Identification and validation of seven prognostic long non-coding RNAs in oral squamous cell carcinoma

TINGTING MIAO*, QINGZONG SI*, YUAN WEI, RUIHONG FAN, JUNJIE WANG and XIAOLI AN

School of Stomatology, Lanzhou University, Gansu, Lanzhou 730000, P.R. China

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Abstract. Oral squamous cell carcinoma (OSCC) is one of the most common malignancies worldwide, due to poor diagnosis and treatment. There is increasing evidence that demonstrates the involvement of long non-coding RNAs (lncRNAs) in carcinogenesis and cancer progression. Therefore, the aim of the present study was to explore potential lncRNA-associated features of patients with OSCC as a valuable and independent prognostic biomarker. A total of 268 lncRNA expression profiles and clinical patient information on OSCC were downloaded from The Cancer Genome Atlas database. The clinical information was exploited for prescreening, using Cox regression analysis, and differentially expressed lncRNAs (DElncRNAs) were identified using edgeR software. Using the ‘caret’ package, the datasets were categorized into test datasets and training datasets, respectively. Through bioinformatics, seven prognostic DElncRNAs were selected. Using the regression coefficients, a risk score based on the seven-DElncRNA signature was developed to assess the prognostic function of key DElncRNAs. According to the median risk score, patients were classified into high-risk and low-risk groups in the training and test datasets. Additionally, receiver operating characteristic (ROC) curve analysis was conducted to evaluate the sensitivity and specificity of the prognostic DElncRNAs, and the optimal cut-off point was obtained from ROC analysis. Based on the optimal cut-off point, the patients were also categorized into high-risk and low-risk groups. Notably, the optimal cut-off point was more sensitive than the median risk cut-off. Additionally, stratified analysis results revealed that the seven-DElncRNAs signature was also independent of OSCC age. Furthermore, the findings of the present study suggested that the seven-DElncRNA signature can be used as a potential prognostic indicator and may have important clinical significance in OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide, with high recurrence rate and metastasis, and remains the leading cause of cancer-associated mortality in the world (1,2). Despite advances in diagnosis and treatment, the 5-year survival rate has not improved significantly, and the prognosis of patients with metastatic disease has remained poor over the past decade (3,4). Currently, surgery and adjuvant therapy remain the most popular means of treatment for patients with OSCC (5,6). Hence, more exact molecular biomarkers should be identified for prognosis prediction and for the development of novel therapeutic strategies for OSCC.

Long non-coding RNAs (lncRNAs) are broadly defined as non-protein coding transcripts >200 nt in length that lack protein-coding ability (7-9). An increasing number of studies have reported that lncRNA is a critical regulator that is involved in multiple cellular biological processes and is associated with tumorigenesis, progression and metastasis (10), such as lncRNA P21-associated non-coding RNA (ncRNA) DNA damage-activated in hepatocellular carcinoma and lncRNA HOX transcript antisense intergenic RNA in 26 human tumor types (11,12). However, limited studies have indicated lncRNA signatures as independent biomarkers can predict OSCC prognosis with high efficiency (13‑15).

The aim of the present study was to detect a lncRNA signature for the identification of OSCC prognostic biomarkers, by mining lncRNA expression profiles and clinical data in a large cohort of patients with OSCC in The Cancer Genome Atlas (TCGA) database. Multiple bioinformatics analyses were performed, such as Kaplan-Meier (K-M) curve survival analysis, univariate and multivariate Cox analyses and robust likelihood-based survival analysis. Seven lncRNAs (LINC01629, AC083967.1, AC067863.1, AC022092.1, AC005532.1, BX323046.1 and PRR29-AS1) were identified as potential novel independent prognostic biomarkers for the prediction of survival in patients with OSCC.
Materials and methods

Clinical information and IncRNA expression profile collection. OSCC IncRNA data and corresponding clinical information were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) using TCGBioblink (R version 3.6) (16). The OSCC datasets samples from the oral cavity were selected, including buccal mucosa, tongue, lip, hard palate, alveolar ridge, floor of the mouth and oral cavity. The latest genome annotation files were downloaded from GENECODE database (https://www.gencodegenes.org/). Moreover, patients were excluded from the present study for the following reasons: i) OSCC plus other malignancies; ii) tissue samples without complete RNA sequencing data; iii) patients receiving radiotherapy and chemotherapy prior to surgery; and iv) missing clinical information values. As a result, a total of 268 OSCC patients and 44 controls were enrolled in the study.

Differential expression analysis. Differentially expressed IncRNAs (DEIncRNAs) screened from TCGA were analyzed using the ‘edgeR’ package (17). In order to improve the screening accuracy and simplify the screening process, the DEIncRNAs were selected with the false discovery rate set at 0.05 and the fold change value > 2-fold.

Identification and selection of prognostic DEIncRNAs. The DEIncRNA expression profile and clinical features were incorporated into the complete dataset and further randomly divided into training datasets and test datasets, using the ‘caret’ R package (version 6.0-84) (http://caret.r-forge.r-project.org/). The association between DEIncRNAs and patients’ OS was analyzed in the training dataset. Univariate Cox regression analysis was utilized to identify significant DEIncRNAs with a P<0.05 in the R environment using ‘survival’ packages (version 3.1-8; https://cran.r-project.org/web/packages/survival/index.html). In order to ensure the reliability of these IncRNAs, a robust likelihood-based survival analysis was conducted using the R packages ‘phenoDiagram’ (version 3.1-8; https://cran.r-project.org/web/packages/phenoDiagram/index.html). To further integrate into a complete dataset and patient samples were randomly divided into training (n=134) and test (n=134) datasets.

Construction of a risk score system for the key DEIncRNAs. According to the results of multivariate Cox regression analysis, the regression coefficients of prognostic DEIncRNAs were calculated and a risk scoring system was constructed. The risk score of each patient was determined based on the following risk formula: Risk score = Σ Coef_{DEIncRNA} x Exp_{DEIncRNA} (19). In the formula, Coef_{DEIncRNA} represents the coefficient of each DEIncRNA and Exp_{DEIncRNA} is the expression of each DEIncRNA.

Validation of the risk score formula. Based on this formula, the risk scores were calculated in the training and test datasets, and the patients were categorized into high-risk and low-risk groups using the median risk score as the cut-off point. To further evaluate the specificity and sensitivity of the seven-IncRNA signature, receiver operating characteristic (ROC) curve analysis was performed and the ROC curve was obtained using the ‘survivalROC’ R package (https://cran.r-project.org/web/packages/survivalROC). Once the sensitivity and specificity reached a maximum, an optimal cut-off point was selected. Based on the cut-off point, the risk levels were used to classify patients into high-risk and low-risk groups in the training datasets, test dataset and the complete dataset. The differences in survival were further assessed by K-M curve analyses and log-rank test analyses, using the ‘survminer’ R package (version, 0.4.6; https://cran.r-project.org/web/packages/survminer/index.html).

Correlation analysis and function enrichment. In order to explore the potential function for the prognostic IncRNA, correlation analysis was performed between gene expression and prognostic IncRNA expression. The correlated genes were screened based on the following criteria: P<0.05 and |Pearson coefficient| > 0.3. The selected genes were further applied to conduct pathway enrichment analysis using the online tool ‘metascape’ (http://metascape.org/gp/index.html). The significant pathways were screened according to the criterion: q value < 0.05

Results

Preprocessing clinical features and screening for DEIncRNAs. The RNA sequencing (RNAseq) expression profile data and detailed clinical information on patients with head and neck squamous carcinoma were downloaded from the TCGA database. To obtain the OSCC datasets, samples from the oral cavity were selected, including buccal mucosa, tongue, lip, hard palate, alveolar ridge, floor of the mouth and oral cavity. After excluding unclear clinical information, a final sample of 268 patients was considered (Table I). The RNAseq expression datasets consisted of coding and non-coding genes. To obtain the IncRNA datasets, the latest genome annotation files (https://www.gencodegenes.org/) were downloaded. Based on the annotation file, expression data of a total of 15,183 IncRNAs were extracted. In addition, 2,157 significant DEIncRNAs (including 1,454 upregulated and 703 downregulated DEIncRNAs) were identified between OSCC tumor and normal tissues (fold change >2; P<0.05; Fig. 1A and B). The expression data and clinical features of 2,157 IncRNAs were further integrated into a complete dataset and patient samples were randomly divided into training (n=134) and test (n=134) datasets.

Identification of prognostic IncRNAs associated with OSCC. In total, 2,157 DEIncRNAs in the training dataset were first used for univariate Cox regression analysis, with P<0.05, and 81 significant DEIncRNAs were identified and further fitted to a robust likelihood-based survival analysis. As Table shown in Table II, 12 feasible and reliable DEIncRNAs were selected (Table II). Furthermore, multivariate Cox regression analysis yielded seven prognosis-associated IncRNAs, including LINCO1629, AC083967.1, AC067863.1, AC022092.1, AC005532.1, BX323046.1 and PRR29-AS1 (Fig. 2). To comprehensively understand the association between the seven-DEIncRNA
signature and the prognosis of OSCC, a seven-DElncRNA risk scoring system was constructed, based on its Cox regression coefficient. Risk score = (0.1448) x ExpAC083967.1 + (0.5187) x ExpAC067863.1 + (0.3046) x ExpAC022092.1 + (0.2533) x ExpAC005532.1 + (-0.4013) x ExpBX323046.1 + (0.6586) x ExpPRR29-AS1. The risk score of the seven-DElncRNA signature was calculated for each sample in the training and test datasets separately. Patients were categorized into the high-risk group (N=67) and low-risk group (N=67), based on the median risk score. As shown in Fig. 3, the K-M curves revealed a significant difference between the high-risk and low-risk groups in the training dataset, which were not significantly associated with OSCC in the test dataset (P=0.092).

**Evaluation and validation of the seven-DElncRNAs signature.** In order to evaluate the sensitivity and specificity of the seven-DElncRNA signature, ROC analysis was performed on the training dataset. Fig. 4A shows a value of the area under the curve 0.827. It has been demonstrated that the cut-off points with the maximal sensitivity and specificity could achieve a good classification (15). The optimal cut-off point reached 1.444, and the optimal cut-off value was used to further divide patient samples into the high-risk group (N=43) and the low-risk group (N=91) in the training dataset (Fig. 4B). K-M curves and log-rank test results suggested that a significant difference existed in survival time between the high-risk and the low-risk group (P<0.0001). In addition, K-M curves and log-rank tests were also performed in the test and complete datasets. The patients were separated into the high-risk and the low-risk group in the test dataset (P=0.0044) and in the complete dataset (P<0.0001), using the same risk formula (Fig. 4C and D). Additionally, compared to the expression level of BX323046.1, the other lncRNAs were at a high expression level in the high-risk group. Most cases of mortality were observed in the high-risk group, and patients with extended survival time were observed in the low-risk group in the complete dataset (Fig. 5). Based on the aforementioned results, age factors were found to be associated with the survival time of OSCC. Therefore, the datasets were regrouped based on the median age (62 years) to investigate the applicability of the seven-DElncRNAs signature. Patients were divided into a younger group (N=141) and an older group (N=127). Using the same formula, the younger group was further categorized into the high-risk (N=31) and the low-risk group (N=110), based on the optimal cut-off (Fig. 6A). Similarly, the older group was divided into the high-risk (N=39) and the low-risk group (N=92; Fig. 6C). The K-M curves demonstrated that the patients in the high-risk group had shorter OS (P<0.0001), and the area under the curve (AUC) values were 0.760 and 0.742 for the younger and older group, respectively, indicating that the seven-DElncRNA signature was independent to age (Fig. 6B and D). Additionally, the association between the seven-DElncRNA signature and clinical phenotypes was investigated. The results demonstrated that the seven-DElncRNA signature can serve as an independent predictor among the clinical phenotypes (Fig. S1). In order to explore the potential functions of the seven DElncRNAs, a correlation analysis between genes and the seven lncRNAs was performed. As a result, a total of 287 genes were screened from the correlation analysis by setting the following criteria: P<0.001 and absolute.

**Table I. Clinical information of patients and pre-screening of the clinical factors associated with the survival of OSCC based on the Cox regression analysis.**

| TCGA OSCC set (n=268) | Cox regression analysis | Hazard ratio (95% CI) | P-value |
|-----------------------|-------------------------|-----------------------|---------|
| Age                   | 1.364 (1.064-1.748)     | 0.014a                |
| Clinical_M            | 1.923 (0.476-7.771)     | 0.359                 |
| Clinical_N            | 1.128 (0.879-1.447)     | 0.346                 |
| Tumor_stage           | 1.178 (0.888-1.563)     | 0.257                 |
| Sex                   | 0.957 (0.736-1.243)     | 0.742                 |

*P<0.05. OSCC, oral squamous cell carcinoma; TCGA, The Cancer Genome Atlas; M, metastasis; N, Node.

**Table II. Prognostic value of lncRNAs was screened by performing forward selection in the training dataset (n=134).**

| lncRNA       | nloglik | AIC   |
|--------------|---------|-------|
| LINC01629    | 606.51  | 1215.01a |
| AP002989.1   | 603.15  | 1210.29a |
| AC073578.1   | 597.29  | 1200.58a |
| AC083967.1   | 592.62  | 1193.23a |
| AC067863.1   | 588.19  | 1186.39a |
| AC069503.1   | 586.91  | 1185.82a |
| HCG14        | 586.51  | 1187.02a |
| AC022092.1   | 585.00  | 1186.00 |
| AC005532.1   | 579.36  | 1176.71a |
| BX323046.1   | 577.73  | 1175.45a |
| PRR29-AS1    | 573.64  | 1169.29a |
| AC130456.2   | 572.02  | 1168.05a |
| BLACE        | 571.05  | 1168.10 |
| LINC00628    | 570.71  | 1169.43 |
| SMARCA5-AS1  | 569.99  | 1169.98 |
| KLHL30-AS1   | 569.56  | 1171.12 |
| AL391001.1   | 569.55  | 1173.11 |
| AC008011.2   | 569.10  | 1174.20 |
| AL035661.1   | 569.03  | 1176.06 |
| AC007786.1   | 567.94  | 1175.88 |
| LINC02453    | 567.09  | 1176.18 |
| AL021026.1   | 567.08  | 1178.15 |
| AC026471.6   | 566.29  | 1178.59 |
| AC093510.2   | 565.64  | 1179.29 |
| BDNF-AS      | 565.43  | 1180.85 |
| MIR503HG     | 562.80  | 1177.61 |
| AC097634.1   | 561.28  | 1176.56 |
| AC090337.1   | 559.59  | 1175.19 |
| CBR3-AS1     | 559.16  | 1176.32 |

*P<0.05. lncRNA, long non-coding RNA; AIC, Akaike information criterion; nloglik, negative log-likelihoods.
value of Pearson correlation coefficient >0.3. Further pathway enrichment analysis for the corresponding genes revealed that these lncRNA may be involved in the ‘PPAR signaling pathway’ and ‘cell cycle’ (P<0.05; Fig. S2).

Discussion

OSCC is an aggressive malignancy of the head and neck, with a 5-year survival rate of 40-50% worldwide (20,21). Although great efforts have been made over the past few decades to develop signatures for prognostic predictions, the lack of specificity and sensitivity to predict survival remains due to the complex molecular and cellular heterogeneity of OSCC. Therefore, the identification of an effective and independent molecular biomarker of OSCC is required.

The development of high-throughput technologies has promoted the discovery and study of ncRNAs, including lncRNAs, which account for only a small proportion (2%) of transcribed genes in eukaryotic species (22). A number of studies have reported that lncRNAs serve a pivotal role in complex biological processes (23-25), including tumor promotion and tumor suppression. For example, Liu et al (26) reported that DiGeorge syndrome critical region gene 5 is involved in cervical cancer progression by modulating the Wnt pathway. Guo et al (27) found that the lncRNA CEBPA-AS1 is associated with poor prognosis and promotes tumorigenesis.
via CEBPA/Bcl2 in OSCC, which may contribute to improving the effects of clinical treatment in OSCC. However, to the best of our knowledge, the prognostic values of lncRNAs in OSCC have not been comprehensively examined. Therefore, it is of great significance to explore the lncRNAs associated with prognosis, which will provide a potential mechanism and help to identify effective therapeutic targets for patients with OSCC.

Figure 3. K-M survival curves of overall survival time in patients with oral squamous cell carcinoma, based on the median cut-off value. (A) K-M curve indicating the significance between the high-risk and low-risk groups, based on the median risk score, in the training dataset. (B) K-M curve demonstrating the significance between the high-risk and low-risk groups, based on the median risk score, in the test dataset. K-M, Kaplan-Meier.

Figure 4. Evaluation of the seven-DElncRNAs signature based on the risk score and classification of different risk level groups and an optimal cut-off point. (A) Evaluation of the accuracy of seven-DElncRNA signature by conducting the receiver operating characteristic analysis, and the red point represents the optimal cut-off. (B) K-M curve showing the significance between the high-risk and the low-risk groups based on the optimal cut-off in the training dataset. (C) K-M curve demonstrating the significance between the high-risk and the low-risk groups based on the optimal cut-off in the test dataset. (D) K-M curve demonstrating the significance between the high-risk and low-risk groups, based on the optimal cut-off of the complete dataset. AUC, area under the curve; K-M, Kaplan-Meier; DElncRNA, differentially expressed long non-coding RNA; TPR, true positive rate; FPR, false positive rate.
The association between dysregulated lncRNAs and the prognosis of OSCC has been studied using a single biomarker or in small-scale studies (28,29). However, compared with single clinical biomarkers, integrating multiple biomarkers...
In conclusion, the present study explored the aberrantly expressed lncRNAs in OSCC profiles from the large-scale TCGA database. Furthermore, a seven-DElncRNA signature was identified, which was associated with OS in OSCC and could act as a potential independent biomarker for the prediction of prognosis in patients with OSCC. Nevertheless, future studies are required to evaluate and validate the molecular mechanisms of these DElncRNAs in prospective clinical trials.

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Availability of data and materials
The datasets used/and or analyzed during the current study are available from the corresponding author on reasonable request. The datasets used/and or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
XA designed the study. YW, RF and JW collected the clinical information, lncRNA expression data, and revised the figures and manuscript. TM and QS analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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