with independent external mRNA expression data are needed to investigate the performance of the signature. In addition, the signature was established based on bioinformatics data. Further experimental studies are needed to validate the functions and predictive value of the 3 ARGs.

Conclusions

This study successfully identified 3 prognostic ARGs and developed a novel scoring system to predict OS in patients with papillary thyroid carcinoma. It may help in clinical decision-making for individualized therapeutic regimen design.

Authors' Note

G.H. and H.-F.F. contributed equally to this work. G.H. and H.Z. conceived and designed the original study. G.H. and H.-F.F. collected and analyzed the data. G.H., H.-F.F., and H.Z. contributed to the interpretation of data. G.H. and H.-F.F. drafted the manuscript. H.Z. revised the manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

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