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Mir-let-7a Differential Expression in Oral Squamous Cell Carcinoma and Oral Lichen Planus: Insights for Early Diagnostic Biomarker Development

Seyedmovahed Seyedhoseini[1], Nooshin Mohtasham[2], Shadi Saghafi[3], Negin Alahkhani[4], Farnaz Mohajertehran*[1], Fahimeh Afzaljavan[5], Mahammad Taghi Shakeri[6], Amirkian Bokharaei*[7]

Abstract

Background: Early detection of oral squamous cell carcinoma (OSCC) is essential for improving treatment outcomes. Oral lichen planus (OLP) is recognized as a premalignant condition that may progress to OSCC. Recently, microRNAs, particularly miR-let-7a, have emerged as promising biomarkers for gene regulation and early disease diagnosis. This study aimed to evaluate the expression level of miR-let-7a in OSCC and OLP patients, and to compare it with healthy controls, to determine its potential as an early diagnostic marker.

Methods: In this cross-sectional study, serum samples were collected from 36 OSCC patients, 38 OLP patients, and 38 healthy controls. Diagnosis of OSCC and OLP was confirmed via biopsy. Serum RNA was isolated, and after quality verification, cDNA was synthesized. Quantitative real-time PCR (qRT-PCR) was performed to assess miR-let-7a expression across the three groups. Statistical analysis was conducted using SPSS version 16.0.

Results: Significant differences in miR-let-7a expression were observed among the groups. Mean expression levels of miR-let-7a were 1.55 ± 1.19 in OSCC, 2.97 ± 2.00 in OLP, and 7.02 ± 4.10 in the control group ($p< 0.001$). Lower miR-let-7a expression in OSCC was notably correlated with adverse clinicopathological features, including higher tumor grade ($p < 0.001$), advanced clinical stage ($p= 0.011$), larger tumor size (T2) ($p< 0.0001$), and lymph node involvement ($p< 0.0001$).

Conclusion: The findings demonstrate that miR-let-7a expression is significantly reduced in OSCC and OLP patients compared to healthy controls, highlighting its potential as an early biomarker for detecting malignant transformation in oral lesions and understanding disease progression in OSCC and OLP.

Keywords: Head and Neck Squamous Cell Carcinoma, Let-7, Lichen Planus, MicroRNAs, Neoplasms, Tumor Biomarkers.

Introduction

Oral Cavity Cancer (OCC) is a type of Head and Neck Squamous Cell Carcinoma (HNSCC) and ranks among the twenty most common types of cancer worldwide. It is most prevalent in Asia,

1: Dental Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.
2: Oral and Maxillofacial Diseases Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.
3: Department of Oral and Maxillofacial Pathology, School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran.
4: Oral and Dental Diseases Research Center, Kerman University of Medical Science, Kerman, Iran.
5: Department of Obstetrics and Gynecology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
6: Department of Epidemiology, School of Public Health, University of Medical Sciences, Mashhad, Iran.
7: Student Research Committee, Faculty of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran.
***Corresponding authors: Farnaz Mohajertehran; Tel: +98 5138067272; E-mail: Mohajertf@mums.ac.ir & Amirkian Bokharaei; Tel: +98 5138067231; E-mail: Bokharaeiak991@mums.ac.ir.**
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with the highest rates in Southeast Asia (1). OCC involves the squamous cells of the lips, tongue, gums, inner cheek lining, roof of the mouth (hard palate), floor of the mouth, and tonsils (2). This malignancy primarily affects individuals over the age of 40 and is more prevalent in men than women (5.8 vs. 2.3 per 100,000 subjects, respectively) (3). Smoking, alcohol consumption, and human papillomavirus (HPV) infection are significant risk factors associated with the development of OCC (4); nevertheless, the fact that only a small quantity of exposed individuals develop OCC suggests that genetic predisposition plays a significant role in modulating the risk of oral cavity cancer (5).

Oral Lichen Planus (OLP) is a persistent inflammatory condition affecting the oral cavity's mucous membranes. Its manifestations include white lace-like patches, redness, and sores (6). According to the World Health Organization (WHO), OLP was classified as a potentially cancerous disease of the mouth in 2005 due to its malignancy rate of 0 to 10% (7). This condition impacts approximately 1-2% of the population, with a higher prevalence among women over 50 (8). The etiology of OLP has been a considerable debate, with immune dysregulation emerging as the most plausible causal factor. Extensive research indicates that lichen planus is an autoimmune condition reliant on T cells, notably CD8+ T cells, which instigate the apoptosis of oral epithelial cells (9).

Oral Cavity Cancer (OCC) diagnosis often relies on biopsies, which can be invasive and subjective. Biomarkers offer a promising alternative as measurable biological indicators that can be analyzed in blood, saliva, or tissue samples. They identify specific molecules associated with oral cavity cancer, potentially improving diagnosis and aiding treatment decisions (10). This approach facilitates the development of individualized treatment plans, enhancing patient outcomes and minimizing unnecessary procedures. Additionally, microRNAs (miRNA), which are about 22 nucleotides long, are noncoding RNAs that control gene expression after transcription by targeting messenger RNAs. They are crucial in physiological processes, and their dysregulation is linked to various human diseases, such as cancer (11).

The initial miRNA discovered in humans, let-7, was first identified in the nematode Caenorhabditis elegans. In humans, 13 precursors of this miRNA family have been described (12). It is widely believed that let-7 functions as a tumor suppressor by regulating various oncogenes such as K-Ras, STAT3, c-Myc, and HMGA2. Research indicates that a decrease in let-7 expression can enhance the tumorigenicity of cancer cells (13). It targets several oncogenes, and its suppression in cancer is associated with poor prognosis. This miRNA family plays a crucial role in regulating various biological processes, including self-renewal, proliferation, apoptosis, and cell metabolism through the controlling of target genes and signaling pathways (14). Therefore, this study evaluated the expression levels of miR-let7a in individuals diagnosed with OSCC and OLP. Furthermore, the research aimed to assess the potential of miR-let7a as a primary diagnostic biomarker by comparing the findings to those of a healthy control group.

Materials and Methods

Ethics

Ethics Committee of Mashhad University of Medical Sciences authorized the research protocol under the ethical approval number IR.MUMS.DENTISTRY.REC.1401.060.
Strict adherence to ethical protocols was maintained throughout all stages of the study. Additionally, all participants provided voluntary written consent to partake in the study and retained the freedom to withdraw at any point.

Study population

In a cross-sectional study, 74 patients referred to the Otolaryngology (ENT) Department of Ghaem Hospital and the Dentistry School of Mashhad University of Medical Sciences between 2020 and 2023 were enrolled. The healthy control group (consisting of 38 healthy

individuals) was selected from, the companions of patients at the Dentistry School and Medical School of Mashhad University of Medical Sciences. Subsequently, 5 ml of peripheral blood samples were obtained from all patients using EDTA (ethylenediaminetetraacetic acid) tubes and promptly stored at -80 °C following serum extraction. Based on the patient's pathology results, the pathologist confirmed the re-test results after the initial diagnosis and confirmation of the disease.

Inclusion and exclusion criteria

Patients were included in the study if they had complete medical records, no history of other malignancies, and had not received any antitumor treatments prior to the sampling. Controls were selected to match patients in terms of age and sex, and were required to have no history of systemic diseases or inflammatory conditions. Exclusion criteria included unwillingness to participate, as well as unclear or low-quality samples. This study followed the latest version of the 2023 NCCN Clinical Practice Guidelines in Oncology.

miRNA extraction

The patient saliva samples were centrifuged at 4 °C for 20 minutes at 14500 RCF (Relative Centrifugal Force) to separate the components. Following centrifugation, 800 µl of RNX-plus (SinaClon, IRAN) reagent was added, and the mixture was homogenized for 15 seconds to ensure even distribution. After a 4-minute incubation period at room temperature (RT), 200 ml of chloroform (Merck Co.) was added, and the samples were centrifuged again at 4 °C for 20 minutes at 14500 RCF. Subsequently, the supernatants were carefully transferred to DNase/RNase-free microtubes. Cold absolute ethanol (Merck Co.) was added to the supernatant, followed by overnight incubation at -20 °C to precipitate RNA. The subsequent centrifugation at 4 °C at 14500 RCF for 20 minutes resulted in RNA pellet formation. This process was repeated twice, adding 1 ml of 80% ethanol each time to wash the pellet thoroughly. Finally, the microtubes were air-dried for 4 minutes at RT to remove residual ethanol. The RNA was then dissolved in 20 ml of DEPC (Diethylpyrocarbonate) water and centrifuged after a 5-minute incubation at RT. Additionally, the concentration and purity of the extracted miRNAs were assessed using the Nanodrop system (Thermo Scientific 2000, USA) by measuring absorption at 260/280 nm wavelength.

cDNA synthesis and quantitative real-time PCR (qRT-PCR)

The isolated miRNAs exhibited an average concentration of 50 ng/µl, and for cDNA synthesis, a range ratio of 1.5-2 was utilized. The Adscript cDNA synthesis kit (REF: 22701, Bio-Tech, addbio, Korea) was employed according to the manufacturer's instructions. The total volume of the qRT-PCR reaction mixture was 20 µl, consisting of 10 µl of SYBR Green (addbio, Korea, Ref 70205HR), two µl of cDNA, 6.8 µl of distilled sterile water, and 0.6 µl of each reverse and forward primers (10 pm). RT-PCR amplification was performed in duplicate, and the cycling process in Light Cycler 96 (Roche, Germany) included a preincubation step at 95 °C for 1 min, 40 cycles at 95 °C for 15 sec, and 61 °C for 40 sec. The relative quantitation of miRNA expression was determined using the ΔΔCT method. Additionally, a melting curve analysis was conducted to validate specific target amplification. Primer sequences are shown in Table 1.

Table 1. The sequences of primers for molecular tests.

Primer Name		Primer Sequence	Accession Number
microRNA Let-7a-5p	Forward	5’-GGTGATGAGGTAGTAGGTTGT-3’	NR_029493.1
	Reverse	5-GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACTAT-3’	
U6	Forward	5'-AAGGATGACACGCAAATTC-3'	NR_004394.1
	Reverse	5'-GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAAAATATGG-3'	

Differentially expressed target genes of miRNAs

The Target genes of miRNAs were identified using miRTarBase (http://mirtarbase.cuhk.edu.cn/). The mutual genes were chosen for the next pathway analysis.

Statistical and pathway analysis

Following the identifying of relevant genes, the gene list was uploaded to the GeneAnalytics online tool for pathway analysis (https://geneanalytics.genecards.org/). The Gene Ontology (GO) database assessed biological pathways, molecular functions, and cellular components. Furthermore, the primary tissues and cells expressing the microRNA's target genes were investigated. The data analysis was done using SPSS version 16 software. The Shapiro-Wilk test was used to check for normal distribution. In the case of a normal distribution, parametric tests such as the analysis of variance and the independent t-test were employed. Non-parametric tests were utilized when dealing with a non-normal distribution. The Pearson test was applied to consider correlated quantitative variables. The significance level of 0.05 was consistently applied to all statistical tests conducted in this research.

Results

Characteristics of study subjects

In this study, the expression of miR-let-7 was examined in 112 blood samples, including 36 cases of OSCC, 38 with OLP, and 38 healthy subjects. Thirty-five men comprised 47% of the sample, while 39 women comprised 53% of the patient participants. The mean age was 50.74 ± 13.53, 54.53±12.30, and 54.39±13.24 in OLP, SSC, and healthy subjects. No considerable correlation was found between gene expression, age, and gender in various groups (Table 2).

Table 2. Demographic information of Participants.

Variables	Age (Mean±SD)	Gender N (%)	
Groups		**Male**	**Female**
OLP	50.74±13.526	18 (50%)	18 (50%)
SCC	54.53±12.304	17 (44.7%)	21 (57.3%)
Control	54.39±13.239	21 (57.3%)	17 (44.7%)
P-value	0.369	0.656	

miR-let-7 expression quantitatively

The miR-let-7 expression levels were 1.55± 1.194, 2.97± 2.00, and 7.02± 4.10 in the OSCC, OLP and healthy groups. A statistically significant difference was observed between groups in the average miR-let-7 expression ($p< 0.001$) (Fig. 1).

miR-let-7 association with OSCC clinicopathological features

The present study found no evidence of statistically significant differences in miR-let-7 expression across the three groups under investigation, as well as between male and female participants ($p> 0.05$). Patients in the early stage significantly expressed more miR-let-7 than those in the advanced stage ($p< 0.001$). Furthermore, the average microRNA expression was considerably lower in grade III compared to grades I, II, and the control group ($p= 0.011$). Additionally, the average expression of miR-let- was substantially lower at the T2 level than at the T1 level ($p< 0.0001$). Regarding the involvement of lymph nodes, there was a significant difference ($p< 0.0001$) in the average expression of miR-let-7 at the N2 level compared to the N0 and N1 levels (Table 3).

Table 3. The comparison of quantitative miR-let-7 expression based on clinopathlogical features in OSCC patients.

Variables		Number	miR-let-7	
			Mean ± SD	P-value*
Grade	I	15	2.02 ± 0.99	0.011
	II	16	1.52± 0.27	
	III	5	0.27± 0.09	
TNM	I	17	2.31±0.84	<0.0001
	II	4	2.63±0.91	
	III	11	0.48±0.43	
	IV	4	0.21±0.09	
Tomour size	(T1) ≤ 2 cm	22	2.20±1.01	<0.0001
	(T2) >2 cm	14	0.54±0.06	
Stage	Early stage	21	2.37±0.84	<0.0001
	Advanced stage	15	0.41±0.39	
Primary lymph node involvement	N0	21	2.37±0.84	<0.0001
	N1	12	0.45±0.42	
	N2	3	0.23±0.09	

* Kruskal–Wallis Test.

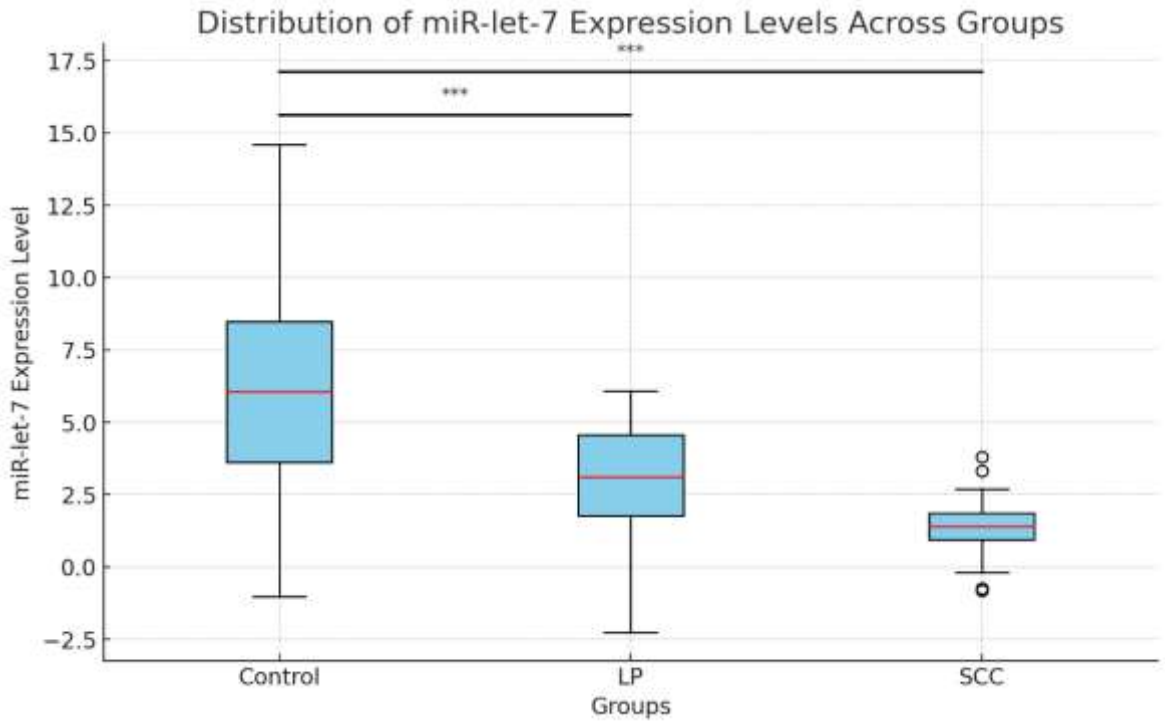


Fig. 1. distribution of miR-let7 expression levels across groups. The miR-let-7 expression levels were 1.55± 1.19 in the OSCC and 2.97± 2.00 in the OLP and 7.02± 4.10 in the healthy group (control group). A statistically significant difference was observed between groups in the average miR-let-7 expression. Applied test was Kruskal–Wallis Test. The *** indicate P< 0.0001.

miRNAs target genes

miRTarBase (http://mirtarbase.cuhk.edu.cn/) was searched to determine the target genes of miR-let-7a. The genes that are mutually relevant were chosen for the next phase of functional analysis.

Pathway analysis

The pathway analysis results, indicating significant expression of the identified genes across various brain tissues, including the cerebral cortex, cerebellum, and medulla oblongata, have intriguing implications for Head and Neck Squamous Cell Carcinoma (HNSCC).

Gene expression, cellular responses to stimuli, and RNA polymerase I opening pathway were the most important pathways linked to the gene list. The first pathway includes 93 genes, of which the ten most important ones were as follows: ZNF555, ZNF556, ZNF566, EIF4A3, NUP155, RANBP2, NUP58CNOT9, INTS12, and DICER1. The most vital genes involved in the cellular responses to stimuli pathway included SYVN1, MAP2K7, RPS24, NUP155, RANBP2, NUP58, MDM4, SP1, MYC, SESN1, and SESN2. Moreover, the third pathway belonged to the RNA polymerase I opening pathway involving forty-five genes (EIF4A3, CNOT9, NCOA3, DICER1, POLR2D, CCND1, MYC, CASP8, EZH2, and CDC25B).

Regarding Gene Ontology (GO) analysis, after putting 614 genes through an online tool, three GO groups with statistical significance (FDR, False Discovery Rate) $P< 0.05$) were identified. The most notable enrichments within the biological process category involve protein binding, G1/S transition of the mitotic cell cycle, and cell cycle process. As for molecular functions, the predominant roles include serving as protein, RNA, and DNA binding. Based on our gene list, the top three important cellular components were the nucleus, cytosol, and cytoplasm indicating genes related to each section.

Discussion

microRNAs have significant potential as diagnostic, prognostic, and therapeutic biomarkers because of their involvement in the genesis and development of different malignancies. The role of miRNAs, a class of noncoding RNAs, in oral cancer regulation by interacting with target mRNAs is essential in promoting or inhibiting cancer (15). Numerous investigations have demonstrated the role of miRNAs in the development and metastasis of oral cancer. Additionally, miRNAs' expression profiles have been linked positively to patient survival, metastasis, and clinical stage in oral cancer, suggesting that these miRNAs may serve as prognostic markers (16-18). Due to the inconsistent results in various ethnicities and backgrounds, this survey was dedicated to examining and comparing the expression of miR-let7a in the serum of patients with OSCC, OLP, and a healthy control group in an Iranian population.

The outcome of the current study demonstrated that there was a considerable variation in the three groups under investigation's levels of miR-let7a expression overall. The observed variation was that individuals with oral carcinoma had reduced expression compared to the other two groups, while patients with oral lichen planus had decreased expression compared to the healthy control group (p-value< 0.001). Subsequently, the correlation between the microRNA's expression level and age and gender was examined. However, the results revealed no statistical significance.

Moreover, results indicated that people with oral squamous cell cancer had lower expression of miR-let-7a; the lower the miR-let-7a expression, the more likely the disease will progress to higher grades and stages.

Helal et al. showed in their research of patients with advanced gastric cancer that increased glycolysis, increased autophagy, and decreased let-7a expression are substantially correlated with poor survival and resistance to pharmacological therapy (19). Gioacchini et al. looked into the expression of miR-let7a in a

subset of sinonasal adenocarcinoma known as intestinal-type adenocarcinoma (ITAC). They found a significant decrease in this microRNA's expression in tumour tissue compared to normal tissue, and the decrease was linked to grade III and poor differentiation. Additionally, they demonstrated that the expression of microRNA varies with disease stage, with less expression in the latter stages (T3–T4) of the illness than in the earlier stages (T1–T2) (20). Research on the tissue of patients with OSCC revealed a strong correlation between the large size of the tumour and the lower expression of miR-let7a. However, the level of miR-let-7a expression did not show any meaningful correlation with other clinical variables (21). Furthermore, Polz et al. found that lesions involving lymph nodes and moderately and poorly differentiated tumours with large dimensions have much lower expression levels of miR-let7a (22).

Fadhil et al. found that the considerable drop in miR-3928 and miR-let7a-5p expression in saliva can be explored as novel non-invasive biomarkers in the early diagnosis and prognosis of this illness in their investigation of the saliva of patients with HNSCC. However, they also demonstrated a correlation between the size of the tumour and the metastasis of lymph nodes by these two microRNAs (23). The association between miR-let-7a-5p and cervical cancer was investigated in a Chinese study in 2022. The results showed that miR-let-7a-5p is a novel, independent anti-oncogene in cervical cancer that can activate the TGF Regulate β1/TGFBR1/pSmad3 cellular pathway and impede the growth of cervical cancer cells (24).

It was determined that miR-let7a-5p could be a novel therapeutic target for the management of cervical cancer. Further studies also examined the function of miR-let7 in inflammatory reactions. When let-7 is suppressed, the TLR4/NF-κB pathway is activated more (25). Activating transcription factors linked to NF-κB triggers the production of several cytokines, including IL-1, IL-2, IL-6, and TNF-α. One cytokine strongly induced and reliant on the NF-κB pathway is IL-6 (26). The most consistent conclusion across the many investigations was the upregulation of pro-inflammatory cytokines, particularly IL-6, which is linked to the inhibition of let-7 (27, 28).

A recent study compared the expression of miR-let7a-5p in the saliva of patients with OSCC, oral lichen planus, and healthy control groups. The results indicated that the expression of this microRNA in the saliva of patients with OSCC was significantly lower than that of the healthy control group. Nevertheless, there was no discernible correlation between the stage and grade of OSCC patients and the reduction in this microRNA's expression in their saliva. Additionally, this microRNA's expression was decreased in the OLP patient group compared to the healthy control group. This observation might be explained by the microRNA's function in inflammation or the possibility of malignancy in this lesion (29). The function of microRNAs in oral lichen planus was examined in a Newcastle University study. This work examined the expression alterations of many microRNAs in OLP, a T-cell-mediated illness primarily defined by the overexpression of IFN-γ and TNF-α as well as apoptotic markers, utilizing NanoString analysis and RT-qPCR confirmation of the data. It was noted that there was a reduction in let-7 expression in OLP tissues (30).

In light of pathway analysis, we found significant expression of the identified genes across various brain tissues, including the he cerebral cortex, cerebellum, and medulla oblongata. Although HNSCC primarily affects the mucosal linings of the head and neck, the observed gene expression patterns in critical brain regions suggest potential neurological involvement or impact. These findings point to the nervous system’s role in the progression or symptomatology of HNSCC, potentially affecting aspects such as pain perception, cognitive function, and motor coordination in patients. The expression of these genes in the

cerebellum and medulla oblongata could relate to the motor and autonomic symptoms sometimes observed in advanced cases.

The pathway analysis of our study highlights significant gene regulation linked to gene expression, cellular responses to stimuli, and the RNA polymerase I opening pathway. Key genes such as ZNF555, EIF4A3, NUP155, and MYC play crucial roles in these pathways (31). Gene Ontology (GO) analysis of 614 genes identified three statistically significant groups, with notable enrichments in biological processes like protein binding, G1/S transition of the mitotic cell cycle, and cell cycle process. The predominant molecular functions of these genes include protein, RNA, and DNA binding, while the most relevant cellular components are the nucleus, cytosol, and cytoplasm. These findings provide a deeper understanding of the molecular mechanisms in HNSCC, suggesting that these pathways and gene functions are crucial in the disease's progression and could be potential targets for therapeutic intervention. The expression patterns and functional enrichments underline the complexity of HNSCC and offer insights into the broader systemic effects of the disease, potentially guiding more effective treatment strategies.

The present study established a significant and qualitative reduction in the expression of miR-let-7a within the serum of patients diagnosed with oral squamous cell carcinoma (OSCC) and those with oral lichen planus (OLP). This notable decrease in miR-let-7a levels among OSCC and OLP patients suggests that this microRNA may play a critical role in the pathogenesis of these conditions. Furthermore, the findings empower this idea that miR-let-7a could be considered as a potential biomarker for assessing the risk of malignant transformation in oral lesions. Given this point, future research with a larger sample size and the investigation of serum and saliva will provide a more thorough knowledge of its possible uses.

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Conflict of Interests

The authors declare no competing interests.

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