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CIRC-0008798 and CIRC-0035967 as novel biomarkers in oral lichen planus and oral squamous cell carcinoma

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ABSTRACT. Emerging evidence has indicated that circRNAs, a novel class of endogenous noncoding RNAs formed by a covalently closed loop, play an important role in oral cancer. However, the role of circ-0008798 and circ-0035967 in oral cancer remains unclear. Oral cancer has been considered as one of the major leading causes of cancer-related mortality. It involves different mechanisms and signaling pathways such as a MAPK pathway that comprises several key signalling components and phosphorylation events in tumorigenesis. The purpose of this study is to investigate the expression profile of circRNA in oral cancer tissue and explore the regulatory mechanism in oral cancer tumorigenesis. the present study, qRT-PCR was used to detect the expression of circ-0008798, circ-0035967 and *map2k1* in 30 pairs of samples of oral cancer tissue and adjacent normal tissue.Significant upregulation of *MAP2K1* gene was noted in the OLP (p value = 0.034), and OSCC (p value = 0.00), specimens compared with healthy controls. The expression level of circ-008798 and circ-0035967 expression was down-regulated in OSCC tissues (p value = 0.23), (p value = 0.00) and OLP tissues (p value = 0.088), (p value = 0.054).Moreover, the area under ROC curves (AUC) of*map2k1* was 0.92, has_circ_0008798 was 1.00 ($p < 0.001$) and has_circ_0035967 was 0.99 ($p < 0.001$). Our data may help researchers to predict the molecular mechanisms of novel circ-0008798 and circ-0035967 in the development and progression of oral cancer comprehensively. Moreover, the present data indicate that novel circ-0008798 and circ-0035967 targets may be a series of promising candidates as biomarkers for oral cancer.

Keywords: circ-0008798; circ-0035967; *map2k1*; oral cancer; lichen planus; OSCC.

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Introduction

Oral Squamous Cell Carcinoma (OSCC)is the most commonly malignant tumour of oral cavitysystem with high recurrence and death rates (Andisheh-Tadbir, Mehrabani, & Heydari, 2008). In addition to genetic risk, lifestyle factors, tobacco, alcoholand diet show conclusive associations with oral cancer incidence (Gupta, Bray, Kumar, & Johnson, 2017). Oral cancer is low prevalence cancer that late diagnosis usually means the disease has already spread within the body, making it less treatable, reducing a patient's chance of survival, and potentially increasing the mortality and morbidity (Dhanuthai et al., 2018). Facing with those urgent challenges, it is important to explore novel biomarker and identify effective therapeutic targets. Although several critical events have been identified that play key roles during oral carcinogenesis, only few of these molecular targets are clinically actionable (Santosh, Jones, & Harvey, 2016). Circular RNA (circRNA), have recently gained widespread attentionas a potentially new layer of biological regulation and delineating the complex mechanisms underlying malignant processes such as tumorigenesis and carcinogenesis (Ouyang, Wang, Zhao, Zhang, & Liao, 2018; Sun et al., 2018a, 2018b). Although mechanisms of circRNA biogenesis have been elucidated in some detail, their biological functionand the exact role of circRNA in human disease is still unclear so, further research is needed (Liang, Zhang, Liu, Jia, & Li, 2017).

The host gene, *MAP2K1*, was suggested to be a crucial gene in Ras/Raf/MEK/ERK pathway against oral cancer (Zhong et al., 2018). Abnormally elevated expression and function of *MAP2K1* are correlated with progression, metastasis and drug resistance of various tumours (Molinolo et al., 2009).

In the current study, we have made first attempts to fill this gap in knowledge to assess the molecular contribution of novel circRNAs, has_circ_0008798 and has_circ_0035967 on the malignant behaviour of oral squamous cell carcinoma cancer. Although increasing evidence has indicated that circRNAs are dysregulated in malignancies, including oral cancer, further studies are still warranted to elucidate their biological

functions and roles in the gene regulatory networks of oral cancer. The present review briefly introduces recent findings regarding classification and biogenesis of circRNAs, summarizes their functions and corresponding mechanisms in oral cancer, and discusses the potential implication of circRNAs in the pathogenesis of oral cancer. We specifically set out to investigate its relevance as a prognostic biomarker and potential therapeutic target. Accordingly, we analyzed the expression level of has_circ_0008798 and has_circ_0035967 in tissues and matched normal tissues.

Material and method

Tissue procurement and isolation of RNA

The tissue samples analysed in this study were derived from patients undergoing a surgical procedure of oral and maxillofacial including, 30 normal, 30 malignant oral cancer and 10 lichen planus tissues that obtained from bank of Imam hospital (Iran National tumour bank). This study has been approved in Ethic Committee of Islamic Azad University Science and Research Branch (Ethical code: IR.IAU.SRB.REC.1399.094). Written and signed informed consents were obtained from each participant prior to tissue collection. Participation of individuals was voluntary and they were aware of the project's purpose.None of these patients had received chemotherapy or radiation therapy before surgery. All OSCC and normal tissues were snap frozen and restored in liquid nitrogen before use.Sections from each specimen were examined by a pathologist and graded histologically. Information concerning patient age, sex, and cancer stage were obtained from patient records (Table 1).

Table 1. Clinic-pathological characteristics of patients with oral cancer.

Characteristics total cases	N of cases 46	CRNDE expression		*P* value
		Low	High	
Age (years)				
⩽ 40	2	1	1	0.7628
> 40	27	9	22	
Gender				
Male	23	16	7	0.3695
Female	7	5	2	
Stage				
I	3	1	2	0.3698
II	3	1	2	
III	7	3	4	
IV	17	8	5	
Grade				
I	17	10	7	0.9821
II	13	8	5	
Tumour size				
⩽3 cm	15	11	4	0.2057
>3 cm	15	8	7	
Lymphatic Invasion				
Yes	5	1	4	0.7644
No	24	20	4	
Unknow	1	1	0	
Necrosis Presence				
Yes	7	4	3	0.0073
No	23	12	11	
Perineural Invasion				
Yes	11	8	3	0.5701
No	19	10	9	
Vascular Invasion				
Yes	6			0.9821
No	24			

Extracting total RNA from tissues was carried out by TRIzol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. Briefly, 1 ml chloroform used to separate organic phase from inorganic phase, 200 µL isopropanol for the precipitation of total RNA at 12,000 × g for 15 min at 4°C washed with 75% ethanol at 7,500 × g for 5 min at 4°C. The extracted RNA was diluted in 30 µL DEPC water (nuclease-

free water) and preserved at −80°C until use. Total RNA concentration was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), at 260 and 280 nm (A260/280) wavelengths.

RNASE R Treatment and CDNA synthesis

mRNA was reverse transcribed into first cDNA strands by reverse transcription kit (biofact) and oligo DT primers. Divergent primer has been designed for circRNA, which specifically amplified the back-spliced and canonical forms of circRNAs, which was resistant to the digestion by RNase R.cDNA was synthesised from 1 µL of 2000 ng μL^{-1} total RNA using Reverse Transcription kit (thermofisher). According to the manufacturer's instructions contain 0.5 µL RNaseR, 1 µL DNTP, 1 µL Random Hexamer and up to 10 µL Nuclease free water.

Real-Time PCR

Quantitative PCR (qPCR) were carried out to quantify the amount of circRNA, mRNA and detect the expression level of circ-0008796, circ-0035967 and *map2k1* by using the SYBR® Green PCR Master Mix as the fluorescent dye (Roche Co., Ltd.), according to the manufacturer's protocol: 10 µL SYBR Green, 1.6 µL cDNA product, 1 µL PCR primers, 6.4 µL DEPC water. The cycling conditions were 95 1C for 10 min, followed by 40 cycles of 95 1C for 15s and 60 1C for 60s in 7500 real-time PCR. All of the reactions were performed in triplicate. After the reactions were completed, the cycle threshold (CT) data were determined using fixed threshold settings, and the mean CT was determined from triplicate PCRs. A comparative CT method was used to compare each condition to the control reactions. For circRNA and mRNA, B-actin was used as an internal control.The primer sequence was listed in Table 2.

Table 2. Primers sequence list.

gene*map2k1*	F:GGTGTTCAAGGTCTCCCACAAG R:CCACGATGTACGGAGAGTTGCA
gene*ACTB*	F:GATCAAGATCATTGCTCCTCCTG R:CTAGAAGCATTTGCGGTGGACC
Circ-0008798	F:GCTCCCCTGCAAAGCC R:GAATACCTGGGCAATG
Circ-0035967	F:CCTTTTTACACATCTCGTTA R:CAATACCCTGGGCAATGACG

Statistical analysis

Data were assessed by Graph-Pad Prism software v.5.0. All data are expressed as mean ± standard deviation (SD) for the experiments. p-value < 0.05 was considered statistically significant for all statistical assessments. SPSS 8.0 software (SPSS Inc., Chicago, IL, USA) has been conducted as well. The T-test was used for checking of data normality distribution. The Shapiro-Wilk test, a nonparametric test, provided by the SPSS softwarewas used as the test for checking of normality distribution.

Result

Amplification of circ-0008798 and circ-0035967

Melt curve analysis is frequently used as a diagnostic tool for assessing qPCR amplicon length with intercalating dye qPCR assays. We first analysed the specificity of the amplified circ-0008798 and circ-0035967 product. Our melting curve analysis indicated only a single narrow peak that corresponded to the RT-PCR product. This means that there was neither non-specific amplification nor primer dimers. The presence of single bands for the circRNAs and housekeeping gene, B-actin, on gel electrophoresis confirmed the specificity of the PCR.

Differential expression of circRNA in tumour tissues

Oral cancer tissue and paired adjacent non- cancerous tissue were collected and screened for dysregulated circRNAs and *map2k1*. To identify the expression of circular RNAs in clinical samples from patients with oral cancer, we applied SPSS software and QRT-PCR was used to verify two typically differential expression circRNAs (hsa_circ_0008798 and hsa_circ_0035967) in OSCC tissues. Each selected circRNA candidate was verified by omitting reverse transcriptase to exclude the potential DNA contamination and treated with RNase R. Next, differential expression of the circRNA candidates were quantified and dysregulated circRNA may provide a

better understanding of the role of circRNA in oral cancer tumorigenesis. circ-0008798 and circ-0035967 were significantly down regulated in the OSCC tissues (p value = 0.23, p value = 0.00) and the level of *map2k1* gene was significantly up regulated in tumour tissues than in normal tissues (p value = 0.00) (Figure 1).

Differential expression of circRNA and map2k1 in oral lichen planus (OLP) tissues

According to Total RNA extracted from both OLP patients and healthy individuals, reductions in circ-0008798 (p value = 0.088) and significant reduction in circ-0035967 levels (p value = 0.034) has been observed in all cases. However, *map2k1* had highest level in OLP tissue. (p value = 0.000) (Figure 1).

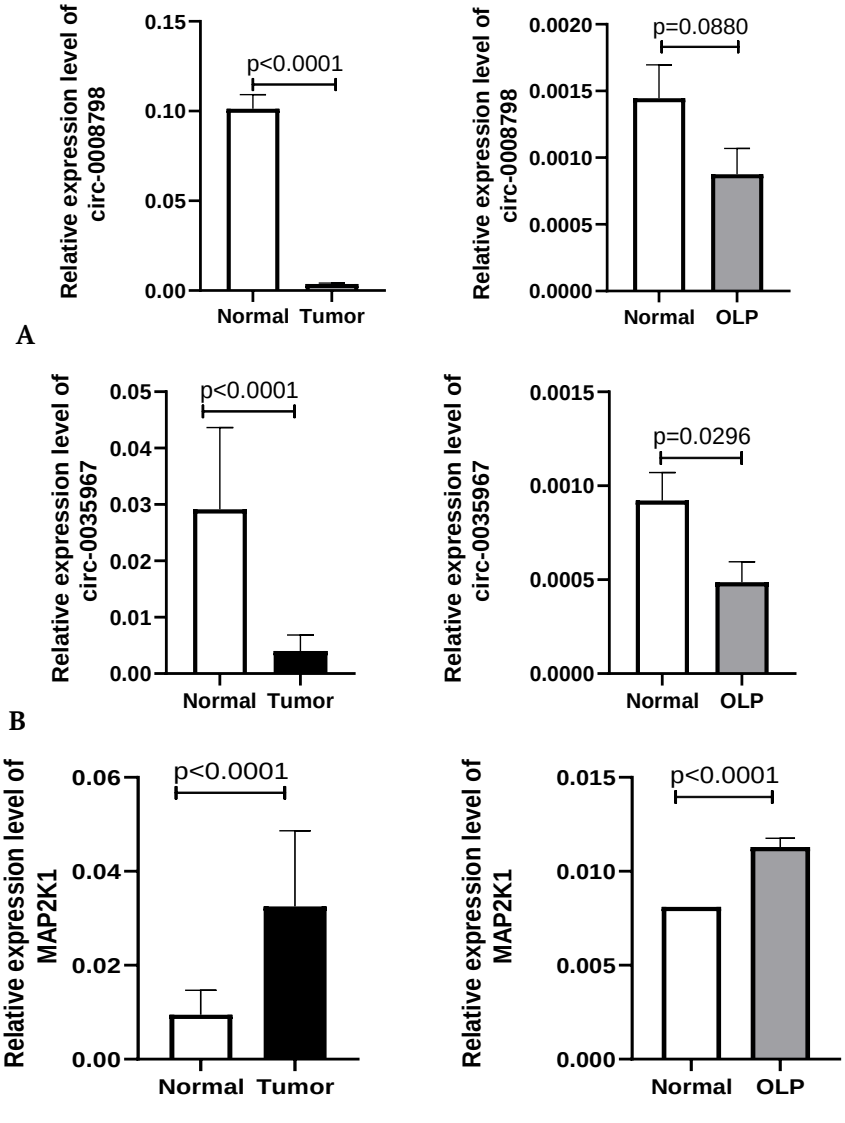

Figure 1. Downregulated expression of circ-0008798 (A) circ-0035967 (B) and upregulated of *MAP2K1* gene (C) in oral squamous cell carcinoma. Data are shown as means ± SD of three separate experiments. *p < 0.05.

Considering the additional decreased circRNAs levels observed in the dysplastic OLP and OSCC samples, this circRNAs may serve as a biomarker for the detection of malignant transformation in OLP patients, which is novel.

CIRC-0008798 AND CIRC-0035967 are an independent prognostic marker for survival of patients with oral cancer

Conventional clinic-pathological evaluation is currently the basis upon which risk stratification is pursued for patients with oral cancer. There are several clinic-pathological characteristics that are classical high-risk factors, including old patient age at the time of diagnosis, gender, large tumour size, lymph node metastasis, distant metastasis, and advanced disease stages. Each of these clinic-pathological risk factors has been shown to be associated with an increased risk for the progression, recurrence, and even morbidity and mortality of

oral cancer. further evaluation were performed for correlation among the circ-0008798 and circ-0035967 expression level and different clinical characteristics features of 30 patients with OSCC (Tables 3 and 4). The results suggested that the low expression of circ-0035967 expression significantly affected lymphatic invasion (p value = 0.012) and near association with vascular invasion (p value = 0.068). These findings suggested that down-regulation of circ-0035967 was involved in the tumorigenesis and progress of OSCC, which may affect the prognosis of OSCC. No other relationship between hsa_circ_0008798 and has_circ_0035967 levels and other clinicopathologic factors were observed.

Table 3. Association of circ-0008798 expression with the clinicopathological characteristics of OSCC.

parameter	Group	Cases	Circ-0008798 expression	p_value
gender	Male female	23 7	0.00±0.490	.497
age	>40 <40	9 21	0.000±0.49	.47
Tumor size	>=5 <2	19 11	0.024±2.53	.232
Tumor necrosis	Yes no	7 23	0.001±0.927	.629
Lymphaticinvasion	_	_	0.000±1.24	.455
stage	stageI stageII stageIII stageIV	3 3 7 17	0.00±1.09	.505
grade	Grade| Grade||		0.00±1.04	.805
Vascularinvasion	_	_	0.00±0.456	.303

Table 4. Association of circ-0035967 expression with the clinicopathological characteristics of OSCC.

parameter	Group	Cases	Circ-0035967 expression	p_value
gender	Male female	23 7	0.025±0.490	.218
age	> 40 < 40	9 21	0.039±0.49	.47
Tumor size	> = 5 < 2	19 11	0.025±2.53	.415
Tumor necrosis	Yes no	7 23	0.026±0.927	.533
Lymphaticinvasion	_	_	0.10±1.24	.012
stage	stageI stageII stageIII stageIV	3 3 7 17	0.036±1.09	.505
grade	Grade| Grade||		0.036±1.04	.235
Vascular invasion	–	–	0.102±0.456	.068

Go enrichment analysis and pathway analysis

The results of GO enrichment analysis of the differentially expressed circRNAs with identified target genes revealed that numerous target genes were involved in the biological processes, such as cellular process, regulation of biological process, metabolic process, etc. These processes were associated with human tumorigenesis and metastasis. To explore the role of abnormally expressed hsa_circ_0008798 and has_circ_0035967 of OSCC tissues in biological processes, cellular components and molecular functions, we performed GO enrichment analysis of the gene symbols of those circRNAs to assess their properties. The lower the P value was, the more meaningful the enrichment was in GO analysis.(KEGG) pathway analysis revealed strong enrichment in the MAPK signalling pathway, and *map2k1* target genes enrichment in MAPK signalling pathway. These processes and pathway were associated with human tumorigenesis and metastasis.

Map2k1 induces complete necrosis of the OSCC tissues

Great efforts have been made in revealing the mechanisms governing cancer resistance and recurrence. However, in this work, the direct effects of *map2k1* on necrosis was analysed by T-test Analysis and the findings revealed that *map2k1* could induce complete necrosis in oral cancer tissues ($p < 0.00$) but no other significant differences in clinico-pathologic characteristics were observed (Table 5).

Table 5. Association of *MAP2K1* expression with the clinicopathological characteristics of OSCC.

parameter	Group	Cases	Map2k1 expression	p_value
gender	Male female	24 6	8.805 + 0.490	.488
age	> 30 < 30	9 21	8.65 + 0.39	.47
Tomur size	> = 5 < 5	19 11	11.07 + 2.53	.350
Tomur necrosis	Yes no	7 23	6.892 + 0.927	0.00
Lymphatic invasion	–	–	9.109 + 1.24	2.69
stage	stageI stageII stageIII stageIV	3 3 7 17	8.49+1.09	.337
Vascular invasion	_	_	8.91+0.455	.698

Source: Direct research.

Potential diagnostic values of circRNAs and *MAP2K1* in oral cancer

To evaluate the potential diagnostic value, a ROC curve was generated for *map2k1* gene levels in tissues. We found that the area under the ROC curve (AUC) was 0.92. The sensitivity and specificity were 76% and 100%, respectively (Figure 2). Furthermore, to determine the diagnostic performance of hsa_circ_0008798 and hsa_circ_0035967 for estimating the therapeutic effect in OSCC, we next performed ROC curve analysis for each circRNA. The analysis was conducted by calculating the sensitivities and specificities at different thresholds of circRNA expression in the validation cohort and plotting the resulting values in the ROC space (Figure 3). At the optimal cut-off value of −0.089, with the values of sensitivity plus specificity considered to be maximal for has_circ_0008798, the sensitivity and specificity were 100% and 96.67%, respectively. At the optimal cut-off value of −1.171 for has_circ_0035967, the sensitivity and specificity were 100% and 66.67%.

The area under ROC curve (AUC) of has_circ_0008798 was 1.00 ($p < .001$), and the area under ROC curve (AUC) of has_circ_0035967 was 0.99 ($p < .001$).

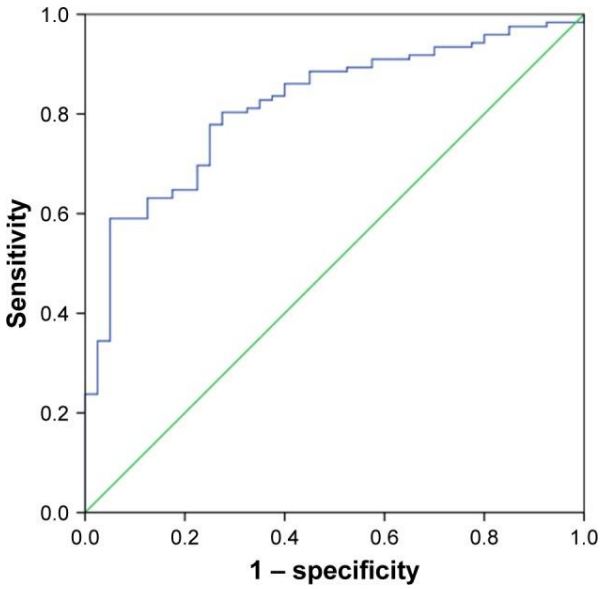

Figure 2. Receiver operating characteristic curve of tissues in *map2k1*.

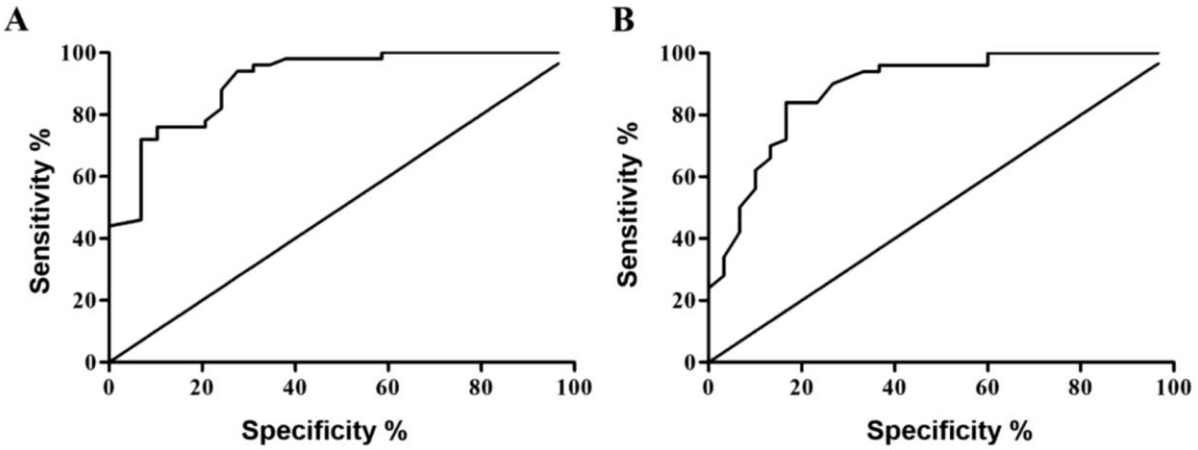

Figure 3. (A) ROC analysis for the expression miR_6824 in 30 paired tissue of OSCC patients. (B) ROC curves of the OSCC tissues for the miR_539 expressions.

Discussion

Oral cancer has become the leading cause of cancer related deaths worldwide with a rising trend of incidence and mortality, and OSCC accounts for 95% of all oral cancer (Bos, 1989). Despite significant advancement in understanding the potential molecular of OSCC, the use of genetic alterations for diagnostic and prognostic purposes remains limited, so this emphasises the high demand for reasonable and systematic therapies. Over the past decade, circRNAs have emerged as a critical factor in various cellular processes. An accumulating number of studies have indicated circRNAs as salient regulators of cancer progress and novel diagnostic tools in therapeutic. Compared with other non-coding RNAs, like miRNAs and long non-coding RNAs (lncRNAs), circRNAs have their own advantages (Yoon, & Seger, 2006).

A group of scientists has found that hsa_circ_0003159 expressions were significantly down-regulated in gastric cancer tissues (Couto et al., 2017). Expression levels of hsa_circ_0004018 in HCC were significantly lower compared with para-tumorous tissue ($p < 0.001$) (Li et al., 2013). circDDX17 was significantly down-regulated in CRC tissues and associated (Pénzváltó et al., 2014). The lower expression level of hsa_circRNA_103809 in colorectal cancer was significantly correlated with lymph node metastasis ($p = 0.021$) and tumour-node-metastasis stage ($p = 0.011$) (Lin, Hsu, & Tsai, 2020).

Our study aimed to clarify the correlation between circRNA expression and clinic-pathological parameters indicated that low expression of circ-0035967 was significantly associated with lymphatic invasion (p value = 0.012). Overall, these results provided important clinical evidence that circ-0035967 had an important clinical value for OSCC prognosis and may serve as a new biomarker for tumour diagnosis in the future. On the other hand, Eukaryotic gene expression is regulated during transcription. In the present work, *map2k1* up-regulation was measured in oral cancer tissues compared to normal oral tissues. Of note, high expression of *map2k1* was found to associate with necrosis. Our data was suggesting that *map2k1* might be a novel diagnostic and prognostic biomarker in oral cancer. Wefurther determined the differentially expressed of circRNA in OLP.Our results showed that the expression levels of circ-0008798 in OLP patients were significantly downregulated than those of healthy controls (p value = 0.08). Additionally, there was a direct but insignificant correlation between circ-0035967 expression levels among patients' group (p value = 0.03). However, due to the limited number of available tissue samples from patients with oral cancer, only 10 paired oral lichen planus tissues and 10 matched tissue samples were analyzed. Studies utilizing a large number of samples in multiple canters should be implemented in future. The study of circ-0008798 and circ-0035967 functions in oral cancer is also likely to improve the understanding of the occurrence and progression mechanisms of oral cancer. The correlation of the circ-0035967 gene and map2k1 gene as well as their future roles have been indicated in Table 6.

Table 6. Target genes' characteristics.

Gene	correlation	Future role
circ-0035967	lymphatic invasion	new biomarker for tumor diagnosis
map2k1	Upregulated in oral cancer and necrosis	diagnostic and prognostic biomarker in oral cancer

Conclusion

The high stability of circRNAs makes them become non-invasive biomarkers for OSCC detection. The identification of has_circ-0008798 and has_circ-0035967 provides evidence of a pivotal role for circRNA in OSCC tumorigenesis and progression. Given that has_circ-0008798 and has_circ-0035967 ae down-regulated in OSCC, the re-introduction of this mature circRNA into tumour tissue could serve as a therapeutic strategy by reducing the expression of target genes. CircRNA-based therapeutics are still in their infancy; however, our finding are encouraging and suggest this circRNA could be targeted for the development of a treatment for patient with OSCC, in the future. Moreover, receiver operating curve analysis indicated that the AUC of these two circRNA were all greater than 0.7, indicating that they might be potential biomarkers in the diagnosis of OSCC. Especially has_circ_0008798 and has_circ_0035968 were proved to be the most accurate tumour markers in distinguishing OSCC patients from healthy controls with AUC ROC reaching 0.99 and 1.00.

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