High hsa_circ_0081621 expression indicates a poor prognosis of laryngeal squamous cell carcinoma: A bioinformatics analysis and retrospective clinical study

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Abstract. Laryngeal squamous cell carcinoma (LSCC) is the second most common malignant tumour of the head and neck with a low 5-year survival rate. There is need to identify novel biomarkers for diagnosis and treatment of LSCC. The present study identified differentially expressed circular RNAs (circRNAs/circs) in LSCC and larynx adjacent non-carcinoma epithelial specimens by analysing the circRNA microarray dataset GSE117001. hsa_circ_0081621 had highest expression among three circRNAs (hsa_circ_0015211, hsa_circ_0023326 and hsa_circ_0081621) in LSCC specimens by reverse transcription-quantitative PCR. The expression levels of hsa_circ_0081621 in 67 LSCC specimens were detected by fluorescence *in situ* hybridization (FISH). Expression levels of hsa_circ_0081621 were analysed in relation to clinicopathological parameters and prognosis of patients with LSCC. According to FISH results, 59.7% of LSCC specimens exhibited high hsa_circ_0081621 expression. In LSCC specimens, hsa_circ_0081621 high expression was associated with lymph node metastasis and high clinical stage. High expression levels of hsa_circ_0081621 were associated with a poor 5-year overall survival rate in patients with LSCC. In addition, high hsa_circ_0081621 expression was an independent prognostic factor for patients with LSCC. hsa_circ_0081621 may participate in malignant progression of LSCC, and its high expression could be used for prognostic assessment of patients with LSCC.

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Introduction

Laryngeal squamous cell carcinoma (LSCC), which originates from the epithelium of the laryngeal mucosa, is the second most common malignant tumour in the head and neck region, and its incidence and mortality are on the rise (1). Most patients with LSCC are diagnosed at an advanced stage due to the occult nature of the disease. The primary reason for the poor prognosis of patients with LSCC is the propensity of LSCC for local invasion, cervical lymph node metastasis and chemoresistance (2). Despite improvement of treatment strategies, including surgery, systemic chemotherapy and local radiotherapy, patients with LSCC have a low survival rate (5-year survival rate was <50% between 2001 and 2004 in Switzerland) (3). Therefore, identification of novel biomarkers for LSCC diagnosis and investigation of effective novel therapeutic targets is required.

Due to their covalently closed loop without 5'-3' poly-adenylation end, circular RNAs (circRNAs/circs) are more stable than linear RNAs (4-6). Growing evidence implicates circRNAs in progression of several cancer types, in which they act as microNA (miRNA/miR) sponges, forming complexes with other RNAs or proteins, regulating RNA transcription and splicing and translation into peptides or microproteins (7-11). circRNAs serve a variety of cell functions in LSCC progression through different mechanisms. For example, circular RNA zinc finger protein 609 promote LSCC progression by upregulating epidermal growth factor receptor via sponging microRNA-134-5p (12). circ_0120175 reportedly promotes LSCC development by upregulating solute carrier family 7 member 11 through miR-330-3p (13). Circ coronin 1C was shown to promote LSCC progression by modulating the let-7c-5p/ PBX homeobox 3 axis (14) In addition to acting as miRNA sponges, circRNAs also regulate LSCC progression by forming complexes with proteins. For example, circ-cyclin D1 promotes the proliferation of LSCC by enhancing cyclin D1 mRNA stability by interacting with
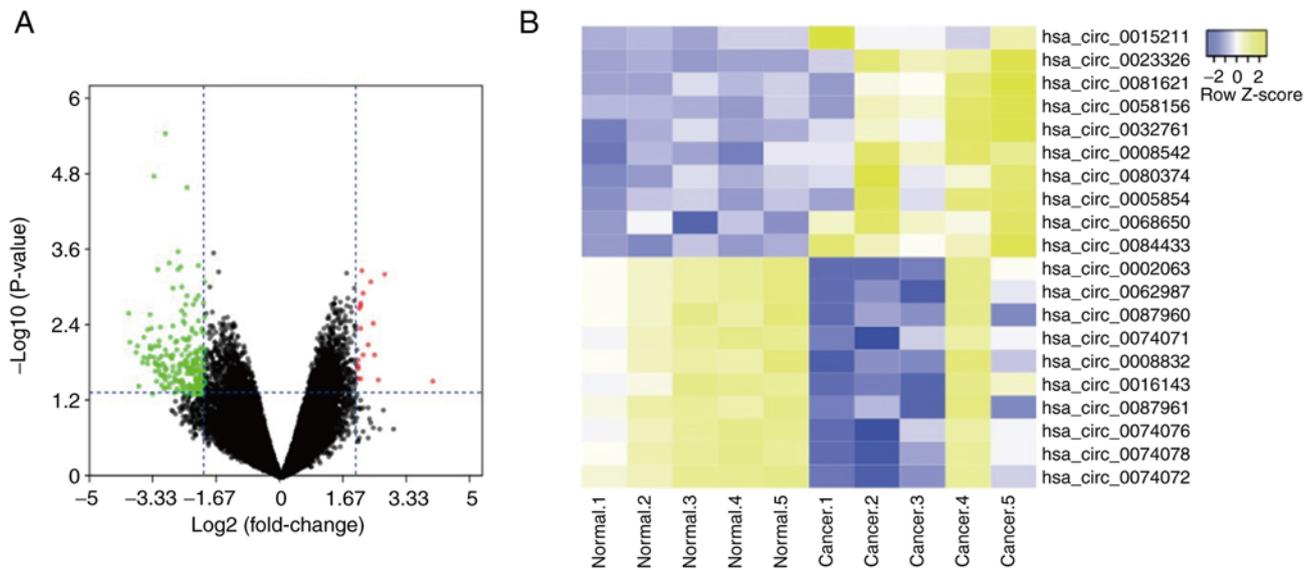
Figure 1. Bioinformatics analysis of differentially expressed circRNAs in five pairs of matched LSCC and normal laryngeal squamous specimens obtained from the GSE117001 dataset in the Gene Expression Omnibus database. (A) Volcano plot of differentially expressed circRNAs between five pairs of matched LSCC specimens and normal laryngeal squamous specimens. Red, upregulation; green, downregulation. (B) Clustered heatmap of 20 significantly differentially expressed circRNAs in LSCC and normal laryngeal squamous tissue. Yellow, upregulation; blue, downregulation. circRNA, circular RNA; LSCC, laryngeal squamous cell carcinoma.

human antigen R protein (15). circRNA microtubule cross-linking factor 1 promotes LSCC progression by directly recruiting complement C1q binding protein (C1QBP) and inhibiting ubiquitin-proteasome-mediated degradation of C1QBP, thereby increasing its expression (16). In addition, studies have demonstrated that circRNAs serve as biomarkers in diagnosis or prognosis of LSCC (12-15). In a pre-clinical study, circRNAs derived from plasma cells were screened in patients with LSCC and circ_0019201, circ_0011773 and circ_0122790 were shown to act as potential biomarkers for the prediction of LSCC prognosis (17).

The present study used dataset GSE117001 to identify candidate circRNAs by identifying DEcircRNAs in the dataset. The present study also investigated the correlation of hsa_circ 0081621 with clinicopathological characteristics, and their association with survival of patients with LSCC.

Materials and methods

Subjects and gene information. The circRNA expression profile (accession no. GSE117001) for LSCC was obtained from the Gene Expression Omnibus (GEO) database (ncbi.nlm.nih.gov/geo/). It included five pairs of matched LSCC specimens (GSM3267212, GSM3267213, GSM3267214, GSE3267215 and GSE3267216) and normal laryngeal squamous specimens (GSM3267217, GSM3267218, GSM3267219, GSM3267220 and GSM3267221). The GEOquery (version 2.68.0; bioconductor.org/packages/release/bioc/html/GEOquery.html) and limma (version 3.56.2; bioconductor.org/packages/release/bioc/html/limma.html) packages in R software (version 3.5.0; http://www.r-project.org) were used to process the expression matrix and differential expression analysis, as previously described (18). P<0.05 and |log2 fold-change (FC)|>2 were set as thresholds to identify circRNAs that exhibited differential expression between LSCC and laryngeal squamous specimens.

Patients and tissue specimens. A total of 77 male patients with LSCC were recruited. The mean age of patients with LSCC was 60.7±7.9 years (range, 43-79 years). No patients had a prior history of cancer, chemotherapy or radiotherapy. All the fresh LSCC and corresponding non-carcinoma tissue was collected from these patients. Non-carcinoma tissues were resected tissues ~1 cm from the tumours and were identified by pathology. The pathological results for all individuals were reviewed by two experienced pathologists. The first cohort comprised 10 patients with LSCC who underwent surgery at The Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between June 2022 and September 2022. The first cohort was used to explore the expression of three candidate circRNAs. Specimens from the first cohort preserved in liquid nitrogen for RNA extraction.The second cohort contained 67 patients untreated LSCC who presented to The Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between January 2014 and November 2015. The second cohort were used to validate the correlation between the expression profiles of hsa_circ_0081621 and the clinicopathological characteristics of patients with LSCC. Specimens from the second cohort were fixed at 4°C for 16 h with 4% formaldehyde after surgery and embedded in paraffin for diagnosis and the subsequent analysis. Patients in the second cohort were followed up for 12-101 months. Informed consent was obtained to participate. The study was approved by the clinical research ethics committee of The Fourth Hospital of Hebei Medical University (approval no. 2020ky198; Shijiazhuang, China).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate total RNA from tissue (19). GoScript™ Reverse Transcription System (Promega Corporation) was
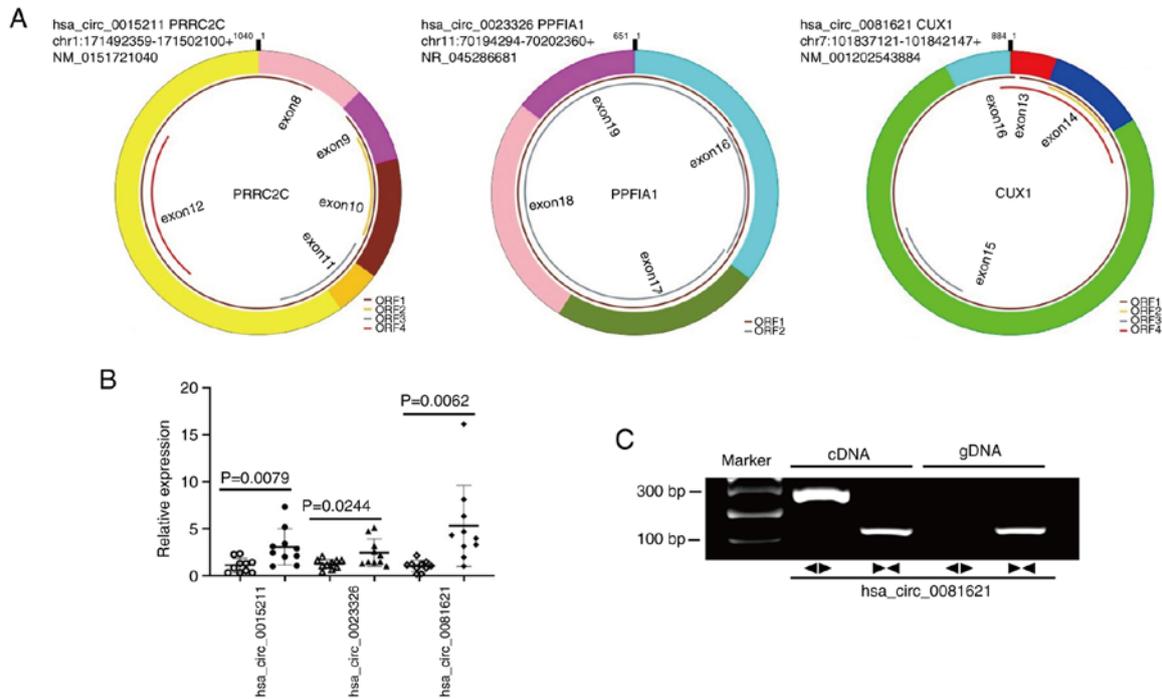
Figure 2. Expression of hsa_circ_0015211, hsa_circ_0023326 and hsa_circ_0081621 detected by RT-qPCR in LSCC tissue. (A) Schematic representation of hsa_circ_0015211, hsa_circ_0023326 and hsa_circ_0081621. (B) Relative expression of hsa_circ_0015211, hsa_circ_0023326 and hsa_circ_0081621 in 10 pairs of matched fresh LSCC and larynx adjacent non-carcinoma epithelial specimens detected by RT-qPCR. (C) Expression of hsa_circ_0081621 in LSCC tissue was detected by RT-PCR. circ, circular RNA; LSCC, laryngeal squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative PCR.

used to prepare cDNA from total RNA according to the manufacturer's instructions. cDNA was used as a template for RT-qPCR using GoTaq® qPCR Master Mix (Promega Corporation). The thermocycling conditions of qPCR were as follows: Initial denaturation, 70˚C for 5 min; annealing, 25˚C for 5 min; extension, 42˚C for 60 min; and denaturation, 70˚C for 15 min. GAPDH was used for normalization. The primer sequences were as follows: hsa_circ_0081621, forward, 5'-AAT AAACTGACTGTTCGTGGCA-3' and reverse, 5'-GCAGCG AGCGGTTCTTCT-3'; corresponding linear RNA, forward, 5'-ATCCGACTCCCAGCCCACAAC-3' and reverse, 5'-TCC GTCAGCACCTCCTTCACC-3' and GAPDH, forward, 5'-GACCACAGTCCATGCCATCAC-3' and reverse, 5'-ACG CCTGCTTCACCACCTT-3'. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (20).

Fluorescence in situ hybridization (FISH) and evaluation. FISH assay was performed using biotin-labelled hsa_circ_0081621 probe (5'-CY3-GACTTCAGAATGCTTCAG ACCCA-3'-CY3) which was purchased from GenePharma Co., Ltd. FISH assay was performed on 5 µm sections of paraffin-embedded tissue specimens. The sections were deparaffinized in xylene and rehydrated using a graded ethanol rinse series (50, 75, 85 and 95%). The slides were incubated at 37˚C for 30 min in 50 µl Proteinase K solution (15 ug/ml), washed with sterile distilled water. After pre-hybridization (1X PBS/0.5% Triton X-100), cells were hybridized in hybridization buffer (40% formamide, 10% Dextran sulfate, 1 x Denhardt's solution, 4 x SSC, 10 mm DDT, 1 mg/ml yeast transfer RNA, 1 mg/ml sheared salmon sperm DNA) with biotin-labelled probes specific to hsa_circ_0081621 at 60˚C overnight. Conjugate Cy™ 5
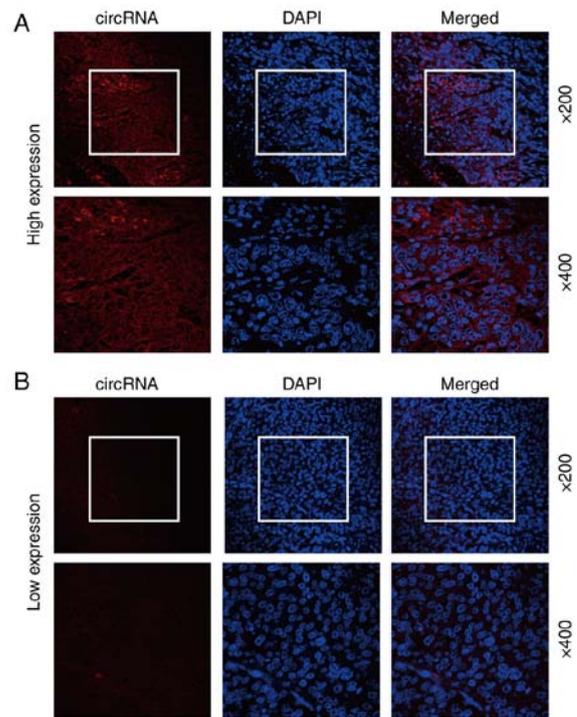

Figure 3. Expression of hsa_circ_0081621 in LSCC tissue. (A) Representative images (A) high and (B) low expression of hsa_circ_0081621 in LSCC tissue. circ, circular RNA; LSCC, laryngeal squamous cell carcinoma.

streptavidin conjugate was used to detect the fluorescence signal of hsa_circ_0081621 (ZyMAXTM Grade; Invitrogen; Thermo Fisher Scientific, Inc.). Cell nuclei were stained with DAPI for 10 min at 37˚C. Images were captured using

Table I. Association between the expression of hsa_circ_0081621 and clinical pathological features in patients with laryngeal squamous cell carcinoma.

| Characteristic | n | hsa_circ_0081621 | | χ^2 | P-value |
		Low	High		
Age, years				0.298	0.585
<60	30	11	19		
≥60	37	16	21		
Smoking status				0.155	0.694
Non-smoker	14	5	9		
Smoker	53	22	31		
Pathological differentiation				2.223	0.136
I	16	9	7		
II and III	51	18	33		
T stage				3.159	0.075
1 and 2	41	20	21		
3 and 4	26	7	19		
Lymph node metastasis				4.161	0.041
No	54	25	29		
Yes	13	2	11		
Clinical stage				6.660	0.010
I and II	45	23	22		
III and IV	22	4	18		

Table II. Univariate and multivariate analyses of prognostic factors in laryngeal squamous cell carcinoma.

| Variable | Univariate analysis | | | Multivariate analysis | | |
	HR	P-value	95% CI	HR	P-value	95% CI
Expression of hsa_circ_0081621, high vs. low	4.749	<0.001	2.01-11.222	3.934	0.002	1.627-9.512
Age, <60 vs. ≥60 years	1.300	0.466	0.642-2.634			
Smoking status, non-smoker vs. smoker	1.224	0.656	0.504-2.974			
Histological grade, I vs. II and III	1.116	0.788	0.501-2.486			
T stage, 1 and 2 vs. 3 and 4	3.259	0.001	1.603-6.627	2.402	0.019	1.155-4.996
Lymph node metastasis, no vs. yes	3.168	0.002	1.513-6.634	2.173	0.047	1.010-4.678
Clinical stage, I and II vs. III and IV	4.716	<0.001	2.318-9.595			

a confocal microscope with a ZEISS LSM 900 lens (Carl Zeiss AG).

Quantitative evaluation of FISH was conducted by examining five randomly selected fields/slide using a high-magnification light microscope (magnification, x400). The percentage area covered by fluorescence staining was assessed using the following scoring system: 0, no staining; 1, 1-25%; 2, 26-50% and 3, 51-100%. The fluorescence staining intensity was scored as follows: 0 (no fluorescence), 1 (weak fluorescence) and 2 (mild fluorescence) and 3 (high fluorescence). hsa_circ_0081621 expression was ranked on a scale of 0-6 based on the sum of its intensity and area. The sample was considered to show low expression in the 0-2 range and high expression in the 3-6 range, with weak positive expression (3 and 4) and strong positive expression (5 and 6).
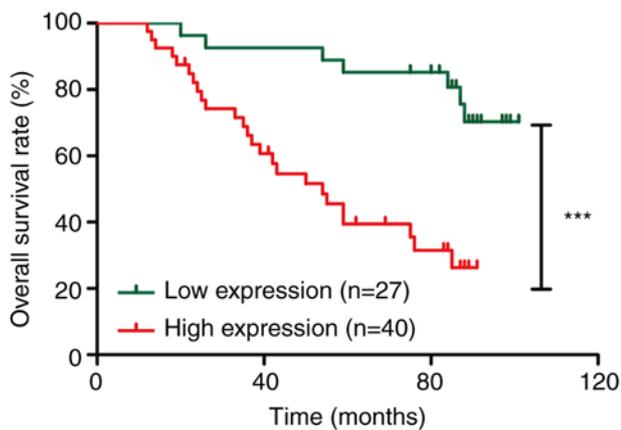
Figure 4. Kaplan-Meier survival analysis of hsa_circ_0081621 expression in patients with laryngeal squamous cell carcinoma using log-rank test. ***P<0.0001.

Statistical analysis. SPSS 22.0 software (IBM Corp.) was used to perform statistical analysis. The RT-qPCR assay repeats three times. Data are presented as the mean ± standard deviation (SD) and compared using Student's t-test. The data were normally distributed. χ^2 test were used to evaluate the potential association between the expression of hsa_circ_0081621 and various clinicopathological factors. Survival analysis was performed using Kaplan-Meier analysis with log-rank test. The Cox regression model was used for univariate and multivariate analysis of overall survival and prognostic factors. The statistical analyses were conducted using two-sided tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of differentially expressed circRNAs. R3.5.0 limma package (P<0.05 and |log$_2$FC|>2) was used to analyse GEO dataset GSE117001. A total of 19 up- and 226 down-regulated circRNAs in LSCC tissue were identified. The three most significantly upregulated circRNAs in LSCC tissue were hsa_circ_0015211, hsa_circ_0023326 and hsa_circ_0081621 (Fig. 1A and B). Fig. 2A shows the basic structure of these circRNAs, which are derived from exonic regions of their respective parent genes. According to qPCR results in 10 paired fresh LSCC tissues and the corresponding non-carcinoma tissue (the first cohort), all aforementioned circRNAs were found to be upregulated in LSCC tissue; hsa_circ_0081621 exhibited the highest expression levels in LSCC tissue (Fig. 2B). Agarose gel electrophoresis demonstrated the presence of hsa_circ_0081621 in LSCC (Fig. 2C).

hsa_circ_0081621 is highly expressed in LSCC specimens. To explore the expression expression of hsa_circ_0081621 in LSCC, a biotin-labelled hsa_circ_0081621 probe, which can recognize the junction site of hsa_circ_0081621, was used to stain 67 LSCC specimens (the second cohort). Representative images of high and low fluorescence staining of hsa_circ_0081621 in LSCC samples are shown in Fig. 3. Overall, 40 of 67 (59.7%) LSCC specimens exhibited high hsa_circ_0081621 and 27 of 67 (40.3%) LSCC specimens exhibited low hsa_circ_0081621 expression. In most specimens, hsa_circ_0081621 expression was primarily observed in the cytoplasm (Fig. 3).

Hsa_circ_0081621 expression is associated with clinicopathological factors indicating poor prognosis of LSCC. To investigate the potential effect of hsa_circ_0081621 in LSCC progression, the association between hsa_circ_0081621 expression and clinicopathological factors of patients with LSCC was evaluated. hsa_circ_0081621 expression was not associated with patient age, smoking status, pathological differentiation or T stage (Table I). hsa_circ_0081621 high expression was more frequent in LSCC samples with lymph node metastasis (11/13; 84.6%) than in those without (29/54; 53.7%). hsa_circ_0081621 high expression was found in 18 out of 22 (81.8%) LSCC specimens with clinical stages III and IV, which was higher than expression in specimens with clinical stages I and II (22/45; 48.9%; P<0.05). These results suggested that high expression of hsa_circ_0081621 may be indicative of a poor prognosis in patients with LSCC.

Hsa_circ_0081621 expression is associated with poor survival of patients with LSCC. To evaluate the prognostic significance of hsa_circ_0081621 expression in patients with LSCC, the association between expression levels of hsa_circ_0081621 and the 5-year overall survival of patients with LSCC was investigated (second cohort). Survival data based on Kaplan-Meier analysis showed that patients with high hsa_circ_0081621 expression had a significantly shorter overall survival than patients with low hsa_circ_0081621 expression (χ^2=15.03; Fig. 4). Furthermore, the present study analysed the association between hsa_circ_0081621 and various clinicopathological factors and overall survival of patients with LSCC. Overall survival of patients with LSCC with high T stage, lymph node metastasis or high hsa_circ_0081621 expression was shorter than that of patients with low T stage, non-lymph node metastasis or low hsa_circ_0081621 expression (Table II).

The prognostic significance of hsa_circ_0081621 expression was assessed in multivariate analyses. Multivariate Cox regression analysis of the association between factors, including patient age, smoking status, histological grade, T stage, lymph node metastasis and hsa_circ_0081621 expression, and overall survival was performed. T stage [hazard ratio (HR), 2.402; 95% CI, 1.155-4.996], lymph node metastasis (HR, 2.173; 95% CI, 1.010-4.678) and hsa_circ_0081621 expression (HR, 3.934; 95% CI, 1.627-9.512) were independent prognostic factors for patients with LSCC (Table II).

Discussion

LSCC is one of the most common types of cancer of the head and neck and has a low 5-year survival rate (2,3). Furthermore, most patients with LSCC are diagnosed at an advanced stage, which limits treatment options (2,3). Therefore, the identification of novel prognostic biomarkers and molecular targets for therapy is required.

Due to the covalently closed circular RNA molecules, circRNAs are unaffected by RNA exonuclease and are more stable than linear RNAs. In addition, circRNAs have tissue specificity, and participate in multiple physiopathological processes. Therefore, circRNAs may serve as biomarkers for

diagnosis and prognosis of various types of disease (21). For example, based on a machine learning classification model, hsa_circ_0005505, circ erb-b2 receptor tyrosine kinase 2 and circ carbohydrate sulfotransferase 12 have been identified as potential diagnostic biomarkers for intracerebral haemorrhage (22). circ meningioma expressed antigen 5 has been identified as a novel premetastatic factor and latent biomarker in osteosarcoma by analysing the expression profile of circRNAs (23). Through high-throughput RNA sequencing, hsa_circRNA_0101388 and hsa_circRNA_0022426 have potential predictive value for malignant transformation of human colorectal inflammation into colorectal cancer (24). In addition, circRNAs are stably enriched in exosomes and show a unique circular structure, high stability, conservation and tissue specificity, thereby exhibiting great potential as tumor biomarkers and anti-tumor targets (25). For example, through analysis of the urinary exosomal circRNA profile, hsa_circ_0001250 derived from urinary extracellular vesicles has been detected as a potential biomarker for idiopathic membranous nephropathy (26).

In the present study, GEO dataset GSE117001 was analysed to explore differentially expressed circRNAs in LSCC; 19 upregulated and 226 downregulated circRNAs were identified in LSCC tissue. Through analysis of the top three circRNAs by RT-qPCR in 10 paired fresh LSCC and corresponding non-carcinoma tissues, the highest expression of hsa_circ_0081621 was found in LSCC tissues. Due to the difficulty of preparing RNA from paraffin-embedded sections, FISH staining was used to analyse hsa_circ_0081621 expression in 67 LSCC samples. The present results revealed high hsa_circ_0081621 expression in 59.7% of patients with LSCC. These results suggested that hsa_circ_0081621 may be associated with the developmental process of LSCC.

Here, high hsa_circ_0081621 expression was associated with lymph node metastasis and high clinical stage. In survival analysis, the overall survival rate of patients with LSCC with high hsa_circ_0081621 expression, as determined by log-rank analysis, was markedly lower than that of patients with low hsa_circ_0081621 expression. In addition, hsa_circ_0081621 expression was an independent prognostic factor for patients with LSCC.

Numerous circRNAs have been identified as diagnostic or prognostic biotargets for patients with LSCC. For example, as an oncogenic RNA, circ coronin 1C can serve as a novel target for LSCC treatment and may act as a diagnostic and prognostic marker for LSCC detection (14). Circ bifunctional apoptosis regulator is associated with poor prognosis and accelerated LSCC progression (27). circ_0067934 is an oncogene promoting LSCC and may be a viable prognostic biomarker and target for diagnosis and treatment (28). Zhao *et al* (29) reported that circ ATP binding cassette subfamily B member 10 promotes LSCC progression and may act as a possible target in LSCC therapy. In addition, circRNA_103862 promotes LSCC proliferation by targeting the miR-493-5p/golgi membrane protein 1 axis and might serve as a potential prognosis marker and therapy target for LSCC (30). The present study demonstrated that hsa_circ_0081621 may be a prognostic marker for patients with LSCC.

The present study has limitations. First, only GSE117001 was analysed. Secondly, the biological functions of hsa_circ_0081621 in LSCC were not investigated and should be validated by *in vitro* cytology experiments and *in vivo* animal experiments.

Through bioinformatics analysis and retrospective study, the present results revealed that high hsa_circ_0081621 expression was associated with poor prognosis of patients with LSCC, and hsa_circ_0081621 may be a target for LSCC treatment. Further research is needed to determine the role of hsa_circ_0081621 in LSCC progression.

Availability of data and materials

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

Authors' contributions

ML and SL designed the study. ML, RZ, XS, YZ, HC and YX performed experiments and data analysis. ML, RZ and SL confirm the authenticity of all the raw data. ML and RZ interpreted the data and wrote the manuscript. ML revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients. The study was approved by the clinical research ethics committee of The Fourth Hospital of Hebei Medical University (approval no. 2020ky198; Shijiazhuang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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