Abstract. Traditional clinicopathological indices are insufficient in predicting the prognosis of patients diagnosed with oral and oropharyngeal squamous cell carcinoma (OSCC/OPSCC). Notably, autophagy and long non-coding RNAs (lncRNAs) regulate the development and progression of various types of cancer. The present study aimed to assess the association between autophagy-related lncRNAs and the prognosis of patients diagnosed with OSCC/OPSCC. Gene sequencing and clinicopathological data of patients with OSCC/OPSCC were downloaded from The Cancer Genome Atlas database, while gene set functional classification was downloaded from the Gene Set Enrichment Analysis database. Out of the 413 transcriptome data samples and 402 clinicopathological data samples retrieved, a total of nine autophagy-related lncRNAs, including PTCSC2, AC099850.3, LINC01963, RTCA‑AS1, AP002884.1, UBAC2‑AS1, AL512274.1, MIR600HG and AL354733.3, were screened. This was geared towards establishing a signature through gene co-expression network, univariate and Least Absolute Shrinkage and Selection Operator Cox regression analyses. Based on this signature, the patients were subdivided into a high-risk group and a low-risk group. Kaplan-Meier survival analysis revealed that the overall survival of the high-risk group was significantly lower than that of the low-risk group. Furthermore, principal components analysis demonstrated that the patients diagnosed with OSCC/OPSCC could be distinguished into low-survival and high-survival groups according to the signature. Univariate and multivariate Cox regression analyses of clinicopathological data and the signature revealed that the signature could potentially be used as an independent prognostic factor for OSCC/OPSCC. In addition, reverse transcription-quantitative PCR analysis of clinical samples demonstrated the validity of the signature. In summary, the present study revealed that the signature based on autophagy-related lncRNAs potentially acts as an independent prognostic indicator for patients with OSCC/OPSCC. Furthermore, it promotes research on targeted diagnosis and treatment of patients diagnosed with OSCC/OPSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumor worldwide (1). Notably, the main types of HNSCC are oral and oropharyngeal squamous cell carcinoma (OSCC/OPSCC), which severely affect the quality of life of patients and endanger their lives (2). Recent studies have demonstrated that the development of OSCC/OPSCC is closely associated with the changes of key genes (3-5). Therefore, it is necessary to identify novel gene-level biomarkers that predict the prognosis of OSCC/OPSCC.

Autophagy, a mechanism maintaining the stability of the internal environment and the balance of protein metabolism in cells, regulates the development and progression of various types of cancer (6). In the early stage, autophagy removes abnormal organelles and proteins in normal cells to prevent the generation of the stress response, protect the genes from being damaged and inhibit the occurrence of tumors; however, autophagy also maintains the survival of tumor cells in the late stage of tumor development under certain conditions, such as hypoxia or nutrient deficiency, thereby sustaining proliferation and progression of tumor cells (7). By investigating autophagy-related molecules, the present study aimed to identify novel biomarkers for the prognosis prediction of OSCC/OPSCC.

Abnormal expression of long non-coding RNAs (lncRNAs) regulates the proliferation, apoptosis and migration of tumor cells, implying that lncRNAs can potentially be used as important biomarkers and therapeutic targets (8). Nonetheless, lncRNAs in OSCC/OPSCC represent an important subject which has not been sufficiently investigated. In the present study, The Cancer Genome Atlas (TCGA) database was used to evaluate the potential value of autophagy-related lncRNAs as a prognostic tool.
for patients with OSCC/OPSCC. Through TCGA data analysis, nine autophagy-related lncRNAs associated with the overall survival (OS) of patients with OSCC/OPSCC were identified, and their independent associations were further verified. Subsequently, the nine lncRNAs were integrated into an independent signature, and it was revealed that their predictive performance for the prognosis of patients with OSCC/OPSCC was higher than that of clinicopathological features (age, sex, grade, T stage, N stage and TNM comprehensive stage). This demonstrated the potential of autophagy-related lncRNAs as biomarkers to predict the prognosis of patients with OSCC/OPSCC.

Materials and methods

Acquisition of autophagy-related lncRNAs and clinicopathological data of patients with OSCC/OPSCC. The level 3 RNA-seq and clinicopathological data (project ID: TCGA-HNSC) of patients diagnosed with OSCC/OPSCC were extracted from TCGA (https://portal.gdc.cancer.gov/), while the autophagy gene set (accession number: M10281) was obtained from the Molecular Signatures Database v7.0 (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). Subsequently, the function of lncRNAs was explored based on the hypothesis that co-expressed genes are more likely to be functionally related (9). Spearman correlation analysis was carried out on the expression of lncRNAs and autophagy genes through the ‘limma’ (version 3.11; https://bioconductor.org/packages/limma/) package of R software (version 3.6.1; R foundation) and lncRNAs with high correlation (Spearman correlation coefficient (cor)>0.4; P<0.001) with autophagy genes were identified as autophagy-related lncRNAs.

Identification of a prognostic multi-lncRNA signature. First, univariate Cox regression analysis was applied to explore lncRNAs associated with the prognosis of patients (P<0.01). Thereafter, Least Absolute Shrinkage and Selection Operator (LASSO) Cox regression analysis was used to optimize the prognostic multi-lncRNA signature (10). Afterwards, the present study substituted the expression of lncRNAs in patients with OSCC/OPSCC into the Cox model, and using the LASSO Cox regression coefficient, the risk score of each patient was calculated. Subsequently, based on the median risk score, the patients were subdivided into a high-risk group and a low-risk group.

The Kaplan-Meier method and log-rank test were performed to evaluate differences in OS between patients in the high- and low-risk groups. The risk score distribution, survival status of patients with OSCC/OPSCC and expression profiles of prognostic lncRNAs were visualized through images. R software (version 3.6.1; R foundation) was used for statistical calculations and data plotting.

Independence of multi-lncRNA signature in predicting prognosis of patients with OSCC/OPSCC. The independent associations of the multi-lncRNA signature-based risk score and clinicopathological factors (age, sex, TNM stage and pathological stage) with the prognosis of patients with OSCC/OPSCC were determined through univariate and multivariate Cox regression analysis using R software. Furthermore, a receiver operating characteristic (ROC) curve was used to evaluate the accuracy of these factors in predicting the prognosis of OSCC/OPSCC.

Validation of the clinical OSCC/OPSCC specimens by reverse transcription-quantitative PCR (RT-qPCR). To further validate the findings from TCGA, RT-qPCR was performed to detect the expression levels of the nine autophagy-related lncRNAs in OSCC/OPSCC samples (n=35). All patients (30 men, 25 women; median age, 46 years; age range, 35-68 years) received lesion excision between January 2015 and October 2018 at the First Affiliated Hospital of Nanchang University (Nanchang, China) where they were pathologically diagnosed with OSCC/OPSCC. Patients with distant metastasis, multiple primary cancers or non-first surgery were excluded from the present study. The collection of the tumor samples from patients with OSCC/OPSCC conformed to the Declaration of Helsinki and current legislation. In addition, clinicopathological information of patients was captured by an investigator through interviews and medical records. The present study was approved by the Ethics Committee of First Affiliated Hospital of Nanchang University (approval no. 2019B0017), and all patients provided written informed consent.

Total RNA was isolated from the tissue samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The GoScript Reverse Transcription kit (cat. no. PRA5000; Promega Corporation) and GoTaq® qPCR Master Mix of Power SYBR® Green (Promega Corporation) was used to synthesize cDNA and for RT-qPCR detection. RT-qPCR was performed using the GeneAmp® PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Additionally, relative fold change results were calculated using the 2^-ΔΔCq method (11). (The thermocycling conditions were as follows; 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. RNA expression was normalized to GAPDH. The following primer sequences were used: PTSC2 forward 5’-CCCTAGGCCCACCGCATTTT-3’ and reverse, 5’-GGGTCGACTGCGGTCTAGAC-3’; AC099850.3 forward 5’-CGTCTTTTCACCCCGCCTT-3’ and reverse, 5’-AAAGACAGAACCTCCTGTTG-3’; LINC01963 forward 5’-CCCCGGTGGTGAGGAATTCGCA-3’ and reverse, 5’-TTTCTCCGCCATTCCGTTT-3’; RTCA-AS1 forward 5’-CGGAGGTGCCGACATTAGA-3’ and reverse, 5’-CCAGGTTCACTCAGCTTA-3’; AP002884.1 forward 5’-TGTTGGTACGAGGGTTTG-3’; UBAC2-AS1 forward 5’-GCAACTCCCGTACCGGATTTT-3’ and reverse, 5’-ATGCCGACATCCCGGATTT-3’; PDCDC2 forward 5’-GCTAAGTCCGTGAGCAG-3’ and reverse, 5’-TTGTTGGTACGAGGGTTTG-3’; UBA2-AS1 forward 5’-TGAAACGATGGGCGCTCAGAG-3’ and reverse, 5’-TCAGGTCTCTCGATGTCAGA-3’; ALS12274.1 forward 5’-AACACAGTGGAGTGTCAGG-3’ and reverse, 5’-CACCCTACAGACGCCAG-3’; MIIR600HG forward 5’-GCCAGTCTCGACGTGAACA-3’ and reverse, 5’-GGCGTCTTCTACAGCGCCATCT-3’; AL354733.3 forward 5’-TCCCCGAGGCTCATTAAATCCT-3’ and reverse, 5’-TCTGCTGTGAGCTTGTTCG-3’; GAPDH

Kruskal-Wallis with Dunn's post hoc test was used to further explore the relationship between the expression levels of the nine autophagy-related lncRNAs and the clinicopathological characteristics of patients with OSCC/OPSCC.

Principal components analysis (PCA) and gene set enrichment analysis (GSEA). Furthermore, PCA was used to test the differentiation of patients in low- and high-risk groups. GSEA (https://www.gsea-msigdb.org/gsea/index.jsp) was used to investigate the functions of autophagy-related genes in low- and high-risk groups. Statistical calculations and data plotting were done using R software (version 3.6.1; R foundation).
forward 5'-GGAAGCTTGTCTACATAATGAAATC-3' and reverse, 5'-TGATGACCCTTTGGCTCCC-3'.

Statistical analysis. Statistical differences were calculated using R software (version 3.6.1; R foundation) and SPSS Statistics software (version 20; IBM Corp.). All experiments were performed in triplicate. For non-parametric analysis, Kruskal-Wallis with Dunn's post hoc test was used. Survival curves were plotted using the Kaplan-Meier method, and differences were analyzed via the log-rank test. The regression analysis of univariate and multivariate Cox proportional hazards analysis was completed using the ‘survival’ package (version 2.36-10, http://cran.r-project.org/web/packages/survival) of R software. Results are presented in the form of hazard ratio and corresponding 95% confidence interval (CI). P<0.05 was considered to indicate a statistically significant difference.

Results

Autophagy-related lncRNAs and clinicopathological data in patients with OSCC/OPSCC. A total of 646 autophagy-related lncRNAs, 402 patients with survival information and 298 patients with complete clinicopathological information were identified (Fig. 1).

Identification of a prognostic autophagy-related lncRNA signature in patients with OSCC/OPSCC. Using univariate Cox regression analysis, 32 autophagy-related lncRNAs associated with the OS of patients with OSCC/OPSCC were identified (Fig. 2).

Figure 1. Process of data selection. Screening process of (A) lncRNA data and (B) clinicopathological data. lncRNA, long non-coding RNA; OSCC/OPSCC, oral and oropharyngeal squamous cell carcinoma.

Figure 2. Autophagy-related long non-coding RNAs affecting overall survival of patients. 95% CI, 95% confidence interval.
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The aforementioned nine lncRNAs and the LASSO Cox regression coefficients were integrated to establish the signature (Table I). Based on the signature, the risk scores of patients with OSCC/OPSCC (n=371; among the 402 patients, 31 patients with no detectable target lncRNA expression were excluded) were calculated and subdivided into a high-risk group (n=185) and a low-risk group (n=186) according to the median value (1.057).

The Kaplan-Meier curve demonstrated that the OS of the high-risk group was significantly poorer than that of low-risk patients (P=5.223x10^{-8}; Fig. 3). The risk score distribution, survival status of patients with OSCC/OPSCC, and expression profiles of the nine prognostic lncRNAs are shown in Fig. 4. It was revealed that the rate of mortality among patients with high-risk scores was higher compared with that in patients with low-risk scores. The expression
levels of AC099850.3, AP002884.1 and UBAC2-AS1 were higher in high-risk patients compared with in patients with low-risk. The expression levels of AL512274.1, PTCSC2, LINC01963, RTCA-AS1, AL354733.3 and MIR600HG were lower in high-risk patients compared with in patients with low-risk.

In order to determine the role of the nine lncRNAs, we extracted and displayed the correlation analysis results between these nine lncRNAs and autophagy-related genes (Fig. S1; Table SI). All of the nine lncRNAs were positively correlated with autophagy genes. PTSC2 exhibited the highest correlation coefficient (cor=0.614), while MIR600HG had the most co-expression genes (n=20).

Association between the nine-lncRNA signature and prognosis in patients with OSCC/OPSCC. The results of univariate Cox regression analysis demonstrated that the nine-lncRNA signature-based risk score, age, T stage, N stage and TNM comprehensive stage were significantly associated with patient survival (Fig. 5A). Furthermore, multivariate Cox regression analysis revealed that the signature-based risk score and age were independent factors associated with the OS of patients with OSCC/OPSCC (Fig. 5B). Due to the absence of M1 in the case data, the M stage was not included in the analysis.

The prediction capability of the signature was further analyzed using ROC curves, and the area under the curve value was 0.710, which was higher than that of the other clinicopathological characteristics (Fig. 6).

Relationship between nine autophagy-related lncRNAs and clinicopathological factors in patients with OSCC/OPSCC. A Kruskal-Wallis test was used to investigate the association between the expression levels of the nine autophagy-related lncRNAs and the clinicopathological characteristics of the patients (Fig. 7). The results demonstrated that AC099850.3 (P<0.01), AL512274.1 (P<0.001) and RTCA-AS1 (P<0.01) were significantly associated with the pathological grade (Fig. 7A), and RTCA-AS1 was significantly associated with T stage (P<0.05; Fig. 7B). However, none of the lncRNAs were significantly associated with the clinical stage or N stage of the patients (Fig. 7C and D).

Autophagy status of low- and high-risk groups. PCA was conducted to investigate differences in the expression of the nine autophagy-related lncRNAs, autophagy-related lncRNAs, autophagy-related genes and all genes between low- and high-risk groups. The patients in the two groups were mixed when the expression of all the genes was used as the spatial indicator. The patients in the two groups were separated gradually when the index was optimized gradually from all genes to autophagy-related genes, autophagy-related lncRNAs and the nine autophagy-related lncRNAs (Fig. 8), suggesting
that the signature established in the present study exhibited an improved discriminative power for prognostic prediction of patients with OSCC/OPSCC compared with other gene indices.

Functional annotation was further performed by GSEA, and the results demonstrated that the difference in gene function between low- and high-risk patients were not significantly enriched in the positive or negative regulation gene sets of autophagy (Fig. 9), indicating that, based on the overall increase or decrease of autophagy, determining the prognosis of the patients was not tenable.

RT-qPCR validation. To assess the validity and reliability of the bioinformatics results, the expression levels of the nine autophagy-related lncRNAs were detected by RT-qPCR in 55 patients with OSCC/OPSCC. Based on the results of RT-qPCR and risk scores of the signature, the patients were divided into a high-risk group and a low-risk group. Kaplan-Meier analysis revealed that the OS of the high-risk group was significantly lower than that of the low-risk group (P=0.021; Fig. 10), and univariate and multivariate Cox regression analysis demonstrated the potential of the signature as an independent prognostic factor for patients with OSCC/OPSCC (Fig. 11), confirming the credibility of the signature.

Discussion

Autophagy is an important material catabolism process in cells, is caused by hypoxia, peroxidation, drug and DNA damage, and regulates cell self-renewal and homeostasis. According to previous studies, autophagy can be a double-edged sword in tumor development due to its ability to kill tumor cells during tumor development (12). However, it also protects tumor cells from being damaged (13).

At present, more than 40 autophagy-related genes have been identified in yeast and mammals (14). Being highly conserved between species, these genes regulate the occurrence and degree of intracellular autophagy via complex regulatory networks, and they are also implicated in the pathophysiology of diseases (15).

Targeted regulation of the autophagy level has become a subject of research focus in the treatment of cancer and other diseases (16). In the complex regulatory network of intracellular genes, regulating the expression of proteins associated with autophagy is a basic method to regulate autophagy (17).

Previous studies have asserted that lncRNAs, such as lncRNA-HOTAIR (18-21) and lncRNA-MALAT1 (22-25), serve a pivotal role in regulating the expression of
autophagy-related proteins through positive or negative effects on mRNAs. Therefore, understanding and utilizing the role of lncRNAs in the regulation of autophagy will advance the clinical diagnosis and treatment of tumors.

With the advent of high throughput RNA sequencing and advanced computer technology, lncRNAs can continuously be investigated. Nevertheless, due to wide expression, complexity of the relationship and functional diversity, research on the functions of most lncRNAs has not matured (26).

Bioinformatics analysis is an effective method for exploring the functions of IncRNAs, and provides a basis for subsequent experimental studies (27,28).

Figure 8. Principal components analysis between low- and high-risk groups. The patients in the two groups were separated gradually when the index was optimized gradually from (A) all genes to (B) autophagy-related genes, (C) autophagy-related lncRNAs and (D) the nine autophagy-related lncRNAs. lncRNA, long non-coding RNA.

Figure 9. Gene Set Enrichment Analysis of the difference of the autophagy status between patients in the low- and high-risk groups. The difference in gene function between low- and high-risk patients were not significantly enriched in the (A) negative or (B) positive regulation gene sets of autophagy. GO, Gene Ontology.

Figure 10. Kaplan-Meier plot of overall survival based on reverse transcription-quantitative PCR analysis of patients. The overall survival of the high-risk group was significantly lower than that of the low-risk group (P=0.021).
Using Cox regression analysis and the Kaplan-Meier method, the present study identified nine autophagy-related IncRNAs which significantly influenced the prognosis of patients with OSCC/OPSCC. Based on the aforementioned findings, a nine-IncRNA signature with satisfactory performance was developed. As a result, the present study further classified the patients with OSCC/OPSCC into a high-risk group and a low-risk group with significantly different prognosis.

By reviewing related literature and the genes exhibiting co-expression relationships with IncRNAs, the functions of the nine IncRNAs in the signature were explored. Zhou et al. (29) revealed that high expression levels of AC099850.3 were closely associated with decreased survival of patients diagnosed with tongue cancer. The present study revealed that AC099850 exhibited the greatest positive co-expression correlation with eukaryotic translation initiation factor 2 alpha kinase 4 (EIF2AK4). Notably, EIF2AK4 belongs to the family of protein kinases that phosphorylates the subunit of eukaryotic translation initiation factor 2 (EIF2) in response to various stress stimuli (30). To activate autophagy-related gene expression in response to cellular stress, activation of the EIF2AK4-EIF2A-activating transcription factor 4 signaling pathway is essential (31).

Zhu et al. (32) reported that UBAC2-AS1 was a potential therapeutic target and a prognostic biomarker of clear cell kidney carcinoma. Furthermore, Chen et al. (33) suggested that UBAC2-AS1 might be implicated in adipogenesis by acting as a competing endogenous RNA or being co-expressed with its targets. The present study demonstrated that UBAC2-AS1 exhibited the greatest positive co-expression correlation with a suppressor of Ty homolog-5, which has been demonstrated to be a novel tumor-specific human telomerase reverse transcriptase promoter-binding protein in colon cancer cells (34).

According to Song et al. (35), MIR600HG is a potential prognostic biomarker in predicting the survival of patients diagnosed with pancreatic ductal adenocarcinoma. The present study demonstrated a positive correlation in the co-expression of MIR600HG and ubiquitin-specific protease 30 (USP30). Furthermore, USP30 potentially reverses depolarization-induced PTEN induced kinase 1-parkin RBR E3 ubiquitin protein ligase-dependent mitophagy and has rapidly emerged as a potential therapeutic target in Parkinson's disease (36).

It was also identified that the PTCSC2, LINC01963, AP002884.1, RTCA-AS1, AL512274.1 and AL354733.3 genes have not been studied in detail. Additionally, a significant positive co-expression relationship was identified between PTCSC2 and Xeroderma pigmentosum group A (XPA). Notably, as a key subunit implicated in the nucleotide excision repair (NER) system, XPA protein is a central organizer in the NER signaling pathway which identifies DNA damage and recruits other NER proteins to DNA lesions (37). A study by Ge et al. (38) demonstrated that XPA potentially promotes cell-protective autophagy in a DNA repair-independent manner by enhancing the activation of poly(ADP-ribose) polymerase 1 (PARP1) in melanoma cells resistant to cisplatin.

It was revealed that LINC01963 harbored the greatest positive co-expression correlation with tuberculosis 2 (TSC2), while AL354733.3 had the greatest positive co-expression correlation with tuberculosis 1 (TSC1). By suppressing mTOR signaling, studies have revealed that TSC1 and TSC2 potentially induce autophagy (39,40).

AP002884.1 and fasciculation as well as elongation zeta/zygin1 (FEZ1) had a significant positive co-expression relationship. FEZ1 potentially acts as an adaptor of cargo transport and may be a scaffold protein; the complexes of FEZ1 formed with unc-51 like autophagy activating kinase 1, short coiled-coil protein, RAB3 GTPase activating protein catalytic subunit 1 or RAB3 GTPase activating non-catalytic protein subunit 2 have been demonstrated to be associated with autophagy (41).

RTCA-AS1 was markedly positively correlated with chromatin-modifying protein 4 (CHMP4). Notably, CHMP4A, CHMP4C and CHMP2B belong to the family of chromatin-modifying protein/charged multivesicular body protein. They are components of the endosomal sorting complex required for transport III involved in the formation of endocytic multivesicular bodies (42). CHMP4A expression is associated with the recurrence of ovarian cancer (43), whereas...
CHMP4C regulates radiation resistance in non-small cell lung cancer (44).

AL512274.1 was significantly positively co-expressed with mitogen-activated protein kinase 3 (MAPK3). Previous studies have identified MAPK3 to be specifically implicated in the control of cell proliferation, differentiation and autophagy (45,46).

To the best of our knowledge, except for AC099850.3, the other eight lncRNAs have not been reported in previous studies on OSCC/OPSCC, implying that these lncRNAs represent a potential target for the treatment of OSCC/OPSCC. In addition, their biological roles in the autophagy of OSCC/OPSCC will be a focus in future studies. The present investigation had some limitations. For instance, the present study was based on profiles of high-throughput RNA-sequencing and data analysis, and therefore, lacked validation in a large clinical sample. A multi-center, large sample, longitudinal study with diverse clinical, radiographic and histopathologic factors is required to further demonstrate the reliability of the present study. Furthermore, the roles of the nine autophagy-related lncRNAs deserve further in vitro and in vivo investigation.

In conclusion, based on nine autophagy-related lncRNAs, a signature that demonstrated the capability to predict the prognosis of patients diagnosed with OSCC/OPSCC was developed. Using this signature, patients with a higher risk of mortality can be predicted, and therefore more priority in treatment should be given to these patients.

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Availability of data and materials

The datasets used can be obtained from TCGA (https://portal.gdc.cancer.gov). The RT-qPCR data generated in the present study is available from the corresponding author upon reasonable request.

Authors' contributions

QJ and JQ conceived and designed the present study. QJ performed the bioinformatics analysis. DX analyzed the data. QJ and DX drafted the initial manuscript. QJ and FS performed tissue tests and followed up patients. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

For the use of human samples, the present study was approved by the Institutional Ethics Committee of First Affiliated Hospital of Nanchang University (Nanchang, China; approval no. 2019B00017), and written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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